

Design and Characterization of Various Lipid, Polymer, and Gemini-Surfactant – Based Vectors and DNA as Non-Viral Delivery Systems for Gene Therapy Applications

by

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Author's Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

I understand that my thesis may be made electronically available to the public.

Statement of Contributions

Gurmeet Kaur Lall was the sole author of Chapters 1, 2, 3, 4 and 6 which were written under the supervision of Dr. Shawn D. Wettig and were not written for publication.

This thesis consists in part of one manuscript written for publication. Exceptions to sole authorship of material are as follows:

Research presented in Chapter 5:

This research was conducted at the University of Waterloo by Gurmeet Kaur Lall and Scott C Gillis under the supervision of Dr. Shawn D. Wettig. Gurmeet Kaur Lall and Scott C Gillis designed the study with consultation from Dr. Shawn D. Wettig and contributed to conducting the experiments, data analysis, and manuscript drafting/editing. Han-Jin Philip Lu and Yilin Qiu contributed to conducting the experiments.

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As second author of Chapter 5, I was responsible for conceptualizing the study, conducting the experiments, carrying out data collection and analysis, and drafting and editing the manuscripts. The first author contributed equally to the work and was responsible for submitting the manuscript. My coauthors contributed to conducting the experiments and provided feedback on draft manuscripts.

Abstract

The bulk of this thesis work is the result of a pharmaceutical collaboration, and it utilizes several proprietary systems. While Chapters 3 and 4 will provide an in-depth discussion of the results, the specific names, structures, and manufacturing processes of the systems cannot be disclosed.

Genes are segments of DNA – the blueprint of all living organisms which contain instructions needed for organisms to grow, survive, and reproduce. However, genes that do not function properly can cause various diseases and genetic defects. Gene therapy has the potential to treat these diseases by correcting the underlying genetic problem. Recent success with the use of lipid nanoparticles have demonstrated that gene delivery by using synthetic vectors is effective and commercially achievable. However, other synthetic carriers, such as polymers and gemini surfactants (GS), continue to face challenges associated to low gene expression which limits their clinical application.

The rational design approach, where individual vector components are selected based on favourable transfection efficiency properties, is often used in non-viral formulation development. Countless studies have used this approach to focus on designing efficient synthetic carriers. However, highly efficient non-viral gene therapeutics require the rational design of all components of the vector – namely the synthetic carrier **and** the DNA cargo. For example, to address the several challenges associated with the use of conventional circular covalently closed (CCC) plasmid DNA (pDNA) vectors, a new generation of DNA vectors have been under investigation, linear covalently closed (LCC) DNA minivectors. Although preliminary studies have shown that the *in vitro* transfection efficiency of formulations incorporating the LCC DNA minivectors is higher compared to formulations incorporating the CCC DNA counterpart, their overall transfection efficiencies still appear to be limited. Key to improving their efficiency is characterizing both the synthetic carrier and the DNA construct and to establish a relationship between molecular interactions, physicochemical properties, and transfection. This approach may optimize LCC DNA minivector-based formulations for future non-viral gene therapy applications.

In collaboration with a pharmaceutical partner, we designed a study to assess the influence of DNA size and topology on physicochemical properties (i.e. size, polydispersity index (PDI), and zeta potential) and *in vitro* transfection efficiency. Cationic lipids (Lipofectamine 3000 and proprietary Lipid 2) complexed with either the proprietary LCC DNA minivectors or CCC pDNA in deionized water were prepared and evaluated. Comparable physicochemical properties were

observed despite the differences in DNA size and topology. Sizes of all lipoplexes were below 325 nm and the positive zeta potentials appeared to increase linearly with increasing N^+/P^- ratio. Transfection efficiency was evaluated by expression of enhanced green fluorescence protein (eGFP) in HEK 293 and ARPE-19 cells *in vitro*, and cell viability was determined by propidium iodide staining. Statistically significant increases in transfection efficiency were observed with samples prepared with the LCC DNA minivectors and higher overall transfection was associated with Lipofectamine 3000/LCC DNA minivector lipoplexes as determined by one-way analysis of variance (ANOVA) and a dependent, two-tailed, two sample Student's t-test (same results obtained by both methods). These findings reveal that a relationship between DNA size, topology, and transfection efficiency exists and should be considered for effective cationic lipid/LCC DNA minivector lipoplex design and formulation development.

In an effort to improve the transfection efficiencies of LCC DNA minivectors for non-viral gene therapy, the relationship between physicochemical properties and transfection efficiency of polymer-LCC DNA minivectors were studied. Formulations comprising the proprietary Polymer 1 or proprietary Polymer 2 incorporating either LCC DNA minivectors or CCC pDNA in deionized water were prepared at several N^+/P^- ratios (2:1, 4:1, and 8:1) and their physicochemical properties (size, zeta potential, and PDI), transfection efficiency (eGFP expression), and cell viability (determined by propidium iodide staining) were evaluated. Higher transfection was achieved with polyplexes forming small (< 150 nm), uniformly distributed ($PDI < 0.2$) nanoparticles with an excess positive charge (zeta potential ~ 25 mV), presumably due to high cell interactions and internalization. The link to transfection efficiency found from this study may contribute to the rational design and optimization of future LCC DNA minivector-based polyplexes.

To briefly provide insight into the low gene expression of GS-DOPE-LCC DNA minivector systems previously determined by our group, the mixing behaviour and interactions between DOPE and two GS (18-7-18 or 18-7NH-18) were characterized at pH 4 and pH 7 at various ratios using Langmuir monolayer methods. The mixing behaviours were derived from π -A curves by examining the excess free energy of mixing (ΔG^E) which was calculated through the surface area additivity rule, while the intermolecular interactions of the mixtures were evaluated using the compressibility modulus (C_s^{-1}). Synergistic interactions dominated in most binary mixtures, with the exception of $X_{GS} = 0.5$ monolayers where a net repulsive force was dominant. This synergism may explain the low gene expression of GS-DOPE-LCC DNA minivectors, as it may attribute to low LCC DNA release from the vector. These findings may be used for future optimization.

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Last but not least, I would like to acknowledge my mother, Harjeet, and my husband, Sanjay. This work was accomplished due to your unconditional love and support.

Dedication

To my late grandmother, Beji. We did it.

To my mother, Harjeet – for your endless love and support. I am who I am all because of you.

To my husband, Sanjay – for always encouraging and believing in me. Thank you for being my best friend and cheerleader.

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List of Abbreviations

16-3-16	N,N'-bis(dimethylhexadecyl)-1,3-propanediammonium dibromide
18-7-18	1,7-heptanediyl-bis(dimethyl-octadecylammonium) dibromide
18-7NH-18	1,9-bis(octadecyl)-1,1,9,9-tetramethyl-5-imino-1,9-nonanediammonium dibromide
ΔG^E	Gibb's excess free energy of mixing
π -A	surface pressure vs mean molecular area isotherm
π_c	collapse pressure (isotherm parameter)
ANOVA	analysis of variance
A_∞	limiting area (isotherm parameter)
A_L	lift off area (isotherm parameter)
A_c	collapse area (isotherm parameter)
ATP	adenosine triphosphate
C_s^{-1}	compression modulus (derived from π -A isotherms)
CCC	circular covalently closed (supercoiled)
CMC	critical micelle concentration
COVID-19	coronavirus disease 2019
CPP	cell-penetrating peptides
DLinDMA	1,2-dilinoleyloxy-n,n-dimethyl-3-aminopropane
DLS	dynamic light scattering
DMAEMA	dimethylaminoethyl methacrylate
DMRIE	dimyristyloxypropyl-3-dimethylhydroxyethyl ammonium bromide
DNA	deoxyribonucleic acid
DOGS	dioctadecyl amino glycyl spermine
DOPE	1,2-dioleoyl- <i>sn</i> -glycero-3-phosphoethanolamine
DOPC	Dioleoylphosphatidylcholine
DORIE	dioleyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide

DOSPA	dioleoyloxysperminecarboxamidoethyl-dimethylpropaniminium
DOTAP	dioleoyloxy-trimethylammonium propane
DOTMA	<i>N</i> -[1-(2,3-dioleyoxy)propyl]- <i>N,N,N</i> -trimethylammonium chloride
DPSC	Distearoylphosphatidylcholine
ECO	(1-aminoethyl)- <i>N</i> -oleicylcysteinyl-1-amino-ethyl) propionamide
EO	ethylene oxide
FDA	Food and Drug Administration (United States)
GAG	Glycosaminoglycan
GS	gemini surfactant
HA	hyaluronic acid
HMW	high molecular weight
HS	heparan sulfate
LCC	linear covalently closed
LMW	low molecular weight
LNP	lipid nanoparticle
MIDGE	minimal immunological defined gene expression vectors
MiLV	micro-linear vectors
miRNA	micro ribonucleic acid
m-s-m, 2Br ⁻	α,ω -alkanediyl-bis(dimethylalkylammonium bromide
MW	molecular weights
N-VGDS	Non-viral gene delivery system
NLS	nuclear localization sequence
NPC	nuclear pore complexes
PC	Phosphatidylcholine
PEG	polyethylene glycol
PEI	polyethyleneimine
PDI	polydispersity index

pDNA	recombinant plasmid DNA
PLL	poly-L-Lysine
PBAE	poly(β -amino ester)
RES	reticuloendothelial system
RGD	arginine-glycine-aspartic acid
RNA	ribonucleic acid
RNAi	RNA interferences
siRNA	silencing ribonucleic acid
SEM	scanning electron microscopy
TEM	transmission electron microscopy
Tf	transferrin (ligand)

Chapter 1

Introduction and Background

1.1 Gene Therapy Uses Genes as Medicine to Treat or Prevent Illnesses

Gene therapy is a powerful technique designed to introduce foreign therapeutic genes into cells to replace, inactivate, or regulate defective genes which contribute to disease development.¹ As such, gene therapy offers the potential to treat several inherited genetic conditions such as cystic fibrosis² and hemophilia³ as well as acquired genetic disorders including cancer⁴ by repairing the genetic problem at the source—the genetic code. This approach is different from conventional drug-based mechanisms, which may treat the symptoms, but not the underlying genetic problem.⁵ However, the introduction of foreign DNA to cells is incredibly challenging.⁶

Gene delivery techniques consist of vector-assisted delivery or physical delivery methods, which will be discussed in more detail in the following sections.⁷ Vector-assisted delivery requires the design of efficient molecular carriers to transport and release the therapeutic gene to the targeted cell. These vectors must be efficient and have flexibility to deliver several genes with no size constraints.^{7,8} Once the vector is inserted into the patient, it should not only improve normal cellular functions, but also correct potential deficiencies or inhibit deleterious activities without inducing an inflammatory response.⁹ In a successful gene therapy treatment, the introduced gene will potentially treat or prevent the progression of genetic diseases at the site of its localization, and remain viable over the patient's lifetime.⁷ However, of the numerous challenges associated with the process, one of the most significant is the design of an efficient vector to deliver therapeutic genes to host cells. Vectors designed for gene therapy can be classified into viral and non-viral gene delivery systems, both in which have their own advantages and disadvantages.¹⁰

1.1.1 Safety Limitations of Viral Gene Delivery Systems

Viral gene delivery systems use viruses and their mechanisms to efficiently target host cells, insert the DNA into the host genome, and use host cellular machinery to replicate. Before gene therapy applications, genes responsible for viral pathogenesis and replication are typically removed from the viral genome and are replaced with therapeutic genes.¹¹ As of 2022 approximately 67.5% of all gene therapy clinical trials have utilized viral vectors, typically retrovirus and adenovirus, mainly due to their efficiency in delivering genes to numerous cell lines (**Figure 1.1-1**).¹² Interestingly, the FDA recently approved Luxturna™, a gene therapy developed by Spark Therapeutics, to treat patients with loss of vision due to inherited retinal dystrophy, a rare genetic disorder of the retina. When administered subretinally, Luxturna™ uses an adeno-associated virus as a carrier to deliver a normal copy of the RPE65 gene to retinal cells which then enables the production of the missing enzyme called all-trans retinyl isomerase. This helps to improve the function of cells in the retina and slow down the progression of the disease.¹³ However, since Luxturna™ is developed using a viral vector-based platform, there may be potential drawbacks associated with their use. Although viral vectors are genetically modified prior to administration, they still have the potential to trigger powerful immune responses which will not only reduce the efficacy of the therapeutic gene, but may also impose detrimental effects onto the administered host.¹⁰ In addition, insertional mutagenesis, the insertion of viral DNA to any arbitrary location in the genome, is also an ongoing safety concern with the use of viral vectors which leads to serious genetic consequences.¹⁴ For example, two children that were enrolled in a clinical trial to treat X-linked severe combined immunodeficiency developed leukemia-like conditions in 2002. Following this occurrence, the trials restarted with a revised protocol in 2005 and a third child developed a similar condition. This condition was caused by retroviral vector integration near an oncogene promoter.¹⁵ Thus, despite being able to effectively deliver therapeutic genes, viral gene therapy is unattractive due to their associated immunogenicity. In addition, there are numerous problems associated with the assembly of viral vectors including constraints on the maximum size of the therapeutic gene, high cost of production, and short shelf-life which limits their application in gene therapy.¹⁰ To overcome the numerous problems associated with viral gene therapy, non-viral gene delivery was introduced as an alternative method which are non-toxic and non-immunogenic in comparison to their viral counterparts.¹⁶

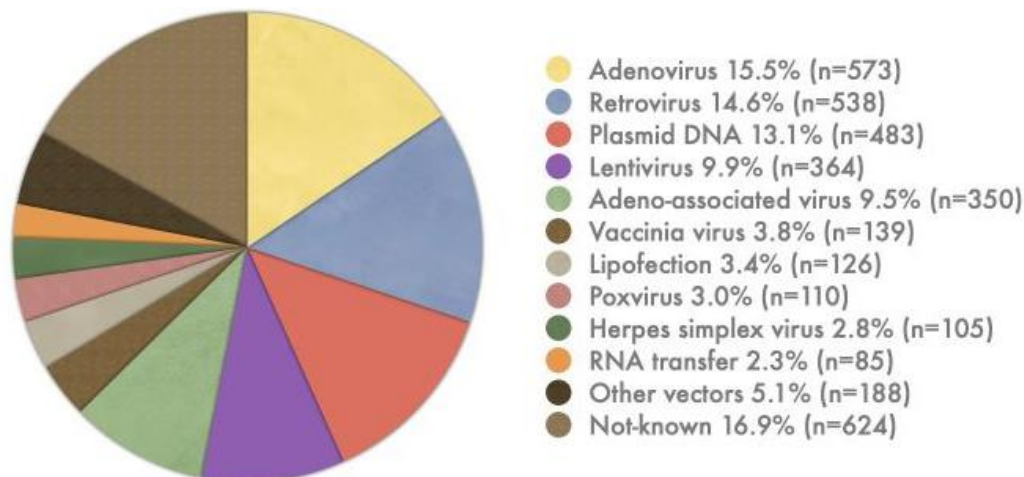


Figure 1.1-1: Viral vectors used in gene therapy clinical trials as of 2022. Adapted from reference¹².

1.2 Non-Viral Gene Delivery is a Safer Alternative to Viral-Based Systems

In view of the cytotoxicity and biocompatibility issues associated with the use of viral vectors, scientific interest to explore non-viral means of gene delivery piqued. Non-viral gene delivery vectors are generally developed from natural or synthetic amphiphilic compounds, such as cationic lipids, polymers, or surfactants, which self-assemble with DNA to form polyelectrolyte complexes called lipoplexes or polyplexes.¹⁶ Typically, an excess amount of the cationic component is required to achieve complete neutralization of the negatively charged phosphate groups of DNA to effectively complex the therapeutic gene.¹⁷ The optimal charge ratio to form this complex is dependent on the chemical structure of the cationic component in the non-viral gene delivery system (N-VGDS).¹⁸ Unfortunately, there is no correlation between charge ratio and transfection efficiency that would apply across all vector systems, though the most commonly used ratios of cationic component to DNA are 2:1, 4:1, and 10:1.^{19,20} At these ratios, the cationic components can readily condense the DNA molecules to form an overall complex possessing a net positive charge. This positive complex charge is essential to facilitate interactions with the negatively charged proteoglycans on the cell surface, which will be discussed later.¹⁸ However, these charge ratios are merely used as a guide to determine the approximate amount of the cationic components required to complex a therapeutic gene. The optimal charge ratio, while typically higher than a N^+/P^- ratio of 1:1, is highly dependent on the chemical structures of the cationic components in the vector system.¹⁹ In some cases, N^+/P^- ratios of 2:1 may be sufficient to achieve a combination of both effective gene complexation and transfection efficiency and safety.²¹ However, other studies

have determined that transfection was optimum when formulations were prepared at a 4:1 or 10:1.²² This is simply because there is more cationic charge available to interact and complex the DNA, while allowing for counterion effects to occur which are associated with some carriers.¹⁸ Consequently, non-viral vector design is significant because a fine balance between all of the requirements of the system must be attained to achieve maximal transfection efficiency.

N-VGDS are attractive because there are no constraints on the maximum size of the therapeutic gene to be delivered.^{20,23} As a result, large fragments of DNA can be introduced to host cells, which would not be able to fit into the capsid of viral vectors. In addition, there is a greater control over the molecular composition of non-viral vectors, allowing for a more simplified production and adaptability for different delivery options (e.g. enhanced circulation times, time-dependent release, targeted delivery, repeat administration).²⁰ Consequently, this approach would enhance the biocompatibility of gene carriers and address the challenges associated with carriers developed from viruses.

Despite these advantages, N-VGDS are inefficient in delivering therapeutic genes to host cells in comparison to viral-based vectors.¹⁸ This inefficiency is correlated to the various barriers encountered between the site of administration to the site of localization in the cell nucleus.^{24–28} The following section will discuss the various physicochemical barriers faced by N-VGDS in systemic delivery.

1.2.1 Physiological Barriers to Non-Viral Gene Delivery

The physiological barriers faced by N-VGDS in systemic delivery have been identified as being both at the extracellular and intracellular level as summarized in **Figure 1.2-1**.²⁴ In general, the N-VGDS must target the appropriate cell type, while protecting the therapeutic gene from extracellular and intracellular degradation, avoid elimination from the body, and traffic the therapeutic gene to the nucleus for transcription.²⁵ The following sections will cover the major barriers in more detail along with typical strategies for addressing them in the context of non-viral vector design.

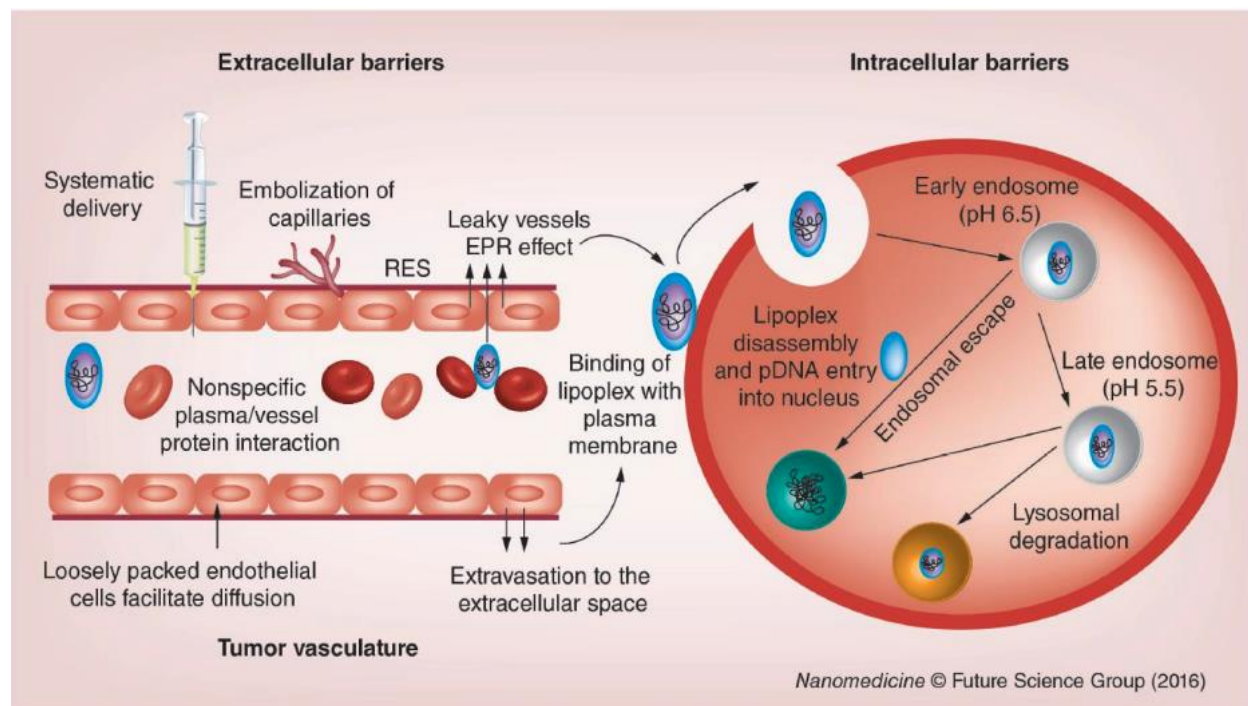


Figure 1.2-1: Overview of some extracellular and intracellular barriers to non-viral gene therapy following systemic delivery. The lipoplexes/polyplexes remain at the early endosome stage for approximately 2-5 mins (pH 6.5), late endosome stage for approximately 10-15 mins (pH 5-6) and in the lysosome for approximately 30-35 mins (pH 4-5). Reproduced from ²⁹ with permission (See Appendix D).

1.2.1.1 Extracellular Barriers

Extracellular barriers to non-viral gene therapy via systemic delivery are obstacles that may be encountered anywhere from the point of delivery to the surface of the cellular target of interest.³⁰ DNA administered intravenously have a very short half-life within serum, approximately within the range of 2 to 21 minutes, as a result of both endo- and exonuclease activity in the plasma.^{24,26} However, an efficient cationic N-VGDS can protect the therapeutic gene against degradation, and improve its circulation time within serum.²⁷ DNA that evade nuclease activity, however, also come into contact with the plasma proteins.³⁰ Negatively charged proteins have the ability to bind to the non-viral vectors to cause aggregation, consequently inhibiting their biological activity and removing the vector from the circulation.²⁶ It is also important to note the interactions between DNA and blood cells in the extracellular environment. The negative surface charge on the blood cells allows for strong electrostatic interactions to form between the cells and the cationic N-VGDS, thereby encouraging removal of the vector from the circulation.³⁰ Strategies to protect

DNA from nucleuses, plasma proteins, and blood cell activity include the incorporation of a cationic helper lipid, such as 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), into N-VGDS formulations, see **Figure 1.2-2**.²⁷ DOPE works to encapsulate the plasmid and shield it from enzymatic activity as well as protein and blood cell interaction in the extracellular environment. As a result, the shielding properties of DOPE will significantly enhance the stability, protection, and half-life of the N-VGDS within serum.³¹

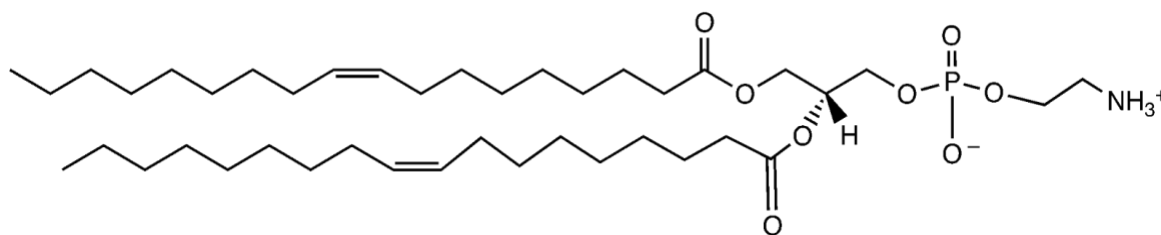


Figure 1.2-2: Chemical structure of the zwitterionic helper lipid DOPE.

Another approach to protect the N-VGDS from degradation is to attach hydrophilic “stealth” molecules, such as polyethylene glycol (PEG), to the surface of the cationic carrier as illustrated in **Figure 1.2-3**.^{32,34} PEG can be incorporated to non-viral formulations to form a dense hydrophilic shell over the molecule to: 1) conserve the nanoparticle core from non-specific hydrophobic interactions with serum proteins in the extracellular environment, 2) enhance nanoparticle stability, and 3) prolong circulation times *in vivo*.³³ Another advantage of PEGylated complexes is that it may increase the hydrodynamic size of the nanoparticles, thus decreasing risks of clearance by the reticuloendothelial system, and degradation by endonucleases in the blood and extracellular space.³² Aside from PEG, high density cell-binding ligands, such as transferrin, can also be used to decorate the surface of the delivery vector in order to mask the positive surface charge of the complex. As a result, these interactions produce stable and near neutral complexes which help to reduce nonspecific interactions with negatively charged serum proteins and enhance cellular uptake.³⁴

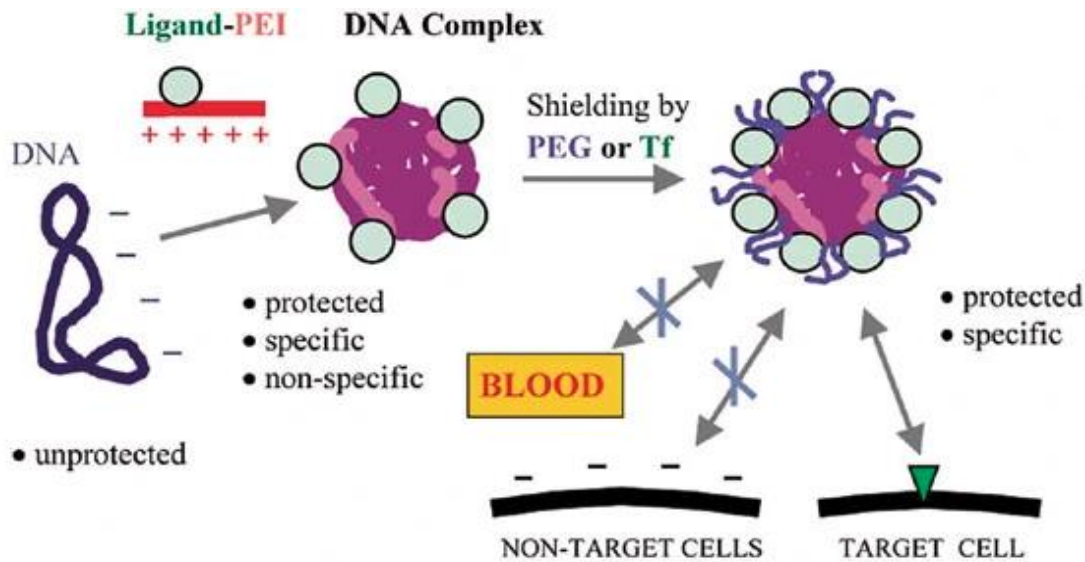


Figure 1.2-3: Protecting DNA transfection complexes from hydrophobic non-specific interactions by incorporating PEG or cell binding ligand, such as transferrin (Tf), at sufficiently high densities. This helps protect the surface charge of transfection complexes and blocks non-specific interactions, thereby shielding the complexes from degradation. Reproduced from ³⁴ with permission from Springer (See Appendix D).

1.2.1.2 Intracellular Barriers

1.2.1.2.1 The Cellular Membrane

Once the N-VGDS has reached the targeted cell, it then encounters a second significant barrier, the cellular membrane.²⁴ Passive diffusion into the cell is not possible due to the size restrictions of transmembrane pores and channels. Therefore, N-VGDS must be internalized into the cell through a process called endocytosis.³⁵

Depending on the design of the N-VGDS, cell internalization of the vector can occur through: non-specific uptake following electrostatic internalization with negatively charged membrane-bound proteoglycans, receptor-ligand binding at the cell surface, lipophilic interactions between lipophilic residues in the delivery vector, and/or cellular membrane phospholipids which will be discussed later in this section.³⁶ N-VGDS, which typically possess a net positive charge, may naturally tend to interact with the negatively charged extracellular matrix components, such as proteoglycans, to enter the cell through endocytosis.³⁵ However, it is important to keep in mind that when designing the transfection complexes, the positive charge of the N-VGDS must be

balanced to prevent permanent attachment to the cell membrane.³⁵ Size of the nanoparticles has also been shown to influence cell internalization. For example, positively charged nanoparticles with a hydrodynamic size under 200 nm have been shown to facilitate cellular internalization.³⁹ Consequently, understanding the relationship between the physiochemical properties (i.e. surface charge and size) of the non-viral components and cell uptake mechanisms are significant as these characteristics may contribute to a more rational design of non-viral formulations.^{38,39}

As previously mentioned, cationic non-viral vectors may interact and internalize into the cell via negatively charged membrane-bound proteoglycans. Proteoglycans consist of a core protein covalently linked to sulfated or carboxylic glycosaminoglycans (GAGs) (e.g. hyaluronic acid, chondroitin sulfate, or heparan sulfate). As such, it may be likely that extracellular GAGs may physically interact with the non-viral vector complex and affect the mobility and the interactions of the nanoparticles with the target cells. For example, non-viral vectors which interact with hyaluronic acid may direct the complexes to a route that is more favourable for transfection, while complexes which interact with heparan sulfate may inhibit transfection (**Figure 1.2-4**).³⁷ Therefore, when considering cell internalization through proteoglycans, it is also important to consider their structure and how nanoparticles can be manipulated to leverage their mechanisms to achieve maximal transfection.

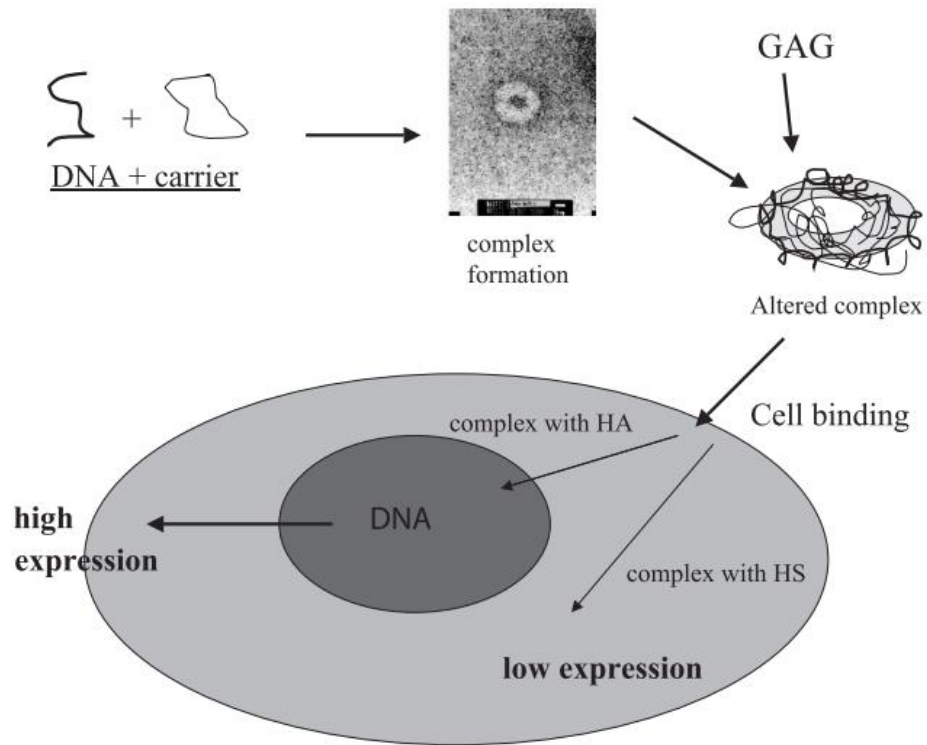


Figure 1.2-4: Effects of GAGs on transfection complexes. Vectors that interact with extracellular GAGs, like hyaluronic acid (HA) and heparan sulfate (HS), may alter the fate of the complexes that are internalized into the cell. HA in the complex may increase transfection while HS inhibits transfection. Reproduced from ⁴⁰ with permission from Elsevier (See Appendix D).

Non-viral gene delivery vectors most commonly utilize the following endocytosis mechanisms to enter the cell: 1) clathrin mediated endocytosis, 2) caveolae-mediated endocytosis, or 3) macropinocytosis as illustrated in **Figure 1.2-5**.⁴¹ These pathways differ with regards to the size of the vesicle, surface charge, and the nature of the cargo being internalized.⁴²

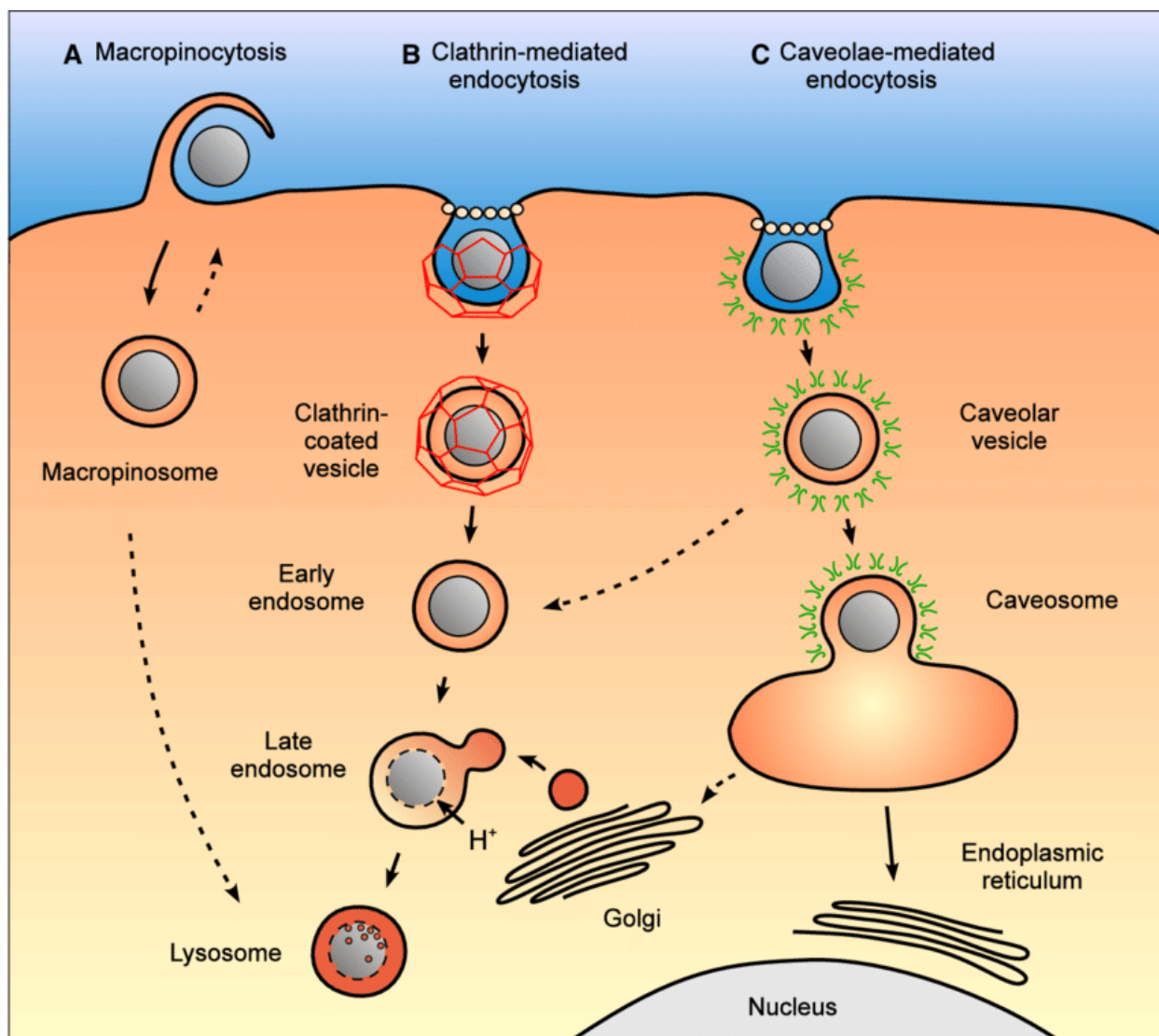


Figure 1.2-5: Intracellular uptake of nanoparticles may occur through three different endocytosis mechanisms: A) Macropinocytosis, B) Clathrin-mediated endocytosis or C) Caveolae-mediated endocytosis. Reproduced from ⁴¹ with permission from Elsevier (See Appendix D).

Clathrin-mediated endocytosis is triggered with the binding of ligands to its high-affinity transmembrane receptors located on the cell membrane.⁴² This ligand-receptor binding triggers the recruitment of clathrin proteins to the cell membrane and leads to the formation of structures known as clathrin-coated pits.^{38,43} These pits then invaginate and are pinched off from the cell membrane, forming intracellular clathrin-coated vesicles that are approximately 150-200 nm in diameter.³⁸ The endocytosed material is then immediately entrapped in an early endosome and are then trafficked into lysosomes which are highly acidic membrane-enclosed organelles comprising approximately 50 degradative enzymes.⁴³ Non-viral vectors must escape from endosomes and be released into the cytoplasm before fusion and degradation by lysosomes.⁴³ Strategies to facilitate endosomal escape will be discussed in the following section.

Caveolae-mediated endocytosis occurs through small hydrophobic domains that are rich in cholesterol and glycosphingolipids. However, this particular route does not follow an acidic or digestive route.^{38,43} As such, the particles that enter into the cell through this route will be transported directly to the Golgi apparatus or endoplasmic reticulum without undergoing degradation.⁴⁴ Due to this attractive feature, caveolae-mediated cell internalization could be advantageous for gene therapy applications since it bypasses lysosomal degradation. However, reportedly, the internalization of the caveolae is typically slower compared to other pathways and their size is more appropriate for vesicles within the range of 50-80 nm.³⁵

Finally, macropinocytosis non-selectively engulfs a large volume of extracellular medium and macromolecules, as well as other chemical substances, into cells by membrane protrusions that collapse and fuse with the plasma membrane.^{35,38} Unlike the mechanisms described above, this process is not directly triggered by receptors or by the cargo, but can be induced by mitogenic factors.^{43,44} The resulting vesicles that enter the cells, known as macropinosomes, are between 0.5 to 10 μm in size.³⁷ Once the macropinosomes enter the cell, it is currently unclear which pathways they may undertake. However, previous studies have shown that the fate of the macropinosomes is cell type dependent.³⁵ For example, in certain cell types, the vesicles may fuse with the lysosome and degrade, while in others, they may remain in the early endosome stage.^{35,42}

Ultimately, the fate of the DNA cargo depends on two factors: 1) the size of the N-VGDS and 2) the endocytosis pathway.⁴⁴ It is also important to keep in mind that although non-viral vectors are developed to possess an overall positive surface charge, after intravenous administration, the vector charge is expected to neutralize following interactions with serum

proteins.⁴⁵ As a result, there may not be sufficient electrostatic interactions which exist between the N-VGDS and the cell membrane for efficient cell internalization.^{45,46} Strategies that can be used to improve cellular uptake is by coupling targeting molecules to gene delivery vectors which bind strongly to cell receptors on cell surfaces to enhance internalization.³⁶ These ligand binding surface markers are cell specific and trigger clathrin-mediated endocytosis. This process generally results in faster cell internalization compared to non-specific uptake (without a ligand) and accumulation of the DNA cargo into the cell, leading to improved cell internalization and transfection efficiency.³⁶ Additionally, it has previously been shown that incorporating ligands into non-viral formulations has decreased non-specific delivery to healthy cells which by extension has decreased the overall cell toxicity, and enhanced the safety profile of the therapeutic for *in vivo* applications.⁴⁷ Although outside the scope of this work, some examples of commonly used ligands in non-viral formulations include: 1) transferrin, which are recognized by transferrin receptors and are generally upregulated in many cancer cells, 2) arginine-glycine-aspartic acid (RGD) peptides which are recognized by tumor vasculature integrins, 3) lactoses which are recognized by hepatocytes, and 4) folate, recognized by certain tumor cells.^{47,48}

Ultimately, to improve selective cell attachment and enhance cellular internalization, it is first essential to understand the nature of the cell surface and then manipulate the vector components accordingly. Surface modifications with targeted ligands and shielding of cationic charges of the vector may provide better efficiency and lower toxicity. For example, the incorporation of peptide-based ligands, such as CAG and REDV, have shown to specifically bind vector complexes to receptors present on the cell surface.⁴⁹ In addition, the surface of gene therapy nanocarriers can be decorated with several short sequences, such as cell-penetrating peptides (CPP), to facilitate and accelerate cell entry. Proteins such as TAT and polyarginine peptides can be used and depending on the CPP, different internalization mechanisms may be used to access the cytoplasm including direct transduction through the lipid bilayer as well as energy-dependently macropinocytosis.⁵⁰ These CPPs have been shown to be particularly efficient in the delivery of small DNAs and synthetic RNAs.⁵⁰ Moreover, physical methods of gene delivery, such as the gene gun, electroporation (electric fields), sonoportation (ultrasound), microinjection and hydrodynamic (high pressure) delivery in combination with N-VGDS may also facilitate the delivery of vectors across the cell membrane.⁵¹

1.2.1.2.2 Endosomal Escape

Non-viral vectors which utilize endocytosis to cross the cell membrane and access the cytoplasm are then moved through the endocytic compartments. The endocytosed material is immediately entrapped in membrane bound endocytic vesicles, which then fuse with early endosomes and become highly acidic vesicles as they mature and develop into late endosomes.²⁶

Figure 1.2-6 illustrates the pH transitions of the endosomal compartments from an early endosome at pH 6.5 to a late endosome at pH 5.5.²⁷ Endosomal acidification occurs through the actions of ATPase proton pumps located on the endosomal membrane, which allows for the transport of protons from the cytosol into the endosome.²⁵ Under these highly acidic conditions, lysosomes, the main degradative compartment of the cell, will trigger complete endosomal degradation.²⁷ Therefore, another significant barrier to successful gene delivery is the release of DNA from the acidic endosomal compartments at an early stage to prevent lysosomal degradation.²² Strategies to facilitate endosomal escape include the incorporation of the zwitterionic helper lipid, DOPE, in N-VGDS formulations.²² Under acidic conditions, the morphology of DOPE transitions from a bilayer structure to an inverted hexagonal structure which is able to fuse to, and consequently destabilize the endosomal membrane, and release the DNA into the cytosol.²⁸

More significantly, endosomal escape, and consequently transfection levels, have been found to be dependent on the nature of the cationic component of the N-VGDS.^{52,53} In particular, cationic components containing pH-sensitive functionalities, specifically nitrogen groups capable of being protonated, tend to resist the acidification of endosomes due to their high buffering capacity.²⁹ Endosomal escape with these cationic components have been found to increase transfection through disruption of the endosomal membrane through the “proton sponge” mechanism as depicted in **Figure 1.2-6**.²⁴ As the endosomal compartment is acidified, the protonable nitrogen groups become protonated, which causes a greater influx of protons to be continuously pumped into the endosomes in the attempt to lower the pH of the endosomal compartment. Subsequently, the accumulation of protons is charge balanced by an influx of chloride ions, which increases the ionic concentration of the endosomal compartment and leads to an influx of water. The increased number of protons and chloride ions lead to osmotic pressure build up which causes osmotic swelling and rupture of the endosome, subsequently releasing the

N-VGDS into the cytosol before the endosomal compartment becomes too acidic.^{24,55} As a result, N-VGDS with nitrogen groups increase the amount of time before passage of DNA to lysosomes, which increases the likelihood of the DNA being released into the cytoplasm, ultimately mediating levels of transfection. Depending on the non-viral vector composition, the DNA may dissociate from the vector at the endosomal-stage or within the cytoplasm following interaction or fusion with the cytosolic membrane network.^{29,55}

Polyethyleneimine (PEI) including the commercially available jetPEI, is a prime example of a gene delivery polymer that can effectively condense DNA into nanosized particles and help in the endosomal escape of DNA through the proton sponge effect.⁵⁴ These gene carriers have shown a wide range of buffering capacities due to the structural presence of three different types of amine groups: primary, secondary, and tertiary.⁵⁴ Examples of other polymers capable of this effect are ammonium chloride, chloroquine, methylamine, poly-L-histidine, polyamidoamines, and polypropylacrylic acid.^{45,48}

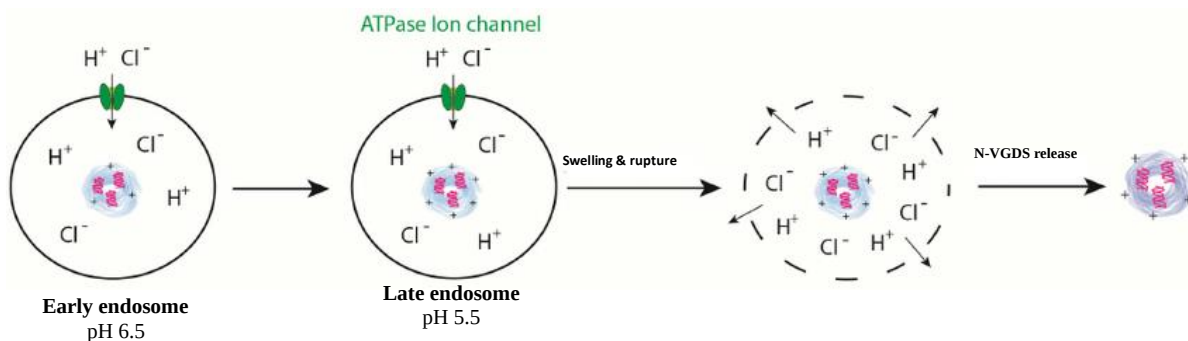


Figure 1.2-6: Schematic representation of the endosomal escape process of pH-sensitive cationic component N-VGDS through the proton sponge mechanism. Adapted from reference ⁵⁵.

1.2.1.2.3 Cytoplasmic Trafficking and Nuclear Import

If the DNA has successfully been transferred to the cytoplasm, it must then traffic to the nucleus in order for gene expression to initiate.³⁰ The cytoplasm itself poses a major barrier to gene transfer. First, the cytoplasm contains a complex network of microtubules along a dense medium of proteins and nucleases which may degrade free DNA by hydrolyzing the phosphodiester bonds that joins the surface residues.³⁰ Additionally, the viscous cytoplasmic environment forms a diffusional barrier for DNA, which significantly decreases the mobility of the macromolecules. Passive transport⁵⁶ and active transport⁵⁷ are the two pathways by which plasmids can reach the nucleus and the movement of molecules through the cytoplasm is size-dependent.^{56,57} For example, when DNA fragments have more than 2000 base pairs, they are completely immobilized in the cytoplasm.⁵⁷ However, several successful transfection studies have revealed that DNA utilizes the microtubule network found throughout the cytoplasm, to facilitate their movement towards the nuclear envelope.³⁰

Once the DNA has successfully reached the nuclear envelope, it must then transport across the nuclear membrane, and enter the nucleus for expression. The nucleus is composed of a double membrane and nuclear pores that regulate passage across the nuclear membrane.³⁰ The size threshold for molecules to freely pass through the nuclear pore complex is approximately < 9 nm in diameter.⁵⁰ Since plasmid DNA, typically used for transfection, is too large to freely pass through the nuclear pores, it may enter the nuclear envelope by one of two mechanisms. During mitosis, the nuclear membrane is temporarily ruptured, which allows the therapeutic gene to diffuse into the nuclear region.⁵⁰ Consequently, *in vitro* studies have shown that gene expression is more efficient in dividing cells compared to non-dividing cell types.⁵⁷ Alternatively, the DNA can be imported through nuclear pore complexes (NPCs) by an ATP dependent pathway.⁵⁸ The NPCs are aqueous channels in the nuclear envelope where only specific molecules are selectively transported in one direction, i.e. cytoplasm to nucleus, or nucleus to cytoplasm.⁵⁷ A strategy to improve overall nuclear import involves coupling nuclear localization sequence (NLS) peptides to the DNA. For example, it has been shown that coupling an SV40-derived NLS-containing peptide to the DNA construct has resulted in an eight-fold increase transfection efficiency compared to the uncoupled DNA construct.⁴⁶ Ultimately, successful DNA nuclear localization will initiate gene expression, and consequently mediate levels of transfection.³⁵

A non-viral gene delivery system which is designed efficiently will be able to overcome all the barriers discussed above. As such, it is important to understand the relationship between vector structure and transfection as it plays a key role in further optimizing non-viral transfection formulations. However, during formulation design and development, a specific vector component can be added to the sample to overcome one barrier, while that same component can also hinder the ability to overcome another barrier.^{16,57} For example, as previously discussed, typically an excess of the cationic component is required to effectively complex the DNA cargo and to obtain an overall positive charge on the complex to facilitate interactions with the cell membrane. However, an excess positive charge may also lead to serum and cell toxicity which may compromise the safety profile of the vector, making it unfavourable for use in clinical applications. Therefore, a fine balance between all the requirements of the system must be struck to achieve maximal transfection efficiency.

1.3 Non-Viral Gene Delivery Systems

Non-viral gene delivery systems can be divided into three major categories: 1) naked DNA vectors, 2) physical delivery systems, and 3) chemical delivery via synthetic vectors.⁵⁹ Each gene delivery system will be discussed in the following sections.

1.3.1 Naked DNA and Physical Delivery Systems

Low gene expression has been achieved with the direct injection of naked DNA vectors into cells mainly due to the presence of repulsive forces acting between the negatively charged cell membrane and the negatively charged DNA. These forces reduce the close interaction required for efficient cellular uptake.⁶⁰ However, improvements have been made to enhance the efficiency to deliver naked DNA into the cytoplasm. This includes pairing naked DNA with physical methods of delivery, as shown in **Figure 1.3-1**, to temporarily disrupt the structural integrity of the cell membrane and allow the DNA to bypass this barrier.⁶¹ However, even if the unprotected DNA vector is successfully delivered to the cytosol, it will then be subject to intracellular nuclease degradation, which further limits the expression of the DNA vector.⁵⁹ As such, since naked therapeutic genes and physical delivery systems are generally inefficient at delivering their payloads, scientists started to think about complexing the DNA with synthetic gene carriers to protect and deliver the therapeutic gene to the cells of interest.¹⁷ Chemical delivery systems using synthetic vectors will be discussed further in the following sections.

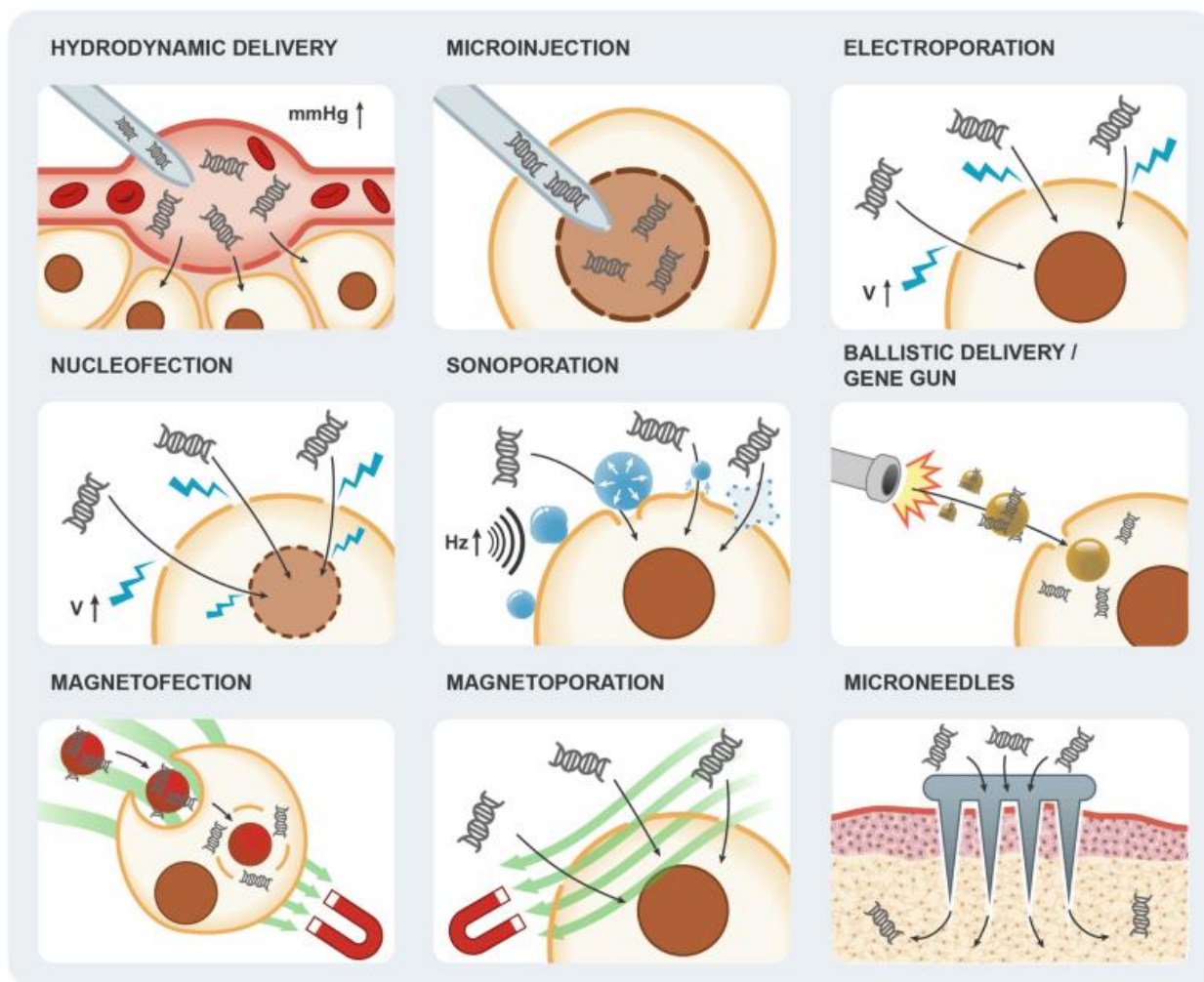


Figure 1.3-1: Physical delivery systems used for gene therapy applications. Various physical methods can be used to enhance transfection efficiency, including: (1) hydrodynamic delivery, which uses hydrostatic pressure enhancement to inject DNA into the circulation, (2) microinjection which directly transfers DNA material into the cell interior, (3) electroporation and (4) nucleofection, both utilize electric pulses which temporarily disrupts the cell membrane and/or the nuclear envelope to deliver the DNA into the cell, (5) sonoporation, utilizes ultrasounds to form cell membrane pores to facilitate DNA delivery, (6) ballistic delivery/gene gun is a needle free method involving the discharge of accelerated DNA-coated gold or tungsten particles of various sizes directly into cell cytoplasm, (7) magnetofection and (8) magnetoporation are both methods based on application of magnetic field which guide the magnetic agent-containing vector to the target cells or promote cell pore formation, and (9) microneedles which are used to deliver DNA across the skin. Reproduced from ⁶¹ with permission from Springer (See Appendix D).

1.3.2 Chemical Delivery Systems Utilizing Synthetic Vectors

Since the 1970s, substantial improvements in the efficiency of synthetic vectors have been made with the application of nanotechnology in the design of these vectors. For example, inorganic materials such as silica, quantum dots, iron oxide, carbon, or gold have been conjugated to the therapeutic nucleic acids or the cationic vectors to aid in DNA complexation.⁶² In 2018 the FDA approved Onpattro (Alnylam Pharmaceuticals, Cambridge, USA), a formulation which uses a lipid-based nanoparticle to deliver therapeutic small interfering RNA to the liver to treat peripheral nerve disease caused by hereditary amyloidosis.⁶³ More recently, lipid nanoparticles (LNPs) were also used to successfully deliver mRNA to cells with the Pfizer-BioNTech and Moderna vaccines now in clinical use against coronavirus disease 2019 (COVID-19).⁶⁴ These exciting breakthroughs validate the use of non-viral gene therapy methods to treat or prevent diseases.

To design efficient non-viral gene carriers, it is first important to understand the relationships between formulation development and transfection efficiency. The preparation of synthetic vector complexes is generally dictated by the N^+/P^- ratio, the ratio of the number of cationic to the number of anionic charges.⁴⁸ As previously described (in section 1.2), an excess of the cationic component is often required to achieve complete neutralization of the negatively charged phosphate group of DNA, so a minimum N^+/P^- charge ratio of at least 1:1 is required.¹⁷ Once in contact with DNA, the cationic component(s) interact with the negatively charged phosphate group of DNA through electrostatic or hydrophobic interactions, resulting in the formation of lipoplexes and polyplexes with an overall positive charge on the complex.¹⁹ The net positive charge on these complexes is essential for: 1) neutralizing the negative charge of the DNA phosphate backbone, 2) protecting the DNA cargo from both extracellular and intracellular degradation, 3) facilitating interactions with proteoglycans, negatively charged proteins, on the cell membrane surface via electrostatic attraction and cellular uptake, and 4) condensing the size of the DNA cargo to enable cellular internalization.¹⁶ The next section will discuss the most common vectors utilized for non-viral gene delivery applications.

1.3.2.1 Cationic Amphiphilic Molecules as Non-Viral Transfection Vehicles

Cationic amphiphilic molecules, such as surfactants and lipids, consist of a hydrophilic head group and a hydrophobic tail group. An important property of the amphiphile, with regard to its application as a vector, is its geometry.⁶⁵ When suspended in aqueous solutions, amphiphiles self-assemble and form various aggregate morphologies due to the hydrophobic effect.⁶⁶ To characterize this behaviour, a fundamental property known as the critical micelle concentration (CMC), the concentration at which amphiphiles begin to assemble into aggregates in solution, is defined for many amphiphilic molecules used in gene therapy.⁶⁷ Although the micelle aggregates, or the small amphiphilic clusters, are typically depicted as spherical, the micellar structure depends on the relative shape and space occupied by the head group and hydrophobic groups (**Figure 1.3-2**).⁶⁸ The expected shape of the clusters or aggregates can be predicted by using the critical packing parameter (P), defined as:

$$P = \frac{v}{a_0 l_c} \quad \text{Equation 1.3-1}$$

where v and l_c are the volume and length of the hydrophobic tail groups, and a_0 is the equilibrium area per molecule of the hydrophilic head group at the aggregate surface.⁶⁸ **Table 1.3-1** outlines the expected value of the packing parameter as well as the expected shape of the aggregate based on the structure of the amphiphile in question.⁶⁵ The packing parameter reveals that the shape of the surfactant or lipid aggregate is highly dependent on their structure. Even slight changes to the structure of the amphiphile can significantly influence the morphology of the aggregates. For example, lipids which contain one alkyl chain and a polar headgroup tend to form micelles.⁶⁵ Conventional spherical micelles are formed when the lipids have a relatively large headgroup area and small hydrophobic volume which exhibits a cone-like shape. Alternatively, phosphatidylethanolamine lipids, like DOPE, that have a smaller head group area in comparison to the lipid tail prefer to aggregate in an inverted (or reversed) hexagonal structure.⁶⁶

The following section will discuss the important features of surfactant and lipid design and how variations to their structures may impact the critical packing parameter and transfection efficiency.

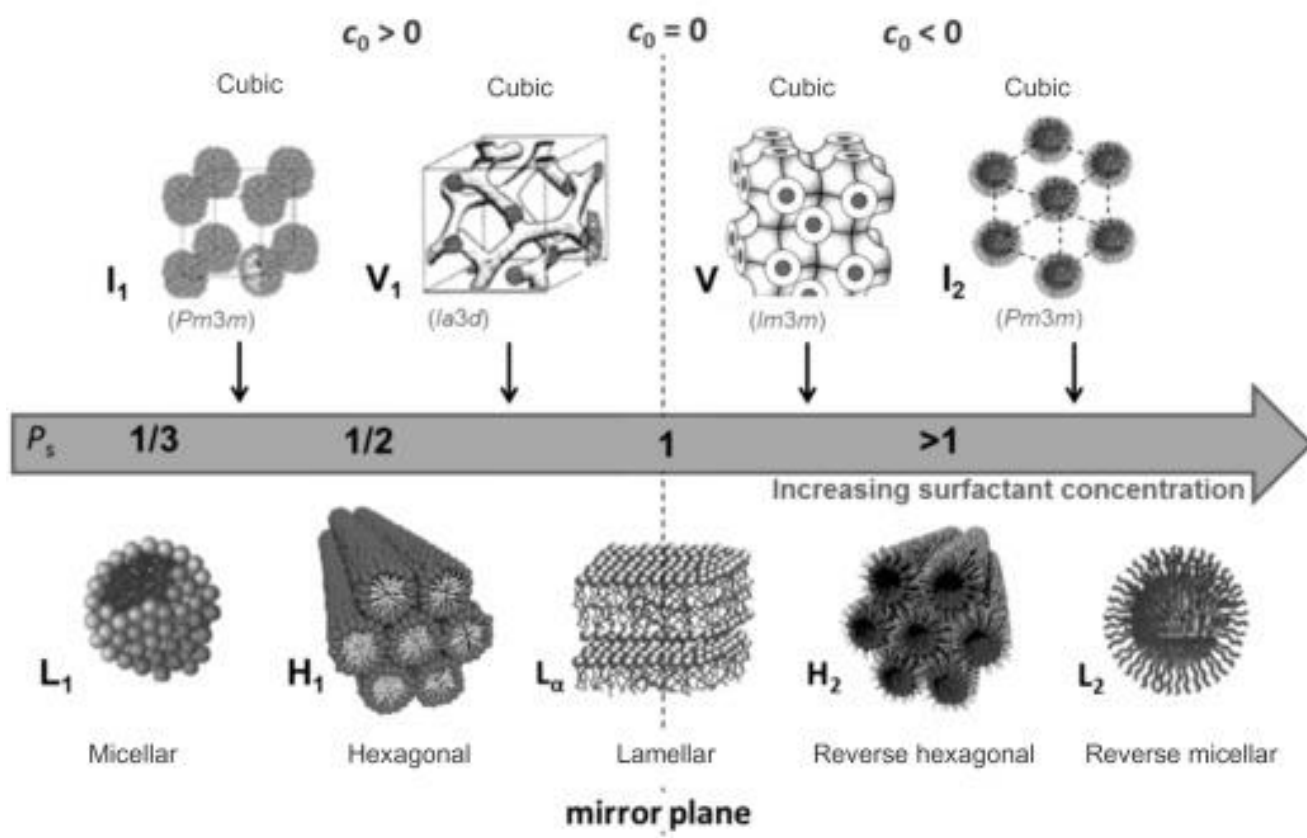
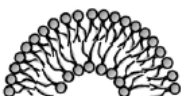
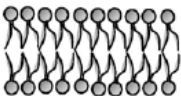


Figure 1.3-2: Idealized sequence of surfactant aggregate structures as a function of surfactant concentration, critical packing parameter (P_s) and preferred surfactant membrane curvature (c_0). Reproduced from ⁶⁷ with permission from Elsevier (See Appendix D).

Table 1.3-1: Expected surfactant geometry and aggregate shape in aqueous media based on the packing parameter ⁶⁷.

Packing Parameter (P)	Surfactant Geometry	Expected Aggregate Shape	Molecular shape	Micellar structure
< 0.33	One chain and relatively large headgroup	Spherical micelles		
$0.33 - 0.50$	Relatively small headgroup, or ionic surfactant in presence of excess electrolyte	Cylindrical micelles		
$0.50 - 1.0$	Two chains (flexible) with large headgroup	Vesicles and flexible bilayer		
1.0	Two chains with small headgroup or immobile chains	Planar bilayer structures		
> 1.0	Two chains with small headgroup, bulky hydrophobic groups	Inverted micelles		

1.3.2.1.1 Gemini Surfactants as Gene Delivery Vehicles

Surface active materials, also known as surfactants, are one group of amphiphiles that are commonly found in most pharmaceutical products where they function in many different roles, including: solubilizers, stabilizers, lubricants, and wetting agents.⁶⁵ For non-viral gene therapy applications, positively charged surfactants are used to complex DNA into nanoparticles. Conventional surfactant molecules are composed of one hydrophobic tail group and one hydrophilic head group, while gemini surfactants (GS) are composed of two conventional surfactant molecules whose head groups are covalently linked by a spacer group as illustrated in **Figure 1.3-3**.⁶⁶ The structure of GS is of particular interest as it has been found to significantly influence the behaviour of these amphiphilic compounds in solution. As such, extensive studies have been conducted by manipulating the tail, head, and spacer regions of the GS structure, and studying their behaviour and interactions with DNA and other components in order to mediate higher levels of transfection.⁶⁵ Since there are few limitations to how the GS structure can be varied, countless related compounds have been synthesized and characterized as components of N-VGDS.

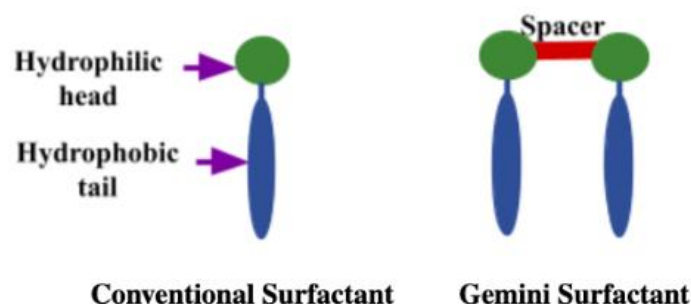


Figure 1.3-3: General structures of conventional surfactants and gemini surfactant.

GS have unique structural, solution properties, and aggregate morphologies in comparison to conventional surfactants which makes them attractive carriers for gene delivery applications.⁶⁷ The most widely studied family of GS are the symmetrical α,ω -alkanediyl-bis(dimethylalkylammonium bromide) surfactant series as shown in **Figure 1.3-4**, denoted as m-s-m, 2Br⁻ GS, where m is the number of carbon atoms in the alkyl tail groups and s is the number of carbon atoms in the alkyl spacer.^{67,69} The spacer group can vary in length and chemical structure, be rigid or flexible, and be hydrophobic or hydrophilic.⁶⁹

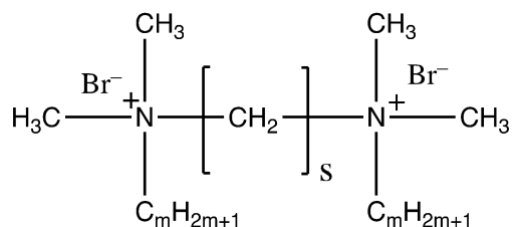


Figure 1.3-4: Chemical structure of m-s-m, 2Br⁻ GS.

As previously discussed, an excess of the cationic component, in this case cationic GS, is required to achieve complete charge neutralization of DNA.¹⁸ For this to occur, GS form aggregates to effectively complex the large therapeutic genes. Atomic force microscopy studies have shown that GS compacts the large loop structure of naked plasmid DNA within 30 seconds of formulation, and more compact nanoparticles are formed within 15 minutes.⁷⁰

Extensive studies have been done on analyzing the physicochemical properties and behaviours of these quaternary ammonium GS as components of N-VGDS. It has been determined that variations in the chemical structure of the surfactant tail group, head group, and spacer group can all have an effect on its interaction properties with DNA, and ultimately transfection efficiency.^{38,71-74} For example, within a given series of conventional surfactants (i.e. their head groups are identical), the critical packing parameter increases as the length of the hydrocarbon tail group increases resulting in the formation of aggregates of low curvature structures, such as extended bilayers or inverse micelles.⁶⁷ The same trend is observed in GS, but the effect of the nature of the spacer group on aggregate shape is far less significant.⁷⁵ GS that form vesicle structures in aqueous solutions are expected to mediate high transfection levels, versus those with micelle structures, due to a higher tendency to directly interact, fuse to, and consequently disrupt, endosomal membranes.^{75,76} Although non-lamellar structures are typically not favourable for binding DNA, they are favourable for releasing DNA once the lipoplex is within the endosome which, as discussed in section 1.2.1.2.2 above, is one of the critical steps of vector-assisted DNA delivery.⁷⁷ Moreover, as previously mentioned, a neutral helper lipid such as DOPE is often added to gemini surfactant-based transfection formulations to enhance transfection efficiency. DOPE drives the formation of mixed aggregates structures, shifting micelle systems towards vesicles or inverted hexagonal structures and vesicle systems toward the inverted hexagonal or a cubic phase.⁷⁸ These morphological changes then aid in destabilization of the endosomal membrane,

consequently, releases the DNA into the cytosol.^{79,79} However, for some GS-based formulations, DOPE may not be required to improve transfection efficiency. For example, short GS tail groups such as the 12-s-12 series do not show transfection without the addition of DOPE, but longer alkyl tails such as those of 16-3-16 or the oleyl 18:1-3-18:1 can transfect in the presence or absence of DOPE.⁷⁷ The requirement of DOPE to improve transfection efficiency is highly dependent on the structure of the surfactant molecule.⁷⁷⁻⁷⁹

To mediate non-viral transfection, it is important to consider the intricate relationship between GS structure and their associated behaviours in solution. For example, the CMC of m-s-m 2Br⁻ GS is generally two or more orders of magnitude lower than that of analogous conventional surfactants of equivalent tail length.⁶² This is because GS are more hydrophobic in nature as they are composed of not one but two hydrophobic tail groups. For example, the CMC of dodecyl trimethyl ammonium bromide, the conventional surfactant analogue of 12-s-12, 2Br⁻ GS is 1.6×10^{-2} M while the CMC of 12-3-12, 2Br⁻ is only 9.1×10^{-4} M and the CMC of 12-16-12, 2Br⁻ is even smaller with a value of 1.2×10^{-4} M.⁸⁰ Similarly, as the length of the GS alkyl tail group increases, the CMC decreases due to increasing hydrophobicity. For example, the CMC of cetyl trimethyl ammonium bromide (CTAB) is 1.0×10^{-3} M and the CMC of the 16-3-16, 2Br⁻ GS is 2.55×10^{-5} M, respectively.⁸⁰ As a result, GS aggregation is favourable at much lower concentrations compared to conventional surfactants, and this is advantageous for the purposes of gene therapy because it means that less material needs to be used to complex the therapeutic gene, ultimately meaning that less foreign material is introduced into patient cells.⁷⁶ Furthermore, it has been determined that the two-tailed GS are more efficient in compacting DNA compared to single-tailed conventional surfactants.⁸¹ This is observed because GS are more hydrophobic compared to conventional surfactants, allowing for the molecules to bind tightly to the DNA in the aggregated form, ultimately increasing DNA compaction efficiency.⁸⁰

In contrast, the effect of the length of the polymethylene spacer group of m-s-m 2Br⁻ GS on CMC is much more complex. For example, the CMC of the 12-s-12, 2Br⁻ GS series increases as s increases from 3 to 4, but continuously decreases as the length of the spacer group increases from 4 to 16 methylene units.⁸¹ However, it has been determined that high efficiency in DNA compaction is achieved when the length of the spacer group is short ($s < 4$) or long ($s > 10$), while spacers of intermediate lengths ($s = 6$) appear to be the least efficient in compacting DNA due to the competition between entropy loss and enthalpy gain.⁸¹⁻⁸²

The complexity of GS structure can be easily increased through the introduction of substitutions to the spacer group. Substitutions are made in a symmetric fashion to spacer groups of odd number of methylene units, e.g. 3, 5 or 7, such that an equal number of methylene units separate the substitution from each of the quaternary ammonium head groups.⁸³ For example, Wettig et al. studied a series of (oligooxa)-alkanediyl- α,ω -bis(dimethyldodecylammonium bromide) GS denoted as 12-EO_x-12, where X = 0 – 3, and compared the CMC value of 12-EO₁-12, 2Br[−] to that of 12-4-12, 2Br[−] and 12-6-12, 2Br[−] GS. Interestingly, the CMC for the 12-EO₁-12, 2Br[−] GS was higher than those obtained for both 12-4-12, 2Br[−] and 12-6-12, 2Br[−] GS. This is due to the increased hydrophilic character of the ethylene oxide (EO) spacer group which correlates to an increase in solubility of the 12-EO₂-12, 2Br[−] GS monomers, relative to the 12-s-12, 2Br[−] GS.⁸⁴ It is important to note that the exact position of the substitution is not often specified in the literature since the central position for the substitution is generally assumed.⁸⁵ For example, hydrophilic aza- (NCH₃) and imino- (NH) substituted spacer groups are typically abbreviated as m-7X-m, 2Br[−], where X = NH or NCH₃.⁸⁵ Consequently, the unique structure of GS offers several advantages like CMC control which is important when designing efficient transfection vectors. For example, a high CMC is important in order to have a high monomer concentration to form DNA complexes with an overall net positive charge. However, a low CMC can enhance micelle stability and keep the delivery complex together during the gene delivery process.⁷⁰ Since the CMC of gemini surfactants is more sensitive to changes in alkyl tail length than variations in the spacer size, their properties can be fine-tuned by manipulating the spacer, with minor effect on the CMC.^{70,85}

Polyamines are pH-sensitive groups which, in the presence of DNA, self-assemble and effectively bind and condense DNA.⁸⁶ As a result, despite having no effect on CMC, quaternary ammonium GS with aza- and imino- substituted spacer groups are of interest in non-viral gene delivery because of the polar region of polyamines.⁸⁶ Wettig et al. studied these interactions, and revealed that the 12-8N-12 polyamine, which closely resembled the spermine structure, most effectively binds and condenses DNA because the separation between nitrogen centers separated by three methylene units, 4.9 Å, is the optimal distance for interactions with the phosphate groups of DNA which are spaced by distances of 6.5 Å–7.1 Å.⁷² As a result, N-VGDS composed of GS whose polar region is polyamine-like are expected to mediate higher levels of transfection than unsubstituted m-s-m, 2Br[−] GS N-VGDS.⁸⁶ From this, it would then be expected that the GS series

like the m-7NH-m, 2Br⁻ and the m-7N-m, 2Br⁻ would result in higher transfection. This prediction is supported by a study which composed N-VGDS of symmetrically substituted 12-7NH-12, 2Br⁻ and 12-7N-12, 2Br⁻ GS, and compared it to those composed of unsubstituted 12-3-12, 2Br⁻ GS.⁸⁷ The results revealed that the imine- and aza- substituted spacer groups showed a 9-fold increase in *in vitro* transfection efficiency resulting in levels comparable to the Lipofectamine Plus commercial control, with 12-7NH-12, 2Br⁻ being the most effective for two reasons. Firstly, DNA interaction is limited by the steric hindrance of the aza group and secondly, the imino group is much more pH-sensitive which may result in increased endosomal escape through the proton sponge mechanism.⁸⁷ Evidently, the nature of the spacer group, and its effects on CMC, DNA compaction, and transfection is far less predictable.

To further understand the GS structure-transfection activity relationship and achieve more favourable effects both *in vitro* and *in vivo*, several modifications to the simple structure of m-s-m, 2Br⁻ GS have been applied and studied. For example, while quaternary ammonium head groups have been most commonly studied, m-s-m 2Br⁻ type GS with imidazolium⁸⁸ and pyridinium⁸⁹ head groups have also been widely explored. In addition, the nature of the linker group connecting the spacer to the charged nitrogen head groups have been studied by comparing GS with amine, amide, ester and carbamate groups.⁵⁰ Although outside the scope of this work, some studies have also designed dissymmetric GS which are composed of two different tail groups abbreviated as m-s-n, 2Br⁻ GS, where m≠n.⁹⁰

1.3.2.1.2 Cationic Lipids as Gene Delivery Vehicles

Lipid-based vectors are one of the most commonly used strategies to introduce exogenous genetic material into host cells due to their ease of use, commercial availability, and low cost.⁸ Similar to GS, lipids are amphiphilic in nature. However, what differentiates lipids from GS is their structure. Lipids have a single head group bound to two tails, while GS have two head groups each of which are bound to a single tail and the two head groups are covalently linked to each other.²⁸ Cationic lipids form lipoplexes by binding to DNA through electrostatic and hydrophobic interactions which then self-assemble into various aggregate structures, such as vesicles and bilayers, to protect the therapeutic gene on their journey to the target site.⁸

Lipids are biocompatible in nature which accounts for their most important advantages as gene carriers, including: 1) low toxicity and antigenicity; 2) their ability to degrade and be metabolized *in vivo*, and 3) control of membrane permeability.^{26,28} Cationic lipids have been examined in safety studies through different routes of administration such as intratumoral⁹¹ intrapulmonary⁹² intracerebral⁹³ and intravenous⁹⁴ with little or no toxicity reported in any of these studies. However, there are several limitations associated with cationic lipids employed in N-VGDS, including their low transfection efficiency, poor stability, as well as their tendency to induce inflammatory responses in the body.⁴² Another limitation with the use of lipids as carriers is that high amounts of cationic lipids are required for high encapsulation efficiency. As a result, this can often lead to an increase in cationic surface charge and which can subsequently lead to serum instability or toxicity.²⁶ Through understanding the structural requirements for successful transfection efficiency, numerous improvements continue to be made to these and other lipids used for the formulation of N-VGDS.

The first successful *in vitro* transfection using a cationic lipid formulation was achieved in 1987 by Felgner and co-workers using the quaternary ammonium cationic lipid, *N*-[1-(2,3-dioleoyoxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA).⁹⁵ The helper lipid, DOPE was incorporated into this formulation to facilitate fusion with the cell. This study revealed that DOTMA/DOPE formulations mediated levels of transfection due to: 1) their ability to form electrostatic interactions between the positively charged DOTMA liposome and negatively charged DNA molecules which results in an efficient condensation of the nucleic acid, 2) spontaneous formation of small, uniform lipoplexes with a net positive charge which promotes the

interaction with negatively charged biological membranes, 3) the fusogenic properties of the lipoplex which induced fusion and/or destabilization of targeted cell membrane, thus facilitating endosomal escape and intracellular release of complexed DNA into the cytosol.⁹⁵ This formulation has since been commercialized as LipofectinTM.

Since then, numerous other cationic lipids, such as dioleoyloxy-trimethylammonium propane (DOTAP)⁹⁶ and dioctadecyl amino glyceryl spermine (DOGS)⁹⁷ have been designed and investigated for application in non-viral gene therapy. These include lipids with various types of head groups (tertiary amine-based, quaternary amine based, univalent and multivalent cationic) and lipophilic moieties (typically consisting of unsaturated alkyl or acyl chains or cholesterol).^{76,96} Although these lipids vary in composition, they do all have one element in common: a cationic head group covalently attached to a hydrophobic moiety through a linker.⁹⁵ The structures of some of these cationic lipids are shown in **Figure 1.3-5**. Typically, cationic lipids can either be formulated into liposomes alone, but more often they are mixed with neutral helper lipids, such as DOPE. This helper lipid plays a key role in lipoplex gene transfer, including improving fusogenicity and/or endosomal escape of the therapeutic gene.⁷⁹ As previously discussed, these functions facilitate DNA release into the cytoplasm.²⁶

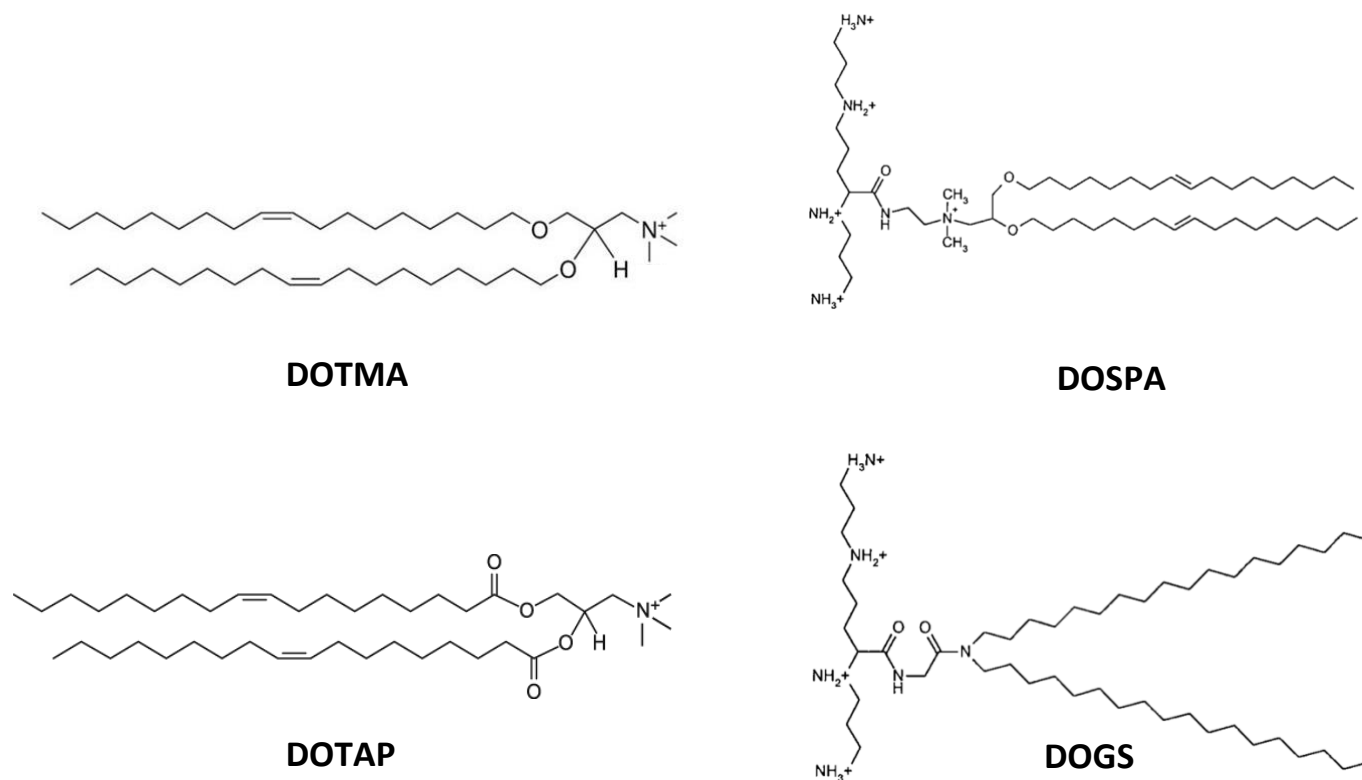


Figure 1.3-5: Structures of commonly used cationic lipids as components of non-viral gene delivery formulations.

To investigate the structural properties of various cationic lipids, Ren et al. produced a series of structural analogues of DOTMA and DOTAP by modifying the relative distance between the hydrophobic chains or between the hydrophobic domain and the cationic headgroup by lengthening the backbone and then studying these lipids *in vitro* and *in vivo*.⁹⁸ From this study, it was revealed that the lipids which comprised a glycerol backbone, where the cationic headgroup and the neighbouring aliphatic chains are in a 1,2 relationship, exhibited the highest *in vivo* transfection efficiency. Interestingly, *in vitro* activities of these same lipids were shown not to be statistically significant.⁹⁸ In another study, Felgner et al. complexed two cationic lipids, dimyristyloxypropyl-3-dimethylhydroxyethyl ammonium bromide (DMRIE) and dioleyloxypropyl-3-dimethylhydroxyethyl ammonium bromide (DORIE) with plasmid DNA and studied their ability to transfect cells to further understand the structure-transfection activity relationship. Here, it was revealed that the hydroxyl group of DMRIE and DORIE increased the

condensation of DNA with the cationic lipids and also improved the interactions of the lipoplex with the cell membranes leading to greater transfection efficient than DOTMA and DOTAP *in vitro*.⁹⁹ Further optimization of these carriers led to the formation of dioeyloxysperminecarboxamindoethyl-dimethylpropanaminium (DOSPA), the first cationic lipid containing quaternary and protonable amines in the headgroup with pH buffering properties which have the ability to condense and protect DNA.¹⁰⁰ The formulation of DOSPA incorporated the helper lipid DOPE and this combination revealed increased *in vitro* transfection efficiency in eukaryotic cells due to the presence of the polyamine region of DOSPA (which is expected to have a pH-dependent transition to promote endosomal escape).¹⁰⁰ This transfection reagent is now commercially available as LipofectamineTM and is the most widely used cationic lipid in *in vitro* transfection experiments.⁷²

The effect of cationic lipid chain length on transfection efficacy was investigated by Felgner et al.⁹⁹ For this study, the chain lengths of a homologous series of hydroxyethyl quaternary ammonium derivatives were modified and evaluated. It was revealed that as the length of the hydrocarbon tail group increases from C14 to C18, transfection efficiency decreases due to an increase in hydrophobicity.⁹⁹ On the other hand, the effect of geometry of the unsaturated double bonds is much more complex. To better understand the importance of geometry, a structure-function study was carried out with two lipids with identical headgroup but varying unsaturated oleoyl chains: one with pure cis double bond and the second with a pure trans double bond. This study revealed that after formulations were prepared with DOPE, the trans derivative were found to be more favourable at transfecting COS-7 cells.¹⁰¹ However, in another study, formation of DOPE mixed with cationic triglycerides bearing cis-oleoyl chains revealed to be more active in transfection CHO cells than its trans analogue.¹⁰² Moreover, polyunsaturated alkyl lipid 1,2-dilinoleyloxy-n,n-dimethyl-3-aminopropane (DLinDMA), which has two linoleyl moieties, has shown increased transfection efficiency compared to DODMA, which has mono-unsaturated alkyl chains, as a component of lipid nanoparticles (LNPs).¹⁰³ The higher number of cis-unsaturation in DLinDMA leads to greater volume of the hydrophobic region of this lipid, resulting in a molecular geometry that promotes membrane fusion and bilayer disruption.¹⁰³ This contradiction in trends suggests that activities displayed by cationic lipids with unsaturated double bonds may be cell-type dependent.

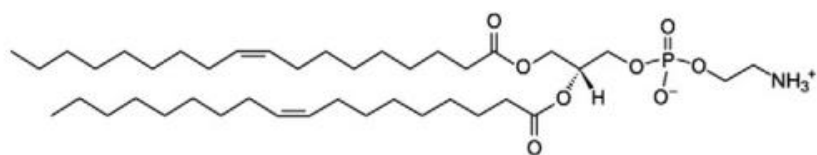
More recently, Miller et al. have demonstrated that modifying the position and degree of unsaturation on the alkyl chains can also influence transfection efficiency. In this study, a series of DOTAP analogues and unsaturated C18 fatty acid chains containing a single alkyne bond at 4-9- and 14- positions were evaluated. Formulations prepared with N-[1-(2,3-(Dioctadec-14-ynoyloxy))propyl]-N,N,N-trimethylammonium methyl sulfate (DS(14-yne)TAP) and cholesterol showed highest transfection efficiency and lowest cytotoxicity both *in vitro* and *in vivo* compared to samples prepared with DOTAP and cholesterol. Additionally, from x-ray diffraction studies, it was determined that the DS(14-yne)TAP compound formed stable liposomes and lipoplexes near physiological temperatures, which protected the DNA molecules from nucleases degradation during intracellular trafficking.¹⁰⁴ These results indicate that it may be important to maintain a balance between degree of acyl/alkyl chain unsaturation in the lipophilic region of a lipid bilayer to enhance DNA protection, promote endosomal escape, and achieve high transfection efficiency.^{103,104}

As can be seen in several recent reviews^{105,106}, LNP composition requires customization to specific applications, route of administrations, and must be optimized based on empirical experimentation. However, there seem to be some general rules that can be used as guidelines for LNP design. For *in vitro* DNA delivery, when LNPs have direct access to the cells, best results are more likely to show with lipoplexes which comprise a high zeta potential and are combined with fusogenic helper lipids. This is in part because, for *in vitro* applications, LNPs can be freshly prepared and so formulation colloidal stability is often not significantly critical.¹⁰⁵ On the other hand, for *in vivo* applications, factors including colloidal stability, shelf-life, interaction with serum protein and blood cells, reticuloendothelial system (RES) clearance, circulation time, extravasation rate, and cytokine induction all must be taken into consideration. As a result, there is typically little correlation between *in vitro* delivery efficiency of a LNP formulation with *in vivo* efficiency.^{105,106} For example, highly cationic LNPs may efficiently interact with the cell membrane to promote cell internalization and consequently increase *in vitro* transfection efficiency. However, highly cationic LNPs can be toxic *in vivo* due to their ability to interact with blood components, resulting in rapid clearance, and to induce cytokine release due to toll-like receptor activation.¹⁰⁴ Therefore, LNPs for *in vivo* delivery generally need to have low (near neutral) zeta potentials and have stabilizing helper lipids such as phosphatidylcholine (PC), cholesterol, and PEGylated lipid to enhance colloidal stability and reduce recognition by the RES.¹⁰⁴⁻¹⁰⁶

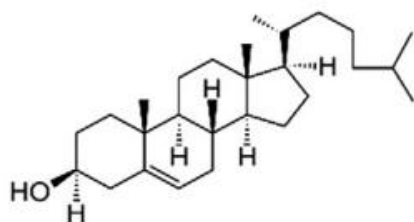
The N^+/P^- charge ratio also impacts the extent to which DNA can be condensed and protected against degradation by cellular nucleases. It is generally observed that as the charge ratio increases, the encapsulation efficiency of the therapeutic gene also increases implying better condensation and protection of the DNA molecules.¹⁰⁵ However, given that the cationic charge carriers in lipids are generally quaternary ammonium groups, lipoplexes prepared at high charge ratios typically also exhibit higher cytotoxicity because of the high number of free quaternary ammonium groups. One strategy recently developed to help overcome this barrier is with the use of ionizable lipids containing an amino head group (pK_a below 7) in combination with helper lipids (such as DOPE) as N-VGDS. Low pK_a allows for ionizable lipids to be positively charged at acidic pH to enhance DNA encapsulation efficiency but are neutral at physiological pH to minimize toxicity. Ionizable lipids in combination with a helper lipid will also interact with the negatively charged membrane of the endosome to aid in membrane disruption and the release of nucleic acid into the cytosol.¹⁰³

1.3.2.2 Incorporation of Helper Lipids to Gemini Surfactant and Lipid-Based Formulations Contribute to Improved Transfection Efficiency

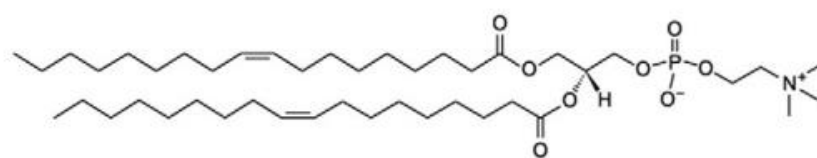
As previously discussed, positively charged N-VGDS often facilitate interactions with the cellular membrane via electrostatic attraction and cellular uptake.²⁶ However, when used alone, cationic GS and lipids often carry a high density of positive charge which can often lead to serum instability or toxicity.¹⁰³ To prevent this from occurring, helper lipids are typically added to cationic GS and lipid transfection complexes to aid in forming stable formulations that are fusogenic in order to promote efficient transgene expression.¹⁰⁷ The transfection efficiency achieved by such lipid carriers has been correlated to the structural transformation of the lipoplex accompanied by their interactions with anionic lipid of cellular membranes. For example, helper lipids with cone-shaped geometry favoring the formation hexagonal II phase, such as DOPE, can promote endosomal escape and enhance transfection efficiency in both *in vitro* and *in vivo* applications.⁷⁹ In a study conducted by Wang et al. the addition of DOPE to GS-based formulations increased the packing parameter of the system which significantly affected the polymorphic features of the lipoplexes in that it promoted the transition from a lamellar to a hexagonal phase, and its presence caused by partial neutralization of cationic charges by the negatively charged phosphodiester of DOPE. Meanwhile, cylindrical-shaped lipid phosphatidylcholine can provide greater bilayer stability, which is important for *in vivo* application of LNPs.¹⁰⁸ Additionally, cholesterol may also be used as a helper lipid to improve intracellular delivery as well as LNP stability *in vivo*.¹⁰⁸ The structures of DOPE, cholesterol, and other commonly used helper lipids such as dioleoylphosphatidylcholine (DOPC) and distearoylphosphatidylcholine (DPSC), are shown in **Figure 1.3-6**.



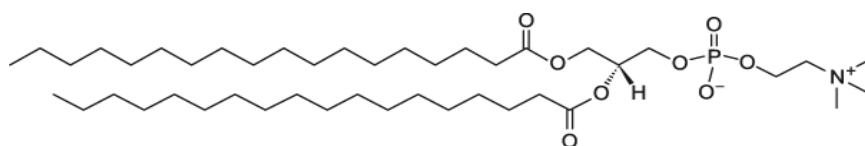
DOPE



CHOLESTEROL



DOPC



DSPC

Figure 1.3-6: Structures of commonly used helper lipids as components of non-viral gene delivery formulations.

1.3.2.3 Cationic Polymeric Molecules as Non-Viral Transfection Vehicles

As previously discussed, cationic polymers when mixed with DNA form nanosized complexes called polyplexes.¹⁰⁷ Cationic components containing pH-sensitive functionalities, specifically polymers with nitrogen groups capable of being protonated, have been found to increase transfection through disruption of the endosomal membrane through the so-called “proton sponge” mechanism.²⁴ Once in contact with DNA, cationic polymers interact with the negatively charged phosphate backbone through electrostatic interactions to form polyplexes with a net positive charge. These cationic polyplexes are then capable of interacting with the negatively charged cell membrane, trigger cell uptake, and mediate transfection efficiency.¹⁰⁷

The physical and chemical properties of cationic polymers can be modified to achieve a dynamic range of characteristics. For example, cationic polymers have the important role of protecting the DNA from nuclease and lysosomal degradation. This role is maintained as long as there is a strong interaction between the DNA and polymer. Weak polymer-DNA interactions may lead to disassembly and release of the DNA from the vector due to aggregation of the polyplexes and non-specific binding with negatively charged serum proteins.¹⁰⁷ To avoid this, modifications to the cationic polymers or polyplexes can be made by the addition of hydrophilic polymers, such as PEG, which can be used as shielding/stealth groups to oppose aggregation or polyplex destabilization.^{109,110} Although outside the scope of this work, other alternatives to PEGylation which appear to introduce shielding effects have also been investigated including: deoxycholate¹¹¹ hydroxyl propyl methacrylate (HPMA)^{112,113} galactosylation¹¹⁴ and various oligosaccharides^{111,115-117}.

The complexation of cationic polymers and DNA has been found to be strongly pH-dependent.¹¹⁸ For example, Rungsardthong et al. demonstrated that DNA binding with the polymer-based carrier dimethylaminoethyl methacrylate (DMAEMA) increased after decreasing the pH from 8 to 6.6.¹¹⁸ This may be due to the protonation of the sensitive imino functional groups of DMAEMA under acidic conditions, which drives DNA-polymer electrostatic interaction and promotes the formation of a polyplex. However, it is important to note that strong binding does not necessarily mean better transfection. Depending on the structure of the vector and DNA components, strong interactions may hinder the transfection by preventing the release of DNA into the cytoplasm.¹¹⁸

Similar to amphiphilic compounds, molecular weight and chain length have a significant effect on cellular uptake, endosomal escape, DNA unpacking and nuclear internalization with polyplexes.¹¹⁹ Typically, high molecular weight (HMW) polymers show better DNA binding, cellular uptake and transfection efficiency, while low molecular weight (LMW) polymers show less cytotoxicity and efficient DNA unloading.^{120,121} Several approaches have been utilized to either increase cellular uptake and stability of LMW polymers or decrease cytotoxicity and enhance DNA unpacking with HMW polymers. For example, decorating the surface of HMW polymers with hydrophilic moieties, such as PEG, have shown to reduce the cytotoxicity of the vector.¹²² More recently, Deng et al. compared the transfection efficiency of LMW vs. HMW compounds. It was determined that high transfection efficiency was observed with LMW (7 kDa) disulfide-PEI compared to HMW (400 kDa) disulfide-PEI, 25k-PEI, and even Lipofectamine 2000.¹²³ Optimization of the molecular weight is important and unique for each type of polymer, as even slight modification to the polymer structure may affect the optimal molecular weight for transfection. An example of this is the self-branched triaccharide-substituted chitosans, which show efficient cell internalization, low cytotoxicity, and high transfection efficiency compared to their linear counterparts.^{122,123} The most commonly studied cationic polymers used in gene therapy formulation development will be further discussed in the following sections.

Although outside of the scope of this work, recently biodegradable natural polymers, such as chitosan⁵⁵ dextran¹¹⁰ and gelatin³³ have gained attention as they can be generally acquired from two major natural sources including proteins and polysaccharides. Due to their unique mechanical properties, low immunogenicity, biocompatibility, and low production costs, natural polymers as a component of N-VGDS are of significant interest for gene therapy applications.

1.3.2.3.1 Poly-L-Lysine (PLL)

The first cationic polymer utilized as a transfection vehicle was Poly-L-Lysine (PLL) conjugated with asialoorosomucoid for hepatocellular gene targeted delivery.¹²⁴ PLL is a linear polypeptide composed of the amino acid lysine as the repeating unit, see **Figure 1.3-7**. At physiological pH, PLL is positively charged and in the presence of DNA, can form cationic polyplexes which may facilitate interactions with the cellular membrane through electrostatic attraction.¹²⁵ Although the peptide linkages are naturally found in proteins, the repeating unit of PLL (the L-lysine amino acid) is not found in nature. As such, administration of PLL nanoparticles

in the circulatory system may cause toxicity, possibly arising from its slow degradation *in vivo*.¹²⁶ Additionally, Akinc et al. determined that after internalization of the complex, most of the PLL polyplexes are retained in the lysosomal pathway instead of being released to the cytoplasm which lowers overall delivery of genes.¹²⁷

Consequently, gene delivery of PLL polyplexes is poor when applied alone or without modification.¹²⁸ To enhance their efficiency, weak bases such as ammonium chloride¹²⁹ spermine¹³⁰ and methylamine¹³¹ have been added to PLL-based transfection formulations since they are known to induce pH buffering in the endosomal vesicles, but they do not seem to improve transgene expression. Alternatively, the design of amphiphilic PLL, by linking PEG¹³² endosomolytic¹³³ (e.g. imidazole) or endolytic agents^{134,135} (e.g. chloroquine or dextran) have shown to increase solubility and reduce toxicity, without compromising their transfection efficiency. More significantly, the conjugation of histidine residues to PLL has been shown to destabilize the lipid bilayers when in a slightly acidic medium and induce fusion upon protonation of the imidazole groups by the increase of interaction between this polymeric cation and the membrane phospholipids, consequently improving transfection efficiency.¹³⁶ Furthermore, recently Kelley et al. has developed nanoparticles comprising a single molecule of plasmid DNA compacted with lysine peptides conjugated to PEG, known as CK30PEG. This study showed that CK30-PEG-DNA nanoparticles are safe and tolerable with multiple (X2) subretinal injections in baboon retina/RPE as gene expression was achieved without triggering immune responses.¹³⁷

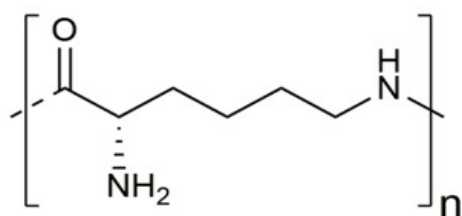


Figure 1.3-7: General structure of PLL.

1.3.2.3.2 Poly (ethylene imine) (PEI)

Poly(ethylene imine) (PEI) was first introduced by Behr in 1995, and has now become one of the gold standards among the non-viral based vectors.¹³⁸ PEI is a cationic vector which contains several pH-sensitive functionalities, specifically nitrogen groups capable of being protonated, which as previously described, have been found to increase transfection through disruption of the endosomal membrane through the so-called “proton sponge” mechanism. Therefore, PEI has been seen to enhance intracellular trafficking by buffering the endosomal compartments, thus protecting the DNA from lysosomal degradation by endosomal release via lysosomal disruption.¹³⁹

PEI can be synthesized in two forms, linear or branched as shown in **Figure 1.3-8**.¹⁴⁰ Compared to the linear structure, branched PEI, including the commercially available reagents jetPEI and ExGen500, have shown to be more efficient in complexing DNA into colloidal and stable nanoparticles. These condensed particles are of spherical shape and have a narrow particle size distribution, which allows high cellular uptake of the vector components.¹⁴⁰ Besides degree of PEI branching, molecular weight is also an important element to consider when designing an effective vector. The transfection efficiency of PEI has been evaluated for a wide range of polymer molecular weights (MW) and has found to increase with the increase of the polymer molecular weight. For example, PEI with MW higher than 25 kDa demonstrated higher transfection efficiency, whereas PEI with MW lower than (800-2000 Da) revealed low transfection efficiency.¹⁴¹ However, the toxicity associated to the transfection process mediated by PEI polymers has also shown to increase with the PEI molecular weight.¹⁴² As such, focus has been drawn on the improvement of PEI transfection efficiency and toxicity profile. One strategy that has been proposed is decorating the surface of PEI with PEG. PEGylation allows the formation of complexes with a more hydrophilic surface, which reduces the interaction of complexes with proteins.¹⁰⁹ *In vivo* studies in mice have shown that PEI-PEG copolymer-based complexes had enhanced blood circulation and reduced toxicity, when injected intravenously.¹⁴³ Additionally, other molecules have been grafted to PEI in order to improve transfection efficiency. For example, the grafting of LMW branched PEI (1.8 kDa) with cholesterol moieties has shown to increase transfection efficiency while reducing cell toxicity, but further optimization is likely needed.¹⁴³ Many alternatives have since been synthesized that outperform PEI in transfection efficacy and maintaining cell viability, one being poly(β -amino ester) (PBAE).¹⁴⁴

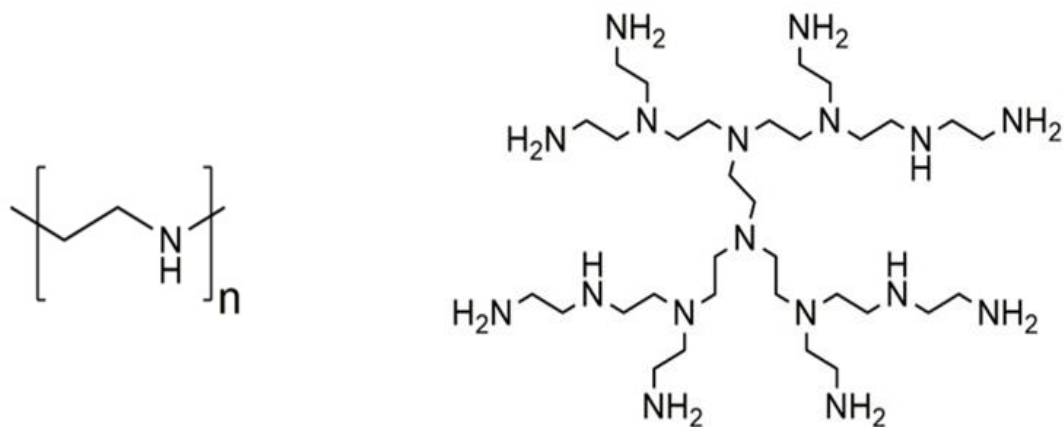


Figure 1.3-8: General structures of linear (left) and branched (right) PEI.

1.3.2.3.3 Poly(β -amino ester)s (PBAE)

PBAEs are a class of polymers first developed by Lynn et al. in 2000.¹⁴⁴ Over the years, a library of PBAE-based derivatives have been synthesized and studied for gene delivery.¹⁴⁵ PBAEs can complex nucleic acids through electrostatic and hydrophobic interactions to form nanoscale positively charged polyplexes that can be internalized into cells due to the positive charge of the polymer. The amine end groups of PBAEs have the ability to buffer the endosomal compartment and release the DNA into the cytoplasm through the proton sponge mechanism, while the ester groups promote degradation of these molecules through hydrolysis within hours under physiological conditions (see **Figure 1.3-9**).¹⁴⁶ Due to these structural properties, combined with their increased biodegradability, biocompatibility, and structural diversity, PBAEs have been the subject of several studies employing them as DNA delivery systems.¹⁴⁷⁻¹⁵⁰

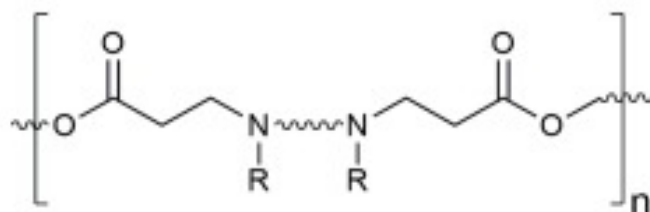


Figure 1.3-9: General structure of polymer PBAE.

Sunshine et al. explored DNA delivery using a large library of PBAE nanocarriers to a wide range of cells, including cancer cells, immune cells, and human stem cells.¹⁵¹ These transfection studies revealed that small molecules conjugated to the ends of the same base polymer modified the gene delivery efficacy. However, some polymer end-group modifications were highly efficient in delivering the nucleic acid in one cell line, while in other cell lines they performed rather poorly.¹⁵¹ Since PBAEs are sensitive to end-group modifications, their properties can be manipulated by fine-tuning their molecular weight, degradability, and hydrophobicity to address the unique challenges of delivering DNA into cells.¹⁴⁶ To support this theory, a more thorough study was carried out by Green et al. where over 2000 PBAEs were developed with varying molecular weights from 2,000 to 50,000 Da. This study showed that diacrylate monomer terminated polymers were unable to promote cellular uptake and the optimal formulations were composed of polymers greater than a molecular weight of 10,000 Da.¹⁵² Similarly in another study, modifications of PBAEs by end-capping with diamines increased the transfection efficiency to levels comparable to adenovirus.¹⁵⁰ More recently, Zhou et al. have reported a new class of degradable amine-co-ester terpolymers which show great promise in tumor targeting and suppression.¹⁵³

Further improvements to the PBAE structure have been made to improve their DNA delivery capabilities. For example, the surface of the polymer has been decorated with PEG to promote biological stability at the systemic and tissue levels, which is a critical property to facilitate efficient *in vivo* utilization and to overcome the extracellular barriers.^{154,155} Kim et al. revealed that non-PEGylated PBAE polyplexes tend to aggregate following 24-hour incubation in acidic and physiological buffers which presents a challenge for therapeutic use. However, PEG-PBAE polyplexes can successfully maintain particle stability, without compromising their therapeutic function for intracellular delivery to human small cell lung cancer cells.¹⁵⁶ Consequently, continuous optimization of polymer properties is important to consider and can contribute to more rational polyplex design.

1.3.3 Other Synthetic Non-Viral Vectors

While the transfection efficiency of non-viral vectors is still low compared to viral vectors, several modifications to the simple structures of GS, lipids, and polymers can be made to enhance the overall efficiency of these carriers. For example, more recently a lipopeptide-based nanoparticle, a lipid connected to a peptide, has been studied for use in retinal gene therapy. The multifunctional (1-aminoethyl)-N-oleicylcysteinyl(1-amino-ethyl) propionamide (ECO), composed of an ethylenediamine head group, two cysteine functional linkers, and two oleoyl lipophilic tails have shown to effectively complex with the therapeutic gene and exhibit high transfection efficiency in the retina. This ‘smart’ hybrid vector is of great interest as it combines the favourable properties of both lipids and polymer carriers: the lipid moieties of these peptides cause them to self-assemble into nanoparticles to efficiently encapsulate the DNA, while the protonable ethylenediamine peptide head group aids in DNA escape from endosomal lysis in the cytoplasm and trafficking to the nucleus. Sun et al. showed that ECO can efficiently deliver the RPE65 gene into the retinal pigmented epithelium in the LCA model of Rpe65^{-/-} mice to restore vision.¹⁵⁷

Extensive studies and reviews on the nature and effectiveness of N-VGDS composed of various other lipids, surfactants, and polymers as well as their derivatives have previously been published. Although outside the scope of this work, some promising non-viral transfection vehicles include: nanoparticles based on gold¹⁵⁸ silica¹⁵⁹ or carbon nanotubes¹⁶⁰, biodegradable polymers such as poly[α -(4-aminobutyl)-L-glycolic acid]¹⁶¹ and poly(4-hydroxy-L-proline ester)¹⁶², asymmetrical gemini surfactants¹⁶³ and a class of water-soluble, non-ionic block copolymers known as Pluronics.¹⁶⁴

Although the design of the synthetic carrier is important to consider, highly efficient gene therapeutics require the rational design of both the synthetic vector **and** the enclosed DNA cargo. The following section will discuss the essential DNA components and structural features required to achieve successful transfection.

1.4 Recombinant Plasmid DNA (pDNA) Vectors

The first successful gene silencing therapeutic strategy with the use of RNA interferences (RNAi) was observed in the late 1980s by Fire et al.¹⁶⁵ This biological process involved the introduction of RNA molecules, including small interfering RNA (siRNA) and microRNA (miRNA), into cells to interfere with the function of endogenous gene through translational or transcriptional repression.¹⁶⁶ The discovery of RNAi won the Nobel prize in 2006 and has since then been utilized extensively to diagnose and treat diseases at both the cellular and molecular levels.¹⁶⁷ However, despite its promising therapeutic potential, RNAi-based clinical trials encountered various challenges, including immune-related toxicities, low stability, transient nature, and low transfection efficiency.¹⁶⁵

To address these challenges, scientific interest in the use of recombinant plasmid DNA (pDNA) vectors for gene therapy applications piqued, the first being recognized by Wolff et al. in 1990.¹⁶⁸ Wolff et al. observed that when pDNA is injected directly into skeletal muscle, it is taken up and expressed for long periods of time by a small percentage of skeletal myocytes in the area of injection.¹⁶⁸ pDNA vectors are circular covalently closed (CCC) vectors which typically consists of two essential components, as shown in **Figure 1.4-1**: 1) a eukaryotic expression cassette which comprises the genetic components essential for transgene expression (e.g. gene of interest, intron, promoter, and transcription termination/polyadenylation signal), and 2) a prokaryotic backbone with an origin of replication for plasmid amplification and an antibiotic resistance gene cassette for selection.¹⁶⁹ The double-stranded covalently closed ring of pDNA comprises no breaks or nicks in the structure which serves to enhance the overall stability of the plasmid during the delivery process.^{168,169}

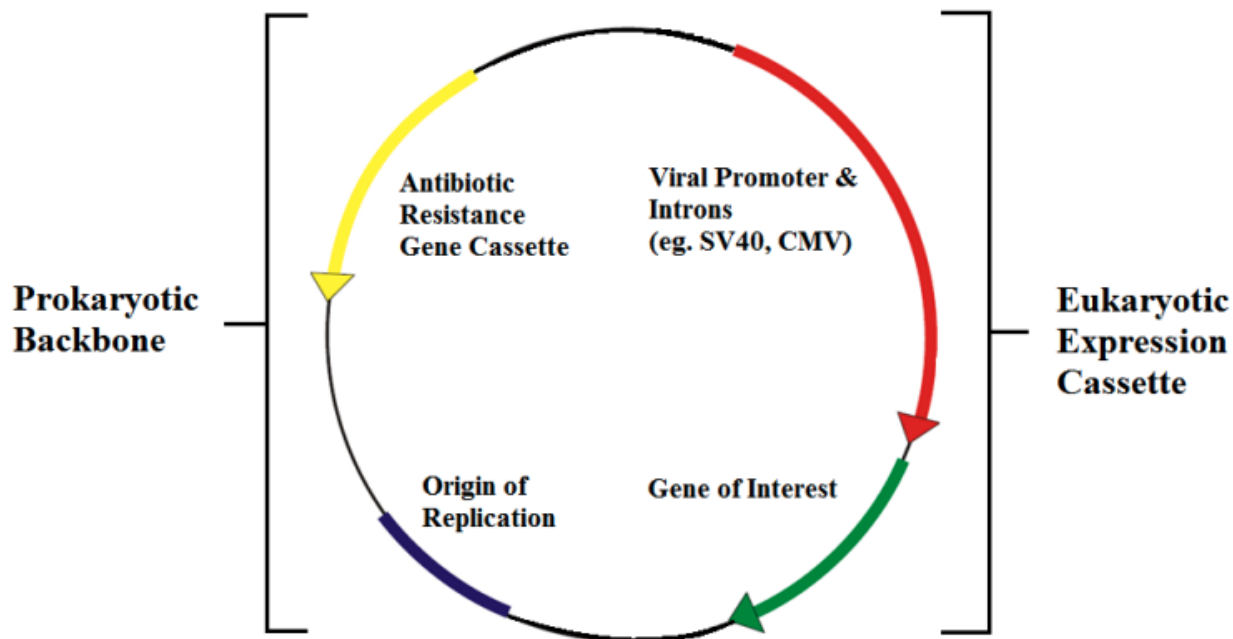


Figure 1.4-1: Schematic of recombinant plasmid DNA (pDNA) vectors. Reproduced from ¹⁷⁰ with permission (See Appendix D).

Compared to viral and RNA-based vectors, pDNA are easier and cheaper to produce in large quantities, are stable, and have a much longer shelf life. Additionally, due to the modular nature of pDNA, they are easy to manipulate and design for therapeutic use.¹⁶⁹ However, there are limitations of using plasmid vectors for gene therapy applications. As previously described, naked pDNA vectors are typically inefficient at delivering their payloads compared to viruses. Therefore, naked pDNA vectors are often combined with other delivery methods, such as synthetic vectors or physical forces, to improve their cellular uptake and nuclear localization. However, when rational vector design is not considered, some of these delivery methods have shown to lead to breakage of the pDNA backbone, which then increases the likelihood of genome integration. Also, if the break occurs in the therapeutic gene sequence, this may lower the efficiency of gene expression.¹⁷⁰ Additionally, pDNA vectors do have their own safety concerns. Either when delivered alone or packaged within a synthetic vector, pDNA comprise a prokaryotic backbone containing bacterial sequences and antibiotic resistance genes which may enable horizontal gene transfers, giving rise to antibiotic resistant pathogens. This would negatively impact longevity of

gene expression and cause silencing by changing the euchromatin format of DNA to heterochromatin format. Additionally, unlike vertebrate animals, bacterial DNA have a higher frequency of unmethylated CpG motifs which may induce immune responses.¹⁷¹ Hyde et al. showed that even the presence of a single CpG motif upon intranasal delivery induced inflammatory responses in BALB/c mice. When delivered to the host, CpG motifs interact with toll-like receptor 9 (TLR9) which are embedded in the endosomal membrane of immune cells.¹⁷² CpG-TLR9 binding then results in the activation of eukaryotic transcription factors and pro-inflammatory cytokine production. Since TLR9 are situated within the endosome, the administration of synthetic vectors may trigger CpG-mediated immunostimulatory responses.¹⁷² However, Wolf et al. found that the deletion of pDNA CpG motifs in PEI/pDNA polyplexes and DOTAP/DOPE/pDNA lipoplexes reduced immune responses and prolonged gene expression.¹⁷³ Consequently, removal of the prokaryotic backbone would eliminate the bacterial DNA and enhance the safety of the vector to satisfy the regulatory requirements for clinical applications.

1.4.1 Introduction of New Generation DNA vectors

As discussed above, the presence of antibiotic resistance genes and unmethylated CpG motifs commonly found in prokaryotic pDNA can trigger immune responses, which decreases their clinical potential.¹⁷¹ In order to preserve the advantages of pDNA and bypass their disadvantages, a new generation of DNA vectors were introduced: DNA minicircles and linear covalently closed (LCC) DNA minivectors, see **Figure 1.4-2**.¹⁷⁰ Removal of the prokaryotic backbone in these new generation of DNA constructs not only eliminates the delivery of unwanted prokaryotic sequences known to induce immune responses, but it also allows for the formation of smaller vectors which may enable efficient DNA packaging into synthetic vectors and rapid intracellular trafficking upon cytoplasmic release.¹⁷⁴

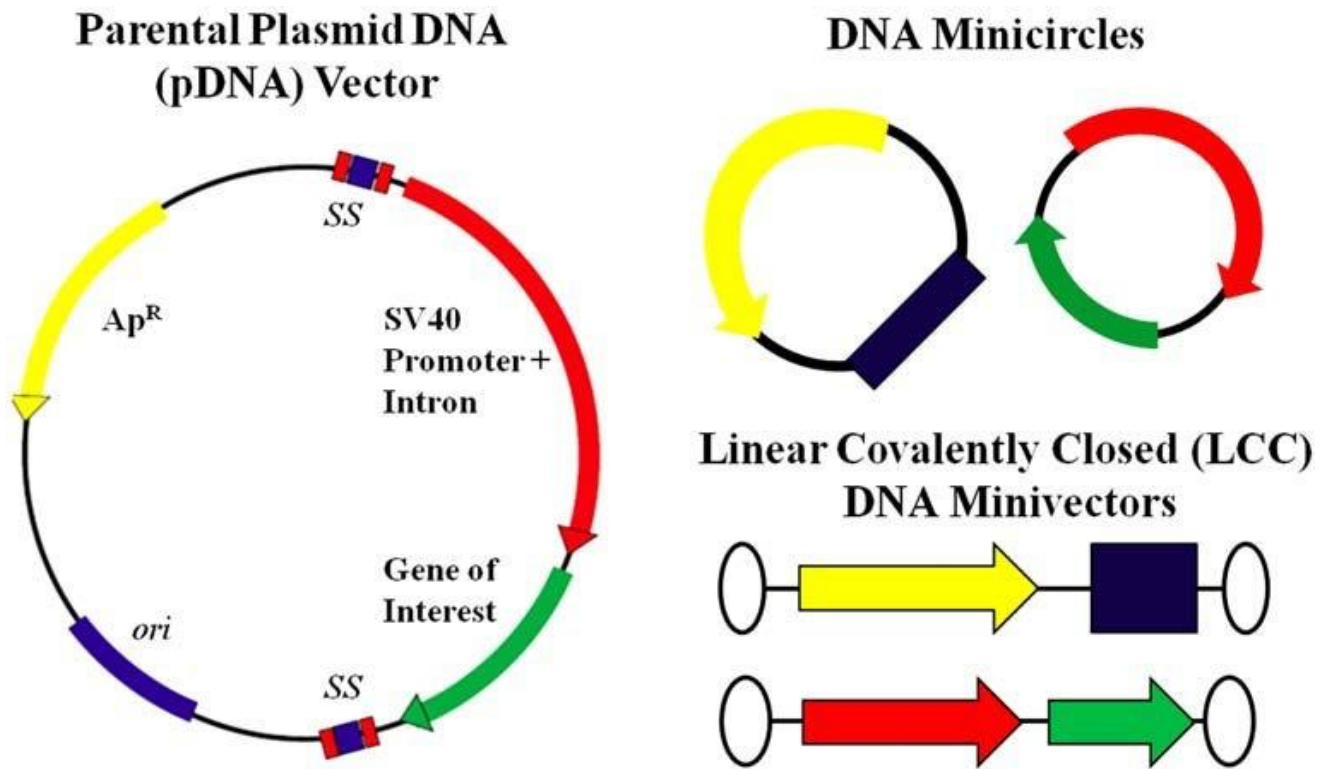


Figure 1.4-2: Schematic of recombinant CCC plasmid DNA (pDNA) vectors, CCC DNA minicircles, and Linear Covalently Closed (LCC) DNA minivectors. Reproduced from ¹⁷⁰ with permission (See Appendix D).

1.4.1.1 DNA Minicircles

In 1997, Darquet et al. used *in vivo* site-specific recombination to turn a single parent plasmid into two well-characterized circular molecules: 1) a discardable miniplasmid comprised of the prokaryotic backbone, and 2) the DNA minicircle containing the essential expression cassette, as shown in **Figure 1.4-3**.¹⁷⁵ Due to the removal of the prokaryotic backbone, CCC minicircles cannot replicate in bacteria and are therefore, no longer considered plasmids.¹⁷⁵ As a result, CCC minicircles are smaller and safer than pDNA as delivery vectors for gene therapy. Although, no clinical trials have been reported to date, several studies have shown that minicircles can provide an approximate 10 to 1000-fold increase in long term transgene expression *in vitro*¹⁷⁶ and in a variety of organ systems *in vivo*, including the liver¹⁷⁷ heart¹⁷⁸ skeletal muscles¹⁷⁹ and lungs¹⁸⁰ over conventional pDNA vectors.¹²

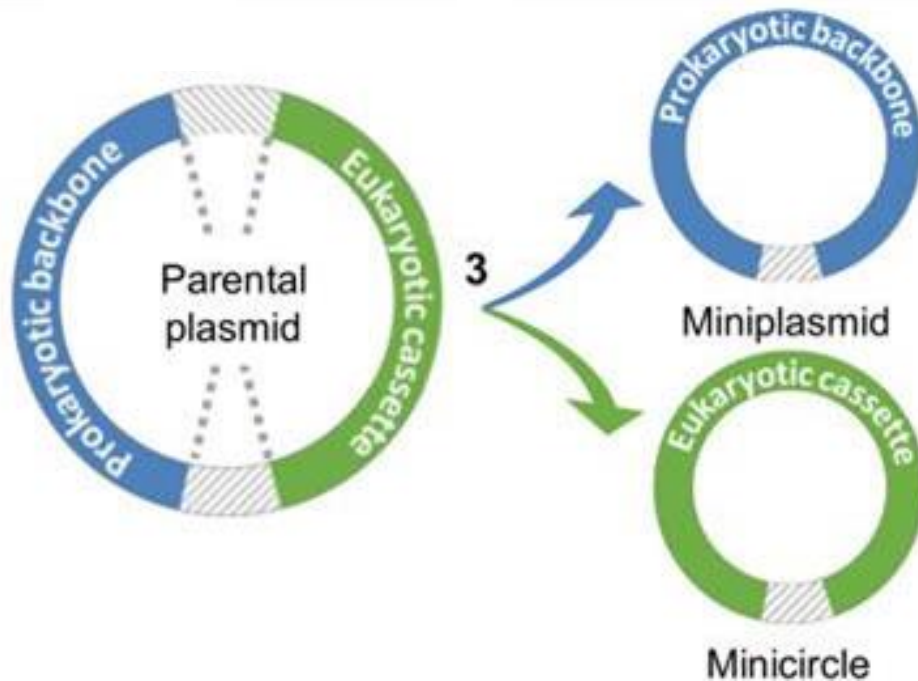


Figure 1.4-3: The production of supercoiled CCC minicircle DNA from parental plasmid *by in vivo* site-specific recombination. Adapted from reference ¹⁸¹.

Although the removal of the prokaryotic sequences in DNA minicircles reduces the delivery of unnecessary prokaryotic sequences to cells (which are known to induce immune responses), they do still pose other safety concerns with regards to undesirable recombination events.¹⁸¹ For example, in the random event of vector integration into a host chromosome, circular DNA vectors, including DNA minicircles and conventional plasmid DNA, promote molecular continuity and vector integration into the host DNA which may lead to insertional mutagenesis.¹⁸² On the other hand, in a similar event, linear DNA vectors with covalently closed terminal ends confer double strand breaks which disrupt the chromosome and initiate cell death pathways. This pathway then removes the mutant cell from the proliferating cell population, thereby providing a safer option for DNA vector-mediated transgene delivery by preventing the manifestation of oncogenic insertional mutagenesis.¹⁸²

1.4.1.2 LCC DNA Minivectors

LCC DNA minivectors are dumbbell-shaped vectors possessing hairpin ends which enclose the essential eukaryotic expression cassette.¹⁸³ These covalently closed hairpin loops provide stability and protection to the vector from exonucleases, a barrier that significantly affects the successful delivery of linear DNA.¹⁸³ Additionally, the minimized size of these vectors allows for the delivery of higher copy numbers of therapeutic genes. For example, like conventional pDNA, LCC DNA minivectors can deliver up to 15-20 kb of DNA to the target cell of interest, which is approximately five times the payload of viral vectors.^{184,185} However, unlike conventional pDNA, DNA LCC DNA minivectors lack prokaryotic sequences which silence the therapeutic cargo. As a result, LCC DNA minivectors can insert therapeutic genes in the genome of target cells to provide long-term transgene expression with less frequent dosing.¹⁸⁵ The linear covalently closed structure of LCC DNA minivectors also reduces the potential of insertional mutagenesis in the event of chromosomal integration.¹⁸⁴ Although the manufacturing of DNA constructs is outside the scope of this work, LCC DNA minivectors may be generated from their parental CCC pDNA substrates through three distinct *in vitro* enzymatic mechanisms: 1) pDNA digestion and ligation, 2) PCR amplification and ligation and 3) protelomerase mediated processing as shown in **Figure 1.4-4**.¹⁸³

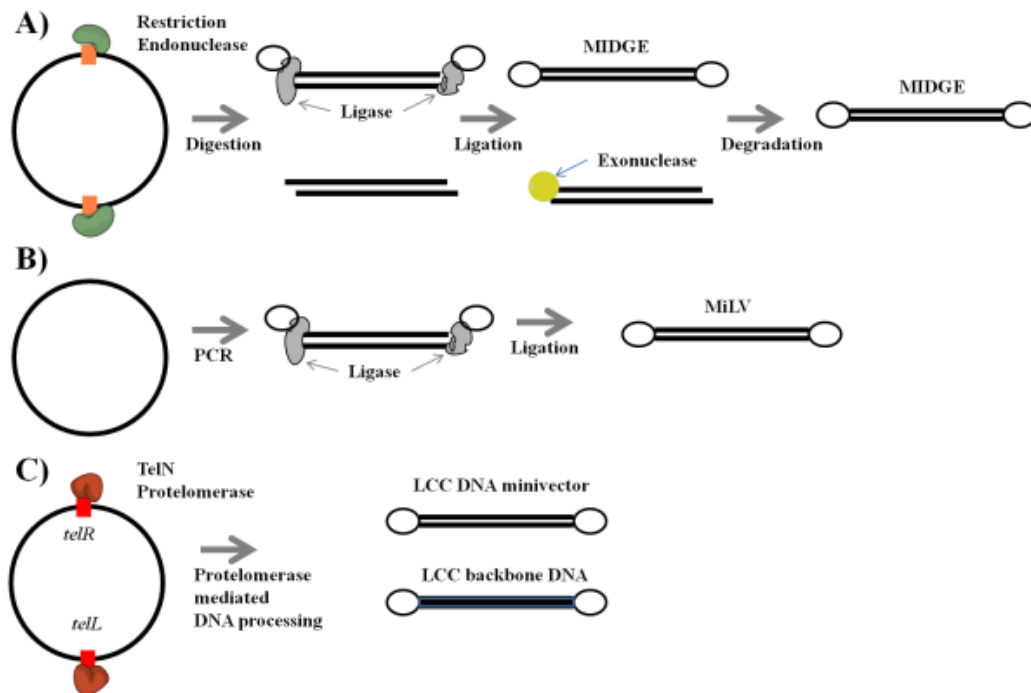


Figure 1.4-4: *In vitro* production methods of double stranded LCC DNA minivectors. A) Production of minimalistic immunogenic defined gene expression vectors (MIDGE) through targeted pDNA digestion with restrictive enzymes and ligation with hairpin oligonucleotides; B) Production of micro-linear vector (MiLV) through polymerase chain reaction (PCR)-mediated amplification of the expression cassette from the plasmid construct and ligation with hairpin oligonucleotides; C) Production of LCC DNA minivectors by utilizing TelN protelomerase from bacteriophage N15 and its target sequences followed by pDNA processing. Reproduced from ¹⁸³ with permission (See Appendix D).

Minimal immunological defined gene expression vectors, or MIDGE, are the first generation of such LCC DNA minivectors generated by Schakowski et al.¹⁸⁶ The production of MIDGE involves restriction endonuclease digestion to separate the eukaryotic expression cassette from the prokaryotic backbone sequences, followed by ligation with hairpin oligonucleotides for the formation of covalently closed ends. The unprotected prokaryotic backbone sequences are then subsequently removed by exonucleases which leads to the generation of MIDGE vectors.¹⁸⁶ By removing the prokaryotic backbone and antibiotic-resistant genes, MIDGE vectors have greatly enhanced immunocompatibility, reduced risk of spreading antibiotic-resistant genes the host, and a transcriptionally active structure.¹⁸³ In addition, due to the linear nature of LCC DNA, risks of insertional mutagenesis from random genomic integration are inhibited.¹⁸⁶ The application of LCC

MIDGE vectors have previously shown improved gene expression in colon carcinoma cells upon complexation with Lipofectamine and PEI.¹⁸⁶ In another study, upon *in vivo* hydrodynamic injection into BALB/c mice, it was shown that MIDGE-based vectors improved transfection efficiency in mammalian cells up to 17-fold to the heart compared to the parental pDNA.¹⁸⁷ MIDGE vectors also show promising characteristics for use in DNA vaccine development. For example, when MIDGE vectors, encoding the LACK antigen, was administered to susceptible BALB/c mice, it offered protection from *Leishmania* infection at lower doses compared to pDNA vectors.¹⁸⁸ In another study, MIDGE was introduced into human mesenchymal stromal cells (hMSC), where even after 55 days post treatment, high stability, cell viability, and protein expression levels were achieved.¹⁸⁹ Further studies have shown that MIDGE, particularly when conjugated with NLS peptides, have greater transfection efficiency as compared to its parental plasmid both *in vitro* and *in vivo*.¹⁸⁷ Interestingly, MIDGE vector systems have also been used successfully in clinical applications as DNA vaccines against advanced stage cancers in conjugation with double stem loop immunomodulator molecules (dSLIM) that serve as effective immune adjuvants.¹⁹⁰ However, despite being more efficient and safer than conventional CCC pDNA vectors, large-scale production of MIDGE through the multistep *in vitro* processes is costly and time consuming making them less desirable for use in clinical applications.¹⁸⁶

As an alternative to MIDGE, micro-linear vectors (MiLV) are mini linear vectors which are generated from PCR amplification of the eukaryotic expression cassette followed by ligation with hairpin oligonucleotides and finally purified by use of PCR cleanup kits.¹⁹¹ MiLV have been investigated to deliver genes both *in vitro* and *in vivo* in Epstein-Barr virus (EBV) positive tumors. In this study it was revealed that MiLV resisted exonuclease degradation, improved transfection efficiency, reduced immunostimulatory effects, and prolonged transgene expression compared to conventional pDNA vectors.¹⁹¹

Continued efforts to optimize LCC minivectors have led to the development of several proprietary DNA conformations for use in gene transfer. These linear constructs have been strategically designed to incorporate specialized sequences to enhance efficiency and safety, while lowering cost of production compared to MIDGE. For example, Nafissi & Slavcev developed a one-step *E. coli* based *in vivo* production system for the generation of bacterial sequence-free LCC DNA minivectors (as illustrated in **Figure 1.4-5**).¹⁹² Briefly, the manufacturing process of LCC DNA minivectors involves passaging the parent plasmid vector through R cells (TelN and Tel

recombinase expressing cells) under temperature-controlled conditions. Compared to enzymatic processes, this *in vivo* approach is more cost effective. Additionally, the recombination-mediated process in *E. coli* cells removes the bacterial sequences from the LCC DNA minivectors, thereby generating a safer alternative relative to conventional plasmid DNA.¹⁹²

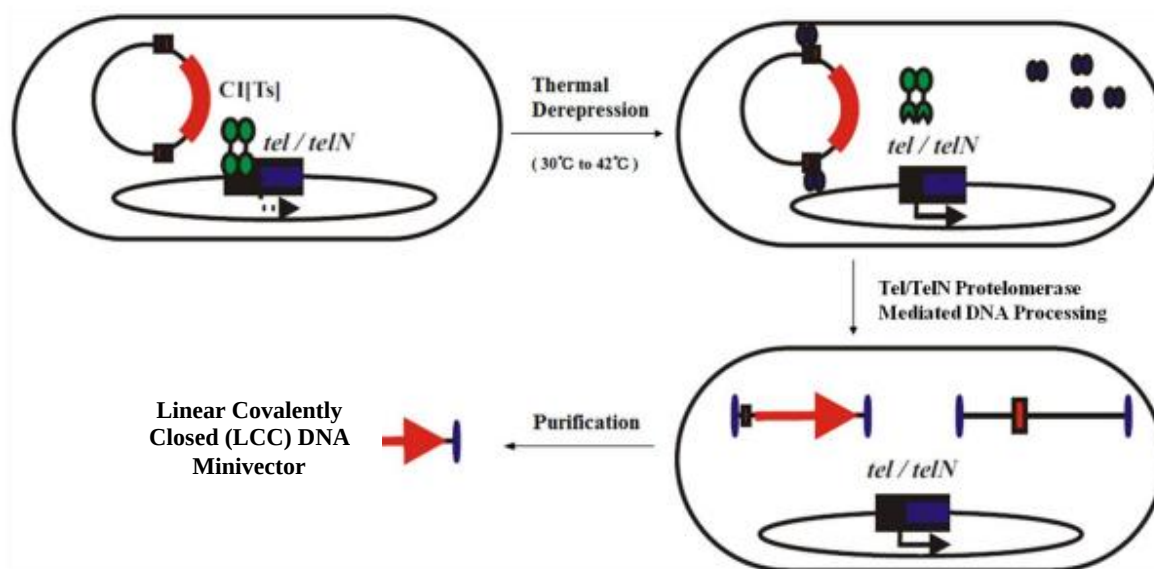


Figure 1.4-5: Schematic of the enzymatic one-step *E. coli* based *in vivo* production system for the generation of LCC DNA minivectors. At temperatures below 37°C, protelomerase is repressed by the *CI[Ts]* repressor. A temperature upshift to 42°C allows for the controlled expression of protelomerase. Then, the enzymatic activity of the expressed protelomerase on the parental plasmid DNA substrate results in the generation of the proprietary LCC DNA minivectors. Adapted from reference ¹⁹².

LCC DNA minivectors are dumbbell-shaped vectors which carry a minimal expression cassette consisting of the eukaryotic promoter, gene of interest, intron, and polyA sequence, and nuclear translocation enhancing sequences (super sequence), which significantly reduces the size of the LCC DNA minivectors compared to the parental pDNA.^{193,194} In an effort to improve the efficiency of LCC DNA minivectors systems for gene transfer, the *in vitro* gene expression after complexation with GS have been previously evaluated.¹⁹⁵ This study showed that although these LCC DNA minivectors produced a statistically significant increase in expression compared to their parental CCC counterparts, the overall gemini-mediated transfection efficiencies are still low with less than 13% cells transfected.¹⁹⁴

Another novel linear DNA construct which has been engineered for efficient transgene delivery are closed-ended DNA construct (ceDNATM).¹⁹⁶ ceDNA are linear, double stranded, covalently closed, DNA constructs which include the gene of interest, regulatory elements to drive gene expression, and the eukaryotic expression cassette flanked by two inverted terminal repeats (ITRs).¹⁹⁵ ceDNA are generated through a cell-free rapid enzymatic synthesis. A promising feature of ceDNA is their ability to deliver large construct payloads (approximately 12 kilobases) to targeted cells. As such, ceDNA-based therapeutics can potentially treat diseases caused by larger genes, such as Stargardt disease or Wilson disease.¹⁹⁶ The most current application of ceDNA has been in a Hemophilia A study in 2021. In this study, upon *in vivo* hydrodynamic injection in CD-1 mice, factor VIII ceDNA showed a greater than 30-fold increase in construct expression when complexed within a lipid nanoparticle delivery system compared to the wildtype mice containing a factor VIII open reading frame (ORF) sequence.¹⁹⁷ A deeper understanding of the molecular interactions between these and other LCC DNA minivectors with synthetic vectors (such as lipids, polymers, and surfactants) is important to further optimize LCC DNA minivectors for clinical applications.

1.5 Non-Viral Gene Delivery Vector Evaluation Techniques

As previously discussed, optimal non-viral vectors for gene therapy applications will consider the design of both the cationic and anionic components of the non-viral delivery system and ensure that they complement each other and work in combination to achieve high gene expression. For example, during formulation development, there must be a fine balance between the cationic charges of the synthetic vector to the anionic charges of the DNA molecules to not only effectively complex the therapeutic gene, but to also avoid formulation instability or toxicity due to excessive cationic charges.²⁶

After successful preparation of the delivery vector, it is then critical to evaluate and characterize the resulting formulation. Depending on their application, there are various analytical techniques which can be used to evaluate non-viral vector formulations. Since the ultimate goal of N-VGDS is to efficiently transfect the host cell and express its gene, these analytical techniques are used as a precursor to deduce a relationship between physicochemical properties of the formulation to the biological properties of the formulation. The most commonly used techniques and procedures to characterize non-viral vectors prior to *in vitro* assessment will be discussed in the following section.

1.5.1 Dynamic Light Scattering

Dynamic light scattering (DLS) is a technique that can be used to determine the size and size distribution profile of small particles in suspension.¹⁹⁸ This technique measures time-dependent fluctuations in the scattering intensity of particles undergoing Brownian motion, the random movement of suspended particles, and relates this to the size of the particles. The speed of the Brownian motion is influenced by the particle size. In general, it is observed that large particles tend to experience slower Brownian motion due to their bulky nature. In contrast, smaller particles are separated further by the solvent molecules which allow for rapid motion (as shown in **Figure 1.5-1**).¹⁹⁸ The velocity of the Brownian motion of the particles in solution is represented by the transitional diffusion coefficient, D , which is used in the Stokes–Einstein equation to calculate the hydrodynamic diameter (dH), a measure of the particles size. The Stokes–Einstein equation is defined as:

$$d(H) = \frac{kT}{3\pi\eta D} \quad \text{Equation 1.5-1}$$

where k is Boltzmann's constant, T is the thermodynamic temperature, and η is the dynamic viscosity of the medium.¹⁹⁹ It is important to note that the size output of the DLS method is the diameter of a sphere that has the same translational diffusion coefficient as the particle being tested. As a result, the translational diffusion coefficient will not only depend on the size of the particle core, but also on surface structure as well as the concentration and the types of ions in solution.¹⁸⁵ In addition to measuring size, DLS can also be used to measure the size distribution of the particles using a value called polydispersity index (PDI). PDI values indicate the homo- or heterogeneity of a sample. The numerical value of PDI ranges from 0 (for a perfectly uniform sample with respect to the particle size) to 1 (for a highly polydisperse sample with multiple particle size populations).¹⁹⁸⁻²⁰⁰

DLS is a rapid method to determine the particle size and size distribution of the samples. However, complex concentrated samples often lead to excess particle-particle interactions and multiple scattering.¹⁹⁸ Therefore, appropriate dilution is often required to determine the size of the micellar structures.²⁰⁰

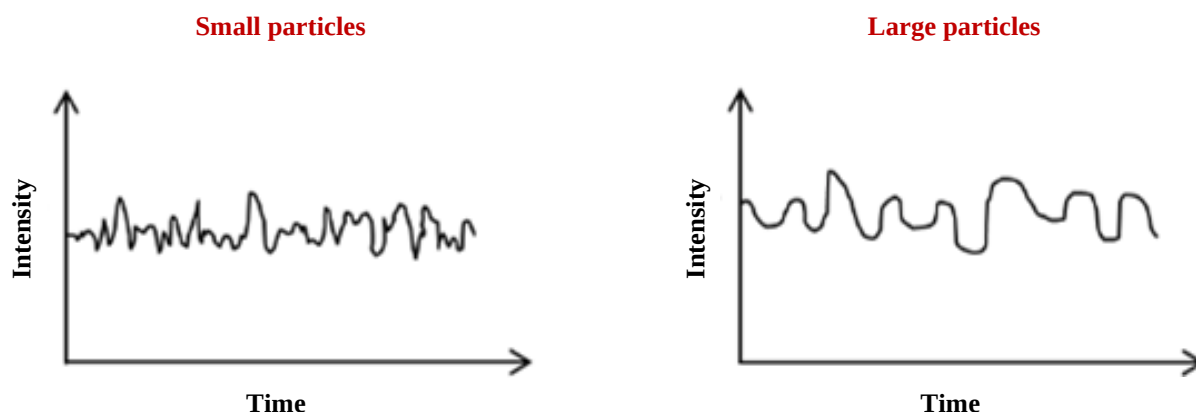


Figure 1.5-1: Differences in intensity of scattered light due to thermal motion of dispersed particles. Smaller particles show faster fluctuations of the scattered light, while larger particles show slower fluctuations. Adapted from reference ²⁰⁰.

1.5.2 Scanning and Transmission Electron Microscopy

As previously described, all nanoparticles are defined by their physical and chemical properties, including particle size, size distribution, shape, and surface features. Since nanoparticle characterization, both qualitative and quantitative, forms an important step in nanotechnology QC as well as research and development, electron microscopy is a valuable technique not only for thorough physicochemical characterization of uncomplexed and complexed materials, but to also explore the effects of nanocomposites on biological systems, providing essential information for the development of efficient therapeutic and diagnostic strategies.²⁰¹ More specifically, electron microscopy can be used to characterize non-viral vectors to further understand how the structure and shape of the DNA and complex systems influence the interactions of the components on biological systems, ultimately learning more about how the complex enters the cell and escapes from the endosome. This technique also validates the reliability of other routine particle sizing techniques, such as DLS.²⁰²

Both scanning electron microscopy²⁰³ (SEM) and transmission electron microscopy²⁰⁴ (TEM) can be used to visualize and accurately characterize the particle size, shape, as well as surface area of hydrophobic and hydrophilic nanoparticles through electron beams. The primary difference in data output between the two techniques is the way in which the nanoparticle images are formed. SEM produces accurate 3D images of particles in the dispersion while TEM produces 2D images that require further interpretation. Although the images are 2D, TEM systems can deliver much greater resolution. TEM views thin specimens such as molecules and tissue sections while the SEM, is designed to directly study the surfaces of solid objects with a beam of focused electrons.²⁰⁵ Such studies were not undertaken in this work, though electron microscopy is valuable and are important to consider for future studies.

1.5.3 Zeta Potential

All particles in an aqueous solution carry an electric charge which may be positive, negative, or neutral.²⁰⁶ The zeta potential is then defined as the electrical potential that exists at the boundary (“slipping plane”) between two layers of ions, namely, the compact and diffuse layers, which surround a particle in solution, as shown in **Figure 1.5-2**.²⁰⁷ The zeta potential is therefore an indication of the surface charge of a particle. It is important to note here that the zeta potential is not the actual surface potential (i.e. surface charge), which would generally be larger in absolute value compared to the zeta potential. Since zeta potential is an indication of surface charge, factors such as ionic strength and pH may have an affect the zeta potential of a nanoparticle.²⁰⁸

Zeta potential measurements allow for further understanding of the colloidal, thermodynamic stability, and interfacial behaviours of the transfection complex in the dispersed medium.²⁰⁶ Values either greater than +30 mV or less than -30 mV are used as a general scale for particle stability. However, recent studies have revealed that a more accurate measure of stability is to evaluate the change in zeta potential of the nanoparticles with time or zeta potential rate.²⁰⁸

Zeta potential can be measured using a DLS instrument which uses a combination of Electrophoresis and Laser Doppler Velocimetry to measure the speed at which particles move in an aqueous medium upon application of an electric field (i.e. its velocity) which is then equated to charge.^{206,208}

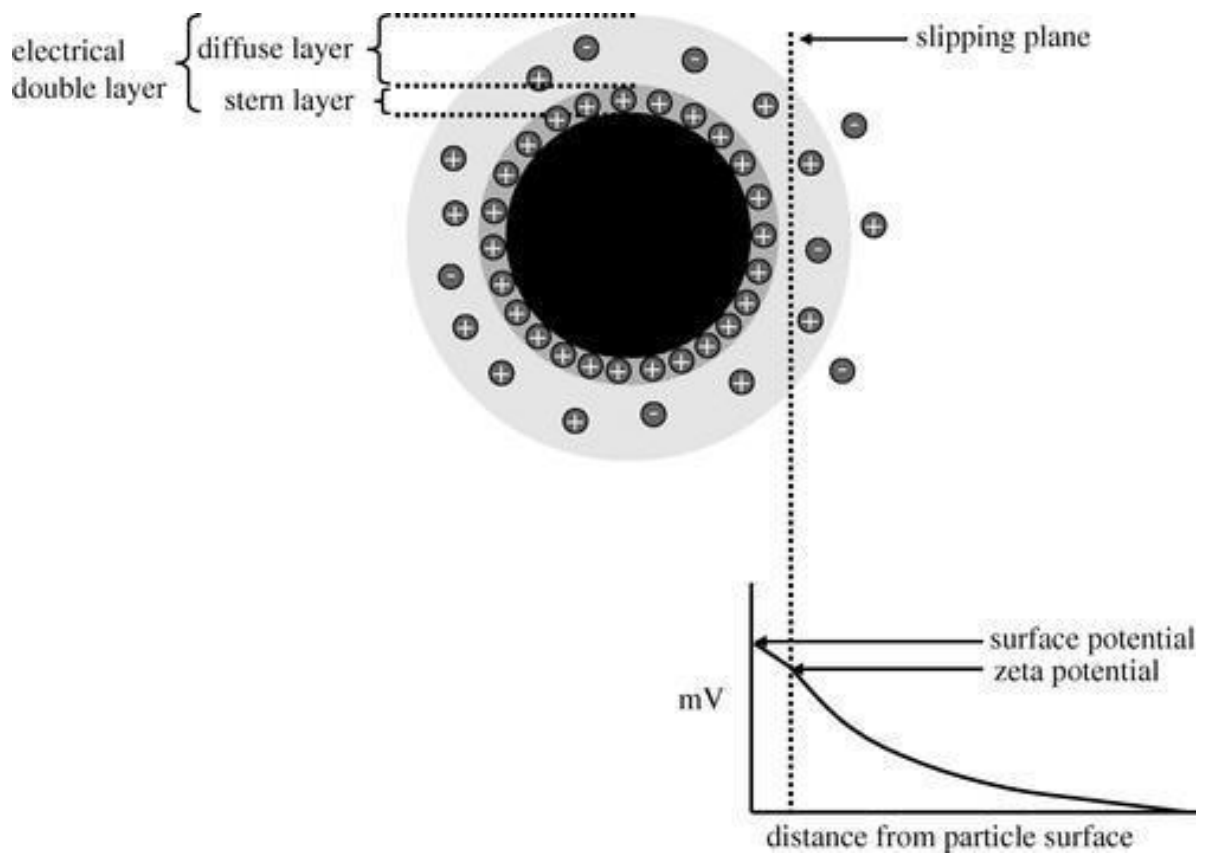


Figure 1.5-2: Schematic showing the electrical double layer (the diffuse and stern layer) which surrounds a particle in a dispersed medium and the position of the slipping plane. The zeta potential is defined as the electrical potential at the slipping plane. Adapted from reference ²⁰⁷.

1.5.4 Langmuir-Blodgett Technique

Endosomal escape is the principal biological barrier to effective transfection.²⁴⁻²⁷ As such, Langmuir-Blodgett studies investigate the interactions between the non-viral vector components.²⁰⁹ This experimental procedure involves filling a Langmuir-Blodgett trough, a long shallow basin with surface barriers on each end, with water, and placing a specific volume of the sample onto the surface of the water to form a monolayer. Surface pressure, the difference between the surface tension of the water and the monolayer phase, is monitored throughout the experiment by using the Wilhelmy plate method.²¹⁰ Initially, the area available to the monolayer is large. As such, the molecules will remain far apart, and there will be minimal interactions between neighboring molecules. Under these conditions, the monolayer has little effect on the surface pressure of water, and the monolayer is considered to be in the gaseous phase. Subsequently, as the barriers of the Langmuir trough are closed, the area available to the monolayer begins to decrease while forcing the molecules to pack closer together. As a result, the surface pressure of the monolayer increases.²¹¹ Further compression of the monolayer continues to decrease the area available to the molecules of the monolayer, resulting in a phase transition from the gaseous state to a liquid expanded state to a liquid condensed state, and finally a solid-like state where the molecules are closely packed together. Continued compression of the monolayer after reaching the solid phase causes the monolayer to collapse at a critical pressure, and this marks the completion of the experiment. Subsequently, a plot of surface pressure vs mean molecular area (π -A isotherms), as shown in **Figure 1.5-3**, is collected from the experiment, which defines the thermodynamic relationship between the area available to each molecule of the monolayer, and the monolayer surface pressure at a constant temperature.^{212,213} Several parameters can be derived from the π -A isotherm including: the lift off area (A_L), the molecular area at which the surface pressure of the monolayer begins to increase; the limiting area (A_∞), the area at which the rate of change in the surface pressure is greatest; the collapse area (A_C); and the collapse pressure (π_C), the mean molecular area and the surface pressure at which the monolayer collapses. When analyzing the isotherms of a system, emphasis is given to the range between 30–35 mN/m since this is the surface pressure of biological cell membranes.²¹³

The phase transitions of the monolayer can be observed using a plot of compression modulus, C_s^{-1} vs surface pressure that are derived from π -A isotherms.²¹¹ C_s^{-1} is a measure of the

changes in monolayer elasticity, which is indicative by the phase transitions of the monolayer. Consequently, the compressibility of the monolayer, and therefore its phase, can be determined using the following equation:

$$C_s^{-1} = -A \left(\frac{\partial \pi}{\partial A} \right)_T \quad \text{Equation 1.5-2}$$

where A is the mean molecular area, T is the temperature, and π is the surface pressure.²¹⁴ For example, C_s^{-1} values less than 12.5 mN/m represent a gaseous state, values between 12.5–50 mN/m represent a liquid-expanded state, values between 50–100 mN/m represent a liquid condensed state, and values greater than 250 mN/m indicate a solid state.²¹³

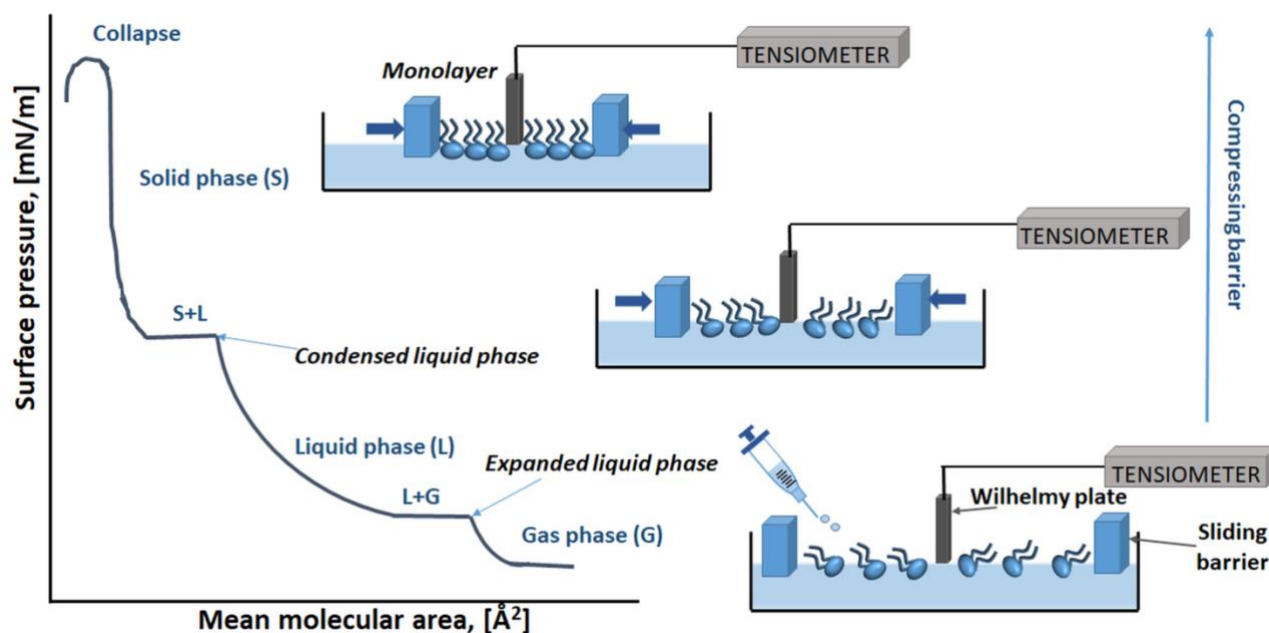


Figure 1.5-3: Theoretical surface pressure-area (π - A) isotherm obtained by compressing a monolayer formed at the air-water interface in different phases. Adapted from reference ²¹².

1.6 Rational Non-Viral Vector Design Must Consider the Transfection Efficiency and Safety of Both the Synthetic Vector and the DNA Cargo

Compared to transfection formulations prepared with pDNA, the small sizes of minicircles and LCC DNA minivectors allows for efficient DNA complexation with synthetic vectors, protection against intracellular and extracellular environments and improved bioavailability.²¹⁵ Although lipoplex size and structure do not vary with increasing pDNA length, the number of pDNA copies incorporated into each lipoplex increases linearly with decreasing pDNA size.^{215,216} Transfection activity of lipoplexes is proportional to the number of enclosed pDNA with the most active lipoplexes possessing the largest amounts of DNA.²¹⁷ To showcase this, Kreiss et al. evaluated and compared the physicochemical properties of various cationic lipid-pDNA lipoplexes with pDNA sizes ranging from 900 to 52 500 bp. It was determined that smaller pDNA attained higher transfection efficiency over larger pDNA when lipoplexes were restricted to possess a single coding cassette in each particle. In other words, the most effective lipoplexes for gene transfer are those containing the shortest plasmid DNA.²¹⁷

With respect to the efficiency of cell uptake between the circular and linear DNA forms, Kamiya et al. did demonstrate that LCC vectors elicit a 5-10-fold increase in transgene expression over CCC pDNA counterpart after microinjection of vectors into the nucleus of COS-7 cells for up to 14 days' post injection. In the same study, size dependent effects on transgene expression were also observed as the GFP expression efficiency increased with decreasing DNA, with the smallest tested LCC DNA minivectors (2.3 kb) showing the highest expression.^{216,217} These results indicate that the most effective complexes for gene transfer are small, linearized DNA constructs that are end capped with hairpin loops.²¹⁷

As previously discussed, efficient non-viral formulations require the rational design of both the DNA cargo as well as the synthetic vector. For gene therapy applications, positively charged carriers are used to complex DNA into nanoparticles to protect the therapeutic gene from enzymatic degradation and safely deliver the DNA into the nucleus for transcription to initiate.¹⁰ Key to improving the transfection efficiency of non-viral vectors is by understanding the structural features of both the synthetic vector and the enclosed DNA cargo and characterizing their physicochemical properties, including particle size, zeta potential, polydispersity index, encapsulation efficiency, and shape as summarized in **Figure 1.6-1**. These properties also contribute to the safety profile of the gene therapy nanoparticles. For example, while safer than

their viral counterparts, non-viral transfection complexes often require a net positive charge for improved cell uptake. This is balanced by the fact that highly cationic materials in the circulatory system can cause toxicity by inducing platelet aggregation in the blood.²¹⁸ For example, synthetic vectors which contain an ether bond in its chemical structure have been shown to increase *in vitro* transfection. However, ether bonds are associated with high cytotoxicity since they are too stable for biodegradation.²¹⁹ On the other hand, synthetic vectors which contain biodegradable amide and ester bonds are associated with less cytotoxicity as they are more susceptible to decomposition in the systemic circulation.²¹⁹ Additionally, recall that the preparation of transfection formulations is guided by the N^+/P^- charge ratio, and it has been observed that although increasing the charge ratio favours DNA compaction and enhanced cellular uptake, it also may lead to cytotoxicity.¹⁸ For example, in study which focused on optimizing 16-3-16/DOPE/pDNA formulations for *in vitro* transfection to PAM 212 murine keratinocytes cells, it was revealed that an N^+/P^- ratio of 10:1 was ideal based on both transfection efficiency and cell viability. Although, a 40:1 N^+/P^- ratio showed the highest percentage of transfected cells, the cell viability was compromised as it was reduced to 20%.⁷⁰ Therefore, a balance between transfection efficiency and cytotoxicity is essential for a more rational design of N-VGDS.

The aim of this thesis is to find this correct balance by designing and characterizing several non-viral gene delivery components by using various techniques in an attempt to predict which structural features of the synthetic vector *and* DNA constructs are essential to promote the release of the DNA cargo from the particles, while not compromising the safety profile of the vector.

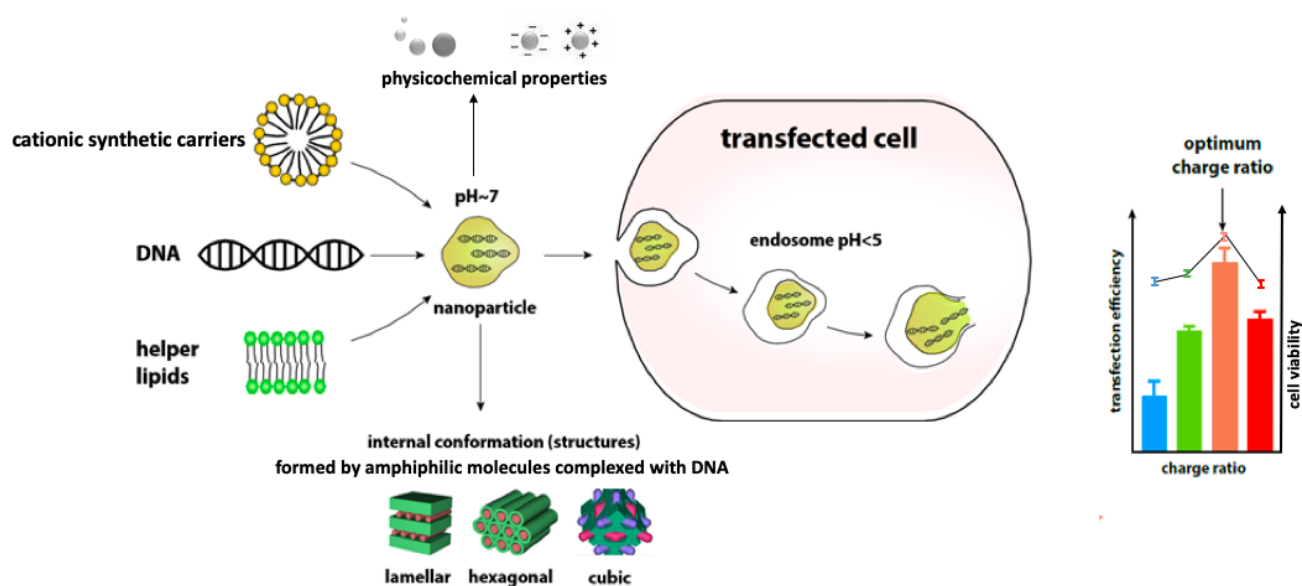


Figure 1.6-1: High level summary of important elements to consider when developing efficient non-viral vectors for gene therapy applications. Rational vector design considers the structural features of both the cationic synthetic carrier and the DNA cargo. These formulations must then be characterized (through physical, chemical, and *in vitro* methods) to ultimately select an optimal sample which not only reveals high transfection efficiency, but also high cell viability. Reproduced from ²²⁰ with permission (See Appendix D).

Chapter 2

Project Aims, Hypothesis, and Objectives

2.1 Rational Design of Non-Viral Gene Delivery Vectors Requires Understanding of the Structural Features and Molecular Interactions Between the Components

Compared to viral methods, non-viral gene delivery is less immunogenic and less toxic, but the transfection efficiency of these vectors is limited. The design of the components that make up the non-viral vector, including both the synthetic carrier **and** enclosed DNA cargo, significantly influences the properties of the system and consequently transfection efficiency. Major barriers faced by non-viral gene therapy to deliver DNA to targeted cells include a lack of understanding of the molecular interactions and structural properties of vector components and their relationship to transfection efficiency. To make informed decisions regarding the optimization of N-VGDS, it is essential to define these features and also consider safety alongside efficacy.

Among the different physical and chemical systems, various lipid and polymer-based synthetic vectors have shown the capacity to overcome physiological barriers due to their chemical composition which favour transfection. For example, lipid materials containing ether linker bonds and polymers which comprise pH-sensitive functionalities have shown to produce greater transfection *in vitro*.¹ Additionally, the rational design of DNA vectors also serves to further improve therapeutic outcomes, allowing for the development of vectors with heightened safety profile while achieving transfection efficiency that is comparable to their viral counterparts. CCC pDNA vectors, alone or when packaged within lipid or polymer synthetic vectors, have revealed a limited safety profile as they often result in the transfer of antibiotic resistance genes as well as other unwanted prokaryotic sequences with CpG motifs.² As an alternative, LCC DNA minivectors are genetically designed to improve intracellular delivery of the DNA cargo leading to enhanced transgene expression. In addition, LCC DNA minivectors offer a heightened safety as they inhibit insertional mutagenesis through covalently closed ends conferring double-strand breaks that cause chromosomal disruption and cell death in the event of chromosomal integration.³ A deeper understanding of the influences of DNA size, topology, physiochemical properties and their relationship to *in vitro* transfection efficiency of formulations prepared with various lipid and

polymer-based synthetic vectors complexed with LCC DNA minivectors is important to consider. These studies can be used to predict which structural characteristics of synthetic vectors favour the release of the DNA cargo from the nanoparticles, ultimately allowing for a more rational design of non-viral gene therapy formulations.

Additionally, gemini surfactants and the helper lipid DOPE possess inherent properties that make them suitable delivery vehicles. Previous studies have revealed that lipoplex formation and their performance in *in vitro* and *in vivo* transfection experiments are strongly influenced by the electrostatic interactions between its components.⁴ The molecular interactions governing several aspects of GS-DOPE liposome mediated transfection, such as the complexation and condensation of DNA by GS⁵⁻⁹ and by DOPE¹⁰ as well as the disruption of cellular membranes by GS-DOPE lipoplexes^{11,12} are well understood. Additionally, a previous comparative study evaluated the physical properties and transfection efficiencies of the GS-DOPE-LCC DNA minivector lipoplexes. Overall low gemini surfactant-mediated transfection efficiencies (13% cells transfected) in OVCAR-3 cell lines were achieved with this system.¹³ To provide insight into these results, it is important to investigate the molecular interactions and surface miscibility of specifically GS-DOPE (in the absence of LCC DNA minivectors) which is currently unknown. The structural activity of the resulting lipoplexes would further contribute to the rational design of novel GS-DOPE-LCC DNA minivectors as synthetic vectors catered to gene therapy.

2.2 Project Aims

The work presented in this thesis focuses on exploring the molecular interactions and structural features of non-viral vector components, including various synthetic vectors (lipids or polymers) and DNA constructs (CCC pDNA or LCC DNA minivectors), and characterizing their behaviour whether through evaluation of physicochemical properties, *in vitro* transfection efficiency, or Langmuir monolayer methods, to select an appropriate vector combination which not only exhibits improved efficacy but at the same time exerts minimal or no toxicity. These results will impact future research which focuses upon the rational design of synthetic vectors incorporating LCC DNA minivectors for gene delivery applications.

2.3 Hypothesis

This thesis consists of two distinct yet related projects: 1) an examination of the combined effects of DNA construct **and** synthetic vector (specifically CCC pDNA vs LCC DNA minivector **and** lipid- or polymer-based vector); and 2) a continuation of our groups work using the GS as a tool to probe lipoplex structure through rational modification to the GS structure. While a single unifying hypothesis that encompasses both projects is challenging to express, both projects imply that a detailed examination of the properties of, and the careful selection of both the carrier component (polymer or lipid) AND the DNA component will lead to the development of non-viral gene delivery formulations capable of improved transfection efficiency.

2.4 Objectives

- 1) Investigate the influences of DNA size and topology on physicochemical properties and transfection efficiency of Lipofectamine 3000 and Lipid 2 incorporating either LCC DNA minivectors or the pDNA (CCC) counterpart
 - (i) Characterize lipoplexes by size, zeta potential, and polydispersity index
 - (ii) Determine the *in vitro* transfection efficiency and cytotoxicity of lipoplexes in HEK 239 and ARPE-19 cell lines
- 2) Determine whether there is a relationship between physicochemical properties and transfection efficiency with formulations prepared with polymer-based vectors (Polymer 1 and Polymer 2) incorporating either LCC DNA minivectors or CCC pDNA
 - (i) Characterize all polyplexes by particle size, zeta potential, and polydispersity index
 - (ii) Determine the *in vitro* transfection efficiency as well as cytotoxicity of each polyplex mixture in HEK 239 and ARPE-19 cell lines
- 3) Synthesize 18-7-18 and 18-7NH-18 GS and investigate the nature of the intermolecular interactions and the surface miscibility behaviour of GS-DOPE binary mixtures (in the absence of LCC DNA minivector) as well as the effects of mixture composition, GS structure, and pH on model biological membranes using Langmuir Monolayer Methods.

Chapter 3

Influence of DNA Size and Topology on Physicochemical Properties and Transfection Efficiency of Lipid-Based Vectors Containing Linear Covalently Closed (LCC) DNA Minivectors

The work in this chapter is the result of a pharmaceutical collaboration. It contains and utilizes a number of proprietary systems. While the introduction will provide context to the results and a relationship to current knowledge in a particular field, the discussion of specific names, chemical structures, and manufacturing processes of the lipid and DNA constructs cannot be disclosed.

3.1 Abstract

Most recently, lipid nanoparticles, specifically those containing ionizable lipids, have been proven to be efficient non-viral vehicles for mRNA-based delivery. This is due to their unique properties of: 1) efficiently encapsulating the DNA cargo; 2) protecting the gene from extracellular and intracellular degradation; and 3) improving the overall delivery of the therapeutic by enabling endosomal escape which allows for the release of the mRNA cargo into the cytoplasm for translation to initiate. However, efficient non-viral DNA-based gene therapy not only require the rational design of the synthetic carrier, but also the enclosed DNA cargo, an area that is often overlooked. Conventional plasmid DNA vectors are most commonly used for transfection and have a significant role in gene therapy; however, the presence of prokaryotic DNA sequences, such as unmethylated CpG motifs and antibiotic selective markers in plasmid DNA vectors lower their biocompatibility and safety for clinical studies. In this comparative investigation, the commercially available reagent Lipofectamine 3000 and the proprietary Lipid 2 (a lipid-based synthetic vector) incorporating either circular covalently closed (CCC) pDNA or linear covalently closed (LCC) DNA minivectors were characterized and compared with respect to particle size, polydispersity index, zeta potential, and *in vitro* transgene delivery efficacy. Despite the inherent differences in DNA size and topology, comparable physicochemical properties were observed. Even with these similarities, LCC DNA minivectors in combination with Lipofectamine 3000 have shown to exhibit improved gene delivery and expression over CCC pDNA counterparts while offering a heightened safety profile.

3.2 Introduction

Lipid nanoparticles (LNP) have been developed and strategically designed as non-viral vectors to treat genetic and acquired disorders in gene therapy.¹ In the presence of DNA, lipids (generally composed of a hydrophobic tail group and a hydrophilic head group) aggregate into clusters known as micelles which offers a feasible means of minimizing the free energy of the systems.² Micellization allows the encapsulation of DNA inside the micelle core, while the hydrophobic tail groups are removed from the water to form the hydrophobic core, and the hydrophilic head groups face outwards to form a shell that remains in contact with water.³ Many LNPs are made up of ionizable lipids, which are positively charged at low pH (enabling efficient complexation of the therapeutic gene) and neutral at physiological pH (reducing potential toxic effects compared to positively charged liposomes).³ The ionizability of the lipids at low pH enables endosomal escape, which allows release of the cargo into the cytoplasm.⁴ The commercially available transfection reagent Lipofectamine (consisting of the helper lipid DOPE and DOSPA) is a notable example of a lipid gene delivery system which is widely accepted as a “gold standard” for the safe and efficient delivery of exogenous DNA or RNA into a broad range of cell lines.⁵ Lipofectamine contains lipid subunits with a pH-dependent transition due to the polyamine region of DOSPA which can form LNPs in an aqueous environment and entrap the therapeutic gene to form a lipoplex with a net positive charge.⁵ This positive charge enhances electrostatic interactions with the negatively charged cellular membrane of cells and improves cellular uptake.⁶ LNPs are also biocompatible in nature which accounts for their most important advantages as gene carriers, including: 1) low toxicity and antigenicity compared to viral vectors; 2) their ability to degrade and be metabolized *in vivo*, and 3) control of membrane permeability.⁷ As such, they can self-assemble in order to encapsulate and protect the therapeutic gene on their journey to the target site.² More recently, lipid nanoparticles have successfully been utilized for the delivery of mRNA to cells and are now in clinical use against coronavirus disease 2019 (COVID-19), an exciting milestone for non-viral gene therapeutics.⁸

While the nature of the cationic component is vital for improved cellular intake and endosomal escape, the overall transfection efficiency, duration of expression, and cell-specific protein expression are all dictated by the design of the therapeutic gene.⁹ Conventional recombinant plasmid DNA are circular covalently closed (CCC) vectors which typically consists

of a eukaryotic expression cassette and a prokaryotic backbone.¹⁰ This prokaryotic backbone comprises bacterial sequences and CpG motifs which attribute to several safety and efficiency limitations: 1) it induces cytotoxic reactions to the host immune system; 2) it compromises the bioavailability of the vector due to its size; 3) it may be genotoxic due to its potential to integrate into the host chromosome, thus potentiating oncogenesis.¹⁰ Removal of the prokaryotic backbone in the new generation of linear covalently closed (LCC) DNA minivectors not only eliminated the delivery of unwanted prokaryotic sequences known to induce immune responses, but also allowed for the formation of smaller vectors which allows for efficient DNA packaging into synthetic vectors and rapid intracellular trafficking upon cytoplasmic release.¹¹⁻¹⁵

The parental CCC pDNA is composed of a eukaryotic expression cassette enclosed by two target sites which act as recognition sites for bacteriophage derived Tel protelomerase.¹² Upon *in vivo* expression of Tel protelomerase and following enzymatic activity for excision of the covalently closed terminal ends, the parental CCC pDNA is converted into two small fragments: 1) a LCC backbone DNA carrying the prokaryotic backbone, and 2) a LCC DNA minivector.¹⁶ LCC DNA minivectors carry a minimal expression cassette consisting of the eukaryotic promoter, gene of interest, intron, and polyA sequence, and nuclear translocation enhancing sequences, which significantly reduces their size compared to conventional pDNA.^{12,16} Although the specific manufacturing process of the small dumbbell-shaped LCC DNA minivectors utilized in this study is proprietary, the reader is referred to a previously described a one-step inducible *in vivo* system for the production of LCC DNA minivectors in *E. coli*.^{12,16}

When designing vectors for gene therapy applications, it is important to consider the size as well as the topology, or the intramolecular shape (circular vs. linear), of the DNA component. LCC DNA vectors were shown to exhibit enhanced transgene expression over CCC pDNA counterparts as demonstrated by cytoplasmic and nuclear microinjections along with transfection using Lipofectamine.^{12,17} Microinjection of LCC vectors into the nucleus of COS-7 cells elicited a 5-10 fold increase in transgene expression relative to its CCC pDNA counterpart.¹⁷ Further Lipofectamine-mediated transfection studies involving cytoplasmic microinjection also demonstrated improved gene expression with LCC DNA minivectors compared to its CCC pDNA counterpart.¹⁷ These results revealed a relationship between nucleic acid size and transgene expression as higher transgene expression was correlated with decreasing LCC vector sizes with the highest expression achieved with the smallest LCC DNA minivector (2.3 kb).¹⁷ Similar results

were obtained in a comparative study where 16-3-16 gemini surfactants incorporating either LCC DNA minivectors or CCC parent plasmid shows minimal differences with respect to physicochemical properties or gemini-mediated transfection efficiencies.¹⁸ However, statistically significant increase in transfection was achieved with LCC DNA minivectors complexed with the positive control Lipofectamine with approximately 19% cells transfected.¹⁸ These studies reveal that nucleic acid size and topology do have an influence on transgene expression and should be defined for every novel transfection formulation. Consequently, to provide further insight into the interactions between LCC DNA minivectors and lipid-based carriers, we collaborated with a pharmaceutical partner and developed a comparative analysis study to evaluate the influence of LCC and CCC DNA size and topology and compare the physical properties and transfection efficiency of the resulting lipoplexes after complexation with either the commercially available reagent Lipofectamine 3000 or the proprietary Lipid 2. Interestingly, despite the size and DNA topology disparities between the 6.3 kb CCC pDNA vectors and 3.2 kb LCC DNA minivectors, lipoplexes of comparable hydrodynamic diameters, polydispersity index, and zeta potential measurements were formed when assessed in deionized water. However, LCC DNA minivectors exhibited improved *in vitro* transfection efficiency when complexed with either Lipofectamine 3000 or Lipid 2 compared to their parental CCC counterparts.

3.3 Materials

Lipofectamine 3000 (Invitrogen) was purchased from Thermo Fisher Scientific (Burlington, ON, Canada). The Lipid 2 is a proprietary carrier and was received through a collaboration with a pharmaceutical partner. The synthesis process, size, structure, and the molecular weight of Lipid 2 is proprietary to the partner.

The CCC parental pDNA and the bacterial sequence-free LCC DNA minivectors carrying the enhanced green fluorescence protein (*eGFP*) expression cassette downstream of a (CAG) promoter was produced via the proprietary manufacturing platform of the pharmaceutical partner. Information on the manufacturing process of these DNA constructs is proprietary to the company.

Physicochemical characterization and transfection experiments used deionized water which was purchased from Thermo Fisher Scientific (Burlington, ON, Canada).

HEK 293 (human embryonic kidney) cells (ATCC CRL-1573) and ARPE-19 (arising retinal pigmented epithelium) cells (ATCC-CLR-2302) were purchased from American Type Culture

Collection, ATCC (Manassas, VA, USA). Dulbecco's Modified Eagle Medium (DMEM) formulated with high glucose and pyruvate (Gibco), DMEM/F-12 without phenol red (Gibco), Canadian-origin fetal bovine serum (FBS) (Gibco), Opti-MEM I Reduced Serum Medium (Gibco) 0.25% Trypsin-EDTA with phenol red, Penicillin-Streptomycin 100X (10,000 U/mL) (Gibco), 15 mL falcon tubes, 75cm² and 175cm² Nunc 114 EasYFlask cell culture flasks and cell culture coated 24-well plates (Nunc, Thermo Scientific) were purchased from Thermo Fisher Scientific (Burlington, ON, Canada).

3.4 Methods

3.4.1 Preparation of transfection lipoplexes

The preparation of the transfection lipoplexes was dictated by the N⁺/P⁻ ratio, the ratio of the number of cationic charges of the lipid nanoparticles to the number of anionic charges of the DNA molecule. Equal amounts (by weight) of LCC DNA minivectors and CCC parent plasmid were used to prepare all samples (see **Table 3.4-1**). All stock solutions and solvents were filtered through a sterile 0.22 μ m polyethersulfone (PES) syringe filter (Sartorius) prior to use.

Lipofectamine 3000 lipoplexes were made at a 2:1, 4:1 and 8:1 N⁺/P⁻ charge ratio in deionized water. Briefly, the transfection lipoplexes were prepared by mixing 1 μ g of diluted nucleic acid into different aliquots of the Lipofectamine 3000 stock solution (2 μ L, 4 μ L, and 8 μ L) all at once. The solution was vortexed immediately and spun down briefly. The mixture was then allowed to incubate for 15 minutes at room temperature.

Lipid 2 formulations were prepared by mixing 1 μ g of diluted nucleic acid into different aliquots of the lipid stock solution all at once. The solution was vortexed immediately and spun down briefly. The mixture was then allowed to incubate for 30 minutes at room temperature to allow complexation.

Table 3.4-1: Preparation of LCC DNA minivectors and CCC pDNA-derived lipoplexes

DNA construct	Nucleic acid Size (Base Pair)	Molarity (pMol) per 1 ug	Molarity (pMol) per N ⁺ /P ⁻ ratio		
			2:1	4:1	8:1
LCC DNA minivector	3170	0.48	0.96	1.91	3.82
CCC pDNA	6280	0.24	0.48	0.97	1.93

3.4.2 Physicochemical characterization of lipoplexes

Hydrodynamic diameter, polydispersity index (PDI), and zeta potential analyses were performed on all Lipofectamine 3000/DNA and Lipid 2/DNA lipoplexes using a Zetasizer Nano-ZS set to 25°C (Malvern Nano series instruments, UK). Particle sizes and PDI in deionized water were determined using the scattered light intensity distribution from dynamic light scattering with a measurement angle of 173°. Disposable Solvent Resistant Micro Cuvettes (ZEN0040) used for particle size and PDI were primed with the sample before loading. Zeta potential in deionized water was determined from laser Doppler electrophoresis. Disposable folded capillary cells (DTS1070) used for zeta potential were primed with the sample before loading. 700 μ L sample was loaded via syringe into a primed Disposable folded capillary cells (DTS1070) and inserted into the cuvette holder in the Malvern Zetasizer Nano-ZS set to 25°C (Malvern Nano series instruments, UK). All samples were freshly prepared, as previously described, immediately prior to measurement. Three individual experiments were completed per study, and each experiment included three independent measurements per sample. The results are expressed as the mean of three separate experiments with three replicates (i.e. means of means) $\pm$ the standard deviation. Uncomplexed DNA (samples without lipid) served as a positive control group for the physicochemical characterization experiments.

3.4.3 Cell culture

HEK-293 cells were cultured with DMEM (with pyruvate and high glucose) supplemented with 10% (v/v) FBS, while ARPE-19 cells were cultured with DMEM/F-12 without phenol red supplemented with 10% (v/v) FBS and 1X penicillin-streptomycin. Cells were incubated at 37°C with 5% carbon dioxide in a tissue culture incubator (Thermo Scientific). Cells were sub-cultured

at least once before use in any transfection and viability experiments. Experiments were carried out with cultures having passage numbers 4 to 10.

3.4.4. *In vitro* transfection assay

Cells were grown at 37°C with 5% carbon dioxide and maintained at 70-90% confluency in 75cm² tissue culture flasks prior to transfection. To initiate transfection experiments, cells were seeded into 24-well plates at a density of 100, 000 cells/well (HEK 293 cells) and 200, 000 cells/well (ARPE-19 cells) and were incubated at 37°C for 24 h before transfection in complete media without antibiotics. One hour before transfection, cells were washed with 1X DPBS and the culture medium was replaced with serum-free medium. Meanwhile, lipoplexes and controls were prepared freshly under as described above. All stock solutions were filtered through a sterile 0.22 μ m PES syringe filter (Sartorius) before use. An appropriate volume of the transfection nanoparticles was added to each well to achieve a plasmid dose of 1 μ g/well. To ensure consistency in plasmid and lipid concentrations between wells, first a volume of medium was removed from each well, then an equal volume of the transfection treatment was added to the wells dropwise. The plates were then rocked to ensure even distribution in the wells before being returned to the incubator. Plates were then incubated at 37° C for 5 hours before the transfection medium was replaced by fresh DMEM or DMEM/F-12 with 10% FBS without antibiotic. Cells were then further incubated at 37° C until 48- hours post-transfection at which point the cells were collected, washed, and resuspended in DPBS. Cells were then harvested for flow cytometry analysis to assess transgene (EGFP) expression and cell viability. Prior to harvesting, the medium was collected from each well and combined with the corresponding harvesting cells.

To select the appropriate positive control group, Lipofectamine 3000 was complexed with LCC DNA minivector pDNA CCC constructs and formulations were prepared in Opti-MEM Medium by following the manufacturer's recommendations and evaluated at three N⁺/P⁻ charge ratios: 2:1, 4:1 and 8:1.¹⁹ Optimization of these samples revealed that highest transfection efficiencies were achieved when formulations were prepared at a ratio of ratio of 1 μ g DNA to 4 μ L of Lipofectamine 3000 (data shown in Table B-1 and Figure B-3 of Appendix B). To ensure consistency between the control and test treatments, a final plasmid dose of 1 μ g/well was used.

All transfection treatments were freshly prepared one hour prior to transfection. Additionally, three independent experiments were completed per cell line and each experiment

included three independent measurements per sample to ensure consistency. The results are expressed as the mean of three separate experiments with three replicates (i.e. means of means) $\pm$ the standard deviation.

3.4.5 Flow cytometry analysis

After 48 hours of transfection, the supernatant medium was collected from each well, then cells were rinsed with 250 μ L of DPBS, detached with 1 mL 0.25% Trypsin-EDTA for 5 minutes followed by neutralization with 2 mL of complete medium. The cells were then collected in a 5 mL round bottom polystyrene tubes (Falcon, Corning Life Sciences) and centrifuged at 4°C for 10 minutes at 125xg (Sorvall Legend RT, Thermo Scientific). The supernatant was then aspirated, and the cells were washed twice with 2 mL DPBS after centrifugation. The final cell pellets were then re-suspended in DPBS and stored on ice. Five minutes before flow cytometry analysis, 10 μ L of a 1 mg/mL propidium iodide (PI) solution (Sigma Aldrich) was added to each tube. Samples were analyzed using a Beckman Coulter CytoFlex S Flow Cytometer (School of Pharmacy, University of Waterloo). Samples were excited with a 488 nm laser, and 10,000 events analyzed per sample. Untreated cells (i.e. cells that did not receive any treatment) and cells that were not stained with PI were used as cell controls. Data files were subsequently analyzed using CytExpert software.

3.4.6 Statistical analysis

Graphs were plotted and statistically analyzed using the Graphpad Prism 6 software. Statistical analysis comparing treatment results to a control group were determined by one-way analysis of variance (ANOVA) and a dependent, two-tailed, two sample Student's t-test. Test assumptions included a normal distribution with equal variance at 95% confidence intervals, where values of $p < 0.05$ were considered statistically significant. Each error bar represents the standard deviation of three independent experiments, where each experiment consisted of three individual replicates per sample.

3.5 Results

3.5.1 Physicochemical characterization of nanoparticles

The mean hydrodynamic diameters, polydispersity index (PDI), and zeta potential of both uncomplexed and complexed nanoparticles in deionized water were evaluated at 25°C using a Malvern Zetasizer Nano-ZS (Malvern Nano series instruments, UK). For hydrodynamic diameter and PDI measurements, samples were diluted 100X with deionized water. Results for the uncomplexed materials are shown in **Table 3.5-1** and the results for the lipoplexes are displayed in **Table 3.5-2** and illustrated in **Figure 3.5-1** and **Figure 3.5-2**.

The results in **Table 3.5-1** shows that despite the differences in size and topology between the 6.3 kb CCC pDNA and 3.2 kb LCC DNA minivector, when equal amounts (by weight) of each nucleic acid is used, only very slight changes in mean hydrodynamic diameters were observed. These differences though small may be attributed to the structural nature of the two DNA constructs, with linear DNA minivectors contributing to more compact conformations than their circular counterparts. With respect to zeta potential, the LCC DNA minivector exhibited a greater (-) value compared to the pDNA (CCC). On the other hand, the PDI of all uncomplexed nanoparticles ranged between 0.30 – 0.57 indicating substantial polydispersity due to the formation of nanoparticles that are highly variable in size.

Table 3.5- 1: Mean hydrodynamic diameter, polydispersity index (PDI), and zeta potential of uncomplexed LCC DNA minivectors, CCC pDNA Lipofectamine 3000, and Lipid 2 prepared in deionized water. Mean values $\pm$ the standard deviation for three experiments, each experiment consisting of three individual replicates per sample are reported.

	Mean hydrodynamic diameter (nm)	Mean PDI	Mean Zeta Potential (mV)
LCC DNA minivector	324 $\pm$ 10	0.39 $\pm$ 0.01	- 32 $\pm$ 5
CCC pDNA	360 $\pm$ 9	0.43 $\pm$ 0.02	- 26 $\pm$ 2
Lipofectamine 3000	212 $\pm$ 9	0.30 $\pm$ 0.02	31 $\pm$ 3
Lipid 2	321 $\pm$ 4	0.57 $\pm$ 0.03	45 $\pm$ 2

The preparation of the lipoplexes were dictated by the N^+/P^- charge ratio. Typically, an excess amount of the cationic lipid component is required to achieve complete neutralization of the negatively charged phosphate group of DNA to effectively complex the therapeutic gene. As such, lipoplexes were formulated at a 2:1, 4:1, and 8:1 N^+/P^- charge ratio via electrostatic and hydrophobic interactions between the cationic lipid carrier and the negatively charged DNA constructs.

Table 3.5-2 and **Figure 3.5-1** show the mean hydrodynamic diameters and PDI results of the Lipofectamine/DNA and Lipid 2/DNA lipoplexes. Like the trend observed with the uncomplexed materials, minimal differences in mean hydrodynamic diameters with lipoplexes prepared with either LCC DNA minivector and CCC pDNA were seen. Compared to the lipoplexes prepared with Lipid 2, samples formulated with Lipofectamine 3000 formed relatively small nanoparticles (between 111 – 158 nm) and more uniform particles as indicated by a PDI of less than 0.3 for all three charge ratios. Based on the literature, gene delivery nanoparticles that form nanoparticles smaller than 200 nm with a uniform particle size distribution (PDI typically less than 0.3) are optimal as they have shown to increase cell uptake and DNA release from the endosome.²⁰ Additionally, small nanoparticles have also shown high transfection efficiency *in vitro* and *in vivo* due to their ability to cross capillary networks, for example in the spleen, lungs, liver, and kidney.²¹ As such, these threshold values are important and were taken into consideration when designing these lipoplexes in order to avoid degradation and for efficient DNA delivery to the nucleus. **Figure 3.5-1** illustrates the trend between the mean hydrodynamic diameter, and PDI for all lipoplexes. Overall, the hydrodynamic diameter decreased as the N^+/P^- charge ratio increased. These results were expected and align well with what has been seen in previous literature reports. The larger hydrodynamic diameter exhibited at lower charge ratios are likely the result of aggregation upon charge neutralization and subsequent addition of more of the cationic lipid carrier component at 4:1 and 8:1 N^+/P^- charge ratios, resulted in a decrease in hydrodynamic diameter with lower PDIs.

Table 3.5-2: Mean hydrodynamic diameter, polydispersity index (PDI), and zeta potential of Lipofectamine 3000 and Lipid 2 prepared in deionized water at varying N⁺/P⁻ ratio measured at 25°C. Mean values ± the standard deviation for triplicate experiments, each experiment consisting of three individual replicates per sample are reported.

Lipid	DNA added	N⁺/P⁻ ratio	Mean hydrodynamic diameter (nm)	Mean PDI	Mean Zeta Potential (mV)
Lipofectamine 3000	no DNA added	0	212 ± 9	0.30 ± 0.02	31 ± 3
	LCC DNA minivector	2:1	144 ± 4	0.26 ± 0.02	15 ± 1
		4:1	121 ± 5	0.16 ± 0.02	17 ± 2
		8:1	111 ± 7	0.13 ± 0.04	23 ± 2
	CCC pDNA	2:1	158 ± 5	0.29 ± 0.04	17 ± 3
		4:1	125 ± 4	0.17 ± 0.01	23 ± 2
		8:1	122 ± 7	0.15 ± 0.02	29 ± 1
Lipid 2	no DNA added	0	321 ± 4	0.57 ± 0.03	45 ± 2
	LCC DNA minivector	2:1	224 ± 5	0.33 ± 0.02	21 ± 3
		4:1	152 ± 8	0.24 ± 0.02	27 ± 1
		8:1	144 ± 8	0.17 ± 0.01	28 ± 2
	CCC pDNA	2:1	321 ± 9	0.43 ± 0.01	24 ± 2
		4:1	209 ± 11	0.29 ± 0.01	29 ± 1
		8:1	153 ± 6	0.17 ± 0.02	39 ± 1

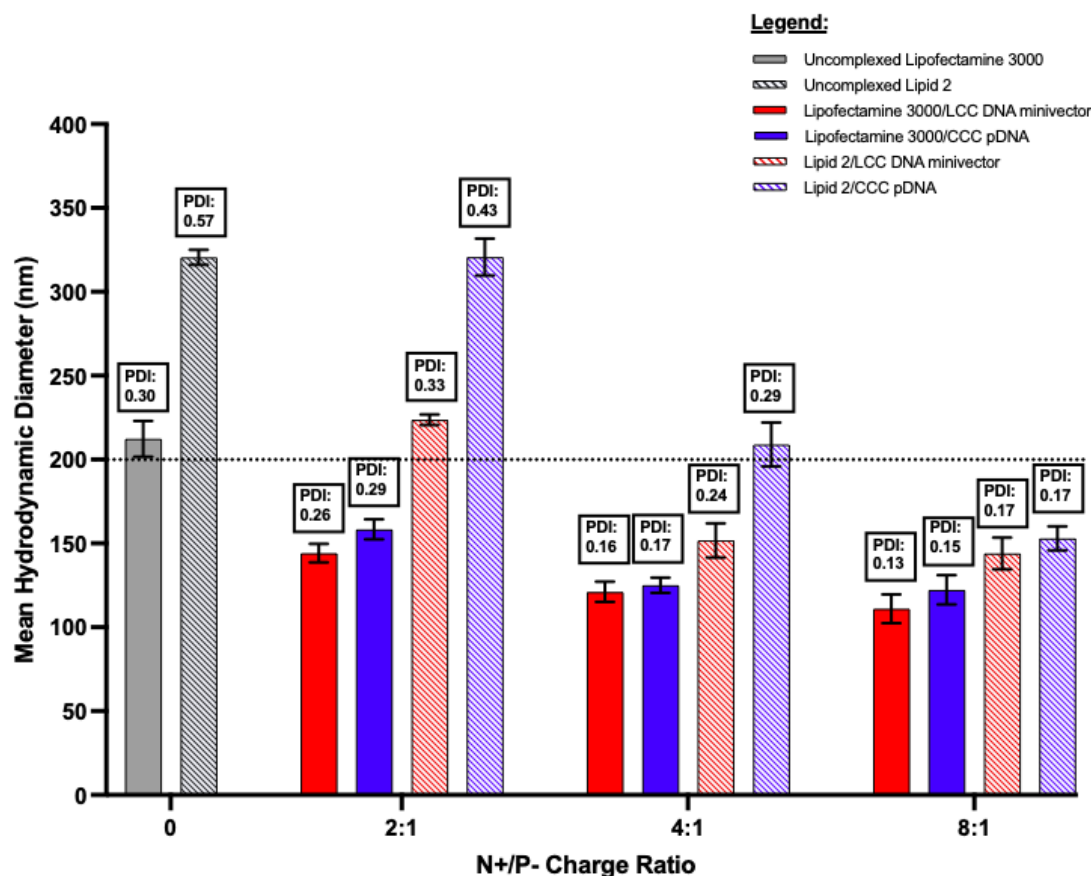


Figure 3.5-1: Mean hydrodynamic diameter and polydispersity indices (PDIs) of uncomplexed lipids and lipid/LCC DNA minivector or lipid/CCC pDNA complexes prepared at varying N⁺/P⁻ ratios of each lipid upon 100X dilution with deionized water. Error bars represent the standard deviation of triplicate experiments, each experiment consisting of three individual replicates per sample. The dotted line represents the hydrodynamic diameter threshold for an optimal gene delivery system (200 nm).

High zeta potential values, whether negative or positive, are indicative of high stability as electrostatically repulsive forces exceed attractive forces, the latter of which lead to coagulation or flocculation and thus, physical instability. For *in vivo* gene delivery application, values above +20 mV are used as a threshold for particle stability.²¹ **Table 3.5-2** and **Figure 3.5-2** show the zeta potential measurements of the Lipofectamine/DNA and Lipid 2/DNA lipoplexes. Interestingly, despite the differences in zeta potential values seen in **Table 3.5.1** with the uncomplexed DNA constructs, lipoplexes formed by LCC DNA minivector and CCC pDNA exhibited comparable zeta potential measurements. Additionally, all lipoplexes showed a very clear trend: the zeta

potential increased linearly as the N^+/P^- charge ratio increased. These results were expected because theoretically as the N^+/P^- ratio increases from 2:1 to 4:1 to 8:1, the amount of the cationic lipid nanoparticles required to complex the DNA construct also increases, resulting in the presence of excess lipid cations in the solution that remains uncomplexed in the solution and attributes to an increase in zeta potential.²² All complexed nanoparticle formulations show zeta potentials between the ranges of 15 – 39 mV which is generally associated with high colloidal stability arising from electrostatic repulsive forces.²² Compared to samples prepared with Lipid 2, Lipofectamine 3000 formulations resulted in a slightly lower zeta potential, presumably due to the shielding of the negatively charged DNA phosphate group by the cationic lipid.

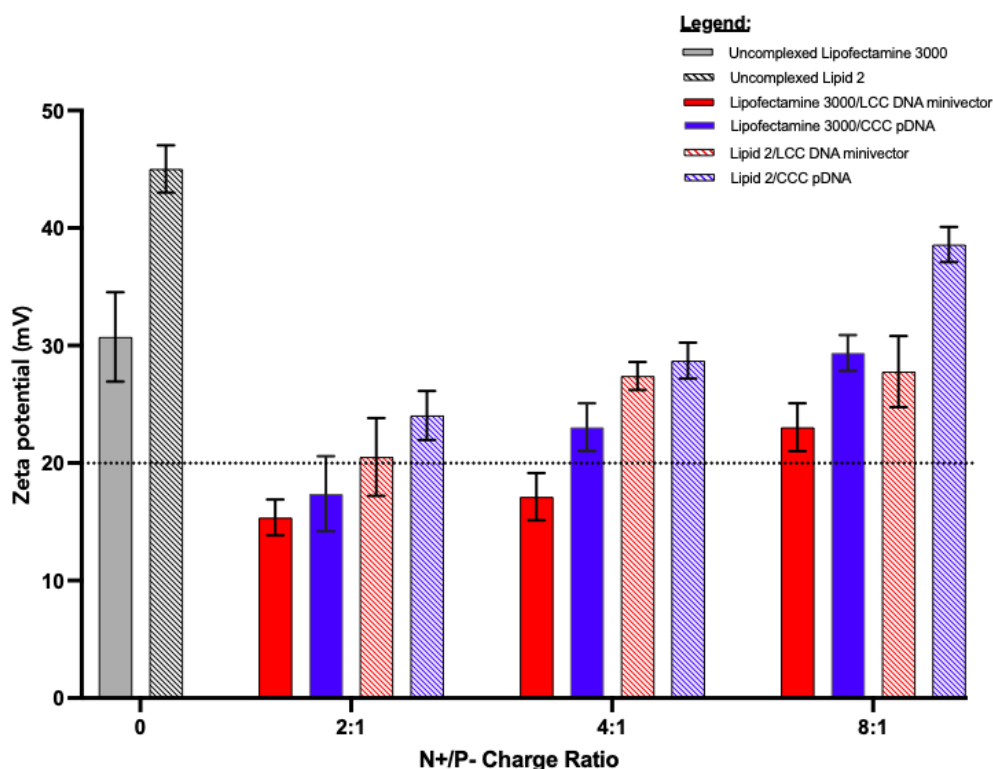


Figure 3.5-2: Mean zeta potential of uncomplexed lipids and lipid/LCC DNA minivector or lipid/CCC pDNA complexes upon 100X dilution with deionized water at varying N^+/P^- ratios of each lipid. Error bars represent the standard deviation of three independent experiments, each experiment consisting of three individual replicates per sample. The dotted line represents the zeta potential threshold for an optimal gene delivery system (+ 20 mV).

3.5.3 *In vitro* transfection efficiency and cell viability of HEK-293 cell lines

Gene expression of the Lipofectamine 3000/DNA and Lipid 2/DNA complexes prepared at a carrier:DNA charge ratios of 2:1, 4:1 and 8:1 in water was assessed *in vitro*. Equal amounts (by weight) of the LCC DNA minivector and CCC pDNA were used to prepare the transfection treatments.

From the results displayed in **Figure 3.5-3**, transfection efficiency determined by EGFP expression in viable HEK-293 cell lines was shown to be dependent on two factors: 1) N^+/P^- charge ratio, and 2) DNA size and topology. Formulations prepared with either the Lipofectamine 3000 or Lipid 2 resulted in an upward trend in transfection as the N^+/P^- ratio increased, with the 8:1 formulation showing the greatest transfection efficiency. Additionally, transfection treatments prepared with Lipofectamine 3000 resulted in a two-fold increase in transfection efficiency compared to Lipid 2-mediated transfection efficiencies. With respect to DNA size and topology, direct comparisons with equal amounts (by weight) of LCC DNA minivectors and CCC pDNA indicated no significant differences with Lipid 2-mediated transfection efficiencies. However, transfection using Lipofectamine 3000 indicated that the 3.2 kb LCC DNA minivector imparted statistically significant increase in transfection compared to their 6.3 kb parental counterparts.

As shown in **Figure 3.5-3**, there was no significant decrease in cell viability compared to non-treated cells. It is also interesting to note that there was a significant improvement in cell viability when the cells were harvested and analyzed 48 hours following transfection treatment as compared to the results obtained the day after treatment (data not shown; see **Table A-1** and **Figure A-3** in **Appendix A**). This may likely be due to recovery of the cell membrane following permeabilization.

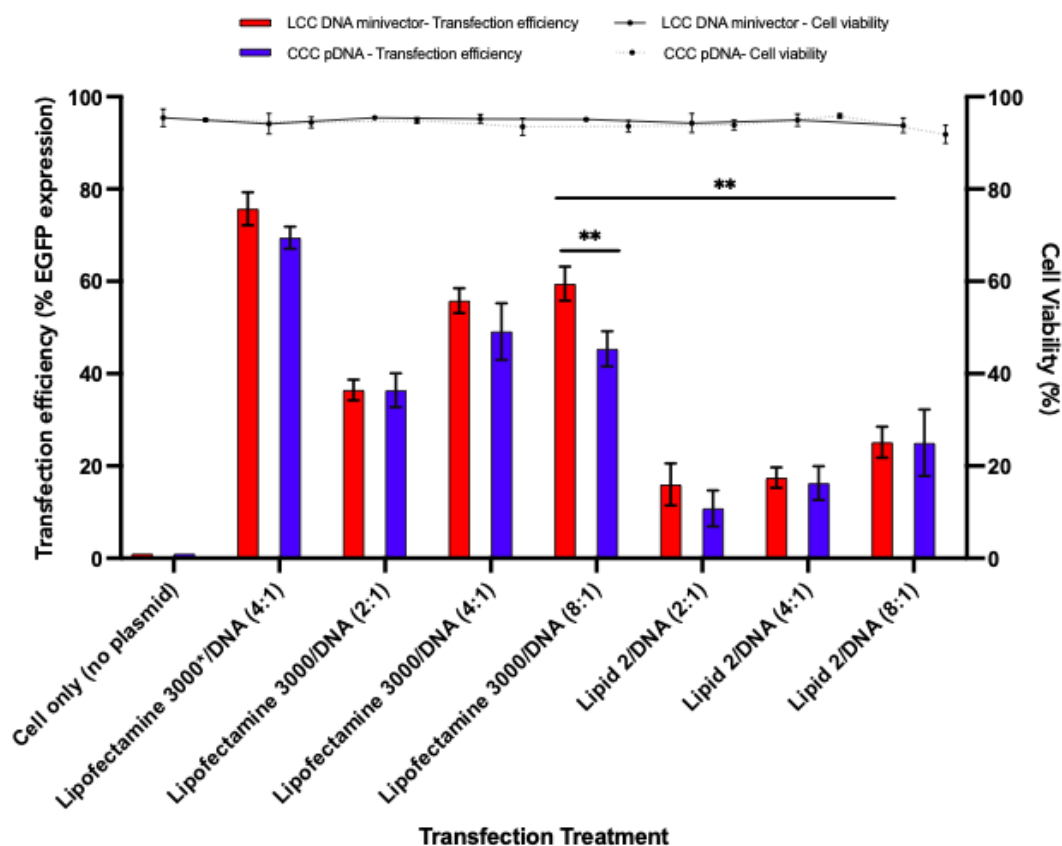


Figure 3.5-3: Transfection efficiency and cell viability in HEK 293 cells treated with lipid/LCC DNA minivector or lipid/CCC pDNA nanoparticles prepared in water at varying N^+/P^- ratios of each lipid. Transfection efficiency is represented by the number of live cells expressing EGFP (as a percentage of total cells analyzed), while cell viability was indicated by positive propidium iodide staining (as a percentage of total cells analyzed). Transfected cells were collected 48 hours post transfection. Means of three separate experiments with three replicates are plotted with error bars representing the standard deviation of triplicate experiments, each experiment consisting of three individual replicates per sample. Stars indicate significantly higher transfection efficiencies of LCC DNA minivectors compared to its parental CCC pDNA. Control conditions consisted of untreated cells (labelled as “cell only (no plasmid)”) and cells treated with Lipofectamine 3000*/LCC DNA minivector OR Lipofectamine 3000*/pDNA (CCC) (N^+/P^- charge ratio of 4:1) were prepared in Opti-MEM Medium by following the manufacturer’s recommendations.

3.5.3 *In vitro* transfection efficiency and cell viability of ARPE-19 cell lines

ARPE-19 cell lines were used to assess the transfection efficiency and cell viability of the Lipofectamine 3000/DNA and Lipid 2/DNA complexes in retinal cell lines. Transfection treatments were prepared at a carrier:DNA N^+/P^- charge ratios of 2:1, 4:1 and 8:1 in water and equal amounts (by weight) of the LCC DNA minivector and CCC pDNA were used to ensure a direct comparison between the two DNA constructs.

Similar to what was observed in HEK 293 cells, transfection efficiency determined by EGFP expression in viable ARPE-19 cells was dependent on N^+/P^- charge ratio as well as DNA size and topology (**Figure 3.5-4**). However, here the effect of N^+/P^- charge ratio on transfection was not consistent for all lipoplexes. For example, in ARPE-19 cells, formulations prepared with Lipid 2 resulted in an upward trend in transfection as the N^+/P^- ratio increased, with the 8:1 formulation showing the greatest transfection efficiency (23.0% cells transfected by LCC DNA minivectors and 16.1% cells transfected by CCC pDNA). On the other hand, relative to Lipid 2 lipoplexes, formulations prepared with Lipofectamine 3000 in water showed greater expression with the highest transfection efficiency achieved at the 4:1 charge ratio (40.9% cells transfected by LCC DNA minivectors and 34.1% cells transfected by CCC pDNA). With respect to DNA size and topology, a statistically significant increase in transfection was observed with formulations prepared with the smaller LCC DNA minivector construct in comparison to their larger parental CCC counterparts. As also shown in **Figure 3.5-4**, cell viability was not significantly reduced by any of the transfection treatments when compared to the negative control group (untreated/cell only, no plasmid).

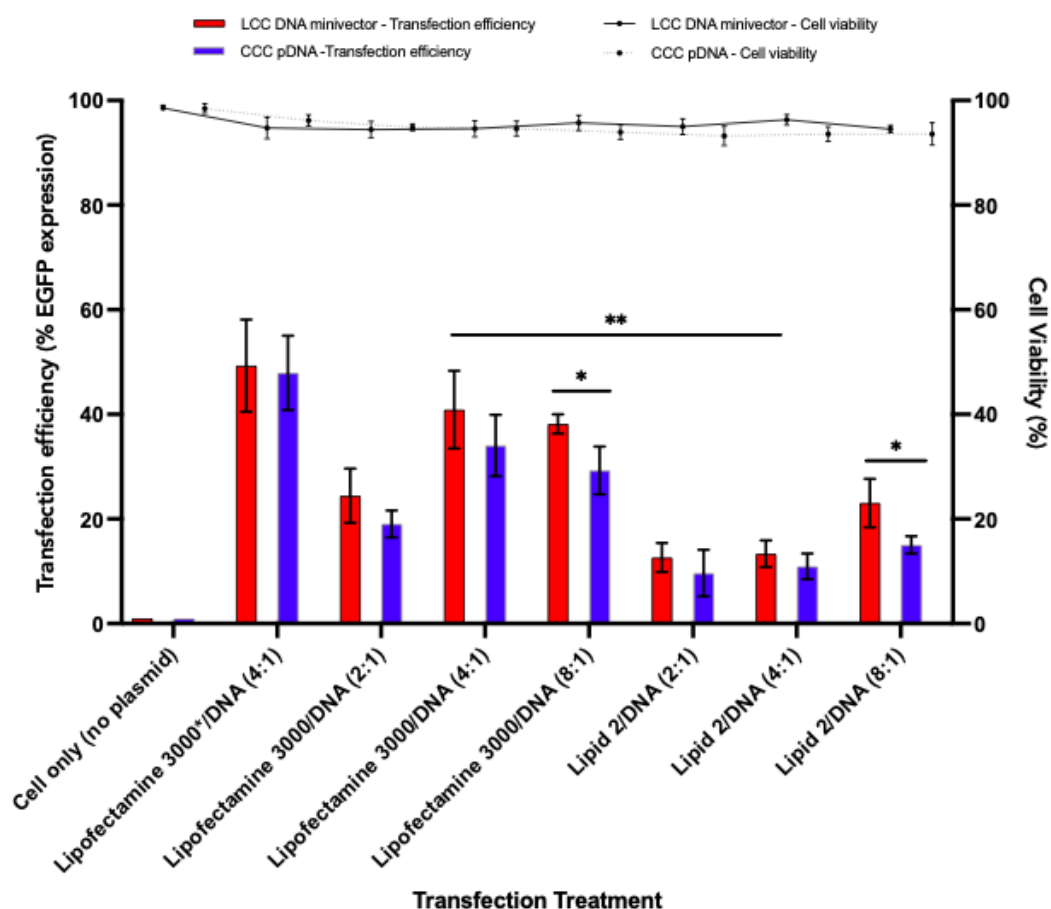


Figure 3.5-4: Transfection efficiency and cell viability in ARPE-19 cells treated with lipid/LCC DNA minivector or lipid/CCC pDNA nanoparticles prepared in water at varying N⁺/P⁻ ratios of 2:1, 4:1 and 8:1. Transfection efficiency is characterized by the number of live cells expressing EGFP as a percentage of the total cells analyzed, while propidium iodide was added to measure the cell viability of the transfection treatments and is shown as a percentage of total cells analyzed. Transfected cells were collected 48 hours prior post transfection. Means of three separate experiments with three replicates are plotted. The error bars represent the standard deviation of triplicate experiments, each experiment consisting of three individual replicates per sample. Stars indicate significantly higher transfection efficiencies of LCC DNA minivectors compared to its parental CCC pDNA. Control conditions consisted of untreated cells (labelled as “cell only (no plasmid)”) and cells treated with Lipofectamine 3000*/LCC DNA minivector) OR Lipofectamine 3000*/pDNA (CCC) (N⁺/P⁻ charge ratio if 4:1) were prepared in Opti-MEM Medium by following the manufacturer’s recommendations.

3.6 Discussion

Compared to viral vectors, non-viral DNA-based gene delivery is a safer alternative method. However, the transfection efficiency of non-viral vectors is lower, likely due to inefficient DNA uptake by cells, poor cytoplasmic diffusion, and low nuclear uptake.²³ Design and optimization of highly efficient synthetic carriers **and** the DNA construct will significantly influence the properties of the system, and consequently improve transfection efficiency. This work aimed to study the influence of DNA size and topology on nanoparticle physicochemical characteristics in deionized water, and *in vitro* transfection efficiency which may inform the development and optimization of LCC DNA minivector formulations.

With respect to the influences of DNA topology on the physicochemical properties and transfection efficiencies, direct comparisons between the LCC DNA minivectors (3.3 kb) and the CCC pDNA (6.3 kb) cannot be made due to the inherent differences in the size of the two vectors. By preparing samples based on equal amounts by weight, the results from this work would translate to more coding units for the DNA minivectors compared to the CCC pDNA. For example, since 1 μ g of each DNA construct was used to prepare the samples and knowing that the average molecular weight of a nucleotide pair is 660 g/mol²⁴ this would translate to approximately 0.48 pMol of LCC DNA minivector/sample and 0.24 pMol of CCC pDNA/sample as outlined in **Table 3.4-1** above. Therefore, a true comparison would result from approximately doubling the concentration of CCC pDNA/sample while keeping the concentration of LCC DNA minivector/sample the same. Nonetheless, results from this study even when equal amounts (by weight) of LCC DNA minivectors and CCC pDNA are compared, reveal certain differences arising from the influences of DNA size and topological conformations.

First, to study the effect of DNA size and topology on physicochemical properties, uncomplexed as well as complexed CCC pDNA and LCC DNA minivectors with either Lipofectamine 3000 or Lipid 2 nanocarriers were prepared in deionized water and assessed. Compaction of the DNA construct through electrostatic and hydrophobic binding of cationic lipids lead to the formation of lipoplexes. Measurements of the mean hydrodynamic diameter, PDI, and zeta potential measurements of the lipoplexed transfection treatments are presented in **Table 3.5-2**, **Figure 3.5-1**, and **Figure 3.5-2**. Mean hydrodynamic diameter values for the lipoplexes prepared with the LCC DNA minivectors ranged between 111 nm to 223 nm, their PDI ranged from 0.13 to 0.33, and their zeta potential measurements ranged from 15 mV to 28 mV. On the

other hand, mean hydrodynamic diameter values for formulations prepared with the parental CCC counterparts ranged between 122 nm to 321 nm, their PDI ranged from 0.15 to 0.43, and their zeta potential measurements ranged from 17 mV to 39 mV. These results suggest that direct comparisons with equal amounts (by weight) of DNA constructs reveal no statistical differences in physicochemical properties despite the distinct differences in DNA size and topology. Nanosized lipoplexes were formed, suggesting efficient compaction of DNA.

Despite having comparable physicochemical properties as lipoplexes, certain differences were observed with the uncomplexed materials which may provide further insights into the influences of DNA size and topology. As shown in **Table 3.5-1**, the mean hydrodynamic diameter of the pDNA CCC DNA ($360 \text{ nm} \pm 9$) was comparable to the LCC DNA minivectors ($324 \text{ nm} \pm 10$) despite being the larger sized plasmid (CCC pDNA: 6.3 kb vs. LCC DNA minivectors: 3.2 kb). DNA supercoiling may have led to the formation of compact conformations which may have attributed to the comparable particle sizes. With respect to zeta potential, lower effective negative surface charge for CCC pDNA (-26 ± 2), was obtained compared to LCC DNA minivectors (-32 ± 5). The supercoiled conformation of CCC pDNA may have shielded a fraction of the negative charge attained from the phosphate groups of DNA, which explains the lower effective negative charge for CCC pDNA. In contrast, the linear structure of LCC DNA minivectors contributed to more prominent surface charges, which may explain the lower negative zeta-potential charge (see **Figure 3.6-1**).²⁵

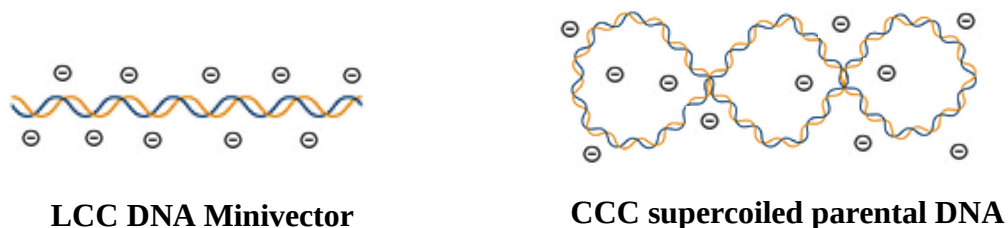


Figure 3.6-1: Schematic of the differences between the negative charge distribution (predicated by the phosphate groups of DNA) of the LCC DNA minivector and the CCC supercoiled parental DNA. Due to the supercoiled nature of the CCC parental DNA, a fraction of the negative charges may be masked whereas, due to the linear structure of LCC DNA minivector, the negative charges are more prominent leading to a higher zeta-potential relative to their CCC counterpart.

In relation to lipoplex formation, if the less negative zeta-potential with the CCC pDNA is due to supercoiling and charge shielding, then it is expected that there will be less lipid binding at lower N^+/P^- charge ratios (lipid not in excess) which would result in the formation of larger particles due to less condensation. This theory aligns with the results displayed in **Table 3.5-2**. From these results, it is evident that these particle sizes were larger at lower charge ratios (lipid not in excess). However, as the concentration of lipids in solution increased at higher charge ratios, this may have altered the supercoiling structure of CCC pDNA such that the previously shielded negative charges have now become more prominent. As such, this may have enhanced interactions between the cationic and anionic components, resulting in efficient complexation between the lipid-DNA construct and the formation of smaller particles with a higher positive zeta potential.

The ultimate goal of these transfection treatments is to efficiently transfect the host cells and express its gene. Therefore, the ability of the LCC DNA minivectors and CCC pDNA complexed with the lipid-based nanocarriers to transfect cells was assessed *in vitro* using HEK 293 and ARPE 19 cell lines. Cell viability was assessed alongside transfection efficiency using flow cytometry. Cell viability for all transfection treatments remained high and comparable to the positive control group which indicates that the tested samples caused minimal toxicity in both HEK 293 and ARPE-19 cell lines. With respect to transfection efficiencies, a number of relationships were identified between formulations that may give further insight into the influences of DNA size and topological conformations. As presented in **Figure 3.5-3** and **Figure 3.5-4**, direct comparison with equal amounts (by weight) of LCC DNA minivectors and CCC parent plasmids revealed no statistically significant differences with Lipofectamine 3000 and Lipid 2-mediated transfection efficiencies at the lower charge ratios of 2:1 and 4:1. However, transfection using Lipofectamine 3000 indicated that LCC DNA minivectors showed significantly higher transfection efficiency compared to their parental CCC counterparts at the 8:1 charge ratio. Similarly, LCC DNA minivectors complexed with Lipofectamine and Lipid 2-mediated transfection efficiencies in ARPE-19 cell lines, specifically at the higher charge ratios of 8:1 (**Figure 3.5-6**), imparted higher transfection efficiency compared to its CCC counterpart. This observation is similar to the findings by Sum et al. where LCC DNA vectors were shown to exhibit improved transgene expression over the CCC pDNA counterparts using Lipofectamine.¹⁸ This may be explained by the fact that the LCC DNA vectors are markedly smaller by weight (CCC pDNA: 6.3 kb vs. LCC DNA minivectors: 3.2 kb)

compared to the corresponding CCC parental counterpart. Due to their smaller size, LCC DNA minivectors allow for efficient packaging of a higher copy number of the cistron into the lipid nanoparticle relative to the CCC pDNA. Although lipoplex size and structure do not vary with increasing DNA length, the number of DNA molecules incorporated into each lipoplex has shown to increase linearly with decreasing DNA size.²⁶ As such, the transfection efficiency of lipoplexes is proportional to the number of enclosed DNA molecules in the lipoplexes, with higher transfection efficiencies achieved with lipoplexes incorporating a higher number of DNA molecules as observed in these results.²⁶ These observations are comparable to those in a previous study by Cameron et al. where smaller pDNA complexes attained higher transfection efficiency over larger pDNA when lipoplexes were restricted to possess a simple coding cassette in each particle.²⁶ Additionally, smaller sized DNA constructs have previously revealed rapid intracellular trafficking upon cytoplasmic release which may also attribute to the increased transfection efficiency observed by the LCC DNA minivectors in this work.¹² With respect to topology, previous atomic force microscopy studies have shown that CCC DNA form compact spherical or cylindrical condensates with cationic lipids whereas LCC DNA minivectors form extended pearl necklace-like structure which may help promote faster cytoplasmic diffusion, nuclear uptake, and gene expression rate relative to its parental CCC counterparts.²⁷ This could also explain the observations made in a previous study on different types of linear closed vectors, where LCC MIDGE vectors improved gene expression level up to 17-fold in specific tissues compared to the corresponding plasmid.¹³ These results suggest that DNA topology is important for *in vitro* transgene expression.

Additionally, based on **Figure 3.5-3** and **Figure 3.5-4**, the transfection efficiencies were also influenced by the nature of the synthetic lipid component. Overall, higher transfection efficiencies were observed with samples prepared with Lipofectamine 3000 compared to samples prepared with the Lipid 2 (chemical structure and composition unknown). This may be attributed to the general structural composition of Lipofectamine. Lipofectamine consists of the lipid 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and dioeyloxysperminecarboxamidoethyl-dimethylpropanaminium (DOSPA).²⁸ The helper lipid DOPE aids in forming stable and fusogenic liposomes which initiates the endosomal escape pathway in the cell.²⁹ The high transfection efficiencies achieved by formulations incorporating DOPE has previously been attributed to the structural transformation of these lipids accompanied by their interactions with the negatively

charged cell membrane. For example, the morphology of DOPE can transition from a bilayer to an inverted hexagonal structure (see **Figure 3.6-2**) which aids to destabilize the endosomal membrane and release the DNA cargo in the cytoplasm.²⁹ Koynova et al. supported this theory suggesting that formulations that form phases with a highly negative interfacial curvature, such an inverted hexagonal, would be the most effective carriers. DOSPA is also expected to have a pH-dependent transition due to the polyamine region which is likely to aid in endosomal release and enhance transfection efficiency.³⁰

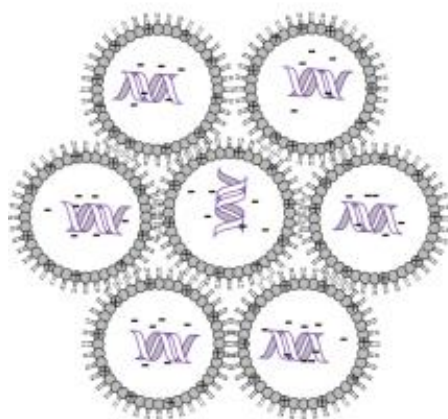


Figure 3.6-2: Prediction of Lipofectamine structure upon lipoplex formation with LCC DNA minivectors in water. In the presence of LCC DNA minivectors, Lipofectamine may self-assemble to form an inverted hexagonal phase structure which may aid in destabilization of the endosomal membrane.

Additionally, based on the results presented in **Table 3.5-2** and **Figure 3.5-3** and **Figure 3.5-4**, it is clear that physicochemical properties have a direct influence on *in vitro* transfection efficiency. Highest transfection efficiency was achieved with smaller lipoplexes with a higher cationic surface charge. These results are in agreement with literature as small nanoparticles can easily enter the cell to exert their function, while the high positive charge allows for favourable interactions with the cell surface consequently improving cell uptake.³¹⁻³³

3.6.1 Effect of N⁺/P⁻ charge ratio on the physicochemical properties of LCC DNA minivector-based lipoplexes

The transfection treatments for this work were prepared and assessed at three different N⁺/P⁻ charge ratios (2:1, 4:1 and 8:1) by systemically increasing the ratio of nitrogen's (from the lipid) while fixing the number of phosphates (from DNA) in the system. Previous studies have shown that the N⁺/P⁻ character of a lipid/DNA complex can influence many lipoplex properties such as its hydrodynamic diameter, particle size distribution, as well as net surface charge.³¹⁻³⁵ These relationships are well defined for lipid/CCC pDNA complexes. However, as previously observed, the prominent negative charge distribution attained from the phosphate groups of DNA LCC minivectors may have influences on DNA-lipid binding. As such, the effect of N/P ratio on physicochemical properties is an important property to further study for linearized DNA constructs.

Based on **Table 3.5-2**, **Figure 3.5-1**, and **Figure 3.5-2**, there are some trends between N⁺/P⁻ charge ratio and physicochemical properties which are clear. Smaller and more uniformly distributed aggregates possessing high positive zeta potentials were formed at higher N⁺/P⁻ charge ratios, of 8:1. Similarly with respect to transfection efficiency, improved gene expression was achieved at higher N⁺/P⁻ ratios of 4:1 and 8:1. At higher N⁺/P⁻ ratios, there is an excess of positive surface charges for extensive electrostatic interactions with the negatively charged phosphate groups on the DNA backbone, leading to reduced lipid head group repulsion and the formation of smaller and more stable lipoplexes through synergistic interactions. Lipoplex stabilities at the higher N⁺/P⁻ ratios (4:1 and 8:1) were exemplified by high zeta potential values (with low variability) for both the LCC and CCC-based lipoplexes (see **Table 3.5-2**). Additionally, previous studies have revealed that when the cationic component is used in enough excess, the overall positive charge aids to facilitate interactions with the cellular membrane via electrostatic attraction and cellular uptake for *in vitro* transfection.²¹ However, for *in vivo* applications, the trade-off to higher gene expression is the higher toxicity and instability that accompanies it. For example, the cationic charge is often necessary for efficient transfection, however, delivery of highly cationic materials, with a high excess of free cationic lipids in the circulatory system, can cause toxicity by adhering to and destabilizing the plasma membrane of red blood cells (hemolysis) which carry a negative surface charge. The presence of particles carrying a high cationic surface charge also have the potential to induce platelet aggregation in the blood.³³ When the excess free lipid is purified

from the complexes, *in vivo* toxicity is drastically reduced. Thus, it is expected that the tendency of the complexes to interact with negatively charge blood cells will also increase with N^+/P^- ratios.³⁶ Consequently, for *in vivo* applications, it is important to understand the implications of using cationic lipids at various N^+/P^- ratios from the context of not only gene expression, but also short and long-term toxicity.

3.7 Conclusion

Despite the inherent structural differences between LCC DNA minivectors and CCC pDNA, the physicochemical properties of the transfection treatments were surprisingly similar. However, statistically improved transfection efficiency was achieved with formulations prepared with LCC DNA minivectors. More specifically, highest transfection efficiencies were associated with Lipofectamine 3000/LCC DNA minivector lipoplexes at higher N^+/P^- charge ratios. These results suggest that a relationship between DNA size, topology, and transfection efficiency do exist, and an excess cationic charge is important for efficient *in vitro* transgene delivery. Further experimentation through additional physical characterization methods, in particular using transmission electron microscopy or scanning electron microscopy methods, would add to the results found in this work and would help to gain more insight into the properties of these transfection nanoparticles. More specifically, the influences of DNA topology can further be investigated through evaluation of nucleic acid condensation or through the evaluation of coiling. These studies would lead to an improved understanding of the effects of DNA size and topology on transfection capacities of lipid-based synthetic vectors.

Chapter 4

Physicochemical Properties and Transfection Efficiency of Cationic Polymer-Based Vectors Incorporating Linear Covalently Closed (LCC) DNA Minivectors

The work in this chapter is a continuation of the work presented in Chapter 3 and is the result of a pharmaceutical collaboration. While the introduction will provide a review of the background literature relevant to this work, the polymeric and nucleic acid components utilized in this work are proprietary and the specific names, chemical structures, and manufacturing processes cannot be disclosed.

Non-viral gene therapies have the potential to overcome the several drawbacks associated with viral gene therapy, including their high immunogenicity, limited packaging capacity, and insertional mutagenesis leading to oncogenesis. Of the various non-viral vectors developed, cationic polymers, specifically those which comprise pH-sensitive functionalities, are promising candidates due to their unique properties of forming polyelectrolyte complexes with genes and their ability to protect DNA from various enzymes to ultimately enhance transfection efficiency by promoting endosomal escape through the proton sponge mechanism. The key to improving their efficiency and to realize their full clinical potential is to rationally design all components of the vector, including both the synthetic vehicle and the therapeutic cargo. Compared to conventional circular covalently closed (CCC) plasmid DNA vectors, linear covalently closed (LCC) DNA minivectors have previously demonstrated higher transfection efficiency and their smaller size allows for the delivery of higher copy numbers to cells. In this study, the proprietary polymeric carriers, linear Polymer 1 and branched Polymer 2, incorporating either the CCC pDNA or the LCC DNA minivectors were formulated and characterized to determine their particle size, polydispersity index, zeta potential, and *in vitro* transfection efficiency. A clear relationship between the transfection efficiency and the particle size and zeta potential was observed, with higher transfection being associated with smaller, uniformly distributed nanoparticles comprising an excess positive charge. Of note, polyplexes composed of branched polymers comprising ester linkages complexed with linear DNA constructs exhibited higher transfection, confirming the importance of rational non-viral vector design.

4.2 Introduction

Successful gene therapy requires the efficient delivery of the therapeutic nucleic acids to the cells of interest to treat diseases.¹ However, challenges still remain with introducing DNA to targeted cells and the mechanisms by which these processes occur are still not well understood.²

There are two types of delivery vehicles that can be used in gene therapy, viral and non-viral vectors, both of which have their own benefits and drawbacks.³ For example, although viral vectors are effective in achieving high efficiency for both gene delivery and expression, limitations of small carrying capacity, toxicity, high cost and immunogenicity make viral vectors unattractive.⁴ On the other hand, compared to viral vectors, non-viral vectors are not only advantageous from a safety perspective, but their properties can also be fine-tuned by manipulation of the molecular composition, ultimately allowing for a more simplified production.⁵ While preferential from a safety and design perspective, non-vectors vectors exhibit low transfection efficiencies, an important obstacle that must be addressed for such systems to be recognized as effective vehicles for gene delivery.⁶ Transfection efficiency can be improved by understanding the general structural features of both the synthetic vector and the enclosed DNA cargo and characterizing their physicochemical and transfection properties. These properties also contribute to the safety profile of the gene therapy nanoparticles.

Early studies have revealed that incorporation of cationic components containing pH-sensitive functionalities, specifically polymers with nitrogen groups capable of being protonated, may enhance transfection through disruption of the endosomal membrane through the so-called “proton sponge” mechanism.⁷ The protonatable carboxyl groups and alkyl chains of these peptides tend to resist the acidification of endosomes due to their high buffering capacity, as a result easing one of the critical steps of the transfection process – endosomal escape.⁸ These functional groups have since been mimicked in synthetic pH-sensitive polymers. The commercially available reagent from Polyplus Transfection, jetPEI® is an engineered linear polymer and is one notable example of a gene delivery material capable of inducing this proton sponge effect under the acidic conditions of the endosome, which has been credited, in part, with the high transfection efficiency achieved by PEI.⁹ To validate this hypothesis, Akinc et al. designed a comparative study to assess the transfection processes of the N-quaternized (and therefore non-proton sponge) version of PEI and native PEI, capable of endosomal escape through the proton sponge mechanism. The results

from this study were consistent with the proton sponge theory and suggested that the transfection efficiency of native PEI vectors is due to their unique ability to avoid acidic lysosomal degradation.¹⁰ Additionally, polymeric vectors also offer numerous other advantages, including: 1) high positive charge for effective DNA compaction at low concentrations to enhance cell uptake; 2) versatility in structural confirmation; 3) high biodegradability; and 4) efficient DNA compaction generating smaller and uniform nanoparticles.¹¹

Since the names and the specific structures of the cationic carriers utilized in this work are proprietary and cannot be disclosed, the generic structural components of the carriers will be described to provide insight to their composition.

The proprietary Polymer 1 is a simple, linear polymer which is broadly based upon a common polyethyleneimine structure.¹¹ Due to the ethylenimine core, Polymer 1 has the potential to adopt a membrane disrupting conformation as the functional groups, such as the secondary and tertiary amines, become protonated with decreases in pH (proton sponge effect).¹⁰ This leads to an increase in osmotic pressure and eventual disruption of the endosomal membrane to facilitate endosomal escape.¹⁰

The branched proprietary Polymer 2 has been synthesized and optimized to possess several unique solution properties that are ideal for gene delivery. For example, due to the presence of several different types of diamines and polymerized esters of amino acids, Polymer 2 can be engineered to be water-soluble or water insoluble, or to have very high or low cationic charge densities. Additionally, the presence of secondary and tertiary amine groups in the Polymer 2 structural backbone contributes to this polymer being suitable for the intracellular delivery of DNA. These amine groups, particularly when positively charged, will not only promote binding to nucleic acids, but it will allow the complexes to remain stable in the cytosol due to their ability to buffer the pH of their surroundings. Also, the buffering capacity of these molecules will also promote the release of the DNA from the endosome into the cytoplasm through the proton sponge mechanism.¹⁰

Rational design of both the synthetic carrier as well as the DNA cargo is essential to consider when designing efficient N-VGDS. Conventional recombinant plasmid DNA, commonly used for transfection, are circular covalently closed (CCC) vectors which are composed of a eukaryotic expression cassette and a bacterial backbone.¹² The eukaryotic expression cassette consists of genetic components such as the gene of interest, intron, promoter, and transfection

termination/polyadenylation signal, all essential for successful gene expression in transfected cells. The bacterial backbone consists of an origin of replication for plasmid DNA replication and an antibiotic resistance gene cassette for selection.¹³ Compared to viral vectors, plasmid DNA are easier and cheaper to produce in large quantities, are stable, and have a much longer shelf life.¹³ Plasmid DNA vectors do however have their own safety concerns. When delivered alone or packaged within a synthetic vector, plasmid DNA can transfer antibiotic resistance genes which may enable horizontal gene transfers, giving rise to antibiotic resistant pathogens. Additionally, unlike vertebrate animals, bacterial DNA have a higher frequency of unmethylated CpG motifs which can induce immune responses.¹⁴ To overcome the various safety concerns associated with plasmid DNA vectors, the dumbbell-shaped proprietary linear covalently closed (LCC) DNA minivectors are introduced as an alternative.¹⁵⁻¹⁹ LCC minivectors have been specifically designed and engineered to provide advantages of efficiently trafficking the nucleic acids into the cell nucleus.²¹ Compared to their supercoiled CCC parental counterpart, LCC DNA minivectors lack a bacterial backbone in its structure, it not only enhances the safety features of the nucleic acid, but also condenses the DNA size to improve intracellular trafficking and cytoplasmic diffusion.²⁰ The linear nature of DNA minivector also minimizes the potential for insertional mutagenesis from random genomic integration.²⁰ Although the manufacturing process of the LCC DNA minivectors is proprietary and cannot be disclosed, the reader is referred to a previously described the production system for LCC DNA minivectors.¹⁵⁻¹⁹

Currently, a great deal of research is being carried out that focuses upon the rational design of efficient synthetic vectors to address the major challenges associated with conventional CCC plasmid DNA gene therapy including, efficient DNA encapsulation, targeted delivery, cellular uptake and internalization, endosomal escape, and nuclear localization. However, as previously described, conventional CCC pDNA suffer from several safety and efficiency limitations so it essential to rationally design an alternative strategy to traditional plasmid vectors, such as LCC DNA minivectors. LCC DNA minivectors complexed with lipid nanoparticles were previously studied in Chapter 3 to gain insight into the influences of DNA size and topology on physiochemical properties and transfection efficiency. Although a clear relationship between these properties were observed, the overall transfection efficiency of the proprietary Lipid 2 in complexation with LCC DNA minivectors (23.2% cells transfected) and CCC pDNA (15.3% cells transfected) were low in APRE-19 cells. To optimize and improve the transfection efficiency of

LCC DNA minivectors, this work was completed in collaboration with a pharmaceutical partner which aims to explore the currently unknown molecular interactions and evaluate the efficiency between LCC DNA minivectors after complexation with either the linear Polymer 1 or the branched Polymer 2 carriers. Polymer 1 and Polymer 2 were selected by the pharmaceutical partner for this work due to their versatile morphological and structural characteristics, as described above, and their transfection efficiency was compared to a well-known transfection reagent, jetPEI. A key finding is that compared to the lipoplexes studied in Chapter 3, transfection using Polymer 2/LCC DNA minivectors reported significantly improved transfection efficiency in ARPE-19 cells (60.1% cells transfected) suggesting that the structural features of both the synthetic carrier and the DNA construct are vital to optimizing non-viral vectors for gene therapy applications. The results from this study also reveal that the molecular interactions and the physicochemical properties between LCC DNA minivector-based polyplexes differ from lipid-based systems.

4.3 Materials

The CCC parental pDNA and bacterial sequence-free LCC DNA minivectors, carrying the enhanced green fluorescence protein (*GFP*) expression cassette and a (CMV) promoter, was received through a collaboration with a pharmaceutical partner. Since these DNA constructs are proprietary, information regarding the manufacturing processes cannot be disclosed.

Similarly, the proprietary Polymer 1 and Polymer 2 carriers were also received through a pharmaceutical collaboration. Information regarding their name, chemical structure, and synthesis process cannot be disclosed.

Physicochemical characterization experiments used either deionized water or 1X Dulbecco's phosphate-buffered saline without magnesium and calcium (DPBS) which was purchased from Thermo Fisher Scientific (Burlington, ON, Canada). Transfection experiments used deionized water.

HEK 293 (human embryonic kidney) cells (ATCC CRL-1573) and ARPE-19 (arising retinal pigmented epithelium) cells (ATCC-CLR-2302) were purchased from American Type Culture Collection, ATCC (Manassas, VA, USA). Dulbecco's Modified Eagle Medium (DMEM) formulated with high glucose and pyruvate (Gibco), Canadian-origin fetal bovine serum (FBS) (Gibco), DMEM/F-12 without phenol red (Gibco), 0.25% Trypsin-EDTA with phenol red,

Penicillin-Streptomycin 100X (10,000 U/mL) (Gibco), 15 mL falcon tubes, 75cm² and 175cm² Nunc 114 EasYFlask cell culture flasks and cell culture coated 24-well plates (Nunc, Thermo Scientific) were purchased from Thermo Fisher Scientific (Burlington, ON, Canada).

4.4 Methods

4.4.1 Preparation of transfection polyplexes

All stock solutions and solvents were filtered through a sterile 0.22 µm polyethersulfone (PES) syringe filter (Sartorius) prior to use. Transfection polyplexes were prepared based on N⁺/P⁻ ratio, the ratio of positively charged polymer amines (N = nitrogen) groups, to the ratio of negatively charged nucleic acid phosphate (P = phosphate) groups. To ensure a direct comparison by weight, an equal mass of LCC DNA minivectors and CCC pDNA were used to prepare all transfection complexes. **Table 4.4-1** below provides insight into the N⁺/P⁻ charge ratio as well as the molar: molar ratio based on the base pair size of each construct.

Polymer 1-based polyplexes were prepared at a 2:1, 4:1 and 8:1 N⁺/P⁻ charge ratio in either deionized water or PBS buffer. Briefly, 1 µg of diluted nucleic acid was mixed into different amounts of the Polymer 1 stock solution all at once. The solution was vortexed immediately and spun down briefly. The complex was then allowed to incubate for 15 minutes at room temperature to allow complexation.

Polymer 2-based polyplexes were made at a 2:1, 4:1, and 8:1 N⁺/P⁻ charge ratio in either deionized water or PBS. Briefly, different aliquots of the 1 mM Polymer 2 polymer stock solution were mixed into 1 µg of DNA. The solution was vortexed immediately and spun down briefly. The mixture was then allowed to incubate for 30 minutes at room temperature.

Table 4.4-1: LCC DNA minivectors and CCC pDNA-derived transfection polyplexes

DNA construct	Nucleic acid Size (Base Pair)	Molarity (pMol) per 1 ug	Molarity (pMol) per N ⁺ /P ⁻ ratio		
			2:1	4:1	8:1
LCC DNA minivector	3439	0.44	0.88	1.76	3.53
CCC pDNA	6506	0.23	0.47	0.93	1.86

4.4.2 Physicochemical characterization of nanoparticles

4.4.2.1 Particle size and polydispersity index of polyplexes

Hydrodynamic diameter along with polydispersity index (PDI) measurements were performed on all Polymer 2/DNA and Polymer 1/DNA polyplexes using a Malvern Zetasizer Nano-ZS set to 25°C (Malvern Nano series instruments, UK). Particle sizes and PDI in deionized water or PBS were determined using the scattered light intensity distribution from dynamic light scattering with a measurement angle of 173°. Samples were diluted 100X based on the instruments recommendations that particle sizes in the rate of 10-1000 nm be prepared as 1% w/w solutions. 500 µL sample was pipetted into a disposable Solvent Resistant Micro Cuvette (ZEN0040) and inserted into the cuvette holder. All samples were freshly prepared, as previously described in section 4.4.1, immediately prior to measurement. Results are expressed as an average of three separate experiments with three independent measurements per sample (i.e. means of means) ± the standard deviation. Graphs were plotted in Graphpad Prism 6 software.

4.4.2.2 Zeta potential of polyplexes

Zeta potential measurements in deionized water or PBS was determined from laser Doppler electrophoresis taking three independent measurements per sample. 700 µL sample was loaded via syringe into a primed Disposable folded capillary cells (DTS1070) and inserted into the cuvette holder in the Malvern Zetasizer Nano-ZS set to 25°C (Malvern Nano series instruments, UK). All samples were freshly prepared, as previously described, immediately prior to measurement. The results are expressed as the average of three independent experiments, with three measurements per sample per experiment ± the standard deviation. Uncomplexed DNA formulations (i.e. samples

prepared under the same conditions but without polymer) served as a positive control group for the physicochemical characterization experiments. Graphs were plotted in Graphpad Prism 6 Software.

4.4.3 Cell culture and *in vitro* transfection of HEK 293 and ARPE-19 cells

HEK-293 cells were cultured with DMEM (with pyruvate and high glucose) supplemented with 10% (v/v) FBS. ARPE-19 cells were cultured with DMEM/F-12 without phenol red supplemented with 10% (v/v) FBS and 1X penicillin-streptomycin. Both HEK 293 and ARPE-19 cell lines were incubated at 37°C with 5% carbon dioxide in a tissue culture incubator (Thermo Scientific) and sub-cultured at least once before initiating transfection or cell viability experiments. Experiments were carried out with cultures having passage numbers 4 to 10.

Prior to transfection, cells were grown at 37°C with 5% carbon dioxide and maintained at 70-90% confluency in 75cm² tissue culture flasks. Cells were then seeded into 24-well plates at a density of 100, 000 cells/well (HEK 293 cells) and 200, 000 cells/well (ARPE-19 cells) and were returned to the incubator at 37°C for 24 h prior to transfection in complete media without antibiotics. One hour prior, cells were washed with 1X DPBS and the culture medium was replaced with serum-free medium. Polyplexes and controls were then prepared freshly as described above. To initiate transfection experiments, first a specific volume of medium was removed from each well, and then an equal volume of the transfection treatment was added dropwise to each well to achieve a plasmid dose of 1 µg/ per well. All transfection treatments were repeated in three independent experiments with freshly made polyplex solutions. Each experiment included three independent measurements per sample. The plates were then rocked gently to ensure even distribution of the transfection treatments in the wells and then were returned to the incubator at 37°C for 5 hours. After 5 hours, the transfection medium was replaced by fresh DMEM or DMEM/F-12 with 10% FBS without antibiotic. Plates were then incubated for an additional 48 hours at 37° C. 48-hours post transfection, the cells were collected, washed, and resuspended in DPBS. Cells were then harvested for flow cytometry analysis to assess gene (GFP) expression and cell viability.

Formulations prepared with *in vitro* jetPEI served as the positive transfection control and were evaluated simultaneously with each transfection experiment. To select the optimal control group, *in vitro* jetPEI complexes were prepared in 50 µL of 150 mM NaCl according to the

manufacturer recommendations at three different N⁺/P⁻ charge ratios: 2:1, 4:1 and 8:1.²¹ The final DNA dose per well was kept consistent between both control and test treatments at 1 µg/well.

4.4.4 Flow cytometry analysis

Cells were harvested for flow cytometry 48 hours after removal of the treatments. The supernatant medium was collected from each well, then cells were rinsed with 250 µL of DPBS, detached with 1 mL 0.25% Trypsin-EDTA for 5 minutes followed by neutralization with 2 mL of complete medium. Following this, the cells were collected in a 5 mL round bottom polystyrene tubes (Falcon, Corning Life Sciences) and centrifuged at 4 °C for 10 minutes at 125xg (Sorvall Legend RT, Thermo Scientific). After centrifugation, the supernatant was then removed, and the cells were washed twice with 2 mL DPBS. The final cells were then re-suspended in DPBS and stored on ice.

Five minutes before analyzing the cells using flow cytometry, 10 µL of a 1 mg/mL propidium iodide (PI) solution (Sigma Aldrich) was added to each tube. Samples were analyzed using a Beckman Coulter CytoFlex S Flow Cytometer (School of Pharmacy, University of Waterloo). Samples were excited with a 488 nm laser, and 10,000 events analyzed per sample. Cells that did not receive any transfection treatments and unstained cells (cells that were not stained with PI) served as controls. Data files were subsequently analyzed using CytExpert software. Graphs were plotted in Graphpad Prism 6 software.

4.4.5 Statistical analysis

All data were statistically analyzed using the Graphpad Prism 6 software. Each error bar on the graphs represents the standard deviation of three separate experiments, where each experiment consisted of three individual replicates per sample. Statistical analysis comparing treatment results to a control group were determined by one-way analysis of variance (ANOVA) and a dependent, two-tailed, two sample Student's t-test. Test assumptions included a normal distribution with equal variance at 95% confidence intervals, where values of $p < 0.05$ were considered statistically significant.

4.5 Results

4.5.1 Physicochemical characterization of nanoparticles

Colloidal stability, cell internalization, and DNA release are all significantly influenced by the particle size, uniformity, and surface charge of the nanoparticles. Consequently, the mean hydrodynamic diameters, polydispersity index (PDI), and zeta potential of both uncomplexed and complexed nanoparticles were evaluated at 25°C using a Malvern Zetasizer Nano-ZS (Malvern Nano series instruments, UK). For hydrodynamic diameter and PDI measurements, samples were diluted 100X with deionized water. Results for the uncomplexed materials are shown in **Table 4.5-1**, while the results for the polyplexes are shown in **Table 4.5-2** and illustrated in **Figure 4.5-1** and **Figure 4.5-2**.

As noted in **Table 4.5-1**, differences in zeta potential are slightly more prominent than the differences in the mean hydrodynamic diameter and PDI of the uncomplexed DNA constructs. As previously discussed in Chapter 3, the DNA topology (linear vs. circular structure of DNA) may attribute to these differences where the DNA supercoiling in pDNA (CCC) masks the negative charge of the DNA phosphate group, thereby shielding the negative charge. On the other hand, the linear nature of the DNA minivector (LCC) allows for a more prominent surface charge distribution of the negative charges. These results align with observations discussed in Chapter 3.

Table 4.5-1: Mean hydrodynamic diameter, polydispersity index (PDI), and zeta potential of uncomplexed LCC DNA minivector and CCC pDNA. Deionized water was used to dilute all samples before measurement at 25°C. Mean values $\pm$ the standard deviation for triplicate experiments, each experiment consisting of three individual replicates per sample are reported.

	Mean hydrodynamic diameter (nm)	Mean PDI	Mean Zeta Potential (mV)
LCC DNA minivector	348 $\pm$ 9	0.41 $\pm$ 0.03	- 31 $\pm$ 6
CCC pDNA	362 $\pm$ 7	0.45 $\pm$ 0.01	- 27 $\pm$ 3

Polyplexes were formulated via electrostatic interactions between the cationic polymer and negatively charged DNA constructs. For *in vivo* applications, previous literature on nanoparticle design and characterization reveal that a nanocarrier diameter of < 200 nm and PDI of < 0.3 are optimal as anything greater are shown to accumulate in the liver and spleen and may also have an impact on cell internalization as well as DNA release from the endosome.²² With respect to zeta potential, nanoparticles are classified to be relatively stable when < + 20 mV as this allows for favourable interactions with the cell surface to improve cellular uptake, well potentially eliminating the excess positive charge which has been known to lead to serum instability or toxicity.²³

The mean hydrodynamic diameters and PDI measurements of the Polymer 1/DNA and Polymer 2/DNA polyplexes are shown in **Figure 4.5-1** and **Table 4.5-2**. All polyplexes formed relatively small (97 – 125 nm) nanoparticles and exhibited the progressive formation of uniformly sized particles as indicated by a PDI < 0.3 for all three charge ratios. A general trend was noted where the hydrodynamic diameter decreased as the N⁺/P⁻ charge ratio increased with nanoparticles prepared with Polymer 1 complexed with either LCC DNA minivector and pDNA (CCC). The larger hydrodynamic diameter exhibited at lower charge ratios was likely the result of aggregation upon charge neutralization as subsequent addition of more Polymer 1 at 4:1 and 8:1 N⁺/P⁻ charge ratios resulted in a decrease in hydrodynamic diameter with lower PDIs. Interestingly, the opposite trend was observed with nanoparticles prepared with Polymer 2 complexed with either LCC DNA minivector and CCC pDNA where we observed that the hydrodynamic diameter slightly increased as the N⁺/P⁻ charge ratio increased. This suggests that DNA complexation by the Polymer 2 carrier may occur more efficiently at lower N⁺/P⁻ charge ratio compared to higher charge ratios (e.g. 8:1).

This may be attributed to the branched nature of Polymer 2 which is promoting the electrostatic interactions between the nucleic acids and the polymer side chains at lower concentrations.

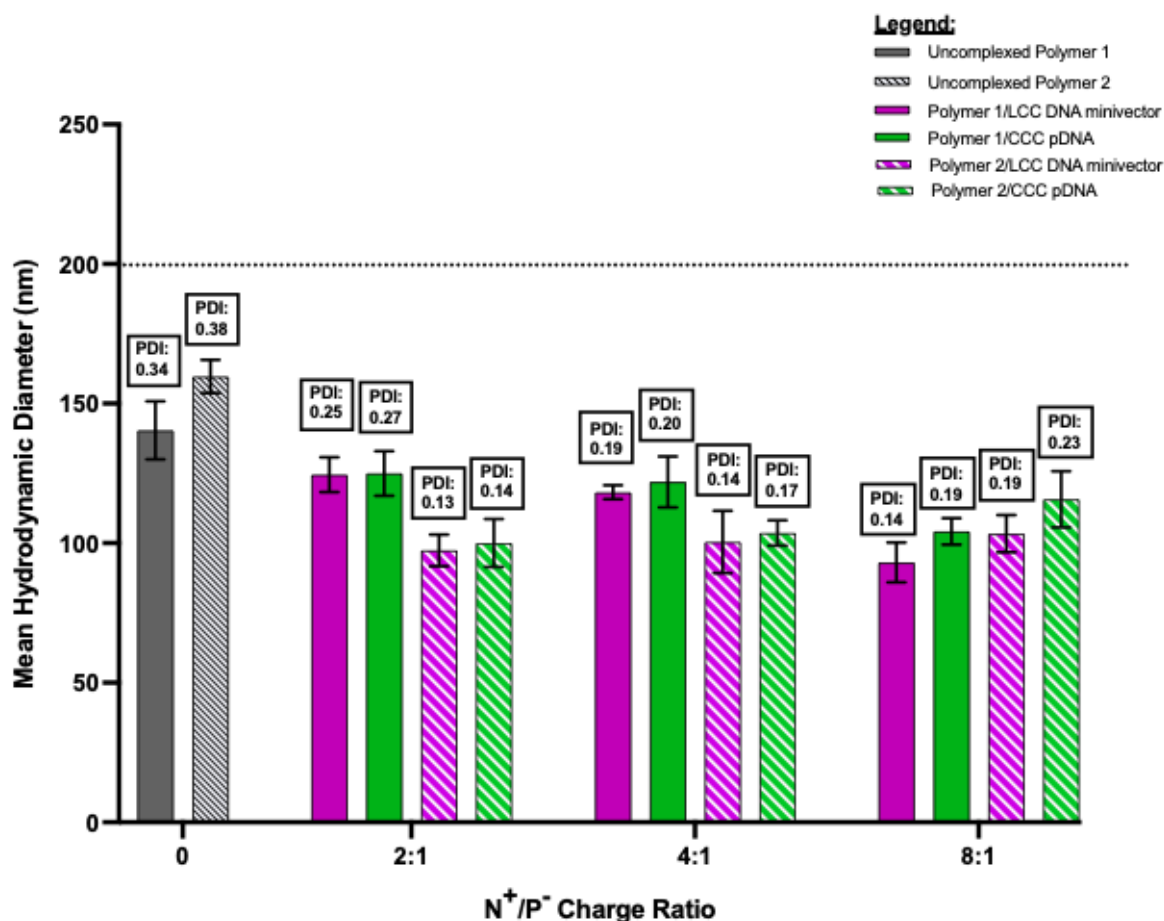


Figure 4.5-1: Mean hydrodynamic diameter and mean polydispersity indices (PDIs) of uncomplexed polymers and complexed polyplexes upon 100X dilution with deionized water at varying N^+/P^- ratios of each polymer. Mean values for three independent experiments, each experiment consisting of the three sample replicates are reported. Error bars represent standard deviation, and the dotted line represents the 200 nm hydrodynamic diameter threshold.

Table 4.5-2 and **Figure 4.5-2** show the zeta potential results for the Polymer 1/DNA and Polymer 2/DNA polyplexes. From these results, a clear relationship between zeta potential and N^+/P^- charge ratio was observed. Unsurprisingly, as N^+/P^- ratio increased from 2:1 to 4:1 to 8:1, there was a presence of an excess number of uncomplexed polycations in solution, resulting in an increase in the zeta potential. Additionally, all polyplexes show zeta potentials between the ranges of 15 – 28 mV, which as previously discussed, is associated with high colloidal stability.

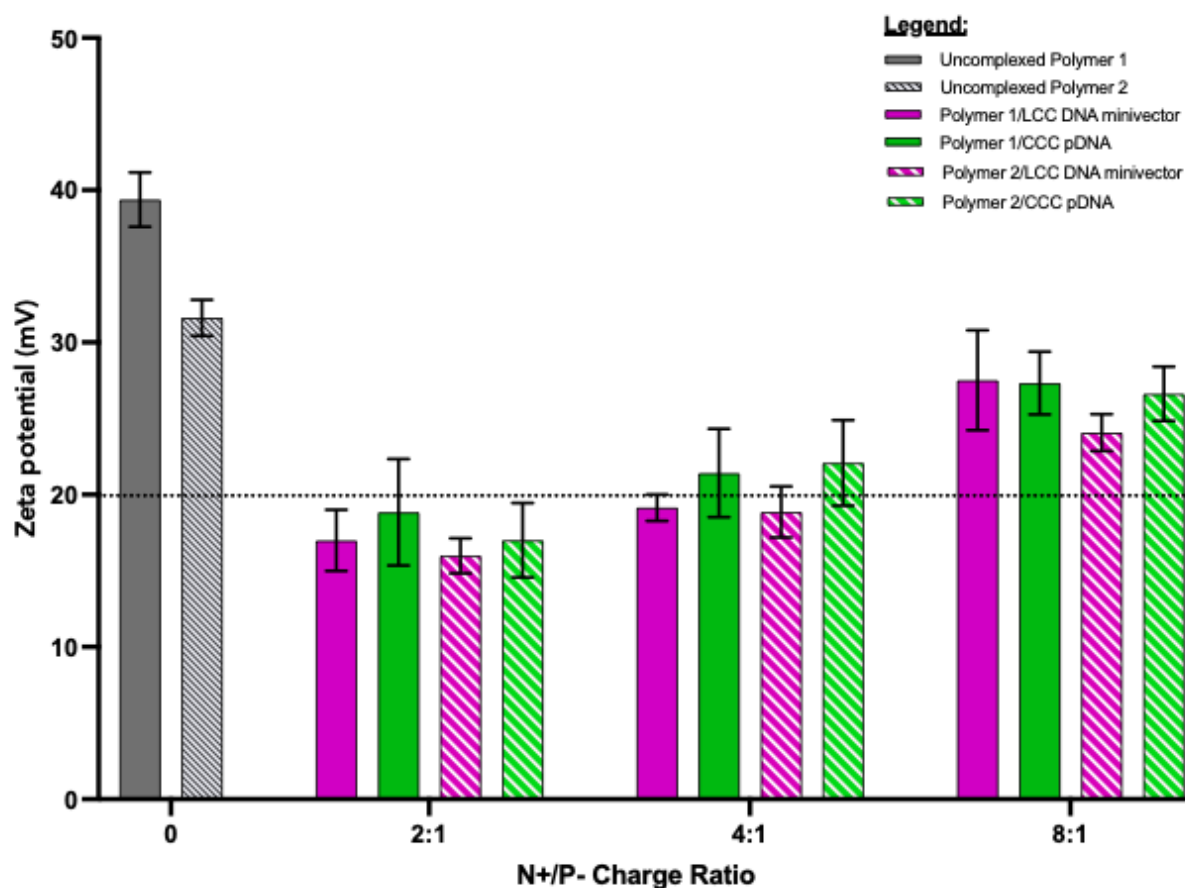


Figure 4.5-2: Mean zeta potential of uncomplexed and complexed polyplexes upon 100X dilution with deionized water at varying N^+/P^- ratios of each polymer. The mean of three separate experiments with three replicates are reported. Error bars represent standard deviation, and the dotted line represents the zeta potential threshold for an optimal gene delivery system.

Ionic strength can have an impact on the physicochemical properties of polyplexes. Salts can disrupt polymer-DNA interactions during nanoparticle formation, which may impact the transport of the DNA cargo to the targeted cell and ultimately affect gene expression.²² As such, to briefly assess the effect of ionic strength on physicochemical properties, the polyplexes were diluted in PBS (to mimic the cellular ionic environment) and the hydrodynamic diameter, PDI, and zeta potential were measured at varying charge ratios and under the same experimental conditions as described above. The results are shown in **Table 4.5-2** and illustrated in **Figure 4.5-3** and **Figure 4.5-4**. Aside from the uncomplexed polymer samples, the mean hydrodynamic diameters, PDI, and zeta potential measurements under these conditions were not significantly different from the measurements obtained in deionized water at 25°C. Therefore, we can conclude that the presence of salts does not significantly influence the physicochemical properties of polyplexes.

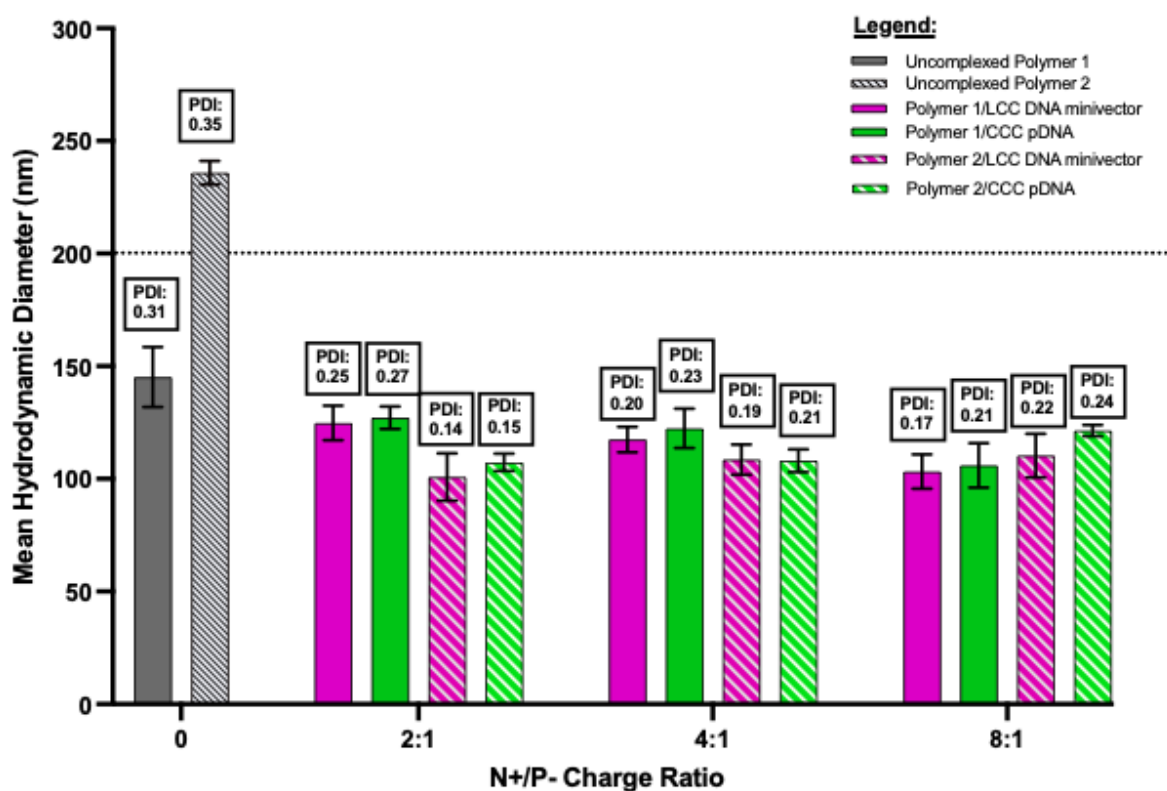


Figure 4.5-3: Mean hydrodynamic diameter and polydispersity indices (PDIs) of uncomplexed and complexed polyplexes complexes upon 100X dilution in PBS with varying N⁺/P⁻ ratios of each polymer. The mean of three separate experiments with three replicates are reported. Error bars represent the standard deviation from triplicate measurements and the dotted line shows the threshold for hydrodynamic diameter (200 nm).

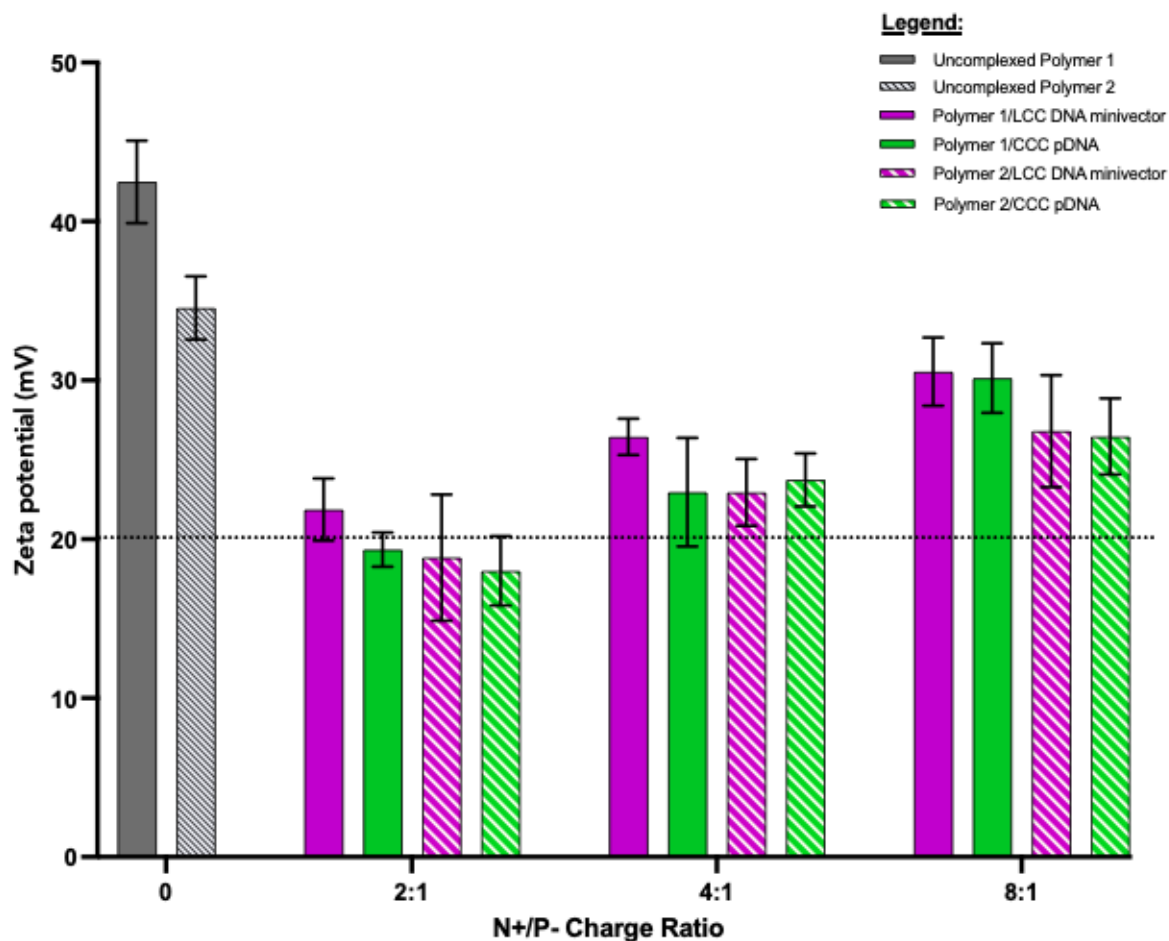


Figure 4.5-4: Mean zeta potential of uncomplexed and complexed polyplexes upon 100X dilution with PBS at varying N⁺/P⁻ ratios of each polymer. The mean of three separate experiments with three replicates are reported. Error bars represent standard deviation, and the zeta potential threshold for a gene delivery system is represented by the dotted line (+ 20 mV).

Table 4.5-2: Mean hydrodynamic diameter ($\langle d_h \rangle$), mean polydispersity index ($\langle PDI \rangle$), and mean zeta potential $\langle ZP \rangle$ of Polymer 1 and Polymer 2 prepared in deionized water or PBS at varying N⁺/P⁻ ratio, the ratio of the number of positive charges of the polymer amine groups, to the number of negative charges of the DNA phosphate groups, measured at 25°C. The mean of three separate experiments with three replicates are reported. $\pm$ the standard deviation are reported.

Polymer	DNA added	N ⁺ /P ⁻	$\langle d_h \rangle$ (nm) (water)	$\langle d_h \rangle$ (nm) (PBS)	$\langle PDI \rangle$ (water)	$\langle PDI \rangle$ (PBS)	$\langle ZP \rangle$ (mV) (water)	$\langle ZP \rangle$ (mV) (PBS)
Polymer 1	no DNA added	0	140 $\pm$ 9	145 $\pm$ 11	0.34 $\pm$ 0.04	0.31 $\pm$ 0.02	39 $\pm$ 1	42 $\pm$ 2
	LCC DNA minivector	2:1	125 $\pm$ 5	125 $\pm$ 6	0.25 $\pm$ 0.02	0.25 $\pm$ 0.04	17 $\pm$ 2	22 $\pm$ 2
		4:1	118 $\pm$ 2	117 $\pm$ 5	0.19 $\pm$ 0.02	0.20 $\pm$ 0.02	19 $\pm$ 1	27 $\pm$ 1
		8:1	93 $\pm$ 6	103 $\pm$ 6	0.14 $\pm$ 0.04	0.17 $\pm$ 0.02	28 $\pm$ 3	31 $\pm$ 2
	CCC pDNA	2:1	125 $\pm$ 7	127 $\pm$ 4	0.27 $\pm$ 0.03	0.27 $\pm$ 0.04	19 $\pm$ 3	19 $\pm$ 1
		4:1	123 $\pm$ 8	122 $\pm$ 7	0.20 $\pm$ 0.01	0.23 $\pm$ 0.03	21 $\pm$ 2	23 $\pm$ 3
		8:1	104 $\pm$ 4	106 $\pm$ 8	0.19 $\pm$ 0.04	0.21 $\pm$ 0.01	27 $\pm$ 2	30 $\pm$ 2
Polymer 2	no DNA added	0	160 $\pm$ 5	236 $\pm$ 4	0.38 $\pm$ 0.04	0.35 $\pm$ 0.04	32 $\pm$ 1	35 $\pm$ 2
	LCC DNA minivector	2:1	97 $\pm$ 5	101 $\pm$ 9	0.13 $\pm$ 0.04	0.14 $\pm$ 0.01	16 $\pm$ 1	19 $\pm$ 3
		4:1	100 $\pm$ 9	109 $\pm$ 6	0.14 $\pm$ 0.03	0.19 $\pm$ 0.02	19 $\pm$ 2	23 $\pm$ 2
		8:1	103 $\pm$ 5	110 $\pm$ 8	0.19 $\pm$ 0.04	0.22 $\pm$ 0.03	24 $\pm$ 1	27 $\pm$ 3
	CCC pDNA	2:1	100 $\pm$ 7	107 $\pm$ 3	0.14 $\pm$ 0.02	0.15 $\pm$ 0.01	17 $\pm$ 2	18 $\pm$ 2
		4:1	110 $\pm$ 9	108 $\pm$ 4	0.17 $\pm$ 0.01	0.21 $\pm$ 0.02	22 $\pm$ 2	24 $\pm$ 1
		8:1	115 $\pm$ 8	121 $\pm$ 2	0.23 $\pm$ 0.04	0.24 $\pm$ 0.03	27 $\pm$ 1	26 $\pm$ 2

4.5.2 *In vitro* transfection efficiency and cell viability of HEK-293 and ARPE-19 cell lines

The commercially available polymer, *in vitro* jetPEI, served as the positive control group for this work. To select the optimal control group, the transfection efficiency of *in vitro* jetPEI complexed with LCC DNA minivectors and CCC pDNA at three different N⁺/P⁻ charge ratios: 2:1, 4:1 and 8:1 were evaluated in HEK 293 cell lines. The results are presented in **Figure 4.5-5**. This optimization data revealed that the highest transgene expression was achieved at a N⁺/P⁻ charge ratio of 4:1 with 67.3% cells transfected by LCC DNA minivectors-base complexes and 57.9% cells transfected CCC pDNA-based complexes. Consequently, the 4:1 N⁺/P⁻ charge ratio (1 µg DNA to 4 µL of *in vitro* jetPEI) served as the positive control for the *in vitro* evaluation of this work.

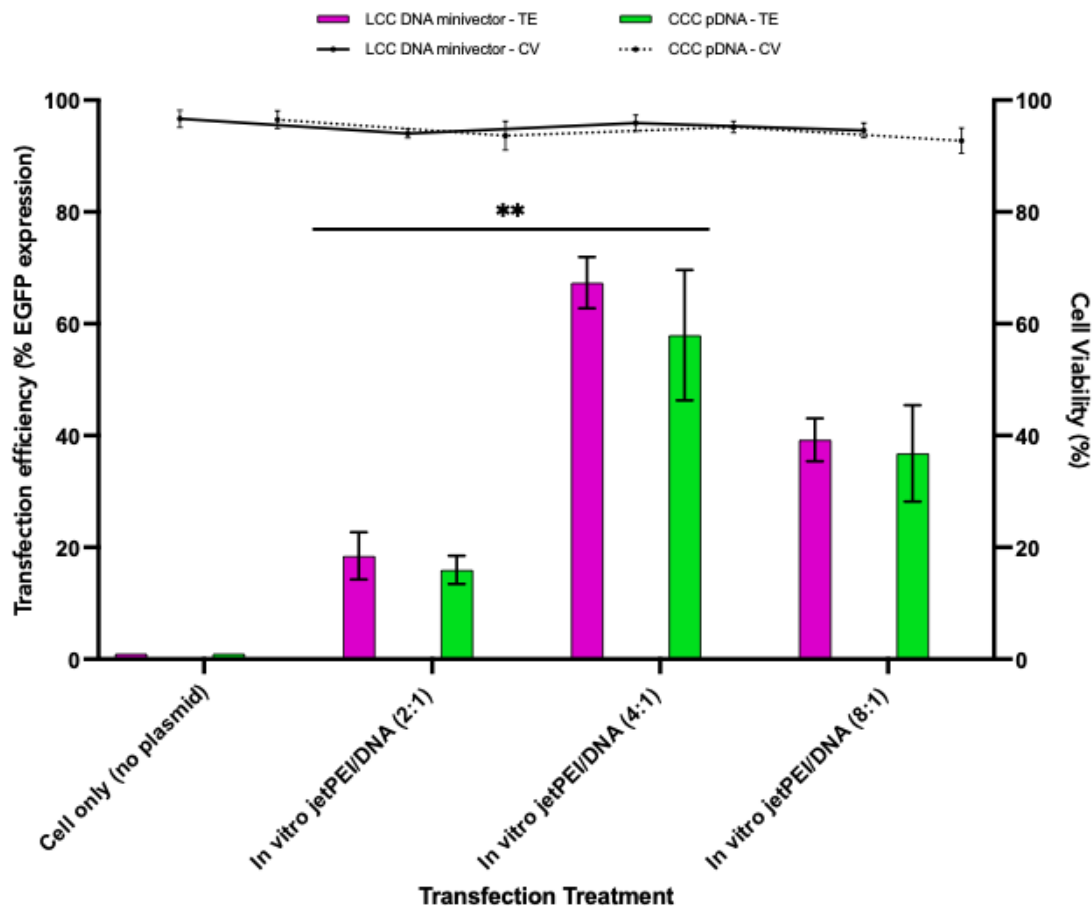


Figure 4.5-5: Transfection efficiency and cell viability in HEK 293 cells treated with in vitro jetPEI/DNA minivectors or in vitro jetPEI/pDNA (CCC) complexes at varying N^+/P^- ratios of each polymer. Transfection efficiency is represented by the number of live cells expressing GFP (as a percentage of total cells analyzed), while cell viability was indicated by positive propidium iodide staining and shown as a percentage of total cells analyzed. Mean values are plotted with error bars representing standard deviation. All treatments were repeated in three independent experiments, each experiment consisting of three replicates per sample. Control conditions consisted of untreated cells (labelled as “cell only (no plasmid)”). No statistically significant differences were observed between the LCC DNA minivector and CCC pDNA.

Results of transfection efficiency determined by GFP expression with the Polymer 1 and Polymer 2 carriers in complexation with LCC DNA minivectors and CCC pDNA in HEK 293 and ARPE-19 cell lines showed a clear relationship between transfection efficiency and N^+/P^- charge ratio. As seen in **Figure 4.5-6** and **Figure 4.5-7**, as the N^+/P^- charge ratio increased from 2:1 to 4:1 to 8:1, the transfection efficiency also increased, with 8:1 formulation showing the greatest transfection efficiency for both polyplexes in HEK 293 and ARPE-19 cell lines.

In HEK 293 cell lines (**Figure 4.5-6**), statistically significant increases in transfection was observed with formulations prepared with the Polymer 2/LCC DNA minivector constructs in comparison to their parental CCC counterparts and these observations were consistent between the 4:1 and 8:1 charge ratios as determined by one-way analysis of variance (ANOVA) and a dependent, two-tailed, two sample Student's t-test (same results obtained by both methods). These differences may be attributed to both the effects of DNA topology on transfection efficiency as previously discussed in detail in Chapter 3 as well as the structural composition of Polymer 2. In comparison to the average transfection efficiency obtained with the in vitro jetPEI positive control (67.3% cells transfected by LCC DNA minivectors and 57.9% cells transfected by CCC pDNA) the 8:1 Polymer 2/DNA formulations outperformed the control group with a transfection efficiency of 74.4% for LCC DNA minivectors and of 59.6% for CCC pDNA. On the other hand, transfection efficiency in ARPE-19 cell lines (**Figure 4.5-7**) showed no statistically significant differences with formulations prepared with the LCC DNA minivector constructs in comparison to their parental CCC counterparts and this was consistent across all transfection treatments. Additionally, higher transfection efficiencies were demonstrated by the 8:1 Polymer 2/DNA-based formulations (60.1% by LCC DNA minivectors and 48.6% by CCC pDNA) compared to the in vitro jetPEI positive control group (43.5% cells transfected by LCC DNA minivectors and 38.2% cells transfected by CCC pDNA). These observations indicate that transfection efficiency is influenced by the nature of the synthetic vector as well as the design of the DNA construct. With respect to cell viability, both **Figure 4.5-6** and **Figure 4.5-7** show that cell viability remained high (> 92%) and was not significantly reduced by any of the transfection treatments compared to the negative control group (untreated/cell only cells).

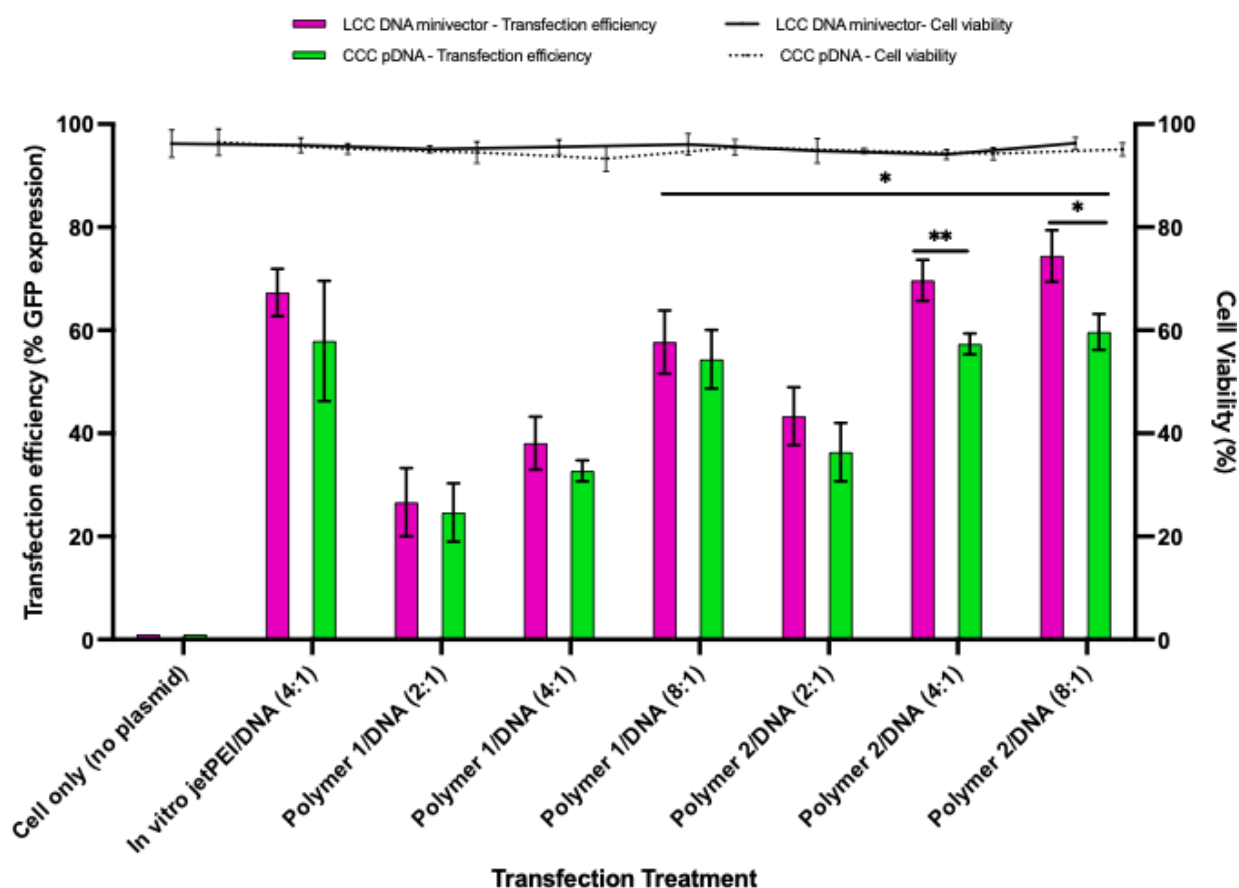


Figure 4.5-6: Transfection efficiency and cell viability in HEK 293 cells treated with polymer/LCC DNA minivectors) or polymer/CCC pDNA complexes at varying N^+/P^- ratios. Control groups consisted of untreated cells (labelled as “cell only (no plasmid)”) and cells treated with in vitro jetPEI/LCC DNA minivector OR jetPEI/CCC pDNA. Transfection efficiency is represented by the number of live cells expressing GFP and as a percentage of total cells analyzed, while cell viability is represented by propidium iodide staining and shown as a percentage of total cells analyzed. Transfected cells were collected and analyzed 48 hours post-transfection. Mean values are plotted with error bars representing standard deviation. All treatments were repeated in three independent experiments, each experiment consisting of three replicates per sample. Statistical differences were determined by one-way analysis of variance (ANOVA) and a dependent, two-tailed, two sample Student’s t-test (same results obtained by both methods). Stars indicate significantly higher transfection efficiencies of LCC DNA minivectors compared to its parental CCC pDNA.

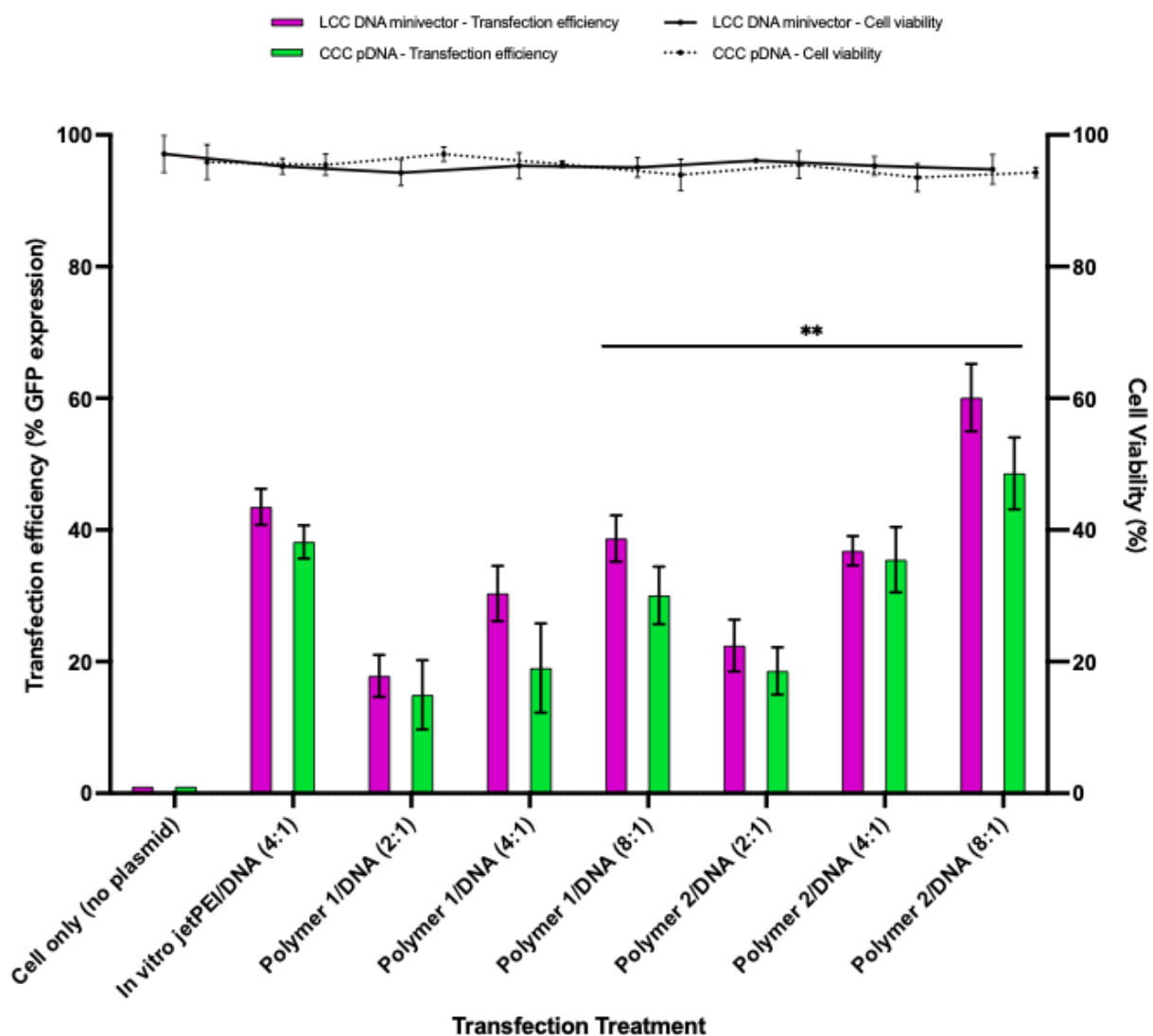


Figure 4.5-7: Transfection efficiency and cell viability in ARPE-19 cells treated with polymer/LCC DNA minivector or polymer/CCC pDNA. Complexes were prepared at N^+/P^- ratios of 2:1, 4:1, and 8:1. Mean values are plotted with error bars representing standard deviation. All treatments were repeated in three independent experiments, each experiment consisting of three replicates per sample. Transfected cells were collected and analyzed 48 hours post transfection. Transfection efficiency is represented by the number of live cells expressing GFP (as a percentage of total cells analyzed), while cell viability was indicated by positive propidium iodide staining and shown as a percentage of total cells analyzed. Control conditions consisted of untreated cells (labelled as “cell only (no plasmid)”) and cells treated with in vitro jetPEI/LCC DNA minivector OR jetPEI/pDNA (CCC). No statistically significant differences were observed between the LCC DNA minivector and CCC pDNA.

4.6 Discussion

To advance clinical application for non-viral gene therapy, optimal design of two key components are required, namely an efficient synthetic vector and an optimal DNA cargo structure. The work in Chapter 3 provided an insight into the influences of DNA size and topology on LCC DNA minivector-based lipoplexes. However, the overall transfection efficiency of these lipoplexes were low. To further improve the efficacy of linear DNA, this work was designed in collaboration with a pharmaceutical partner to assess the physicochemical properties and transfection efficiency of LCC DNA minivectors when complexed with linear and branched polymeric carriers. Ultimately, these studies would allow us to select an optimal synthetic carrier and move forward with developing efficient LCC DNA minivectors for gene therapies in pre-clinical and clinical settings.

Polyplex size and surface charge are important vector design features which drive several biological phenomena with discrete cut-off size ranges, including circulation half-lives, extravasation through leaky vasculature and macrophage uptake.²⁰ Although the geometry drives initial internalization of the nanoparticle, size and surface charge ultimately determine survival of the non-viral vector from extracellular and intracellular cell barriers.²⁰ Large plasmids (> 30 kb) offer the advantage of the delivery of larger sized therapeutics, while smaller plasmids (> 5 kb) have shown to enhance cellular and nuclear uptake.²⁰ Anti-cancer gene therapy nanomedicines aim to develop gene nanocarrier sizes which range approximately between 20 – 200 nm.²⁴ Nanoparticles within this size range would not only avoid renal clearance *in vivo*, which removes particles smaller than 30 nm from circulation, but would also contribute to longer blood circulation times.²² In this study, the physicochemical properties and transfection efficiency of two different plasmids were evaluated in complexation with Polymer 1 and Polymer 2: 1) LCC DNA minivectors (3.4 kb) and 2) parental CCC plasmid (6.5 kb). **Table 4.5-2**, **Figure 4.5-1**, and **Figure 4.5-2** show the physical characteristics of the polyplex nanocarrier systems in deionized water. Resulting hydrodynamic size, PDI, zeta potential, and transfection efficiency for Polymer 2/LCC DNA minivector polyplexes show promising characteristics for the use in gene therapy application and these characteristics are discussed below.

Polymer 1 and Polymer 2-based polyplexes will both be stabilized by similar molecular interactions, as the amine groups of the polymer molecules primarily interacted with the nucleic acid phosphate groups through electrostatic and hydrophobic interactions.²⁵ Although only small

(statistically insignificant) differences were observed when comparing the mean hydrodynamic sizes for polyplexes across the different charge ratios as presented in **Table 4.5-2** and **Figure 4.5-1**, some general trends can be noted. The results confirm that a relationship between polymer structure and physicochemical properties exist, where linear polymers (Polymer 1) form smaller and more uniform particle sizes compared to branched polymers (Polymer 2) at higher N^+/P^- charge ratios, of 8:1. This is because linear polymers have a simple structure comprising no side chains.²⁶ As a result, in the presence of DNA, Polymer 1 can form tightly packaged polyplexes through electrostatic interactions compared to Polymer 2, as evidenced by a small particle size.²⁴ Additionally, at lower concentrations, linear Polymer 1 monomers may have only partially dissociated upon dilution in water, resulting in weak electrostatic interactions with DNA forming “loose” aggregate structures, as evidenced by the larger particle size. On the other hand, the 8:1 N^+/P^- condition with the branched Polymer 2-based polyplexes may have represented a situation where there is excess polycations from uncomplexed side chains which remained in solution, leading to the formation of slightly larger and variable particle sizes.²⁵ Similar trends were observed when these polyplexes were prepared and assessed in PBS, as shown in **Table 4.5-2**, **Figure 4.5-3**, and **Figure 4.5-4**, suggesting that variations in ionic strength do not significantly influence the properties of the polyplexes.

The zeta potential of nanoparticles is also an important vector design parameter that can be tailored to prolong circulation time and selectively enhance accumulation at specific sites of interest. Non-viral vectors with a positive zeta potential are often desirable in order to facilitate interactions with the cell membrane.²⁷ As presented in **Table 4.5-2** and **Figure 4.5-2**, some general trends with respect to zeta potential were noted. Increasing the N^+/P^- ratio increased the net positive charge of the system and these results agreed with literature.^{28,29} If the amount of DNA is fixed, and the amount of added polymer is systemically increased, this increases the ratio of nitrogen's (from the polymer) to phosphates (from the DNA construct) in the system. Therefore, increasing the net cationic charge of the polyplex.²⁹ These results also provide insight into the thermodynamic stability of the transfection complexes in solution. In general, polymeric complexes prepared at the N^+/P^- charge ratio of 8:1 formed highly stable nanoparticles, as evidenced by the high zeta potential values (26 mV – 31 mV) at this concentration.

Previous work have shown high cell internalization of positively charged non-viral vectors, compared to their negatively charged counterparts, in different cancer cell types, such as HeLa

cells²⁷ MCF-7 cells²⁸ and endothelial cells.²³ Particularly, a particle size of 100 -150 nm is generally optimal to facilitate endocytosis.³¹ All polyplex sizes reported here are within this range and carry a net positive charge when complexed with either the CCC pDNA and LCC DNA minivectors. However, not surprisingly, formulations prepared at an N⁺/P⁻ charge ratio of 8:1 formed particles of smaller sizes with a higher zeta potential and these samples achieved the highest transfection efficiencies (**Figure 4.5-6** and **Figure 4.5-7**). These results confirm trends found in previous work in this field that transfection influenced by the physicochemical properties of non-viral formulations and also composition (e.g. the N⁺/P⁻ charge ratio) of polymer/DNA complexes.^{30,32}

It is however important to note that although non-viral formulations with an excess positive charge may be a desirable property for efficient *in vitro* application, but there are several other elements to consider when optimizing these samples for *in vivo* applications.²⁹ *In vivo*, delivery of vehicles with a high positive charge may promote interactions with negatively charged opsonin proteins through a process called opsonization. Particularly at high N⁺/P⁻ charge ratios where there is likely to be an excess of free polycations, these nonspecific interactions are expected to be even more apparent than at N⁺/P⁻ charge ratios closer to the neutralization point of the complexes where the excess charges are minimal.²⁹ Yamamoto et al. showed the advantage of designing neutral (1.3 mV) and anionic (-10.6 mV) polyplexes to enhance systematic circulation. The group also demonstrated that negatively charged nanoparticles resulted in lower accumulation in the liver and spleen.³³

On the other hand, as previously described, the positive surface charge of nanoparticles is also important as it facilitates endosomal release through mechanisms such as the proton sponge effect for most polymer-based vectors (including Polymer 1 and Polymer 2), hindering degradative effects of the endosomal compartment on the DNA cargo.¹⁰ In order to preserve the advantages of neutral and charged nanoparticles, while overcoming the disadvantages, the work done by Yuan et al. was of great significance which designed zwitterionic nanoparticles with a changeable charge based on environmental stimuli, such as pH, in order to prolong circulation, maximize tumor accumulation, and enhance cellular uptake.³⁴ Upon administration to tumor sites with lower pH values of ~6.8, the anionic component of the surface was shed, leaving a positive surface charge on nanoparticles which improved tumor cell entry and enhanced *in vivo* responses.³⁴ Therefore, for application *in vivo*, these polyplexes would need to be further tailored to precise dimensions of

high monodispersity, while also possessing an initial neutral or slightly negative nanoparticle surface charge which then switch to a positive charge upon arrival at the target site.

In this work, transfection samples prepared at an N^+/P^- ratio of 8:1 with LCC DNA minivectors and the branched Polymer 2 achieved the highest transfection efficiency in both HEK 293 and ARPE-19 cell lines compared to samples prepared with the linear Polymer 1 and CCC pDNA (see **Figure 4.5-6** and **Figure 4.5-7**). These results reveal a structure-transfection activity relationship. As previously described, Polymer 1 is a linear polymer and its chemical structure is broadly based on an ethylenimine core, whereas Polymer 2 is a branched polymer comprising hydrolysable ester linkages and secondary and tertiary amine groups.^{35,36} There are currently mixed findings in the literature regarding the *in vitro* transfection efficiency of both linear and branched polymers in complexation with conventional recombinant plasmid DNA.^{26,35-37} For example, some findings have shown improved transfection efficiency with linear polymers due to improved cytoplasmic diffusion and intracellular trafficking.³⁵ Whereas some have revealed that the branching structure of the polymer has more favourable properties due to the presence of the side chains which may help to further protect the encapsulated DNA from degradation by endosomal enzymes.^{36,37}

Our results reveal that compared to the linear Polymer 1/LCC DNA minivector polyplex, interactions between the branched Polymer 2 and linearized DNA minivectors appear to be more favourable for transfection. For example, the branched nature of the polymer may have efficiently protected the LCC DNA construct from intracellular degradation and promote cellular uptake. Then, once the LCC DNA minivector was released into the cytoplasm, the linear nature of the DNA construct may have promoted a fast cytoplasmic diffusion, nuclear uptake, and ultimately enhanced transfection efficiency compared to their CCC pDNA counterpart.¹⁵ Additionally, a key structural feature which is present in Polymer 2 and not present in both the control group (in vitro jetPEI) or Polymer 1 are hydrolysable ester linkages. Under transfection conditions, these ester linkages may have interacted with water to undergo hydrolysis. This may have led to the degradation of these functional groups and maximized the density of the buffering amines to facilitate and improve endosomal escape through the proton sponge effect resulting in improved transfection.⁷ As such, the combination between the branched polymer and the linear DNA, in addition to the presence of these ester linkages in Polymer 2 may have worked together to improve the transfection efficiency of these polyplexes.

4.7 Conclusion

A clear relationship between polyplex physicochemical properties and transfection efficiency was observed, with highest transfection associated with the 8:1 Polymer 2/LCC DNA minivector sample which formed small, uniformly distributed nanoparticles with a high zeta potential. Of note, DNA topology also influenced the complexation of the polymer nanocarriers during polyplex formation. Such differences may be attributed to the supercoiling effect for CCC pDNA and variations in particle size. Additionally, the branched nature of Polymer 2 as well as the presence of ester linkages in their structure may have contributed to the high transfection efficiency of these polyplexes. The trends revealed here as well as the trends discussed in Chapter 3 may lead to greater understanding of the influences of both the synthetic vehicle and DNA size, topology and physicochemical properties on transfection efficiency of synthetic based vectors. Further investigation, through electron microscopy studies or small angle X-ray scattering, is warranted to fully understand the relationships found in this work, ultimately allowing for a more rational approach to the formulation of LCC DNA minivector polyplexes for future gene therapy *in vivo* applications.

Chapter 5

Surface miscibility of Gemini Surfactants and DOPE in binary mixed monolayers

This side project was designed to briefly assess the impact of lipid-surfactant energetics to provide insight into the low transfection efficiencies achieved by GS-DOPE-LCC DNA minivectors, a system which was previously studied and published in our group.¹

5.1 Abstract

Surface pressure (π)–molecular area (A) isotherms were gathered to characterize the packing of binary mixed Langmuir monolayers of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and one of two gemini surfactants, 18-7-18 or 18-7NH-18. Information about miscibility behaviour was derived from the π – A curves by examining the excess free energy of mixing (ΔG^E) that was calculated through the surface area additivity rule. Compressibility modulus (C_s^{-1}) was also used to characterize intermolecular interactions. Attractive interactions due to GS condensation by DOPE dominate at most monolayer compositions leading to the formation of condensed monolayers which indicates a high degree of miscibility between these compounds. An exception to this is that a net repulsive force is found in all $X_{GS} = 0.5$ monolayers studied. The prevalence of attractive synergistic interactions in the monolayers studied differs from a previous finding of antagonistic mixing behaviour in GS/DOPE micelles. Due to the previously determined low transfection efficiency of these lipoplexes, further transfection studies with LCC DNA minivectors were not undertaken in this particular work. Nonetheless, these results contribute to the understanding of GS-lipid interactions and packing that are critical to the stability of liposomes composed of these molecules used for non-viral gene therapy applications.

5.2 Introduction

Several types of vectors are used in gene therapies for the prevention and treatment of a variety of inherited and acquired genetic diseases.² Viral vectors transfect cells with high efficiency but serious safety concerns including cytotoxicity and the risk of severe immune responses to the viral vector and their transgene products remain unresolved.³ In contrast, non-viral vectors formulated from a mixture of neutral and cationic lipids and/or surfactants are generally non-immunogenic, non-toxic, and inexpensive to produce.^{4,5} In addition, there is no limit on plasmid size and they allow for the incorporation of other components that impart beneficial features to the formulation such as enhanced circulation times, targeted delivery, and stimuli-dependent release.⁶⁻⁸ However, plasmid DNA delivery using synthetic gene therapy carriers continue to suffer from poor transfection efficiency.⁹

In Chapters 3 and Chapter 4, the importance of the protonatable amine group was clear in the transfection results. To further study their importance, gemini surfactants (GS), a class of surfactants compounds, with pH-sensitive amine groups provide an opportunity to control the chemical structure of the cationic component of the delivery system very accurately. This allows for a unique insight into possible origins of interactions within the lipoplex (or polyplex) system when DNA is introduced. Consequently, the structure of GS is of interest, particularly for gene therapy applications, due to their favourable solution behaviour and biocompatibility¹⁰⁻¹². The addition of a pH-sensitive amine group to the spacer group of m-7-m GS, the m-7NH-m series of GS, led to higher transfection efficiencies compared to the unsubstituted GS.^{8,9} This is a consequence of both the ability of the m-7NH-m lipoplex to adopt various complex morphologies and the proton sponge mechanism of endosomal escape.⁹ Yet, compared to viral vectors, transfection mediated by m-7NH-m GS is still poor. Only upon the addition of the helper lipid DOPE to the lipoplex do transfection efficiencies slightly increase for both m-s-m and m-7NH-m type GS.⁹ This is attributed to DOPE's fusogenic properties causing significant disruption to the endosomal membrane allowing the escape of the genetic load into the cytosol.¹³

Numerous studies have shown that lipoplex formation and performance in *in vitro* and *in vivo* transfection experiments are strongly influenced by the electrostatic interactions between its components.¹⁴ The molecular interactions governing several aspects of GS-DOPE liposome mediated transfection, such as the complexation and condensation of DNA by GS¹⁵⁻²² and DOPE²²⁻²⁴ as well as the disruption of cellular membranes and transfection efficiencies by GS-

DOPE-DNA lipoplexes^{25,26} are well understood. In spite of this there is a distinct lack of information specifically about GS-DOPE molecular interactions and surface miscibility. These features of GS-DOPE mixtures are related to surface molecular packing and are crucial for liposome stability and effective DNA transfection.

Consequently, since there is a distinct lack of information specifically about GS-DOPE molecular interactions and surface miscibility, a systematic study was designed to investigate the nature of the intermolecular interactions and the surface miscibility behaviour of GS-DOPE binary mixtures as well as the effects of mixture composition, GS structure, and pH on this behaviour. To accomplish this, the molecular organization and structure-packing behaviour of 18-7-18-DOPE and 18-7NH-18-DOPE mixtures were studied using Langmuir monolayers as a confined two-dimensional environment at the air/water interface. These methods have often been used to investigate packing in binary and ternary mixtures of various lipids but, GS containing mixtures have not been extensively studied in this way. A clearer understanding of GS-DOPE intermolecular interactions could guide the rational design of such lipoplexes comprising LCC DNA minivectors for non-viral gene therapy applications.

5.3 Materials

1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) (>99% purity) is a synthetic lipid that was purchased from Avanti Polar Lipids and used without further purification.

The synthesis of the gemini surfactants used in this study has previously been described.^{27,28} Briefly, the synthesis of N,N-bis(dimethyloctadecyl)-1,7-nonanediammonium dibromide (18-7-18) involved a single bis-Menschutkin reaction between 1 equivalent of the spacer group 1,7-dibromoheptane (Sigma-Aldrich), and 2.1 equivalents of the tail group N, N-dimethyloctadecylamine (ACROS Organics). The reaction was carried out in HPLC-grade acetonitrile (CH₃CN) under reflux for 48 hours at 82°C. Subsequently, the solvent was removed, and the acquired product was purified by recrystallization from hexane-ethyl-acetate to obtain the pure 18-7-18 GS. Moreover, the synthesis of 1,9-bis(octadecyl)-1,1,9,9-tetramethyl-5-amino-1,9-nonanediammonium dibromide (18-7NH-18) involved a single bis-Menschutkin reaction between 1 equivalent of the spacer group 3,3'-iminobis(N,N-dimethylpropylamine) (Sigma-Aldrich) with 2.1 equivalents of 1-bromooctadecane (ACROS Organics). The reaction was carried out in HPLC-

grade acetonitrile under reflux for 48 hours at 82°C. Subsequently, the solvent was removed, and the acquired product was purified by recrystallization from hexane–ethyl acetate to obtain the pure 18-7NH-18 GS.

5.4 Methods

5.4.1 Monolayer measurements

One-component and mixed monolayers were prepared by spreading 20 μL of their chloroform solutions (2.5 mM total concentration) over either a pure water (pH 7) or acetic acid–acetate (pH 4) buffer subphase contained in a large Langmuir trough (KSV Instruments, Helsinki, Finland) using a micro-syringe (GASTIGHT®, Hamilton-Bonaduz, Schweiz, Switzerland). Note that the buffer subphase was left to equilibrate for 30 minutes before the addition of the sample. After the addition of the sample, 15 min were allowed for solvent evaporation and surface pressure stabilization. The monolayer was then compressed by moving the lateral barriers at a rate of 10 mm/min. Surface pressure was monitored using a metal Wilhelmy plate and recorded as a function of mean molecular area. The experiments were performed at 25.0 ± 0.5 °C. The isotherms were recorded at least in triplicate using a fresh sample for each trial to avoid chemical modification. The extracted parameters described in Section 5.4.2 represent the averages $\pm$ standard deviation of the parameters obtained for each of the triplicate measurements. Differences in isotherm measurements resulted from random error, particularly solution preparation and sample delivery to the surface.

5.4.2 Data analysis

The measured isotherms were analyzed through the main features of monolayers: lift-off area (A_L), minimum mean molecular area ($A_{\min}$), collapse pressure (π_{col}), and surface compressional modulus (C_s^{-1}).^{29,30} Also, the deviation of the mixed monolayers from the ideal surface mixture from the molecular surface area additivity rule and excess free energy of mixing (ΔG^E) were calculated^{31,32} as described below.

5.4.3 Excess free energy of mixing (ΔG^E)

The interactions between GS and DOPE in the binary monolayers were investigated by calculating the excess free energy of mixing according to **Equation 3.4-1** and **Equation 3.4-2**. ΔG^E were then plotted as a function of monolayer composition at several surface pressures.

$$\Delta G^E = \int_{\pi_L}^{\pi} (A_{12} - A_{12}^{id}) d\pi \quad \text{Equation 3.4-1}$$

$$A_{12}^{id} = X_1 A_1 + X_2 A_2 \quad \text{Equation 3.4-2}$$

where, A_{12} is the measured mean molecular area, A_{12}^{id} is the ideal mean molecular area, and A_1 and A_2 are the mean molecular areas of pure GS and pure DOPE monolayers, respectively. ΔG^E was then plotted as a function of monolayer composition at several surface pressures.

Because GS have significant surface activities, Gibbs excess energies were not calculated from $\pi = 0$ mN/m but rather from the surface pressure at the lift off area, A_L , as determined from the respective isotherms of the pure GS monolayers. Determination of ΔG^E allows for the identification of attractive and repulsive intermolecular interactions in the mixed monolayer. Attractive interactions are indicated by a negative value of ΔG^E and the more negative the value the more stable the mixed monolayer is compared to the pure films. Conversely, positive values of ΔG^E indicate repulsive intermolecular interactions and mixed monolayer instability.

5.4.4 Compressibility modulus (C_s^{-1})

C_s^{-1} was derived from the isotherms according to **Equation 3.4-3** and plotted as a function of surface pressure. The resulting curves were smoothed through adjacent averaging. The value of C_s^{-1} gives information about the molecular packing in the monolayer and the higher the value the more condensed the monolayer.

$$C_s^{-1} = -A \left(\frac{d\pi}{dA} \right) \quad \text{Equation 3.4-3}$$

5.5 Results

5.5.1 18-7-18/DOPE binary monolayers (pH 7)

The π -A isotherms for 18-7-18/DOPE binary mixtures under pH 7 conditions are presented in **Figure 5.5-1A**. The isotherm profiles of all 18-7-18/DOPE binary mixtures are characteristic of expanded liquid phases and do not exhibit phase transitions. In general, the higher the GS molar fraction, the more expanded the monolayer becomes as can be seen from the shift of the isotherm to larger molecular areas; no other change in the isotherms is observed. In addition, due to their high surface activity, the higher the GS molar fraction generally the higher the initial surface pressure of the GS-DOPE mixture. All mixed monolayers are more condensed than the pure 18-7-18 monolayer but more expanded than the pure DOPE monolayer except for the $X_{18-7-18} = 0.1$ monolayer which is also more condensed than the pure DOPE monolayer. The monolayers become more condensed as the proportion of 18-7-18 decreases, as seen in the general trend of decreasing values for lift-off area (A_L) and minimum cross-sectional area (A_{min}) which are approximately 211 Å² and 130 Å² respectively for the $X_{18-7-18} = 0.9$ mixed monolayer and approximately 93 Å² and 60 Å² respectively for the $X_{18-7-18} = 0.1$ mixed monolayer (See **Table C-1 in Appendix C**). The $X_{18-7-18} = 0.5$ monolayer is significantly more expanded than expected with A_L and A_{min} values similar to those of the pure 18-7-18 monolayer (approximately 250 Å² and 166 Å², respectively). The collapse pressure, π_{col} , of the mixed monolayers remains relatively unchanged, and similar to that of the monolayers of the pure components, ranging from 38 to 41 mN/m, when $X_{18-7-18} = 0.4$ –0.9. But π_{col} of the binary mixtures increases to ~ 43 –44.5 mN/m when $X_{18-7-18} \leq 0.3$ with π_{col} increasing with increasing 18-7-18 molar fraction.

The excess free energy of mixing (ΔG^E) plots (**Figure 5.5-1B**) shows negative values for GS-rich ($X_{18-7-18} \geq 0.6$) and GS-poor ($X_{18-7-18} \leq 0.4$) 18-7-18/DOPE mixed monolayers at all surface pressures studied, with ΔG^E increasing in magnitude with increasing surface pressure. This indicates that attractive interactions between 18-7-18 and DOPE dominate in the mixed monolayer at these compositions. The magnitude of ΔG^E is usually larger in the GS-rich monolayers than the GS-poor monolayers, for all surface pressures studied. ΔG^E reaches a minimum (~ -5 kJ/mol) for the $X_{18-7-18} = 0.9$ mixed monolayer at $\pi = 35$ mN/m. Notably, a significantly large positive value of ΔG^E (~ 7 kJ/mol at $\pi = 35$ mN/m) was found at $X_{18-7-18} = 0.5$, which indicates that there is a net

repulsive force in the equimolar mixed monolayer and that 18-7-18 and DOPE are immiscible at this particular composition.

The influence of DOPE on 18-7-18 monolayers was also evaluated from C_s^{-1} – π curves, as presented in **(Figure 5.5-1C)**. When $X_{18-7-18} \geq 0.5$, the mixed monolayers exist in the liquid expanded phase with C_s^{-1} ranging between 32 mN/m and 45 mN/m when $\pi = 30$ –35 mN/m, which are pressures similar to those of biological membranes.³¹ At the same surface pressure range, $X_{18-7-18} \leq 0.4$ mixed monolayers are in the liquid expanded-liquid condensed coexistence phase with higher C_s^{-1} values ranging from 51 mN/m to 78 mN/m. Pure 18-7-18 and pure DOPE monolayers have maximum C_s^{-1} values of 39 mN/m and 72 mN/m, respectively. The maximum C_s^{-1} values of the mixed monolayers when $X_{18-7-18} = 0.1$ –0.3 is slightly greater than that of pure DOPE but when $X_{18-7-18} \geq 0.5$ the maximum C_s^{-1} values of the mixed monolayers are similar to those of the pure GS monolayer (**Table C-1 in Appendix C**).

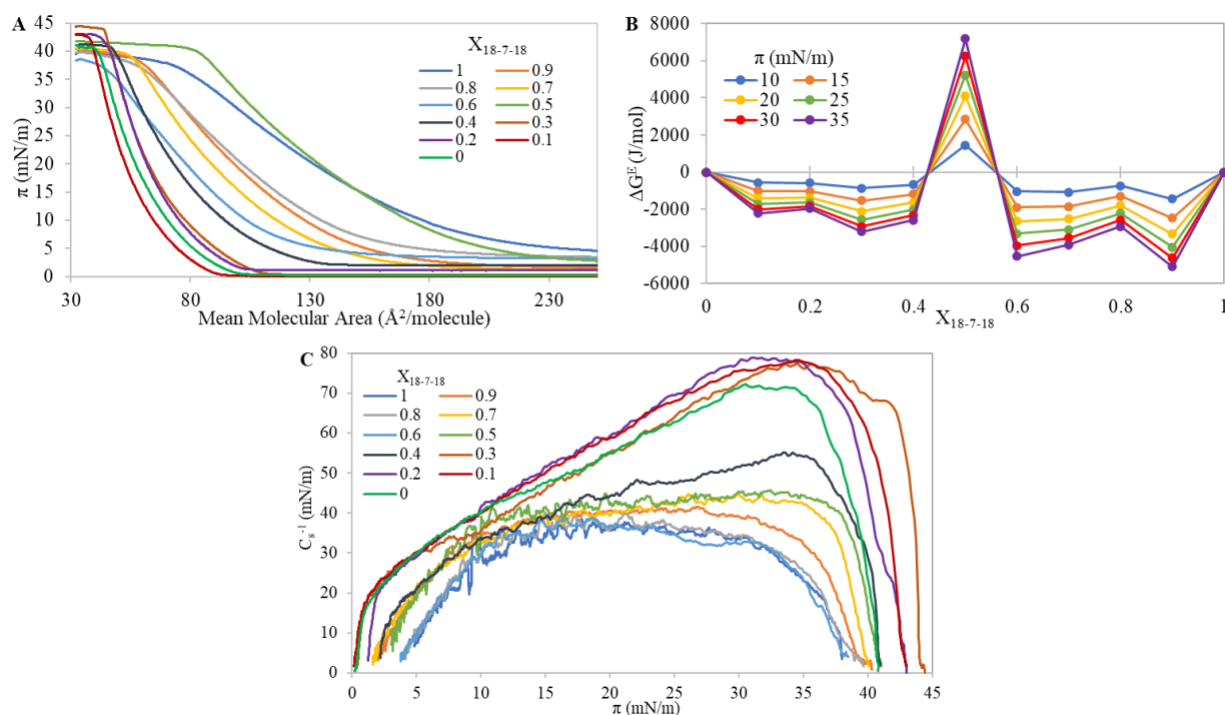


Figure 5.5-1: 18-7-18/DOPE one-component and binary mixed monolayers under pH 7 conditions: **(A)** Surface pressure–area (π –A) isotherms of 18-7-18/DOPE mixed monolayers formed at the air/water interface. **(B)** The excess free energy of mixing (ΔG^E) vs. composition for 18-7-18/DOPE mixed monolayers at various constant surface pressures. **(C)** Compressibility modulus (C_s^{-1})– π curves for 18-7-18/DOPE mixed monolayers.

5.5.2 18-7-18/DOPE binary monolayers (pH 4)

The π -A isotherms for 18-7-18/DOPE mixtures in pH 4 conditions are presented in **Figure 5.5-2A**. The isotherm profiles for these binary mixtures are again characteristic of expanded liquid phases with no observable phase transitions. The 18-7-18/DOPE mixed monolayers tend to be more expanded at pH 4 than at pH 7 as indicated by larger values of A_L and A_{min} ; although when $X_{18-7-18} \leq 0.3$, A_{min} is smaller at pH 4 than at pH 7 (see **Appendix C**, Table C-1). A_L and A_{min} were approximately $240 \text{ \AA}^2$ and $151 \text{ \AA}^2$ respectively for the $X_{18-7-18} = 0.9$ mixed monolayer and approximately $115 \text{ \AA}^2$ and $56 \text{ \AA}^2$ respectively for the $X_{18-7-18} = 0.1$ mixed monolayer under acidic conditions. In addition, the $X_{18-7-18} = 0.5$ monolayer is significantly more expanded than the pure 18-7-18 monolayer. pH had little effect on the π_{col} of the 18-7-18/DOPE mixed monolayers; at pH 4 π_{col} ranges between 37 mN/m and 44 mN/m for $X_{18-7-18} = 0.9$ –0.4 with no clear trend with composition. However, no collapse point is reached for $X_{18-7-18} \leq 0.3$ indicating that these mixed monolayers could be compressed further than allowed for by the experimental setup.

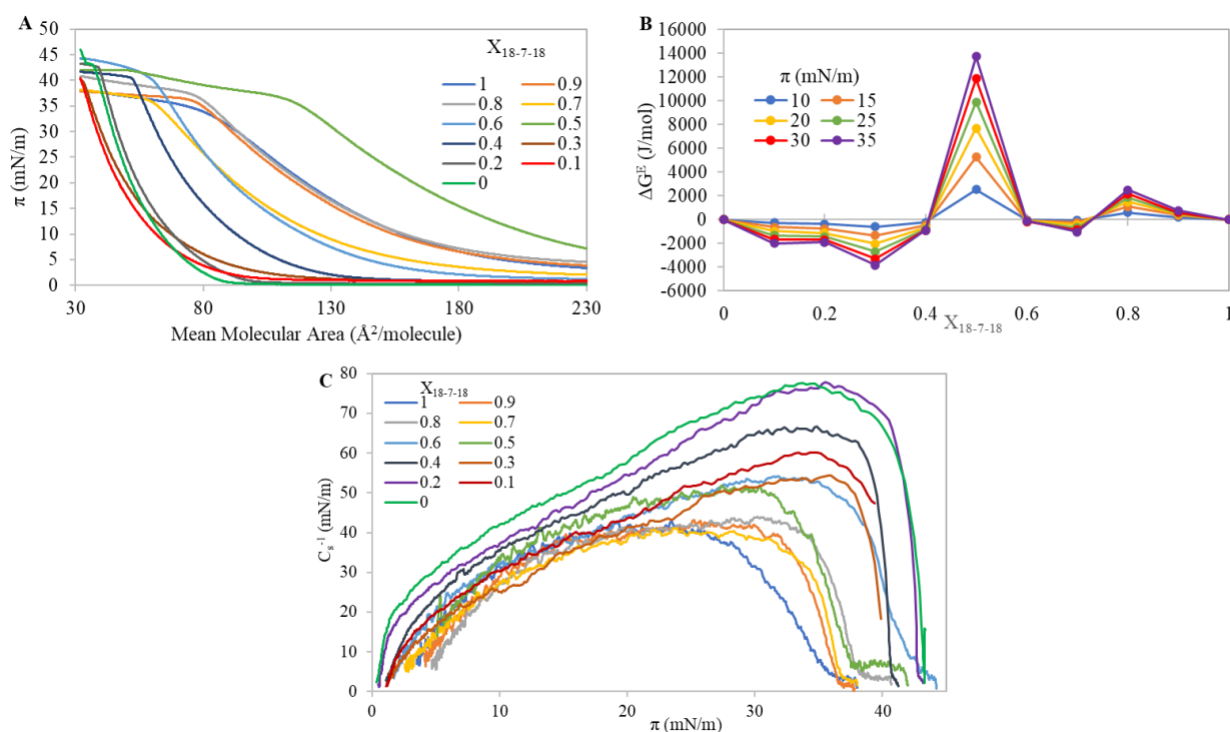


Figure 5.5-2: 18-7-18/DOPE one-component and binary mixed monolayers under pH 4 conditions: (A) Surface pressure–area (π -A) isotherms of 18-7-18/DOPE mixed monolayers formed at the air/water interface. (B) The excess free energy of mixing (ΔG^E) vs. composition for 18-7-18/DOPE mixed monolayers at various constant surface pressures. (C) Compressibility modulus (C_s^{-1})- π curves for 18-7-18/DOPE mixed monolayers.

Notably, the isotherms of $X_{18-7-18} = 0.9$ and 0.8 mixed monolayers are much more similar to the pure 18-7-18 monolayer under pH 4 conditions than under neutral conditions. This indicates that the expected condensation effect upon adding a small amount of DOPE to the GS monolayer is not as strong under acidic conditions. In fact, monolayer expansion is confirmed by positive rather than negative values of ΔG^E for $X_{18-7-18} = 0.8$ and 0.9 ($\Delta G^E \sim 2.5$ kJ/mol and $\Delta G^E \sim 0.75$ kJ/mol, respectively, see **Figure 5.5-2B**, indicating that repulsive interactions predominate in these mixed monolayer compositions. In addition, under pH 4 conditions, ΔG^E is about 0 J/mol at $X_{18-7-18} = 0.6$ and at $X_{18-7-18} = 0.7$ it is slightly negative. Negative values of ΔG^E are observed for all GS-poor ($X_{18-7-18} \leq 0.4$) monolayers confirming net attractive interactions between 18-7-18 and DOPE in the monolayers and their miscibility only at these compositions. A minimum value of ΔG^E (~ -4 kJ/mol) is achieved when $X_{18-7-18} = 0.3$. Again, the value of ΔG^E of the $X_{18-7-18} = 0.5$ mixed monolayer is highly positive (~ 14 kJ/mol at $\pi = 35$ mN/m) indicating very strong net repulsive GS-DOPE interactions in the monolayer at this particular composition.

The C_s^{-1} - π plots for 18-7-18/DOPE mixed monolayers at pH 4 are presented in **Figure 5.5-2C**. Again, all monolayers are in the liquid expanded phase ($X_{18-7-18} \geq 0.7$) or the liquid expanded-liquid condensed coexistence phase ($X_{18-7-18} \leq 0.6$) when $\pi = 30$ – 35 mN/m. The maximum C_s^{-1} values of the pure 18-7-18 and pure DOPE monolayers are slightly higher in acidic conditions, 43 mN/m and 78 mN/m, respectively. Like the neutral conditions presented above, maximum C_s^{-1} values generally increase with decreasing $X_{18-7-18}$ ranging between 43 mN/m and 79 mN/m under acidic conditions.

5.5.3 18-7NH-18/DOPE binary monolayers (pH 7)

The π -A isotherms for 18-7NH-18/DOPE mixtures in pH 7 conditions are presented in **Figure 5.5-3A** and are again representative of expanded liquid phases and do not show any phase transitions. Compared to the monolayers composed with the unsubstituted GS, 18-7NH-18/DOPE mixed monolayers are more condensed at a given composition as indicated by smaller A_L and A_{min} values. These monolayer parameters were approximately $157 \text{ \AA}^2$ and $88 \text{ \AA}^2$ respectively for the $X_{18-7NH-18} = 0.9$ mixed monolayer and approximately $101 \text{ \AA}^2$ and $61 \text{ \AA}^2$ respectively for the $X_{18-7NH-18} = 0.1$ mixed monolayer. The isotherms of $X_{18-7NH-18} = 0.8, 0.9$ are more condensed than the pure 18-7NH-18 monolayer but are very similar to each other with nearly identical A_{min} values of $\sim 85 \text{ \AA}^2$. Also, the profiles of the GS-poor monolayers ($X_{18-7NH-18} = 0.1$ – 0.4) are similar to that of

the one-component DOPE monolayer, with the $X_{18-7NH-18} = 0.1$, and 0.2 monolayers being slightly more condensed. π_{col} of the pure 18-7NH-18 monolayer is 43.5 mN/m which is higher than that of 18-7-18 under neutral conditions. π_{col} of all the 18-7NH-18/DOPE mixed monolayers ranges from 43 mN/m to 49 mN/m which is slightly higher than the collapse pressures of the 18-7-18/DOPE mixed monolayers.

Like the 18-7-18/DOPE mixed monolayers at pH 7, **Figure 5.5-3B** shows negative values of ΔG^E minimums when $X_{18-7NH-18} \geq 0.6$ and $X_{18-7NH-18} \leq 0.4$, confirming the miscibility of the monolayer components, at all surface pressures studied. However, the magnitude of the ΔG^E values are smaller in the 18-7NH-18/DOPE mixed monolayers. Minimum ΔG^E values are achieved at $X_{18-7NH-18} = 0.7$ for GS-rich monolayers and at $X_{18-7NH-18} = 0.4$ for GS-poor monolayers ($\Delta G^E \sim -4.5$ kJ/mol and $\Delta G^E \sim -3.5$ kJ/mol, respectively, at $\pi = 35$ mN/m). Also, the magnitude of ΔG^E at $X_{18-7NH-18} = 0.5$ is smaller ($\Delta G^E \sim 4.4$ kJ/mol at $\pi = 35$ mN/m) compared to the $X_{18-7-18} = 0.5$ mixed monolayer under neutral conditions.

The C_s^{-1} - π plots (**Figure 5.5-3C**) show that all monolayers, pure and mixed, exist in the liquid expanded-liquid condensed coexistence phase at $\pi = 30$ –35 mN/m. C_s^{-1} ranges from ~ 55 mN/m to 100 mN/m in this range of surface pressures with C_s^{-1} generally increasing as the proportion of GS decreases. The maximum C_s^{-1} values of the 18-7NH-18/DOPE mixed monolayers are greater than that of the 18-7-18/DOPE mixed monolayers, at a given composition, ranging from 59 mN/m to 100 mN/m and increasing with decreasing $X_{18-7NH-18}$.

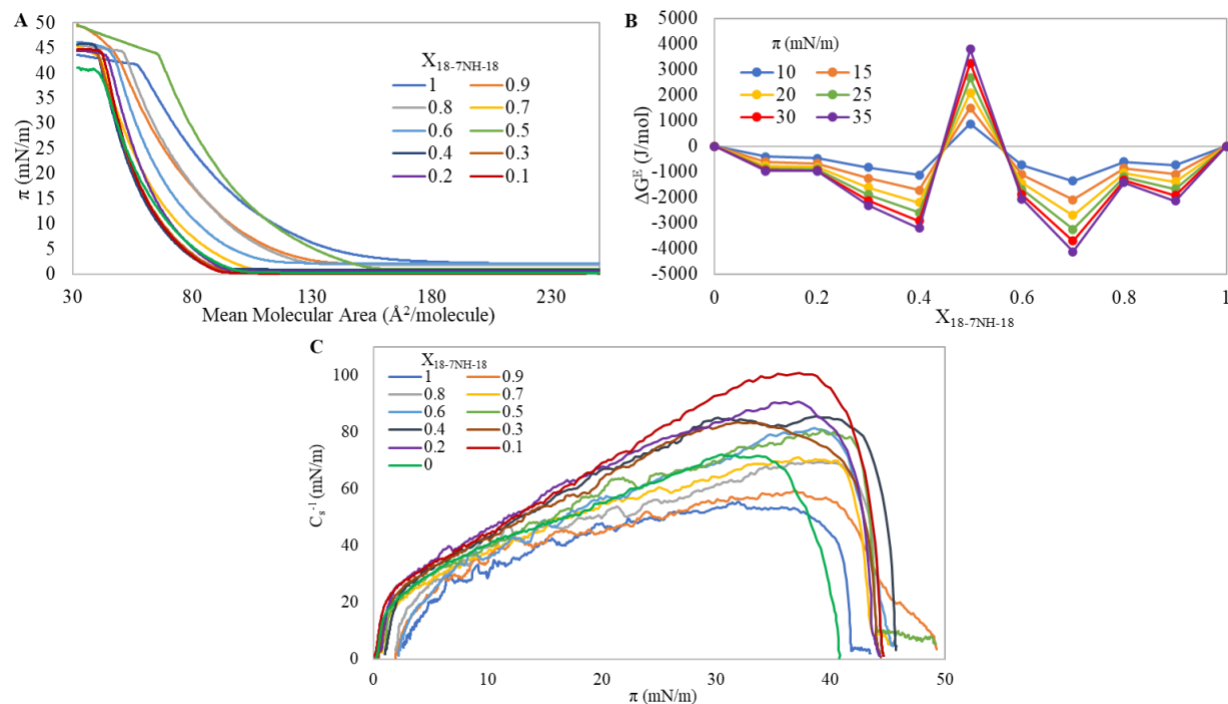


Figure 5.5-3: 18-7NH-18/DOPE one-component and binary mixed monolayers under pH 7 conditions: (A) Surface pressure–area (π –A) isotherms of 18-7NH-18/DOPE mixed monolayers formed at the air/water interface. (B) The excess free energy of mixing (ΔG^E) vs. composition for 18-7NH-18/DOPE mixed monolayers at various constant surface pressures. (C) Compressibility modulus (C_s^{-1})– π curves for 18-7-18/DOPE mixed monolayers.

5.5.4 18-7NH-18/DOPE binary monolayers (pH 4)

The π -A isotherms for 18-7NH-18/DOPE mixtures in pH 4 conditions are presented in **Figure 5.5-4A**. The isotherm profiles of all these binary mixtures are again characteristic of expanded liquid phases with no observable phase transitions. The 18-7NH-18/DOPE mixed monolayers have smaller values of A_L and A_{min} at a given X_{GS} than the 18-7-18/DOPE mixed monolayers under acidic conditions. This indicates that the substituted GS mixed monolayers are more condensed. A_L and A_{min} were determined to be approximately $185 \text{ \AA}^2$ and $102 \text{ \AA}^2$ respectively for the $X_{18-7NH-18} = 0.9$ mixed monolayer and approximately $94 \text{ \AA}^2$ and $55 \text{ \AA}^2$ respectively for the $X_{18-7NH-18} = 0.1$ mixed monolayer at pH 4. Also, though still more expanded than expected, unlike other monolayers of this composition, the $X_{18-7NH-18} = 0.5$ mixed monolayer in this series is more condensed than the pure 18-7NH-18 monolayer as indicated by smaller values of A_L and A_{min} . All the isotherms with 18-7NH-18 molar fractions in the range of 0.1–0.4 have very similar isotherms which are comparable to that of pure DOPE. None of the GS-rich 18-7NH-18/DOPE monolayers reached a collapse point in acidic conditions meaning they could be compressed further than the experimental setup allowed. π_{col} of the GS-poor 18-7NH-18/DOPE monolayers was similar to that of pure DOPE at pH 4 ranging from $\sim 44 \text{ mN/m}$ to 45 mN/m .

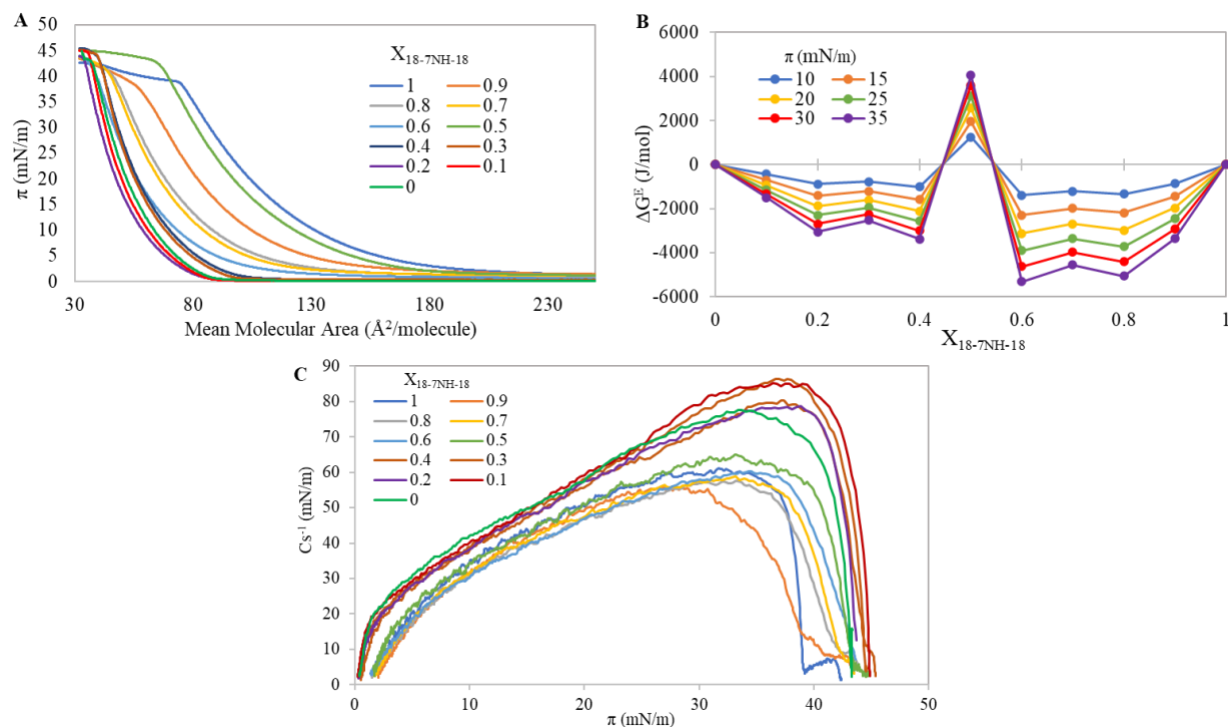


Figure 5.5-4: 18-7NH-18/DOPE one-component and binary mixed monolayers under pH 4 conditions: (A) Surface pressure–area (π –A) isotherms of 18-7NH-18/DOPE mixed monolayers formed at the air/water interface. (B) The excess free energy of mixing (ΔG^E) vs. composition for 18-7NH-18/DOPE mixed monolayers at various constant surface pressures. (C) Compressibility modulus (C_s^{-1})– π curves for 18-7NH-18/DOPE mixed monolayers.

Again, negative values of ΔG^E were determined for $X_{18-7NH-18} \leq 0.4$ and $X_{18-7NH-18} \geq 0.6$ mixed monolayers, as seen in **Figure 5.5-4B**, indicating a net attractive interaction between 18-7NH-18 and DOPE in these mixed monolayers. A minimum is reached at $X_{18-7NH-18} = 0.6$ ($\Delta G^E \sim -6$ kJ/mol) and a maximum at $X_{18-7NH-18} = 0.5$ ($\Delta G^E \sim 4.5$ kJ/mol). Also, like all the other series, there is a significant positive deviation for $X_{18-7NH-18} = 0.5$ suggesting a net repulsive interaction at this particular monolayer composition.

The C_s^{-1} – π plots of the 18-7NH-18/DOPE mixed monolayers under pH 4 conditions are presented in **Figure 5.5-4C**. Like at neutral conditions, under acidic conditions all the 18-7NH-18/DOPE mixed monolayers are in the liquid expanded-liquid condensed coexistence phase when $\pi = 30$ – 35 mN/m with C_s^{-1} values ranging from 54 mN/m to 84 mN/m in this surface pressure range. Maximum C_s^{-1} values are slightly lower (56–86 mN/m) at pH 4 than at pH 7 but also generally increase with decreasing amount of 18-7NH-18.

5.6 Discussion

18-7-18 and 18-7NH-18 are synthetic gemini surfactants with essentially the same molecular structure except for the substitution of the central methylene (CH_2) unit in the spacer group of 18-7-18 for an amine (NH) group in 18-7NH-18. This slight modification was made so that the head group of 18-7NH-18 would resemble the structure of spermidine which is known to condense DNA more effectively than other polyamines.⁹ In addition, the inclusion of the pH-sensitive amine group in the GS-DOPE lipoplex is thought to promote the proton sponge mechanism of endosomal escape in a similar way as other polyethylenimine-based non-viral vectors.³³⁻³⁵ However, the 7NH spacer is less flexible than the $s = 7$ spacer due to a hydrogen bond formed between the amine substitution and the aqueous subphase. Zwitterionic lipids, like DOPE, are commonly used as helper lipids in non-viral vectors. DOPE has a relatively small polar headgroup compared to its C(18:1 Δ^9):C(18:1 Δ^9) hydrophobic tail group. As such DOPE has a truncated cone form and tends to aggregate in the inverted hexagonal phase in aqueous dispersions.

The monolayers of pure 18-7-18 and pure 18-7NH-18 are extremely expanded due to the large size of the head groups of these molecules, consisting of two cationic quaternary ammonium groups as well as the GS spacer group, and strong intermolecular repulsion between these cationic charges (**Figure 5.6-A**). In contrast, the highly condensed nature of the monolayer of pure DOPE is a consequence of the ability of phosphatidylethanolamine (PE) lipids to form lateral intra- and intermolecular hydrogen bonds. These lateral interactions reduce the hydration of the PE headgroup resulting in densely packed monolayers³⁰ (**Figure 5.6-1B**). The behaviour of the mixed GS/DOPE monolayers depends on the balance between repulsive and attractive intermolecular interactions and can be explained in a similar way as the behaviour of cationic lipid/DOPE mixed monolayers.³⁰

The significant degree of monolayer condensation that is observed upon adding a small amount of DOPE to the pure 18-7-18 monolayer when $X_{18-7-18} \geq 0.6$ (**Figure 5.5-1A**) results from the reduction of repulsive forces between the cationic quaternary ammonium head groups of the GS through the introduction of attractive interactions between the GS headgroups and DOPE's phosphate group (see **Figure 5.6-1D**). The interspersions of DOPE molecules between GS molecules significantly reduces the mean area occupied per molecule as reflected in the negative deviations from the surface area additivity rule and large negative values of ΔG^E (**Figure 5.5-1B**) over this range of monolayer composition. In contrast, the addition of a small amount of 18-7-18

($X_{18-7-18} \leq 0.4$) expands the pure DOPE monolayer. The presence of a small quantity of cationic polar head groups disrupts intermolecular PE-PE interactions and the formation of hydrogen bonds (**Figure 5.6-1E**). However, even when the GS content of the monolayer is low, the effect of attractive interactions due to the condensation of 18-7-18 by DOPE is strong enough that there is a net attractive interaction in GS-poor monolayers. As such, negative deviations from ideality and negative values of ΔG^E are also observed over this range of monolayer composition, though the magnitude of these values is smaller than observed for the GS-rich compositions. The equimolar composition, $X_{GS} = 0.5$, is the only composition at which the repulsive interactions due to the disruption of DOPE packing by cationic charges due to the presence of GS is stronger than the attractive interactions resulting from GS condensation by DOPE. This leads to a net repulsive force in the monolayer at only this composition resulting in the formation of highly expanded monolayers.

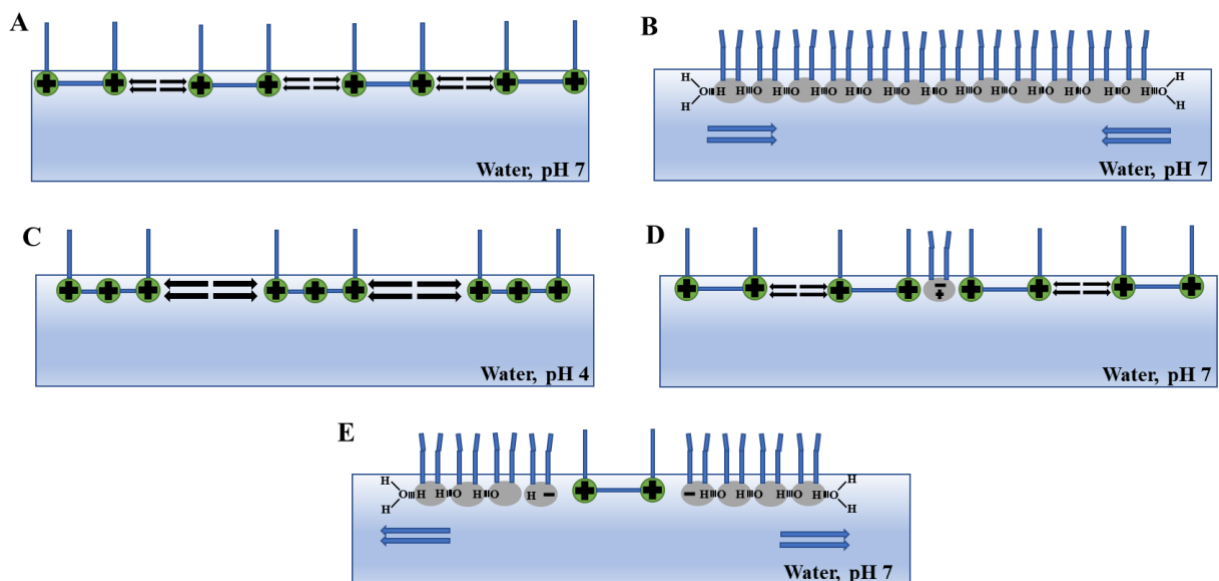


Figure 5.5-5: Schematic representation of 18-7-18, 18-7NH-18, and DOPE one-component and mixed monolayers at the air/water interface. (A) One-component 18-7-18 monolayer. The cationic quaternary headgroups of the GS are represented by green circles. The parallel arrows represent electrostatic repulsion between headgroups. (B) One-component DOPE monolayer. The zwitterionic headgroup is represented by grey circles. Intramolecular interactions that result in a densely packed monolayer are depicted (compression represented by blue arrows). (C) One-component 18-7NH-18 monolayer under acidic conditions. The amine substitution in the spacer group of this GS is protonated in a higher proportion of molecules under acidic conditions than under neutral conditions resulting in increased intermolecular repulsion and a more expanded monolayer. (D) GS-rich 18-7-18/DOPE mixed monolayers. A small addition of DOPE reduces GS headgroup repulsion (arrows represent remaining repulsive interactions in monolayer). The same effect occurs under pH 4 conditions and in GS-rich 18-7NH-18/DOPE mixed monolayers. (E) GS-poor 18-7-18/DOPE mixed monolayer. A small addition of GS disrupts intermolecular PE-PE interactions (arrows represent monolayer expansion). The same effect occurs under pH 4 conditions and in GS-poor 18-7NH-18/DOPE mixed monolayers.

The balance of forces is altered when the aqueous subphase of the monolayers is changed from neutral pH to acidic conditions. Under acidic conditions, the phosphate group of a higher proportion of DOPE molecules is protonated than under neutral conditions. Without the anionic charge, the protonated DOPE molecules are unable to condense 18-7-18 and instead there is a repulsive interaction. As such, when the amount of DOPE in the monolayer is small (10%–20%), the attractive effect of 18-7-18 condensation by unprotonated DOPE molecules is outweighed by repulsive DOPE–GS interactions resulting in a monolayer that is more expanded than expected. This change in behaviour is evident in the results of the $X_{18-7-18} = 0.8$ – 0.9 mixed monolayers whose

isotherms are similar to that of the pure GS (**Figure 5.5-2A**) and have positive deviations from the additivity rule and positive ΔG^E (**Figure 5.5-2B**) values under acidic conditions whereas these metrics are negative under neutral conditions. When the proportion of DOPE is increased (30%–40%), the number of unprotonated DOPE molecules is also correspondingly increased and sufficient to reduce intermolecular repulsion between the cationic quaternary ammonium head groups of some GS molecules such that this attractive force just outweighs the protonated DOPE–GS repulsion force. As such, at $X_{18-7-18} = 0.6–0.7$ very slight negative deviations from surface area additivity and very small negative values of ΔG^E are observed. As the amount of DOPE increases in the mixed monolayers there is a sufficient number of unprotonated DOPE molecules present to minimize intermolecular repulsion between the relatively small number of 18-7-18 molecules in GS-poor monolayers. Like under pH 7 conditions, this attractive force outweighs the DOPE monolayer expansion effect resulting from the disruption of intra- and intermolecular PE-PE interactions by the presence of a small amount of polar cationic GS head groups. Thus, when $X_{18-7-18} = 0.1–0.3$, negative deviations from ideality and negative values of ΔG^E with magnitudes similar to those at pH 7 are observed. Again, the results obtained for the equimolar composition, $X_{18-7-18} = 0.5$, under acidic conditions indicate that repulsive interactions are dominant at this composition. The magnitude of deviation from ideality and ΔG^E is however much greater under pH 4 conditions due to the decreased ability of DOPE to minimize repulsion between the cationic GS head groups and increased GS-DOPE repulsion due to phosphate group protonation on a greater proportion of DOPE molecules.

The behaviour of the $X_{18-7-18} = 0.8$ and 0.9 mixed monolayers is particularly interesting. At pH 7, typical liposome preparation conditions, 18-7-18 and DOPE show synergistic behaviour (negative deviations from ideality, and large negative values of ΔG^E , (**Figure 5.5-1B**) but under acidic conditions, like those encountered in the endosome, the monolayers of these mixtures are more expanded than under neutral conditions. Antagonistic mixing behaviour predominates under acidic conditions as seen from positive deviations from ideality and positive ΔG^E values (**Figure 5.5-2B**). This observation is a critical and important finding of this study as it suggests that there is some degree of 18-7-18/DOPE aggregate disassembly in acidic conditions which may ultimately promote the release of the genetic load from the GS/DOPE liposome and improve transfection efficiency.

The results of the 18-7NH-18/DOPE mixed monolayers can be explained in a similar way to the results of the 18-7-18/DOPE mixed monolayers previously discussed. When 18-7NH-18 is the major component of the mixed monolayer, attractive forces between DOPE's phosphate group and the cationic quaternary head groups of the GS dominate, effectively reducing the mean molecular area occupied per molecule resulting in more condensed monolayers. In general, the magnitude of the deviations from ideality and ΔG^E values are smaller for 18-7NH-18/DOPE mixed monolayers than the 18-7-18/DOPE monolayer of the same composition. This is likely simply since the pure 18-7NH-18 monolayer is itself more condensed than the pure 18-7-18 monolayer. A minimum in ΔG^E is observed at $X_{18-7NH-18} = 0.7$ indicating that the optimum balance between the induced dipoles from the zwitterionic DOPE and the cationic charges from the GS head groups is achieved at this mixed monolayer composition. The repulsive effect of the disruption of DOPE packing by the cationic charge of 18-7NH-18 is again outweighed by the large attractive force of 18-7NH-18 condensation by DOPE in GS-poor monolayers leading to an overall net attractive force as seen from negative deviations from surface additivity and negative values of ΔG^E (**Figure 5.5-3B**) when $X_{18-7NH-18} = 0.1-0.4$. Again, the equimolar composition is the only one where repulsive 18-7NH-18/DOPE interactions dominate leading to an expanded monolayer, positive deviations from additivity, and a positive value of ΔG^E .

The balance of forces is again different in the 18-7NH-18/DOPE mixed monolayers under acidic conditions than in the other series studied. At pH 4, the amino substitution in the spacer group of 18-7NH-18 is protonated in a greater number of molecules than at pH 7. As such, it is unsurprising that the pure monolayer of 18-7NH-18 is more expanded at pH 4 than at pH 7 due to increased intermolecular repulsion as a result of the increased charge of the molecule (**Figure 5.6-1C**). Unlike the 18-7-18/DOPE series, attractive GS–DOPE interactions outweigh the repulsive nature of intermolecular GS interactions and the disruption of DOPE packing by GS at all 18-7NH-18/DOPE compositions, except $X_{18-7NH-18} = 0.5$, under acidic conditions. Thus, the condensation effect of DOPE on the 18-7NH-18 monolayer is stronger under acidic conditions than neutral conditions leading to larger magnitudes of the negative deviations from ideality and ΔG^E values. Again, the equimolar composition is the only one where repulsive 18-7NH-18/DOPE interactions dominate leading to an expanded monolayer, a positive deviation from additivity, and a positive value of ΔG^E . Though the magnitude of this positive deviation is larger under acidic

conditions than neutral conditions due to the increased intermolecular repulsion between triply charged 18-7NH-18 molecules.

An evaluation of the transfection efficiency of 18-7-18 and 18-7NH-18 GS in combination with DOPE has been previously completed in PAM 212 cell lines using a flow cytometric approach.³⁷ Overall low transfection efficiency was observed with both 18-7-18/DOPE (3.4 % cells transfected) and 18-7NH-18/DOPE (3.9% cells transfected) lipoplexes. Interestingly, comparable transfection efficiency was observed between the two lipoplexes despite the presence of the pH-sensitive functional group of the 18-7NH-18 GS. However, when comparing the different series of GS, the 18 series GS exhibited the highest transfection efficiency followed by the 16 series (2.3% cells transfected) and the lowest transfection efficiency was revealed by the 12 series GS (0.9% cells transfected). This trend was attributed to the C18 GS having longer alkyl tails, resulting in efficient encapsulation of the nucleic acid and thereby improved gene delivery.³⁷ Considering the overall low transfection achieved by the 18-7-18/DOPE and 18-7NH-18/DOPE lipoplexes, these formulations were not further studied *in vitro* for this work. However, the results of this work do provide insight into the results of a previous study conducted in our group which investigated the physicochemical properties and transfection efficiency between GS-DOPE-LCC DNA minivectors.¹ Overall, low transgene expression was observed with these systems (13% cells transfected) compared to the Lipofectamine control group. The results obtained from this work may now be used to explain the low transfection efficiencies of GS-DOPE-LCC DNA minivectors.¹ The molecular interactions between the GS-DOPE may be synergistic which may have resulted in an insufficient release of the LCC DNA minivector from the vector complex, resulting in low transgene expression.

It is also important to note that the results obtained in this study, namely that attractive interactions predominate in GS/DOPE mixed monolayers at nearly all monolayer compositions for both 18-7-18 and 18-7NH-18 GS and under both neutral and acidic conditions, differ from a previous study by our group.³⁶ This earlier study examined 16-s-16 and 16-7NH-16 mixing behaviour with DOPE in mixed micelles and concluded that interactions were predominately antagonistic due to a difference in the preference of curvature of the GS and DOPE monomers. GS tend to aggregate in micellar structures whereas DOPE aggregates in inverted hexagonal structures or bilayers in aqueous solution. The characterization of GS/DOPE mixed aggregates is key to advancing our understanding of the liposome structure of GS-based lipoplexes but the stark

difference in conclusions that are drawn from the study of monolayer and micellar structures underscores the importance of morphological considerations when interpreting the electrostatics of mixed GS/DOPE systems.

5.7 Conclusions and Future Directions

We have studied the interaction between the phosphatidylethanolamine lipid DOPE and two gemini surfactants, 18-7-18 and 18-7NH-18, used for non-viral vector formulation in gene therapy applications. Strong attractive interactions resulting from the condensation of GS by DOPE dominate in most of the binary mixtures studied indicating the miscibility of GS and DOPE. Repulsive interactions dominate in $X_{GS} = 0.5$ monolayers resulting in highly expanded monolayers. The results obtained for $X_{18-7-18} = 0.8, 0.9$ mixed monolayer indicate the potential for GS/DOPE lipoplex disassembly which may promote transfection. However, considering the low transfection efficiency achieved by GS-DOPE based lipoplexes from previously published work in our group, this system was not pursued as a viable transfection product. Nonetheless, the results achieved are interesting from the perspective of monolayer structure, fundamental chemistry, and lipid-surfactant energetics and contributes to the understanding of GS/DOPE interactions for future projects involving GS-based lipoplexes.

Chapter 6

Conclusions, Future Directions, and Scientific Contribution

6.1 Conclusions

Non-viral gene delivery systems require a rational design approach, where each of the vector components are selected based on properties which are known to favour transfection efficiency. This thesis has focused on the importance of a detailed understanding of both main components of a potential gene therapy: the carrier **and** the therapeutic cargo – namely the DNA. Given the enormous range of materials that have been investigated and reported on in the literature, this is particularly important for non-viral gene delivery systems; however, this work also demonstrates the importance of DNA topology. In this work, interactions between synthetic carriers (lipids and polymers) and nucleic acids (LCC DNA minivectors or CCC plasmid DNA) were evaluated to further understand which structural features are involved in the formation of these nanoparticles. From our observations, the rational design approach should not only be considered for the design of the synthetic cationic component, but it should also extend out to the design of the DNA construct as well since DNA size and topology do influence transfection efficiency.

Until recently, most efforts have focused on the rational design of synthetic vectors for improved delivery of the therapeutic gene to the target cell. However, efficient gene therapeutics also require the rational design of the enclosed DNA cargo such that optimal gene expression and the corresponding therapeutic effect can take place in the cell. Modifying the composition of the DNA cargo may also contribute to improving their bioavailability, biocompatibility, durability, and safety. Prokaryotic DNA sequences, such as CpG motifs, origins of replication and antibiotic selectable markers, in recombinant CCC plasmid DNA have the potential for eliciting unwanted immunostimulatory responses in the administered host which reduces their biocompatibility, safety and consequently their clinical potential.¹ Removal of the unnecessary plasmid sequences in the design of LCC DNA minivectors enhances their overall safety profile compared to recombinant CCC plasmid DNA.¹ Upon exploring the interactions between various lipid and polymer based nanocarriers complexed with LCC DNA minivectors and the parental CCC pDNA

in Chapter 3 and Chapter 4, some important relationships between DNA size, topology, physicochemical properties and transfection efficiency have come to light. Most notably, no differences were seen for particle size and polydispersity index measurements that could be attributed to influences from different DNA sizes or topological conformations. Most of the formulations evaluated produced nanoparticles uniformly distributed (PDI values below 0.2) with sizes below 200 nm, which is desirable for future *in vivo* applications to reduce clearance by glomerular filtration. Furthermore, compared to LCC DNA minivectors (zeta potential: - 32 mV) the supercoiling effect shielded a fraction of the negative charges of CCC pDNA which led to slightly lower (-) surface charges (zeta potential: - 26 mV). Of note, the N⁺/P⁻ charge ratio used to prepare the transfection treatment influenced their physicochemical properties. Generally, increasing the N⁺/P⁻ charge ratio to 8:1 produced smaller and uniformly distributed nanoparticles with a high positive zeta potential. These characteristics are desirable to facilitate interactions with cell membranes and enhance cell internalization. In terms of *in vitro* transfection efficiency in HEK 293 and APRE-19 cells, gene expression varied significantly depending on the physicochemical properties of the nanocarrier and the N⁺/P⁻ charge ratio of the formulations and this trend aligns with previously reported literature. Transfection showed a general increase with smaller and more positive zeta potentials which are known to facilitate interactions with cell membranes and improve cell uptake.

From Chapter 3, transfection treatments prepared with Lipofectamine 3000 at higher charge ratios (4:1 and 8:1) showed greater transfection, while a statistically significant increase in transfection efficiency was achieved with formulations prepared with Polymer 2 at the 8:1 N/P charge ratio as seen in Chapter 4. Considering the findings reported in Chapter 3 and Chapter 4, it is clear that regardless of the type of carrier or size, formulations prepared with the LCC DNA minivectors exhibited statistically significant higher transfection efficiency confirming that DNA properties such as size, composition, and topology do play a role in gene delivery and should not be ignored. The differences in transfection efficiencies observed in these systems reflect some of the trends previously reported in the literature between intracellular trafficking and DNA topology. In particular, due to their linear topology, LCC DNA minivectors are known to show improved cytoplasmic diffusion and intracellular trafficking which is expected to help promote gene expression.

In Chapter 5, synergistic interactions dominated in most of the binary 18-7-18 or 18-7-18 - DOPE mixtures which provided insight into the low transfection efficiencies observed by a previous study with GS-DOPE-LCC DNA minivectors. An exception to this was the $X_{GS} = 0.5$ monolayers which showed antagonistic mixing. Based on the changes in mixing behaviour detected using Langmuir monolayer methods under both neutral and acidic conditions, when $X_{GS} \geq 0.6$ and $X_{GS} \leq 0.4$, attractive interactions dominate due to GS condensation by DOPE which led to the formation of condensed monolayers, indicating miscibility between these two compounds. The magnitude of the synergistic interactions responsible for the mixed micelle stability was influenced by the composition of the mixture. Conversely, repulsive intermolecular interactions were highly favourable at $X_{GS} = 0.5$ monolayers suggesting that DOPE packing is significantly influenced by the cationic charges of GS. Interestingly, under neutral conditions, 18-7-18 and DOPE at mole fractions of $X_{18-7-18} = 0.8$ and 0.9 showed a typical synergistic behaviour, while under acidic conditions, the monolayers of these mixtures were more expanded and show antagonistic mixing. This is an important finding as it reveals the ability of 18-7-18/DOPE aggregate disassembly under acidic conditions which may promote the release of the genetic load from the GS/DOPE liposome and should be investigated further.

Based on the result from Chapter 3 and Chapter 4, statistically significant increase in LCC DNA minivector expression was observed with formulations prepared with the Polymer 2 nanocarrier. While the proprietary nature of Polymer 2 does not allow for an in-depth analysis of specific structural features, our results over all demonstrated the greatest levels of transfection for the LCC DNA/Polymer 2 combination and suggests that polymer nanocarriers have higher compatibility with LCC DNA minivectors than lipid nanoparticles. As such, the Polymer 2/LCC DNA minivector mixtures should have the greatest potential for maximized transfection efficiency and this formulation is recommended for further evaluation *in vivo* to assess the biodistribution of this combination in living organisms.

6.2 Future directions

To bridge the gap between this thesis work and clinical applicability, additional mixtures and techniques have been identified as possible extensions of this work to further investigate the interactions between synthetic carriers and DNA constructs and optimize these formulations for gene therapy applications.

Synthetic gene carrier and DNA shape is an important property to characterize as it also contributes to the efficiency and safety profile of gene therapy nanoparticles. As such, electron microscopy studies would be a valuable addition to the physical characterization of the lipid and polymer-based transfection treatments. Both scanning electron microscopy (SEM) and transmission electron microscopy (TEM) can be used to visualize the shape as well as surface area of hydrophobic and hydrophilic nanoparticles through electron beams, while also further confirming the particle sizes of the nanoparticles.² Additionally, pH is a critical factor to consider in the rational design of non-viral nanocarriers because it has an impact on nanoparticle solubility, stability, and efficiency. All nanoparticles possess different physicochemical properties at varying pH conditions.³ For example, at pH 4, imino functionalities are expected to be protonated which are known to induce endosomal escape through the proton sponge mechanism, while at pH 7, these imino groups will not be protonated.³ The current study focused on evaluating the mixtures at pH 7. However, characterizing the transfection treatments at pH 4 would allow for an improved understanding of how the nanoparticles will interact in the endosome to facilitate endosomal escape - one of the critical steps of the transfection process. Evaluating non-viral formulations under various conditions that contribute to higher or lower transfection efficiency is valuable in further optimizing more successful non-viral gene delivery vectors.

The work presented in Chapter 3 and Chapter 4 focused on testing various carrier-DNA candidates using *in vitro* cell culture systems. Previously reported literature has found a weak correlation between *in vitro* and *in vivo* transfection efficiency and so further studies must be carried out to optimize non-viral formulations.⁴ As an example, a study using a high-throughput *in vivo* approach which pairs carrier candidates with plasmid vectors containing unique DNA barcodes would allow for the evaluation of various carriers in combination with DNA of different topologies simultaneously in the same animal *in vivo*, allowing for direct comparison of their transfection ability in the target tissue of interest.⁵ For this technique, using high-throughput microfluidic mixing, 25-30 carrier candidates can be formulated, with each one carrying a unique

DNA barcoded plasmid. Meaning that carrier candidate 1 can carry DNA barcode A, and carrier candidate 2 will carry DNA barcode B, etc. Following formulation, each carrier-DNA candidate is QC tested to ensure proper particle formation ($20\text{ nm} < \text{nanoparticle diameter} < 300\text{ nm}$). Carrier-DNA candidates that pass QC are then used for *in vivo* testing. Each carrier-DNA is kept separate until just prior to *in vivo* injection, to avoid prolonged mixing of unique barcoded carriers and the potential for fusion into hybrid carriers, which would contaminate downstream DNA barcode analysis. Just prior to *in vivo* injection, the carrier-DNA samples are pooled together in PBS. To determine the best performing carrier molecules for each tissue of interest, pooled carrier-DNA candidates are delivered to specific organ systems using commonly used routes of administration. At the end of the study, tissues are harvested, and cells are lysed to make a DNA library extract. These DNA libraries are then prepared for deep sequencing and DNA barcode analysis.^{5,6} Consequently, DNA barcoding has the potential to advance the science of selecting nanoparticles for delivering gene therapeutics as it allows for researchers to skip the cell culture screening altogether.

Finally, the relationships between GS molecular structure and interactions with DOPE could be strengthened by testing various other GS. This is especially important because the prevalence of attractive interactions found in this work contradicts a previous study that examined GS-DOPE micelles. As such, additional data will help confirm the effect of intermolecular interactions and the surface miscibility behaviour of GS-DOPE binary mixtures as well as the effects of mixture composition and GS structure on this behaviour. Furthermore, the results obtained for $X_{18-7-18} = 0.8, 0.9$ mixed monolayer indicate the potential for GS/DOPE lipoplex disassembly which may promote transfection. As such, these lipoplexes should be further investigated *in vitro* after complexation with LCC DNA minivectors as these results could be used to further optimize LCC DNA minivectors for gene therapy applications.

6.3 Scientific contribution

With respect to the contribution of this work to formulation science, the results clearly demonstrate that researchers designing and optimizing safe non-viral vectors for gene therapy **must** consider the rational design of both the synthetic carrier and the DNA cargo since the combination of these two components have shown to influence transgene expression. Current gene therapy studies typically overlook optimizing the design of the therapeutic gene which, as the results from this work show, may compromise the safety and transfection efficiency of the gene delivery system. As such, an increased focus on important DNA design factors such as molecular weight, size, topology, composition, and even DNA mixing behaviours will circumvent limitations associated with conventional plasmid DNA vectors and lead to the development and optimization of highly efficient synthetic carriers comprising the LCC DNA minivectors for future gene therapy formulations.

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Chapter 1

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Appendices

Appendix A

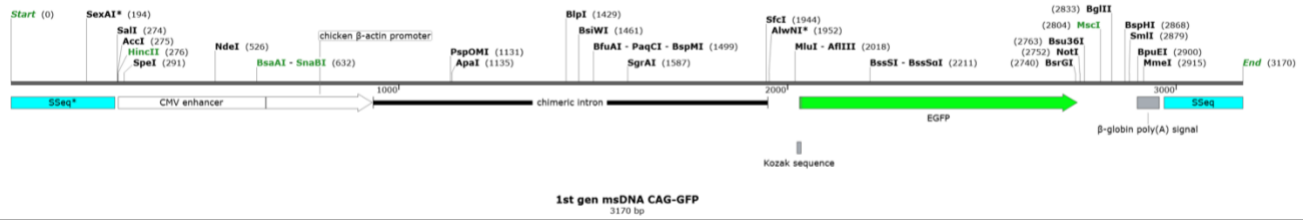


Figure A-1 1st generation LCC DNA minivector CAG plasmid encoding enhanced green florescence protein (eGFP).

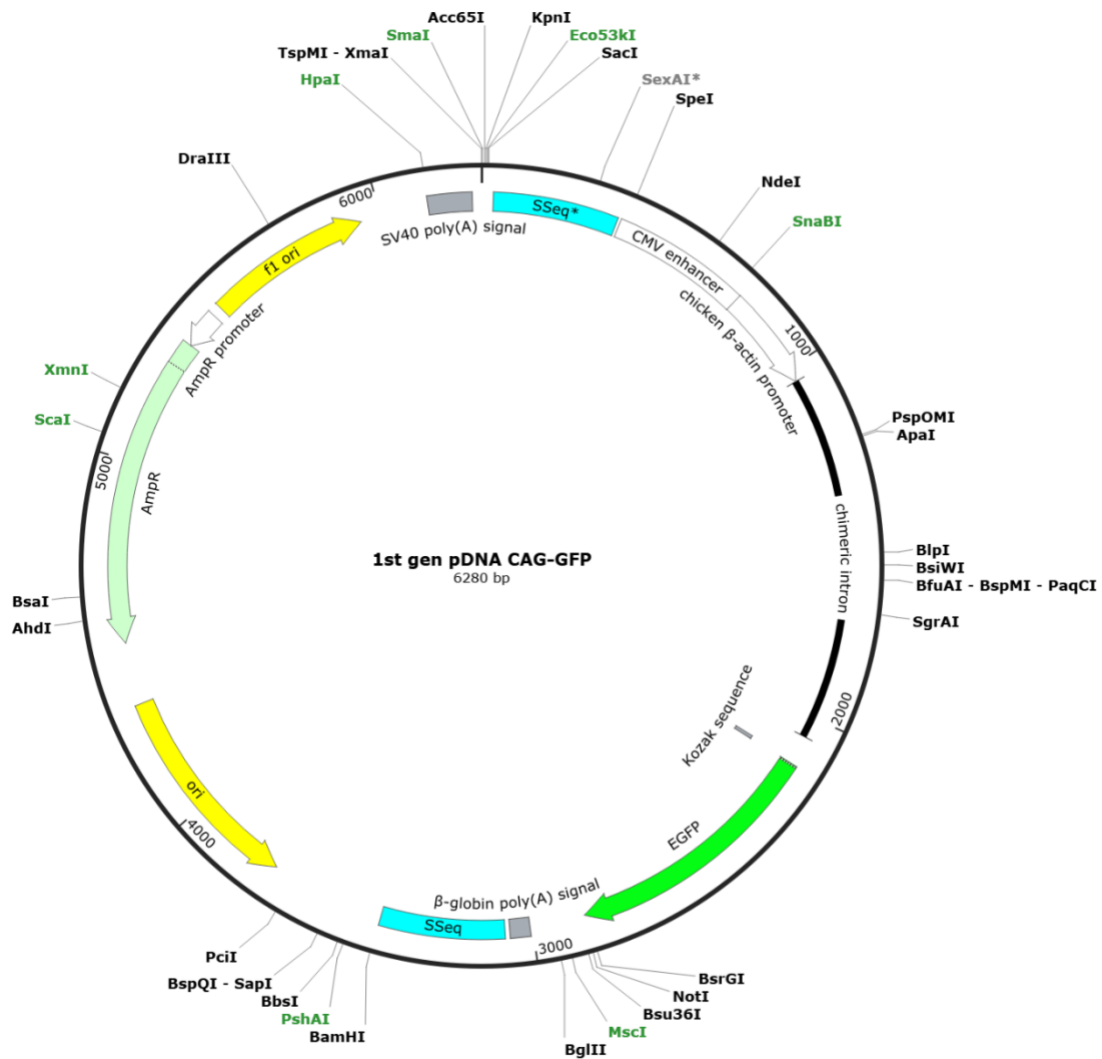


Figure A-2 1st generation CCC pDNA CAG plasmid encoding enhanced green florescence protein (eGFP).

Table A-1 Control group selection for Chapter 3. Transfection efficiency and cell viability in HEK 293 cells treated with Lipofectamine 3000 complexed with either LCC DNA minivector and in vitro jetPEI/CCC pDNA complexes at N⁺/P⁻ ratios 2:1, 4:1 and 8:1. Formulations were prepared in Opti-MEM Medium by following the manufacturer's recommendations. Transfection efficiency is represented by the number of live cells expressing GFP and as a percentage of total cells analyzed, while cell viability is indicated by PI staining and represented as a percentage of total cells analyzed. Mean values are reported $\pm$ standard deviation. Control conditions consisted of untreated cells (labelled as "cell only (no plasmid)").

N ⁺ /P ⁻ ratio	Treatment	Transfection efficiency	Cell viability
0	Cell only (no plasmid)	0.0 (n = 1)	96.3 (n = 1)
2:1	Lipofectamine 3000*/LCC DNA minivector	59.9 $\pm$ 9.1	95.3 $\pm$ 1.2
2:1	Lipofectamine 3000*/CCC pDNA	48.6 $\pm$ 8.5	94.1 $\pm$ 1.5
4:1	Lipofectamine 3000*/LCC DNA minivector	75.7 $\pm$ 3.6	94.2 $\pm$ 2.3
4:1	Lipofectamine 3000*/CCC pDNA	69.5 $\pm$ 2.4	94.4 $\pm$ 1.2
8:1	Lipofectamine 3000*/LCC DNA minivector	71.6 $\pm$ 2.3	95.3 $\pm$ 1.6
8:1	Lipofectamine 3000*/CCC pDNA	56.3 $\pm$ 5.4	93.8 $\pm$ 2.1

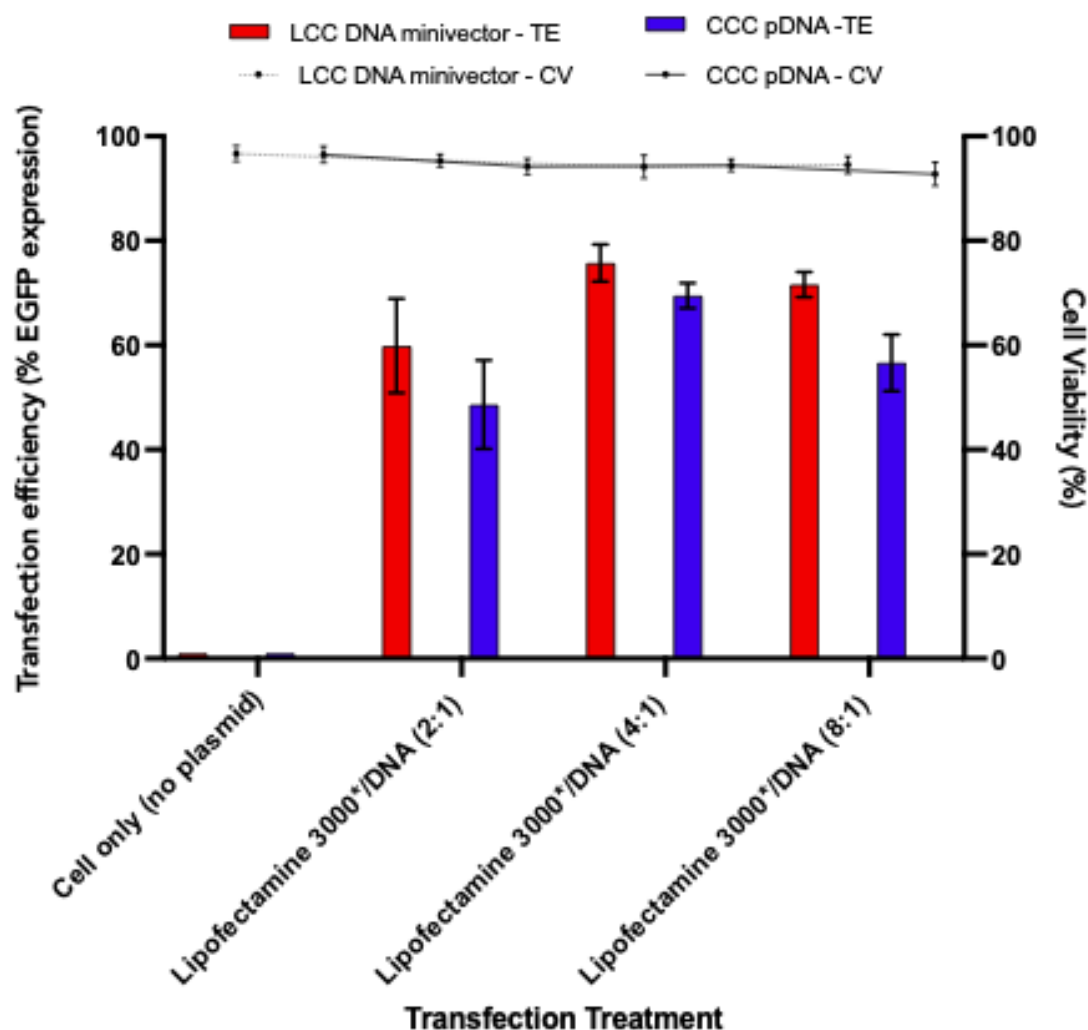


Figure A-3 Control group selection for Chapter 3. Transfection efficiency and cell viability in HEK 293 cells treated with Lipofectamine 3000 complexed with either LCC DNA minivector and in vitro jetPEI/pDNA (CCC) complexes at N^+/P^- ratios 2:1, 4:1 and 8:1. Formulations were prepared in Opti-MEM Medium by following the manufacturer's recommendations and the transfected cells were collected and analyzed 48 hours post transfection. Transfection efficiency is represented by the number of live cells expressing GFP (as a percentage of total cells analyzed), while cell viability was indicated by positive propidium iodide staining and shown as a percentage of total cells analyzed. Mean values of three independent experiments (each experiment consisting of three measurements per sample) are plotted with error bars representing standard deviation. Control conditions consisted of untreated cells (labelled as "cell only (no plasmid)"). Optimization data revealed a N^+/P^- ratio of 4:1 (1 μ g DNA to 4 μ L of Lipofectamine 3000) achieved the highest transgene expression and was selected as the control group for transfection studies in Chapter 4.

Table A-2 Transfection efficiency and cell viability in HEK 293 cells treated with Lipofectamine/LCC DNA minivector or CCC pDNA and Lipid 2/LCC DNA minivector or CCC pDNA prepared at N⁺/P⁻ ratios of 2:1, 4:1 and 8:1 in deionized water. Transfection efficiency is represented by the number of live cells expressing GFP and as a percentage of total cells analyzed, while cell viability is indicated by PI staining and represented as a percentage of total cells analyzed. Cells were collected and analyzed 48 hours post transfection. Mean values of three independent experiments (each experiment consisting of three measurements per sample) are reported $\pm$ standard deviation. Control conditions consisted of untreated cells (labelled as “cell only (no plasmid)”).

N ⁺ /P ⁻ ratio	Treatment	Transfection efficiency	Cell viability
0	Cell only (no plasmid)	0.0 (n = 1)	95.4 (n = 1)
2:1	Lipofectamine 3000/LCC DNA minivector	38.2 $\pm$ 2.2	95.4 $\pm$ 0.2
2:1	Lipofectamine 3000/CCC pDNA	36.4 $\pm$ 3.7	94.8 $\pm$ 0.6
4:1	Lipofectamine 3000/LCC DNA minivector	55.8 $\pm$ 2.7	95.2 $\pm$ 0.9
4:1	Lipofectamine 3000/CCC pDNA	49.1 $\pm$ 6.1	93.5 $\pm$ 1.9
8:1	Lipofectamine 3000/LCC DNA minivector	59.5 $\pm$ 3.7	95.1 $\pm$ 0.2
8:1	Lipofectamine 3000/CCC pDNA	45.4 $\pm$ 3.8	93.6 $\pm$ 1.2
2:1	Lipid 2/LCC DNA minivector	16.2 $\pm$ 4.6	94.3 $\pm$ 2.1
2:1	Lipid 2/CCC pDNA	10.8 $\pm$ 3.9	93.9 $\pm$ 1.1
4:1	Lipid 2/LCC DNA minivector	17.5 $\pm$ 4.6	94.3 $\pm$ 2.3
4:1	Lipid 2/CCC pDNA	16.3 $\pm$ 3.7	95.8 $\pm$ 0.5
8:1	Lipid 2/LCC DNA minivector	26.4 $\pm$ 3.1	93.8 $\pm$ 1.6
8:1	Lipid 2/CCC pDNA	25.2 $\pm$ 7.2	91.8 $\pm$ 2.3

Table A-3 Transfection efficiency and cell viability in ARPE-19 cells treated with Lipofectamine/LCC DNA minivector or CCC pDNA and Lipid 2/LCC DNA minivector or CCC pDNA at varying N⁺/P⁻ ratios in deionized water. Transfection efficiency is represented by the number of live cells expressing GFP and as a percentage of total cells analyzed. Cell viability is indicated by positive propidium iodide staining and shown as a percentage of total cells analyzed. Cells were collected and analyzed 48 hours post transfection and mean values of three independent experiments (each experiment consisting of three measurements per sample) are reported $\pm$ standard deviation. Control conditions consisted of untreated cells (labelled as “cell only (no plasmid)”).

N ⁺ /P ⁻ ratio	Treatment	Transfection efficiency	Cell viability
0	Cell only (no plasmid)	0.0 (n = 1)	96.2 (n = 1)
2:1	Lipofectamine 3000/LCC DNA minivector	24.5 $\pm$ 5.2	94.4 $\pm$ 1.6
2:1	Lipofectamine 3000/CCC pDNA	19.1 $\pm$ 2.6	94.8 $\pm$ 1.3
4:1	Lipofectamine 3000/LCC DNA minivector	40.9 $\pm$ 7.4	94.6 $\pm$ 1.7
4:1	Lipofectamine 3000/CCC pDNA	34.1 $\pm$ 5.9	94.2 $\pm$ 1.4
8:1	Lipofectamine 3000/LCC DNA minivector	38.2 $\pm$ 1.8	95.7 $\pm$ 1.2
8:1	Lipofectamine 3000/CCC pDNA	29.3 $\pm$ 4.6	93.2 $\pm$ 1.8
2:1	Lipid 2/LCC DNA minivector	12.6 $\pm$ 2.8	95.2 $\pm$ 1.5
2:1	Lipid 2/CCC pDNA	9.7 $\pm$ 4.4	93.8 $\pm$ 1.4
4:1	Lipid 2/LCC DNA minivector	13.4 $\pm$ 2.6	96.5 $\pm$ 1.7
4:1	Lipid 2/CCC pDNA	10.9 $\pm$ 2.5	93.6 $\pm$ 1.3
8:1	Lipid 2/LCC DNA minivector	23.2 $\pm$ 4.6	94.5 $\pm$ 0.7
8:1	Lipid 2/CCC pDNA	15.3 $\pm$ 1.7	93.6 $\pm$ 2.1

Appendix B

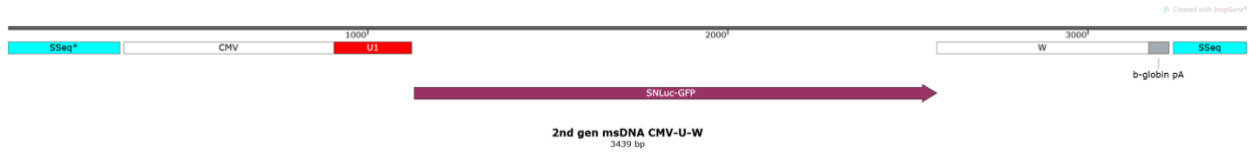


Figure B-1 2nd generation LCC DNA minivector CMV-U-W plasmid encoding enhanced green fluorescence protein (GFP).

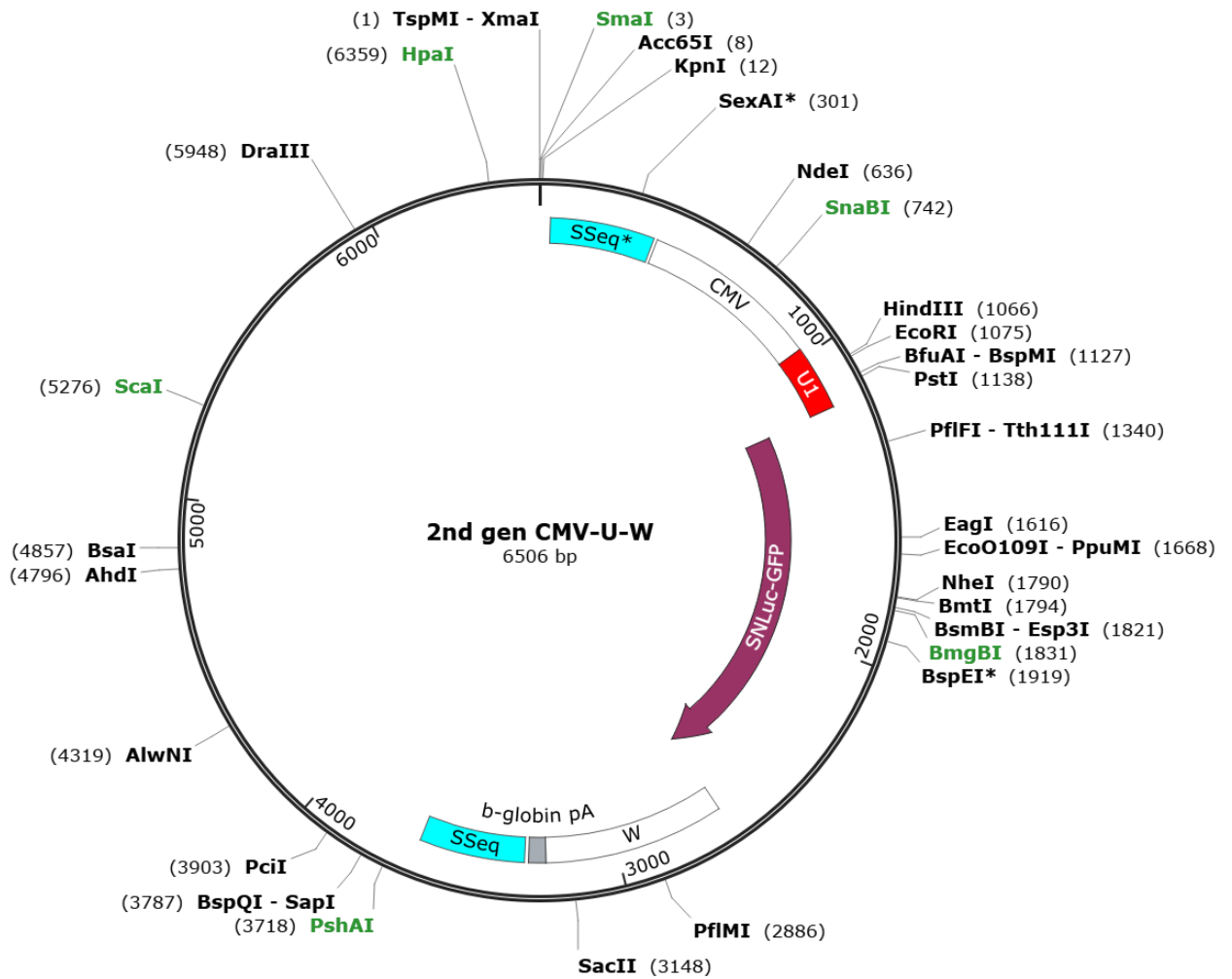


Figure B-2 2nd generation CCC pDNA CMV-U-W plasmid encoding enhanced green fluorescence protein (GFP).

Table B-1 Control group selection for Chapter 4. Transfection efficiency and cell viability in HEK 293 cells treated with in vitro jetPEI/LCC DNA minivector and in vitro jetPEI/pDNA (CCC) complexes prepared at a 2:1, 4:1 and 8:1 N⁺/P⁻ ratios of each polymer. Transfection efficiency is represented by the number of live cells expressing GFP and as a percentage of total cells analyzed. Cell viability is represented by positive propidium iodide staining and shown as a percentage of total cells analyzed. Cells were collected and analyzed 48 hours post transfection. Mean values of three individual experiments, each experiment consisting of three independent replicas per sample $\pm$ standard deviation. Control conditions consisted of untreated cells (labelled as “cell only (no plasmid)”).

N ⁺ /P ⁻ ratio	Treatment	Transfection efficiency	Cell viability
0	Cell only (no plasmid)	0.0 (n = 1)	96.7 (n = 1)
2:1	In vitro jetPEI/ LCC DNA minivector	18.5 $\pm$ 4.2	94.2 $\pm$ 0.8
2:1	In vitro jetPEI/CCC pDNA	16.2 $\pm$ 2.5	93.6 $\pm$ 2.6
4:1	In vitro jetPEI/ LCC DNA minivector	67.3 $\pm$ 4.6	95.3 $\pm$ 1.6
4:1	In vitro jetPEI/CCC pDNA	57.9 $\pm$ 11.7	94.8 $\pm$ 1.8
8:1	In vitro jetPEI/ LCC DNA minivector	39.2 $\pm$ 3.8	94.6 $\pm$ 1.3
8:1	In vitro jetPEI/CCC pDNA	36.8 $\pm$ 8.6	92.8 $\pm$ 2.2

Table B-2 Transfection efficiency and cell viability in HEK 293 cells treated with Polymer 1/LCC DNA minivector or CCC pDNA and Polymer 2/LCC DNA minivector or CCC pDNA prepared at a 2:1, 4:1 and 8:1 N⁺/P⁻ ratios of each polymer. Transfection efficiency is represented by the number of live cells expressing GFP (as a percentage of total cells analyzed), while cell viability was indicated by PI staining and shown as a percentage of total cells analyzed. Mean values of three individual experiments, each experiment comprising three independent replicas per sample, are reported $\pm$ standard deviation. Control conditions consisted of untreated cells (labelled as “cell only (no plasmid)”).

N ⁺ /P ⁻ ratio	Treatment	Transfection efficiency	Cell viability
0	Cell only (no plasmid)	0.0 (n = 1)	96.2 (n = 1)
2:1	Polymer 1/LCC DNA minivector	26.6 $\pm$ 6.6	95.1 $\pm$ 0.7
2:1	Polymer 1/CCC pDNA	24.6 $\pm$ 5.6	94.5 $\pm$ 2.1
4:1	Polymer 1/LCC DNA minivector	38.1 $\pm$ 5.1	95.6 $\pm$ 1.4
4:1	Polymer 1/CCC pDNA	32.7 $\pm$ 2.2	93.3 $\pm$ 2.5
8:1	Polymer 1/LCC DNA minivector	57.7 $\pm$ 6.2	96.1 $\pm$ 2.1
8:1	Polymer 1/CCC pDNA	54.4 $\pm$ 5.7	95.5 $\pm$ 1.5
2:1	Polymer 2/LCC DNA minivector	43.2 $\pm$ 5.6	94.8 $\pm$ 2.4
2:1	Polymer 2/CCC pDNA	36.4 $\pm$ 5.7	94.8 $\pm$ 0.7
4:1	Polymer 2/LCC DNA minivector	69.7 $\pm$ 4.1	94.8 $\pm$ 2.4
4:1	Polymer 2/CCC pDNA	57.4 $\pm$ 2.4	94.2 $\pm$ 1.2
8:1	Polymer 2/LCC DNA minivector	74.4 $\pm$ 5.4	95.3 $\pm$ 1.1
8:1	Polymer 2/CCC pDNA	59.7 $\pm$ 3.5	95.1 $\pm$ 1.3

Table B-3 Transfection efficiency and cell viability in ARPE-19 cells treated with Polymer 1/LCC DNA minivector or CCC pDNA and Polymer 2/LCC DNA minivector or CCC pDNA prepared at a 2:1, 4:1 and 8:1 N⁺/P⁻ ratios of each polymer. Transfection efficiency is represented by the number of live cells expressing GFP (as a percentage of total cells analyzed), while cell viability was indicated by positive PI staining and shown as a percentage of total cells analyzed. Mean values of three individual experiments, each experiment comprising three independent replicas per sample, are reported $\pm$ standard deviation. Control conditions consisted of untreated cells (labelled as “cell only (no plasmid)”).

N ⁺ /P ⁻ ratio	Treatment	Transfection efficiency	Cell viability
0	Cell only (no plasmid)	0.0 (n = 1)	97.1 (n = 1)
2:1	Polymer 1/LCC DNA minivector	17.8 $\pm$ 3.2	94.3 $\pm$ 0.7
2:1	Polymer 1/CCC pDNA	15.2 $\pm$ 5.3	96.1 $\pm$ 1.1
4:1	Polymer 1/LCC DNA minivector	30.4 $\pm$ 4.2	95.3 $\pm$ 2.1
4:1	Polymer 1/CCC pDNA	19.2 $\pm$ 6.8	95.6 $\pm$ 0.5
8:1	Polymer 1/LCC DNA minivector	38.7 $\pm$ 3.5	95.2 $\pm$ 1.5
8:1	Polymer 1/CCC pDNA	30.1 $\pm$ 4.4	93.9 $\pm$ 2.4
2:1	Polymer 2/LCC DNA minivector	22.4 $\pm$ 3.9	96.1 $\pm$ 0.4
2:1	Polymer 1/CCC pDNA	18.6 $\pm$ 3.6	95.5 $\pm$ 2.1
4:1	Polymer 2/LCC DNA minivector	36.8 $\pm$ 2.1	95.3 $\pm$ 1.4
4:1	Polymer 1/CCC pDNA	35.5 $\pm$ 5.2	93.5 $\pm$ 2.3
8:1	Polymer 2/LCC DNA minivector	60.1 $\pm$ 5.1	94.8 $\pm$ 2.2
8:1	Polymer 1/CCC pDNA	48.6 $\pm$ 5.5	94.3 $\pm$ 0.8

Appendix C

Table C-1 Monolayer properties of GS/DOPE binary mixed monolayers.

X_{GS}	pH	A_L (Å ² /molecule)	A_{min} (Å ² /molecule)	π_{col} (mN/m)	C_{s max}⁻¹ (mN/m)	ΔG^E (kJ/mol)
<i>18-7-18/DOPE</i>						
1	4	237.4 ± 15.5	164.1 ± 3.4	38.0 ± 0.2	42.8 ± 0.1	NA
0.9	4	227.9 ± 9.9	151.4 ± 0.02	36.7 ± 0.1	43.2 ± 0.3	0.77 ± 0.2
0.8	4	228.6 ± 5.7	154.2 ± 8.0	40.3 ± 0.6	44.0 ± 0.2	2.51 ± 0.9
0.7	4	213.1 ± 10.4	127.7 ± 5.4	37.8 ± 0.4	41.0 ± 0.9	-1.02 ± 0.8
0.6	4	192.9 ± 3.3	110.7 ± 0.9	44.1 ± 0.1	54.2 ± 0.2	0.09 ± 0.2
0.5	4	255.1 ± 0.2	203.9 ± 1.7	41.5 ± 0.6	51.9 ± 0.7	13.75 ± 0.9
0.4	4	166.4 ± 18.6	85.1 ± 0.7	41.7 ± 0.7	66.7 ± 0.5	0.91 ± 0.7
0.3	4	131.2 ± 6.0	59.5 ± 2.4	NA*	54.3 ± 0.4	3.82 ± 0.5
0.2	4	113.7 ± 4.1	62.7 ± 1.3	NA*	77.8 ± 0.1	3.82 ± 0.1
0.1	4	114.8 ± 0.3	55.9 ± 1.6	NA*	60.2 ± 0.5	1.98 ± 0.5
0	4	98.9 ± 4.9	60.0 ± 0.7	43.2 ± 0.2	77.6 ± 0.4	NA
1	7	236.9 ± 3.7	177.6 ± 4.4	38.0 ± 0.01	38.7 ± 0.7	NA
0.9	7	211.5 ± 1.0	129.8 ± 3.3	40.5 ± 0.2	41.6 ± 0.2	5.10 ± 0.5
0.8	7	198.7 ± 4.6	139.1 ± 1.9	39.4 ± 0.3	40.1 ± 0.4	2.92 ± 0.4
0.7	7	192.7 ± 2.0	112.6 ± 4.0	39.9 ± 0.3	44.7 ± 0.7	3.93 ± 0.6
0.6	7	162.5 ± 1.7	104.1 ± 0.3	38.7 ± 0.3	38.7 ± 0.8	4.54 ± 0.3
0.5	7	246.3 ± 12.0	166.0 ± 1.2	41.4 ± 0.5	45.7 ± 0.2	7.18 ± 0.5
0.4	7	134.6 ± 5.9	85.1 ± 1.5	41.5 ± 0.7	55.2 ± 0.3	2.59 ± 0.1
0.3	7	117.2 ± 2.3	70.3 ± 0.6	44.4 ± 0.01	77.4 ± 0.9	3.11 ± 0.2
0.2	7	106.9 ± 2.4	71.8 ± 0.5	42.7 ± 0.5	78.9 ± 0.4	1.97 ± 0.9
0.1	7	97.3 ± 0.3	60.1 ± 1.1	NA*	78.3 ± 0.2	2.22 ± 0.5
0	7	113.0 ± 8.1	68.4 ± 2.3	40.5 ± 0.4	72.3 ± 0.8	NA
<i>18-7NH-18/DOPE</i>						
1	4	206.4 ± 6.8	123.8 ± 1.8	41.8 ± 0.9	61.1 ± 0.5	NA
0.9	4	185.2 ± 0.7	102.3 ± 1.5	30.8 ± 0.3	55.9 ± 0.2	3.37 ± 0.6
0.8	4	169.7 ± 1.8	82.9 ± 0.1	34.0 ± 0.2	57.6 ± 0.6	5.07 ± 0.2
0.7	4	144.5 ± 12.7	77.6 ± 1.4	37.2 ± 0.2	58.8 ± 0.2	4.56 ± 0.1
0.6	4	132.9 ± 3.5	65.7 ± 0.3	36.7 ± 0.1	60.3 ± 0.8	5.31 ± 0.4
0.5	4	199.4 ± 5.9	113.4 ± 0.4	45.0 ± 0.4	65.0 ± 0.9	4.05 ± 0.4
0.4	4	116.1 ± 4.1	61.4 ± 2.3	42.9 ± 3.5	80.3 ± 0.4	3.38 ± 0.8
0.3	4	101.8 ± 4.6	60.1 ± 2.2	44.6 ± 0.01	86.5 ± 0.7	2.54 ± 0.5
0.2	4	97.8 ± 4.9	55.5 ± 3.6	42.8 ± 4.5	78.7 ± 0.1	3.05 ± 0.6
0.1	4	94.5 ± 2.2	55.3 ± 0.5	41.1 ± 5.3	85.2 ± 0.2	1.51 ± 0.2
0	4	98.9 ± 4.9	60.0 ± 0.7	43.2 ± 0.2	77.6 ± 0.6	NA
1	7	173.2 ± 0.4	101.7 ± 4.4	41.0 ± 3.5	55.4 ± 0.4	NA
0.9	7	157.1 ± 1.0	88.9 ± 3.2	44.3 ± 7.1	59.4 ± 0.7	2.15 ± 0.1
0.8	7	134.1 ± 0.8	83.3 ± 1.0	41.4 ± 4.5	69.5 ± 0.1	1.42 ± 0.9
0.7	7	111.2 ± 1.7	68.4 ± 1.2	40.7 ± 0.2	71.1 ± 0.2	4.13 ± 0.7
0.6	7	124.7 ± 3.2	72.7 ± 0.8	45.2 ± 0.9	81.3 ± 0.5	0.72 ± 0.2

0.5	7	164.7 ± 3.4	103.7 ± 2.6	49.1 ± 0.2	80.8 ± 0.6	3.80 ± 0.2
0.4	7	93.7 ± 2.2	61.1 ± 0.7	45.6 ± 0.2	85.7 ± 0.7	3.22 ± 0.9
0.3	7	106.6 ± 4.1	65.0 ± 0.8	40.5 ± 5.5	83.4 ± 0.6	2.31 ± 0.6
0.2	7	101.5 ± 3.8	68.6 ± 2.4	44.5 ± 0.1	90.7 ± 0.2	0.97 ± 0.7
0.1	7	101.4 ± 5.7	60.8 ± 1.5	44.9 ± 0.4	100.9 ± 0.1	0.97 ± 0.4
0	7	113.0 ± 8.1	68.4 ± 2.3	40.5 ± 0.4	72.3 ± 0.4	NA

*NA – The mixed monolayer did not reach a collapse point in experiments.

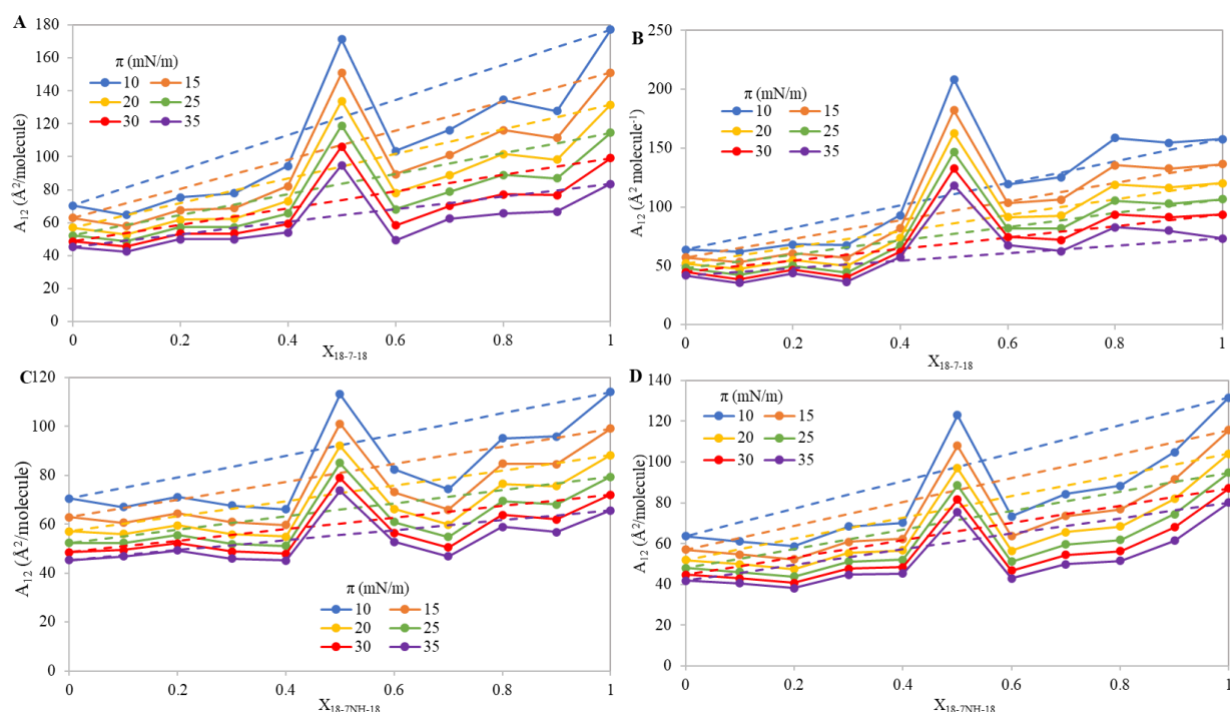


Figure C-1 Mean molecular area (A_{12}) vs. composition plots for GS/DOPE mixed monolayers; the dashed lines were drawn based on the additivity rule. **(A)** 18-7-18/DOPE mixed monolayers under pH 7 conditions. **(B)** 18-7-18/DOPE mixed monolayers under pH 4 conditions. **(C)** 18-7NH-18/DOPE mixed monolayers under pH 7 conditions. **(D)** 18-7NH-18/DOPE mixed monolayers under pH 4 conditions.