

Development of Amino acid-Substituted Gemini Surfactant-Based Non-invasive Non-Viral Gene Delivery Systems

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ABSTRACT

Gemini surfactants are versatile gene delivery agents because of their ability to bind and compact DNA and their low cellular toxicity. The aim of my dissertation work was to develop non-invasive mucosal formulations of novel amino acid-substituted gemini surfactants with the general chemical formula $C_{12}H_{25}(CH_3)_2N^+-(CH_2)_3-N(AA)-(CH_2)_3-N^+(CH_3)_2-C_{12}H_{25}$ (AA= glycine, lysine, glycyl-lysine, lysyl-lysine). These compounds were formulated with a model plasmid DNA, encoding for interferon- γ and green fluorescent protein, in the presence of helper lipid, 1,2 dioleoyl-sn-glycero-phosphatidyl-ethanolamine. Formulations were assessed in Sf 1 Ep epithelial cells. Among the novel compounds, plasmid/gemini/lipid (P/G/L) nanoparticles formulated using glycine- and glycyl-lysine substituted gemini surfactants achieved significantly higher gene expression than the parent unsubstituted compound.

The key physicochemical properties, e.g. size, surface charge, DNA binding, and toxicity of P/G/L complexes were correlated with transfection efficiency. The presence of amino-acid substitution did not interfere with DNA compaction and contributed to an overall low toxicity of all P/G/L complexes, comparable to the parent gemini surfactant.

A cellular uptake mechanistic study revealed that both clathrin- and caveolae-mediated uptake were major uptake routes for P/G/L nanoparticles. However, amino acid substitution in the gemini surfactant imparted high buffering capacity, pH-dependent increase in particle size, and balanced DNA binding properties. These properties may enhance endosomal escape of P/12-7NGK-12/L resulting in higher gene expression.

Finally, the P/G/L complexes were incorporated into an *in-situ* gelling dispersion containing a thermosensitive polymer, poloxamer 407, and a permeation enhancer, diethylene glycol monoethyl ether (DEGEE). A 16% w/v poloxamer concentration produced a dispersion that gelled at body temperature and exhibited sufficient yield value to prevent formulation leakage from the vaginal cavity. The formulations were prepared with a model plasmid, encoding for red fluorescent protein, and administered topically to rabbit vagina. In agreement with our *in vitro* results, confocal microscopy revealed that glycyl-lysine substituted gemini surfactant exhibited higher gene expression compared to the parent unsubstituted gemini surfactant. This provided proof-of-concept for use of amino acid-substituted gemini surfactant in non-invasive mucosal (vaginal) gene delivery systems with potential therapeutic applications.

These formulations will be developed with therapeutically relevant genes to assess their potential as genetic vaccines. In addition, new gemini surfactants will be developed by grafting other amino acids via glycine linkage to retain conformation flexibility and enhance endosomal escape of DNA complexes for higher transfection efficiency.

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DEDICATION

This work is dedicated to:

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Frequent abbreviations

AAV	Adeno-associated virus
Ad	Adenovirus
CD	Circular dichroism
CLSM	Confocal laser scanning microscopy
DAPI	4',6-diamidino-2-phenylindole
DEGEE	Diethylene glycol monoethyl ether
DOGS	Dioctadecylamidoglycylspermine
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
DOPE	1,2-dioleoyl-sn-glycero-3-phosphoethanolamine
DOSPA	2,3-dioleoyloxy-N-[2(spermine- carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate
DOTAP	1,2-dioleoyloxy-3-(trimethylammonium)propane
DOTMA	N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride
FACS	Fluorescence activated cell sorting
GFP	Green fluorescent protein
IFNγ	Interferon-gamma
MTT	Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
P/G	Plasmid/gemini surfactant
P/G/L	Plasmid/gemini surfactant/co-lipid
PEI	Polyethyleneimine
Pol	Poloxamer
SAINT-2	N-methyl-4(dioleoyl)methylpyridinium chloride
TEM	Transmission electron microscopy

1. Introduction

With the advancement of diagnostic tools and the introduction of more sensitive instruments and new analytical techniques, we are unraveling the hidden secrets of living cells. The discovery of DNA is one such achievement. The development of better molecular techniques (e.g. gel electrophoresis, polymerase chain reaction, DNA cloning and DNA sequencing) and highly sensitive instruments (flow cytometry, HPLC and mass spectrometry) have allowed us to learn more about the genetic information and how mutations or changes in it cause disorders.

The ability to create DNA structures *in vitro* and manipulate the DNA in living cells has provided yet another tool to enable humans to improve various disease conditions. This area of medical therapeutics is known as gene therapy [1]. It involves the delivery of genetic material to patients to prevent or treat a disease. Therapy is achieved by replacing a defective gene, by inserting a new gene, by silencing a gene, or by regulating gene expression. In theory, gene therapy provides a treatment option for a number of incurable diseases of today. Early studies conducted in 1990 led to the first gene therapy procedure in a patient carrying a defective gene that makes an essential enzyme adenosine deaminase (ADA), required for normal working of the immune system [2]. The study was considered to be a success leading to a rise in the number of clinical trials involving gene therapy (**Figure 1.1.**) [3].

Although significant research efforts were directed towards gene therapy for the last two decades, the field is still in its development phase. A total of 1786 gene therapy clinical trials have been conducted worldwide, with the majority (~95%) in Phase I/II stage [4]. All the promises of gene therapy have been challenged by one common hurdle: a safe and efficient method of gene delivery. Unfortunately, gene sequences cannot be administered as such. Therefore, different methods of gene delivery have been evaluated, broadly classified into two groups – viral and non-viral methods. A viral method utilizes a virus' natural ability to infect cells to deliver a target gene where, the viral vectors are devoid of any virulence (disease causing ability) [5]. The non-viral approaches use physical methods (e.g. electroporation and iontophoresis) or chemical agents such as lipids, surfactants, polymers, dendrimers and other agents for delivery of genes to the target cells [6]. All delivery systems have their own set of advantages and limitations. The delivery vectors need to overcome various challenges posed by our body to successfully transfer the genes of interest to the target cells.

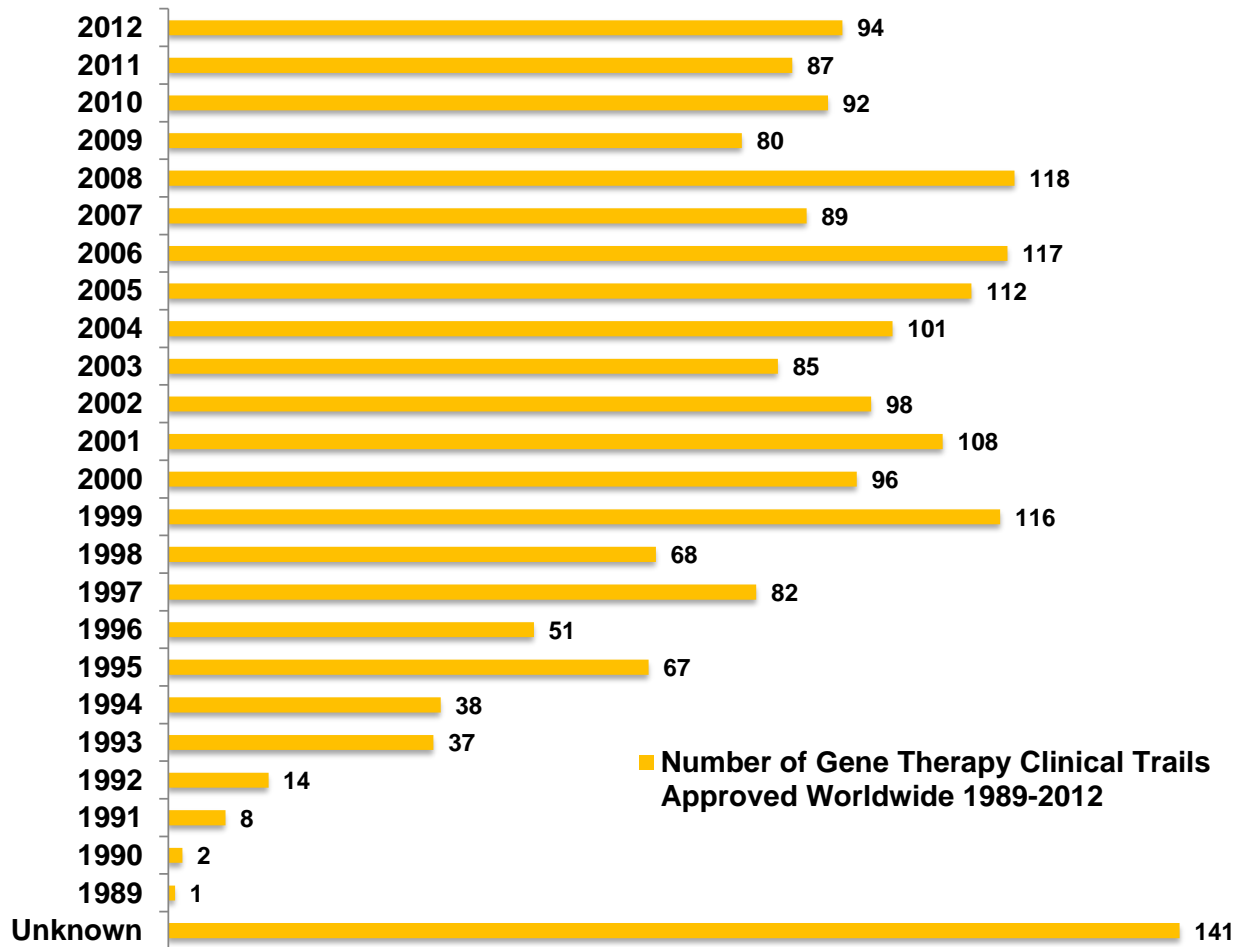


Figure 1.1: Number of gene therapy clinical trials conducted worldwide 1989-2012.

In the following chapters, I will be discussing different delivery vectors that are developed as gene delivery systems. These studies have begun to help us understand the complexity of the gene therapy and develop models to rationally design safe and efficient delivery systems. I will discuss the extra- and the intracellular barriers to gene delivery, current methods of gene delivery with focus on non-viral vectors, and physicochemical properties that influence their transfection efficiency. In the literature review, I identify the need for understanding the physiochemical properties of a delivery system in association with the cellular uptake mechanism and their influence on the ultimate fate of delivery particles. There is a need for systematic evaluation of each delivery system to build more predictable models that can help in rational design of that delivery system to attain better transfection efficiencies. Therefore, I focus my attention on gemini surfactants that are easy to modify and have shown promising potential as excellent gene delivery systems [7-10]. Although the gemini surfactants can be classified under the broad category of cationic lipids, I am presenting a more detailed literature review on this area as a separate chapter in order to build the rationale of my study with these molecules. The long term goal of this study is to develop non-invasive mucosal (vaginal) gene delivery systems using these substituted-gemini surfactants; thus, I am presenting literature on the challenges in vaginal drug delivery, methods to overcome it and a rationale for the choice of an animal model for this study.

2. Methods of gene delivery

2.1. Barriers to gene delivery

Gene based systems encounter a series of challenges before they can reach their intended target cell. Composition of the formulation, its stability and shelf life are relevant concerns for all pharmaceutical delivery systems. The instability of the biotechnology products compared to small chemical drugs adds additional challenges for the pharmaceutical scientists. Moreover, drug absorption, first pass metabolism, plasma stability, distribution to target tissues, body clearance, and immune responses to biotechnology drugs and/or delivery systems, are also different from small molecules. While small molecules with molecular weight in the range of less than 1 kD are delivered to their target relatively, easily, for macromolecules such as plasmid DNA with molecular weight exceeding 1 MDa (**Figure 2.1 & 2.2**), the extracellular barriers represent a significant obstacle [11].

The high molecular weight greatly affects the permeability of macromolecules, resulting in poor absorption across biological membranes. The highly acidic and enzyme-rich conditions in the gastric lumen can degrade DNA very rapidly. This situation is generally avoided by administering DNA through parenteral routes to allow absorption directly into the blood. However, blood plasma proteins bind the DNA and elicit non-specific immune response through opsonization. The binding of plasma proteins also increases the overall size of the DNA particle and impedes its circulation through the microvascular region. Additionally, the naked DNA is rapidly engulfed and extensively degraded by non-parenchymal cells in the liver [12]. The circulation half-life of DNA in mice was reported as 10-15 min [12]. A gene delivery vector needs to protect DNA while avoiding any interactions with plasma proteins or entrapment in tissue vasculature.

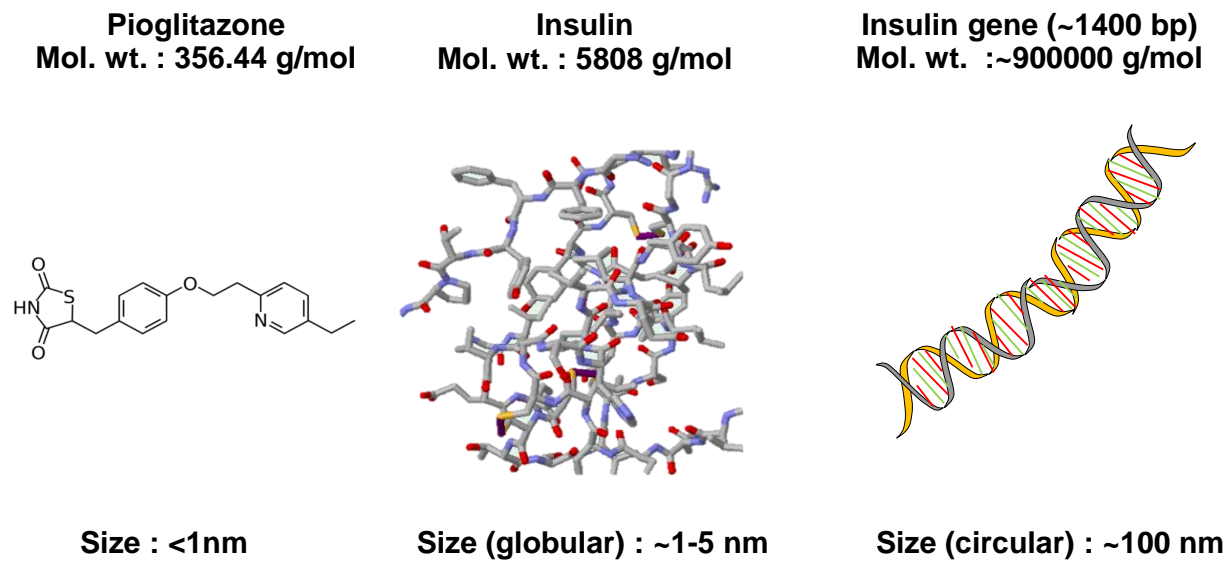
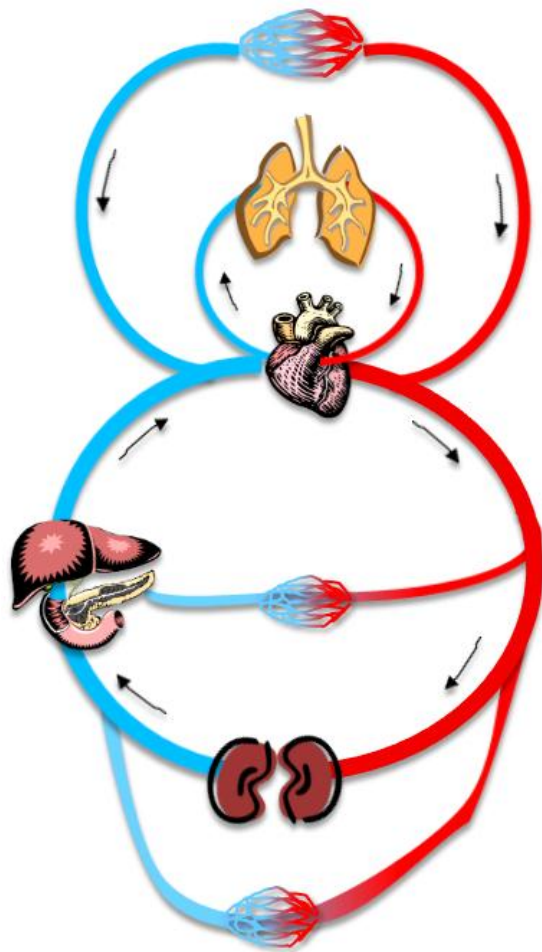


Figure 2.1: Small molecules versus macromolecules. Pioglitazone, a small molecule, that is used for treatment of diabetes has size of less than 1nm while macromolecules such as insulin has a globular diameter of 1-5 nm and, the insulin gene in a circular configuration will have a size of 100 nm.

Early experiments with cationic lipids did not improve the systemic circulation of lipoplexes. Significant amounts of LipofectACE/DNA and Lipofectin/DNA were cleared from systemic circulation by the Kupffer cells of the liver after intravenous injection. [13]. Conjugation of lipoplexes with polyethylene glycol (PEG) decreased their interaction with serum proteins and improved their systemic circulation. A successful application of stealth liposomes (PEGylated liposomes) was demonstrated in the delivery of doxorubicin; a drug used in the treatment of Kaposi's sarcoma [14]. PEGylation of the liposomes reduced adhesion to cells, blood vessel walls and other surfaces and resulted in increased vascular permeability of the stealth liposomes compared to conventional liposomes. The steric stability significantly increased circulation time of the encapsulated drug, leading to increased accumulation in tumor tissue due to its leaky vasculature [15]. To achieve similar results with gene delivery vectors, DOTAP/DOPC/DNA liposomes were prepared in the presence of PEG-lipids. At high PEG to lipid molar ratio, the high molecular weight PEG2000-lipids exhibited this steric stabilization, compared to low molecular weight PEG400-lipids, [16]. However, an increased steric stabilization led to poor cell surface interaction, resulting in low transfection efficiency of high molecular weight PEG2000-lipids. The engineering of long-circulating stealth lipoplexes show promising results but still further improvement is needed to increase plasma circulation to substantial levels. Gaining access to target cells in the tissue is the next challenge for delivery systems. The permeability of the vasculature and the particle size determine the availability of nanoparticles at the cellular level.

Cellular uptake, endosomal escape and nuclear localization are three major challenges at the cellular level (**Figure 2.3**). A large particle size and negative charge on the DNA (zeta potential of -30 to -70 mV) limits its interaction with the negatively charged cell surface [17]. Complexation of DNA with cationic delivery agents neutralizes the negative charge and condenses its size in the range of 100-200 nm [18]. The neutralization of negative charge and compaction enable the particle to interact with the cell surface and facilitate its uptake.



Frequent routes of DNA delivery

i.v., transdermal, transmucosal, pulmonary, oral

Extracellular barriers to gene delivery

Poor stability (acidic pH, enzymes) and poor permeability of plasmid DNA via oral route.

Aggregation of DNA molecules due to interaction with serum proteins

Degradation of plasmid DNA by phagocytosis

Poor tissue targeting

Poor tissue permeability

Entrapment in the mucous

Figure 2.2: Extracellular barriers to gene delivery.

Once inside the cell, enclosed in the endosomes, the DNA molecules can be transported to lysosomes for final degradation by lysosomal enzymes in an acidic environment. Therefore, endosomal escape becomes crucial for successful transport of the DNA to the nucleus [19]. Endosmotropic compounds such as chloroquine that disrupt endosomes have shown to increase gene expression significantly [20]. However, the high toxicity of chloroquine restricts its clinical applications [21]. The majority of research efforts in the last decade have focused on the synthesis of new delivery agents that can efficiently escape endosomes by destabilizing its membrane. The stability of DNA particles may also be compromised by interaction with intracellular proteins and degradation by cell nucleases [22]. Nuclear localization, the final step in realizing successful gene transfer occurs via passive diffusion through the nuclear pore complex, which restricts the entry of big molecules such as DNA [23]. For this reason, gene expression is higher in rapidly dividing cells as the DNA is transferred inside the nucleus during the disassembly of the nuclear envelop. Small amino acid sequences known as nuclear localization signals (NLS) bind to intracellular transport receptors and facilitate transfer of certain proteins, including viruses, through the nuclear membrane [24]. Covalent and non-covalent conjugation of these signaling peptides to DNA has been explored. However, much work needs to be done to successfully utilize these peptides in clinical settings.

The following sections discuss the two types of vectors (viral and non-viral) that have been developed for gene delivery, their advantages and their limitations in overcoming extracellular and intracellular barriers. An ideal gene delivery system should be able to achieve adequate gene expression in target cells without eliciting any toxic or immune response. Ease of production and storage stability are other important components of an optimal gene delivery system.

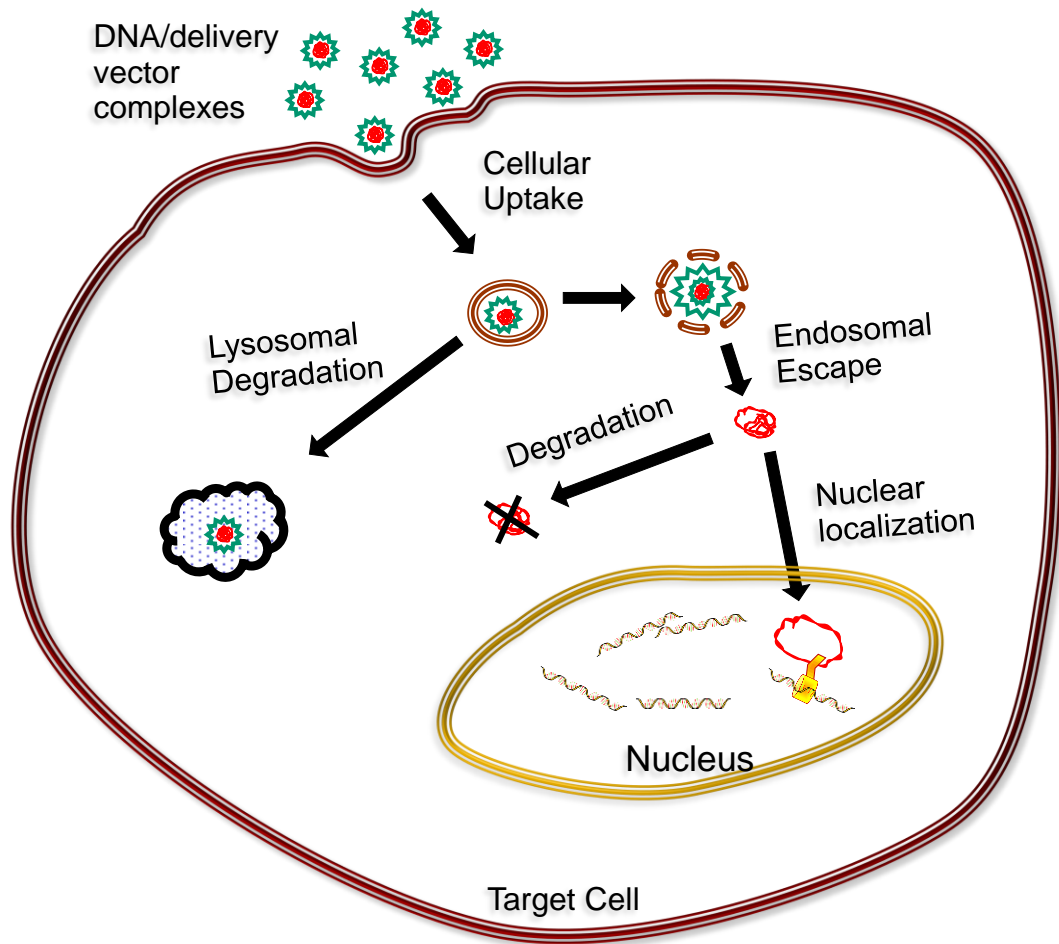
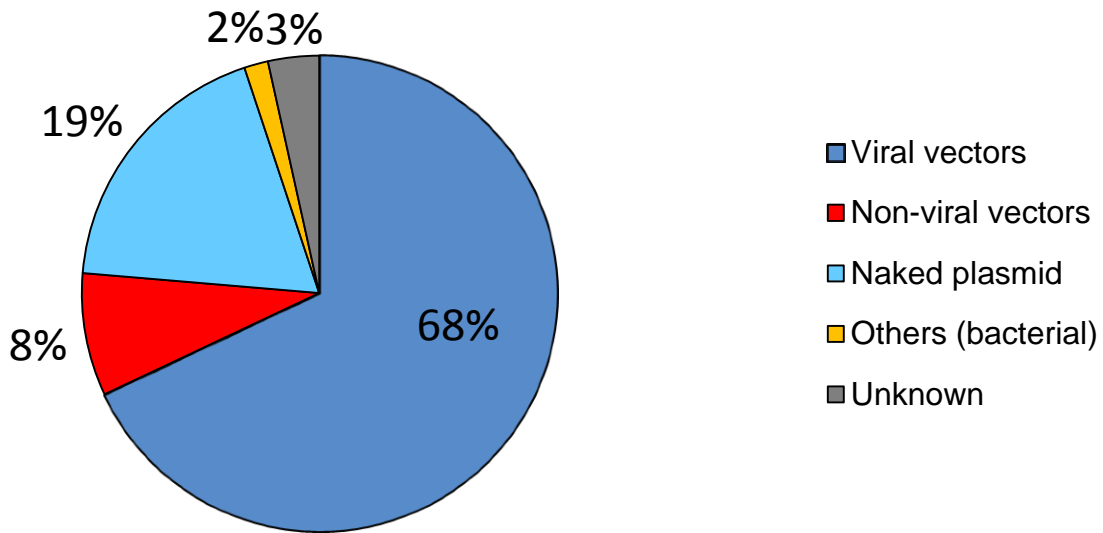


Figure 2.3: Schematic representation of intracellular barriers in effective gene delivery.

2.2. Viral Vectors

Viruses are the ultimate gene delivery systems. They have evolved over millions of years to show efficient transduction of viral DNA to different cells to produce their own replicas and to evade the body's innate immune system. Although the natural evolution of viral propagation was not aimed at therapeutic benefit to humans, about half a century of exploration has contributed to the gain of knowledge to reengineer these viruses as biotechnology drugs. Some of the viruses can be modified to deliver genes of interest while removing their ability to replicate and harm the target cells [25]. Viral vectors have several advantages such as efficient cellular entry [26], nuclear targeting [27] and regulated gene expression (transient or stable) [28]. Viruses constitute a majority (~68%) of the gene delivery vectors that have been used in gene therapy clinical trials (**Figure 2.4**) [29].

Viruses (**Table 2.1.**) that have shown some promising results are adenoviruses (Ad), adeno-associated viruses (AAV) and retroviruses/lentiviruses. Adenoviruses are non-enveloped viruses with a 36-kb double-stranded DNA (dsDNA) that can transduce both dividing and non-dividing cells. The replication-deficient Ad vectors have been used for gene transfer to respiratory, eye, liver and urinary tracts [30]. The lengthy production, non-specificity, preexisting immunity, or possible vector immunogenicity limits its wide application. The adeno-associated virus is also a non-enveloped virus with single-stranded DNA (ssDNA) of 4.7 kb [31]. This virus is not associated with any human disease and can transduce both dividing and non-dividing cells for long-term gene expression. A small genome packaging size, preexisting immunity and gene transfer to off-target cells are potential limitations of this vector [31]. Retroviruses and lentiviruses are enveloped viruses containing 7-12 kb ssRNA [32]. Low immunogenicity and large packaging capacity renders these vectors attractive for gene transfer. However, insertional mutagenesis is a major risk associated with these gene carriers. Low production yields and instability of the envelop protein are two other limitations of the retroviruses [32].



The Journal of Gene Medicine, © 2012 John Wiley and Sons Ltd. www.wiley.co.uk/genmed/clinicaltrials

Figure 2.4: Vectors used in gene therapy trials. Adapted from The Journal of Gene Medicine clinical trial website [29].

Table 2.1: Viral vectors as gene delivery systems.

Viral System	Overview	Advantages	Limitations
<p>Adenovirus (Ad) [30]</p>	<ul style="list-style-type: none"> • non-enveloped • 36 kb dsDNA • transduce dividing and non-dividing cells • most studied type: Ad5 	<ul style="list-style-type: none"> • replication-deficient Ad vectors can be produced • gene transfer to respiratory, eye, liver and urinary tracts 	<ul style="list-style-type: none"> • lengthy production • preexisting immunity • non-specificity • possible vector immunogenicity
<p>Adeno-associated Virus (AAV) [31]</p>	<ul style="list-style-type: none"> • non-pathogenic non-enveloped virus • 4.7 kb ssDNA • requires the presence of helper virus to complete its life cycle • most studied type: AAV2 	<ul style="list-style-type: none"> • not associated with any human disease • long term gene expression in dividing and non-dividing cells 	<ul style="list-style-type: none"> • a small genome packaging size • preexisting immunity • poor transduction of some cells • gene transfer to off-target cells
<p>Retrovirus/ Lentivirus [32]</p>	<ul style="list-style-type: none"> • enveloped viruses • 7-12 kb ssRNA 	<ul style="list-style-type: none"> • low immunogenicity • large packaging capacity 	<ul style="list-style-type: none"> • low production yields • instability of envelop protein • risk of insertional mutagenesis

In 2003, Gencidine (developed by Shenzhen SiBiono GenTech) became the first viral based gene therapy that was approved in China. It consists of a recombinant human p53 tumor suppressor gene, delivered using adenovirus, for treatment of head and neck squamous cell carcinoma. The viral particles enter the tumor cells by binding to coxsackie adenovirus receptors and encode the exogenous p53 gene, causing cell cycle arrest and apoptosis. The medication showed significant improvement in patients with late stage head and neck squamous cell carcinoma when co-administered with chemo and radio therapies, with no patient relapses over a 3 year follow-up [33]. Two years later, another recombinant adenovirus oncolytic virus engineered to express wild-type p53 (Oncorine; H101) developed by Shanghai Sunway Biotech was approved in China [34, 35]. In contrast to these, Epeius Biotechnologies developed a viral based gene delivery formulation that could be delivered intravenously to target tumor cells [36]. The medication uses a tumour targeted retroviral delivery vector that encodes a mutant form of cytotoxic cyclin-G1 gene inside the cell. The formulation is already approved in the Philippines for chemotherapy-resistant tumor types and, has received US orphan drug status and is undergoing clinical trials [36, 37]. In spite of these successes, the scientific community is doubtful about the stringency of regulatory affairs in eastern countries. A total of 120 patients participated in clinical development of Gencidine in China which was significantly lower than the total number of patients that had participated in clinical trials in the U.S.A. Moreover, The State Food and Drug Administration (SFDA) of China accepts reduction in the tumor size as a clinical significance rather than the survival rate of patients post-treatment, which is not considered the best indicator of efficiency of oncology medication. The drug has not received approval from US FDA, yet.

In spite of the high transfection efficiency, the viral vectors run a risk of triggering immune response or can be neutralized by a pre-existing immunity [38, 39]. Development of immunity against viral antigens may hinder multiple successive administration of a viral vector. A major disadvantage of the viral delivery is the lack of comprehensive knowledge about the viral systems, namely their ability to convert into replication-competent virulent viruses and cause insertional mutagenesis [40, 41]. The death of a subject enrolled in a clinical trial, treated for ornithine transcarboxylase deficiency using adenovirus based gene delivery vectors, was a major setback in gene therapy clinical trials using viral vectors [42]. The adenovirus invaded organs other than the intended target (liver), triggering a severe immune response leading to multiple

organ failures. In another study, three children developed cancer after treatment for X-linked severe combined immunodeficiency disease using retroviral vectors [43]. These incidents raised serious concerns about the safety of the virus-based gene delivery systems. In addition, viral systems suffer from limited gene carrying capacity [44] and require lengthy and complicated production methods [45]. Attempts to improve the gene carrying capacity of these viral vectors further complicate the already complex and inefficient methods of viral production [46]. This has led scientists to focus their research on the development of alternative, non-viral gene delivery systems that are safer and equally efficacious (**Table 2.2**).

Table 2.2: Comparison of viral and non-viral carriers of gene delivery.

Viral Vectors	Non-viral Vectors
High cost of production	Low cost of production
Difficult production	Ease of production
Limited gene carrying capacity	No size constraints
High risk of immune response	Low risk of immune response
High transfection efficiency	Low transfection efficiency

2.3. Non-viral vectors

Plasmids are the simplest non-viral vectors which can be used for the delivery of target genes. These are single- or double-stranded circular or linear extra-chromosomal DNA structures that were first observed in bacteria. Later, the plasmids were modified to produce proteins of interest in eukaryotic cells and were evaluated for their use in gene therapy. Like other macromolecules, plasmids suffer from poor absorption, biodistribution to relevant cells, and are sensitive to environmental conditions such as pH, ionic strength, presence of proteins and nucleases [47]. Thus, there is need of additional agents that can carry the plasmid DNA to the target cells.

Physical methods such as electroporation [48], ultrasound [49] and microporation [50] facilitate cellular uptake of the plasmid DNA. However, these applications are limited to accessible tissues such as skin and mucosa. They are invasive in nature and may cause permanent cell damage. For example, electroporation creates numerous pores temporarily in the cell membranes, which carry a risk of permanent damage to cell DNA or its other organelles [7]. The ease of usage, scale-up of the technology and cost of devices are also major hurdles for the wider applicability of these technologies [51].

Another approach involves use of **chemical agents** like lipids, polymers, dendrimers, etc. for delivery of plasmids containing target genes. Some of the examples of various chemical agents (**Table 2.3**) that have been evaluated for gene delivery are as follows:

2.3.1. Cationic Lipids

The cationic lipid-based vectors are the most studied and promising non-viral vectors for gene delivery. The lipid vectors are characterized by three structural domains – a cationic head group which interacts, electrostatically, with the DNA molecules, a hydrophobic tail which imparts lipophilicity to the molecule and facilitates endosomal escape and a linker group joining the head group and the hydrophobic tail.

Examples of cationic lipid based vectors include N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) [52], dioctadecylamidoglycylspermine (DOGS) [53], 2,3-dioleyloxy-N-[2(spermine-carboxamido)ethyl]-N,N-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA) [54], 1,2-dioleyloxy-3-(trimethylammonium)propane (DOTAP) [55] and others. Cationic liposomes and DNA form complexes referred to as lipoplexes. Electrostatic

Table 2.3: Examples of non-viral (chemical) vectors used for gene delivery

Agent	Examples
Lipid based vectors	e.g. DOTMA, DOGS, DOSPA, DOTAP
Polymeric vectors	e.g. Poly(L-lysine), Polyethyleneimine (PEI), Polymethacrylate, β -cyclodextrin, Chitosan, Poly(glycoamidoamine), Schizophyllan, Poly(amido-amine)
Dendrimer based vectors	Polyamidoamine Dendrimers (PAMAM), Poly(propyleneimine) Dendrimers, Poly(L-lysine) Dendrimers, Phosphorous-containing Dendrimers
Polypeptide vectors	e.g. Tat-Based peptides, Antennapedia Homeodomain peptide, MPG Peptide, Transportan Peptide

interaction between the positively charged lipids and negatively charged DNA facilitate the self-assembly of these particles. In addition, the lipoplexes are sufficiently small due to compaction of DNA to promote cellular uptake. In recent years, optimization of these lipids focused on improving stability, cell-specific targeting and facilitating endosomal escape.

Addition of neutral co-lipids, such as 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) has been shown to increase transfection efficiency. These lipids may improve gene transfer by increasing the fluidity of lipoplexes and facilitate gene expression by improving endosomal escape of the particles. Inverted hexagonal supra-molecular structures, formed by DOPE, have negative spontaneous curvature that facilitates endosomal escape by spontaneous flip-flop reorganization of liposomes [56, 57].

Modifications of cationic head group, spacer length and hydrophobic tail affect transfection efficiency significantly. Replacement of nitrogen in the head group with arsonium and phosphonium resulted in reduced cytotoxicity and enhanced gene expression [58, 59]. The increase in efficiency was attributed to reduced charge densities due to the formation of larger cationic complexes. Incorporation of a hydroxylalkyl chain onto the ammonium group reduced the stability of lipoplexes [60]. However, this instability allowed the spontaneous fusion of the lipoplexes with the cell surface membrane, increasing overall gene transfer [60, 61]. The smaller distance between the cationic head groups in a multivalent lipid resulted in lower transfection efficiency [62-64], proposed to be a result of poor distribution of surface charge density.

The hydrophobic carbon tail of cationic lipids can influence their ability to form bilayer structures and fluidity that may be needed for endosomal escape of plasmid DNA [65, 66]. Felgner *et. al.* synthesized a series of homologous lipids that exhibited transfection efficiency in the order of $C_{14} > C_{16} > C_{18}$ while Byk *et.al.* found C_{18} to be the most efficient among lipids containing double alkyl chain lengths of 12, 13, 14, or 18 carbon atoms [61, 67]. Trilysine-based gemini surfactants showed an increase in gene expression, associated with increasing tail length from C_{12} to C_{18} [68]. Heyes *et. al.* synthesized diether lipids with variable asymmetric two carbon tails from C_{12} to C_{18} and basicity of head group by grafting different amino acids [65]. Interestingly, the lipids with shorter carbon tail and asymmetric dialkyl tail showed better transfection efficiency compared to other analogs. The authors proposed this difference as due to their contribution to fluidity of tertiary complexes with DC-Chol and DOPE, used as co-lipids

for formulating lipoplexes. In conclusion, these studies suggest that the effect of hydrophobic tail length needs to be evaluated in relation with the type of cationic head group and co-lipids.

2.3.2. Polymers

Complexes formed by interaction of polymers with DNA are known as polyplexes. Positively charged groups on the cationic polymers condense the negatively charged DNA. The electrostatic interactions, similar to cationic lipids, produce compact DNA complexes that promote cellular entry [69], protect the DNA from enzymatic degradation by nucleases and allow the release of DNA for localization in the nucleus for gene expression.

Polyethyleneimine (PEI) is a notable example in this class [70]. PEI is a simple repetition of the ethyleneimine motif which can be controlled to produce linear or branched polymer structures [71, 72]. The high transfection efficiency of the PEI might be due to its high buffering capacity in acidic pH, also known as ‘proton sponge effect’. At physiological pH, some of the amine groups are positively charged that interact with DNA. Upon gaining entry into the cell, the protonation of remaining amine groups prevents the drop in pH inside endosomes [70]. This results in the entry of a large volume of fluid, causing membrane disruption and endosomal escape of DNA particle [73].

Poly(L-lysine), PLL is another important polymer vector that has good plasmid DNA condensation capability [74]. The protonation of the majority of amine groups leads to a strong DNA binding and compaction. However, the poor buffering capacity of the PLL does not promote endosomal escape [75]. On one hand, the ability to condense the DNA increases with an increase in molecular weight of the PLL. On the other hand, a significant increase in cytotoxicity and tendency to aggregate has also been reported with the increased chain length [76].

Examples of other polymers evaluated for gene delivery include β -cyclodextrin [77], chitosan [78], poly(glycoamidoamine) [79] and schizophyllan [80].

A number of modifications were introduced in these polymers to improve their transfection efficiency and decrease cytotoxicity. Non-covalent PEGylation of PEI improved *in vivo* transfection efficiency by reducing interaction with biomolecules [81]. Conjugation of chitosan with cell-specific ligands such as galactose, lactose or trisaccharide improved its transfection

efficiency [82-84]. Introduction of acid-labile ester linkages in PEI structures reduced its cytotoxicity [85].

2.3.3. Dendrimer-based vectors

Dendrimers are highly branched 3-dimensional monodisperse, usually spherical, macromolecules. Examples of dendrimers used in gene delivery include polyamidoamine dendrimers (PAMAM) [86], poly(propyleneimine) dendrimers [87], PLL dendrimers [88], and phosphorous-containing dendrimers [89]. These vectors showed good transfection efficiency due to improved complexation, cell targeting and intracellular escape [90-92].

Dendrimers form stable and water dispersible complexes with DNA. It was proposed that these complexes were internalized via endocytosis [93]. The low pKa value of amines imparts high buffering capacity (proton sponge capability) to the DNA/dendrimer complexes leading to an efficient endosomal escape [94]. Dendrimer generation, i.e., the number of repeated branching cycles that are performed during its synthesis, affected the transfection efficiency of the dendrimers [95]. Usually, high generation number (>G5) led to a high transfection efficiency. However, an increased cytotoxicity was also observed with an increase in dendrimer generation, especially with >G7 [86].

2.3.4. Polypeptides

Non-viral gene delivery vectors suffer from lack of nuclear targeting. Polypeptides improve both cellular uptake and nuclear targeting. These peptides are used alone or in conjunction with lipoplexes or polyplexes. Some examples of these types of targeting moieties include transactivating transcriptional activators (TAT) peptides [96], *antennapedia homeodomain* peptide [97], MPG peptide [98] and transportan peptide [99]. Amino acid residues of these peptides interact electrostatically with DNA to produce complexes and interact with the cell surface to increase cellular uptake [100, 101].

The TAT protein is an 86-102 amino acid sequence which promotes cellular uptake and nuclear localization. A smaller sequence of TAT protein (47-57) facilitates cellular entry. Incorporation of the TAT-peptide in liposomes enhanced gene expression [100]. Similar results were found on combining TAT peptides with PEI/DNA complexes [101]. The TAT-peptide

improved cellular entry and nuclear localization. However, it exhibits poor endosomal escape ability [102].

A 16-amino acid peptide sequence from *antennapedia* homeodomain was shown to promote cellular entry [97]. Destabilization of the cell membrane via electrostatic interaction with the amino acid residues of the peptides was proposed as the mechanism of cellular entry [102, 103]. Although the *antennapedia* peptide-conjugated DNA exhibited gene expression and low cytotoxicity, the gene expression was further improved after conjugation with another water soluble peptide (L4F) [97]. Antennapedia-L4F conjugated with Lipofectamine 2000/DNA has exhibited significantly higher gene expression than complexes without the peptides [97].

MPG is a synthetic fusion peptide which promotes cellular uptake and nuclear localization [98]. MPG forms transmembrane pore-like structures which facilitate cellular entry via an energy independent manner [98, 104]. Low toxicity and high gene expression were achieved by the MPG/DNA complexes [104]. Transportan, derived from wasp venom peptide toxin, is another peptide that promotes cellular uptake via energy independent pathway [99]. Transportan 10, an analogue of transportan, was used to enhance gene expression of the PEI-DNA complexes [99].

3. Factors affecting transfection efficiency of non-viral vectors

3.1. Physicochemical properties

Physicochemical factors that affect the transfection efficiency of a non-viral gene delivery systems are – surface charge, particle size, DNA topology, morphology and surface charge density.

3.1.1. Cationic Charge

DNA is a negatively charged molecule and, therefore, cationic chemical agents are able to interact electrostatically with plasmid DNA to form complexes [105]. These interactions (complex formation) influence the stability and transfection efficiency of the delivery systems. The cationic agents neutralize the negative charges on the plasmid DNA and facilitate cellular uptake by improving interaction with negatively charged cell surface molecules [106]. Moreover, an overall cationic charge on the DNA complexes provides necessary repulsion between particles to avoid aggregation. This net positive complex surface charge is measured as zeta potential [107, 108].

Although a net cationic charge on the DNA/delivery vector complexes is essential, it is necessary to maintain an optimum charge ratio. On one hand, an excessively strong interaction between the delivery agent and DNA can hinder the release of the DNA inside the cell for gene expression. Transfection efficiency of (poly(2-dimethylamino)ethyl methacrylate) [p(DMAEMA)] was higher than poly(2-(trimethylamino)ethyl methacrylate chloride) [p(TMAEMA)] [109], although there was no difference between the two agents regarding their ability to protect the DNA against DNase I degradation. However, DNA dissociation studies suggested that a strong association between p(TMAEMA) and DNA may hinder DNA release for gene expression. Similar results were observed with DNA complexes formed with different lengths of PLL in the presence of chloroquine (as endosomotropic agent) [110]. On the other hand, weak complexes can cause premature release of DNA on interaction with the cell membrane, or provide insufficient protection against nucleases. These electrostatic interactions can be manipulated by controlling the cationic agent to DNA ratio [108, 111].

3.1.2. Particle Size

The size of the DNA complexes is another important factor that can influence transfection efficiency of delivery systems [112]. The increase in cellular uptake, higher number of intracellular DNA particles per cell, better DNA release and better nuclear targeting are associated with nanoscale particles, leading to high transfection efficiency [108, 112]. A particle size up to ~150 nm promotes transfection [112-115]. The physicochemical properties (surface charge, chemical structure etc.) of the delivery vectors greatly influence the DNA complex size [108, 116, 117].

3.1.3. DNA topology

Plasmid DNA can occur in linear (l-DNA), open circular (c-DNA) or supercoiled (sc-DNA) form. Among these, the complexes containing supercoiled form exhibit higher gene expression than open circular or linear forms [116-118]. Transfection studies using circular and linear forms of EGFP-DNA (encoding for green fluorescent protein) with PEI showed that PEI/c-DNA has 90% EGFP positive cells while only 60% cell population was positive with cells incubated with PEI/l-DNA [116]. Similar results were obtained with cationic lipid agents, DOTAP/DOPE, that exhibited higher transfection efficiency with sc-DNA > c-DNA > l-DNA [119].

DNA topology had a significant effect on the particle size of DNA complexes. PEI produced particles in the range of 150-300 nm with c-DNA while the size was 700-770 nm with l-DNA [116]. Similar differences in particle size with c-DNA and l-DNA were observed with palmitic acid-grafted PLL and lipofectamine-2000. This may be because c-DNA in circular form collapses easily into a supercoil when the negative charges on phosphate groups of DNA are neutralized by the cationic charges of the delivery vectors. However, l-DNA will not offer this conformational constraint and therefore, forms loose aggregates of large size. Although, the difference in DNA topology had a significant effect on the particle size, it did not affect the level of DNA uptake [116]. This suggests that the presence of cationic charge facilitates the internalization of plasmid DNA but confers poor intracellular stability and protection to the linear form of DNA resulting in poor gene expression.

Dissociation studies suggested that a strong association exists between l-DNA and cationic lipids compared to its circular form, while the topology had no effect on DNA binding with

cationic polymers. In another study using p(DMAEMA) with different topologies of DNA, transfection efficiency decreased in the order of sc-DNA > c-DNA > l-DNA [117]. However, the DNA topology had no effect on toxicity, particle size, and DNA binding (association or dissociation) properties of p(DMAEMA). Transfection efficiency of DOTAP/DOPE was higher with sc-DNA than c-DNA and l-DNA [119].

The ionic headgroups on cationic lipids interact electrostatically with the negatively charged phosphate groups of DNA. These interactions are constrained by the distance between the adjacent phosphate groups of DNA, depending upon its topology. Clearly, the helical structure of DNA is highly constrained when present as supercoiled and circular DNA, compared to linear form. Neutralization of these charges should collapse DNA strands into small particles; however, interaction of cationic lipids is also influenced by the attractive and repulsive forces between ionic charges. In cationic lipids, in addition to the ionic interactions, the molecules are also ordered by the hydrophobic interactions among hydrocarbon tails which may be absent in a polymer based vector. The total surface area and total volume of molecules can also influence its interaction with DNA and formation of polymorphic structures. The presence of additional formulation components such as co-lipids, salt concentration etc. can influence the overall interaction. This is the reason for a need to optimize these factors, specific to each delivery system, in order to achieve high transfection efficiency. However, the studies with polymers and cationic lipids clearly indicate that supercoiled and circular form of DNA is advantageous for transfection as it facilitates formation of stable small-sized particles.

3.1.4. Supra-molecular structures

Another important parameter affecting the transfection efficiency of delivery systems is the supramolecular assembly of the DNA complexes. Multilamellar structures with DNA sandwiched between cationic lipids and inverted hexagonal structures with DNA encapsulated within lipid monolayer tubes were the two most frequently observed lipoplex structures [120-122]. The presence of neutral lipids improves transfection efficiency. Liposomes containing 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) as neutral lipid had higher transfection efficiency compared to other neutral lipids [61].

In one study, the transfection efficiency of DNA/DC-Chol/DOPE was reduced significantly when DOPE was replaced by DOPC as neutral lipid [123]. This was attributed to the inverted

hexagonal structures obtained by liposomes containing DOPE, even at low helper lipid to cationic lipid ratio. X-Ray diffraction (XRD) studies revealed an inverted hexagonal structure for DOPE vesicles while DOPC exhibited multilamellar structures [124]. At the same mole fraction of 0.67-0.69 of total lipid content, transfection efficiency of DNA/DOTAP/DOPE was 100 times higher than DNA/DOTAP/DOPC. The inverted hexagonal structures formed more stable lipoplexes due to close interaction of all cationic head groups around DNA molecules. The elastically-frustrated hexagonal lipid membranes interacted more easily with the cell surface and endosomal membranes improving cell entry and endosomal escape [124].

3.1.5. DNA complex charge density

While DOPE containing formulations showed better transfection than DOPC containing formulations, the transfection efficiency of DOPC formulations could be further improved by focusing on DNA complex surface charge density rather than net complex charge [124]. The study found a positive correlation between DNA complex surface charge density and transfection efficiency in DOPC formulations. In DOPE formulation, the driving force for DNA escape was the supra-molecular structure (hexagonal) of the complexes, while in the DOPC-containing formulations the cationic charge influenced the interaction of the DNA complexes with the anionic cell membrane. Therefore, a large number of cationic headgroups per unit surface area of DNA complex translated into higher interaction and greater fusion with the cell membranes. The surface charge density can be modified by use of neutral lipids, such as DOPE or DOPC in combination with cationic lipids or, by modifying the number or size of cationic head groups in delivery vectors.

In summary, favorable physicochemical characteristics are needed for efficient gene delivery. The optimization of these physicochemical factors can be achieved by chemical modification of the molecules, use of combination of chemical agents, optimizing the molar and charge ratios of the various agents and developing optimal methods of preparation. The chemical modification can be introduced to modulate DNA binding properties such as additional cationic groups into the vectors or to optimize the length of the spacer/tail groups. The use of helper lipids (such as DOPE, cholesterol) with cationic lipids can facilitate desirable morphological structures. This gives an opportunity to create a variety of complexes without chemical modification. However, optimization of the ratio of plasmid to delivery agent, ratio between

delivery agents, and a method of preparation is needed as it can affect these important physicochemical properties.

3.2. Mechanism of cellular uptake

At the cellular level, the plasma membrane is the first barrier to delivery of non-viral gene delivery. Due to their large size and hydrophilic nature, the non-viral particles cannot diffuse into the cell. Endocytosis is the process of cellular uptake of macromolecules or solutes that are impermeable to plasma membrane; the plasma membrane invaginates to engulf these molecules (**Figure 3.1**) [125]. Endocytosis has been established as the main route of internalization of the non-viral delivery particles into the cells [126, 127]. Special mammalian cells can engulf large solid particles (phagocytosis) such as cell debris, while the majority of other mammalian cells utilize a more common method to engulf fluid or solutes (pinocytosis). Pinocytosis is divided into clathrin-mediated uptake, caveolae-dependent uptake, macropinocytosis, and clathrin/caveolae-independent pathway [125].

3.2.1. Clathrin-mediated uptake

In clathrin-mediated uptake, the particle binds to clathrin-coated pits on the cell surface and internalized as clathrin-coated vesicles with the help of adaptor proteins [128]. The particles in clathrin-coated vesicles experience a drop in pH as they travel towards the late endosomes, creating acidic conditions (pH 5-6) before merging with lysosomes [129]. Plasmid DNA has more success of survival if it is able to escape the endosome before reaching the lysosomes which have a high concentration of enzymes. To this effect, the use of pH-sensitive delivery vectors such as pH-sensitive liposomes which can destabilize endosomal membrane and release enclosed molecules, have been found to be more efficient than non-pH-sensitive vectors. Legendre *et. al.* prepared pH-sensitive liposomes by combining cholesteryl hemisuccinate and DOPE at 2:1 molar ratio, which exhibited higher transfection efficiency compared to non-pH-sensitive liposomes. [130]. The difference in transfection efficiency was due to the ability of pH-sensitive liposomes to prevent lysosomal degradation by escaping from the endosomes. It was interesting to note that the cationic liposomes formulated with DOPE exhibited even higher transfection efficiency than non-cationic pH-sensitive liposomes because of strong binding with the cell membrane and were able to deliver more plasmids than pH-sensitive lipids. However,

this work did not take into account that DOPE, when combined with cationic lipids, exhibits a pH-dependent structural transition which is crucial to its role in endosomal escape, therefore imparting pH-sensitive character to the cationic liposomes [131].

Similarly, polyethyleneimine (PEI) exhibits high transfection efficiency due to its proton sponge effect in acidic conditions inside an endosome, provoking water influx, endosomal swelling, and disintegration [70]. In addition, lysosomotropic agents such as chloroquine can prolong the fusion of endosomes to lysosomes or cause the disruption of lysosomes, thus avoiding DNA degradation [19, 132]. However, these agents are associated with high toxicity which limits their use in clinical settings. Another strategy is to incorporate fusogenic pH sensitive peptides such as GALA (WEAALAEALAEALAEHLAEALAEALEALAA) into the DNA complexes which enhance endosomal escape in response to low pH in the endosomes [133].

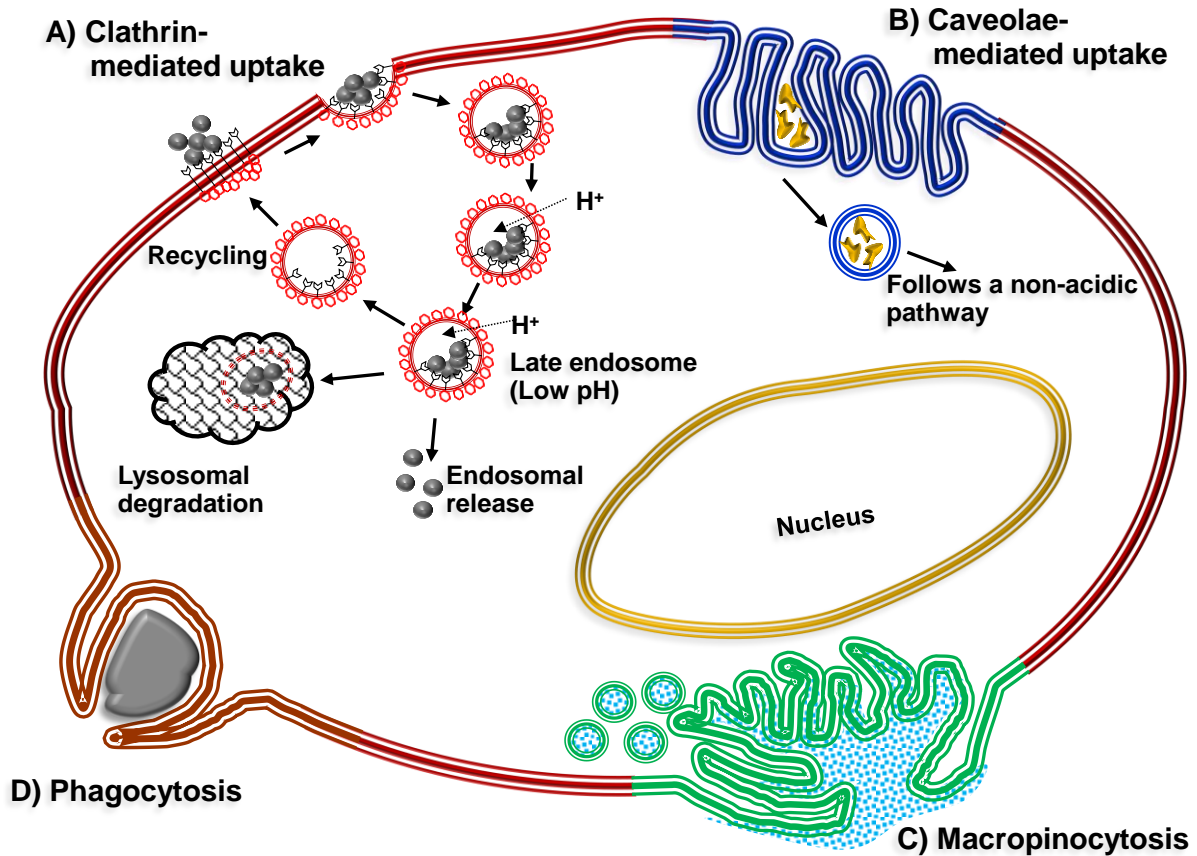


Figure 3.1: Schematic representation of four major uptake pathways in a mammalian cell.

A) Clathrin-mediated uptake follows an acidic pathway, leading to release of cargo into lysosomes, B) Caveolae-mediated uptake follows a non-acidic, non-digestive pathway, C) Macropinocytosis involves non-specific intake of fluids (solutes) and follows a non-acidic pathway, D) Phagocytosis is the engulfing of large solid particles including pathogens, cell debris. Special mammalian cells such as macrophages exhibit this behaviour.

3.2.2. Caveolae-mediated uptake

The study of SV40 virus revealed a clathrin-independent uptake pathway which provided entry to the virus through caveolin-containing vesicles [134]. Due to the association with caveolin proteins, the endocytic vesicles were called caveosomes and the pathway is referred to as caveolae-mediated uptake pathway. Caveolae form smaller vesicles of size 50-60 nm and have a lower rate of uptake, compared to clathrin-coated pits [125]. Particles engulfed via caveolae-mediated uptake avoid degradation in the lysosomes by following a non-acidic, non-digestive pathway to reach the Golgi complex or endoplasmic reticulum [135, 136]. Due to the absence of lysosomes, this pathway has been a target for the delivery of plasmid DNA.

Rejman *et. al* found that lipoplexes composed of DOTAP/FITC–poly-L-lysine-labeled DNA proceeded via clathrin-mediated endocytosis while polyplexes containing PEI/FITC–poly-L-lysine-labeled DNA were internalized via both caveolae-mediated and clathrin-mediated pathways [137]. Interestingly, PEI/DNA polyplexes that were internalized by clathrin-mediated pathway reached lysosomes while polyplexes endocytosed via caveolae lead to gene expression. Even though the caveolae-mediated uptake seemed to be an attractive route of delivery for DNA complexes, this pathway is dependent on the distribution of caveolae on the cell surface, which is cell type dependent. Due to the absence of endogenous caveolins in HepG2 cells, the polyplexes could not use the caveolae-mediated pathway and, therefore, degraded in lysosomes [138, 139].

Gersdorff *et. al.* tested polyplexes using linear PEI and branched PEI in different cell lines and determined the influence of cellular uptake pathways on gene expression [140]. Interestingly, both cell line and polyethylenimine polyplex type affected gene expression. Branched PEI polyplexes were able to mediate transfection via both pathways in HUH-7 cells and HeLa cells whereas linear PEI polyplexes succeeded mainly via the clathrin-dependent route. In COS-7 cells, the clathrin-dependent pathway was the main contributor to the transfection process. In contrast, a study by Van der Aa *et. al.* with pDMAEMA/DNA and PEI/DNA revealed caveolae-mediated and clathrin-mediated uptake for these polymers; however, caveolae-mediated pathway was the major route that lead to gene expression in COS-7 cells [141].

3.2.3. Macropinocytosis

Macropinocytosis is a non-selective engulfing of extracellular fluids by invagination of the plasma membrane, forming macropinosomes of variable shapes and sizes [142]. In special mammalian cells such as macrophages, macropinosomes can be as large as 5 μm and travel towards lysosomes while shrinking their size and gaining acidity [142, 143]. However, in other mammalian cells they may not fuse with lysosomes. The non-specific engulfing of large volumes of extracellular medium that does not end up in lysosomes render macropinocytosis an attractive target for macromolecules such as plasmid DNA. TAT, derived from human immunodeficiency virus-type 1, containing six arginine and two lysine residues, has promoted macropinocytosis uptake [144]. Based on the high arginine content within the TAT sequence, scientists tested polypeptides containing solely arginine residues and found that macropinocytosis was the major route of entry [145, 146]. Khalil *et. al.* studied the effect of octaarginine density on lipoplex surfaces and found that liposomes modified with a high peptide density were internalized mainly through macropinocytosis and were less subject to lysosomal degradation [147]. However, lipoplexes containing low density of octaarginine followed clathrin-mediated pathway. Recently, a similar study with lipoplexes prepared with decaarginine-polyethylene glycol-lipid found that cellular uptake was through macropinocytosis at low N/P ratio, while complexes formed at high ratio also followed a pathway different from macropinocytosis [148]. For lipoplexes prepared at lower N/P ratio, the disruption of the macropinosomal membrane was a critical step for efficient gene delivery.

In addition to the composition and type of biomaterials, the size and shape of the particle influences the uptake behaviour. Rejman *et. al* demonstrated that particles with size less than 200 nm were internalized via clathrin-mediated pathway while larger particles endocytosed via caveolae-mediated pathway [149]. Particle shape and surface charge in addition to particle size plays an equally important role in influencing the uptake behaviour [150]. Rod-like particles (height to diameter ratio > 3) were internalized faster than the symmetrical particles. Positively charged particles of similar size and shape were internalized significantly more (~84% of cells) compared to negatively charged particles (<5% cells). The uptake of cylindrical particles of sizes 150 nm and 200 nm was reduced by ~60-70% on inhibiting either clathrin-mediated or caveolae-mediated uptake. This suggests both pathways are involved in the uptake of particles that were smaller than 200 nm.

In an effort to improve transfection efficiency of gene carriers, significant efforts have been directed towards designing new carriers that can improve endosomal escape. Recently, the importance of the contribution of certain pathways in the uptake of non-viral vectors and their effect on transfection efficiency has become evident. A few studies have revealed that composition, size, shape, surface charge, and surface chemistry influence the uptake mechanism of particles. However, a holistic understanding of these physicochemical properties in association with each other and their influence on uptake mechanism can help in designing gene delivery systems that can improve the intracellular fate of the particles.

4. Gemini surfactants as efficient gene delivery systems

Surfactants are amphiphilic structures having unique interfacial properties. Conventionally, the surfactants consist of a single hydrophobic tail with an ionic or a polar head group. The wide variety of application of these molecules along with the ease of structural modification has attracted the attention of many scientists. Menger et al [151] reported novel dimeric surfactant molecules which contained two ionic head groups and two hydrocarbon chains linked by a spacer (**Figure 4.1**). This new generation of surfactants was named as ‘gemini surfactants’.

In the absence of a third component, e.g. salt, conventional monovalent surfactants generally form spherical micelles at the critical micelle concentration, whereas gemini surfactants can form a variety of structures ranging from thread-like micelles for short spacers, (2-3 carbon atoms), to spherical, rod-like and lamellar shapes as the spacer length increases. These dimeric molecules have several advantages compared to the conventional surfactants such as lower critical micelle concentration, higher efficiency in reducing surface tension and greater tendency to self-assemble [152, 153].

The presence of a cationic head group enables a strong electrostatic interaction of the gemini surfactants with DNA to form nanoparticles and aid in gene delivery. The opportunity to modify their structure creates flexibility in designing suitable delivery systems for enhanced gene transfer into mammalian cells [154].

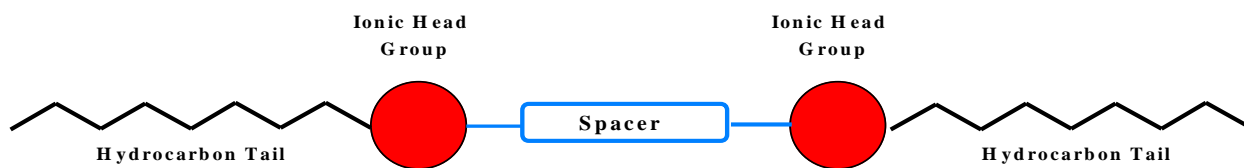
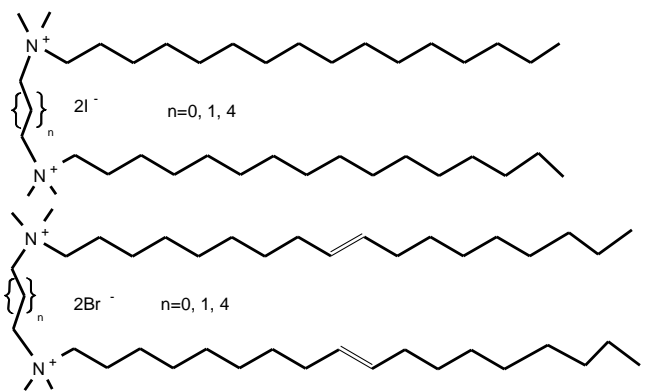
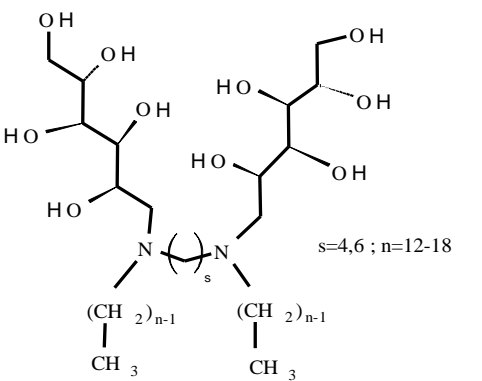
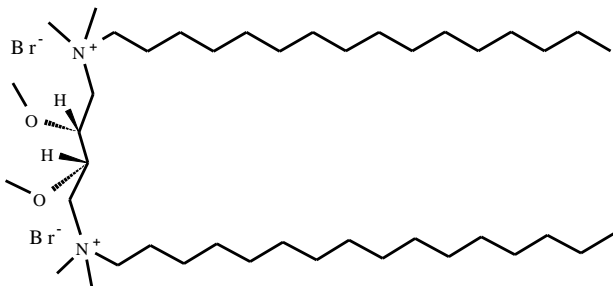
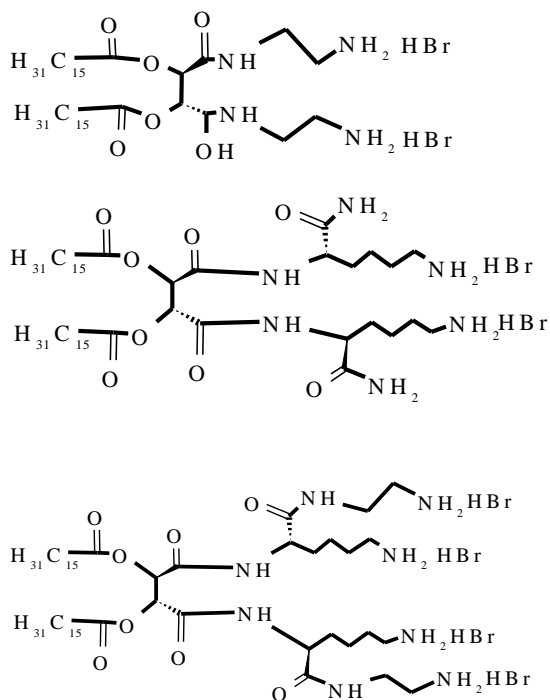


Figure 4.1: General structure of gemini surfactants.

Table 4.1: Gemini surfactants used as gene delivery systems.

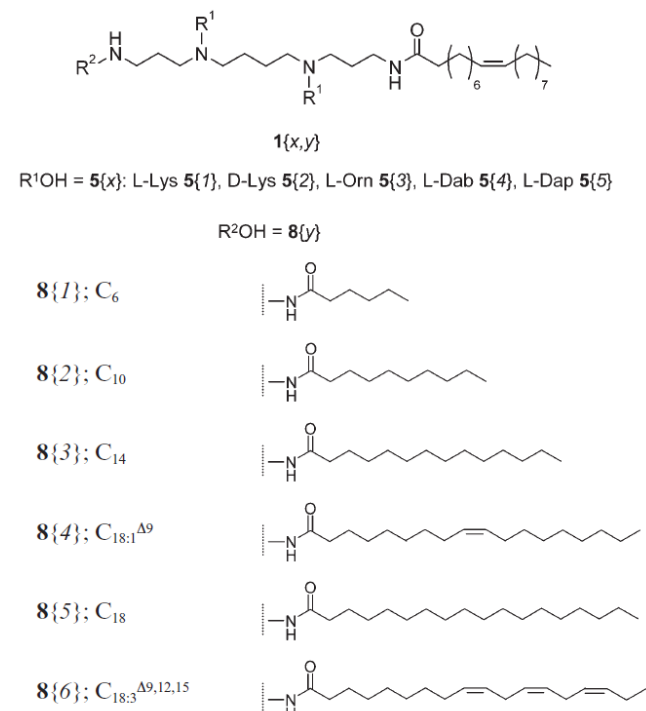
Surfactant Type	Results
<p>1. Amine based m-s-m type [155]</p> 	<p>A six methylene group spacer (n=4) between quaternary ammonium groups exhibited the highest transfection efficiency. These compounds showed reduced transfection efficiency in presence of neutral lipid (DOPE) or serum proteins.</p>
<p>2. Sugar based m-s-m type [156]</p> 	<p>16-carbon tail yielded the highest gene expression. Incorporation of unsaturation in 18-carbon tail increased transfection greater than 16-carbon and Lipofectamine</p>
<p>3. Amine based m-s-m type [157]</p> 	<p>Good transfection efficiency when gemini to helper lipid ratio > 0.5</p>

8. Tartaric acid based gemini lipids [161]



Lysine and lysine/ethylene diamine substituted derivatives showed transfection activity; however, the results were inconclusive due to high toxicity of these compounds.

9. Spermine-based gemini surfactants [162]



Lysine-substituted derivatives yielded the highest gene expression.

Two lipophilic tails of 18-carbon length were optimum for high transfection efficiency.

Rosenzweig *et. al.* demonstrated the potential of diquatery ammonium compounds (**Table 4.1.1**) as gene delivery vectors by conducting transfection studies in BHK-21 hamster kidney cells [155]. Saturated derivatives favored lamellar-type structures and exhibited high transfection with six methylene groups in the spacer, compared to two or three methylene groups. Diquatery ammonium compounds containing an unsaturated bond in the tail formed micelles and liposomes, depending on the ionic strength, and yielded high transfection capability with greater than two methylene groups in the spacer. Fielden *et. al.* experimented with sugar-based gemini surfactants, bis- α,γ -(alkyl-1'-deoxyglucitylamino)-alkanes, (**Table 4.1.2**) again by varying the tail (C_{12} to C_{16}) length and spacer (C_4 to C_6) length [156]. Both the tail length and distance between the cationic amino groups had an impact on the transfection efficiency of the compounds. The derivatives with 16-carbon tail yielded the highest gene expression among the saturated derivatives; with slightly higher gene expression with six methyl groups in the spacer compared to four. The incorporation of unsaturation in the 18-carbon tail generated gene expression higher than saturated 16-carbon tails and about 2.7 fold higher than Lipofectamine. At physiological pH, the sugar-based gemini surfactants formed bilayer structures and attained a slight positive charge that helped in DNA compaction. The authors found that the derivatives with high transfection efficiency continued to protonate in endosomal pH range 6.0 – 4.0 and, proposed that it would induce a bilayer to micelle transition which must be facilitating the endosomal escape of DNA complexes. Later, in a comparative study between the saturated and unsaturated 18-carbon tail sugar based gemini surfactants, the authors observed that the unsaturation in the tail influenced lipoplex morphology in a pH-dependent manner [163]. Initially, at basic to neutral pH, the unsaturated 18-carbon tail derivative formed lamellar structures which experienced a further transition to inverted hexagonal morphology in the endosomal pH range. This variation in morphology upon a change in pH provided a critical opportunity for the lipoplex to escape degradation, which was absent in the saturated derivative.

Further, Ryhänen *et. al.* explored the role of surface charge density on transfection efficiency of a binary lipid system [157]. Liposomes prepared using gemini surfactant (2S,3R)-2,3-dimethoxy-1,4-bis(N-hexadecyl-N,N-dimethylammonium)butane dibromide (**Table 4.1.3**) and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) exhibited high transfection when gemini to helper lipid ratio was > 0.5 . The study revealed that the surface charge density that influenced the DNA condensation by liposomes might be the primary reason for this behaviour.

Fisicaro *et. al.* investigated the transfection efficiency of ester linked diquatary ammonium gemini surfactants (**Table 4.1.4**) with C10, C12, C14 tail lengths [158]. All the derivatives showed high transfection efficiency when formulated with helper lipid (DOPE), except the C14-tail derivative which had transfection activity even without DOPE; though, it was lower than the positive control. The helper lipid improved the stability of DNA/gemini surfactant complexes; however, their interaction with the phospholipid bilayers was dependent on the tail length. The derivatives with C10 and C12-tail showed a slight fluidizing effect causing destabilization of phospholipid bilayers while C14-tail derivative increased the stability of DPPC liposomes (mimicking cellular membranes). This was due to the ability of the gemini surfactant to form hydrocarbon tail associations below the critical micellar concentration (CMC) levels.

Gaucheron *et. al.* were able to show transfection potential of tetraalkyl cationic lipids (**Table 4.1.5**) - alkyl gemini derivatives with two oleyl tails attached to each positively charged nitrogen joined by a hydrocarbon tether three or six carbons in length [159]. At +/- charge ratio < 2, the derivatives with six methyl groups in the spacer gave gene expression levels higher than the C3-spacer derivative and its monomeric equivalent *N,N*-dioleoyl-*N,N*-dimethylammonium chloride (DODAC). The C6-spacer derivative exhibited an improved ability to induce non-bilayer structure (ion-pairing) in mixtures with anionic lipid (mimicking cellular membranes) which had been proposed to correlate with improved transfection activity of delivery vectors. The gene expression levels of tetraalkyl derivatives were increased on co-formulating with helper lipid (DOPE).

Bombelli *et. al.* introduced the role of stereochemistry in the transfection ability of gemini surfactants [160]. Stereoisomers of C16-tail gemini surfactants with four methylene groups between the quaternary ammonium cations were synthesized by introducing two methoxy groups in the spacer region (Table 4.1.6). On formulation with helper lipid (DMPC), all gemini surfactants exhibited transfection activity, whereas no gene expression was observed with its monovalent counterpart; thus re-confirming the superiority of gemini surfactants over single chain single headgroup surfactants. Methoxy substituted-gemini derivatives with (2R, 3S) configuration showed the highest transfection among all the derivatives, even higher than commercial positive control. Circular dichroism experiments revealed that the gemini surfactants were able to induce structural transition from a B-form of the plasmid to a ψ -phase, indicating

DNA condensation. This conformation change was more prominent for the (2R, 3S) isomer and unsubstituted derivative, while it was missing for the monovalent derivative.

McGregor *et. al.* synthesized peptide based gemini surfactants (Table 4.1.7) by varying the nature of tail groups, and peptide (lysine) substitution in the head [68]. In agreement with previous studies with gemini surfactants, the results showed that the transfection efficiency improved with increase in tail length and presence of unsaturation in the tail. The nature of the head group, particularly, trilycine linkage, also affected the transfection efficiency. The compounds with lysine residues attached together via the ϵ -amino group, rather than the α -nitrogen, yielded highest transfection.

Buijsters *et. al.* synthesized gemini surfactants starting from tartaric acid with ethylenediamine, lysine and lysine/ethylenediamine substitution in the head group (**Table 4.1.8**) [161]. Ethylenediamine and lysine substituted Gemini surfactants formed bilayer structures but had poor aqueous solubility whereas no structures were observed with lysine/ethylenediamine derivative. Transfection studies in CHO-K1 cells showed activity for lysine and lysine/ethylenediamine derivatives, however, the results were inconclusive due to high toxicity of compounds.

Castro *et. al.* introduced an interesting twist to the tale by synthesizing gemini surfactants with different diamino acid headgroups (**Table 4.1.9**), containing symmetrical (identical) and unsymmetrical (different) tail groups [162]. Interestingly, the study also found that two lipophilic tails of 18-carbon length was optimum for mediating high transfection activity and the presence of diamino acid residues in the polar head groups were well tolerated in cells depending on the compound concentration. Though lysine-substituted compounds exhibited the highest transfection efficiency at low concentration, less potent L-diaminopropyl (L-Dap) generated comparable gene expression at high concentration. Increasing the concentration of lysine-substituted gemini correlated with higher cytotoxicity while L-Dap derivatives were well tolerated at higher concentrations. In addition to this, co-formulation of gemini surfactants with helper lipid (DOPE) yielded a transfection efficiency higher than what was achieved with commercial reagent (Lipofectamine 2000).

In general, a gene delivery vector should be able to interact with DNA, condense it into nanostructures, interact with cell membranes, protect the plasmid DNA inside the cell, and ultimately deliver it to the nucleus for gene expression. The studies so far have shown that the

cationic gemini surfactants possess all the qualities of a successful gene delivery carrier. In addition, the possibility to modify their properties by tweaking the nature and the length of both tail and head groups provides a continued opportunity to design better and efficient delivery vectors.

The long term goal of the study is to develop non-invasive mucosal (vaginal) gene delivery systems. Therefore, in the next chapter, I describe the vaginal route for drug delivery and the challenges it presents to our gene delivery systems. In addition, I will discuss the rationale for the animal model for vaginal delivery.

5. Vaginal gene delivery

Vaginal mucosa, like other mucosal surfaces, provides a potential route for drug administration. It offers a relatively large surface area for drug permeation, high vascularization, a low enzymatic activity and ease of access with potential for self-administration. Predominately, vaginal drug delivery has been explored for local administration of anti-infective, anti-hemorrhagic, spermicidal agents and hormones [164]. The vaginal route has also been explored for delivery of therapeutic genes, examples include intratumoral administration of the soluble CD40 ligand gene [165], DNA encoding for cytokines such as IL-2, IL-12, GM-CSF or IFN- γ [166], small interfering RNA targeting HPV E6/E7 oncogene [167], and immunomodulation with CpG oligonucleotides [168]. Mucosal surfaces, including the vagina, represent a primary route of entry of pathogens into the body. Immunization through intravaginal route has also been explored [169]. This is even more important as there is evidence that systemic immunization does not elicit a strong mucosal immunity while topical vaccines trigger both local and systemic immunity [170].

5.1. Challenges to vaginal drug delivery

Effective mucosal delivery faces the challenges to chemical (proteases, nucleases) and physical (dilution, entrapment or exclusion) barriers presented by mucous secretions in addition to the cellular barriers of gene delivery [171]. Pharmaceutical agents that adhere to mucosal tissue increase the efficiency of drug permeation into the tissue [172]. Examples of mucoadhesive delivery systems include poloxamer (Pol)/polyethylene oxide (PEO) [173], Pol/polyacrylic acid (PC) [172], Pol/HPMC [174], among others. The incorporation of DNA into the mucoadhesive agents can overcome the challenges of gene delivery by increasing the residence time of the formulation in the vaginal cavity. The development of vaginal gene delivery systems has the potential to improve cancer treatment (such as cervical cancer), vaccines and conditions such as dyskeratosis congenital [175] and lichen planus [176] which affect both the oral and vaginal mucosa.

5.2. Animal models for vaginal delivery

Although *in vitro* and *ex vivo* testing facilitate the screening of a considerable number of nanoparticles and topical formulations, these systems are void of the complex makeup of the

vaginal tissue, immune system, underlying blood flow and active secretion. Ultimately, animal models are needed to evaluate the efficiency of the topical mucosal delivery and gene expression of the DNA nanoparticles. Rabbits are frequently used in efficacy studies of vaginally delivered anti-infective agents [177] and vaccines [178], and represent the standard for toxicity evaluation of vaginal dosage forms [179]. By using a rabbit model, the efficacy and toxicity of the mucosal nanoparticles delivery systems can be assessed simultaneously. Moreover, the similarities between human and rabbit mucosal tissue warrant the use of this model [180]. In addition, the rabbit vagina is suitable to be installed in the diffusion cells for the *ex vivo* experiments, keeping the model consistent. Intravaginal uptake of macromolecules in animal models, such as rodents and primates, depends on the stage of the menstrual cycle, thus administration of the vaginal treatment must be appropriately staged [181]. A major advantage of using rabbits is that they are induced ovulators and do not have an estrous cycle, reducing the need for chemically synchronizing the animals within the group.

6. Research Focus

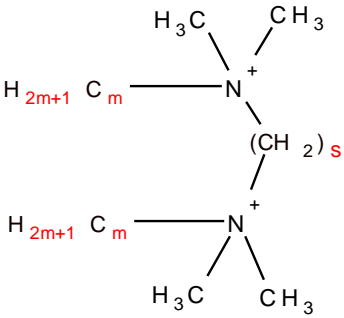
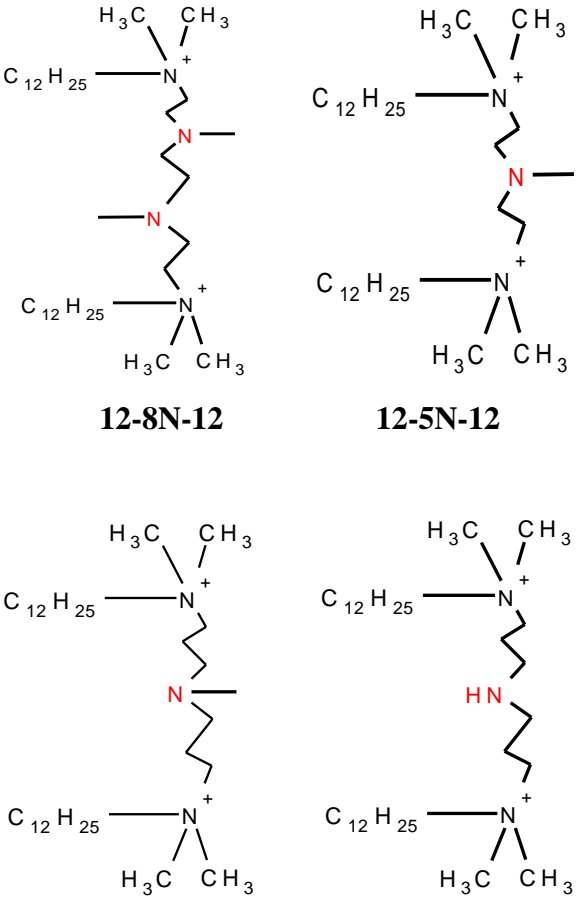
6.1. Rationale for the study

Non-viral vectors offer a non-immunogenic and safe method of gene delivery. However, they have poor transfection efficiency compared to their viral counterparts. Due to this reason, current research efforts are directed towards understanding the various physiochemical parameters that affect the transfection efficiency and using this knowledge to rationally design improved and safer delivery vectors.

Our research group has focused their research efforts in the development of cationic N,N-bis(dimethylalkyl)- α,ω -alkanediammonium gemini surfactants (**Table 6.1.1**) as gene delivery vectors. These are m-s-m type of bisquaternary ammonium gemini surfactants where m and s refer to the number of carbon atoms in the alkyl tails and in the polymethylene spacer groups, respectively. As an example, a compound with two 16 carbon polymethylene tails and two cationic centres (quaternary ammonium) linked through a 3-carbon long polymethylene linker is designated as 16-3-16.

In previous studies, a series of cationic gemini surfactants, with different length of alkyl groups in the tail and spacer region, were synthesized and their gene transfer capability was evaluated (**Table 6.1.1**). The length of both tail and spacer groups had considerable effect on transfection efficiency [9]. A plasmid to gemini charge ratio of 1:10 was optimum for transfection due to high toxicity of lipoplexes at higher gemini concentrations. Transfection efficiency was inversely related to the length of spacer in 12-s-12 series, with the highest transfection efficiency obtained with the 12-3-12 surfactant. An increase in the tail length from 12-carbon to 16-carbon (16-3-16) further increased the transfection efficiency of the cationic diquaternary ammonium gemini surfactants.

Table 6.1: Gemini surfactants designed by our research group.

Gemini surfactants	Rationale & Results
<p>1. First generation [9, 182]</p>  <p>$m = 12, 16 \quad s = 2, 3, 4, 5, 6, 8, 10, 12, 16$</p>	<ul style="list-style-type: none"> • Structure-activity relationship study. • An increase in the length of the spacer groups had considerable effect on transfection efficiency. • Plasmid to gemini charge ratio of 1:10 was optimum for transfection efficiency.
<p>2. Second generation [10]</p>  <p>12-8N-12 12-5N-12</p> <p>12-7N-12 12-7NH-12</p>	<ul style="list-style-type: none"> • Improve the interaction between DNA and gemini surfactants. • The distance between the nitrogen centres was important for interaction. 12-7NH-12 gave the highest transfection efficiency among the second generation gemini surfactants. • Particle size of P/12-7NH-12/DOPE complexes showed pH dependence which may be aiding endosomal escape.

The ability of the DNA/gemini complexes to form polymorphic structures was also an important factor in gene transfection [182]. Second generation gemini derivatives were synthesized including amino groups in the spacer region (**Table 6.1.2**) [10]. The presence of additional amino groups in the spacer improved the transfection efficiency compared to 12-3-12. Among the second generation derivatives, 12-7NH-12 (**Table 6.1.2**) showed the highest transfection [10]. The absence of steric hindrance by the methyl substitution on the nitrogen in the spacer and the optimum spacing between the nitrogen centers lead to favorable binding between the gemini surfactant and adjacent phosphate groups of DNA. Additionally, the particle size of P/12-7NH-12/DOPE complexes showed a pH dependent increase in size which may have contributed to the endosomal escape of the lipoplexes.

Based on the information gathered from these previous experiments, a series of third generation gemini surfactants were developed by introducing different amino acid groups at the N position of the spacer of the previously optimized compound, 12-7NH-12. The rationale for amino-acid substitution was rooted in the understanding that these are bio-compatible and non-toxic moieties and would not increase the cytotoxicity of gemini surfactants. We hypothesized that glycine, the simplest of all the amino acid, is needed for conformational flexibility for DNA binding and lysine, employed as polymers known to exhibit excellent DNA binding and condensing properties could modulate gene transfection efficiency of the gemini surfactants [74, 183]. Additionally, the amino acid substitution could increase interaction with DNA via hydrogen bonding and presence of ionizable amino groups. The general chemical formula for this new generation of amino acid/peptide-substituted gemini surfactants is represented by **Figure 6.1** where R represents glycine (G), lysine (K), glycyl-lysine (GK), lysyl-lysine (KK).

The purpose of my dissertation research was to develop these new amino acid-substituted gemini surfactants as gene delivery vectors and correlate their physiochemical properties with their transfection efficiency. Finally, vaginal administration was evaluated as a non-invasive route of delivery for these delivery systems. The key challenges were to generate high levels of gene expression *in vitro* and *in vivo*. Rabbit epithelial cells (ATCC CCL-68) and pGT.IFN.GFP (encoding green fluorescent protein and interferon- γ), a model reporter plasmid, were used as the *in vitro* model and New Zealand White rabbit and pGT.tdTomato (encoding red fluorescent protein), a reporter plasmid, were used as the *in vivo* model.

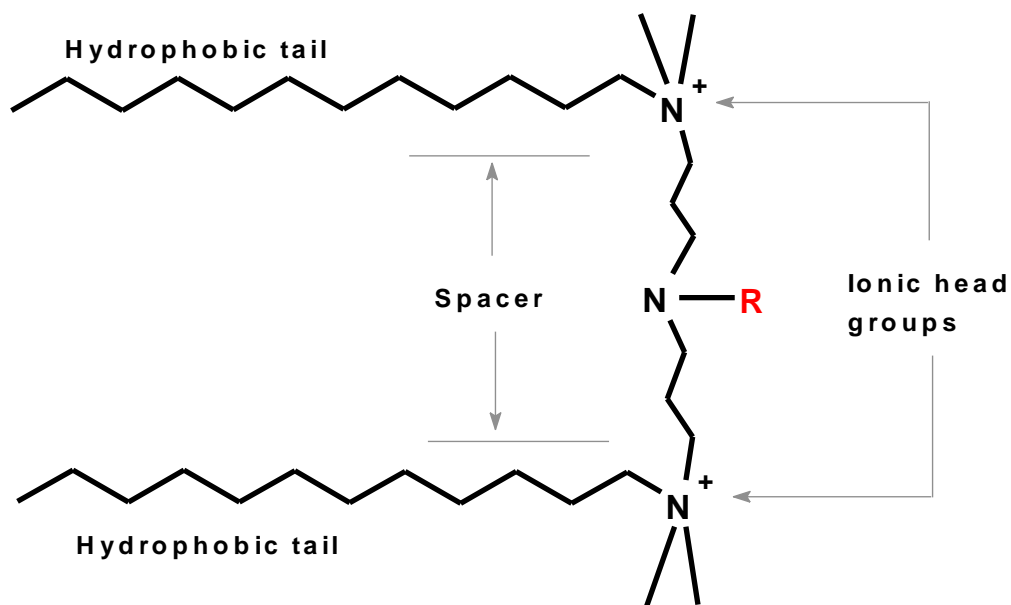


Figure 6.1: General structure of a new family of gemini surfactants. R represents glycine (G), lysine (K), glycyl-lysine (GK), or lysyl-lysine (KK) for amino acid/peptide substitution or hydrogen (H) for unsubstituted parent compound.

6.2. Hypothesis

- a) The amino acid (glycine, lysine, glycyl-lysine, lysyl-lysine) substitution in the gemini surfactants will improve its DNA binding properties without increasing toxicity and will generate a high level of gene expression in epithelial cell culture.
- b) Non-invasive vaginal application of amino acid-substituted gemini surfactant-based DNA delivery systems will generate a high level of gene expression in an *in vivo* animal model.

6.3. Objective

Overall Objectives

- a) To formulate novel amino acid-substituted gemini surfactant based gene delivery systems and evaluate their efficiency *in vitro* and *in vivo*.
- b) To characterize these novel formulations and correlate the physicochemical characteristics with gene transfection efficiency.

Specific Objectives

In vitro evaluation

- a) To formulate novel gene delivery systems with amino acid/peptide-substituted cationic gemini surfactants.
- b) To transfect *in vitro* cottontail rabbit epithelial cells and evaluate the extent and duration of gene expression.
- c) To establish correlation between physicochemical characteristics and transfection efficiency of these formulations.
- d) To evaluate the cellular uptake mechanism of plasmid/gemini/lipid particles and its effect on gene expression.

Ex vivo evaluation

- a) To assess the extent of penetration of the DNA into the vaginal tissue using the amino acid-substituted gemini surfactant that showed the best performance *in vitro*.

In vivo evaluation

- a) To develop new mucosal delivery systems using the amino acid-substituted gemini surfactant with the best performance from *in vitro* evaluation.
- b) To evaluate gene expression in vaginal tissue after non-invasive vaginal application in rabbit animal model.

7. Enhanced gene expression in epithelial cells transfected with amino-acid substituted gemini nanoparticles

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This published manuscript describes in detail the synthesis, characterization and formulation development of amino acid-substituted gemini surfactants. I was responsible for formulation development of amino acid-substituted gemini surfactants as gene delivery systems and the evaluation of their transfection efficiency in cell culture. I also determined key physicochemical properties and correlated with their transfection efficiency. The contents that relate to my contribution in this study are presented here.

This manuscript addresses the first three specific objectives of my research

- To formulate novel gene delivery systems with amino acid/peptide-substituted cationic gemini surfactants
- To transfect *in vitro* cottontail rabbit epithelial cells and evaluate the extent and duration of gene expression.
- To establish correlation between physicochemical characteristics and transfection efficiency of these formulations

7.1. Abstract

Gemini surfactants are versatile gene delivery agents because of their ability to bind and compact DNA and their low cellular toxicity. Through modification of the alkyl tail length and the chemical nature of the spacer, new compounds can be generated with the potential to improve the efficiency of gene delivery. Amino acid (glycine and lysine) and dipeptide (glycyl-lysine and lysyl-lysine) substituted spacers of gemini surfactants were synthesized and their efficiency of gene delivery was assessed in epithelial cells for topical cutaneous and mucosal applications.

Epithelial cell line Sf 1 Ep cells were transfected with plasmid DNA encoding for interferon gamma and green fluorescent protein complexed with the amino acid-substituted gemini compounds in the presence of 1,2 dioleoyl-sn-glycero-phosphatidyl-ethanolamine as a helper lipid. Gene expression was quantified by ELISA. Size, zeta potential and circular dichroism measurements were used to characterize the plasmid-gemini (PG) and plasmid-gemini surfactant-helper lipid (PGL) complexes.

Gene expression was found to increase up to 72 h and then declined by the 7th day. In general, the glycine-substituted surfactant showed consistently high gene expression in all three cell lines. Results of physicochemical and spectroscopic studies of the complexes indicate that substitution of the gemini spacer does not interfere with compaction of the DNA.

The superior performance of these spacer-substituted gemini surfactants might be attributed to their better biocompatibility compared to the surfactants possessing unsubstituted spacers.

7.2. Introduction

Topical gene delivery is an important approach in the quest to find treatment or cure for a multitude of genetic and acquired disorders, such as scleroderma, epidermolysis bullosa, cystic fibrosis, wound healing and others. To correct the imbalances created by a missing or incorrect gene, efficient non-invasive gene delivery systems should be employed. Interferon gamma (IFN- γ) is a secreted cytokine with immunomodulatory, antiviral, antifibrotic and antitumour activity. Based on these properties its therapeutic potential in conditions such as scleroderma [184], atopic dermatitis [185], and post-operative fibrosis [186] have been discussed and delivery of IFN- γ gene into epidermal skin and mucosal epithelial tissue can be considered as a treatment for these diseases. Gemini surfactants, N,N-bis(dimethylalkyl)- α,ω -alkanediammonium dibromide derivatives (**Figure 7.1**) have been shown to be versatile vectors for non-viral gene delivery [187][9, 10, 155, 156]. This stems from the fact that there are many possibilities, through chemical modification of the alkyl tails and the spacer group of these compounds, to modulate their physicochemical behaviour in a way that can enhance the efficiency of gene transfer. For example, modification of the length, degree of unsaturation, and substitution of different functional groups in the spacer and alkyl tails can provide opportunities to tailor their chemical structure for specific needs [158, 188].

Cationic gemini surfactants, are capable of binding DNA [189] and have several advantages compared to classic monovalent surfactants: lower cellular toxicity [155], lower critical micelle concentration (cmc), generally one or two orders of magnitude higher efficiency in reducing surface tension, and greater tendency to self-assemble [190]. The higher-order assembly of the gemini surfactants depends on the shape of the molecule (i.e., nature of the tail and spacer) and ranges from micellar to inverted micellar or bilayer structures. In the presence of the DNA, these supramolecular particles become more complex, resulting in polymorphic phase behaviour with the appearance of inverted micellar and cubic structures [182]. To be efficient transfection agents they have to fulfill several criteria: ability to bind DNA and create complexes of a certain size and morphology, the vector-plasmid complexes must retain an overall positive surface charge so as to facilitate interaction with the negative cell surface and enhance cellular uptake (in the absence of specific targeting), induce changes in the DNA structure favorable for efficient delivery, protect the genetic material against intracellular degradation, have the ability

to undergo polymorphic phase changes, and undergo facile release of the plasmid once inside the cell to effect protein expression.

Gemini surfactants with C3-C12 alkyl spacers are able to compact the DNA into 100-200 nm particles with positive surface charge [9], which enables interaction with the cell surface and endocytosis. The transfection efficiency of these agents depends on the spacer length, the 3-carbon spacer (12-3-12) being the most efficient. CD spectra of the plasmid-gemini surfactant complexes have previously been discussed in terms of the formation of highly compact Ψ -DNA [9]. Addition of a helper lipid, 1,2 dioleoyl-*sn*-glycero-phosphatidylethanolamine (DOPE), induces polymorphic phase behaviour [182] which correlates with increased transfection efficiency [9]. Cellular toxicity of the transfection complexes was significantly lower compared to the commercial Lipofectamine Plus [9]. Substitution of the alkyl spacer with pH sensitive amino groups increases the transfection efficiency of the gemini surfactants [10]. The 1,9-bis(dodecyl)-1,1,9,9-tetramethyl-5-amino-1,9-nonanediammonium dibromide surfactant (12-7NH-12) transfected cells at a 9-fold higher level compared to the alkyl spacer derivatives. This improvement was attributed to its pH sensitivity and ability to form multiple phases, which enable membrane fusion and release of the DNA in the cells [10].

We report here the results of a study focusing on the modification of this derivative by chemically coupling amino acid moieties to the amino-spacer so as to further increase and prolong gene expression. It has been shown that small molecular weight peptides, such as arginine-rich protamine fragments are able to bind and condense DNA into 100 nm particles. Transfection of cells resulted in prolonged gene expression for up to 12 days [191]. We hypothesized that an additive effect could be achieved by coupling the highly efficient amino-substituted spacer of a gemini surfactant with amino acid/dipeptide residues.

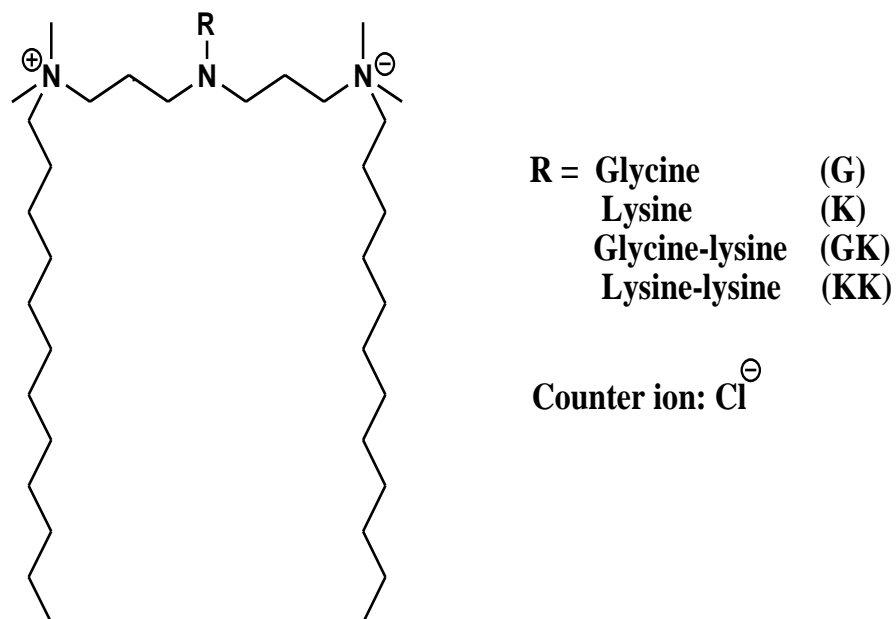


Figure 7.1: General structure of gemini surfactants. The two hydrophobic alkyl chains (tail) with charged head groups (ion) are connected with a carbon spacer [187].

7.3. Materials and methods

7.3.1. Preparation of the gene delivery systems

The pGT.IFN-GFP plasmid was used for the transfection experiments. The murine interferon-gamma (IFN- γ) and green fluorescent protein (GFP) genes were inserted into the pGT backbone as a bicistronic system[9]. Transfection agents of plasmid/gemini surfactant (PG) complexes and plasmid/gemini surfactant/helper lipid (PGL) particles were prepared as previously described [9]. The plasmid:gemini surfactant charge ratio was 1:10. DOPE was used as a helper lipid.

7.3.2. Transfection of Sf 1 Ep cells using amino acid/dipeptide-substituted gemini surfactants and DOPE

Sf 1 Ep cottontail rabbit epithelial cells (ATCC, CCL-68) were seeded on Falcon 96-well tissue culture plates (BD, Mississauga, ON) at a density of 2×10^5 cells/well. Three plates were seeded with each cell line at the same passage number after initiation of the cultures. Transfection experiments were carried out using 100ng plasmid DNA/well. Supernatants were collected every 24 h and replaced with fresh cell culture medium. The results are the average of three plates of triplicate wells.

7.3.3. ELISA

ELISA was used to detect IFN- γ secreted into the supernatant and was performed according to the BD Pharmingen protocol (Cat # 558861). Protein concentration was calculated from a standard curve using recombinant IFN- γ .

7.3.4. Fluorescence microscopy

Fluorescent images of transfected cells were registered by using an Olympus IX71 inverted microscope (Olympus Corporation, Japan). The excitation and emission wavelengths for GFP were 488 and 507 nm (built-in FITC filter), respectively. Light and fluorescent micrographs were merged to create composite images by using Image-Pro 5.1 software.

7.3.5. Size and zeta potential measurements

Size and zeta potential measurements of PG and PGL complexes, prepared at 1:10 plasmid:gemini surfactant charge ratio, the same as for the transfection experiments, were carried out by using a Zetasizer Nano ZS instrument (Malvern Instruments, Worcestershire, UK). The results reported are the average of triplicate measurements on a % volume basis. Size measurements of gemini compounds at two concentrations above the cmc were also carried out on % volume basis.

7.3.6. Circular dichroism

PG and PGL complexes were prepared at the same charge and molar ratio as for transfection experiments, using 20 μ g/mL plasmid DNA. Spectra were recorded using a Π *180 instrument (Applied Photo Physics, UK), with 2-nm slit under a N₂ atmosphere.

7.3.7. Statistics

Results are expressed as the mean of $n \geq 3 \pm$ standard deviation. One way analysis of variance (ANOVA) and Bonferroni post hoc tests were used for statistical analyses (SPSS version 16.0). Significant differences were considered at $p < 0.05$ level.

7.4. Results

7.4.1. Transfection of cells with amino acid/peptide-substituted gemini surfactants

The study revealed a time-dependent increase in the gene expression for all the amino acid/peptide-substituted cationic gemini surfactants (**Figure 7.2 & 7.3**). At 72 hours, the amino acid/peptide-substituted gemini compounds showed higher gene expression than the unmodified derivative and commercial Lipofectamine. There was little or no protein detected after 1 week (results not shown). Glycine (12-7NG-12) and glycyl-lysine (12-7NGK-12) substituted gemini surfactants exhibited highest transfection efficiency with IFN- γ levels (per 2×10^5 cells) of 495 ± 283 pg and 555 ± 266 pg, respectively. This was significantly higher than lysine and lysyl-lysine substituted, and unsubstituted parent compounds ($p < 0.001$).

7.4.2. Size and zeta potential measurements

P/G/L complexes were prepared in a similar manner to those used for the transfection experiments. Particle sizes for all complexes were in the 100-150 nm range (**Table 7.1**). The plasmid/DOPE complexes showed smaller particle size, slightly less than 100 nm. The zeta potential of -32.7 ± 0.6 mV of the plasmid/DOPE complexes shifted to $+20-40$ mV.

7.4.3. Circular Dichroism

Circular dichroism was used to evaluate compaction of the DNA by the substituted gemini surfactants. P/G and P/G/L complexes were prepared in a similar manner to those for the transfection experiments. The plasmid DNA alone showed two positive peaks at 295 and 255 nm, while all P/G complexes had a blue shift of the 295nm peak and depression of the 255-nm peak (**Figure 7.4A**). The P/12-7NG-12/L particles show a flattening of the 295-nm peak and the other compounds, including the parent 12-7NH-12 surfactant, show a slight depression (**Figure 7.4B**). The pattern of the plasmid-DOPE mixture had a positive peak at 275 nm.

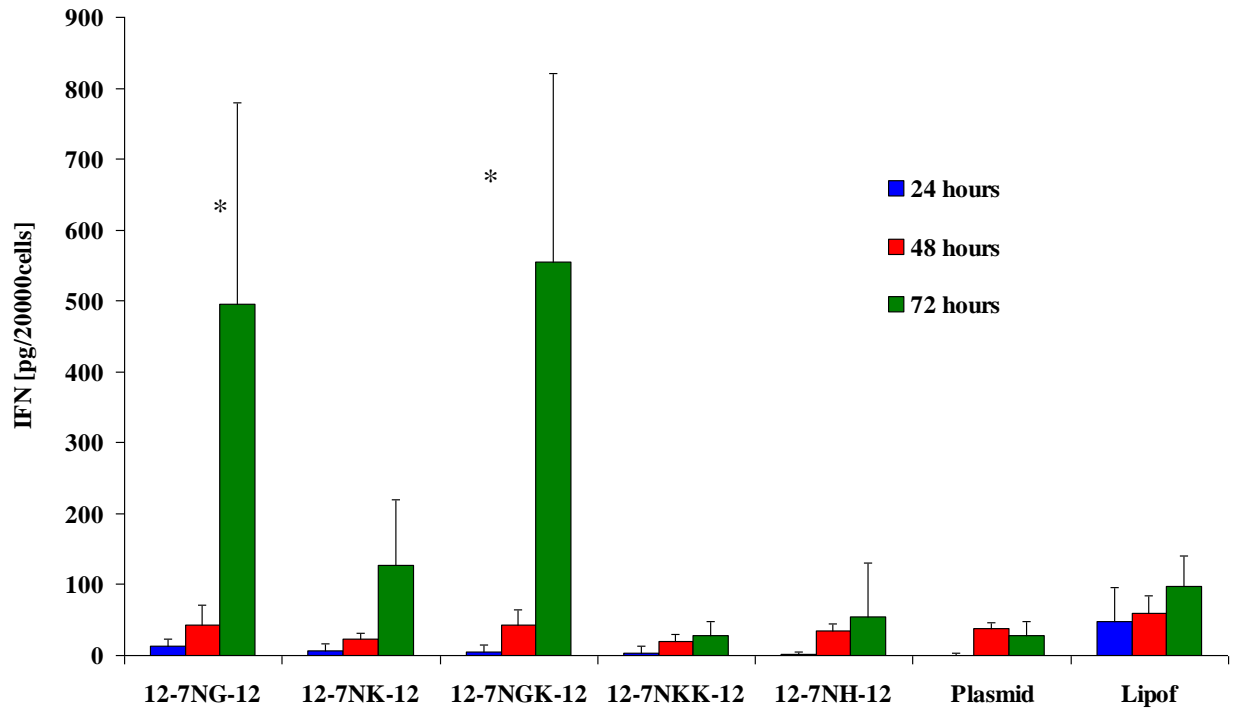


Figure 7.2: Gene expression of plasmid/gemini/lipid complexes in Sf 1 Ep cells. Amino acid-substituted gemini surfactants were formulated with a model plasmid and were evaluated in rabbit epithelial cells in the presence of a helper lipid (DOPE). The secreted IFN γ was measured using ELISA at 24h, 48h and 72h. At 72h, glycine and glycyl-lysine substituted gemini surfactants showed significantly higher gene expression than the unmodified compound. *n=9, p<0.05, one-way ANOVA

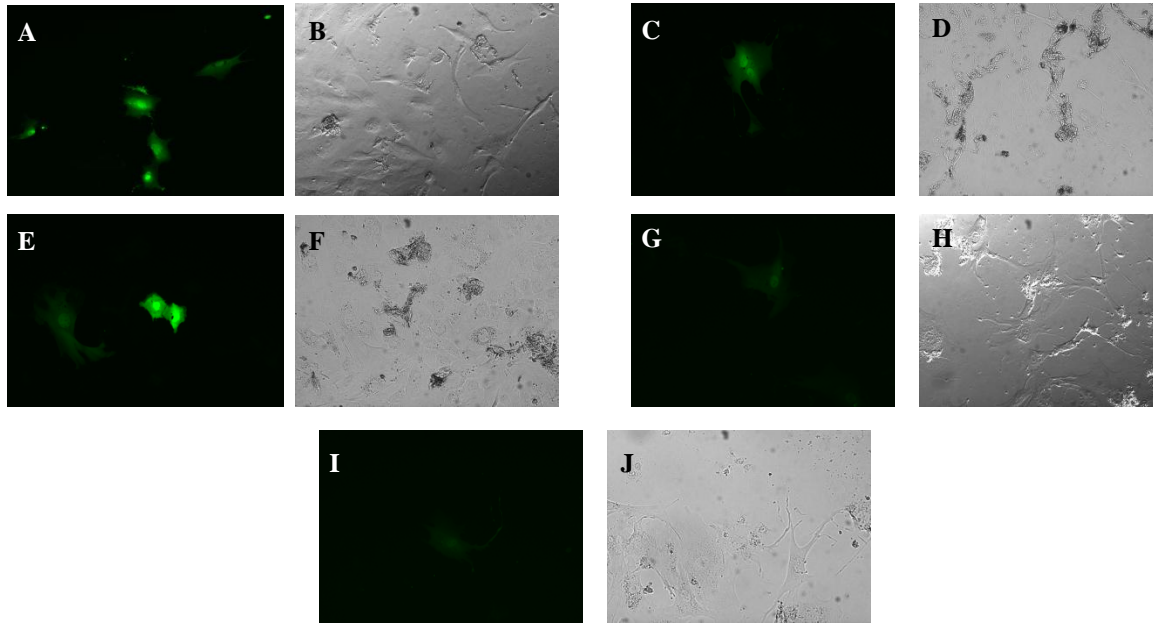


Figure 7.3: Fluorescence microscopic evaluation of GFP expression in cotton tail rabbit epithelial cell lines transfected with 0.2 μg pGT.IFN-GFP plasmid using glycine substituted (A, B), lysine substituted (C, D), glycyL-lysine substituted (E, F), and lysyl-lysine substituted (G, H) and unmodified (I, J) 12 carbon tail gemini surfactants.

Table 7.1: Size and zeta potential measurements. Values are average of four measurements \pm STD. Polydispersity (PDI) index is indicated for the size measurements as average of four measurements \pm STD in brackets.

PGL	Size [nm] (PDI)	Zeta potential [mV]
P/12-7NG-12/DOPE	116.3 \pm 1.3 (0.252 \pm 0.008)	32.6 \pm 1.8
P/12-7NK-12/DOPE	112.7 \pm 1.7 (0.265 \pm 0.006)	28.6 \pm 3.6
P/12-7NGK-12/DOPE	117.3 \pm 0.6 (0.232 \pm 0.012)	34.5 \pm 5.6
P/12-7NKK-12/DOPE	105.3 \pm 0.4 (0.245 \pm 0.008)	31.3 \pm 4.0
P/12-7NH-12/DOPE	133 \pm 2.8 (0.279 \pm 0.006)	24.9 \pm 3.4
P/DOPE	98.2 \pm 0.3 (0.224 \pm 0.008)	-32.7 \pm 0.6

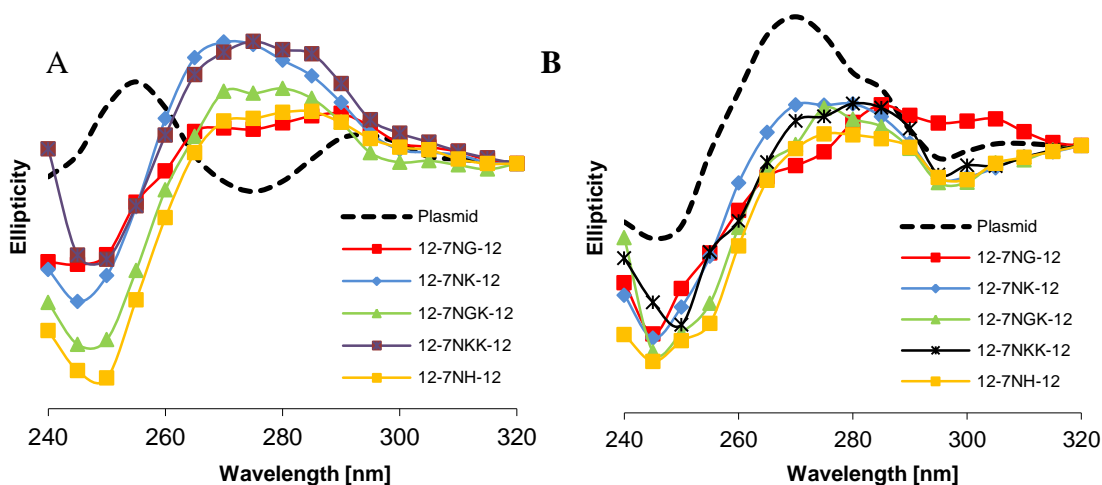


Figure 7.4: CD spectra of the plasmid/gemini surfactants (P/G) complexes (A) and the plasmid/gemini surfactants/co-lipid (P/G/L) particles (B). Values are average of three measurements.

7.5. Discussion

7.5.1. *In vitro* transfection efficiency of the amino acid/peptide-substituted gemini compounds

The *in vitro* transfection efficiency of novel amino acid/peptide-substituted gemini surfactants was evaluated in rabbit epithelial cells. These epithelial cell lines were used primarily to evaluate the performance of these compounds with potential application in development of vaginal gene delivery systems. Gemini surfactants self-assemble into higher order structures and their aggregation depends on the length and chemical nature of the spacer [192] that also determines the fit between the positively charged nitrogens of the head group and the negatively charged phosphate backbone of the DNA [193]. Previously, the transfection efficiency of the alkyl-spacer gemini surfactants was found to vary inversely with their head group area determined from Gibbs interfacial energy [9]. Nitrogen substitution in the spacer introduced a better fit with the DNA and conferred pH sensitivity to the gemini surfactants [10], which could reduce cytotoxicity and facilitate endosomal escape of the DNA. The amino acid/peptide-substitution in the spacer region of 12-7NH-12 gemini surfactant showed higher gene expression compared to the unsubstituted parent compound (12-7NH-12). Cationic agents non-specifically bind the cell surface through ionic interactions with membrane-associated proteoglycans [106]. Transgene expression depends not only on compaction by the cationic agent and delivery of the genetic material into the cells but also on endosomal escape and release of the DNA from the complexes.

Grafting of amino acids or peptides on the gemini surfactant molecule could improve the delivery and release of the DNA in the cytoplasm. It should be noted that a weak binding in the P/G complex could not protect and compact sufficiently, whereas strong binding would not facilitate the release of the DNA. While gemini surfactants bind the DNA via electrostatic interactions, the nature of binding by the amino acid/peptide-substituted gemini surfactants might be 'softened' by van der Waals and hydrogen bonding forces[194], thus increasing the flexibility and plasticity of the supramolecular structures [195]. Increased hydrophilicity of the amino acid/peptide-substituted gemini surfactants compared to the parent compound might translate into better biocompatibility.

The increased biocompatibility of these peptidomimetic particles could lead to lower cytotoxicity. The gene expression pattern showed similarities with higher gene expression in the cells transfected with glycine- and glycy-l-lysine substituted gemini surfactants. Complex sizes of 100-150 nm are optimal for endocytotic internalization by the cells [115]. Zeta potential of the particles was approximately +30mV for all compounds, which indicated that the system was colloiddally stable [196]. There was no significant difference between the zeta potential of the unsubstituted 12-7NH-12 and amino acid/peptide-substituted gemini surfactants, indicating that substitution did not shield the surface charge on the particles. The CD spectra obtained were consistent with those previously reported for gemini surfactants [8, 10, 182]. Such changes in the native DNA structure were interpreted in terms of the surfactants inducing formation of Ψ DNA [197], which usually required high salt concentrations. The changes observed for the gemini surfactants are more consistent with a B-DNA conformation in which both the helicity and base stacking are perturbed as a result of compaction of the DNA molecule by the surfactant [198]. The similarity between the profiles obtained for the amino acid/peptide-substituted versus unsubstituted gemini surfactants indicates that the substitution on the spacer does not interfere with the ability of the gemini surfactant to compact DNA.

7.6. Conclusion

Amino acid- and peptide- substituted amino spacer of gemini surfactants transfect epithelial cells at higher efficiency compared to unsubstituted spacers. The substitution does not increase the size of the particles or reduce zeta potential, nor weaken DNA compaction. The higher transfection efficiency is attributed to the higher biocompatibility and flexibility of the amino acid/peptide-substituted gemini surfactants and demonstrates the feasibility of using amino acid substituted gemini surfactants as gene carriers for the treatment of diseases affecting epithelial tissue.

7.7. Acknowledgements

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8. Amino Acid-Substituted Gemini Surfactant-Based Nanoparticles as Safe and Versatile Gene Delivery Agents

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This published manuscript provides information on the key physicochemical properties and cytotoxicity of amino acid-substituted gemini surfactants as gene delivery systems. A detailed analysis of these properties and their correlation to the toxicity and transfection efficiency of these molecules is discussed.

Based on the conclusions in this study, the glycyl-lysine-substituted gemini surfactant was selected as optimal vector for *in vivo* formulation development. I conducted all experiments and prepared the manuscript. The article is presented here in its entirety with minor modifications.

This manuscript relates, more specifically, to the third specific objective of my research:

- To establish correlation between physicochemical characteristics and transfection efficiency of these formulations.

8.1. Abstract

Gene based therapy represents an important advance in the treatment of diseases that heretofore have had either no treatment or cure. To capitalize on the true potential of gene therapy, there is a need to develop better delivery systems that can protect these therapeutic biomolecules and deliver them safely to the target sites.

Recently, we have designed and developed a series of novel amino acid-substituted gemini surfactants with the general chemical formula $C_{12}H_{25}(CH_3)_2N^+-(CH_2)_3-N(AA)-(CH_2)_3-N^+(CH_3)_2C_{12}H_{25}$ (AA= glycine, lysine, glycyl-lysine and, lysyl-lysine). These compounds were synthesized and tested in rabbit epithelial cells using a model plasmid and a helper lipid. Plasmid/gemini/lipid (P/G/L) nanoparticles formulated using these novel compounds achieved higher gene expression than the nanoparticles containing the parent unsubstituted compound.

In this study, we evaluated the cytotoxicity of P/G/L nanoparticles and explored the relationship between transfection efficiency/toxicity and their physicochemical characteristics (such as size, binding properties, etc.). An overall low toxicity is observed for all complexes with no significant difference among substituted and unsubstituted compounds. An interesting result revealed by the dye exclusion assay suggests a more balanced protection of the DNA by the glycine and glycyl-lysine substituted compounds. Thus, the higher transfection efficiency is attributed to the greater biocompatibility and flexibility of the amino acid/peptide-substituted gemini surfactants and demonstrates the feasibility of using amino acid-substituted gemini surfactants as gene carriers for the treatment of diseases affecting epithelial tissue.

KEYWORDS

Amino acid-substituted, cytotoxicity, gemini surfactants, gene delivery, non-viral, physicochemical properties

8.2. Introduction

Gene based therapy holds a promising future with the possibility of improving current levels of medical treatment of incurable diseases. To capitalize on the true potential of gene therapy, there is a need to develop better delivery systems that can protect these therapeutic biomolecules and deliver them safely to the target sites. Currently, there are two methods of gene delivery: viral and non-viral. The viral method capitalizes on the viruses' natural ability to infect cells to efficiently introduce the target gene into the cells; these viruses are engineered to minimize virulence (disease causing ability) [5]. While viral vectors are currently the most effective means of gene delivery, they run the risk of triggering severe immune responses [38, 39], of reverting into replication competent virulent viruses and of potential insertional mutagenesis [40, 41]. Severe adverse effects causing death in clinical trials using viral gene delivery vectors has raised many doubts about its future applications [42, 43]. Viral systems also suffer from the limitation of gene carrying capacity [44] and require lengthy and difficult production methods [45]. On the other hand, non-viral vectors provide an easy to produce, non-immunogenic and safe method of gene delivery. However, the major disadvantage of non-viral vectors is their low transfection efficiency. These factors have led scientists to focus on the development of novel non-viral vectors with better transfection efficiency.

Cationic gemini surfactants are one such class of non-viral delivery vectors that can self-assemble in the presence of DNA into cationic DNA nanoparticles and aid in gene delivery. The gemini compounds have several advantages compared to the classic monovalent surfactants: lower cellular toxicity, lower critical micelle concentration, higher efficiency in reducing surface tension and greater tendency to self-assemble. The opportunity to modify their structure creates flexibility in designing suitable delivery systems for enhanced gene transfer into mammalian cells [154].

Our research group has focused on the optimization of cationic N,N-bis(dimethylalkyl)- α,ω -alkanediammonium gemini surfactants or m-s-m, where m and s refer to the number of carbon atoms in the alkyl tails and in the polymethylene spacer groups, respectively. Initially, we designed a series of cationic gemini surfactants with different length of alkyl groups in the tail and spacer region and evaluated their effect on gene transfer capability. The length of both tail and spacer groups were found to have considerable effect on transfection efficiency [9]. The ability of the DNA/gemini complexes to form polymorphic structures was also found to be an

important factor in gene transfection [182]. Based on this information, a second generation of gemini derivatives was synthesized having 12-carbon tails and 5 to 8 carbon spacers. The latter was modified to include amino groups in an effort to improve the DNA condensation properties of these agents [10]. Among the second generation derivatives, 12-7NH-12 (**Figure 8.1**) showed the highest transfection with improved DNA binding properties [10]. More recently, a series of third generation gemini surfactants has been developed by introducing different amino acid groups at the N position of the spacer of this previously optimized compound (12-7NH-12) [199]. The general chemical formula for this new generation of amino acid-substituted gemini surfactants is represented by (**Figure 8.1**) where, R represents glycine (G), lysine (K), glycyl-lysine (GK), lysyl-lysine (KK). In a previous study, these new derivatives achieved higher gene expression than the parent unsubstituted compound [199].

In this study, we evaluated the cytotoxicity of the gemini-DNA nanoparticles and assessed which physicochemical parameters of these novel delivery systems most affect transfection efficiency. The objective is to develop an understanding of the effect of amino acid-substitution on critical properties [106-108, 111, 112] of these novel delivery systems which will aid in the rational design of new derivatives (using new amino acid/peptide substitution) with improved transfection efficiency and safety profile.

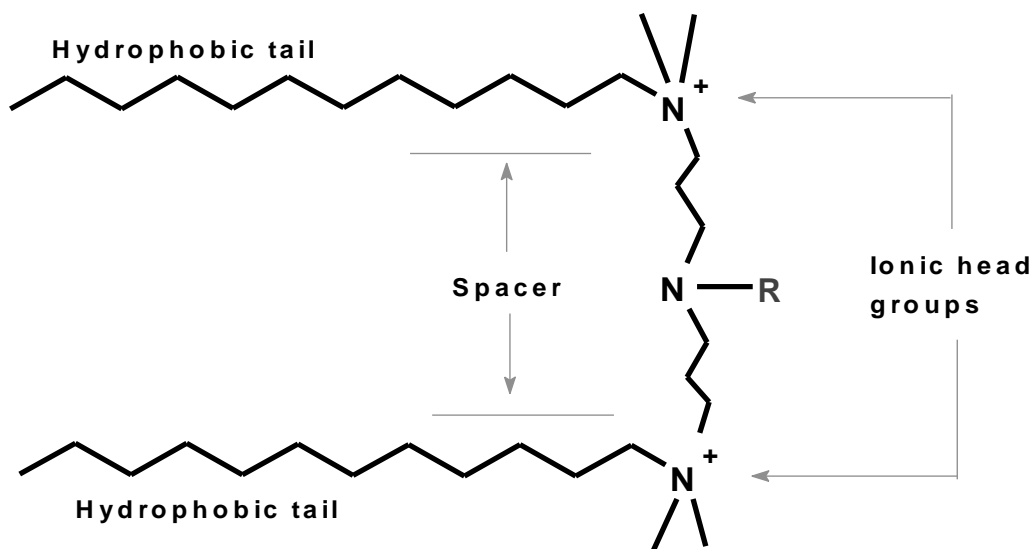


Figure 8.1: General Structure of a new family of gemini surfactants where R represents glycine (G), lysine (K), glycyl-lysine (GK) and lysyl-lysine (KK) for amino acid substitution or hydrogen (H) for unsubstituted parent compound.

8.3. Materials and Methods

8.3.1. Cell culture and propagation

Cotton tail rabbit epithelial cells, Sf 1 Ep (CCL-68, ATCC, VA, U.S.A.) were grown in Minimum Essential Medium (MEM, GIBCO, N.Y., U.S.A.) supplemented with 10% FBS and antibiotic/antimycotic agents, respectively. All cells were grown to 70-80% confluence and trypsinized to prepare a cell suspension. The cells were plated at a cell concentration of 1.5×10^4 cells per well in 96-well and 3×10^5 cells per well in 6-well tissue culture plates (Falcon BD, ON, Canada), respectively. The cells were incubated for 24 hours at $37^\circ\text{C}/5\% \text{CO}_2$ prior to transfection.

8.3.2. Formulations

Amino acid-substituted gemini surfactants were formulated with a model plasmid, pGTmCMV.IFN-GFP in the presence of a helper lipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE ; Avanti Polar Lipids, AL, U.S.A.) creating plasmid/gemini surfactant/lipid (P/G/L) nanoparticles. DNA/Gemini surfactant complexes were prepared at a charge ratio of 1:10 and incorporated in DOPE at a final concentration of 1mM (as used for transfection [199]). Transfection was performed using 100 ng DNA per well for the 96-well plates and $1\mu\text{g}$ DNA per well for the 6-well plates. Lipofectamine PlusTM (Invitrogen, CA, U.S.A.) was used as a control and formulations were prepared as per manufacturer's recommendation.

8.3.3. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

assay

This assay was used to evaluate cell toxicity of gemini derivatives in Sf 1 Ep cells. P/G/L nanoparticles prepared as described in Formulations section for 96-well plates. A sterile 5 mg/mL solution of MTT (Invitrogen, OR, U.S.A.) in phosphate buffered saline (PBS) was prepared. Gemini surfactants were incubated (in triplicate) with the epithelial cells for 5 hours before replacing with a fresh media. A $10\mu\text{L}$ aliquot of 5 mg/mL MTT solution was added in each well and incubated at $37^\circ\text{C}/5\% \text{CO}_2$ for 4 hours. Supernatants were removed and the cells were washed with PBS. The purple formazan formed was dissolved in dimethyl sulfoxide

(Sigma, MO, U.S.A.) on a plate shaker for 10 minutes. The plates were incubated for 10 minutes at 37 °C to eliminate any air bubbles. Absorbance was measured at 550 nm using a plate reader (Biotek Microplate Synergy HT, VT, U.S.A.).

8.3.4. Fluorescence activated cell sorting (FACS)

FACS (BD Biosciences, CA, U.S.A.) was used to evaluate cell toxicity of P/G/L nanoparticles in Sf 1 Ep cells. P/G/L nanoparticles were prepared as described in Formulations section for 6-well plates. Nanoparticles were incubated for 5 hours at 37 °C/5% CO₂ with epithelial cells plated in 6-well plate (two sets of plates in triplicate). After 5 hours, for one set of plates the media was removed and cells were washed twice with 0.02% EDTA in PBS. The cells were detached with 0.02% trypsin and suspended in 2% FBS in PBS for analysis. The cell sorter was calibrated with non-transfected cells and 1x10⁴ cells of each sample were counted. For the second set of plates, the media was removed at 5 hours and replaced with fresh media. This set was incubated for 72 hours before carrying out FACS at 37 °C/5% CO₂ with media replacement every 24 hours. A similar method was used to detach cells incubated for 72 hours before the FACS study. Again, the cell sorter was calibrated with non-transfected cells and 1x10⁴ cells of each sample were counted.

8.3.5. Dye binding assay

A 200 µg/mL concentration of plasmid (pGTmCMV.IFN-GFP) was complexed with the gemini surfactants at a +/- charge ratio of 10 in the presence or absence of DOPE as described earlier on 96-well plates, with samples prepared in triplicate. Ethidium bromide (Sigma) was added to the samples at 1 µg/mL final concentration. The samples were incubated for 10 minutes at room temperature. Fluorescence excitation was carried out at 530 nm and emission measured at 590 nm using a plate reader (Biotek Microplate Synergy HT, VT, U.S.A.). The relative fluorescence of the P/G/L and P/G complexes was expressed as a percentage of fluorescence of a 200 µg/mL plasmid solution. The fluorescence of the P/G/L and P/G complexes were compared to its parent compound.

8.3.6. Statistical analyses

Results are expressed as the mean of $n \geq 3 \pm$ standard deviation. One way analysis of variance (ANOVA), Pearson's correlation coefficient and Scheffé/Dunnett's post hoc tests, were used for statistical analyses (SPSS version 17.0). Significant differences were considered at $p < 0.05$ level.

8.4. Results

8.4.1. Cytotoxicity study

An MTT assay was carried out after incubating the cells for 5 hours with gemini derivatives alone while a FACS study was designed to assess cell toxicity of P/G/L nanoparticles at 5 hours (length of incubation of the cells with the transfection agents) and 72 hours (highest expression of the green fluorescent protein was observed among gemini derivatives at this time [199]). Lipofectamine, a commercial transfection agent, was evaluated for comparison. The results indicated, in the absence of plasmid or helper lipid that all derivatives, including the parent unsubstituted gemini surfactant, exhibited high cytotoxicity with cell viability of 10-20% (**Figure 8.2.A**). When the gemini surfactants were incorporated into nanoparticles, cell viability increased significantly.

After 5 hours of incubation, the P/G/L nanoparticles containing glycine and lysyl-lysine substituted gemini surfactants had cell viability of $93.9\% \pm 1.9$ and $93.1\% \pm 2.4$, respectively, whereas it was greater than 99% for all other derivatives including parent 12-7NH-12 compound (**Figure 8.2.B**). There was no significant difference in cytotoxicity among these novel delivery systems (one-way ANOVA, $p < 0.05$). Conversely, cell viability of the complexes formulated using the commercial agent was $82\% \pm 1.7$ and it was significantly lower than all the formulations containing gemini derivatives ($p < 0.05$, one-way ANOVA).

At 72 hours, cell viability of P/G/L nanoparticles containing the parent gemini surfactant was $87.9\% \pm 6.5$ while it was greater than 90% for all amino acid-substituted derivatives (**Figure 8.3**). Cell viability of the formulation containing commercial agent was drastically reduced to about $55.8\% \pm 4.4$ which was significantly lower than all the P/G/L formulations (one-way ANOVA, $p < 0.05$). A similar trend was observed for both transfected (GFP positive) and non-transfected (GFP negative) cells.

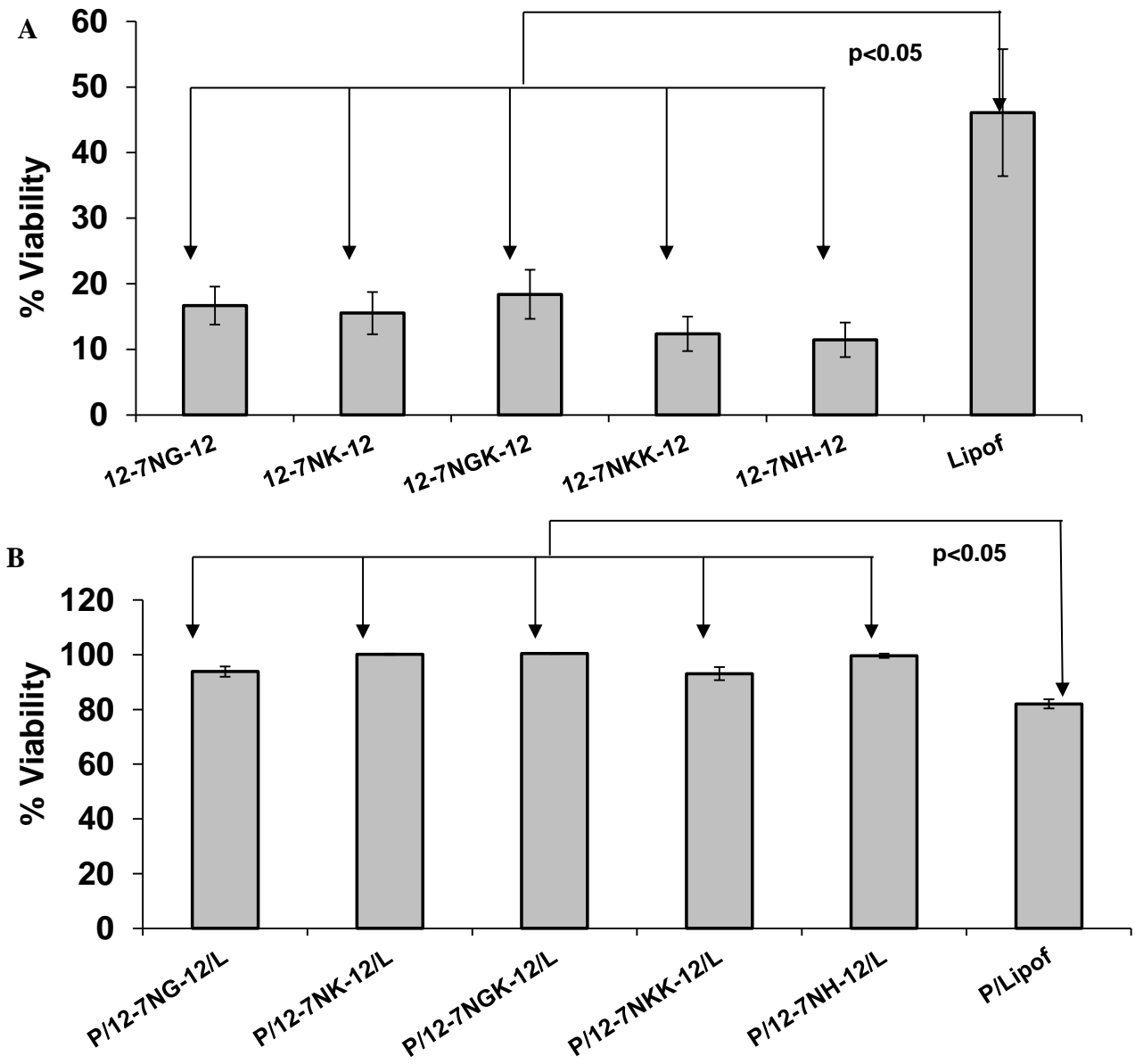


Figure 8.2: Evaluation of cell viability of (A) amino acid-substituted gemini surfactants using MTT assay and (B) P/G/L complexes using FACS, after incubation with the cells for 5 hours (equal to the length of incubation of the cells with transfection agents). While the gemini surfactants exhibited high cytotoxicity with cell viability as low as 10-20%, these values were significantly improved to greater than 93 % after formulation as P/G/L nanoparticles. Cell viability of P/G/L nanoparticles was significantly higher than the commercial Lipofectamine. (p<0.05, one-way ANOVA)

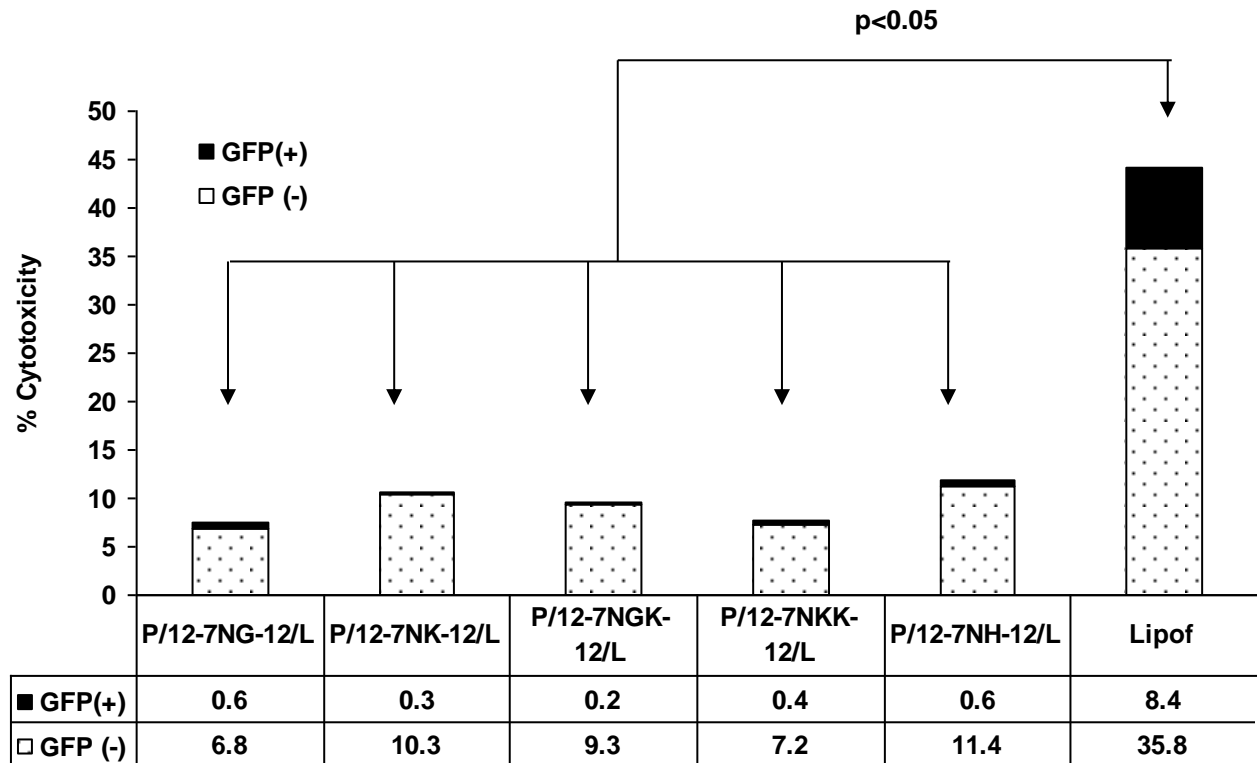


Figure 8.3: Cell toxicity of the plasmid/gemini/lipid (P/G/L) complexes after 72 hours was evaluated using FACS. Cell toxicity of P/G/L nanoparticles in transfected and non-transfected cells was significantly lower than the commercial Lipofectamine. (n=6, $p < 0.05$, one-way ANOVA)

8.4.2. Dye binding assay

P/G and P/G/L nanoparticles were prepared and incubated with ethidium bromide dye to evaluate the protection conferred to the DNA by the amino acid-substituted gemini derivatives. A strong electrostatic interaction between the gemini surfactants and the DNA prevented the penetration of the dye into the complexes, reducing the fluorescence.

The fluorescence emission values presented in **Figure 8.4** were standardized as percentage of total fluorescence achieved using naked (uncomplexed) plasmid. In the absence of the helper lipid, the glycyl-lysine substituted gemini derivative exhibited the highest ($9.5\% \pm 0.5$) fluorescence emission whereas the parent gemini had the lowest ($4.5\% \pm 0.4$). The fluorescence increased in the presence of helper lipid to $16.9\% \pm 1.4$ (highest) for the glycine-substituted, and $7.3\% \pm 0.4$ (lowest) for the lysyl-lysine gemini derivative. Overall, the fluorescence was lower in P/G complexes in comparison to P/G/L complexes. The presence of helper lipid may have increased the fluidity of lipid vesicles resulting in increased dye penetration and thus, higher fluorescence values.

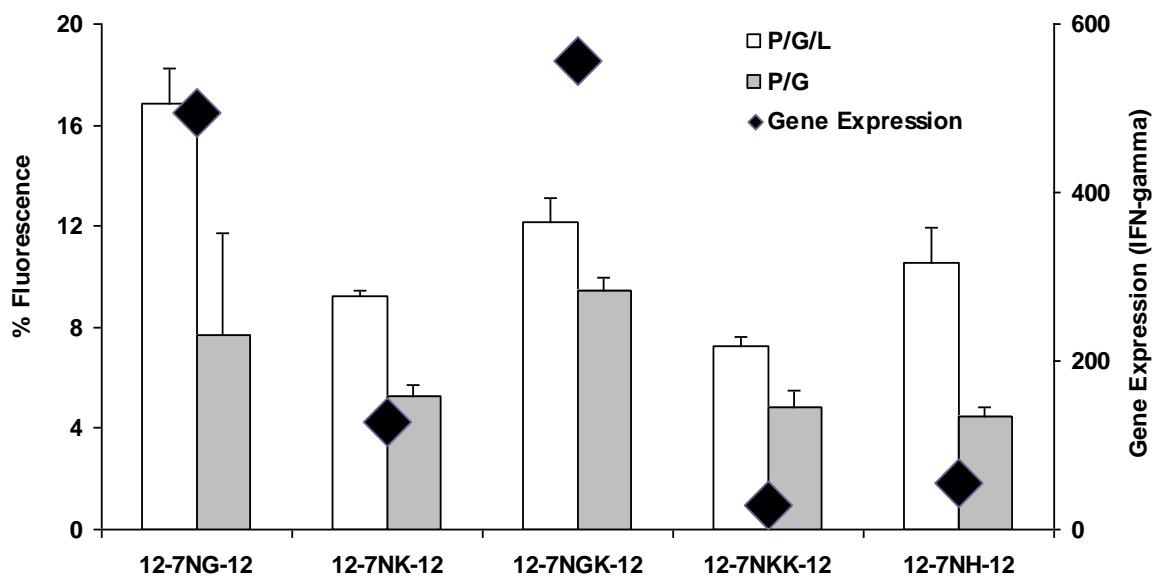


Figure 8.4: Ethidium bromide dye exclusion assay to evaluate the ability of the gemini derivatives to protect the DNA. A low fluorescence value indicates stronger binding affinity. Lysine and lysyl-lysine substituted gemini derivatives showed stronger binding to DNA than glycine and glycyL-lysine substituted gemini derivatives. Gene expression levels at 72 hours (from [199]) are shown for comparison (diamonds).

8.5. Discussion

8.5.1. Amino acid-substitution improved transfection efficiency without increasing cytotoxicity of P/G/L nanoparticles

Cytotoxicity plays a critical role in the overall transfection efficiency of the delivery vectors. In order to deliver the DNA into the cells, the cationic nanoparticles bind to the cell surface by electrostatic interaction, promote endocytosis and release the genetic material inside the cell. The greater the number of DNA molecules that are able to reach the cell nucleus, the higher the protein expression [112, 200]. Unfortunately, while high concentrations of the delivery agents imply increased chance of the DNA to penetrate the cell nucleus, it can also interfere with physiological processes within the cell inducing cell death. Thus the focus is to design gene delivery agents that are able to deliver DNA into the cells with minimal toxicity.

The Sf 1 Ep cells were selected for the cytotoxicity evaluation to establish groundwork for *in vivo* assessment of gemini-nanoparticle based gene delivery in rabbits. All gemini derivatives were found to be highly toxic when incubated without plasmid and helper lipid (**Figure 8.2.A**). This could be caused by the large cationic charge of the molecules which was not neutralized by the negatively charged plasmid DNA. Thus, in the absence of DNA, these positively charged gemini derivatives may aggregate on cell surfaces or bind strongly to negatively charged cell surface proteins impairing important membrane and other cellular functions [201]. An alternative hypothesis is that the free lipids may bind to the cell surface stronger than the nanoparticles, contributing to increased toxicity. It has been demonstrated that in gene delivery systems prepared at high positive to negative charge ratios, the free cationic lipids present in the system may compete with the nanoparticles for binding/uptake and can also be associated with increased toxicity [202]. It was shown that increasing the charge ratio between DNA and gemini surfactants beyond 1:10 increased cellular toxicity and reduced protein expression [9].

P/G/L nanoparticles exhibited significantly higher cell viability in comparison to the commercial agent at 5 hours (**Figure 8.2.B**) and 72 hours (**Figure 8.3**). Overall cell viability of P/G/L after 72 hours was above 87% with greater than 90% levels for nanoparticles formulated using amino acid-substituted gemini compounds. This was true for both transfected (GFP positive) cells and non-transfected (GFP negative) cells. The inclusion of amino acid substitution at the N position of the spacer was found to improve transfection efficiency of the gemini

surfactants without increasing the cytotoxicity of the P/G/L nanoparticles. This may arise because the biomolecules (amino acids) grafted onto the gemini surfactants are inherently biocompatible and biodegradable. This indicates that the addition of these amino acid moieties may have improved the interaction between the gemini surfactants and DNA. We further conducted dye binding assays and examined the correlations between the ability of the nanoparticles to protect the genetic material and the transfection efficiency/cellular toxicity. These results in conjunction with the results of physicochemical parameters reported in our earlier publication [199], serve to facilitate the identification of the crucial parameters that play an important role in improving the gene transfer profile of these delivery systems.

8.5.2. Glycine substitution contributed to conformation flexibility and leads to balanced (protection and release) DNA binding properties

Cationic lipids compact DNA and reduce its flexibility, thus preventing intercalation of the ethidium bromide between the base-pairs [203]. The gemini surfactants electrostatically bind the plasmid DNA causing a collapse of the helical structure into a compact particle. Upon addition of ethidium bromide, fluorescence is quenched due to the inability of the dye to penetrate between the nucleotides. The better the shielding of the DNA by the cationic gemini surfactant, the more pronounced the fluorescence quenching. The highest fluorescence emission was observed for glycine and glycyl-lysine substituted gemini derivatives in the absence of helper lipid, $7.7\% \pm 4$ and $9.5\% \pm 0.5$ of the non-complexed DNA, respectively, indicating the lowest protection of DNA among all derivatives (**Figure 8.4**). In the presence of helper lipid, the fluorescence values increased to $16.9\% \pm 1.4$ and $12.1\% \pm 0.9$ for glycine and glycyl-lysine derivatives, respectively, indicating a further decrease in binding efficiency. Poly (Lysine) is known for good DNA binding and DNA condensation properties [74]. We have observed a similar trend in lysine ($9.2\% \pm 0.2$ fluorescence) and lysyl-lysine ($7.3\% \pm 0.4$ fluorescence) substituted gemini derivatives which have exhibited stronger protection, in the presence of helper lipid, against dye penetration than other amino acid derivatives. A dye binding assay of the gemini surfactants with alkyl spacer indicated that stronger dye exclusion correlated with higher gene transfection efficiency [204]. However, the amino acid-substituted derivatives did not show similar behaviour: the strong fluorescence quenching of the 12-7NK-12 and 12-7NKK-12 was associated with lower transfection efficiency (**Figure 8.4**) [199].

We believe that the glycine and the glycyL-lysine substitution has provided a conformational flexibility to the molecule to affect a balanced binding, sufficient to provide protection against enzymatic degradation without hampering the intracellular release of DNA for gene expression. This may be due to the difference in interplay among the nature of bonding forces such as van der Waals, hydrogen bonding, in addition to electrostatic interactions for different amino acid-substituted derivatives [195].

Amino acid substitution in the spacer group of gemini surfactants has also increased the number of amino groups, especially terminal amino groups, which can affect the transfection efficiency of the delivery system [205]. This may be because of increased hydration of these molecules (as reported previously [199]) resulting in improved DNA interactions and cell membrane [206]. Furthermore, the derivatization of the carboxylic group of amino acids for substitution at the N of the spacer position of gemini surfactants leave terminal amino group(s) with theoretical pKa values greater than 9.5. These terminal amino groups are highly protonated at physiological pH contributing to efficient DNA-gemini electrostatic interactions and DNA compaction. This is exhibited by a strong inverse correlation (Pearson's correlation coefficient = -0.933, $p < 0.05$) between the number of terminal amine groups and P/G/L particle size [199].

Table 8.1: The number of terminal amino groups present in corresponding amino acid substituted gemini derivatives used for calculating correlation.

Delivery System	No. of terminal amino groups
P/12-7NG-12/L	1
P/12-7NK-12/L	2
P/12-7NGK-12/L	2
P/12-7NKK-12/L	3
P/12-7NH-12/L	0
P/L	–

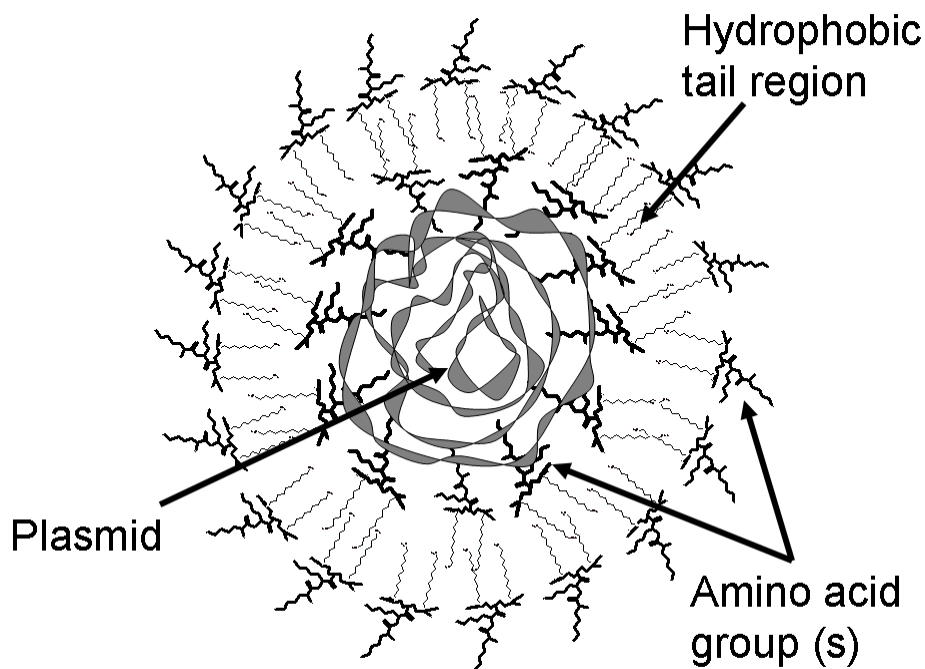


Figure 8.5: Schematic design of biphasic structure of plasmid/gemini derivatives exposing amino acid substitution outwardly.

The lysyl-lysine gemini nanoparticles having three terminal amine groups have the smallest mean diameter (105 nm) among all derivatives (**Table 8.1**), but also the lowest transfection efficiency, indicating once again the need of a balanced interaction between the DNA and gemini surfactant [199].

Cationic gemini surfactants are found to assemble into bilayer vesicles in presence of DNA in aqueous medium as depicted in **Figure 8.5** [154]. This conformational orientation of amino acid molecules (amine rich molecules) would improve cell surface interaction through hydrogen bonding in addition to electrostatic interactions. The terminal primary amine groups can also act as strong polycations at physiological or acidic pH and drive liposomal fusion in a ‘flip-flop’ manner by exposing hydrophobic regions after strong association with the negatively charged surface molecules [207-209]. This may have important significance for cellular uptake as well as endosomal escape, and may be an additional factor in higher transfection efficiency of glycine and glycyl-lysine substituted derivatives.

Moreover, the terminal lysine substitution has introduced extra secondary amine groups, in addition to tertiary amine in the spacer, which may also aid in endosomal escape by improving the buffering capacity of these derivatives. This ‘proton sponge’ effect helps in the disruption of the endosomal membrane enhancing the availability of more DNA nanoparticles for nuclear transport and gene expression [210].

8.6. Conclusion

The insertion of amino acid moieties in the spacer of the gemini surfactants has improved the transfection efficiency without increasing the cytotoxicity of the compounds. The amino acid substituted-derivatives have shown increased DNA condensation relative to the parent 12-7NH-12 gemini. By striking a balance among various interaction forces (hydrogen bonding, van der Waals forces), the glycine substitution has provided conformation flexibility to the derivatives. In addition, the terminal lysine of the glycyl-lysine derivative might act as a proton sponge, leading to overall balanced (protection and release) binding properties. The amino acid-substituted gemini surfactants, in general, exhibit low cytotoxicity and further structural modifications will be designed to enhance gene expression. One of the options will be to graft

amino acid residues to provide both flexibility to the binding moiety and release of the DNA inside the transfected cells.

8.7. Acknowledgements

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9. Evaluation of cellular uptake and intracellular trafficking as determining factors of gene expression for amino acid-substituted gemini surfactant-based DNA nanoparticles

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In this manuscript, I evaluated the cellular uptake mechanism of DNA complexes of the glycyl-lysine-substituted gemini surfactant. A comparison to the parent compound helped in determining the effect of amino acid substitution, specifically glycyl-lysine, on cellular uptake mechanism and its effect on transfection efficiency. I built a model to illustrate factors that contribute to the increase in gene expression of amino acid-substituted gemini surfactant compared to the parent compound. The *in vitro* studies were concluded with this work and laid the foundation for the development of *in vivo* formulation for mucosal application. This manuscript is based on my original idea. I designed and conducted all experiments and prepared the manuscript. The article is presented here in its entirety.

This manuscript relates to the fourth and final objective of my *in vitro* studies:

- To evaluate the cellular uptake mechanism of plasmid/gemini/lipid particles and its effect on gene expression.

9.1. Abstract

Background

Gene transfer using non-viral vectors offers a non-immunogenic and safe method of gene delivery. Cellular uptake and intracellular trafficking of the nanoparticles can impact the transfection efficiency of these vectors. Therefore, understanding the physicochemical properties that may influence the cellular uptake and the intracellular trafficking can aid the design of more efficient non-viral gene delivery systems. Recently, we developed novel amino acid-substituted gemini surfactants that showed higher transfection efficiency than their parent compound. In this study, we evaluated the mechanism of cellular uptake of the plasmid/gemini surfactant/helper lipid nanoparticles and their effect on the transfection efficiency.

Results

Nanoparticles were incubated with Sf 1 Ep cells in the presence of different endocytic inhibitors and gene expression (interferon- γ) was measured using ELISA. Clathrin-mediated and caveolae-mediated uptake were found to be equally contributing to cellular internalization of both P/12-7NH-12/L (parent gemini surfactant) and P/12-7NGK-12/L (amino acid-substituted gemini surfactant) nanoparticles. The plasmid and the helper lipid were fluorescently tagged to track the nanoparticles inside the cells, using confocal laser scanning microscopy. Transmission electron microscopy images showed that the P/12-7NGK-12/L particles were cylindrical while the P/12-7NH-12/L particles were spherical which may influence the cellular uptake behaviour of these particles. Dye exclusion assay and pH-titration of the nanoparticles suggested that high buffering capacity, pH-dependent increase in particle size and balanced DNA binding properties may be contributing to a more efficient endosomal escape of P/12-7NGK-12/L compared to the P/12-7NH-12/L nanoparticles, leading to higher gene expression.

Conclusion

Amino-acid substitution in the spacer of the gemini surfactant did not alter the cellular uptake pathway, showing a similar pattern to the unsubstituted parent gemini surfactant. Glycyl-lysine substitution in the gemini spacer improved buffering capacity and imparted a pH-dependent increase of particle size. This property conferred to the P/12-7NGK-12/L nanoparticles the ability to escape efficiently from clathrin-mediated endosomes. Balanced binding properties (protection and release) of the 12-7NGK-12 in the presence of polyanions could contribute to the facile release of the nanoparticles internalized via caveolae-mediated

uptake. A more efficient endosomal escape of the P/12-7NGK-12/L nanoparticles leads to higher gene expression compared to the parent gemini surfactant.

Keywords: cellular uptake, endosomal escape, non-viral gene delivery, clathrin-mediated endocytosis, caveolae-mediated endocytosis

9.2. Background

Gene therapy is based on the delivery of therapeutic genes to prevent or treat a disease. The method includes replacing a nonfunctional gene, introducing a new or missing gene, silencing a gene, or regulating gene expression. Gene-based therapy could offer an improved therapeutic solution and a cost-effective option to the treatment of many diseases, including cancer and infectious diseases [211, 212]. Among the available gene transfer technologies, non-viral vectors offer a non-immunogenic and safe method of gene delivery. However, they have generally lower transfection efficiency compared to their viral counterparts.

For successful gene expression, a delivery vector needs to overcome three major challenges (**Figure 9.1**): cellular uptake, endosomal/lysosomal escape and nuclear localization [213]. Cellular uptake is an important process as it determines the number of particles that are internalized and available for gene expression. Moreover, the mechanism of uptake may determine the intracellular pathway and the final fate of the vectors [125].

Clathrin-mediated, caveolae-mediated uptake and macropinocytosis are the most common uptake pathways utilized by mammalian cells to engulf macromolecules or solutes impermeable to the plasma membrane [125]. We assessed the effect of these three cellular uptake pathways on the gene transfer efficiency of the gemini surfactant-based nanoparticles. The clathrin-mediated uptake involves special membrane structures called clathrin-coated pits [128]. When ligands bind to these receptors, the coated pits form a polygonal clathrin lattice with the help of adaptor proteins. These clathrin-coated pits are pinched off from the plasma membrane and internalized to form intracellular clathrin-coated vesicles ranging in size from 100 to 150 nm in diameter [128]. Inside the cell, the clathrin coat depolymerizes to form early endosomes which then fuse with late endosomes and proceed to finally fuse with lysosomes. Particles internalized via this pathway experience a drop in pH, towards acidic conditions (pH 5-6), as they travel towards late endosomes, before merging with lysosomes [129]. Chlorpromazine and potassium depletion can dissociate clathrin from the surface membrane and inhibit clathrin-mediated endocytosis [214, 215].

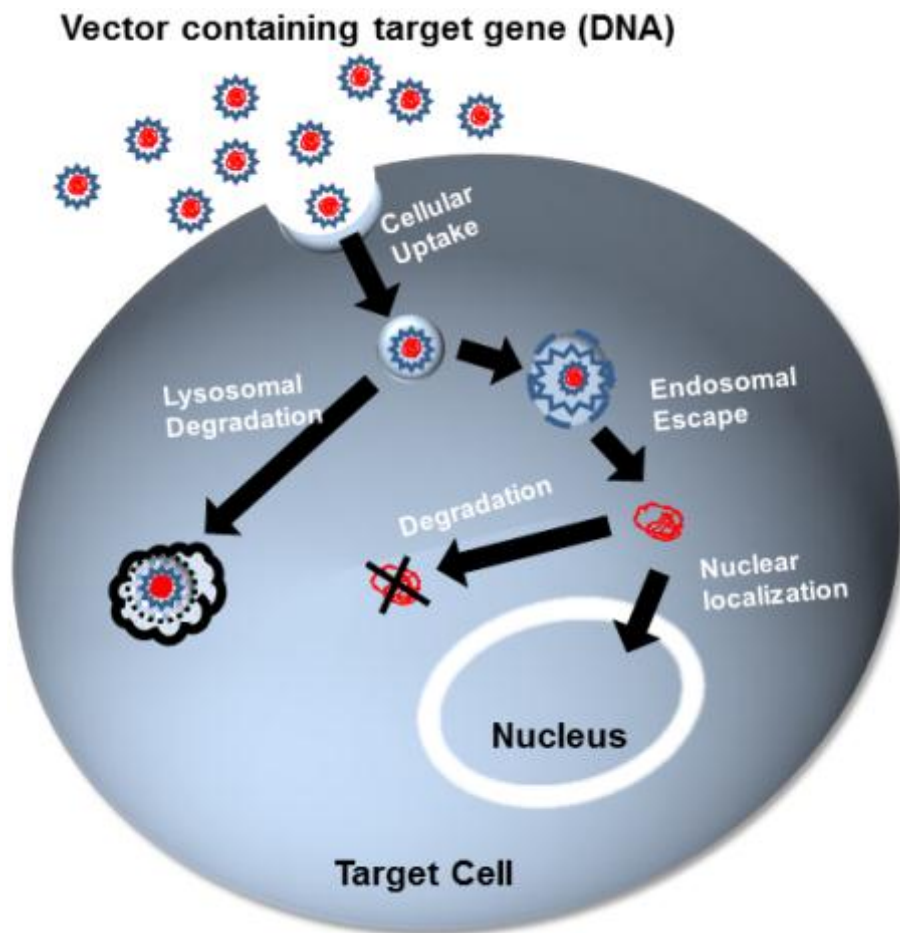


Figure 9.1: Intracellular trafficking of DNA-delivery vector complexes. This schematic representation indicates the critical barriers in successful gene delivery: cellular uptake, endosomal escape and nuclear localization. A delivery vector interacts with the cell membrane for internalization. Once inside the cell, the delivery vector should facilitate endosomal escape and avoid DNA degradation in lysosomes. Cytoplasmic stability of plasmid DNA and localization in the nucleus are final steps to successful gene transfer

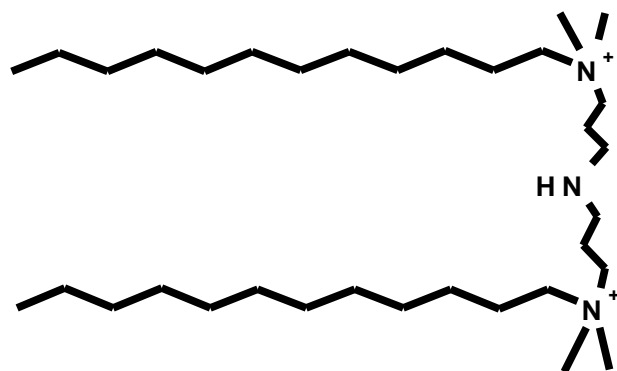
Caveolae-mediated uptake is another important pathway that involves small hydrophobic domains that are rich in cholesterol and glycosphingolipids [216]. Contrary to clathrin-mediated uptake, the caveolae-dependent pathway follows a non-acidic and non-digestive intracellular route. Filipin III inhibits caveolae-mediated uptake by binding to 3β -hydroxysterol, a major component of glycolipid microdomains and caveolae [217]. Genistein also inhibits caveolae-mediated uptake by local disruption of the actin network and by preventing the recruitment of dynamin II, both necessary for this type of cellular uptake [218]. Water-soluble methyl- β -cyclodextrin forms inclusion complexes with cholesterol and is known to inhibit both clathrin-mediated and caveolae-dependent uptake by depleting cholesterol from the plasma membrane [219-221]. Macropinocytosis is a non-selective internalization of large volumes of extracellular medium through cell membrane protrusions that collapse onto and fuse with the cell membrane [142]. The large endocytic vesicles are neither coated nor concentrated before internalization. Phosphatidylinositol 3 kinase and rho family GTPase activities influence macropinocytosis by regulating actin rearrangements. Wortmannin, a phosphatidylinositol 3 kinase inhibitor, can be employed to inhibit macropinocytosis [222].

Understanding the physicochemical properties of the gene delivery vectors in association with these uptake pathways can lead to rational design of nanoparticles that target those pathways and improve the intracellular fate of the particles. For example, the design of delivery agents that respond to the change in pH can escape from the endosomes and evade degradation in lysosomes, thus improving the transfection efficiency of these non-viral vectors [10]. To capitalize on the pH sensitivity of these nanoparticles, clathrin-mediated uptake should be targeted to utilize this phenomenon.

Our research group has focused on the optimization of cationic N,N-bis(dimethylalkyl)- α,ω -alkanediammonium gemini surfactants, m-s-m as transfection vectors, where m and s refer to the number of carbon atoms in the alkyl tails and in the polymethylene spacer groups, respectively [9, 182]. More recently, a series of third generation gemini surfactants has developed by introducing an amino acid/dipeptide group at the N position of the spacer of a previously studied compound (12-7NH-12) (**Figure 9.2.A**). The glycyl-lysine substituted derivative (12-7NGK-12, **Figure 9.2.B**) achieved the highest gene expression among the series, performing significantly better than the unsubstituted parent compound [199, 223].

In this study, we evaluated the cellular uptake behaviour of plasmid/gemini/lipid (P/G/L) nanoparticles and investigated their impact on the transfection efficiency of these nanoparticles. We also explored the physicochemical properties that may have led to the difference in cellular uptake and intracellular trafficking of these nanoparticles resulting in the difference in transfection efficiency between these vectors.

A



B

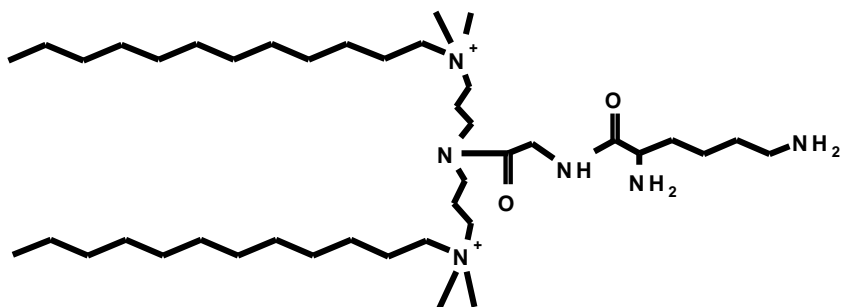


Figure 9.2: Chemical structure of gemini surfactants. (A) Parent unsubstituted gemini surfactant (12-7NH-12) and (B) glycyl-lysine substituted gemini surfactant (12-7NGK-12).

9.3. Materials and Methods

9.3.1. Reagents for uptake studies

Genistein, filipin III, methyl- β -cyclodextrin, chlorpromazine hydrochloride and wortmannin were purchased from Sigma-Aldrich, Oakville, Canada.

9.3.2. Formulations

A model plasmid pGT.IFN-GFP that encodes for interferon (IFN)- γ and green fluorescent protein was used for all formulations. Gemini surfactants were formulated with pGT.IFN-GFP in the presence of a helper lipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE ; Avanti Polar Lipids, AL, U.S.A.) creating P/G/L nanoparticles. A plasmid to gemini surfactant charge ratio of 1:10 was used for all formulations (as described earlier [199]). A quantity of 100 ng of plasmid DNA per well was used in the 96-well plates for transfection.

9.3.3. Cell culture and propagation

Cotton tail rabbit epithelial cells, Sf 1 Ep (CCL-68, ATCC, VA, USA) were grown in Minimum Essential Medium (MEM, GIBCO, NY, USA) supplemented with 10% v/v fetal bovine serum and antibiotic/antimycotic agents, respectively. All cells were grown to 70-80% confluency. The cells were seeded at a concentration of 20,000 cells per well in 96-well tissue culture plates (Falcon BD, Mississauga, Canada) and were incubated for 24 hours at 37 °C/5% CO₂ prior to transfection.

9.3.4. Cell toxicity assay

Genistein (200 μ M), filipin III (5 μ g/mL and 1 μ g/mL), methyl- β -cyclodextrin (10 mM and 5 mM), chlorpromazine hydrochloride (10 μ g/mL and 5 μ g/mL), wortmannin 50 nM were incubated with the cells for 1, 2, 3 and 4 hours [137, 219, 224, 225]. For potassium depletion, the cells were washed three times with potassium free buffer containing 140 mM NaCl, 20 mM HEPES pH 7.4, 1 mM CaCl₂, 1mM MgCl₂ and 1 mg/mL D-glucose, followed by 2 minutes incubation with hypotonic buffer (potassium free buffer diluted with water 1:1) [137, 215] for 1, 2, 3 and 4 hours. At the end of the incubation, cells were washed three times with PBS and incubated with fresh supplemented media at 37 °C/5% CO₂ for 24 hours. A 10 μ L aliquot of 5

mg/mL (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Invitrogen, Burlington, Canada) aqueous solution was added in each well and the plates were incubated at 37 °C/5% CO₂ for 3 hours. Supernatants were removed and the cells were washed with PBS. The formed purple formazan solid was dissolved in 200 µL dimethyl sulfoxide (DMSO, Sigma) on a plate shaker for 10 minutes. The plates were incubated for 10 minutes at 37 °C to eliminate air bubbles. Absorbance was measured at 550 nm using a plate reader (Biotek Microplate Synergy HT, VT, USA). Cell toxicity of the chemically treated/potassium depleted cells were expressed as a percentage of dead cells compared to the untreated normal cells.

$$\% \text{ dead cells} = \frac{Abs_{control} - Abs_{treated}}{Abs_{control}} \times 100$$

9.3.5. Transfection

In a 96-well plate, cells were incubated (in triplicate) for 30 minutes with genistein 200 µM, methyl-β-cyclodextrin 5 mM, wortmannin 50 nM, filipin III 5 µg/mL and for 60 minutes with filipin III 1 µg/mL, chlorpromazine hydrochloride 5 µg/mL and potassium free buffer prior to addition of P/G/L nanoparticles. The nanoparticle formulations were prepared as described above and incubated with the cells for 2 hours. After 2 hours the media was removed and cells were washed three times with PBS and replenished with fresh media. Supernatants were collected at 24, 48 and 72-hour intervals. At 72 hours, an MTT assay was performed to determine the combined toxicity of P/G/L nanoparticles and chemical treatment/potassium depletion on cell viability. ELISA was used to detect the IFN-γ secreted into the supernatant and was performed according to the BD Pharmingen protocol. Protein concentration was calculated from a standard curve using recombinant IFN-γ.

9.3.6. Confocal laser scanning microscopy (CLSM)

The model plasmid pGT.IFN-GFP was labeled with tetramethyl-rhodamine (ex/em wavelengths: 546 nm/ 576 nm) using Label IT® Tracker™ Kit (Mirus, WI, U.S.A.) according to the manufacturer's protocol. N-(7-nitro-2-1,3-benzoxadiazol-4-yl) labeled DOPE (ex/em wavelengths: 460 nm/535 nm) was purchased from Avanti Polar Lipids, AL, U.S.A.

P/G/L nanoparticles were prepared as described in the Transfection section using tetramethyl-rhodamine labeled plasmid with 10% w/w of DOPE in the formulation being substituted with fluorescent tagged DOPE.

Sf 1 Ep cells were treated with chemical inhibitors – genistein, chlorpromazine, wortmannin and methyl- β -cyclodextrin as described under the section ‘Transfection’, prior to incubation with fluorescent P/G/L nanoparticles. Additionally, an aliquot of 30 μ M DAPI was added to the cells for staining the nucleus. After 2 hours, the cells were washed with PBS three times and images were taken using a Leica TCS SP5 laser scanning confocal microscope (Leica Microsystems Inc., Bensheim, Germany). LAS AF Lite 2.4.1 (Leica Microsystems CMS GmbH) and ImageJ 1.44p (National Institute of Health, U.S.A.) were used for image processing.

9.3.7. pH titration and particle size measurement

P/12-7NH-12/L and P/12-7NGK-12/L nanoparticles were prepared as described previously. An MPT-2 autotitrator connected to a ZetaSizer Nano ZS (Malvern Instruments, Worcestershire, UK) was used to determine changes in particle size as a function of pH. Briefly, P/G/L nanoparticles (9 mL) were placed in the titration cell of the auto-titrator and titrated over basic (0.05 M NaOH) to acidic (0.1 M HCl) pH range at 0.2-0.5 pH unit intervals.

9.3.8. DNA/gemini surfactant interactions

Nanoparticles were prepared using plasmid and gemini surfactants at a +/- charge ratio of 10 in the presence of DOPE as described earlier, with samples prepared in quadruplicate. P/G/L nanoparticles were incubated with ethidium bromide (1 μ g/mL) and heparin buffer (20mM HEPES, 5.2% glucose, 0 U/mL, 5 U/mL, 10 U/mL or 20 U/mL heparin, pH 7.0) for 10 minutes at room temperature. Fluorescence excitation was carried out at 528/20 nm and emission measured at 590/35 nm using a multiplate reader (Biotek Microplate Synergy HT, VT, U.S.A.). The relative fluorescence of the P/G/L complexes was expressed as a percentage of the fluorescence of a plasmid solution in the presence of corresponding (0 U/mL, 5 U/mL, 10 U/mL, 20 U/mL) heparin buffer.

$$\%fluorescence = \frac{(fluorescence_{P/G/L \text{ with heparin}} - fluorescence_{P/G/L})}{fluorescence_{plasmid \text{ in presence of buffer}}} \times 100$$

9.3.9. Transmission electron microscopy (TEM)

Formulations were prepared as described earlier and loaded onto formvar-coated 300-mesh copper grids using a 5 μ L aliquot of each sample. The samples were incubated for 2 minutes and the liquid was blotted with absorbent tissue. The specimens were stained with 1% phosphotungstic acid for 15 seconds and blotted with tissue. The dried samples were examined with a Philips CM 10 electron microscope at an accelerating voltage of 100 kV, and pictures taken on 3 ¼" x 4" Kodak Electron Microscope Film 4489.

9.3.10. Statistical analyses

Results are expressed as the mean of $n \geq 3 \pm$ standard deviation. One way analysis of variance (ANOVA) and Bonferroni/Dunnett's-T3 post hoc tests were used for statistical analyses (PASW Statistics 18.0). Significant differences were considered at $p < 0.05$ level.

9.4. Results

9.4.1. Toxicity of chemical inhibitors or potassium depletion in Sf 1 Ep cells

In order to optimize the concentration and the exposure time of the endocytosis inhibitors, we evaluated their intrinsic toxicity on Sf 1 Ep cells after 1, 2, 3 and 4 hours of incubation period (**Figure 9.3**). With the exception of 10 mM methyl- β -cyclodextrin and 10 μ g/mL chlorpromazine concentrations, toxicity was under 20% at the 3-hour time point. Therefore, we selected a lower concentration of these inhibitors, 5 mM for methyl- β -cyclodextrin and 5 μ g/mL for chlorpromazine which retained more than 80% cell viability up to 3 hours.

9.4.2. Combined toxicity of P/G/L nanoparticles and chemical inhibitors/potassium depletion in Sf 1 Ep cells

The inhibition of endocytosis by chemical treatments is a reversible process. Therefore, it is important to maintain these inhibitors in cell media while transfecting the cells with the DNA nanoparticles [214, 215, 217, 219]. Caveolae-mediated inhibitors (filipin and genistein) with P/12-7NH-12/L (parent gemini surfactant) exhibited an increase in cytotoxicity with 20-40% dead cells (**Figure 9.4**).

In the presence of P/12-7NGK-12/L (amino acid-substituted gemini surfactant), filipin caused a $89.1 \pm 0.8\%$ cytotoxicity. A decrease of the filipin concentration to 1 μ g/mL did not improve cell viability significantly ($82.4 \pm 5.2\%$ dead cells). Thus filipin could not be used as an endocytosis inhibitor due to the significant combined toxicity with the P/G/L nanoparticle. However, genistein, another inhibitor of the caveolae-mediated uptake had a cell toxicity of $41.2 \pm 1.1\%$ in the presence of P/12-7NGK-12/L, in the range of other agents. Methyl- β -cyclodextrin which inhibits both clathrin- and caveolae- dependent endocytosis also increased cytotoxicity with $26.3 \pm 8.9\%$ and $40.6 \pm 5.4\%$ cell death in the presence of P/12-7NH-12/L and P/12-7NGK-12/L nanoparticles, respectively.

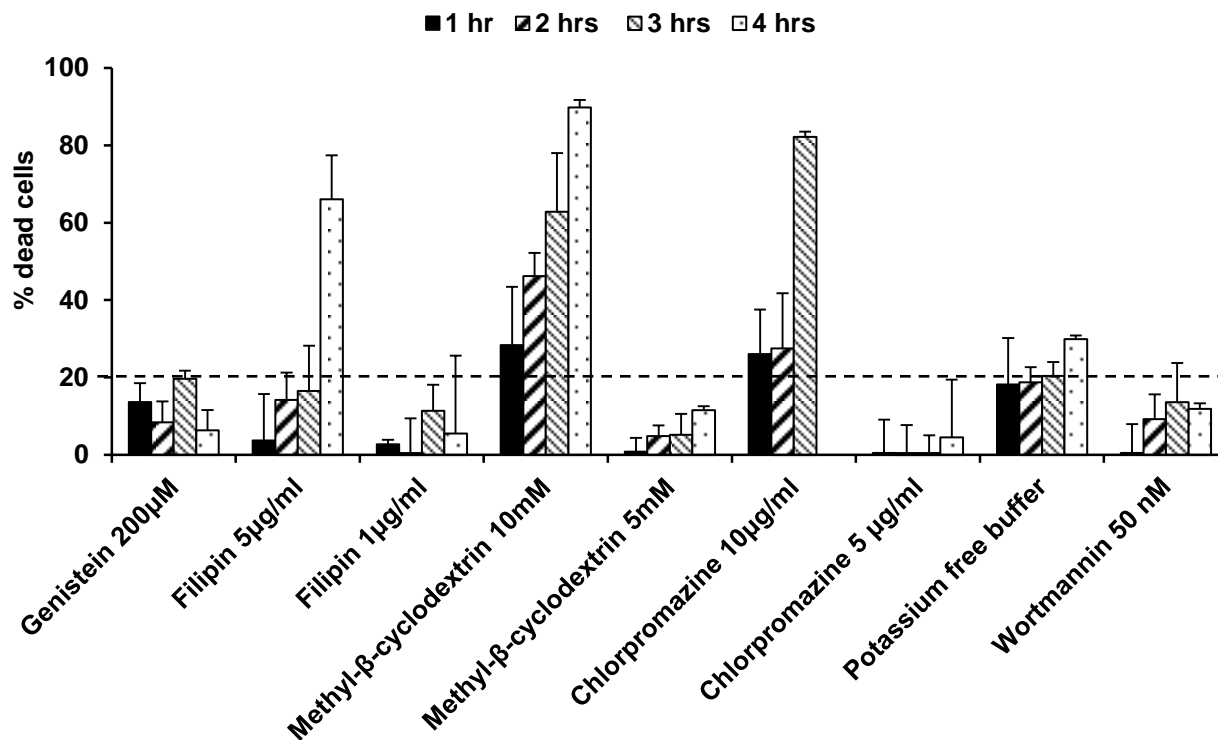


Figure 9.3: Cellular toxicity of the endocytic inhibitors in Sf 1 Ep cells. Cellular toxicity of chemical inhibitors/potassium depletion in Sf 1 Ep cells was measured at 1, 2, 3 and 4 hours. Conditions regarding concentration of the inhibitor and incubation time that retained cell viability greater than 80% were selected for the cellular uptake study.

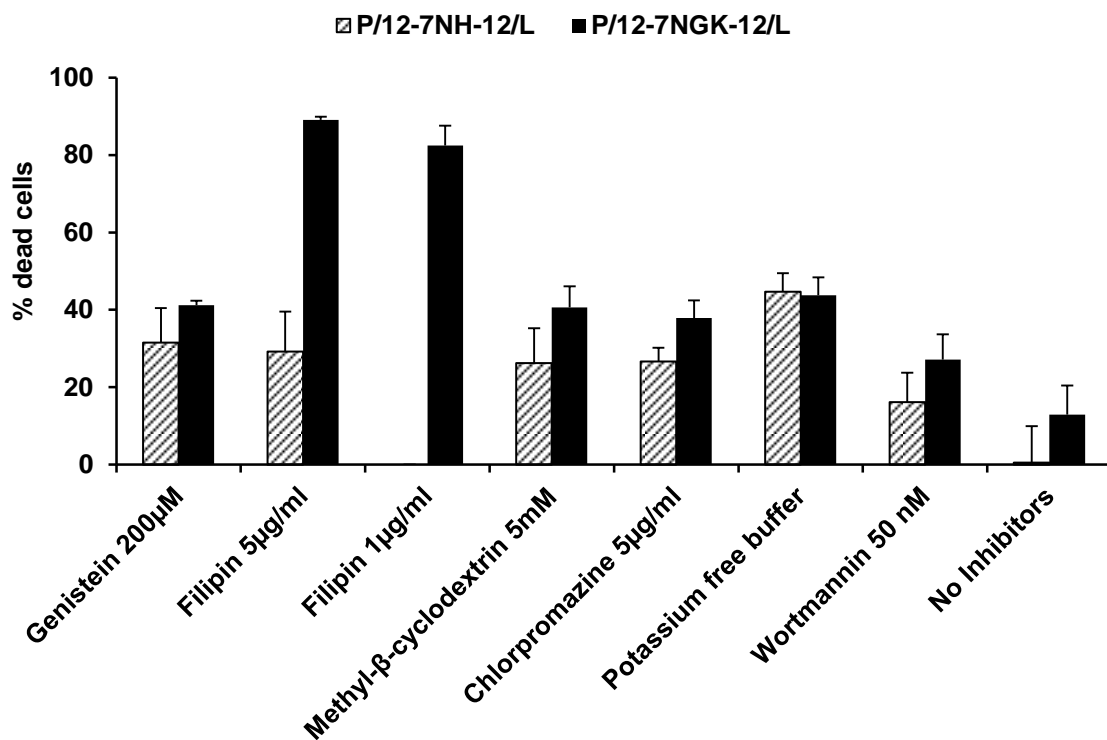


Figure 9.4: Cellular toxicity of the endocytic inhibitors in the presence of P/G/L nanoparticles in Sf 1 Ep cells. Combined cellular toxicity of P/12-7NH-12/L or P/12-7NGK-12/L in the presence of chemical inhibitors/potassium free buffer in Sf 1 Ep cells was measured at 72 hours. Filipin had a significantly high combined toxicity; therefore, it was not selected for gene expression study of the P/12-7NGK-12/L nanoparticles.

Clathrin-mediated uptake inhibition (potassium depletion, chlorpromazine) along with P/G/L nanoparticles showed an increase in toxicity to 20-45% cell death. In potassium depleted cells, the cytotoxicity values were $44.6\pm 4.8\%$ of dead cells in the presence of P/12-7NH-12/L and $43.8\pm 4.7\%$ dead cells after treatment with P/12-7NGK-12/L. Cytotoxicity was $26.6\pm 8.9\%$ in cells incubated with chlorpromazine and P/12-7NH-12/L, while it was $37.9\pm 4.6\%$ in the presence of P/12-7NGK-12/L. Wortmannin marginally increased cytotoxicity to $16.1\pm 7.6\%$ and $27.2\pm 6.5\%$ in the presence of P/12-7NH-12/L and P/12-7NGK-12/L, respectively. With the exception of filipin in the presence of P/12-7NGK-12/L, the cell toxicity among endocytosis inhibitors was not significantly different, thus their intrinsic toxicity was not considered as a modifying factor in gene expression. Due to the higher toxicity of filipin, genistein was selected to correlate gene expression, through the caveolae-dependent pathway, of the P/12-7NGK-12/L nanoparticles.

9.4.3. Cellular uptake of P/G/L nanoparticles in cells treated with chemical inhibition/potassium depletion

P/G/L nanoparticles were prepared using TM-rhodamine (red) labeled plasmid in the presence of fluorescent (green) labeled helper lipid. DAPI (blue) was used as fluorescent stain for the cell nuclei. The objective was to determine, qualitatively, whether materials were transferred into the cells and whether the presence of inhibitors induced a difference in the cellular uptake of P/12-7NH-12/L and P/12-7NGK-12/L nanoparticles. Interestingly, we observed that the inhibition of caveolae-mediated (genistein) or clathrin-mediated (chlorpromazine) uptake pathways did not have any effect on cellular uptake of P/12-7NGK-12/L or P/12-7NH-12/L nanoparticles (**Figure 9.5: B & G, C & H**). However, almost no particles were internalized by the cells treated with either P/12-7NGK-12/L or P/12-7NH-12/L nanoparticles when both clathrin-mediated and caveolae-mediated uptake pathways were inhibited by treatment with methyl- β -cyclodextrin (**Figure 9.5: D & I**). This suggests that both clathrin-mediated and caveolae-mediated pathways are playing an important role in uptake of these particles. Wortmannin (**Figure 9.5: E & J**) showed no difference in the cellular uptake of P/12-7NGK-12/L or P/12-7NH-12/L nanoparticles.

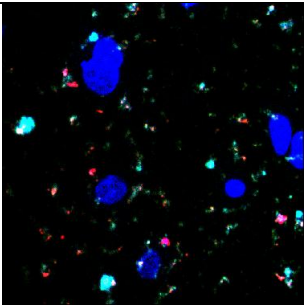
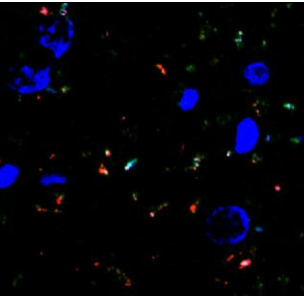
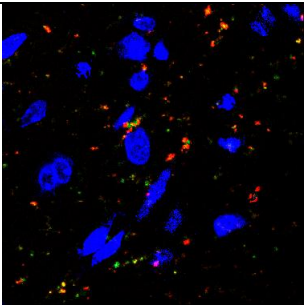
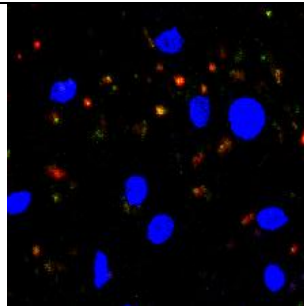
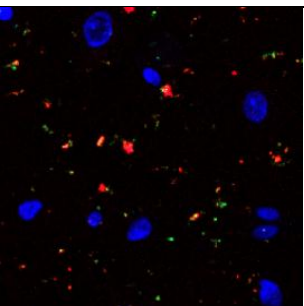
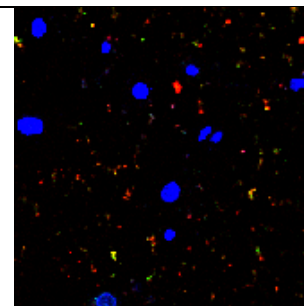
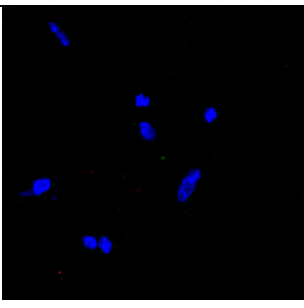
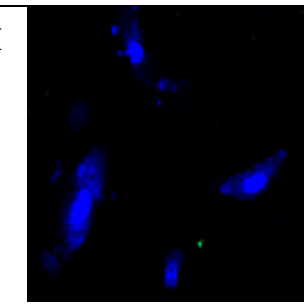
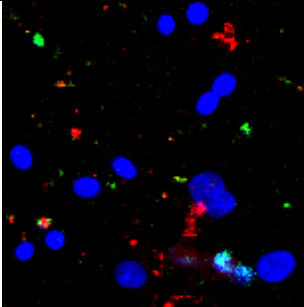
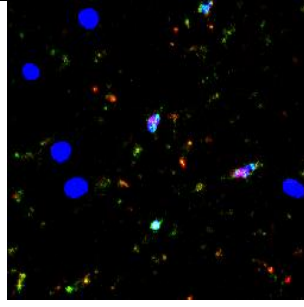
<div style="text-align: center;">Nanoparticles</div> <div style="text-align: left;">Inhibitors</div>	P/12-7NGK-12/L	P/12-7NH-12/L
No chemicals	A 	F 
Genistein	B 	G 
Chlorpromazine	C 	H 
Methyl- β -cyclodextrin	D 	I 
Wortmannin	E 	J 

Figure 9.5: Laser scanning confocal microscopy (LSCM) images of Sf 1 Ep cells. Cells were pretreated with genistein (B & G), chlorpromazine (C & H), methyl- β -cyclodextrin (D & I) and wortmannin (E & J). Cells (A to E) were incubated with P/12-7NGK-12/L and Cells (F to J) were incubated with P/12-7NH-12/L for 2 hours. DAPI was used for staining the cell nuclei. At the end of the study, cells were washed with PBS and images were recorded. DNA (red), DOPE (green) and cell nuclei (blue).

9.4.4. Gene expression of P/G/L nanoparticles in cells treated with chemical inhibition/ potassium depletion

In our previous studies, we found that the amino acid-substitution in the spacer of the parent gemini surfactant increased its transfection efficiency [223]. Here, we evaluated, quantitatively, the effect of each uptake pathway on gene expression of P/12-7NH-12/L and P/12-7NGK-12/L. Nanoparticles internalized via caveolae-mediated, clathrin-mediated and macropinocytosis encounter different intracellular conditions; however, in each case the particles should escape degrading conditions such as lysosomes for successful gene expression. The gene expression levels achieved after inhibiting each cellular uptake pathway, separately, provides information on efficiency of nanoparticles in escaping these detrimental conditions.

Caveolae-mediated uptake

In the absence of any inhibitor treatment to the cells, the gene expression in the P/12-7NH-12/L transfected cells was 183 ± 17.5 pg IFN γ /20000 cells (**Figure 9.6**). Gene expression after P/12-7NH-12/L nanoparticle transfection was 84 ± 9.4 pg IFN γ /20000 cells and 31 ± 14.9 pg IFN γ /20000 cells in the presence of genistein and filipin, respectively, significantly lower ($p < 0.05$) compared to the cells treated with P/G/L nanoparticles in the absence of the inhibitors.

The cells treated with the glycyl-lysine substituted nanoparticles, P/12-7NGK-12/L, exhibited gene expression of 749 ± 15.2 pg IFN γ /20000 cells in the absence of inhibitors. This gene expression was not significantly affected by inhibition of caveolae-mediated uptake (genistein), exhibiting protein levels of 601 ± 200 pg IFN γ /20000 cells. Unfortunately, due to high toxicity of filipin in the presence of P/12-7NGK-12/L (**Figure 9.4**), the gene expression data was not used for analysis. Methyl- β -cyclodextrin, that inhibits both clathrin- and caveolae-mediated uptake, reduced significantly ($p < 0.05$) gene expression in cells transfected with P/12-7NH-12/L and P/12-7NGK-12/L nanoparticles to 6 ± 2.2 pg IFN γ /20000 cells and 50 ± 21.1 pg IFN γ /20000 cells (**Figure 9.6**), respectively.

In conclusion, this suggests that caveolae-mediated uptake was crucial for successful gene expression of P/12-7NH-12/L nanoparticles. However, inhibition of caveolae-mediated uptake alone lowered slightly, but not significantly, the gene expression in the cells transfected with the P/12-7NGK-12/L nanoparticles.

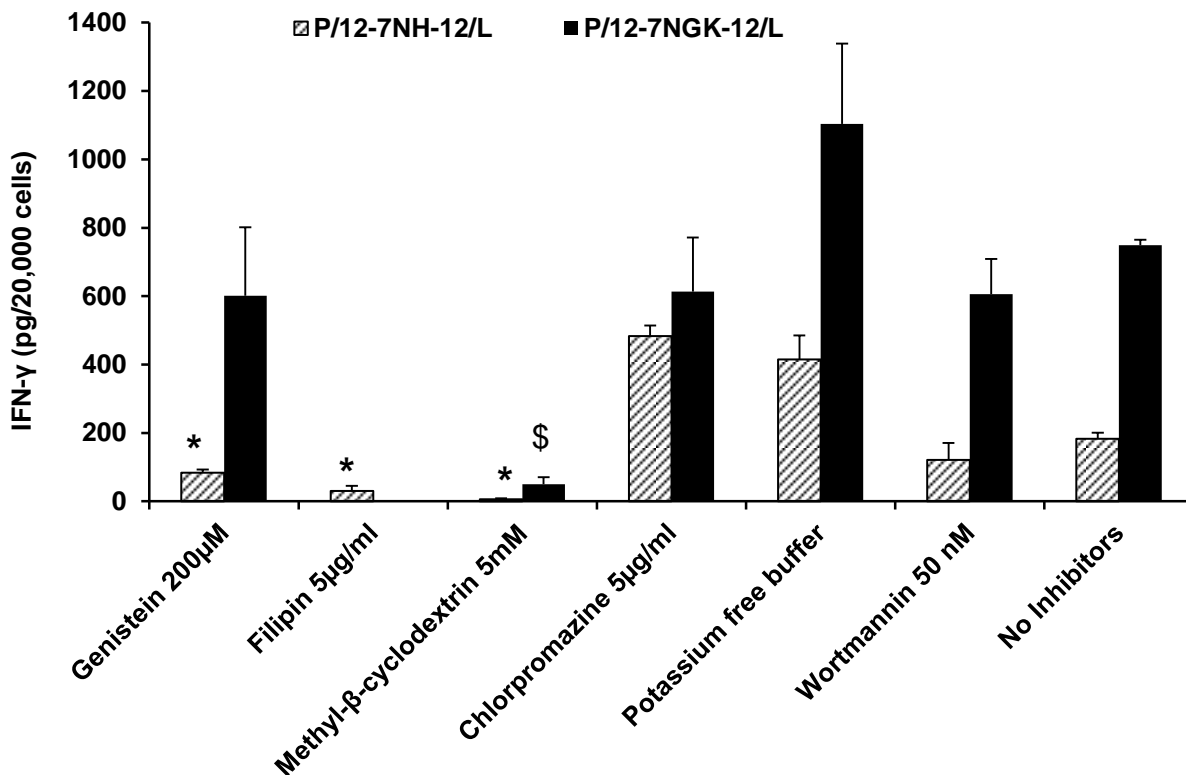


Figure 9.6: Effect of endocytic inhibitors on gene expression of P/G/L nanoparticles. Interferon (IFN)- γ expression was measured at 72 hours in cells incubated with P/G/L nanoparticles in the presence of endocytic inhibitors. In the P/12-7NH-12/L-treated cells, gene expression in cells pre-treated with caveolae-mediated uptake inhibitors (genistein, filipin) was significantly lower than the untreated (no inhibitors) cells, whereas gene expression was significantly higher on inhibiting clathrin-mediated uptake (chlorpromazine). For the P/12-7NGK-12/L nanoparticles, gene expression was significantly lower in the presence of methyl- β -cyclodextrin (clathrin- and caveolae-mediated uptake inhibitor). * represents significant difference, $p < 0.05$, compared to ‘No inhibitors’ control cells treated with P/12-7NH-12/L nanoparticles (No inhibitors black bar). \$ represents significant difference, $p < 0.05$, compared to ‘No inhibitors’ control cells treated with P/12-7NGK-12/L nanoparticles (No inhibitors dashed-line bar)

Clathrin- mediated uptake

It was interesting to note that gene expression of P/12-7NH-12/L nanoparticles was significantly higher ($p < 0.05$) after clathrin-mediated uptake inhibition, compared to cells without inhibitors (**Figure 9.6**). The gene expression increased to 483 ± 30.6 pg IFN γ /20000 cells in the cells pre-treated with chlorpromazine. Potassium depletion, which also inhibits clathrin-mediated uptake, increased gene expression of P/12-7NH-12/L to 415 ± 70 pg IFN γ /20000 cells.

Inhibition of clathrin-mediated uptake by chlorpromazine slightly reduced gene expression of P/12-7NGK-12/L to 613 ± 159 pg IFN γ /20000 cells while gene expression was moderately increased to 1104 ± 235 pg IFN γ /20000 cells after potassium depletion. However, the effect of inhibition of clathrin-mediated uptake on gene expression of P/12-7NGK-12/L was not significant compared to cells without inhibitors. As stated above, inhibition of both clathrin-mediated and caveolae-mediated uptake with methyl- β -cyclodextrin reduced the gene expression of P/12-7NGK-12/L significantly ($p < 0.05$) compared to the control transfected cells.

In conclusion, P/12-7NH-12/L nanoparticles internalized via clathrin-mediated uptake were not a major contributor to overall gene expression. On the contrary, P/12-7NGK-12/L nanoparticles following either clathrin- or caveolae- mediated pathway resulted in successful gene expression.

Macropinocytosis

Macropinocytosis inhibitor, wortmannin, did not significantly modify gene expression of either P/12-7NH-12/L or P/12-7NGK-12/L (**Figure 9.6**). Protein levels of 121 ± 49 pg IFN γ /20000 cells and 606 ± 104 pg IFN γ /20000 cells were measured for P/12-7NH-12/L and P/12-7NGK-12/L, respectively.

9.4.5. Particle size measurement of P/G/L nanoparticles as a function of pH

The pathway of cellular uptake determines the intracellular fate of the engulfed particle [125]. Cellular internalization of the particles via clathrin-coated endosomes proceeds with an early influx of H^+ before fusion with the late endosome, finally merging with the lysosomes. In order to simulate these conditions, we evaluated the behaviour of the P/G/L complexes over the pH range of 8.0-3.0. Particle size increased in the pH range 8.0-5.5 followed by a decreasing trend (**Figure 9.7**), fitting a second order polynomial (quadratic) relationship ($R^2 > 0.9$) between pH and particle size of P/12-NH-12/L nanoparticles. The P/12-7NGK-12/L nanoparticles followed a third order polynomial (cubic) curve exhibiting an exponential increase in particle size from neutral (~ 7.4) to acidic (~ 4) pH. The increase in size of P/12-7NGK-12/L nanoparticles could be attributed to the accumulation of H^+ ions due to the higher buffering capacity of the amino acid-substituted gemini surfactant.

The volume of acid (0.1 M HCl) used per unit pH value provided a relative comparison of the buffering ability of 12-7NGK-12 and 12-7NH-12. The pH titration curve revealed that P/12-7NGK-12/L nanoparticles had considerably higher buffering capacity than P/12-7NH-12/L at neutral (~ 7.4), slightly acidic (6-5) and strongly acidic (~ 4) pH values (**Figure 9.8**).

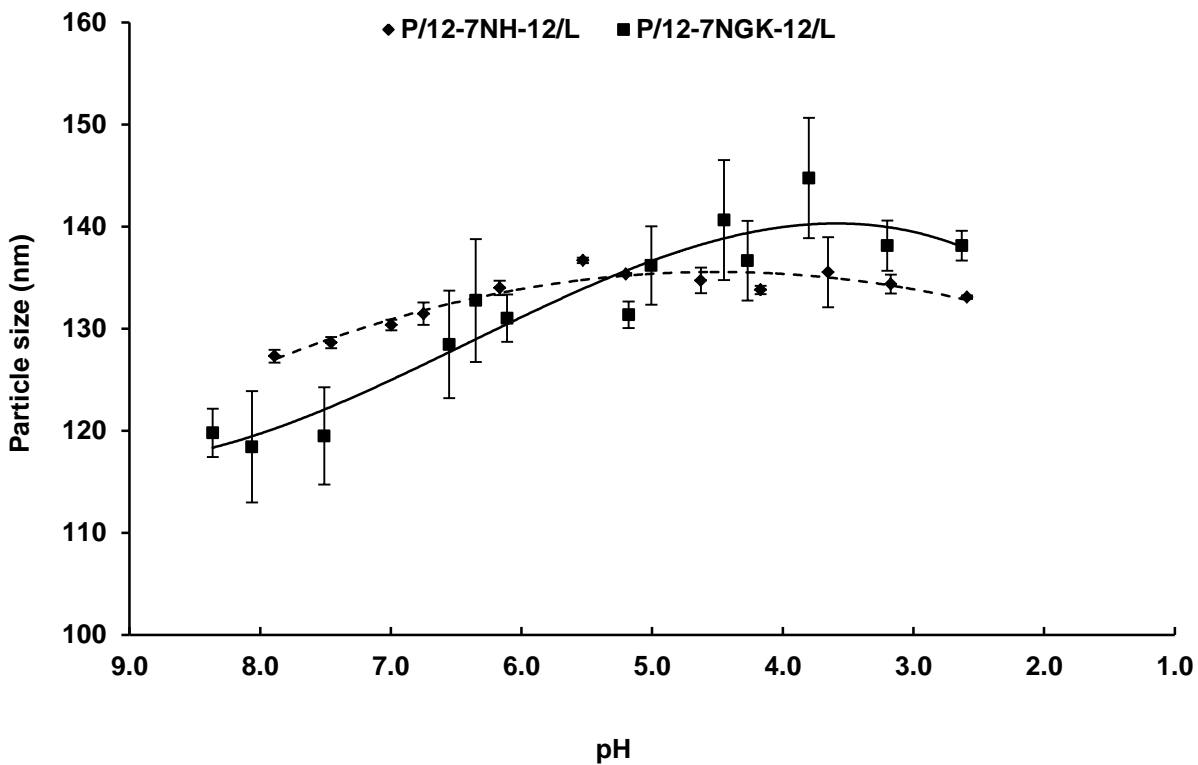


Figure 9.7: Particle size measurement of P/12-7NH-12/L and P/12-7NGK-12/L at different pH. A second order polynomial curve (solid line) was found to be a best fit ($R^2 > 0.9$) for P/12-7NH-12/L while a third order polynomial (dashed line) curve ($R^2 > 0.9$) described the effect of pH on P/12-7NGK-12/L particle size. Stronger acidic conditions resulted in a significant increase in P/12-7NGK-12/L particle size.

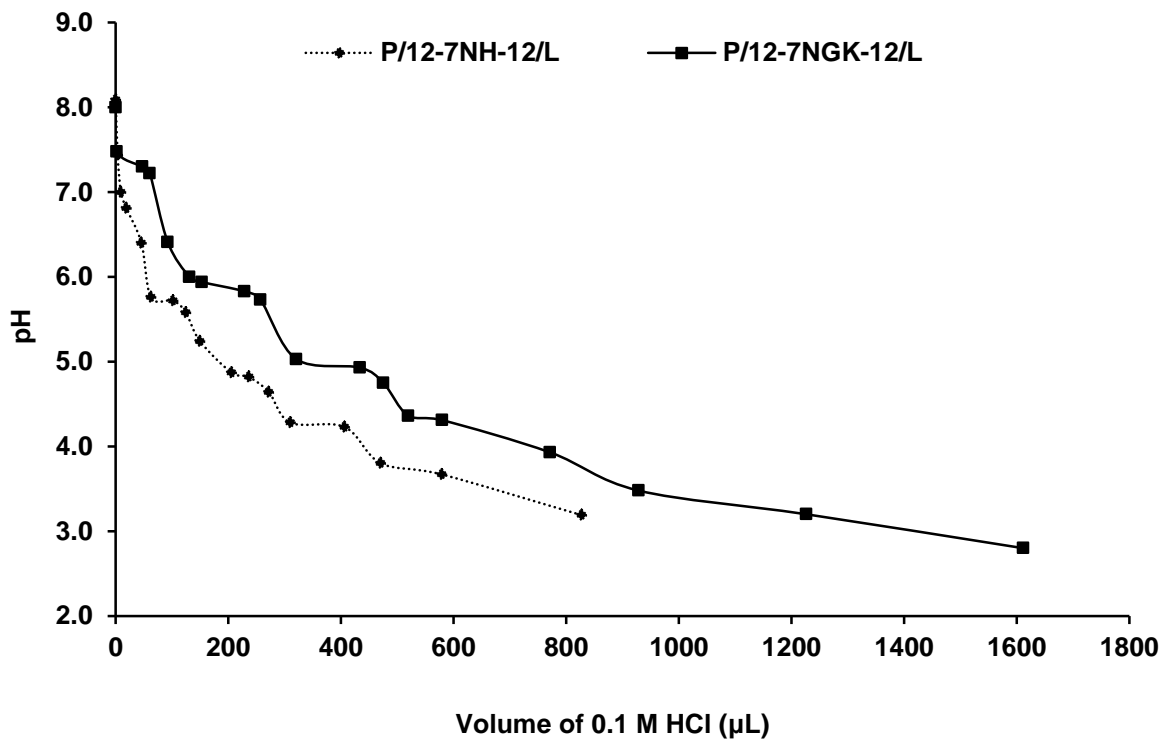


Figure 9.8: Comparison of buffering capacity of P/G/L nanoparticles. A pH titration curve of P/G/L nanoparticles exhibited considerably higher buffering capability of P/12-7NGK-12/L compared to P/12-7NH-12/L in neutral, slightly acidic and strongly acidic pH.

9.4.6. Interactions between the DNA and gemini surfactants

Ethidium bromide exclusion assay is a useful technique to study DNA/delivery vector interaction. The assay was performed in the presence of the polyanionic heparin, which competes with DNA to bind with the cationic gemini surfactants. The interaction between the gemini surfactants and heparin could cause a dissociation of the P/G/L complexes, allowing the ethidium bromide to intercalate in the DNA strand and emit fluorescence. Lower fluorescence values indicate stronger binding between DNA and gemini surfactants and a higher protection of the genetic material. Also, stronger binding properties ensure that DNA is not released prematurely, from the lipoplexes, upon interaction with negatively charged cell surface proteins.

At the 1:10 charge ratio of DNA to gemini surfactant in the presence of DOPE, a small increase in fluorescence ($8.9\pm 0.8\%$) was observed in P/12-7NGK-12/L nanoparticles upon addition of polyanions (5 U/mL heparin), exhibiting a stronger protection of DNA compared to the parent gemini surfactant which had an increase of $52.3\pm 2.3\%$ fluorescence (**Figure 9.9**). A similar trend occurred at higher concentrations of 10 U/mL and 20 U/mL of heparin. Interestingly, a steady increase in fluorescence was observed in P/12-7NGK-12/L nanoparticles with a significant spike in fluorescence ($32.9\pm 5.7\%$) occurring in the presence of 20 U/mL heparin. This indicates that 12-7NGK-12 has balanced DNA binding properties which ensures protection of DNA as well as an ability to interact with a highly negative charged membrane, such as the inside of caveolae endosomes. These balanced binding properties (protection and release) may be instrumental in efficient endosomal escape leading to higher transfection efficiency of P/12-7NGK-12/L compared to the P/12-7NH-12/L nanoparticles (**Figure 9.6**).

9.4.7. Transmission electron microscopy (TEM) imaging

Particle shape and particle aggregation behaviour can also influence the gene expression of nanoparticles. Micrographs of the P/12-7NH-12/L and P/12-7NGK-12/L nanoparticles showed consistency with the previous light scattering measurement, with particles of 100-150 nm in diameter being observed. The TEM images showed no aggregation behaviour of the particles (**Figure 9.10**). However, the P/12-7NGK-12/L nanoparticles were found to be more cylindrical shaped, while the P/12-7NH-12/L particles showed spherical morphology.

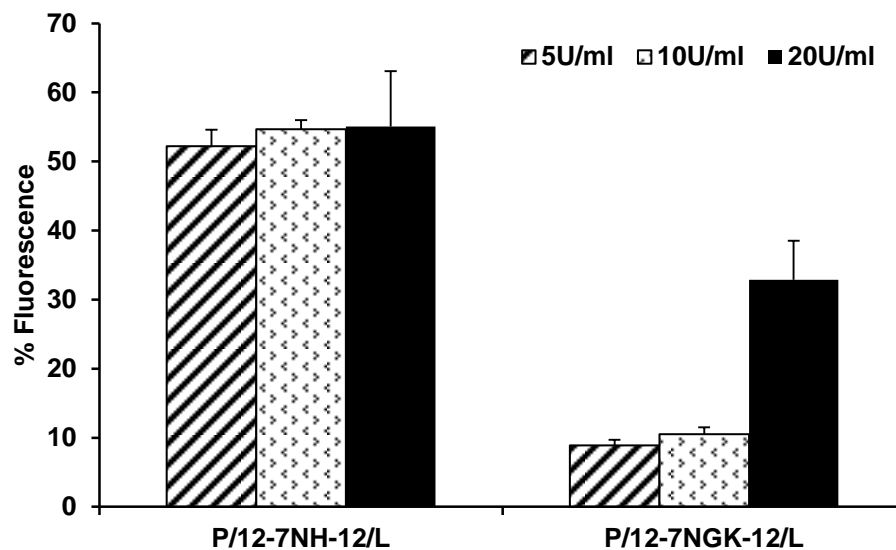


Figure 9.9: DNA-gemini surfactant binding properties. An ethidium bromide dye exclusion assay was performed to determine the binding efficiency of 12-7NH-12 and 12-7NGK-12 with plasmid DNA in the presence of DOPE and heparin (5 U/mL, 10 U/mL and 20 U/mL). At lower concentration of heparin (polyanions), amino-acid substituted gemini exhibited stronger DNA binding compared to unsubstituted gemini surfactant. At higher concentration (20 U/mL heparin), a significant increase in fluorescence was observed, indicating dissociation of DNA and 12-7NGK-12.

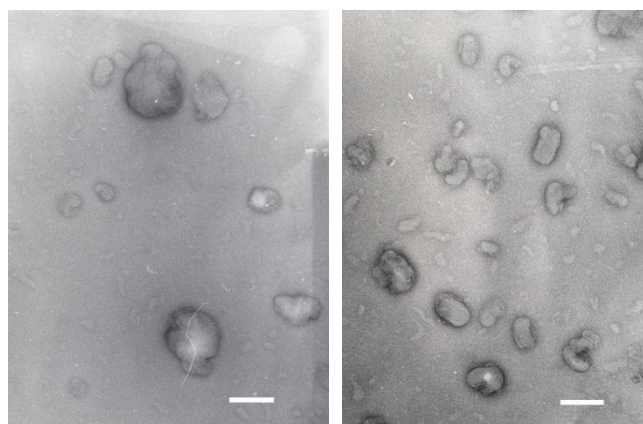


Figure 9.10: Transmission electron microscopy (TEM) images of P/G/L complexes. (A) P/12-7NH-12/L nanoparticles (B) P/12-7NGK-12/L nanoparticles. P/12-7NGK-12/L nanoparticles are cylindrical while P/12-7NH-12/L are spherical in shape. Scale bar corresponds to 200 nm.

9.5. Discussion

In non-viral gene delivery, cellular uptake of the DNA nanoparticles can influence the overall gene expression due to the number of particles that are internalized and the intracellular trafficking of the engulfed particle. In our previous work we found that glycyl-lysine substitution in the spacer region of the gemini surfactants improved gene expression, and the transfection efficiency correlated with DNA binding properties of gemini surfactants [223]. In this study, we evaluated whether the pathway of the cellular uptake of the P/12-7NH-12/L and P/12-7NGK-12/L nanoparticles in Sf 1 Ep cells is a major factor affecting the transfection efficiency of these novel delivery systems. Clathrin-mediated uptake, caveolae-mediated uptake and macropinocytosis are known to be three of the most important uptake pathways in mammalian cells [125]. Treatment with chemical inhibitors such as genistein, filipin, methyl- β -cyclodextrin, chlorpromazine, wortmannin, and potassium free buffer are routinely used to determine the effect of each pathway on cellular uptake of nanoparticles [137, 215, 219, 224, 225]. Upon treatment of the Sf 1 Ep cells with these chemicals at different concentrations, we selected those agents that showed high cell viability with no significant difference among the inhibitors, thus minimizing the impact of the agents on the overall gene expression. Conditions regarding concentration of the inhibitor and incubation time that retained cell viability greater than 80% were selected for further experiments (**Figure 9.3**). In previous studies, we found that the P/G/L nanoparticles were safe, inducing minimal toxicity (9-12%) to the cells [223]; however, the combined toxicity of P/G/L nanoparticles and chemical inhibitors/potassium depletion was unknown. The cell toxicity induced by the chemical treatment in the presence of P/G/L nanoparticles increased to 30-40% (**Figure 9.4**). Without a significant difference in the toxicity among various agents, the cellular uptake study in Sf 1 Ep cells was found to provide meaningful insight of the mechanism of uptake and shed light on the crucial relationship between the physicochemical parameters and the difference in the transfection efficiency of the parent and glycyl-lysine substituted gemini nanoparticles.

In the cellular uptake study using CLSM, it was observed that the inhibition of caveolae-mediated (genistein) or clathrin-mediated (chlorpromazine) uptake did not influence significantly the uptake of P/12-7NGK-12/L or P/12-7NH-12/L nanoparticles. However, no particles were internalized upon inhibition of both clathrin-mediated and caveolae-mediated pathways, simultaneously, by methyl- β -cyclodextrin (**Figure 9.5: D & I**). This suggests that P/G/L

nanoparticles were able to recruit both clathrin-mediated and caveolae-mediated uptake. In previous studies, cationic lipid-DNA complexes (using DOTAP and SAINT-2/DOPE) have been suggested to preferentially internalize via clathrin-mediated endocytosis [137, 226]. However, more recent studies emphasized that the physiochemical parameters of the DNA complexes (such as particle size, shape and surface chemistry) as well as cell type can influence the uptake behaviour [140, 149, 150]. Polyethylenimine (PEI)/DNA complexes were found to internalize via clathrin- or caveolae-mediated mechanisms depending on both PEI type (linear or branched) and cell line [140]. Interestingly, another study using cationic lipid, Amphiphile 1 and DNA complexes found macropinocytosis to be the major pathway leading to gene transfection in CHO cells [227]. Caveolae-mediated uptake was also found to be important for transfection of human serum albumin coated DOTAP/DOPE complexes [228]. In the present study, the role of caveolae-mediated uptake in cationic gemini surfactant based DNA nanoparticles was confirmed by a drop in gene expression in cells transfected with P/12-7NH-12/L in the presence of genistein and filipin, 54% (genistein) and 83% (filipin), compared to the control. Inhibition of clathrin-mediated uptake (chlorpromazine, potassium depletion) did not reduce the gene expression of P/12-7NH-12/L; on the contrary, it led to an increase (more than double) in gene expression. There may be two reasons for this: (a) inhibition of the clathrin-mediated uptake results in an up-regulation of other uptake pathways as observed in certain mammalian cell types [229] or (b) inhibition of the clathrin-mediated pathway leads to the availability of more nanoparticles to follow the caveolae-mediated uptake, or a combination of both. P/12-7NH-12/L nanoparticles following the intracellular route via clathrin-mediated uptake may not be contributing substantially in overall gene expression. No gene expression was observed upon inhibition of both clathrin-mediated and caveolae-mediated pathway by methyl- β -cyclodextrin. Macropinocytosis (wortmannin) had no significant effect on the gene expression in cells transfected with the P/12-7NH-12/L nanoparticles. While there was only a modest decrease in the gene expression in the P/12-7NGK-12/L transfected cells after inhibition of clathrin- or caveolae-mediated uptake, gene expression decreased by 93% when both pathways were blocked simultaneously by methyl- β -cyclodextrin. This suggests that P/12-7NGK-12/L nanoparticles can be internalized via either clathrin-mediated or caveolae-mediated routes and avoid degradation, in turn releasing DNA at appropriate time for successful gene expression.

Analysis of gene expressions also pointed to an interesting fact: glycyl-lysine substitution not only improved the overall transfection efficiency of P/12-7NGK-12/L but also the gene expression level via each route (clathrin-mediated or caveolae-mediated) was higher than P/12-7NH-12/L. This difference in gene expression may be due to the number of particles that are internalized or improved endosomal escape of P/12-7NGK-12/L particles. Particle size and shape may affect cellular uptake, significantly [150]. As reported previously, the particle size of the P/12-NGK-12/L and P/12-NH-12/L nanoparticles was 117.3 ± 0.6 nm and 133.0 ± 2.8 nm, respectively [199]. The smaller particle size of P/12-7NGK-12/L might be advantageous in improving the rate of cellular uptake. Rod-like or cylinder shaped particles were found to be internalized faster than the spherical particles [150]. The effect of cylindrical morphology is more significant on the cellular uptake when the aspect ratio (diameter to width) is high, especially in particles with diameter >150 nm [150]. TEM images showed that P/12-7NH-12/L particles were cylindrical while P/12-7NGK-12/L particles were spherical (**Figure 9.10**). A large number of particles ensure availability of more DNA particles inside the cell. However, this does not guarantee higher gene expression since the DNA can be degraded in the cytosol. Therefore, we further analyzed the important physicochemical properties that may be contributing to more efficient intracellular trafficking of these nanoparticles.

9.5.1. Glycyl-lysine substitution improves endosomal escape in clathrin-mediated pathway

Intracellular trafficking of an engulfed particle is influenced by the type of uptake. In clathrin-mediated endocytosis, the nanoparticles are entrapped in endosomes, transported to late endosomes, and finally merged with lysosomes for complete degradation. This latter step, degradation of the genetic material in the lysosomes, can significantly decrease the overall availability of plasmid DNA for translocation to the nucleus for gene expression [230]. However, several scenarios can be envisioned to overcome this challenge by utilization of an interesting phenomenon that happens during the transfer of cargo from early endosomes to late endosomes. In mammalian cells, there is an early drop in pH in the clathrin-coated endosomes as they travel towards the late endosomes, creating strong acid conditions before merging with the lysosomes[129]. These conditions can be used in an advantageous manner by designing pH-sensitive delivery vectors that can either absorb H^+ -ions or unfold (relax) in an acidic

environment. In the former case, a significant increase in H^+ -ions will trigger an increased inflow of aqueous medium leading to swelling and then bursting of endosomes, releasing the DNA nanoparticles into the cytoplasm [231]. In the latter case, the nanoparticles can unfold and increase their size, significantly, leading to a mechanical disruption of the endosomal membrane and rescuing the DNA from lysosomal degradation [94].

In the clathrin-mediated uptake, we found that buffering capacity (**Figure 9.8**) and particle size increment (**Figure 9.7**) over neutral to acidic pH may be two important properties that could be causing P/12-7NGK-12/L nanoparticles to escape more efficiently than the P/12-7NH-12/L, and lead to timely release of plasmid for nuclear localization and gene expression. In the slow but steady decrease of pH, the P/12-7NGK-12/L nanoparticles could absorb more protons triggering an inflow of Cl^- . The flow of ions also brings more fluid inside the endosomal vesicle, ultimately leading to its breakdown and release of the DNA into the cytoplasm. This scenario is supported by the fact that, at physiological pH, the size of the P/12-7NGK-12/L nanoparticles was 119.5 ± 4.8 nm which then increased significantly with a drop in pH (**Figure 9.7**). Maximum particle size of 144.8 ± 5.9 nm was found at strongly acidic conditions (\sim pH 4), a net increase of 21.2% compared to the original particle size. On the other hand, the parent compound had a particle size of 128.6 ± 0.6 nm at physiological pH with a maximum size of 136.7 ± 0.3 nm around 5-5.5 pH (**Figure 9.8**). This is an overall increase of only 6.3% compared to the original particle size which may not have been instrumental in endosomal escape. The decrease in pH in the endosomes could allow for additional interaction with the endosomal membrane and promote DNA escape/release (**Figure 9.11**). This increase in particle size could be an indication of the relaxation of the nanoparticles, facilitating the availability of more 12-7NGK-12 molecules for absorption of H^+ , thus, increasing overall buffering capacity of the system. The increase in particle size may also be an indication of reduced interaction between the DNA and 12-7NGK-12, facilitating DNA release from the complexes after endosomal escape, as suggested by increased ethidium bromide intercalation in the presence of high concentrations of polyanion (**Figure 9.9**).

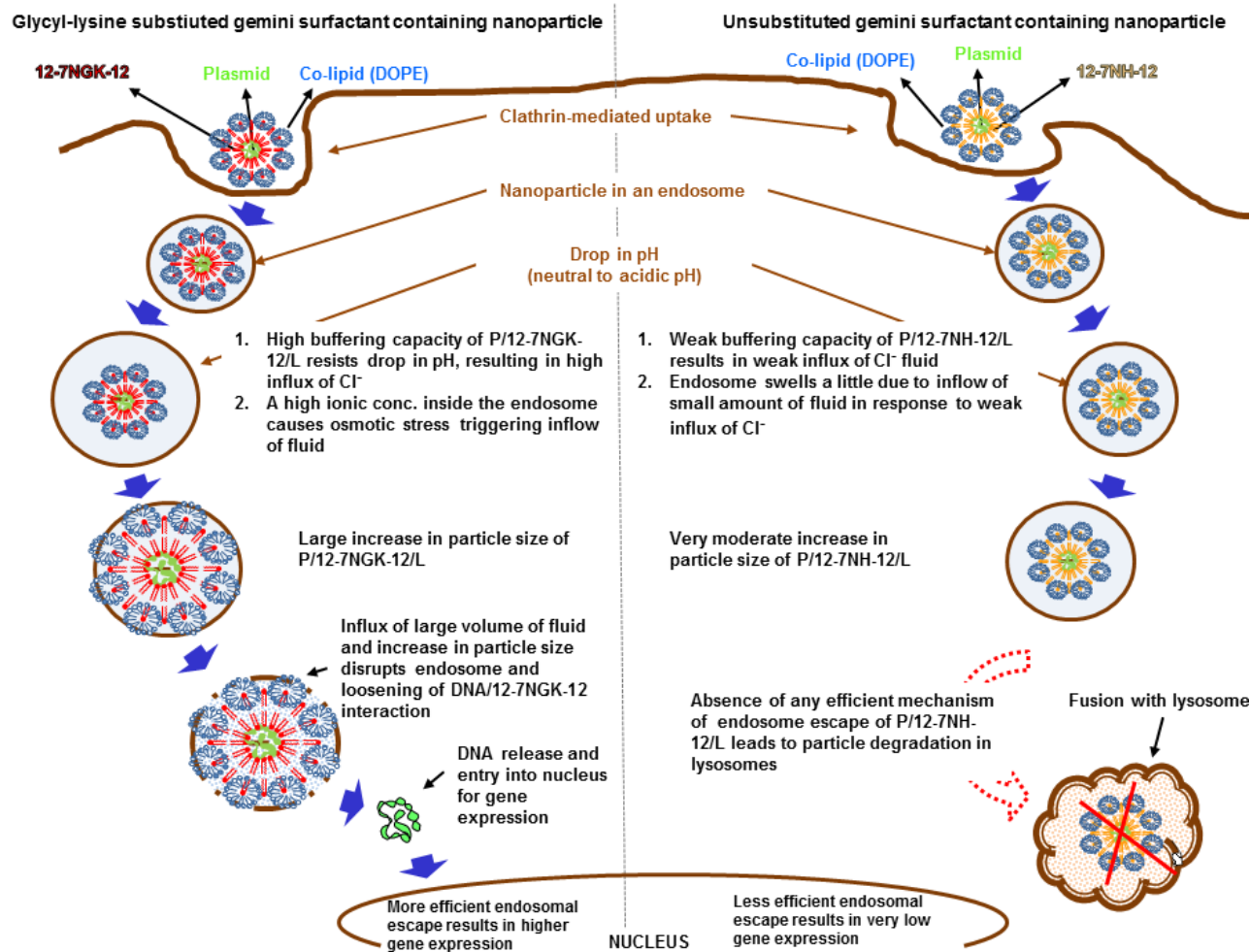


Figure 9.11: Intracellular trafficking of P/G/L complexes via clathrin-mediated pathway. A schematic comparison of crucial steps in endosomal escape of P/12-7NGK-12/L (on the left side of the image) and P/12-7NH-12/L (on the right side of the image) nanoparticles in clathrin-mediated cellular uptake.

9.5.2. Glycyl-lysine substitution improves DNA/gemini surfactant binding properties

Previously, we found that DNA/gemini surfactant interactions play a crucial role in the gene delivery ability of these molecules[223]. Conformational flexibility achieved by insertion of a glycine moiety as a linking molecule between the spacer region and terminal lysine leads to a balance between DNA binding and release behaviour of the P/12-7NGK-12/L nanoparticles. In this study, we further explored this idea in the context of intracellular trafficking following caveolae-mediated uptake of the P/G/L nanoparticles.

In caveolae-mediated uptake, there is no gradual pH transition that could influence the interaction between the gemini surfactants and DNA. Therefore, we mimicked this pathway by evaluating the behaviour of the nanoparticles in the presence of a polyanion (heparin) which could potentially compete with the DNA for the electrostatic interaction with the cationic charges on the gemini surfactants and determined its effect on DNA binding properties of the gemini surfactants (**Figure 9.12**). We found that low concentrations of heparin with P/12-7NGK-12/L did not affect binding of the gemini surfactant to DNA, significantly, compared to nanoparticles formulated with the parent gemini surfactant. The shielding is crucial for the initial interaction of P/G/L nanoparticles with the cell surface membrane and uptake, thus avoiding premature release of the DNA at the cell surface. However, at higher concentration of heparin, the accessibility of the DNA in P/12-7NGK-12/L lipoplexes increased significantly (**Figure 9.9**). This suggests that the 12-7NGK-12/L lipid system provides balanced DNA binding properties that enable the 12-7NGK-12/L system to protect the DNA as well as interact readily with the anionic proteins coating the inner surface of the caveolae-mediated vesicle (endosome). This balance may be instrumental in the efficient disruption of the vesicle membrane leading to DNA escape.

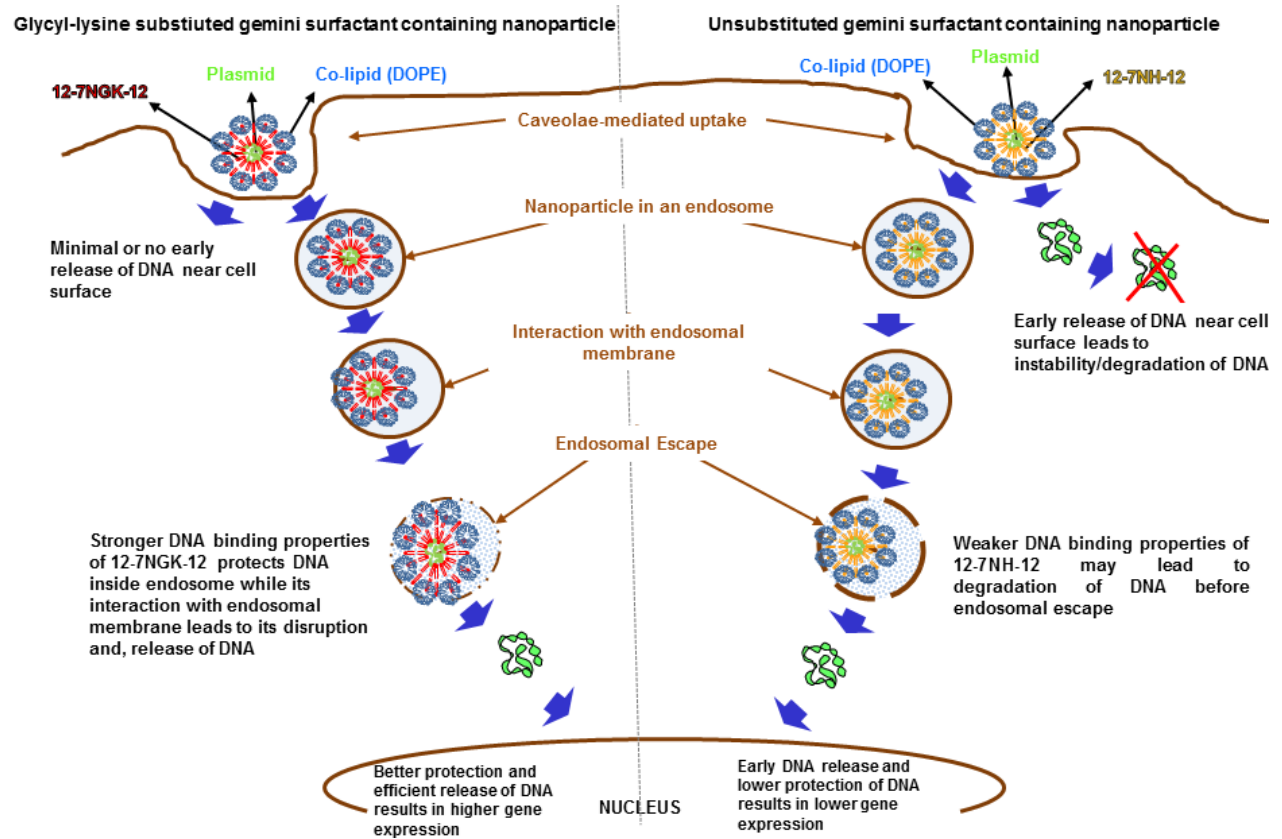


Figure 9.12: Intracellular trafficking of P/G/L complexes via caveolae-mediated pathway. A schematic comparison of crucial steps in endosomal escape of P/12-7NGK-12/L (on the left side of the image) and P/12-7NH-12/L (on the right side of the image) nanoparticles in caveolae-mediated cellular uptake.

9.6. Conclusion

Amino-acid substitution in the spacer of the gemini surfactant did not alter the cellular uptake pathway, showing a similar pattern to the unsubstituted parent gemini surfactant. Both clathrin-mediated and caveolae-mediated pathways were equally important routes of uptake for P/G/L nanoparticles. Glycyl-lysine substitution in the gemini spacer improved buffering capacity and imparted a pH-dependent increase of particle size. This property confers to the P/12-7NGK-12/L nanoparticles the ability to escape efficiently from clathrin-mediated endosomes and leads to higher transfection efficiency compared to the parent gemini surfactant. More balanced binding properties (protection and release) of P/12-7NGK-12/L in the presence of polyanions play an important role in the release of nanoparticles internalized via caveolae-mediated uptake. These factors (balanced binding properties and improved intracellular endosomal escape) will be taken into consideration in the design of further structural modifications, such as attachment of other basic amino acids via glycyl linkage to the gemini surfactants of the P/G/L nanoparticles.

9.7. Competing interests

The authors declare that they have no competing interests.

9.8. Author's contribution

Conceived and designed the experiments: JS, IB. Performed the experiments: JS, DM. Analyzed the data: JS. Contributed materials: JMC, REV. Wrote the paper: JS, IB. All authors read and approved the final manuscript

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10. Development of amino acid-substituted gemini surfactant-based mucoadhesive gene delivery systems for potential use as non-invasive vaginal genetic vaccination

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This manuscript presents the *ex vivo* evaluation of optimal amino acid-substituted gemini surfactant based gene delivery systems and formulation of these delivery systems for mucosal application. The optimized formulations were tested in rabbits by non-invasive administration into the vagina and the gene expression was evaluated. The results of this study were compared to *in vitro* results and overall conclusions were drawn. This manuscript concludes the overall objectives of my dissertation work. I conducted all experiments and prepared the manuscript.

This manuscript addresses my last two research objectives:

- To assess the extent of penetration of the DNA into the vaginal tissue using the amino acid-substituted gemini surfactant that showed the best performance *in vitro*.
- To develop novel mucosal delivery systems using the amino acid-substituted gemini surfactant with the best performance from *in vitro* evaluation.
- To evaluate gene expression in vaginal tissue after non-invasive vaginal application in a rabbit animal model.

10.1. Abstract

Diquaternary ammonium-based gemini surfactants have been utilized as gene delivery vectors. Recently, we developed amino acid- and peptide-substituted spacers of gemini surfactants, 'biolipids' that exhibited high transfection efficiency in rabbit epithelial cells. In this study, we developed non-invasive vaginal mucoadhesive gene delivery systems containing these gemini surfactants using poloxamer 407 and diethylene glycol monoethyl ether (DEGEE). Poloxamer at 16% w/v concentration in DEGEE aqueous solution produced dispersions that gelled near body temperature and had a high yield value, preventing leakage of the formulation from the vaginal cavity. Intravaginal administration in rabbits showed that the glycyl-lysine substituted gemini surfactant formulated in 16% poloxamer 407 led to a higher gene expression compared to the parent unsubstituted gemini surfactant. This provides a proof-of-concept that amino acid-substituted gemini surfactants can be used as non-invasive mucosal (vaginal) gene delivery systems with potential applications in diseases associated with mucosal epithelia.

Keywords: Gemini surfactant, non-viral gene delivery, mucosal, intravaginal administration, gelling temperature, gene expression.

10.2. Introduction

The female reproductive tract is vulnerable to a wide range of sexually transmitted pathogens such as human immunodeficiency virus, human papillomavirus and herpes simplex virus. For protection, an effective local immune response at the first point of contact i.e., cervical epithelium, should detect and deter any pathogenic entry through this route. The traditional vaccine approaches, such as denatured viral protein, fail to retain proper conformation of antigenic epitopes required for a stronger humoral and cellular immune response [232]. Recently, plasmid DNA based vaccines used to generate antigenic proteins *in situ*, were evaluated as a novel approach for inducing strong immune protection against these pathogens. Plasmid DNA vaccine can present these antigens in a similar conformation and repetitive ordered arrangement as that of native viral epitopes [233]. This would help to induce cytotoxic T cell lymphocyte protection against virus particles that may escape from recognition by IgG or IgA antibodies. In addition, vaginal gene delivery systems have the potential to improve cancer treatment (such as cervical cancer) and can be utilized in conditions such as congenital dyskeratosis and lichen planus which affect both the oral and vaginal mucosa [175, 176].

Some of the advantages of vaginal gene delivery are that the vaginal mucosa offers a relatively large surface area for drug permeation, high vascularization, a low enzymatic activity and ease of access with potential for self-administration [171, 234]. Nevertheless, intravaginal delivery of plasmid DNA suffers from poor permeability into tissues, degradation by enzymes, dilution or entrapment in the mucous, and leakage from the vagina [171]. To address some of the deficiencies of gene delivery using non-viral vectors, we have designed and developed a series of gemini surfactant-based nanoparticles.

Cationic gemini surfactants have shown promising results as non-viral gene delivery agents due to their structural versatility and polymorphism [7-9, 182, 235]. A number of modifications have been introduced in the cationic gemini surfactants to improve their transfection efficiency and to reduce toxicity. Recently, we designed amino acid-functionalized gemini surfactants, ‘biolipids’ and evaluated their ability to form nanostructures in the presence of DNA, their efficiency to deliver the genetic material into the cells and compared their relative safety to previous generations of gemini compounds *in vitro* [10, 199, 223]. Among these derivatives, the glycyl-lysine substituted spacer of a gemini surfactant (12-7NGK-12, **Figure 10.1A**) exhibited the highest gene expression among the series, performing significantly better than its parent

compound (12-7NH-12, **Figure 10.1B**). Cellular uptake studies revealed that the glycyl-lysine substitution in the spacer of a gemini surfactant did not alter the cellular uptake pathway; however, it imparted a high buffering capacity, a pH-dependent particle size increase and balanced binding properties, leading to high gene expression [236].

The *in vitro* studies were carried out in rabbit epithelial cells as a cellular model to develop non-invasive vaginal (mucosal) formulations [199, 223]. To further overcome barriers to intravaginal delivery, permeation enhancers in combination with *in situ* gelling polymers, such as poloxamers, were used to increase the residence time of the formulation and penetration of plasmid DNA in the vaginal cavity, thereby, increasing delivery efficiency [172, 174].

In this study, we reported the development of vaginal formulations that incorporate the DNA/biolipid nanoparticles using a gelling agent, poloxamer 407, and permeation enhancer diethylene glycol monoethyl ether (DEGEE). The composition was optimized to achieve temperature-dependent in-situ gelling. Finally, the formulations containing the model plasmid were tested in rabbits and their gene expression was determined using confocal microscopy.

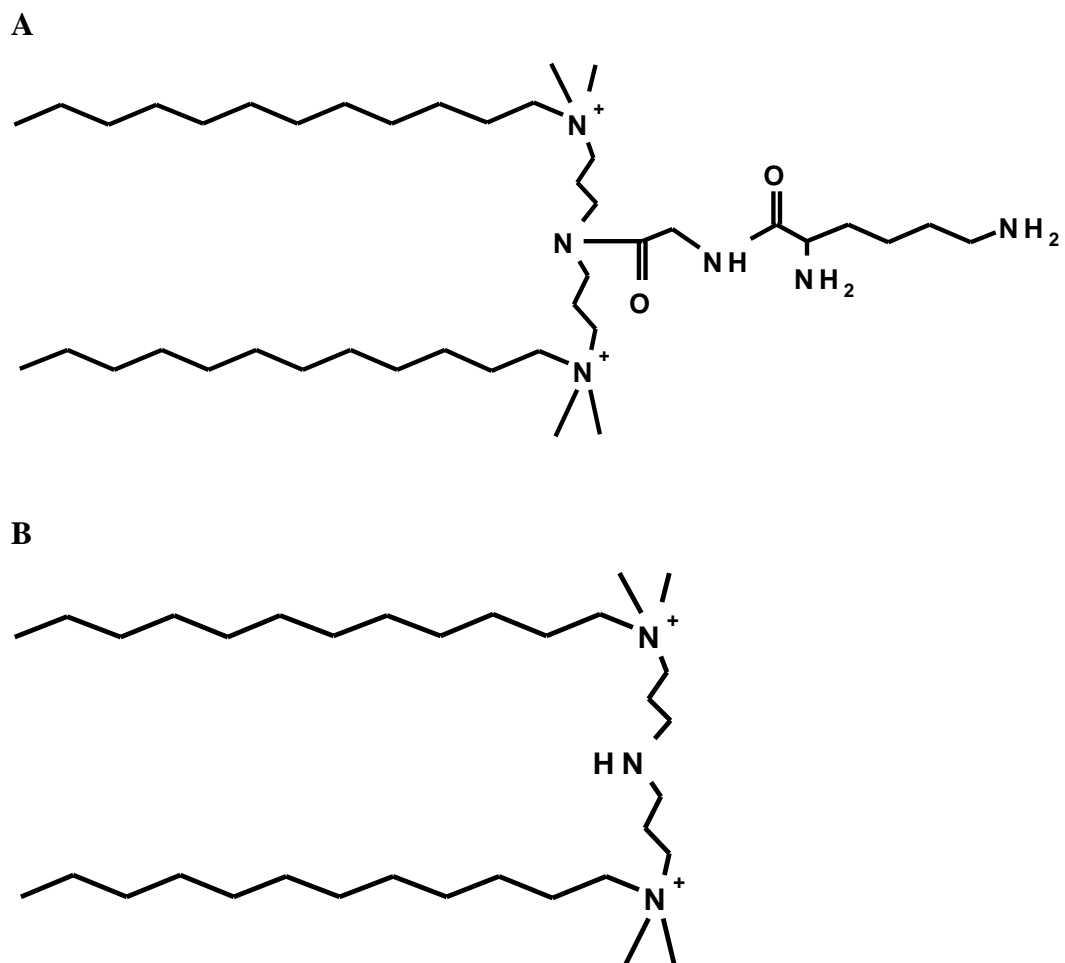


Figure 10.1: Chemical structure of 12-carbon tail gemini surfactants. (A) glycyl-lysine substituted gemini surfactant (12-7NGK-12) and (B) Parent unsubstituted gemini surfactant (12-7NH-12).

10.3. Materials and methods

10.3.1. Plasmid construction

A model plasmid was constructed by inserting tdTomato gene, cloned from commercially available pCMV-tdTomato (Clontech Laboratories, Inc., Mountain View, U.S.A.), into previously optimized pGT backbone [9]. Forward primer 5'-AGAATTCCGCCACCATGGTGAGCAAGGGC-3' and reverse primer 5'-AGTAGATCTCTACTTGTACAGCTCGTCCATGC-3' were used to amplify the tdTomato gene sequence. Purified tdTomato segment and pGT were digested, separately, in presence of restriction endonucleases – EcoR1 and Bgl II; both purchased from New England Biolabs, Canada. The tdTomato fragment was ligated into the pGT vector and amplified in a bacterial system. The gene expression (red fluorescent protein) of plasmid pGT.tdTomato was tested in rabbit epithelial cells using fluorescence microscopy (Olympus IX71 inverted microscope, Olympus Corporation, Japan).

10.3.2. Formulation development

The synthesis and characterization of these gemini surfactants were reported previously [199, 237, 238]. For the *ex vivo* study, gemini surfactants were formulated with a model plasmid, pGTmCMV.IFN-GFP, expressing interferon gamma (IFN) and green fluorescent protein (GFP) in a bicistronic system in the presence of a helper lipid, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE; Avanti Polar Lipids, AL, U.S.A.), creating plasmid/gemini surfactant/lipid (P/G/L) nanoparticles [9]. DNA/gemini surfactant complexes were prepared at a charge ratio (-/+) of 1:10, incorporated in 2mM DOPE and dispersed by sonication (as used for *in vitro* transfection [14]).

Poloxamer 407 (Pluronic® F127) was purchased from Spectrum Chemicals, Toronto, Canada. A series of poloxamer 407 dispersions (15% w/v, 16% w/v, 16.5% w/v, 17% w/v, and 18% w/v) were prepared by mixing the polymer with water and diethylene glycol monoethyl ether (Transcutol® P, Gattefossé, Toronto, Canada) at 10% v/v of total formulation. The polymer dispersions were incubated at 4 °C for 24 hours to ensure complete hydration [239].

For animal studies, gemini surfactants were formulated with plasmid, pGT.tdTomato, in the presence of a helper lipid, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE; Avanti Polar

Lipids, AL, U.S.A.), creating plasmid/gemini surfactant/lipid (P/G/L) complexes. Plasmid DNA/gemini surfactant complexes were prepared at a charge ratio (-/+) of 1:10 and incorporated with DOPE at DOPE/gemini surfactant molar ratio of 8.3; as used in previous *in vitro* studies [14]. DOPE was dispersed by sonication in poloxamer/DEGEE/water and incorporated into plasmid DNA/gemini surfactant complexes. The formulation was incubated at 4 °C for 24 hours to ensure complete hydration of poloxamer in the formulation.

10.3.3. Rheology

The rheological properties of poloxamer dispersions were determined using a digital rheometer (DV-III Programmable Rheometer, Brookfield, Middleboro, U.S.A.). The measurements were performed in a cone-and-plate jacketed system (cone diameter of 24 mm, cone angle 1.565°) at shear rates of 1-50 sec⁻¹. The measurements were performed at 37±0.2 °C to simulate the effect of normal body temperature on the rheological behaviour of the poloxamer dispersion. The sample was incubated for 5 minutes at 37 °C before measurements. The rheological behaviour of the formulation was evaluated by plotting shear stress versus shear rate. A yield value, minimum force required to induce flow, of the polymer dispersions was calculated by extrapolating the curve at low shear rates (in the linear range).

The reversible sol-gel transition temperature of the poloxamer dispersions was determined by increasing temperature from 15 °C to 44 °C and measuring viscosity at a shear rate of 5 sec⁻¹.

10.3.4. *Ex vivo* permeation

This work was approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

Glycyl-lysine substituted gemini surfactant, exhibiting the highest gene expression *in vitro*, was selected for further formulation development for studies in the rabbit model.

Vaginal tissues were collected from fresh carcasses of seven New Zealand White rabbits available at Lab Animal Services Unit (University of Saskatchewan, Saskatoon, Canada) and mounted in an in-line diffusion cell apparatus (PermeGear Inc., Hellertown, U.S.A.) with the mucous surface facing the donor compartment. The diffusion cells were maintained at 37 °C. The tissue samples were allowed to equilibrate for 10 minutes by circulating fresh PBS buffer in the receptor compartment, maintained at 37 °C, through the diffusion cells. The buffer solution

was circulated with a peristaltic pump (Model IPC, Ismatec, Germany) at a flow rate 30 $\mu\text{L}/\text{min}$ and collected in a tube. Formulations were prepared, as described, using pGT.tdTomato plasmid and were placed in the receptor compartment on the mucous surface of the tissue. A total of 2 μg DNA per well (exposed tissue surface area of 0.63 cm^2) was used. After 24 hours, the tissue samples were removed from the in-line diffusion cell and washed 5-6 times with PBS buffer. The tissue sample surface that was not exposed to the formulation was trimmed and the samples were stored at -80 $^{\circ}\text{C}$.

For sample analysis, the tissue samples were incubated in 22 Kunitz/mL DNase I solution (Bio-Rad Laboratories, Mississauga, Canada) for 10 minutes at 37 $^{\circ}\text{C}$ to remove any traces of DNA from the sample surface. The enzyme was inactivated by heating at 95 $^{\circ}\text{C}$ for 5 minutes, and then cooled on ice. A 25 mg of tissue was weighed from each sample and total DNA was extracted using QIAamp DNA Mini Kit (Kit (Qiagen Inc., Toronto, Canada). A 2 μg quantity of total DNA was used for estimation by amplifying kanamycin-resistance gene using nested PCR. External primers, 5'–ACT CAC CGA GGC AGT TCC AT–3' and 5'–GGT AGC GTT GCC AAT GAT GT–3' amplified a 540 bp fragment. DNA bands were separated on 1% agarose gel and images were taken using AlphaImager (ProteinSimple, Toronto, Canada). The 540 bp fragments were purified from the gel using PureLink™ Quick Gel Extraction Kit (Invitrogen, Burlington, Canada). The second set of primers, 5'–ATG GCA AGA TCC TGG TAT CG–3' and 5'–TTA TGC CTC TTC CGA CCA TC–3', amplified a 459-bp fragment within the previous fragment. The presence of 459 bp fragments confirmed the amplification of the correct gene sequence.

10.3.5. Animal Study

This work was approved by the University of Saskatchewan's Animal Research Ethics Board and adhered to the Canadian Council on Animal Care guidelines for humane animal use.

Twenty female New Zealand White rabbits (Charles River Laboratories, Canada) weighing around 2.4-2.7 kg were randomly divided into four groups (n=5 per group). Group 1 (control) was treated with poloxamer/DEGEE, group 2 with plasmid DNA/poloxamer/DEGEE, group 3 with formulation containing parent gemini surfactant, and group 4 was treated with formulation containing glycyl-lysine substituted gemini surfactant. Thermosensitive formulations (100 μL

containing 100 µg pGT.tdTomato plasmid for each animal) were administered intravaginally using plastic feeding tube (18ga X 30mm, Instech Laboratories, PA, U.S.A.) connected to a 1 mL syringe. Control animals were treated with 100 µL of 16% w/v poloxamer/DEGEE in water. The animals were manually restrained during the application and for additional 2 minutes to allow the formulation to gel. Treatments were repeated for four consecutive days with a 24-hour interval. Each animal received a total dose of 400 µg pGT.tdTomato plasmid. On the fifth day, the animals were anesthetized with isoflurane and 30 mL of blood was collected by cardiac puncture. The animals were euthanized under anesthesia and tissue samples (vagina, lymph nodes, spleen) were collected. Tissue samples were stored at -80 °C before analysis. The gene expression was determined using confocal microscopy.

10.3.6. Confocal laser scanning microscopy (CLSM)

Tissue-TEK O.C.T. and Tissue-Tek cryomold were purchased from Sakura Finetek USA, Inc., Torrance, U.S.A. Rabbit vaginal tissue samples were frozen in Tissue-Tek O.C.T. using Tissue-Tek cryomold. Sections (30 µm) of the tissue were placed on lysine treated slides and coverslips were mounted with Prolong Gold antifade reagent containing DAPI (Life technologies Inc., Burlington, Canada). DAPI was used for cell nucleus staining. Images were acquired using a Leica TCS SP5 laser scanning confocal microscope (Leica Microsystems Inc., Bensheim, Germany) and LAS AF Lite 2.4.1 (Leica Microsystems CMS GmbH) was used for image processing. Excitation/Emission wavelengths for tdTomato (red fluorescence) were 554/581 nm.

10.4. Results

Nanoparticles prepared by glycyl-lysine substituted gemini surfactant have shown significantly higher gene expression than unsubstituted parent gemini surfactant in rabbit epithelial cells, Sf 1 Ep Cells [223]. In the long term, the plan is to develop these nanoparticles into non-invasive mucosal DNA-based vaccines for human sexually transmitted infectious diseases. Therefore, we formulated the nanoparticles for vaginal administration in rabbit as an animal model. We optimized the polymer concentrations and included a permeation enhancer for easy administration and improved *in vivo* delivery of the P/G/L particles.

10.4.1. Plasmid construction

As a model, a plasmid DNA was built by inserting the tdTomato gene into a plasmid backbone, pGT, previously used in our lab [9]. The pGT.tdTomato structure was confirmed by plasmid digestion (Supplementary information S1). Fluorescence microscopic evaluation of gene expression of pGT.tdTomato in rabbit epithelial cells showed red fluorescent cells, confirming the correct construction of plasmid (**Figure 10.2**).

10.4.2. Evaluation of rheological properties and gelation temperature

To prevent the loss of the nanoparticles by leakage from the vaginal cavity, poloxamer 407 was incorporated as a vehicle to induce *in-situ* temperature-dependent gelling. Poloxamer dispersions containing 16-20% w/v poloxamer 407 have been reported to gel between room temperature and body temperature [240]. We evaluated the rheological properties of poloxamer 407 gels in the 15-18% w/v concentrations in the presence of 10% v/v DEGEE aqueous solution.

An increase in polymer concentration resulted in a decrease in gelling temperature of poloxamer dispersions (**Figure 10.3**). High concentrations of polymer, 18% and 17% in DEGEE/water, gelled near room temperature at 22 °C and 25 °C, respectively. A lower concentration of polymer containing 15% poloxamer produced dispersions that exhibited sol-gel transition at 35 °C, near normal body temperature. A polymer concentration range between 16-16.5% imparted a sol-gel transition temperature around 28 °C providing an optimal temperature gap between ambient temperature of administration and *in situ* gelling temperatures. Based on these results, we excluded the 18% poloxamer dispersion, which gelled at 22 °C, from further rheological evaluation.

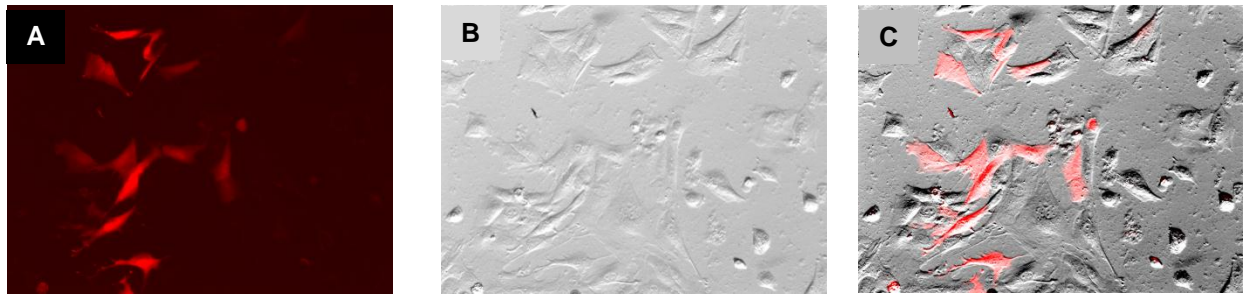


Figure 10.2: Gene expression of pGT.tdTomato in Sf 1 Ep cells. Fluorescent (A), white light (B) and overlay (C) images of rabbit epithelial cells electroporated with pGT.tdTomato (encoding red fluorescent protein).

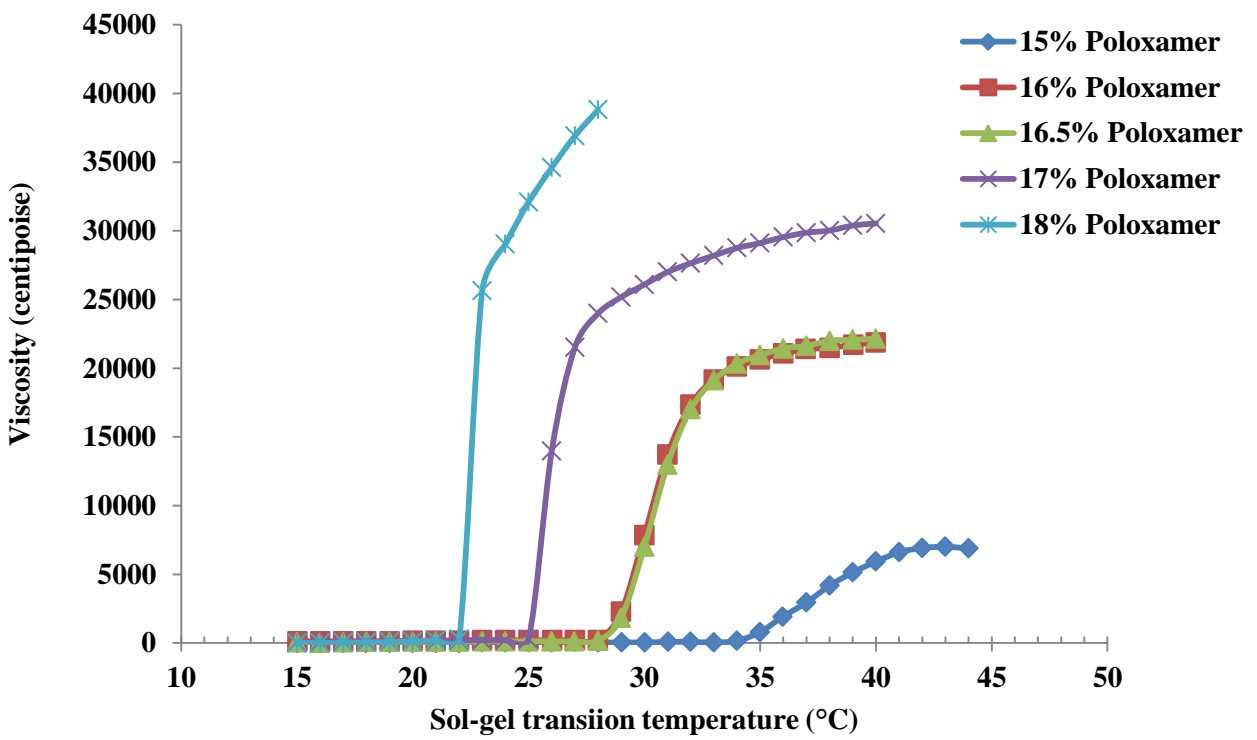


Figure 10.3: Sol-gel transition temperature. Effect of poloxamer 407 concentration on gelling temperature of polymer dispersion in 10% v/v DEGEE aqueous solution.

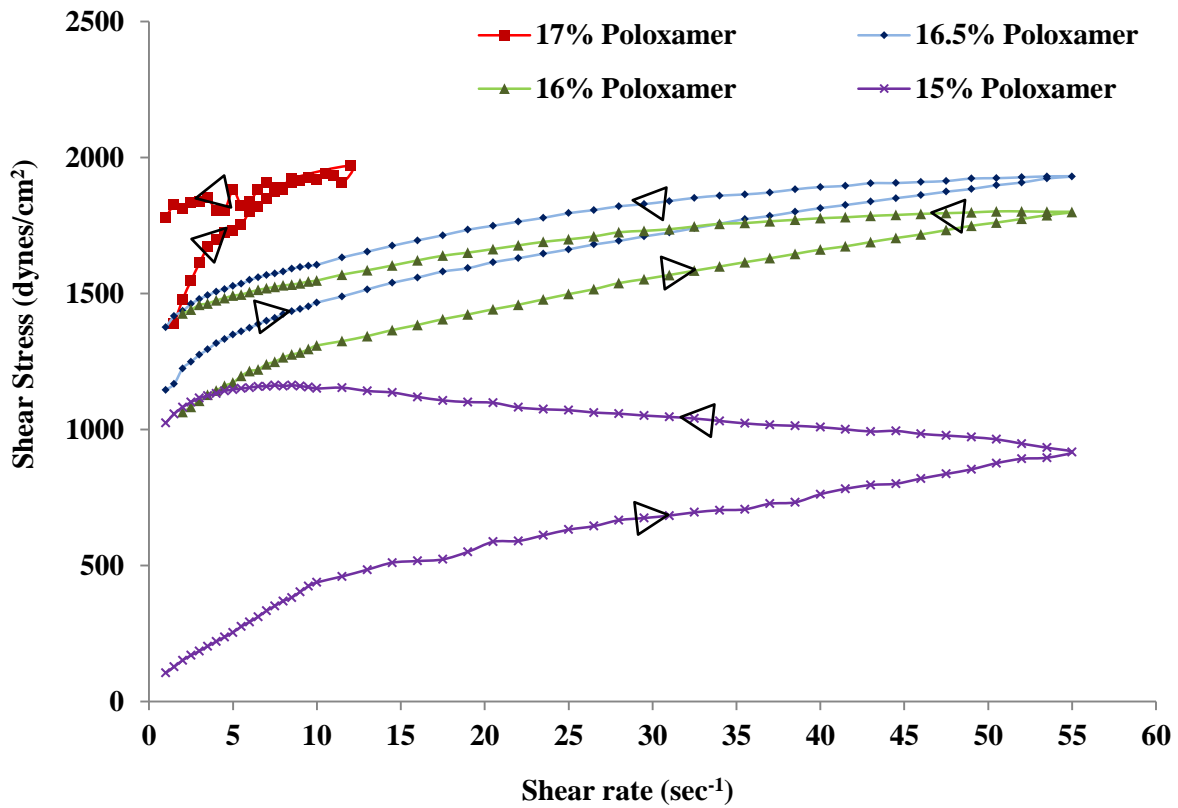


Figure 10.4: Rheogram of poloxamer dispersions in DEGEE aqueous solution at 37 °C. Poloxamer dispersions exhibited a pseudo plastic behaviour, showing an increase in shear stress with an increase in shear rate. \blacktriangleright indicates increasing shear rate and \blacktriangleleft indicates decreasing shear rate.

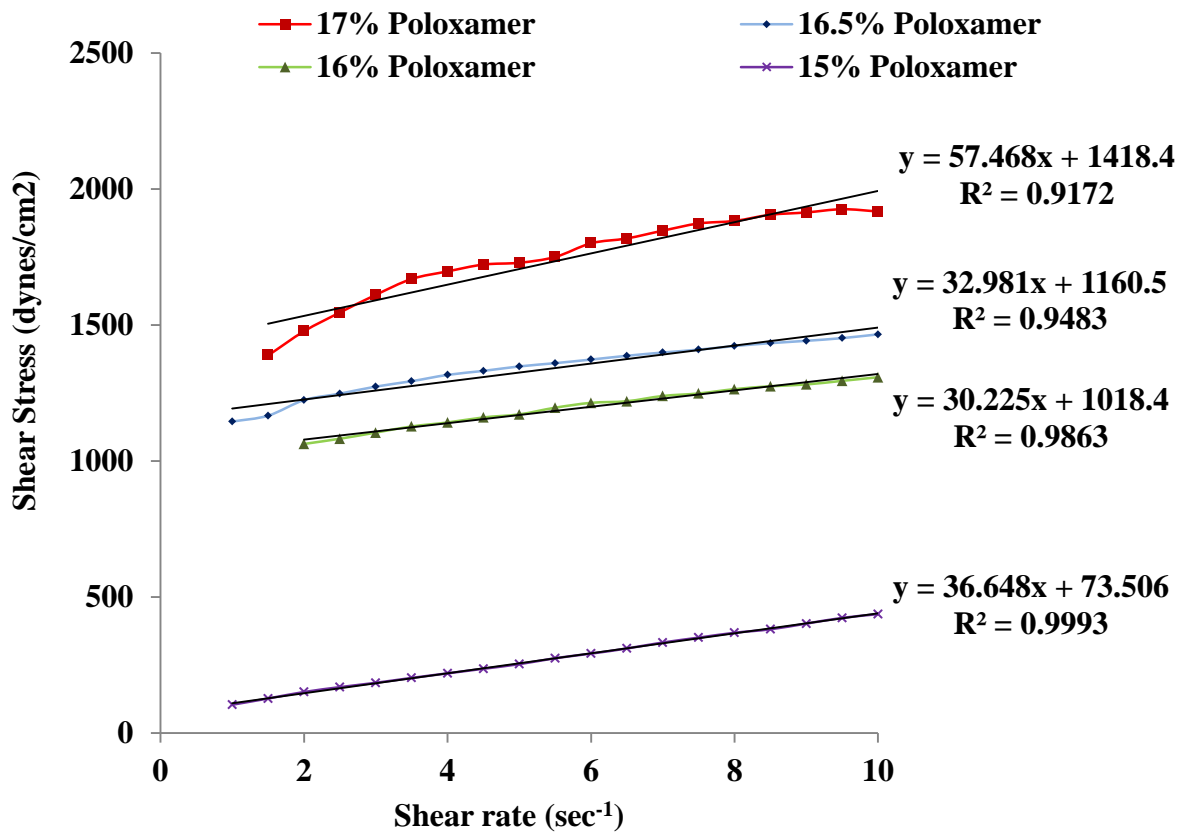


Figure 10.5: Rheogram of poloxamer dispersions at low shear rate (1-10 sec⁻¹) at 37 °C. Shear stress- shear rate rheograms of 16%, 16.5% and 17% poloxamer dispersions in DEGEE aqueous solutions exhibited a yield value > 900 dynes/cm² while it was low (~73 dynes/cm²) for 15% poloxamer dispersion.

At body temperature, above the gelling temperature, poloxamer dispersions exhibited a non-newtonian pseudoplastic behaviour (Figure 11.4). The dispersions behaved as a fluid on applying shear stress and regained consistency rapidly upon lowering the shear rate, indicating thixotropic behaviour. Flow curve analysis also showed that a yield value greater than 900 dynes/cm² is required to induce flow in polymer dispersions with poloxamer 407 concentrations greater than 16% (Figure 11.5). The 15% poloxamer dispersion in DEGEE aqueous solution had a yield value ~73 dynes/cm². Since the 16% poloxamer dispersions achieved sufficient viscosity and considerable high yield stress of 1018 dynes/cm², this concentration was selected to incorporate the P/G/L nanoparticles for further development as a mucosal gene delivery system.

Lipoplexes built with the unsubstituted gemini surfactant in 16% polymer dispersion in DEGEE aqueous solution exhibited sol-gel transition temperature around 27 °C which was 2 °C lower than the polymer in DEGEE/water alone (**Figure 10.6**). However, the incorporation of lipoplexes containing glycyl-lysine substituted gemini surfactant significantly raised this transition temperature to 34 °C. We further explored the effect of the presence of lipoplexes on the flow properties of these formulations at ambient temperature and body temperature. Similar to the polymer vehicle, at room temperature, both formulations exhibited low viscosity and good flow. On increasing the temperature to body temperature, the formulation exhibited a high viscosity above the gelling point. Both formulations showed a non-Newtonian pseudoplastic behaviour. Flow curve analysis of gemini surfactant formulations was conducted in a range of shear rates of 0.2 to 6 sec⁻¹. We observed a decrease of yield value to ~773 dynes/cm² for the unsubstituted gemini surfactant formulation in comparison to 1018 dynes/cm² for the 16% polymer dispersion without lipoplexes (**Figure 10.7A**). The glycyl-lysine substituted gemini surfactant formulation retained high yield value of ~1051 dynes/cm², comparable to 16% polymer dispersion without lipoplexes (**Figure 10.7B**).

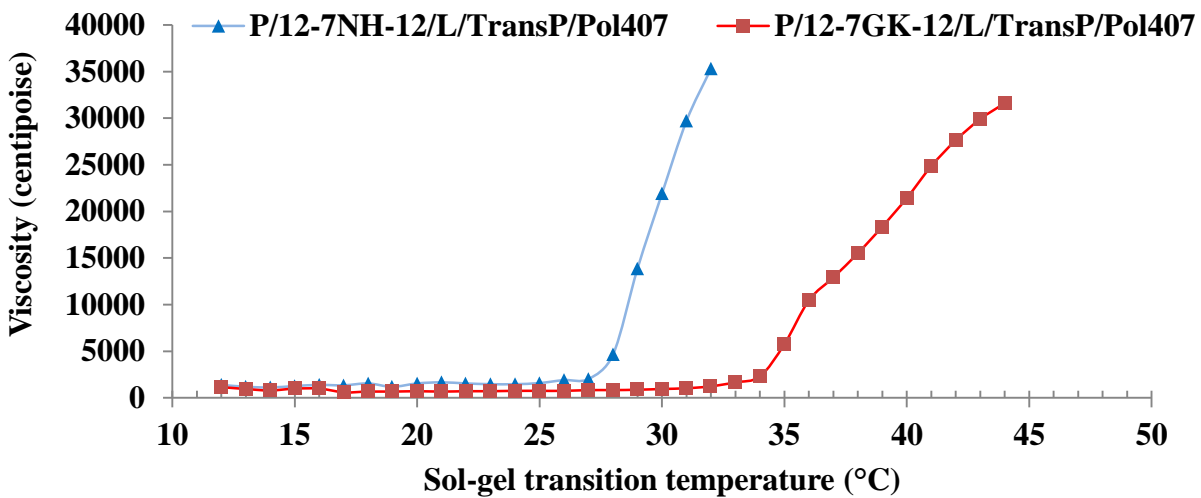
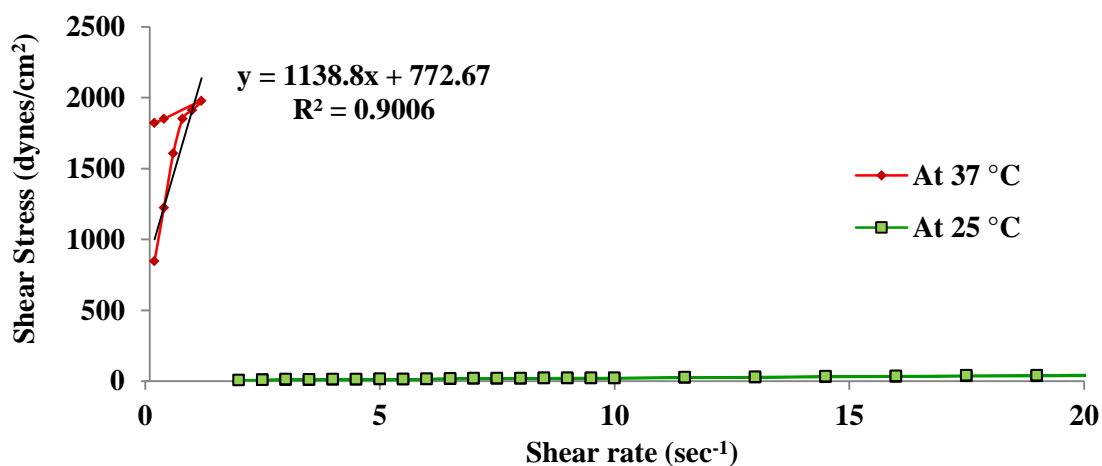


Figure 10.6: Sol-gel transition temperature. Formulation containing the parent gemini surfactant gelled at 27 °C while formulation containing glycyl-lysine substituted gemini surfactant gelled near 34 °C.

A



B

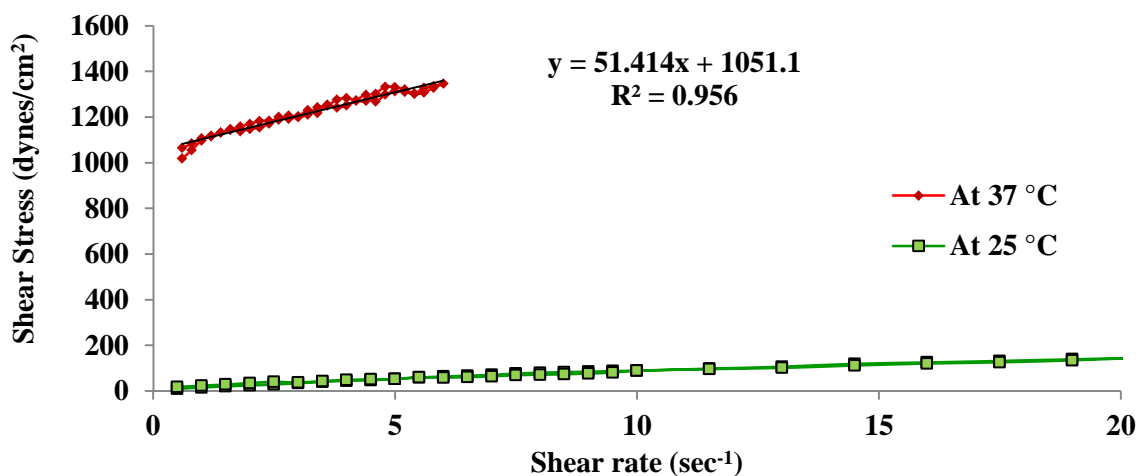


Figure 10.7: Rheogram of formulations (A) P/12-7NH-12/DEGEE/Pol407 (B) P/12-7NGK-12/DEGEE/Pol407. At 37 °C, the formulation containing parent gemini required a yield value of about 773 dynes/cm² while for formulation containing glycyl-lysine substituted gemini required a yield value of 1051 dynes/cm² to induce flow.

10.5. Evaluation of gene delivery systems in *ex vivo* model

Penetration study of P/G/L nanoparticles was conducted *ex vivo* in fresh rabbit vaginal tissue. The delivery of plasmid DNA into the tissue was determined qualitatively by amplifying kanamycin resistance gene sequence present in our plasmid. The 540 bp long fragments (**Figure 10.8A**) were purified from the gel and a nested PCR was performed using another set of primers. The presence of 436 bp fragments in the gel confirmed the correct DNA sequence, unique to our plasmid (**Figure 10.8B**). The *ex vivo* penetration study suggested that both glycyl-lysine-substituted and unsubstituted parent gemini surfactants were able to deliver plasmid DNA into the rabbit vaginal tissue.

10.5.1. *In vivo* gene delivery

Confocal microscopy revealed no fluorescent cells in vaginal tissue samples collected from untreated animals and animals treated with naked DNA, only (**Figure 10.9**). A limited number of fluorescent cells were observed in the tissue of the animals treated with formulations containing the unsubstituted (parent) gemini surfactant (12-7NH-12). In comparison to the parent compound, the vaginal tissue of the animals treated with formulations containing glycyl-lysine substituted gemini surfactant (12-7NGK-12) showed more red fluorescent cells

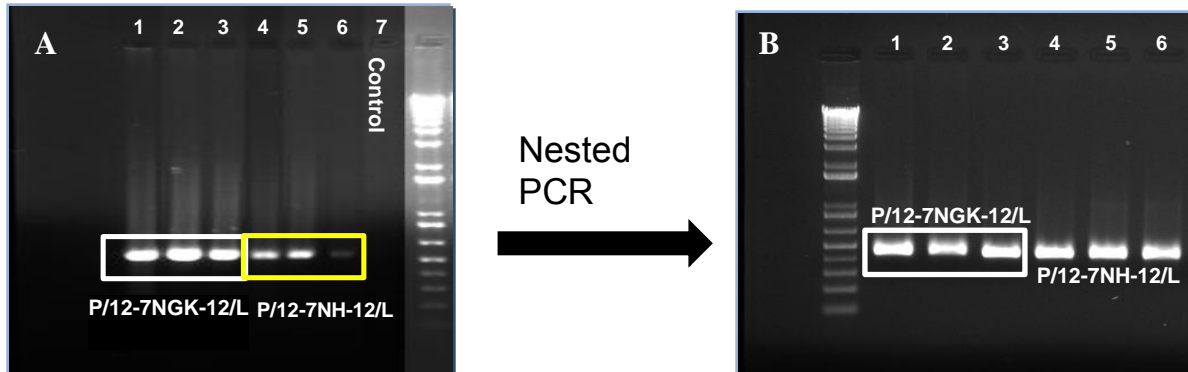
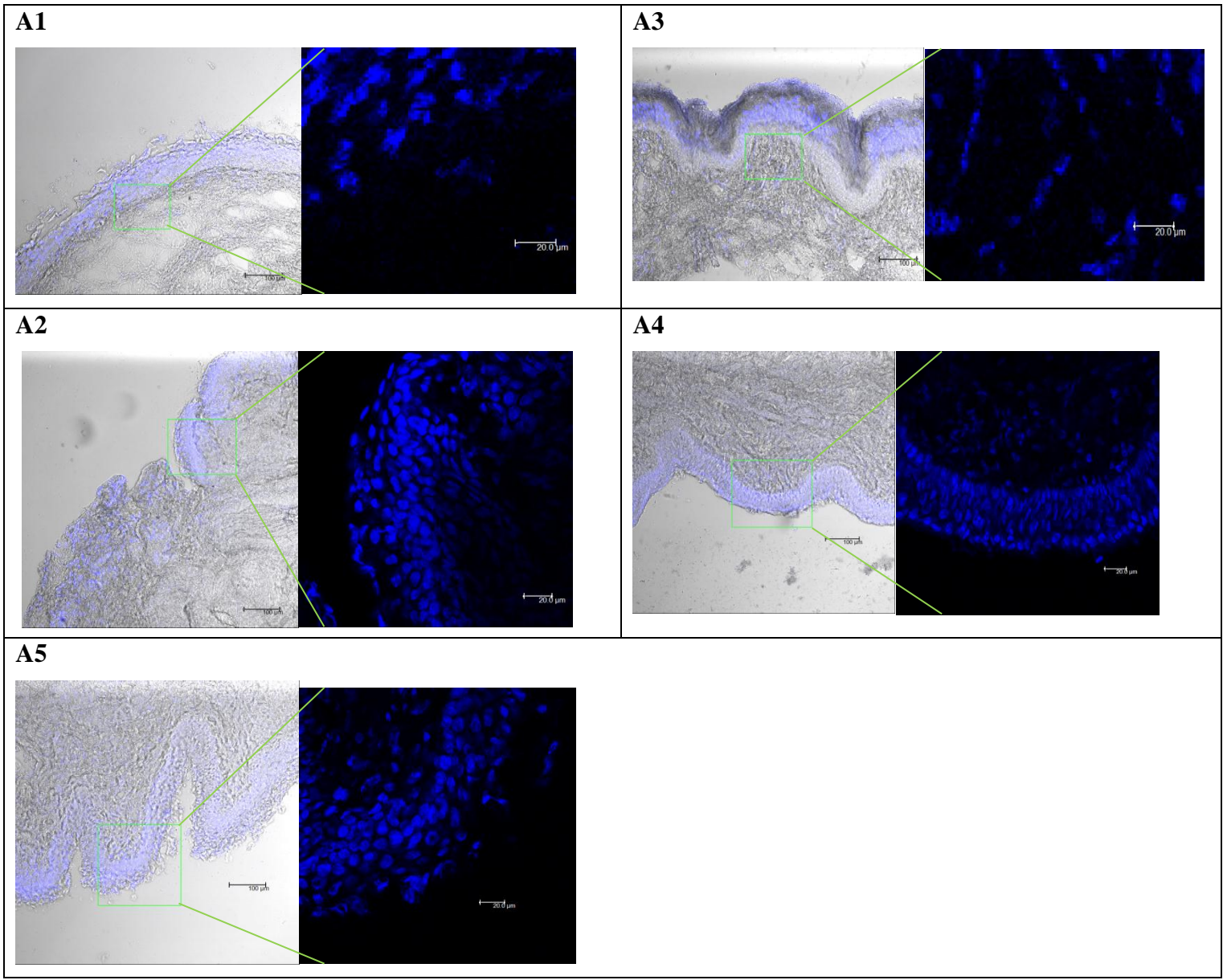
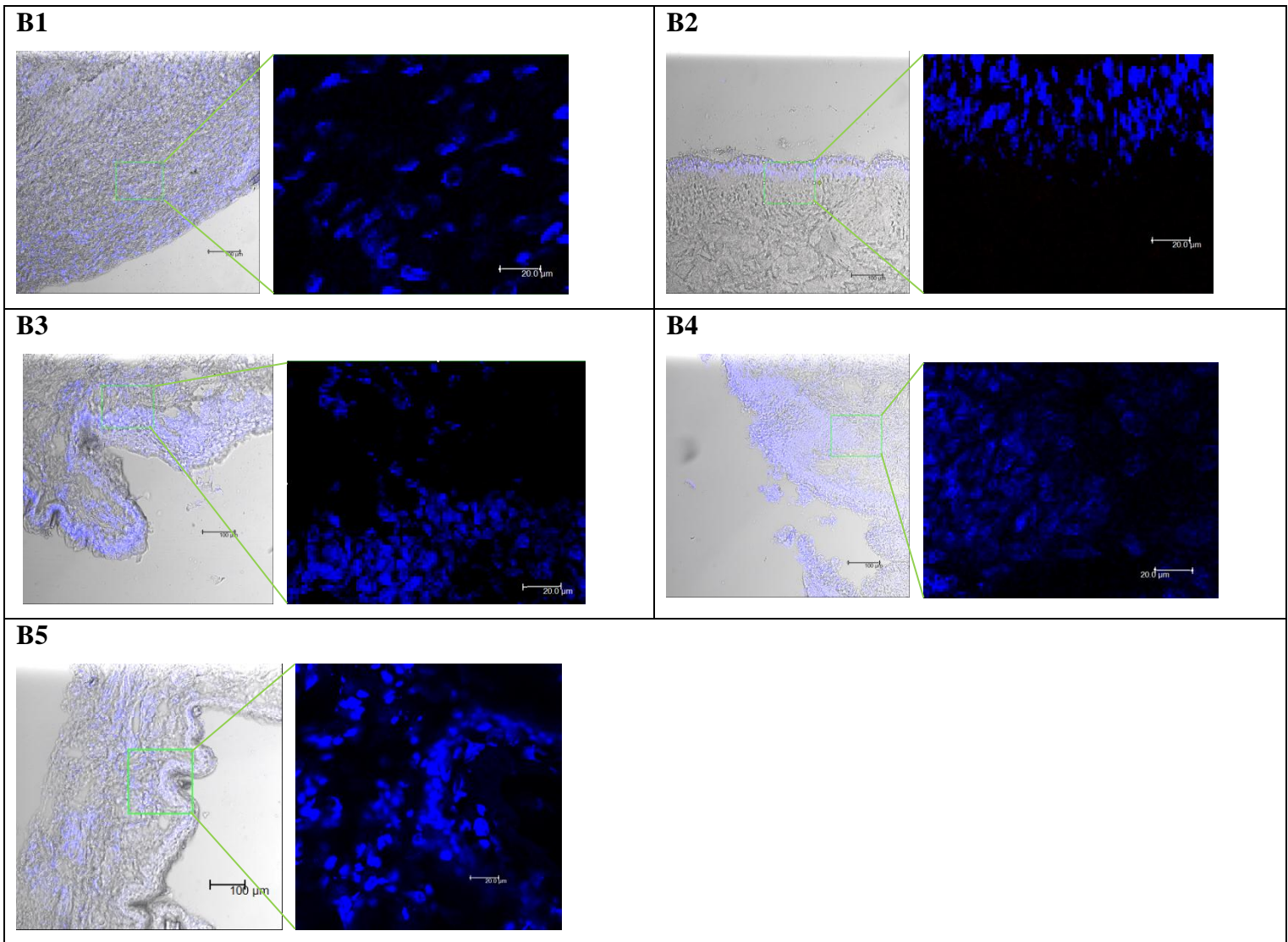
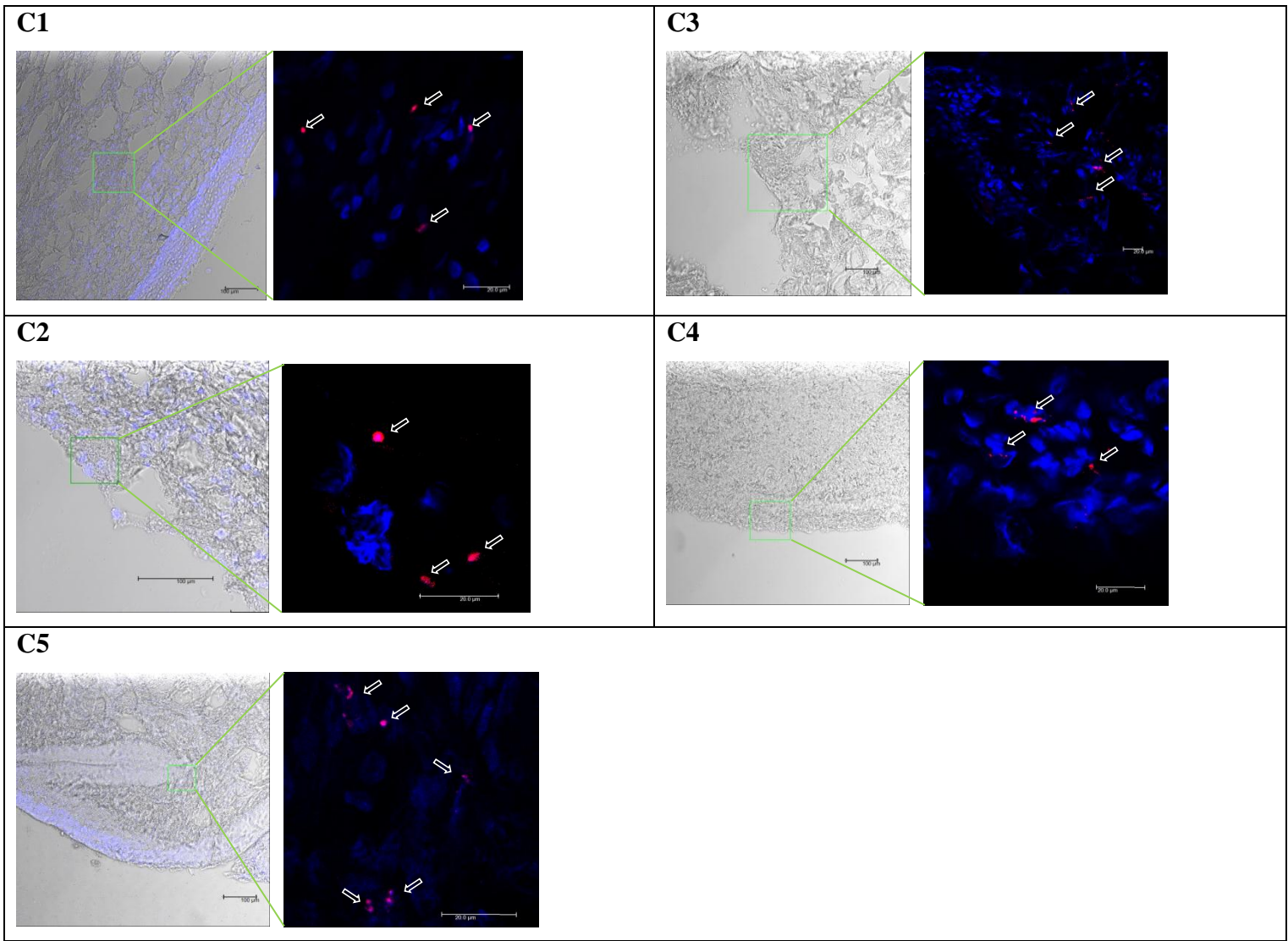


Figure 10.8: *Ex vivo* penetration study of plasmid/gemini/lipid nanoparticles. DNA was extracted from the tissue and nested PCR was performed to confirm the penetration of pGT.tdTomato into the tissue. Gel A: (P/12-7NGK-12/L in lane 1, 2, 3); (P/12-7NH-12/L in lane 4, 5, 6); No treatment control in lane 7. Gel B: (P/12-7NGK-12/L in lane 1, 2, 3); (P/12-7NH-12/L in lane 4, 5, 6)







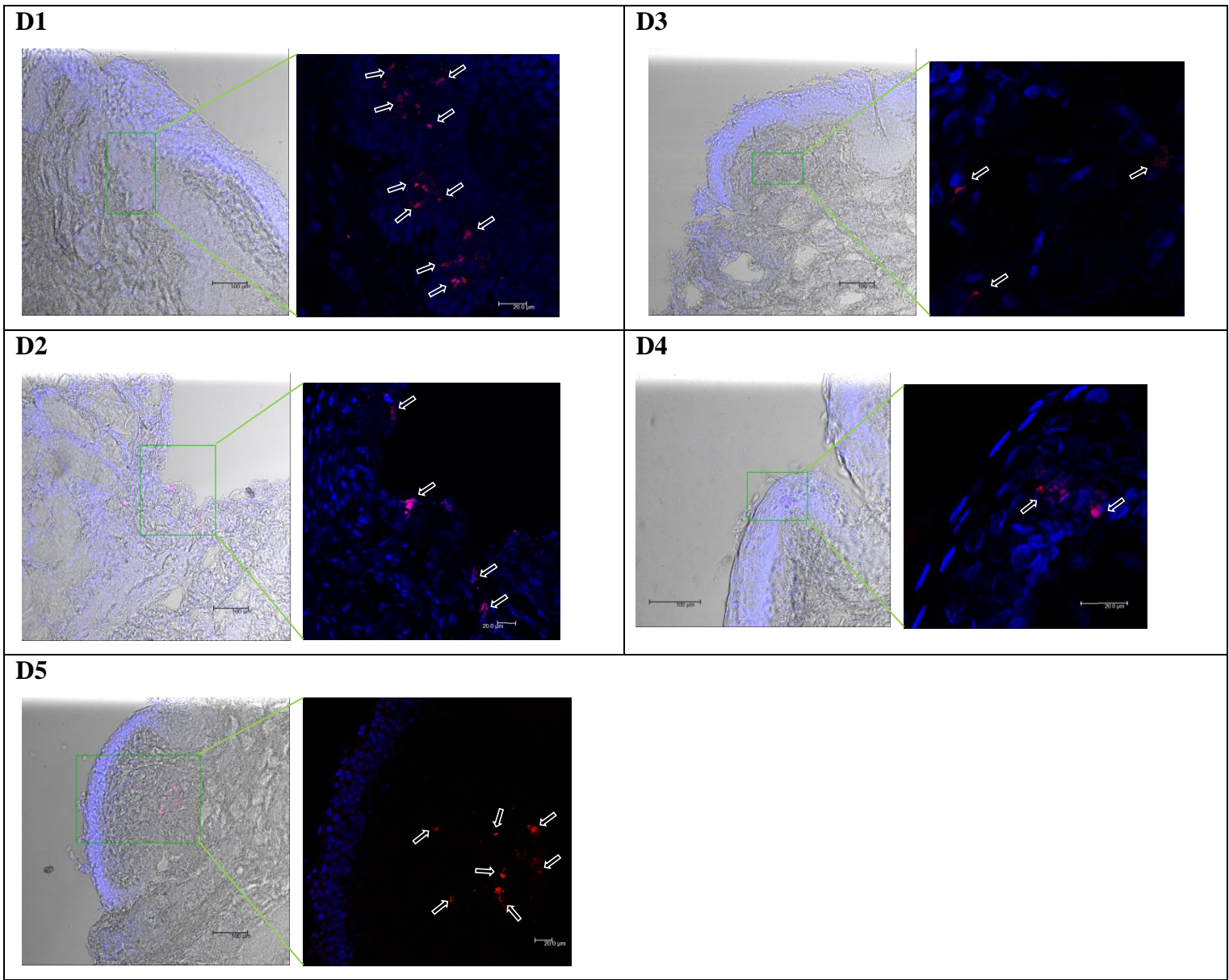


Figure 10.9: Confocal microscopic evaluation of tdTomato gene expression in rabbit vaginal tissue sections. No fluorescent cells were observed in the untreated and DNA only vaginal tissues. Compared to the unsubstituted parent compound formulation, more red fluorescent cells, indicating tdTomato expression, were observed in animals treated with glycyl-lysine substituted gemini surfactant formulation. **A1-5:** Pol407/DEGEE; **B1-5:** pDNA/Pol407/DEGEE; **C1-5:** pDNA/12-7NH-12/L/Pol407/DEGEE; **D1-5:** pDNA/12-7NGK-12/L/Pol407/DEGEE. DAPI (blue) was used as nuclear stain. The area of interest, with red fluorescent cells, is marked with a green box and its enlarged view is presented beside the image. The red fluorescent cells are marked with white arrows. (Scale bar: 100 μ m for tissue overlay image; 20 μ m for the high magnification region).

10.6. Discussion

The need for non-viral gene delivery system arises out of the high risk of viral carriers to trigger immune reactions that may prove to be fatal [42, 241]. For successful gene expression of plasmid DNA, the non-viral delivery system should be able to overcome the cellular barriers leading to cellular uptake, endosomal escape and nuclear translocation [230]. One way to address these challenges is by engineering non-viral delivery systems that can condense a plasmid DNA into stable particles with well-defined properties (e.g. size, surface charge, etc.), protect it from degradation and release the transgene for expression in the cellular nucleus. In addition, extracellular barriers in the vaginal cavity such as penetration through epithelial layer, degradation in mucosal secretions or leakage from the cavity necessitate the use of additional formulation components, e.g. penetration enhancers or mucoadhesive polymers, to improve drug delivery [242].

Previous to this work, we demonstrated that the glycyl-lysine substituted gemini surfactant (12-7NGK-12) showed the highest gene expression among several amino acid-substituted gemini surfactants and, significantly higher than the unsubstituted (parent) gemini surfactant (12-7NH-12) in rabbit epithelial cells [199, 223]. We also showed that grafting a glycyl-lysine moiety showed excellent safety profile with >90% cell viability after 72 hours [223].

Having high transfection efficiency and low toxicity, we selected the glycyl-lysine-substituted gemini surfactant lipoplexes for the assessment of gene delivery efficiency *in vivo*. A vaginal route of delivery was explored in rabbits, an animal model selected for our long-term goals to develop non-invasive vaccines against sexually transmitted pathogens.

For the *in vivo* experiments a new plasmid was constructed using tdTomato gene sequence and previously optimized pGT vector [9]. tdTomato encodes a non-aggregating tandem dimer protein which is an exceptionally bright red fluorescent protein; 6-times brighter than EGFP [243-245]. The emission wavelength (581 nm), its brightness and minimal interference with the autofluorescence of tissue collagen make it ideal for imaging studies [243-245].

The *ex vivo* penetration studies in rabbit vaginal tissue showed that both the amino acid substituted and unsubstituted gemini surfactants were able to deliver the plasmid DNA into the tissue. However, the poor *ex vivo* viability of tissue and the absence of innate defense mechanisms limited the evaluation of gene expression, thus an *in vivo* model was needed for

assessment of gene expression. In an effort to improve residence time and permeation of our formulation in the vaginal cavity, we incorporated poloxamer 407 and diethylene glycol monoethyl ether (DEGEE).

Poloxamer 407, a triblock co-polymer made of poly(oxy ethylene) and poly(oxy propylene), exhibits inverse thermo-gelling properties in a concentration dependent manner [240]. Poloxamers have been used as drug delivery vehicles for a variety of routes of administration. Their thermo-gelling property imparts a unique ability of easy handling and administration of formulations in body cavities such as the vagina [174, 246]. The poloxamer exhibits newtonian flow below its gelling temperature and non-newtonian behaviour above it [246, 247]. The viscosity of poloxamer dispersions is not only affected by polymer concentration but also by the presence of other components and co-solvents [247, 248]. Therefore, for this study we conducted flow studies (shear stress vs. shear rate) to evaluate the effect of other components such as DEGEE and lipoplexes on the viscosity and gelling ability of poloxamer 407. We selected diethylene glycol monoethyl ether (DEGEE) as a penetration enhancer because it is an excellent skin penetration enhancer and it is non-toxic, non-irritating, biocompatible with the skin and miscible with polar solvents [249]. It is approved for use in human pharmaceutical products for topical application [250]. Contrary to other penetration enhancers that disrupt the skin layer, DEGEE gently seeps into the intercellular lipid bilayers and causes swelling to increase penetration and thereby, causing less damaging effects [251]. DEGEE forms clear gels with poloxamers and does not hinder the release rate of lipophilic drugs [252]. Interestingly, DEGEE (24.8% v/v) was shown to reduce the gelling temperature and gelling strength of 27% poloxamer gel; however, this effect was dramatically reversed in the presence of other formulation components such as drug and co-penetration enhancer [253]. Therefore, we studied the effect of DEGEE at lower concentration (10% v/v) and at different concentrations of poloxamer gel with the aim to achieve *in situ* gelling at body temperature. Generally, the aqueous solutions of poloxamer 407 at 18% concentration exhibit sol-gel transition near 30 °C [247]. This thermo-gelation behaviour of poloxamer 407 aqueous solutions is due to the dehydration of the hydrophobic propylene oxide blocks at higher temperature causing aggregation of copolymer molecules into micelles [254]. Poloxamer concentration of 18% w/v in DEGEE aqueous solution gelled below room temperature (**Figure 10.3**). This may be due to the presence of DEGEE and a decreased fraction of aqueous environment causing early sol-gel

transition at comparatively lower temperature. Solutions with lower concentrations of poloxamer (15%, 16%, 16.5% and 17%) exhibited sol-gel transition temperatures above room temperature and therefore were selected for further evaluation. The rheological studies showed that the poloxamer dispersions in DEGEE aqueous solution exhibit non-newtonian pseudoplastic behaviour at 37 °C (**Figure 10.4**). All systems showed thixotropic behaviour. With the exception of 15% concentrations, all poloxamer dispersions exhibited a yield value greater than 900 dynes/cm², the minimum force required to induce flow in polymer dispersions above their sol-gel transition temperature (**Figure 10.5**). A minimum concentration of 16% poloxamer lead to *in situ* thickening near body temperature and exhibited a high yield value. Therefore, the 16% poloxamer formulation was combined with P/G/L nanoparticles for the development of mucosal gene delivery system.

The incorporation of lipoplexes into the polymer dispersion did not increase the viscosity of the formulation at room temperature and retained useful characteristics such as ease of extrusion from a syringe into the vaginal cavity (**Figure 10.6**). The presence of lipoplexes containing unsubstituted gemini surfactant did not affect the sol-gel transition temperature significantly, decreasing the gelling temperature by only 2 °C compared to the 16% polymer/DEGEE aqueous dispersion. On the contrary, the sol-gel transition temperature rose to 34 °C for formulations containing glycyl-lysine substituted gemini surfactant. It is hypothesized that this difference between the behaviour of the formulation in the presence of unsubstituted gemini nanoparticles and the glycyl-lysine substituted nanoparticles is due to the nature of their interaction with the polymer.

The sol-gel transition of the poloxamer proceeds by formation of micelles with the hydrophobic core containing polyoxypropylene surrounded by a polyoxyethylene shell [255, 256]. As the temperature increases, it promotes micellar entanglement leading to gel formation and increased viscosity. Environmental changes can trigger modifications in the size, shape or arrangement of the micelles that can impact the gelling properties of poloxamer. In general, the addition of solutes to poloxamer 407 dispersion produces a decrease in its gelation temperature in a concentration-dependent manner. However, the nature of the solutes determines the overall impact on the gelling temperature and gelling strength (viscosity) [248]. Glycyl-lysine substitution on gemini surfactant may have been able to interact with poloxamer chains through hydrogen bonding or by increasing hydration of the hydrophobic core forming mixed micelles.

This may delay the growth of micelles and hinder association among poloxamer micelles, leading to an increase in gelation temperature compared to the parent gemini compound with less ability for hydrogen bonding.

Rheology measurements at 37 °C of the P/G/L formulations revealed an increase in viscosity compared to polymer dispersions at a similar temperature (**Figure 10.7**). However, the presence of unsubstituted gemini surfactant lowered the yield value to 773 dynes/cm² while it stayed around 1051 dynes/cm² for the glycyl-lysine substituted gemini surfactant formulation. By comparison the yield was 1018 dynes/cm² for the 16% poloxamer/DEGEE dispersion. A low yield value indicated more fluidity under high shear stress. However, it did not result in leakage as other factors such as mucoadhesion could retard the flow [257]. We did not observe any post-administration leakage of unsubstituted gemini surfactant formulation from the vaginal cavity. Interestingly, both gemini surfactant formulations exhibit a small hysteresis loop above its gelling temperature indicating fast recovery after administration. The hysteresis loop was smaller for the glycyl-lysine substituted gemini surfactant compared to the parent compound. This indicates that these formulations regain gel strength faster than the formulations with the unsubstituted gemini surfactant, preventing leakage from the vaginal cavity.

Evaluations of interactions of plasmid DNA with poloxamers 407 showed no effect on the gelling temperature [258]. This suggests that in our studies the presence of gemini surfactants and co-lipid may be responsible for the overall changes in sol-gel transition temperature of the formulations.

To assess the *in vivo* efficiency of the thermosensitive DNA nanoparticles containing glycyl-lysine substituted gemini surfactant or unsubstituted gemini surfactant, the formulations were administered into the vaginal cavity of the animals. On visible inspection, no toxicity was apparent in vaginal tissues in either the treated or control animals. No signs of erythema, discoloration or ulceration were apparent (Supplementary information S2). For gene expression analysis, western blotting, RT-PCR/PCR and confocal microscopy were originally proposed. Unfortunately, due to the high background noise (non-specific bands) in western blot, no conclusive results could be drawn (Supplementary Information S3). Quantitative RT-PCR could not be performed due to the dimer nature of the gene; long fragments (~800-1200 bp) were amplified in addition to the fragments of interest (Supplementary Information S3). Therefore, evaluation of gene expression of red fluorescent protein was performed using confocal

microscopy. DNA in the poloxamer dispersion in the presence of permeation enhancer but absence of gemini surfactants/co-lipid was not able to generate gene expression (**Figure 10.9**). Fluorescence was observed in the vaginal tissue of all animals treated with DNA formulations containing 12-7NH-12 or 12-7GK-12. This is in agreement to the previous studies where gemini surfactants were able to deliver plasmid DNA *in vivo* [9]. A larger number of cells, expressing red fluorescent protein, were observed in the case of the glycyl-lysine substituted gemini surfactant, compared to the unsubstituted gemini surfactant formulation (**Figure 10.9**).

These findings are in agreement with studies conducted *in vitro*. Evaluation of the P/G/L complexes in rabbit epithelial cells demonstrated that the glycyl-lysine gemini surfactants exhibited the highest gene expression compared to parent gemini surfactant and other amino acid-substituted analogs [199, 223]. These results suggest that there might be an *in vitro/in vivo* correlation between the delivery efficiency of these gemini formulations. In the future, we will evaluate other substituted gemini surfactants to assess whether this *in vitro* model can be used as a reliable indicator for *in vivo* gene expression models for topical delivery.

10.7. Conclusion

DNA formulations, prepared using poloxamer 407 and permeation enhancer, diethylene glycol monoethyl ether, gelled at a temperature near to body temperature and exhibited pseudoplastic behaviour. This contributed to the ease of handling of the formulation, especially administration of formulation at room temperature. The *in situ* gelling and a high shear-stress yield value retarded the leakage of the formulation from the vaginal cavity. Confocal microscopic evaluation showed that both glycyl-lysine substituted and unsubstituted gemini surfactants were able to transfect cells *in vivo*. However, the glycyl-lysine substituted gemini surfactant exhibited higher efficiency compared to the parent compound. The results suggest that this *in vitro* model can be used for optimizing and predicting *in vivo* transfection efficiency of novel non-viral vectors for topical delivery. In the future, these formulations will be used to deliver therapeutically relevant plasmid DNA to evaluate their potential in the treatment of diseases affecting mucosal epithelial cells.

10.8. Future perspective

Our research group has focused on the development of cationic N,N-bis(dimethylalkyl)- α,ω -alkanediammonium gemini surfactants as gene delivery vectors. The systematic evaluation of these vectors had led to rational development of a new generation of molecules, biolipids, with superior transfection efficiencies. We believe that gemini surfactant-based gene delivery systems have the potential to generate adequate gene expression for therapeutically relevant applications. Going forward, we need to develop models to better understand how physicochemical properties of these molecules in association with cellular uptake and intracellular trafficking can target a specific pathway for efficient protection and release of the genetic material nearby the nucleus, leading to a higher gene expression. The intent is to select therapeutically relevant genes in order to measure the direct benefit of gene therapy and genetic vaccination.

10.9. Executive summary

- Nanoparticles prepared by glyceryl-lysine substituted gemini surfactant have shown significantly higher gene expression than unsubstituted parent gemini surfactant in rabbit epithelial cells, Sf 1 Ep Cells.
- A plasmid DNA was constructed by cloning tdTomato gene, amplified from commercially available pCMV.tdTomato vector, into a pGT backbone, specifically designed for gene therapy. The tdTomato gene encodes a red fluorescent protein which has brighter (6 times) fluorescence than GFP and minimal interference with the autofluorescence of tissue collagen, making it ideal for imaging studies.
- *Ex vivo* penetration studies of P/G/L showed that the delivery system was able to deliver the plasmid into the tissue.
- P/G/L nanoparticles were formulated with a gelling agent, poloxamer 407, and permeation enhancer, diethylene glycol monoethyl ether (DEGEE), and the composition optimized to achieve temperature-dependent in-situ gelling.
- 16% poloxamer dispersions achieved sufficient viscosity and considerable high yield stress to retard leakage of the formulation from the vaginal cavity. Therefore, this concentration was selected to incorporate the P/G/L nanoparticles for further development as mucosal gene delivery system.

- Finally, the formulations were tested in rabbits by non-invasive administration to the vaginal cavity. The studies revealed that the glyceryl-lysine substituted gemini surfactant (12-7NGK-12) continue to exhibit high transfection efficiency compared to formulations containing parent gemini surfactant (12-7NH-12).

10.10. Competing interests

The authors declare that they have no competing interests.

10.11. Funding

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11. General Discussions and Final Conclusions

Gene therapy is a novel technique that promises to provide a treatment option for both inherited and acquired diseases by manipulation of cell's genetic machinery. Most commonly, these genetic manipulations require a delivery of exogenous genes to generate a therapeutic response [259]. A successful gene therapy requires the delivery of these therapeutic genes in a safe and efficient manner to target cells. At cellular level, a gene delivery system needs to aid cellular uptake, protect the DNA from degradation and facilitate its translocation to nucleus for gene expression [260]. Virus-based gene delivery systems use the innate ability of the viruses to overcome these challenges; however, the risk of immune response and insertional mutagenesis makes this a challenging drug delivery method [40, 41]. Alternatives to viral delivery systems, physical and chemical methods of gene delivery have been explored. The physical methods, thus far, depend on the disruption of cellular membranes through mechanical penetration (microinjection, gene gun etc.) or electric field (electroporation) [261, 262]. The transfection efficiency of physical methods is comparable to viral vectors; however, the disruption force impact significantly upon the cell viability and provides less control on the amount of genetic material that can enter the cell [7, 51].

Chemical methods e.g. cationic lipids, polymers, etc., offer a non-immunogenic and, relatively, a safe method to form DNA complexes that can facilitate gene delivery into the cell [263-266]. Both polymers and cationic lipids have their unique features that make them attractive as gene delivery systems. Polymers are hydrophilic in nature, offer strong DNA binding and produce DNA complexes of narrow size range [267, 268]. Although, a strong DNA binding and small particle size helps in gene transfer, it does not necessarily translate into gene expression. In addition, low biodegradability and a high density of the cationic charges on polymers result in stronger association with cell membranes and therefore, result in high toxicity [269]. In comparison to polymers, cationic lipids offer flexible physicochemical properties due to the presence of hydrophilic and hydrophobic moieties in a single molecule. More interestingly, cationic lipids containing two hydrocarbon tails self-assemble at low concentrations and form bilayer structures that help in efficient gene transfer into the cells [121].

Among the available arsenal of cationic lipids, gemini surfactants offer adequate flexibility for the design of novel delivery vectors through modification of the alkyl tail length and the

chemical nature of the spacer. The gemini surfactants, a term coined by Menger *et. al.*, are surfactant molecules containing two ionic head groups covalently linked at or near the headgroup through a spacer [151]. The gemini compounds have several advantages compared to the classic monovalent surfactants: lower cellular toxicity, lower critical micelle concentration, higher efficiency in reducing surface tension and greater tendency to self-assemble [152, 153]. Several research groups have experimented with these molecules by modifying hydrocarbon tail length, size of head group, spacer length, and degree of unsaturation. With regards to the length and nature of the tail, a longer hydrocarbon chain, generally greater than C₁₂, is required for transfection efficiency and it improves as the length increases [156]. For gemini surfactants, transfection efficiency increases with an increase in tail length; however, the nature in the head group has more profound effect on the overall transfection efficiency of the molecules [235]. The incorporation of unsaturation in the tail improves transfection efficiency of cationic lipids significantly, especially for unsaturated C₁₈ [155][155][155, 155][155, 155][155, 155]. The length and unsaturation of the tail modifies the fluidity and organization of the cationic lipids. For example, unsaturation in phospholipids modifies its molecular shape and aggregate structure, favoring the formation of hexagonal phase [270]. In case of gemini surfactants, the unsaturation in the tail reduces the resistance of pH-dependant vesicle transitions, lamellar to non-lamellar structures, which increases the fluidity of the complexes and therefore, facilitate their efficient escape from the endosomes [271, 272]. Di-unsaturated lipophosphoramidate has shown moderate increase in transfection efficiency compared to mono-unsaturated; however, a further increase in the unsaturation results in reduced gene expression [273, 274]. Currently, no studies for poly-unsaturated gemini surfactants are available. Asymmetrical tails in diether lipids increased transfection efficiency when prepared in presence of co-lipids DOPE and DcChol [65]. The hydrophobic interaction among the asymmetrical tails of the gemini surfactants is disrupted in the presence of DNA which results in weaker interaction of gemini surfactants with DNA; needed for DNA condensation [275]. The nature of the asymmetry, i.e., phantyl or pyrene substitution in one tail vs. difference in chain length of two tails, have more high impact on the transfection efficiency of these molecules. In m-3-n type diquatery ammonium based gemini surfactant, the molecules showed high transfection efficiency when m= phytanyl and n=12, 16, 18, compared to symmetric 16-3-16 gemini surfactants [276]. The presence of phytanyl

substitution is believed to favor a higher order bilayer structure which is known to be advantageous for DNA transfection.

The modifications in the head group are conducted to study the effect of distance between the two cationic centres and the presence of additional substitutions such as amines, sugar, on DNA binding, condensation and transfection efficiency of these molecules. For unsubstituted diquatery ammonium based gemini surfactants, a short spacer groups ($n=2-4$) between cationic centres yield high transfection efficiency [182]. In addition, the presence of hydrophilic groups favors the location of head groups in the aqueous phase in micelles, improving interaction with DNA and facilitating formation of DNA/lipid complexes [154]. In all, a balance of DNA binding, DNA condensation, morphological structures and presence of pH-sensitive groups in the head group have shown to exhibit high transfection efficiency.

Early experiments with cationic N,N-bis(dimethylalkyl)- α,ω -alkanediammonium gemini surfactants in our research group demonstrated that these molecules were able to bind to DNA and compact it for gene transfer [9, 182]. Interestingly, the gemini surfactants with short spacer group ($s=2-4$) resulted in excellent transfection. Computational modeling studies revealed that the presence of three methylene groups created a distance of 4.9 Å between the quaternary nitrogen centres equivalent to the spacing between two adjacent phosphate groups of DNA [10]. This could help the dimeric surfactants to interact with DNA molecules more easily, creating lipoplexes with optimal characteristics for transfection. In agreement with observations by other groups, an increase in hydrocarbon tail from C_{12} to C_{16} improved transfection efficiency of these molecules [9].

A low critical micellar concentration (CMC) value could increase the stability of the DNA/gemini surfactant complex due to micellar aggregation that could keep the complex together during the delivery process. Similar findings were found for ornithine-based gemini surfactants with tail length between C_{12} - C_{16} , where low CMC was associated with high transfection efficiency [277]. The 12-s-12 series of diquatery ammonium based gemini surfactants followed a parabolic relation between CMC and the transfection efficiency [9]. First, the transfection efficiency decreased with an increase in CMC values for a polymethylene spacer length of 3-8, followed by a slight increase in gene expression from for spacer length of 10-16. It may be because high CMC values of gemini surfactants would allow more molecules to interact with DNA before forming self-aggregating micelles [9]. It is important to note that for

transfection the gemini surfactants generally need the presence of another co-lipid such as DOPE that can facilitate formation of non-lamellar phases. Building on the understanding developed in first generation molecules, a second generation of gemini surfactants were synthesized with amino groups in the spacer [10]. This extra N-atom was placed three methylene groups apart from other amino groups in order to maintain a distance equivalent to the spacing between adjacent phosphate groups of DNA. Among this novel series of gemini surfactants, 1,9-bis(alkyl)-1,1,9,9-tetramethyl-5-amino-1,9-nonanediammonium gemini surfactant (12-7NH-12) showed the highest transfection efficiency compared to first-generation compounds. The presence of a methyl substitution at N-atom in the spacer significantly reduced transfection. The reduction in efficiency could be due to increased steric hindrance to N-atom for interaction with phosphate group. Additionally, SAXS and pH-titration experiments revealed that the secondary amine-containing gemini surfactants exhibited a pH-sensitive character and a polymorphic organization, including lamellar and cubic phases. These might be key physicochemical properties imparting higher transfection efficiency of these molecules compared to the first generation alkyl-spacer gemini surfactants [182, 238].

Findings from the previous work led to a hypothesis that the presence of additional amino groups – from bio-compatible amino acids – in the spacer could improve DNA condensing ability of these molecules and impart pH-sensitivity that would help in cellular uptake and endosomal escape for efficient gene delivery. In addition, the incorporation of the bio-compatible and non-toxic amino acid/dipeptide substitution could maintain the low cytotoxicity. My work focused on this novel family of non-viral gene delivery agents. We hypothesized that glycine, the simplest of all the amino acid, is needed for conformational flexibility for DNA binding and lysine, employed as polymers known to exhibit excellent DNA binding and condensing properties could modulate gene transfection efficiency of the gemini surfactants [74, 183]. A novel generation of amino acid-substituted gemini surfactants were synthesized by incorporation of glycine, lysine, glycyl-lysine and lysyl-lysine in the spacer region of 1,9-bis(alkyl)-1,1,9,9-tetramethyl-5-amino-1,9-nonanediammonium gemini surfactant (12-7NH-12).

The amino acid-substituted gemini surfactants were formulated with a model plasmid (expressing green fluorescent protein (GFP) and interferon- γ) in the presence of a helper lipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE). In agreement to the previous work with diquaternary ammonium based gemini surfactants, a DNA to gemini surfactant charge ratio

of 10 was found to be optimum with molar ratio of 8.3 between gemini surfactants and helper lipid [9]. Selection of the *in vitro* and *in vivo* models was based on our long term goal of the study, namely to develop non-invasive mucosal (vaginal) formulations to treat sexually transmitted diseases. The animal model for our long term studies was rabbit; therefore, the DNA formulations were tested in Sf 1 Ep rabbit epithelial cells. The presence of amino acids in the spacer of gemini surfactant improved their DNA condensing ability without increasing toxicity. Glycine and glycyl-lysine -substituted gemini surfactants showed higher transfection efficiency, compared to lysine-, lysyl-lysine- and un-substituted gemini surfactant. While gemini surfactants bind the DNA via electrostatic interactions, the nature of binding by the amino acid/peptide-substituted gemini surfactants might be 'softened' by van der Waals and hydrogen bonding forces [194], thus increasing the flexibility and plasticity of the supramolecular structures [195]. The increased hydrophilicity of the amino acid/peptide-substituted gemini surfactants, compared to the parent compound, translated into better biocompatibility. All amino acid-substituted gemini surfactants maintained high cell viability, greater than 90%.

Amino-acid/dipeptide substitution did not interfere with interaction of cationic N in the gemini surfactants with DNA as evidenced by the similarities of the DNA condensation in circular dichroism spectra. Instead, amino acid substitution in the gemini surfactants increased the number of terminal amino groups that were highly protonated at physiological pH, contributing to efficient DNA-gemini electrostatic interactions and DNA compaction [205]. The presence of this cationic charge on plasmid/gemini/co-lipid complexes facilitated their interaction with cell surface and also conferred colloidal stability to the formulation [196]. Complex sizes of 100-150 nm were obtained, considered as optimal size range for endocytosis by the cells [115]. The smallest particle size of 105 nm was obtained by lysyl-lysine substitution, with the maximum number of amino groups. Though a strong DNA interaction and smaller particle are helpful in gene transfer ability, this does not necessarily translate into high gene expression. A stronger DNA interaction can protect the DNA; however, it may also hinder its timely release for translocation into nucleus leading to poor transfection efficiency. The DNA binding properties of the amino acid-substituted gemini surfactants revealed that the presence of glycine provided conformational flexibility leading to overall balanced binding properties. These balanced binding properties were able to protect the DNA and release it at an appropriate time for gene expression.

Clathrin-mediated, caveolae-mediated uptake and macropinocytosis are the most common uptake pathways utilized by mammalian cells to engulf macromolecules or solutes impermeable to plasma membrane [125]. Clathrin-mediated pathway experience a drop in pH in early endosomes finally merging with acidic enzyme-rich lysosomes, while caveolae-mediated pathway proceeds via a non-acidic, non-digestive route [129] [135, 136]. In previous studies, cationic lipid-DNA complexes (using DOTAP and SAINT-2/DOPE) have been suggested to preferentially internalize via clathrin-mediated endocytosis [137, 226]. However, more recent studies emphasized that the physicochemical parameters of the DNA complexes (such as particle size, shape and surface chemistry) as well as cell type can influence the uptake behaviour [140, 149, 150]. Polyethylenimine (PEI)/DNA complexes were found to internalize via clathrin- or caveolae-mediated mechanisms depending on both PEI type (linear or branched) and cell line [140]. While another study using cationic lipid, Amphiphile 1 and DNA complexes found macropinocytosis to be the major pathway leading to gene transfection in CHO cells [227]. Caveolae-mediated uptake was also found to be important for transfection of human serum albumin coated DOTAP/DOPE complexes [228]. Understanding the physicochemical properties of the gene delivery particles in association with mammalian uptake pathways can lead to rational design of nanoparticles to target those pathways that improve their intracellular fate. Incorporation of pH-sensitive groups can help in destabilization of endosomes following a clathrin-mediated pathway, leading to efficient escape of DNA complexes. Similarly, an optimum fluidity of DNA complexes can maintain the integrity and stability of particle to avoid premature release while uptake and escape caveosomes by repeated interaction with its anionic membranes. We demonstrated that our amino acid substituted gemini surfactants possess characteristics to facilitate cellular uptake and endosomal escape in a more efficient manner compared to the previous generations of gemini surfactants. The key challenge in the design of this experiment was the optimization of endocytic inhibitors that had significant toxicity when incubated with cells for longer hours. In addition, an optimization of experimental conditions was required to compensate for the combined toxicity of endocytic inhibitors and P/G/L complexes. Based on the toxicity studies, a time-period of 3 hours for total incubation of these chemicals was chosen, at concentrations that retained more than 80% cell viability. Recently, alternative approaches such as siRNA targeting of proteins involved in the uptake pathways is being utilized to avoid the direct effect of presence of endocytic inhibitors on the transfection

efficiency of DNA complexes [278, 279]. However, these approaches also require successful targeting of these proteins without inflicting high toxicity that would indirectly affect the results on these experiments.

The amino-acid substituted gemini surfactant (12-7NGK-12) was able to trigger both clathrin- and caveolae-mediated cellular uptake, similar to the unsubstituted parent gemini surfactant (12-7NH-12). Interestingly, the 12-7NGK-12 particles, following either clathrin-mediated or caveolae-mediated pathways, contributed to the overall gene expression while 12-7NH-12 particles internalized via clathrin-mediated uptake were not a major contributor to the overall gene expression. This suggests that the 12-7NGK-12 particles were superior in escaping endosomal degradation compared to the parent compound. In addition, the 12-7NGK-12 particles exhibited high buffering capacity, pH-dependent particle size increase and balanced binding properties that helped in its endosomal escape, resulting in high transfection efficiency. Active targeting of these pathways can be achieved by incorporating receptor specific ligands such as transferrin that internalize via clathrin-mediated uptake [133, 280, 280]. This might be a further direction of research in this field.

Based on the *in vitro* studies, we selected the best performing formulations for the development of non-invasive mucosal gene delivery system for *in vivo* vaginal administration. The availability of non-invasive mucosal (vaginal) formulations, such as DNA vaccine against human papilloma virus, could have better therapeutic outcome and higher patient compliance. The development of mucosal DNA vaccines, that can be applied locally, could confer stronger local and systemic immunity against sexually transmitted pathogens [232, 233]. In addition, the vaginal mucosa offers a large surface area, high vascularization, a low enzymatic activity and ease of access [171, 234]. The painless, non-invasive administration could lead to higher patient compliance and the possibility of self-administration could be conducive to implementation of large-scale self-immunization programs.

Initially, we conducted studies to assess whether gemini surfactants can delivery plasmid DNA into the vaginal tissue non-invasively. The *ex vivo* penetration studies in rabbit vaginal tissue showed that both substituted and unsubstituted gemini surfactants were able to deliver the plasmid DNA into the tissue. For non-invasive administration, a low-viscosity formulation was required that could be administered using a syringe but would not leak out of vaginal cavity. In order to address the issue of low residence time, an *in situ* thermogelling polymer, poloxamer

407, was incorporated. Poloxamers that maintain low viscosity at room temperature and form highly viscous gels at body temperature [255, 256]. We also incorporated a topical permeation enhancer diethylene glycol monoethyl ether (DEGEE), to improve permeability of macromolecular complexes [249].

For *in vivo* experiments, a new plasmid was constructed by cloning tdTomato gene sequence in an already optimized pGT vector. The need for creating a new plasmid DNA for *in vivo* experiments stemmed from low brightness of green fluorescent protein and interference from autofluorescence of tissue collagen, compared to tdTomato (red fluorescent protein) [243-245]. The pGT backbone was also optimized, previously, for *in vivo* application by removing CpG motifs that will reduce any immune responses against plasmid DNA formulations [9, 281]. The polymer concentrations were optimized so that the topical vaginal formulations exhibited low viscosity at room temperature, contributing to the ease of intravaginal administration, and gelled near body temperature preventing their leakage from the vaginal (mucosal) cavity. Based on sol-gel transition temperature and flow curve analysis, 16% polymer concentration was selected for incorporation into the P/G/L complexes in presence of DEGEE.

The formulations were tested in rabbits via non-invasive vaginal administration. More importantly, the animals did not require anesthesia avoiding exposure to additional chemicals and thus, reduced any discomfort. The presence of gemini surfactants lead to gene expression, while no gene expression was observed in the absence of the cationic complexing agent. Formulations containing amino acid-substituted gemini surfactant exhibited higher transfection efficiency compared to parent compound. This provides a proof of concept that the amino acid-substituted gemini surfactants can be used as non-invasive mucosal (vaginal) gene delivery systems with potential applications in diseases associated with mucosal epithelia.

My research presents a systematic development of a novel series of novel amino acid-substituted gemini surfactants. The key challenges were to formulate these new molecules as gene delivery systems that can generate high gene expression. I demonstrated for the first time, that these gemini surfactants performed better, compared to previous generations of gemini surfactants. Based on the results of transfection study, key physicochemical parameters contributing to high transfection such as size, surface charge, DNA compaction and DNA binding properties were pinpointed. This presented an opportunity for a rational understanding for the difference in their efficiency as gene delivery vectors. I conducted experiments to

evaluate the cellular uptake mechanism of DNA/gemini surfactant complexes and their impact on their overall gene expression. To my knowledge, no such studies have been conducted before to understand the effect of physicochemical properties on transfection ability of gemini surfactants. Further, models were built, for each uptake pathway, to explain how difference in physicochemical properties of these amino acid-substituted gemini surfactants were contributing to their high transfection ability. Going forward, single cell experiments could be conducted to determine the intracellular kinetics of DNA complexes as further proof of our findings.

In the existing literature, the majority of the studies with gemini surfactants are concluded at *in vitro* level and scientists have been spending more time in understanding the biophysical properties of these molecules. Although, these findings are crucial for continuous improvement of these molecules, there is a need to build *in vitro/in vivo* correlation to translate our findings to clinical settings. I believe that my work fills this niche in the quest of finding non-viral gene therapies and genetic vaccines. This study clearly demonstrates the feasibility of using gemini surfactants as gene delivery systems, especially for topical applications. In addition, my study suggests that the current *in vitro* model might be useful for optimization and predicting *in vivo* transfection efficiency of novel non-viral vectors for topical delivery.

12. Future directions

My work demonstrates that glycyL-lysine substituted gemini surfactants can be used to deliver plasmid DNA to generate protein of interest *in vitro* and *in vivo*. Rabbit epithelial cell cultures were used to optimize formulations and the final formulations were tested through mucosal (vaginal) administration in the rabbit. In my *in vitro* studies, I found that the transfection efficiency of glycyL-lysine Gemini surfactants was higher than the parent compounds and a similar trend was observed in *in vivo* studies. Such outcomes suggest that the current *in vitro* model could be used for optimization of similar molecules for gene delivery in epithelial cells but this has to be confirmed in the future with other series of gemini surfactants.

12.1. Development of non-invasive mucosal DNA based vaccine

Human Papillomavirus is a sexually transmitted virus infecting the cervix of the female reproductive system [282]. It is important to develop an effective local immune response at the first point of contact, that is, cervical epithelium. The vagina is lined by mucous secretions which carry a locally produced immunoglobulin A (IgA). IgA is a dimeric or multimeric secretory immunoglobulin and is resistant to a protease-rich environment. IgA provides protection against invading pathogens through entrapment and exclusion [283]. An intravaginal application of HPV L1 virus-like particles in mouse has shown to increase the production of IgA in vagina [173]. The vaginal delivery of DNA vaccine may improve IgA induction locally, as opposed to its systemic administration [170]. Further, the use of DNA vaccine will ensure induction of cytotoxic T cell lymphocyte protection against virus particles that may escape from IgG or IgA antibodies. Thus, it may be advantageous to consider intravaginal mucosal (non-invasive) delivery of DNA vaccine to elicit cellular as well as local and systemic humoral immunity [233].

Therefore, I propose that the model plasmid be replaced with a therapeutically relevant plasmid that expresses an antigenic viral protein. The papillomavirus viral components that can be targeted for invoking antigen specific immune response include: L1 and L2 – viral capsid proteins [286, 287] and E6 and E7 – viral oncogenic proteins [288] (**Figure 12.1**). In addition, our *in vitro* and *in vivo* models can be used for the design and development of HPV DNA vaccine as cottontail rabbit is a natural host to a cottontail rabbit papillomavirus and, therefore, is able to mimic the pathogenesis of papillomavirus infection [289, 290].

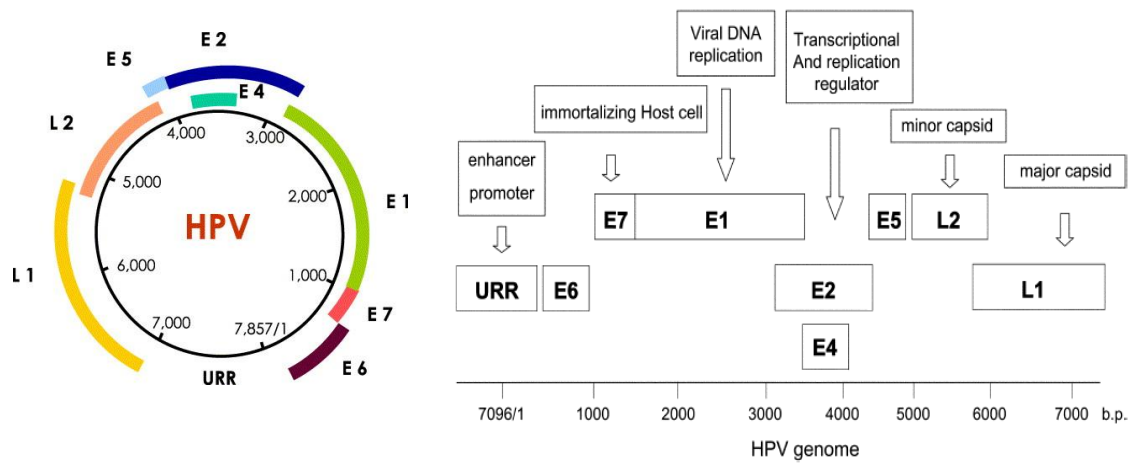


Figure 12.1: Schematic Representation of structure of HPV [284]; HPV genome [285]

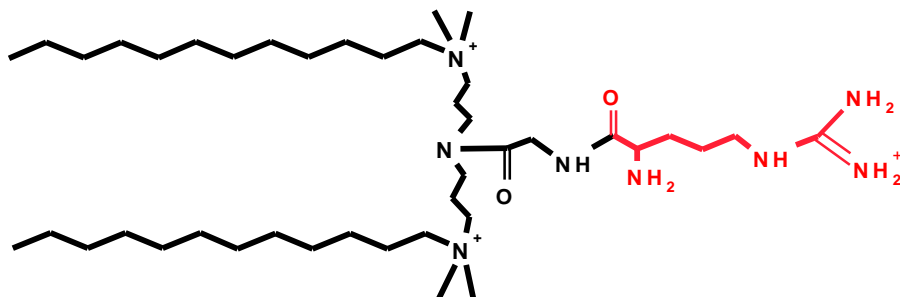
Based on my studies, I believe that glycyl-lysine substituted gemini surfactant is a good candidate for development of genetic vaccine for sexually transmitted diseases. However, further studies will be needed to assess if this delivery system can generate sufficient gene expression of the antigenic protein to elicit protective immunity.

12.2. Development of gemini surfactants

My studies have clearly indicated the role of balanced binding properties and pH-sensitivity of molecules in eliciting higher gene expression [223, 236][223][223]. I believe that the presence of glycine is contributing to the conformational flexibility that is required for balanced binding. Also, the presence of additional tertiary and secondary amino groups is providing the necessary pH-sensitivity. Therefore, future molecules can be designed by grafting other basic amino acids such as histidine, arginine via glycyl linkage to gemini surfactants (**Figure 12.2**).

The arginine residue has a pKa value > 9.0 which will impart strong cationic charges, at neutral pH, needed for electrostatic interaction with plasmid DNA. A lower amount of these molecules may be required for DNA condensation in a suitable particle range, thus, lowering cytotoxicity. The presence of secondary and tertiary nitrogen can still impart pH-sensitivity to the complexes. Histidine is another interesting choice because of its pKa value near 6.5. The permanent cationic charges on gemini surfactant will help in electrostatic interaction with plasmid DNA while a low pKa will exhibit a high pH-sensitivity in acidic endosomes. However, the presence of ring structure (imidazole group) in the histidine may impart rigidity to the DNA complexes. The presence of rigid complexes may reduce the cellular uptake but its overall effect on the transfection efficiency needs to be evaluated. The rigidity of DNA complexes can be addressed (reduced) by exploring different concentrations of co-lipids to gemini surfactants. Finally, structure-activity relationships and *in vitro/in vivo* correlations could be established by comparing these molecules to the previously generated amino acid-substituted gemini surfactants.

(A)



(B)

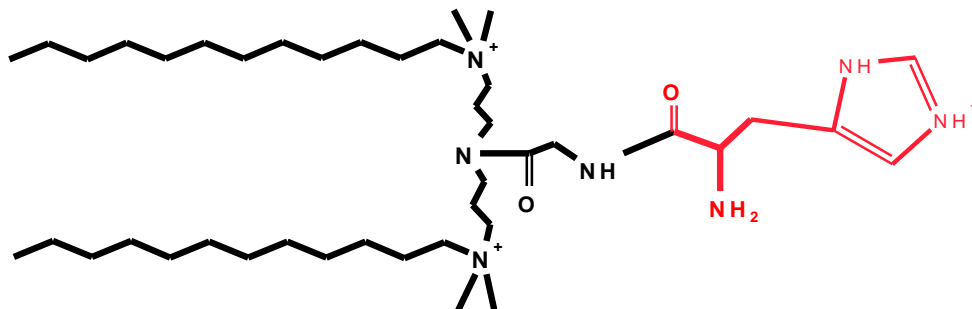


Figure 12.2: (A) Glycyl-arginine substituted gemini surfactant and (B) Glycyl-histidine substituted gemini surfactant

13. Appendix

13.1. Supplementary Information S1

Plasmid construction

Plasmid DNA, pGT.tdTomato was amplified in E. Coli (One Shot® TOP10 Electrocomp™ E. coli, Invitrogen, Burlington, Canada) and purified using Qiagen Plasmid Giga Kit (Qiagen Inc., Toronto, Canada).

The plasmid digestion in the presence of BssSI (New England Biolabs Ltd., Whitby, Canada) at 37 °C for 1 hour gave three fragments; 3273 bp, 1421 bp and 726 bp, which confirmed the correct assembly of the model plasmid (**Figure 13.1**).

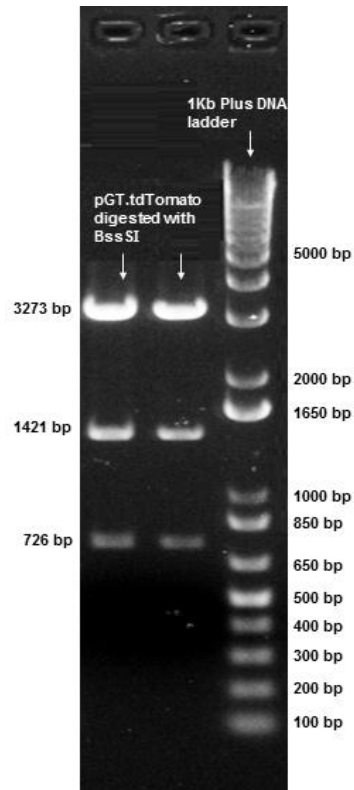


Figure 13.1: Digestion of pGT.tdTomato using BssSI restriction endonuclease. pGT.tdTomato had two restriction sites for BssSI that gave rise to three fragments. This confirmed the correct orientation of tdtomato fragment within the pGT vector.

13.2. Supplementary Information S2

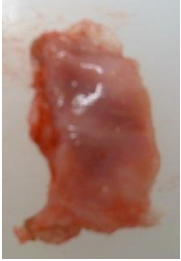
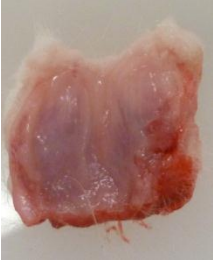
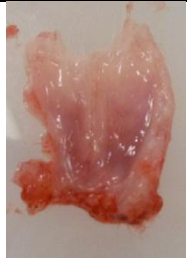

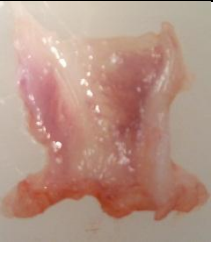

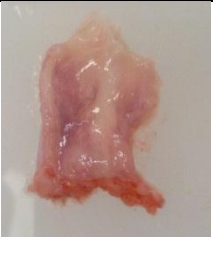
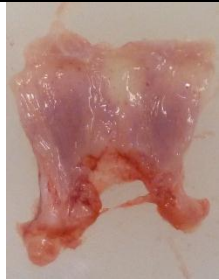



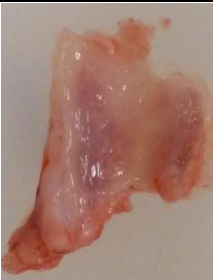
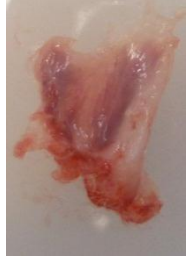
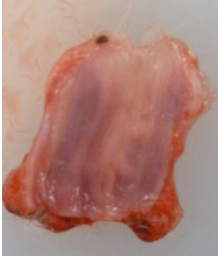


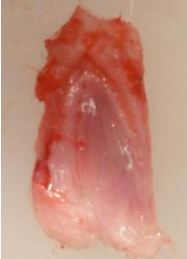
Control 1	Control 2	Control 3	Control 4	Control 5
Picture not taken	Picture not taken	Picture not taken		
DNA 1	DNA 2	DNA 3	DNA 4	DNA 5
				
12-7NH-12 (1)	12-7NH-12 (2)	12-7NH-12 (3)	12-7NH-12 (4)	12-7NH-12 (5)
				
12-7NGK-12 (1)	12-7NGK-12 (2)	12-7NGK-12 (3)	12-7NGK-12 (4)	12-7NGK-12 (5)
				

Figure 13.2: Vaginal tissue showing no visible signs of erythema, discoloration or ulceration post-treatment with gemini surfactant formulations

13.3. Supplementary Information S3

13.3.1. Method and Materials

Real time-PCR

The RNA was isolated and purified from the tissue sample using RNeasy Lipid tissue kit (Qiagen Inc., Toronto, Canada). The integrity of RNA was determined using Agilent 2100 Bioanalyzer (Agilent Technologies, Mississauga, Canada) and cDNA was synthesized from RNA using High Capacity RNA-to-cDNA Master Mix (Applied Biosystems, Burlington, Canada). The cDNA was used to perform real-time PCR using 7300 RT-PCR (Applied Biosystems, Burlington, Canada) and a variety of primer sets for tdTomato gene. The bands were separated on 2% agarose gel. For nested PCR, fragments were isolated from the gel using PureLink™ Gel Extraction Kit (Invitrogen, Burlington, Canada) and a nested PCR was run using a set of internal primers. Rabbit β -Actin (NCBI RefSeq Accession # AF404278), was used as a housekeeping gene and primer sets 5'-GGACCTGACCGACTACCTCA-3' and 5'-GGCAGCTCGTAGCTCTTCTC-3' were used to amplify an 180bp long fragment.

Western blotting

Tissue samples were homogenized and lysed in RIPA buffer containing protease inhibitors. The lysate were kept under constant agitation for 2 hours at 4 °C and then centrifuged at 12000 rpm for 20 min. The supernatant was used for protein determination by the Bradford procedure (Bio-Rad) and Western blotting. The proteins were resolved on 12% SDS-polyacrylamide gels, transferred onto nitrocellulose membranes, and incubated with the appropriate antibodies. Anti-tdTomato antibodies (DsRed mAB and DsRed polyclonal) was purchased from Clontech Laboratories, Inc., Mountain View, U.S.A. and used at 1:500 dilution. Secondary goat anti mouse-AKP conjugate (Sigma-Aldrich, Oakville, Canada) was used at 1:5000 dilution. BCIP/NBT Blue Liquid Substrate detection system (Sigma-Aldrich, Oakville, Canada) was used to develop the bands according to the manufacturer's instructions. tdTomato protein gives a band near 50 kDa with western blot.

13.3.2. Results

Real-time PCR

The evaluation of the integrity of RNA is important in order to compare gene expression of different delivery systems in a meaningful manner. Conventional UV/VIS spectrophotometry methods using the optical density provides a good estimate of quantity and quality of RNA integrity [291] . However, the presence of genomic DNA, proteins and use of buffers may compromise the accuracy of these results resulting in under or over estimation of RNA [292]. Modern, more sensitive instrumental methods such as fluorescence detection are used to determine the quality and quantity of RNA separated using microcapillary electrophoresis. Based on this method, Agilent has developed a 2100 Bioanalyzer that provides a RNA integrity number (RIN) to compare the quality of RNA among different samples. The RIN algorithm calculates a value between 1 (most degraded) to 10 (most intact) using electropherograms of RNA samples [293].

Gel-like densitometer plots of RNA samples isolated from rabbit vaginal samples are shown in **Figure 13.3**. Based on electropherograms, RNA integrity numbers ranged between 8 and 10 indicating excellent integrity of isolated RNA. A similar RIN number indicate that the RT-PCR results used to estimate gene expression profiles can be compared in a meaningful way.

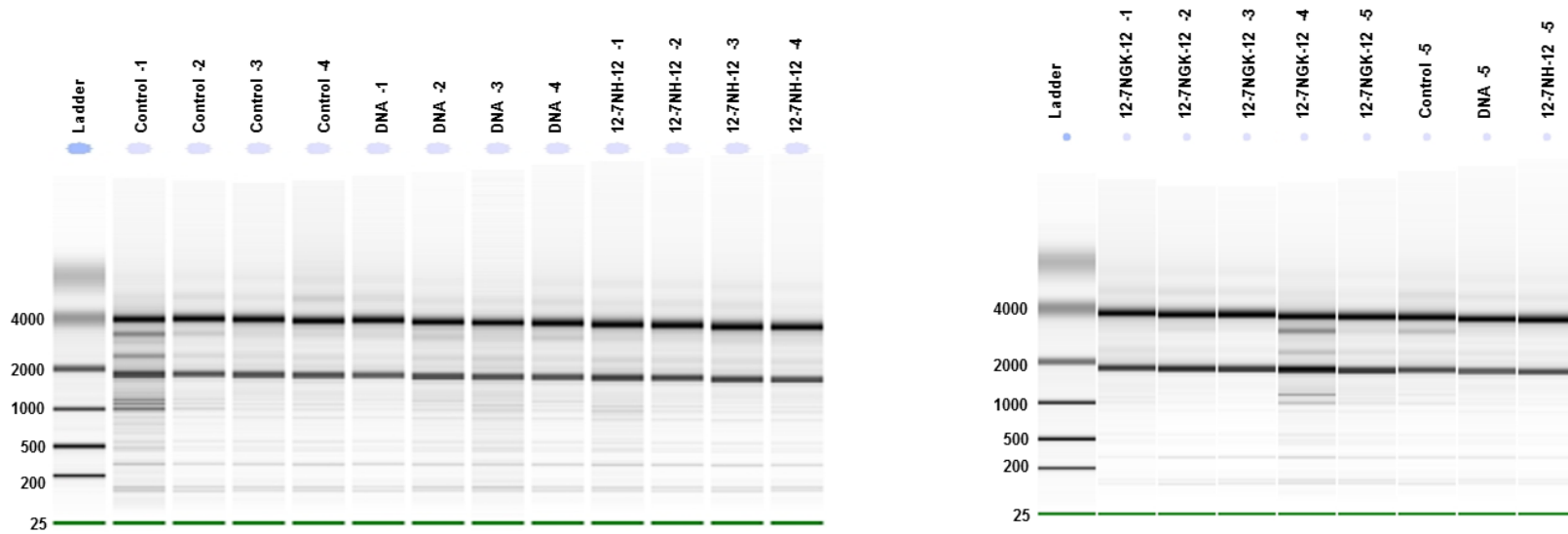
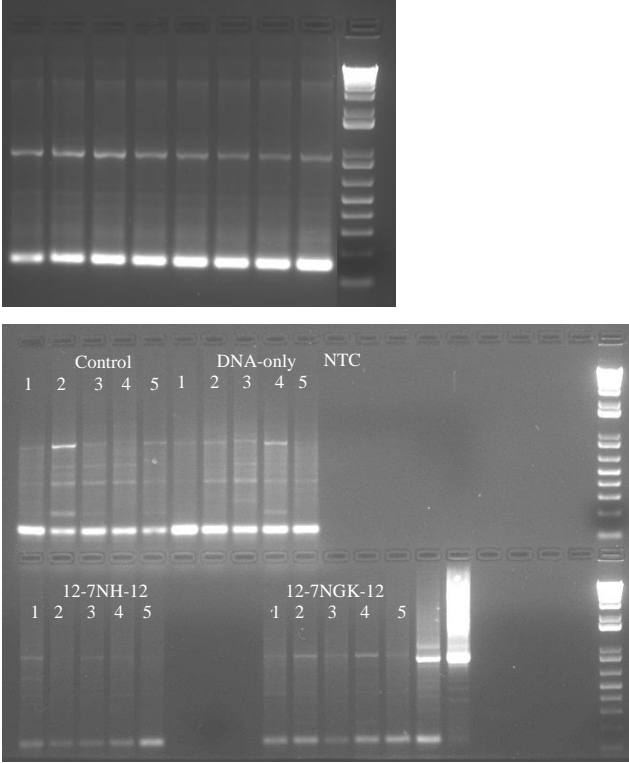
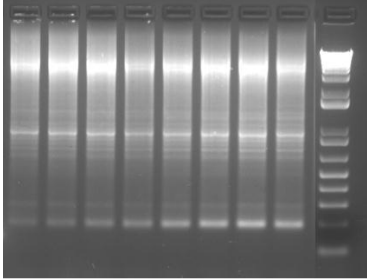


Figure 13.3: Gel-like densitometer plot of RNA isolated from rabbit vaginal samples. RNA isolated from rabbit vaginal tissue show RNA integrity numbers (8-10) for all samples, indicating good quality of RNA.

No meaningful results could be obtained using real time-PCR as a single set of primers designed for tdTomato gave two products because of two tandem repeated sequences in tdTomato cDNA [294]. The different pairs of primers that were tested for fragment amplification within the tdTomato region are listed in **Table 13.1**.

<p>pTOM-Forward2 5'-GCTGAAGGGCGAGATCCA-3'</p> <p>pTOM-Reverse2 5'-GTGGGAGGTGATGTCCAGCTT-3'</p>	<p>871 bp & 145 bp</p>	<p>Gradient PCR between 50 °C and 60 °C using tdTomato plasmid</p>  <p>Control 1 2 3 4 5 DNA-only 1 2 3 4 5 NTC</p> <p>12-7NH-12 1 2 3 4 5 12-7NGK-12 1 2 3 4 5</p>
<p>pTOM-Forward3 5'-CCCGGCTACTACTACGTGGA-3'</p> <p>pTOM-Reverse3 5'-GCCCATGAACTCTTTGATGA-3'</p>		 <p>A gradient PCR between 50 °C and 60 °C as annealing temperatures was run using tdTomato plasmid. Due to high gel streaking in the final PCR products, these primers were not selected further.</p>

Western blotting

Since tdTomato is a two tandem dimer of Dsred protein, antibodies against DsRed can be used to detect tdTomato levels. Both polyclonal and monoclonal antibodies were used for detection of tdTomato in the total protein isolated from the sample tissues. The antibodies showed poor specificity and exhibited high background noise, i.e., detecting a number of other bands. The presence of a stronger band from control samples in the same region as tdTomato protein leads to inconclusive results (**Figure 13.4 & Figure 13.5**).

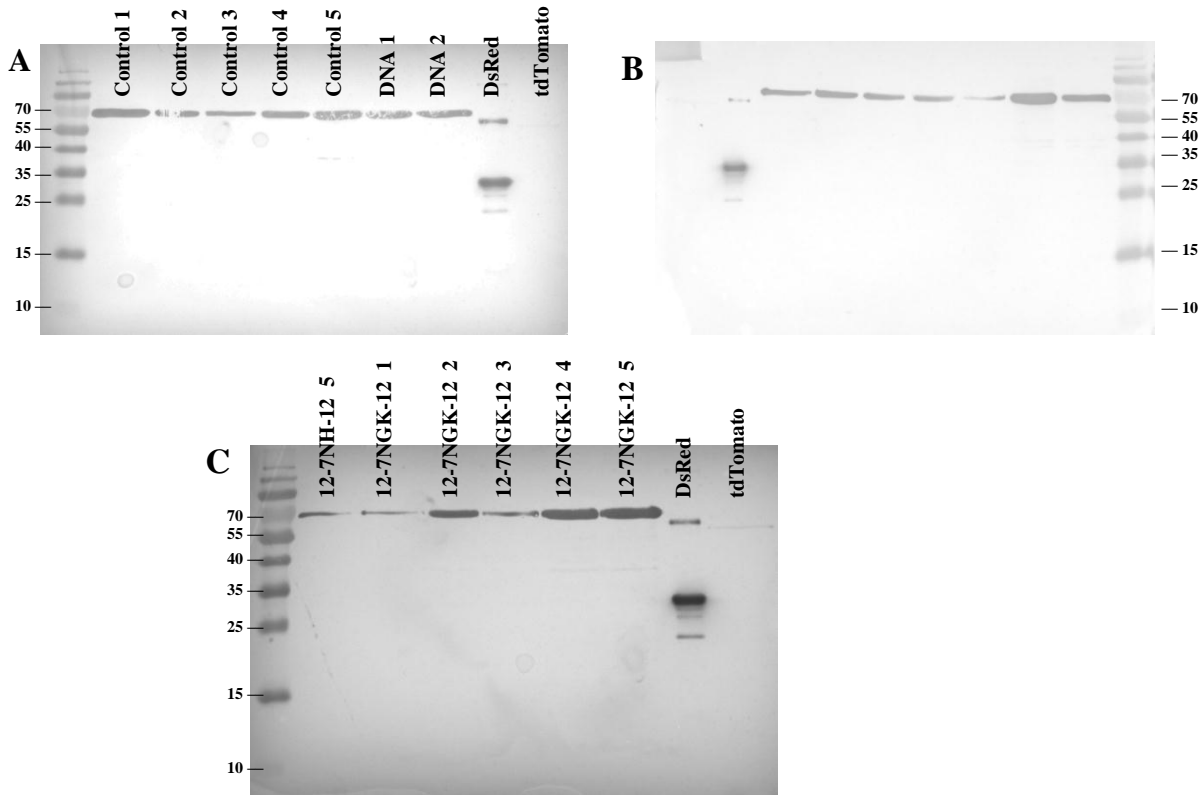


Figure 13.4: Western blot detection of tdTomato protein using polyclonal anti-DsRed antibodies on nitrocellulose membranes. Control: untreated animals; DNA: animals treated with plasmid only; 12-7NH-12: animals treated with DNA formulation containing unsubstituted gemini surfactant; 12-7NGK-12: animals treated with DNA formulation containing glycyl-lysine substituted gemini surfactant; DsRed: commercially available purified DsRed protein; tdTomato: cell lysate from Sf 1 Ep cells (rabbit epithelial cells), electroporated with pGTtdTomato, expressing tdTomato protein.

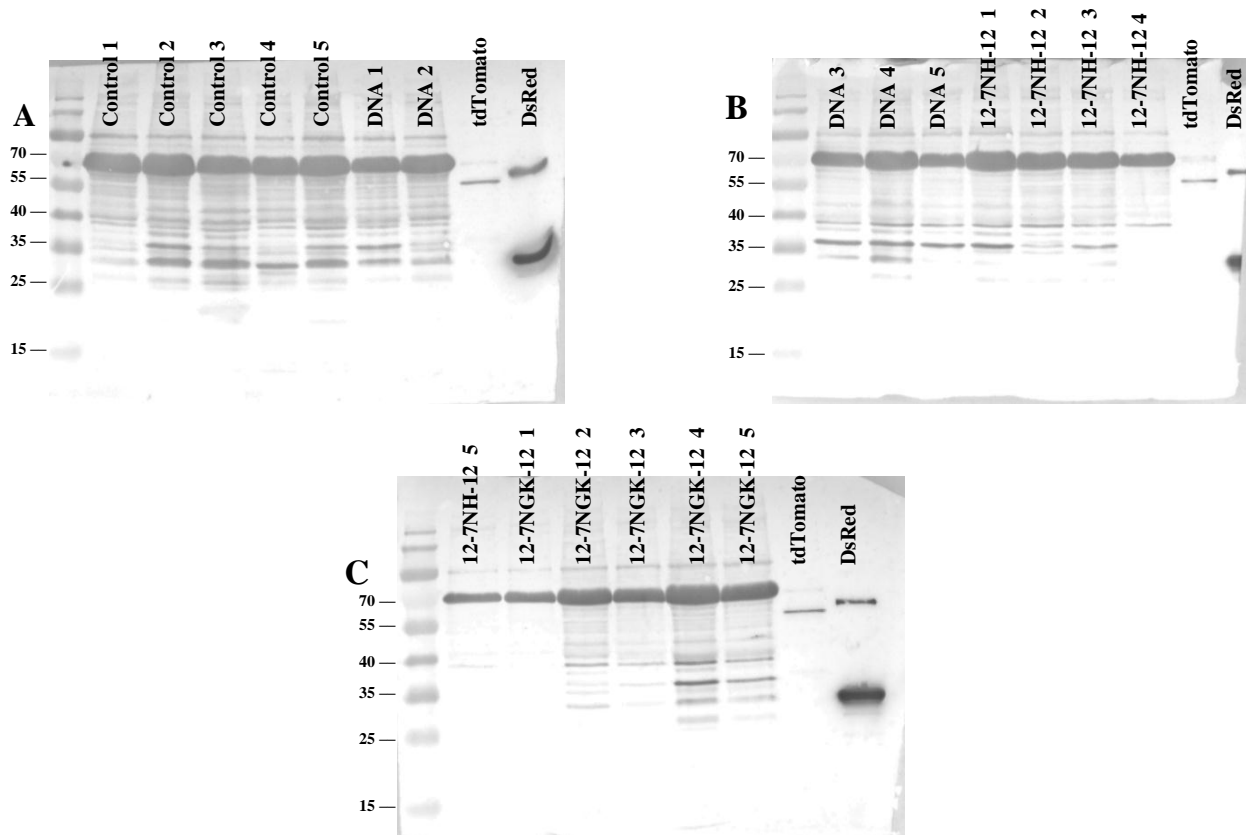


Figure 13.5: Western blot detection of tdTomato protein using monoclonal anti-DsRed antibodies on nitrocellulose membranes. Control: untreated animals; DNA: animals treated with plasmid only; 12-7NH-12: animals treated with DNA formulation containing unsubstituted gemini surfactant; 12-7NGK-12: animals treated with DNA formulation containing glycyl-lysine substituted gemini surfactant; tdTomato: cell lysate from Sf 1 Ep cells (rabbit epithelial cells), electroporated with pGTtdTomato, expressing tdTomato protein; DsRed: commercially available purified DsRed protein.

14. References

1. Schaffer, D. V.; Zhou, W. Gene Therapy and Gene Delivery Systems as Future Human Therapeutics. *Advances in Biochemical Engineering & Biotechnology*, 2005, 99, 1.
2. Anderson, W. F. The best of times, the worst of times. *Science*, 2000, 288(5466), 627.
3. Number of gene therapy clinical trials approved worldwide. Available at <http://www.abedia.com/wiley/years.php> (Accessed on July, 2012).
4. Phases of gene therapy clinical trials. Available at <http://www.abedia.com/wiley/phases.php> (Accessed on July, 2012).
5. Bouard, D.; Alazard-Dany, D.; Cosset, F. L. Viral vectors: from virology to transgene expression. *British Journal of Pharmacology*, 2009, 157(2), 153.
6. Kawakami, S.; Higuchi, Y.; Hashida, M. Nonviral approaches for targeted delivery of plasmid DNA and oligonucleotide. *Journal of Pharmaceutical Sciences*, 2008, 97(2), 726.
7. Badea, I.; Shaterian, N.; Foldvari, M. Topical Gene Delivery in Mice Using Gemini Surfactant - Lipid Nanoparticles With and Without Tape Electrode Electroporation. *Drug Delivery Letters*, 2011, 1(1), 62.
8. Badea, I.; Wettig, S.; Verrall, R.; Foldvari, M. Topical non-invasive gene delivery using gemini nanoparticles in interferon- γ -deficient mice. *European Journal of Pharmaceutics and Biopharmaceutics*, 2007, 65(3), 414.
9. Badea, I.; Verrall, R.; Baca-Estrada, M.; Tikoo, S.; Rosenberg, A.; Kumar, P.; Foldvari, M. In vivo cutaneous interferon- γ gene delivery using novel dicationic (gemini) surfactant-plasmid complexes. *Journal of Gene Medicine*, 2005, 7(9), 1200.
10. Wettig, S. D.; Badea, I.; Donkuru, M. D.; Verrall, R. E.; Foldvari, M. Structural and transfection properties of amine-substituted gemini surfactant-based nanoparticles. *The Journal of Gene Medicine*, 2007, 9(8), 649.
11. Cheng, X.; Camp, D. G.; Wu, Q.; Bakhtiar, R.; Springer, D. L.; Morris, B. J.; Bruce, J. E.; Anderson, G. A.; Edmonds, C. G.; Smith, R. D. Molecular weight determination of plasmid DNA using electrospray ionization mass spectrometry. *Nucleic Acids Research*, 1996, 24(11), 2183.
12. Kawabata, K.; Takakura, Y.; Hashida, M. The fate of plasmid DNA after intravenous injection in mice: involvement of scavenger receptors in its hepatic uptake. *Pharmaceutical Research*, 1995, 12(6), 825.
13. Mahato, R. I.; Kawabata, K.; Nomura, T.; Takakura, Y.; Hashida, M. Physicochemical and pharmacokinetic characteristics of plasmid DNA/cationic liposome complexes. *Journal of Pharmaceutical Sciences*, 1995, 84(11), 1267.

14. Gabizon, A.; Martin, F. Polyethylene glycol-coated (pegylated) liposomal doxorubicin. Rationale for use in solid tumours. *Drugs*, 1997, 54, 15.
15. Working, P.; Newman, M.; Huang, S.; Mayhew, E.; Vaage, J.; Lasic, D. Pharmacokinetics, biodistribution and therapeutic efficacy of doxorubicin encapsulated in Stealth® liposomes (Doxil®). *Journal of Liposome Research*, 1994, 4(1), 667.
16. Martin-Herranz, A.; Ahmad, A.; Evans, H. M.; Ewert, K.; Schulze, U.; Safinya, C. R. Surface functionalized cationic lipid-DNA complexes for gene delivery: PEGylated lamellar complexes exhibit distinct DNA-DNA interaction regimes. *Biophysical Journal*, 2004, 86(2), 1160.
17. Ledley, F. D. Pharmaceutical approach to somatic gene therapy. *Pharmaceutical Research*, 1996, 13(11), 1595.
18. Bloomfield, V. A. DNA condensation by multivalent cations. *Biopolymers*, 1997, 44(3), 269.
19. Wattiaux, R.; Laurent, N.; Wattiaux-De Coninck, S.; Jadot, M. Endosomes, lysosomes: their implication in gene transfer. *Advanced Drug Delivery Reviews*, 2000, 41(2), 201.
20. Wagner, E. Effects of membrane-active agents in gene delivery. *Journal of Controlled Release*, 1998, 53(1-3), 155.
21. Zhang, X.; Sawyer, G. J.; Dong, X.; Qiu, Y.; Collins, L.; Fabre, J. W. The in vivo use of chloroquine to promote non-viral gene delivery to the liver via the portal vein and bile duct. *The Journal of Gene Medicine*, 2003, 5(3), 209.
22. Lechardeur, D.; Sohn, K. J.; Haardt, M.; Joshi, P. B.; Monck, M.; Graham, R. W.; Beatty, B.; Squire, J.; O'Brodovich, H.; Lukacs, G. L. Metabolic instability of plasmid DNA in the cytosol: a potential barrier to gene transfer. *Gene Therapy*, 1999, 6(4), 482.
23. Ludtke, J. J.; Zhang, G.; Sebestyén, M. G.; Wolff, J. A. A nuclear localization signal can enhance both the nuclear transport and expression of 1 kb DNA. *Journal of Cell Science*, 1999, 112(12), 2033.
24. Bremner, K. H.; Seymour, L. W.; Pouton, C. W. Harnessing nuclear localization pathways for transgene delivery. *Current Opinion in Molecular Therapeutics*, 2001, 3(2), 170.
25. Thomas, C. E.; Ehrhardt, A.; Kay, M. A. Progress and problems with the use of viral vectors for gene therapy. *Nature Reviews Genetics*, 2003, 4(5), 346.
26. Greber, U. F.; Willetts, M.; Webster, P.; Helenius, A. Stepwise dismantling of adenovirus 2 during entry into cells. *Cell*, 1993, 75(3), 477.
27. Zennou, V.; Petit, C.; Guetard, D.; Nerhbass, U.; Montagnier, L.; Charneau, P. HIV-1 Genome Nuclear Import Is Mediated by a Central DNA Flap. *Cell*, 2000, 101(2), 173.

28. Benihoud, K.; Yeh, P.; Perricaudet, M. Adenovirus vectors for gene delivery. *Current Opinion in Biotechnology*, 1999, 10(5), 440.
29. Vectors used in gene therapy clinical trials. Available at <http://www.abedia.com/wiley/vectors.php> (Accessed on July, 2012).
30. Bachtarzi, H.; Stevenson, M.; Fisher, K. Cancer gene therapy with targeted adenoviruses. *Expert Opin. Drug Deliv.*, 2008, 5(11), 1231.
31. Daya, S.; Berns, K. I. Gene Therapy Using Adeno-Associated Virus Vectors. *Clinical Microbiology Reviews*, 2008, 21(4), 583.
32. Spirin, P. V.; Vilgelm, A. E.; Prassolov, V. S. Lentiviral vectors. *Molecular Biology*, 2008, 42(5), 814.
33. Pearson, S.; Jia, H.; Kandachi, K. China approves first gene therapy. *Nature Biotechnology*, 2004, 22(1), 3.
34. Garber, K. China approves world's first oncolytic virus therapy for cancer treatment. *Journal of the National Cancer Institute*, 2006, 98(5), 298.
35. Yu, W.; Fang, H. Clinical trials with oncolytic adenovirus in China. *Current Cancer Drug Targets*, 2007, 7(2), 141.
36. Galanis, E.; Carlson, S. K.; Foster, N. R.; Lowe, V.; Quevedo, F.; McWilliams, R. R.; Grothey, A.; Jatoui, A.; Alberts, S. R.; Rubin, J. Phase I trial of a pathotropic retroviral vector expressing a cytotoxic cyclin G1 construct (Rexin-G) in patients with advanced pancreatic cancer. *Molecular Therapy*, 2008, 16(5), 979.
37. List of Orphan Products Designated and Approved as of December 31, 2003. Available at <http://www.fda.gov/ohrms/dockets/dailys/04/June04/060304/84n-0102-1st0101-01.pdf> (Accessed on August, 2012).
38. Worgall, S.; Wolff, G.; Falck-Pedersen, E.; Crystal, R. Innate immune mechanisms dominate elimination of adenoviral vectors following in vivo administration. *Human Gene Therapy*, 1997, 8(1), 37.
39. Tripathy, S. K.; Black, H. B.; Goldwasser, E.; Leiden, J. M. Immune responses to transgene-encoded proteins limit the stability of gene expression after injection of replication-defective adenovirus vectors. *Nature Medicine*, 1996, 2(5), 545.
40. Russell, D. W. AAV vectors, insertional mutagenesis, and cancer. *Molecular Therapy*, 2007, 15(10), 1740.
41. Tenenbaum, L.; Lehtonen, E.; Monahan, P. E. Evaluation of risks related to the use of adeno-associated virus-based vectors. *Current Gene Therapy*, 2003, 3(6), 545.

42. Marshall, E. Clinical Trials:Gene Therapy Death Prompts Review of Adenovirus Vector. *Science*, 1999, 286(5448), 2244.
43. Check, E. Gene therapy put on hold as third child develops cancer. *Nature*, 2005, 433(7026), 561.
44. Grieger, J. C.; Samulski, R. J. Packaging Capacity of Adeno-Associated Virus Serotypes: Impact of Larger Genomes on Infectivity and Postentry Steps. *The Journal of Virology*, 2005, 79(15), 9933.
45. Dormond, E.; Meneses-Acosta, A.; Jacob, D.; Durocher, Y.; Gilbert, R.; Perrier, M.; Kamen, A. An efficient and scalable process for helper-dependent adenoviral vector production using polyethylenimine-adenofection. *Biotechnology and Bioengineering*, 2009, 102(3), 800.
46. Yan, Z.; Zhang, Y.; Duan, D.; Engelhardt, J. F. Trans-splicing vectors expand the utility of adeno-associated virus for gene therapy. *Proceedings of the National Academy of Sciences of the United States of America*, 2000, 97(12), 6716.
47. Nishikawa, M.; Takakura, Y.; Hashida, M. Theoretical considerations involving the pharmacokinetics of plasmid DNA. *Advanced Drug Delivery Reviews*, 2005, 57(5), 675.
48. Aihara, H.; Miyazaki, J. Gene transfer into muscle by electroporation in vivo. *Nature Biotechnology*, 1998, 16(9), 867.
49. Kusumanto, Y. H.; Mulder, N. H.; Dam, W. A.; Losen, M. H.; Meijer, C.; Hospers, G. A. P. Improvement of in vivo transfer of plasmid DNA in muscle: comparison of electroporation versus ultrasound. *Drug Delivery*, 2007, 14(5), 273.
50. Bramson, J.; Dayball, K.; Eveleigh, C.; Wan, Y.; Page, D.; Smith, A. Enabling topical immunization via microporation: a novel method for pain-free and needle-free delivery of adenovirus-based vaccines. *Gene Therapy*, 2003, 10(3), 251.
51. Kobayashi, N.; Rivas-Carrillo, J. D.; Soto-Gutierrez, A.; Fukazawa, T.; Chen, Y.; Navarro-Alvarez, N.; Tanaka, N. Gene delivery to embryonic stem cells. *Birth Defects Research Part C: Embryo Today: Reviews*, 2005, 75(1), 10.
52. Felgner, P. L.; Gadek, T. R.; Holm, M.; Roman, R.; Chan, H. W.; Wenz, M.; Northrop, J. P.; Ringold, G. M.; Danielsen, M. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proceedings of the National Academy of Sciences of the United States of America*, 1987, 84(21), 7413.
53. Behr, J. P.; Demeneix, B.; Loeffler, J. P.; Perez-Mutul, J. Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine-coated DNA. *Proceedings of the National Academy of Sciences of the United States of America*, 1989, 86(18), 6982.
54. Gebeyehu, G.; Jessee, J. A.; Ciccarone, V. C.; Hawley-Nelson, P.; Chytil, A. Cationic lipids. US patent number 5,334,761 (issued 02 August, 1994).

55. Stamatatos, L.; Leventis, R.; Zuckermann, M. J.; Silviu, J. R. Interactions of cationic lipid vesicles with negatively charged phospholipid vesicles and biological membranes. *Biochemistry*, 1988, 27(11), 3917.
56. Xu, Y.; Szoka, F. C. Mechanism of DNA Release from Cationic Liposome/DNA Complexes Used in Cell Transfection. *Biochemistry*, 1996, 35(18), 5616.
57. Zelphati, O.; Szoka, F. C. Mechanism of oligonucleotide release from cationic liposomes. *Proceedings of the National Academy of Sciences of the United States of America*, 1996, 93(21), 11493.
58. Guénin, E.; Hervé, A. C.; Floch, V.; Loisel, S.; Yaouanc, J. J.; Clément, J. C.; Férec, C.; des Abbayes, H. Cationic Phosphonolipids Containing Quaternary Phosphonium and Arsonium Groups for DNA Transfection with Good Efficiency and Low Cellular Toxicity. *Angewandte Chemie*, 2000, 112(3), 643.
59. Floch, V.; Loisel, S.; Guenin, E.; Hervé, A. C.; Clément, J. C.; Yaouanc, J. J.; des Abbayes, H.; Férec, C. Cation substitution in cationic phosphonolipids: a new concept to improve transfection activity and decrease cellular toxicity. *Journal of Medicinal Chemistry*, 2000, 43(24), 4617.
60. Bennett, M. J.; Aberle, A. M.; Balasubramaniam, R. P.; Malone, J. G.; Malone, R. W.; Nantz, M. H. Cationic lipid-mediated gene delivery to murine lung: correlation of lipid hydration with in vivo transfection activity. *Journal of Medicinal Chemistry*, 1997, 40(25), 4069.
61. Felgner, J. H.; Kumar, R.; Sridhar, C.; Wheeler, C. J.; Tsai, Y. J.; Border, R.; Ramsey, P.; Martin, M.; Felgner, P. L. Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. *Journal of Biological Chemistry*, 1994, 269(4), 2550.
62. Geall, A. J.; Blagbrough, I. S.; Taylor, R. J.; Earll, M. E.; Eaton, M. A. W. Synthesis of cholesterol-polyamine carbamates: pKa studies and condensation of calf thymus DNA. *Chem. Commun.*, 1998(13), 1403.
63. Geall, A. J.; Eaton, M. A. W.; Baker, T.; Catterall, C.; Blagbrough, I. S. The regiochemical distribution of positive charges along cholesterol polyamine carbamates plays significant roles in modulating DNA binding affinity and lipofection. *FEBS Letters*, 1999, 459(3), 337.
64. Ewert, K.; Ahmad, A.; Evans, H. M.; Schmidt, H. W.; Safinya, C. R. Efficient synthesis and cell-transfection properties of a new multivalent cationic lipid for nonviral gene delivery. *Journal of Medicinal Chemistry*, 2002, 45(23), 5023.
65. Heyes, J. A.; Niculescu-Duvaz, D.; Cooper, R. G.; Caroline, J. Synthesis of novel cationic lipids: effect of structural modification on the efficiency of gene transfer. *Journal of Medicinal Chemistry*, 2002, 45(1), 99.

66. Balasubramaniam, R.; Bennett, M.; Aberle, A.; Malone, J.; Nantz, M.; Malone, R. Structural and functional analysis of cationic transfection lipids: the hydrophobic domain. *Gene Therapy*, 1996, 3(2), 163.
67. Byk, G.; Dubertret, C.; Escriou, V.; Frederic, M.; Jaslin, G.; Rangara, R.; Pitard, B.; Crouzet, J.; Wils, P.; Schwartz, B. Synthesis, activity, and structure-activity relationship studies of novel cationic lipids for DNA transfer. *Journal of Medicinal Chemistry*, 1998, 41(2), 224.
68. McGregor, C.; Perrin, C.; Monck, M.; Camilleri, P.; Kirby, A. J. Rational approaches to the design of cationic gemini surfactants for gene delivery. *Journal of the American Chemical Society*, 2001, 123(26), 6215.
69. Bloomfield, V. A. DNA condensation. *Current Opinion in Structural Biology*, 1996, 6(3), 334.
70. Boussif, O.; Lezoualc'h, F.; Zanta, M. A.; Mergny, M. D.; Scherman, D.; Demeneix, B.; Behr, J. P. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proceedings of the National Academy of Sciences of the United States of America*, 1995, 92(16), 7297.
71. Klotz, I. M.; Royer, G. P.; Sloniewsky, A. R. Macromolecule-small molecule interactions. Strong binding and cooperativity in a model synthetic polymer. *Biochemistry*, 1969, 8(12), 4752.
72. Brissault, B.; Kichler, A.; Guis, C.; Leborgne, C.; Danos, O.; Cheradame, H. Synthesis of linear polyethylenimine derivatives for DNA transfection. *Bioconjugate Chemistry*, 2003, 14(3), 581.
73. Akinc, A.; Thomas, M.; Klibanov, A. M.; Langer, R. Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis. *The Journal of Gene Medicine*, 2005, 7(5), 657.
74. Zauner, W.; Ogris, M.; Wagner, E. Polylysine-based transfection systems utilizing receptor-mediated delivery. *Advanced Drug Delivery Reviews*, 1998, 30(1-3), 97.
75. Akinc, A.; Langer, R. Measuring the pH environment of DNA delivered using nonviral vectors: implications for lysosomal trafficking. *Biotechnology and Bioengineering*, 2002, 78(5), 503.
76. Kwok, D. Y.; Coffin, C. C.; Lollo, C. P.; Jovenal, J.; Banaszczyk, M. G.; Mullen, P.; Phillips, A.; Amini, A.; Fabrycki, J.; Bartholomew, R. M. Stabilization of poly-lysine/DNA polyplexes for in vivo gene delivery to the liver. *Biochimica Et Biophysica Acta (BBA)-Gene Structure and Expression*, 1999, 1444(2), 171.
77. Hwang, S. J.; Bellocq, N. C.; Davis, M. E. Effects of Structure of β -Cyclodextrin-Containing Polymers on Gene Delivery. *Bioconjugate Chemistry*, 2001, 12(2), 280.

78. Borchard, G. Chitosans for gene delivery. *Advanced Drug Delivery Reviews*, 2001, 52(2), 145.
79. Liu, Y.; Wenning, L.; Lynch, M.; Reineke, T. M. New Poly(d-glucaramidoamine)s Induce DNA Nanoparticle Formation and Efficient Gene Delivery into Mammalian Cells. *Journal of the American Chemical Society*, 2004, 126(24), 7422.
80. Sakurai, K.; Shinkai, S. Molecular Recognition of Adenine, Cytosine, and Uracil in a Single-Stranded RNA by a Natural Polysaccharide: Schizophyllan. *Journal of the American Chemical Society*, 2000, 122(18), 4520.
81. Xiong, M. P.; Forrest, M. L.; Karls, A. L.; Kwon, G. S. Biotin-Triggered Release of Poly(ethylene glycol)-Avidin from Biotinylated Polyethylenimine Enhances in Vitro Gene Expression. *Bioconjugate Chemistry*, 2007, 18(3), 746.
82. Gao, S.; Chen, J.; Xu, X.; Ding, Z.; Yang, Y.; Hua, Z.; Zhang, J. Galactosylated low molecular weight chitosan as DNA carrier for hepatocyte-targeting. *International Journal of Pharmaceutics*, 2003, 255(1-2), 57.
83. Hashimoto, M.; Morimoto, M.; Saimoto, H.; Shigemasa, Y.; Sato, T. Lactosylated Chitosan for DNA Delivery into Hepatocytes: The Effect of Lactosylation on the Physicochemical Properties and Intracellular Trafficking of pDNA/Chitosan Complexes. *Bioconjugate Chemistry*, 2006, 17(2), 309.
84. Issa, M. M.; Köping-Höggård, M.; Tømmeraas, K.; Vårum, K. M.; Christensen, B. E.; Strand, S. P.; Artursson, P. Targeted gene delivery with trisaccharide-substituted chitosan oligomers in vitro and after lung administration in vivo. *Journal of Controlled Release*, 2006, 115(1), 103.
85. Ahn, C.; Chae, S. Y.; Bae, Y. H.; Kim, S. W. Biodegradable poly(ethylenimine) for plasmid DNA delivery. *Journal of Controlled Release*, 2002, 80(1-3), 273.
86. Haensler, J.; Szoka, F. C. Polyamidoamine cascade polymers mediate efficient transfection of cells in culture. *Bioconjugate Chemistry*, 1993, 4(5), 372.
87. Kabanov, V. A.; Zezin, A. B.; Rogacheva, V. B.; Gulyaeva, Z. G.; Zansochova, M. F.; Joosten, J. G. H.; Brackman, J. Interaction of Astramol Poly(propyleneimine) Dendrimers with Linear Polyanions. *Macromolecules*, 1999, 32(6), 1904.
88. Shah, D. S.; Sakthivel, T.; Toth, I.; Florence, A. T.; Wilderspin, A. F. DNA transfection and transfected cell viability using amphipathic asymmetric dendrimers. *International Journal of Pharmaceutics*, 2000, 208(1-2), 41.
89. Loup, C.; Zanta, M.; Caminade, A.; Majoral, J.; Meunier, B. Preparation of Water-Soluble Cationic Phosphorus-Containing Dendrimers as DNA Transfecting Agents. *Chemistry - A European Journal*, 1999, 5(12), 3644.

90. Chen, W.; Turro, N. J.; Tomalia, D. A. Using Ethidium Bromide To Probe the Interactions between DNA and Dendrimers. *Langmuir*, 2000, 16(1), 15.
91. Zhang, Z.; Smith, B. D. High-Generation Polycationic Dendrimers Are Unusually Effective at Disrupting Anionic Vesicles: Membrane Bending Model. *Bioconjugate Chemistry*, 2000, 11(6), 805.
92. Wood, K. C.; Azarin, S. M.; Arap, W.; Pasqualini, R.; Langer, R.; Hammond, P. T. Tumor-Targeted Gene Delivery Using Molecularly Engineered Hybrid Polymers Functionalized with a Tumor-Homing Peptide. *Bioconjugate Chemistry*, 2008, 19(2), 403.
93. Manunta, M.; Tan, P. H.; Sagoo, P.; Kashefi, K.; George, A. J. T. Gene delivery by dendrimers operates via a cholesterol dependent pathway. *Nucleic Acids Research*, 2004, 32(9), 2730.
94. Tang, M. X.; Redemann, C. T.; Szoka Jr, F. C. In vitro gene delivery by degraded polyamidoamine dendrimers. *Bioconjugate Chemistry*, 1996, 7(6), 703.
95. Kukowska-Latallo, J. F.; Bielinska, A. U.; Johnson, J.; Spindler, R.; Tomalia, D. A.; Baker, J. R. Efficient transfer of genetic material into mammalian cells using Starburst polyamidoamine dendrimers. *Proceedings of the National Academy of Sciences*, 1996, 93(10), 4897.
96. Ignatovich, I. A.; Dizhe, E. B.; Pavlotskaya, A. V.; Akifiev, B. N.; Burov, S. V.; Orlov, S. V.; Perevozchikov, A. P. Complexes of Plasmid DNA with Basic Domain 47-57 of the HIV-1 Tat Protein Are Transferred to Mammalian Cells by Endocytosis-mediated Pathways. *Journal of Biological Chemistry*, 2003, 278(43), 42625.
97. Ou, J.; Geiger, T.; Ou, Z.; Ackerman, A. W.; Oldham, K. T.; Pritchard Jr., K. A. AP-4F, antennapedia peptide linked to an amphipathic α helical peptide, increases the efficiency of Lipofectamine-mediated gene transfection in endothelial cells. *Biochemical and Biophysical Research Communications*, 2003, 305(3), 605.
98. Morris, M.; Chaloin, L.; Mery, J.; Heitz, F.; Divita, G. A novel potent strategy for gene delivery using a single peptide vector as a carrier. *Nucleic Acids Research*, 1999, 27(17), 3510.
99. Kilk, K.; EL-Andaloussi, S.; Järver, P.; Meikas, A.; Valkna, A.; Bartfai, T.; Kogerman, P.; Metsis, M.; Langel, Ü. Evaluation of transportan 10 in PEI mediated plasmid delivery assay. *Journal of Controlled Release*, 2005, 103(2), 511.
100. Torchilin, V. P.; Levchenko, T. S.; Rammohan, R.; Volodina, N.; Papahadjopoulos-Sternberg, B.; D'Souza, G. G. M. Cell transfection in vitro and in vivo with nontoxic TAT peptide-liposome-DNA complexes. *Proceedings of the National Academy of Sciences*, 2003, 100(4), 1972.

101. Rudolph, C.; Plank, C.; Lausier, J.; Schillinger, U.; Müller, R. H.; Rosenecker, J. Oligomers of the arginine-rich motif of the HIV-1 TAT protein are capable of transferring plasmid DNA into cells. *Journal of Biological Chemistry*, 2003, 278(13), 11411.
102. Hashida, H.; Miyamoto, M.; Cho, Y.; Hida, Y.; Kato, K.; Kurokawa, T.; Okushiba, S.; Kondo, S.; Dosaka-Akita, H.; Katoh, H. Fusion of HIV-1 Tat protein transduction domain to poly-lysine as a new DNA delivery tool. *British Journal of Cancer*, 2004, 90(6), 1252.
103. Derossi, D.; Chassaing, G.; Prochiantz, A. Trojan peptides: the penetratin system for intracellular delivery. *Trends in Cell Biology*, 1998, 8(2), 84.
104. Deshayes, S.; Gerbal-Chaloin, S.; Morris, M. C.; Aldrian-Herrada, G.; Charnet, P.; Divita, G.; Heitz, F. On the mechanism of non-endosomal peptide-mediated cellular delivery of nucleic acids. *Biochimica Et Biophysica Acta (BBA)-Biomembranes*, 2004, 1667(2), 141.
105. Gershon, H.; Ghirlando, R.; Guttman, S. B.; Minsky, A. Mode of formation and structural features of DNA-cationic liposome complexes used for transfection. *Biochemistry*, 1993, 32(28), 7143.
106. Mislick, K. A.; Baldeschwieler, J. D. Evidence for the role of proteoglycans in cation-mediated gene transfer. *Proceedings of the National Academy of Sciences of the United States of America*, 1996, 93(22), 12349.
107. Takeuchi, K.; Ishihara, M.; Kawaura, C.; Noji, M.; Furuno, T.; Nakanishi, M. Effect of zeta potential of cationic liposomes containing cationic cholesterol derivatives on gene transfection. *FEBS Letters*, 1996, 397(2-3), 207.
108. Kreiss, P.; Cameron, B.; Rangara, R.; Mailhe, P.; Aguerre-Charriol, O.; Airiau, M.; Scherman, D.; Crouzet, J.; Pitard, B. Plasmid DNA size does not affect the physicochemical properties of lipoplexes but modulates gene transfer efficiency. *Nucleic Acids Research*, 1999, 27(19), 3792.
109. Arigita, C.; Zuidam, N. J.; Crommelin, D. J.; Hennink, W. E. Association and dissociation characteristics of polymer/DNA complexes used for gene delivery. *Pharm Res*, 1999, 16(10), 1534.
110. Schaffer, D. V.; Fidelman, N. A.; Dan, N.; Lauffenburger, D. A. Vector unpacking as a potential barrier for receptor-mediated polyplex gene delivery. *Biotechnol Bioeng*, 2000, 67(5), 598.
111. Sakurai, F.; Inoue, R.; Nishino, Y.; Okuda, A.; Matsumoto, O.; Taga, T.; Yamashita, F.; Takakura, Y.; Hashida, M. Effect of DNA/liposome mixing ratio on the physicochemical characteristics, cellular uptake and intracellular trafficking of plasmid DNA/cationic liposome complexes and subsequent gene expression. *Journal of Controlled Release*, 2000, 66(2-3), 255.

112. Prabha, S.; Zhou, W. Z.; Panyam, J.; Labhasetwar, V. Size-dependency of nanoparticle-mediated gene transfection: studies with fractionated nanoparticles. *International Journal of Pharmaceutics*, 2002, 244(1-2), 105.
113. Desai, M. P.; Labhasetwar, V.; Walter, E.; Levy, R. J.; Amidon, G. L. The mechanism of uptake of biodegradable microparticles in Caco-2 cells is size dependent. *Pharmaceutical Research*, 1997, 14(11), 1568.
114. Xu, D. M.; Yao, S. D.; Liu, Y. B.; Sheng, K. L.; Hong, J.; Gong, P. J.; Dong, L. Size-dependent properties of M-PEIs nanogels for gene delivery in cancer cells. *International Journal of Pharmaceutics*, 2007, 338(1-2), 291.
115. Radwan Almofti, M.; Harashima, H.; Shinohara, Y.; Almofti, A.; Baba, Y.; Kiwada, H. Cationic liposome-mediated gene delivery: biophysical study and mechanism of internalization. *Archives of Biochemistry and Biophysics*, 2003, 410(2), 246.
116. Hsu, C.; Uludag, H. Effects of size and topology of DNA molecules on intracellular delivery with non-viral gene carriers. *BMC Biotechnology*, 2008, 8(1), 23.
117. Cherng, J. Y.; Schuurmans-Nieuwenbroek, N.; Jiskoot, W.; Talsma, H.; Zuidam, N. J.; Hennink, W. E.; Crommelin, D. J. Effect of DNA topology on the transfection efficiency of poly((2-dimethylamino)ethyl methacrylate)-plasmid complexes. *J Control Release*, 1999, 60(2-3), 343.
118. Kamiya, H.; Yamazaki, J.; Harashima, H. Size and topology of exogenous DNA as determinant factors of transgene transcription in mammalian cells. *Gene Ther*, 2002, 9(22), 1500.
119. Remaut, K.; Sanders, N. N.; Fayazpour, F.; Demeester, J.; De Smedt, S. C. Influence of plasmid DNA topology on the transfection properties of DOTAP/DOPE lipoplexes. *Journal of Controlled Release*, 2006, 115(3), 335.
120. Lasic, D. D.; Strey, H.; Stuart, M. C. A.; Podgornik, R.; Frederik, P. M. The structure of DNA-liposome complexes. *Journal of the American Chemical Society*, 1997, 119(4), 832.
121. Rädler, J. O.; Koltover, I.; Salditt, T.; Safinya, C. R. Structure of DNA-cationic liposome complexes: DNA intercalation in multilamellar membranes in distinct interhelical packing regimes. *Science*, 1997, 275(5301), 810.
122. Koltover, I.; Salditt, T.; Safinya, C. R. Phase diagram, stability, and overcharging of lamellar cationic lipid-DNA self-assembled complexes. *Biophysical Journal*, 1999, 77(2), 915.
123. Farhood, H.; Serbina, N.; Huang, L. The role of dioleoyl phosphatidylethanolamine in cationic liposome mediated gene transfer. *Biochimica Et Biophysica Acta (BBA) - Biomembranes*, 1995, 1235(2), 289.

124. Lin, A. J.; Slack, N. L.; Ahmad, A.; George, C. X.; Samuel, C. E.; Safinya, C. R. Three-Dimensional Imaging of Lipid Gene-Carriers: Membrane Charge Density Controls Universal Transfection Behavior in Lamellar Cationic Liposome-DNA Complexes. *Biophysical Journal*, 2003, 84(5), 3307.
125. Conner, S. D.; Schmid, S. L. Regulated portals of entry into the cell. *Nature*, 2003, 422(6927), 37.
126. Labat-Moleur, F.; Steffan, A. M.; Brisson, C.; Perron, H.; Feugeas, O.; Furstenberger, P.; Oberling, F.; Brambilla, E.; Behr, J. An electron microscopy study into the mechanism of gene transfer with lipopolyamines. *Gene Therapy*, 1996, 3(11), 1010.
127. Friend, D. S.; Papahadjopoulos, D.; Debs, R. J. Endocytosis and intracellular processing accompanying transfection mediated by cationic liposomes. *Biochimica Et Biophysica Acta (BBA)-Biomembranes*, 1996, 1278(1), 41.
128. Takei, K.; Haucke, V. Clathrin-mediated endocytosis: membrane factors pull the trigger. *Trends in Cell Biology*, 2001, 11(9), 385.
129. Maxfield, F. R.; McGraw, T. E. Endocytic recycling. *Nature Reviews Molecular Cell Biology*, 2004, 5(2), 121.
130. Legendre, J. Y.; Szoka Jr, F. C. Delivery of plasmid DNA into mammalian cell lines using pH-sensitive liposomes: comparison with cationic liposomes. *Pharmaceutical Research*, 1992, 9(10), 1235.
131. Mochizuki, S.; Kanegae, N.; Nishina, K.; Kamikawa, Y.; Koiwai, K.; Masunaga, H.; Sakurai, K. The role of the helper lipid dioleoylphosphatidylethanolamine (DOPE) for DNA transfection cooperating with a cationic lipid bearing ethylenediamine. *Biochimica Et Biophysica Acta (BBA) - Biomembranes*, 2013, 1828(2), 412.
132. De Duve, C.; De Barse, T.; Poole, B.; Trouet, A.; Tulkens, P.; Van Hoof, F. Commentary. Lysosomotropic agents. *Biochemical Pharmacology*, 1974, 23(18), 2495.
133. Kakudo, T.; Chaki, S.; Futaki, S.; Nakase, I.; Akaji, K.; Kawakami, T.; Maruyama, K.; Kamiya, H.; Harashima, H. Transferrin-modified liposomes equipped with a pH-sensitive fusogenic peptide: an artificial viral-like delivery system. *Biochemistry*, 2004, 43(19), 5618.
134. Pelkmans, L.; Kartenbeck, J.; Helenius, A. Caveolar endocytosis of simian virus 40 reveals a new two-step vesicular-transport pathway to the ER. *Nature Cell Biology*, 2001, 3(5), 473.
135. Pelkmans, L.; Helenius, A. Endocytosis via caveolae. *Traffic*, 2002, 3(5), 311.
136. Ferrari, A.; Pellegrini, V.; Arcangeli, C.; Fittipaldi, A.; Giacca, M.; Beltram, F. Caveolae-mediated internalization of extracellular HIV-1 tat fusion proteins visualized in real time. *Molecular Therapy*, 2003, 8(2), 284.

137. Rejman, J.; Bragonzi, A.; Conese, M. Role of clathrin-and caveolae-mediated endocytosis in gene transfer mediated by lipo-and polyplexes. *Molecular Therapy*, 2005, 12(3), 468.
138. Kichler, A.; Leborgne, C.; Coeytaux, E.; Danos, O. Polyethylenimine-mediated gene delivery: a mechanistic study. *The Journal of Gene Medicine*, 2001, 3(2), 135.
139. Fujimoto, T.; Kogo, H.; Nomura, R.; Une, T. Isoforms of caveolin-1 and caveolar structure. *Journal of Cell Science*, 2000, 113(19), 3509.
140. Von Gersdorff, K.; Sanders, N. N.; Vandenbroucke, R.; De Smedt, S. C.; Wagner, E.; Ogris, M. The internalization route resulting in successful gene expression depends on both cell line and polyethylenimine polyplex type. *Molecular Therapy*, 2006, 14(5), 745.
141. van der Aa, M. A. E. M.; Huth, U.; Häfele, S.; Schubert, R.; Oosting, R.; Mastrobattista, E.; Hennink, W.; Peschka-Süss, R.; Koning, G.; Crommelin, D. J. A. Cellular uptake of cationic polymer-DNA complexes via caveolae plays a pivotal role in gene transfection in COS-7 cells. *Pharmaceutical Research*, 2007, 24(8), 1590.
142. Swanson, J. A.; Watts, C. Macropinocytosis. *Trends in Cell Biology*, 1995, 5(11), 424.
143. Meier, O.; Greber, U. F. Adenovirus endocytosis. *The Journal of Gene Medicine*, 2004, 6(S1), S152.
144. Kaplan, I. M.; Wadia, J. S.; Dowdy, S. F. Cationic TAT peptide transduction domain enters cells by macropinocytosis. *Journal of Controlled Release*, 2005, 102(1), 247.
145. Nakase, I.; Niwa, M.; Takeuchi, T.; Sonomura, K.; Kawabata, N.; Koike, Y.; Takehashi, M.; Tanaka, S.; Ueda, K.; Simpson, J. C. Cellular uptake of arginine-rich peptides: roles for macropinocytosis and actin rearrangement. *Molecular Therapy*, 2004, 10(6), 1011.
146. Nakase, I.; Tadokoro, A.; Kawabata, N.; Takeuchi, T.; Katoh, H.; Hiramoto, K.; Negishi, M.; Nomizu, M.; Sugiura, Y.; Futaki, S. Interaction of arginine-rich peptides with membrane-associated proteoglycans is crucial for induction of actin organization and macropinocytosis. *Biochemistry*, 2007, 46(2), 492.
147. Khalil, I. A.; Kogure, K.; Futaki, S.; Harashima, H. High density of octaarginine stimulates macropinocytosis leading to efficient intracellular trafficking for gene expression. *Journal of Biological Chemistry*, 2006, 281(6), 3544.
148. Kobayashi, S.; Hattori, Y.; Osakada, H.; Toma, K.; Maitani, Y.; Hiraoka, Y.; Haraguchi, T. Early entry and deformation of macropinosomes correlates with high efficiency of decaarginine-PEG-lipid-mediated gene delivery. *The Journal of Gene Medicine*, 2012.
149. Rejman, J.; Oberle, V.; Zuhorn, I. S.; Hoekstra, D. Size-dependent internalization of particles via the pathways of clathrin-and caveolae-mediated endocytosis. *Biochemical Journal*, 2004, 377(Pt 1), 159.

150. Gratton, S. E. A.; Ropp, P. A.; Pohlhaus, P. D.; Luft, J. C.; Madden, V. J.; Napier, M. E.; DeSimone, J. M. The effect of particle design on cellular internalization pathways. *Proceedings of the National Academy of Sciences*, 2008, 105(33), 11613.
151. Menger, F.; Littau, C. Gemini surfactants: a new class of self-assembling molecules. *Journal of the American Chemical Society*, 1993, 115(22), 10083.
152. Bai, G.; Wang, J.; Yan, H.; Li, Z.; Thomas, R. K. Thermodynamics of molecular self-assembly of cationic gemini and related double chain surfactants in aqueous solution. *J.Phys.Chem.B*, 2001, 105(15), 3105.
153. Hait, S.; Moulik, S. Gemini surfactants: A distinct class of self-assembling molecules. *Curr.Sci*, 2002, 82(9), 1101–1111.
154. Wettig, S. D.; Verrall, R. E.; Foldvari, M. Gemini Surfactants: A New Family of Building Blocks for Non-Viral Gene Delivery Systems. *Current Gene Therapy*, 2008, 8(1), 9.
155. Rosenzweig, H. S.; Rakhmanova, V. A.; MacDonald, R. C. Diquaternary ammonium compounds as transfection agents. *Bioconjugate Chemistry*, 2001, 12(2), 258.
156. Fielden, M. L.; Perrin, C.; Kremer, A.; Bergsma, M.; Stuart, M. C.; Camilleri, P.; Engberts, J. B. F. N. Sugar-based tertiary amino gemini surfactants with a vesicle-to-micelle transition in the endosomal pH range mediate efficient transfection in vitro. *European Journal of Biochemistry*, 2001, 268(5), 1269.
157. Ryhänen, S. J.; Säily, M. J.; Paukku, T.; Borocci, S.; Mancini, G.; Holopainen, J. M.; Kinnunen, P. K. J. Surface charge density determines the efficiency of cationic gemini surfactant based lipofection. *Biophysical Journal*, 2003, 84(1), 578.
158. Fiscaro, E.; Compari, C.; Duce, E.; Donofrio, G.; Rózycka-Roszak, B.; Woźniak, E. Biologically active bisquaternary ammonium chlorides: physico-chemical properties of long chain amphiphiles and their evaluation as non-viral vectors for gene delivery. *Biochimica Et Biophysica Acta (BBA)-General Subjects*, 2005, 1722(2), 224.
159. Gaucheron, J.; Wong, T.; Wong, K. F.; Maurer, N.; Cullis, P. R. Synthesis and properties of novel tetraalkyl cationic lipids. *Bioconjugate Chemistry*, 2002, 13(3), 671.
160. Bombelli, C.; Faggioli, F.; Luciani, P.; Mancini, G.; Sacco, M. G. Efficient transfection of DNA by liposomes formulated with cationic gemini amphiphiles. *Journal of Medicinal Chemistry*, 2005, 48(16), 5378.
161. Buijnsters, P. J. J. A.; García Rodríguez, C. L.; Willighagen, E. L.; Sommerdijk, N. A. J. M.; Kremer, A.; Camilleri, P.; Feiters, M. C.; Nolte, R. J. M.; Zwanenburg, B. Cationic gemini surfactants based on tartaric acid: synthesis, aggregation, monolayer behaviour, and interaction with DNA. *European Journal of Organic Chemistry*, 2002, 2002(8), 1397.

162. Castro, M.; Griffiths, D.; Patel, A.; Patrick, N.; Kitson, C.; Ladlow, M. Effect of chain length on transfection properties of spermine-based gemini surfactants. *Organic & Biomolecular Chemistry*, 2004, 2(19), 2814.
163. Bell, P. C.; Bergsma, M.; Dolbnya, I. P.; Bras, W.; Stuart, M. C. A.; Rowan, A. E.; Feiters, M. C.; Engberts, J. B. F. N. Transfection mediated by gemini surfactants: engineered escape from the endosomal compartment. *Journal of the American Chemical Society*, 2003, 125(6), 1551.
164. Acarturk, F. Mucoadhesive Vaginal Drug Delivery Systems. *Recent Patents on Drug Delivery & Formulation*, 2009, 3(3), 193.
165. Kikuchi, T.; Crystal, R. G. Anti-tumor immunity induced by in vivo adenovirus vector-mediated expression of CD40 ligand in tumor cells. *Human Gene Therapy*, 1999, 10(8), 1375.
166. Öhlschläger, P.; Quetting, M.; Alvarez, G.; Dürst, M.; Gissmann, L.; Kaufmann, A. M. Enhancement of immunogenicity of a therapeutic cervical cancer DNA-based vaccine by co-application of sequence-optimized genetic adjuvants. *International Journal of Cancer*, 2009, 125(1), 189.
167. Hong, D.; Lu, W.; Ye, F.; Hu, Y.; Xie, X. Gene silencing of HPV16 E6/E7 induced by promoter-targeting siRNA in SiHa cells. *British Journal of Cancer*, 2009, 101(10), 1798.
168. Ashkar, A. A.; Bauer, S.; Mitchell, W. J.; Vieira, J.; Rosenthal, K. L. Local delivery of CpG oligodeoxynucleotides induces rapid changes in the genital mucosa and inhibits replication, but not entry, of herpes simplex virus type 2. *Journal of Virology*, 2003, 77(16), 8948.
169. Echchannaoui, H.; Bianchi, M.; Baud, D.; Bobst, M.; Stehle, J. C.; Nardelli-Haeffliger, D. Intravaginal immunization of mice with recombinant *Salmonella enterica* serovar Typhimurium expressing human papillomavirus type 16 antigens as a potential route of vaccination against cervical cancer. *Infection and Immunity*, 2008, 76(5), 1940.
170. Klavinskis, L. S.; Barnfield, C.; Gao, L.; Parker, S. Intranasal immunization with plasmid DNA-lipid complexes elicits mucosal immunity in the female genital and rectal tracts. *Journal of Immunology*, 1999, 162(1), 254.
171. Hussain, A.; Ahsan, F. The vagina as a route for systemic drug delivery. *Journal of Controlled Release*, 2005, 103(2), 301.
172. Han, I. K.; Kim, Y. B.; Kang, H. S.; Sul, D.; Jung, W. W.; Cho, H. J.; Oh, Y. K. Thermosensitive and mucoadhesive delivery systems of mucosal vaccines. *Methods*, 2006, 38(2), 106.
173. Park, J. S.; Oh, Y. K.; Kang, M. J.; Kim, C. K. Enhanced mucosal and systemic immune responses following intravaginal immunization with human papillomavirus 16 L1 virus-

- like particle vaccine in thermosensitive mucoadhesive delivery systems. *Journal of Medical Virology*, 2003, 70(4), 633.
174. Aka-Any-Grah, A.; Bouchemal, K.; Koffi, A.; Agnely, F.; Zhang, M.; Djabourov, M.; Ponchel, G. Formulation of mucoadhesive vaginal hydrogels insensitive to dilution with vaginal fluids. *European Journal of Pharmaceutics and Biopharmaceutics*, 2010, 76(2), 296.
175. Abdel-Karim, A.; Frezzini, C.; Viggor, S.; Davidson, L. E.; Thornhill, M. H.; Yeoman, C. M. Dyskeratosis congenita: a case report. *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology*, 2009, 108(2), e20.
176. Cooper, S. M.; Haefner, H. K.; Abrahams-Gessel, S.; Margesson, L. J. Vulvovaginal lichen planus treatment: a survey of current practices. *Archives of Dermatology*, 2008, 144(11), 1520.
177. D'Cruz, O. J.; Uckun, F. M. Preclinical evaluation of a dual-acting microbicidal prodrug WHI-07 in combination with vanadocene dithiocarbamate in the female reproductive tract of rabbit, pig, and cat. *Toxicologic Pathology*, 2007, 35(7), 910.
178. Curran, R. M.; Donnelly, L.; Morrow, R. J.; Fraser, C.; Andrews, G.; Cranage, M.; Malcolm, R. K.; Shattock, R. J.; Woolfson, A. D. Vaginal delivery of the recombinant HIV-1 clade-C trimeric gp140 envelope protein CN54gp140 within novel rheologically structured vehicles elicits specific immune responses. *Vaccine*, 2009, 27(48), 6791.
179. Dhondt, M. M. M.; Adriaens, E.; Roey, J. V.; Remon, J. P. The evaluation of the local tolerance of vaginal formulations containing dapivirine using the Slug Mucosal Irritation test and the rabbit vaginal irritation test. *European Journal of Pharmaceutics and Biopharmaceutics*, 2005, 60(3), 419.
180. Harris, D.; Robinson, J. R. Drug delivery via the mucous membranes of the oral cavity. *Journal of Pharmaceutical Sciences*, 1992, 81(1), 1.
181. Kanazawa, T.; Takashima, Y.; Hirayama, S.; Okada, H. Effects of menstrual cycle on gene transfection through mouse vagina for DNA vaccine. *International Journal of Pharmaceutics*, 2008, 360(1-2), 164.
182. Foldvari, M.; Badea, I.; Wettig, S.; Verrall, R.; Bagonluri, M. Structural characterization of novel gemini non-viral DNA delivery systems for cutaneous gene therapy. *Journal of Experimental Nanoscience*, 2006, 1(2), 165.
183. Zhou, X.; HUANG, L. DNA transfection mediated by cationic liposomes containing lipopolylysine: characterization and mechanism of action. *Biochimica Et Biophysica Acta.Biomembranes*, 1994, 1189(2), 195.
184. Freundlich, B.; Jimenez, S. A.; Steen, V. D.; Medsger Jr, T. A.; Szkolnicki, M.; Jaffe, H. S. Treatment of systemic sclerosis with recombinant interferon- γ . A phase I/II clinical trial. *Arthritis & Rheumatism*, 1992, 35(10), 1134.

185. Buys, L. M. Treatment options for atopic dermatitis. *American Family Physician*, 2007, 75(4), 523.
186. Emmez, H.; Kardes, O.; Dogulu, F.; Kurt, G.; Memis, L.; Baykaner, M. K. Role of antifibrotic cytokine interferon-[gamma] in the prevention of postlaminectomy peridural fibrosis in rats. *Neurosurgery*, 2008, 62(6), 1351.
187. Menger, F. M.; Keiper, J. S. Gemini surfactants. *Angewandte Chemie International Edition*, 2000, 39(11), 1906.
188. Zana, R. Dimeric (gemini) surfactants: effect of the spacer group on the association behavior in aqueous solution. *Journal of Colloid and Interface Science*, 2002, 248(2), 203.
189. Karlsson, L.; van Eijk, M. C. P.; Söderman, O. Compaction of DNA by gemini surfactants: Effects of surfactant architecture. *Journal of Colloid and Interface Science*, 2002, 252(2), 290.
190. Wang, C.; Li, X.; Wettig, S. D.; Badea, I.; Foldvari, M.; Verrall, R. E. Investigation of complexes formed by interaction of cationic gemini surfactants with deoxyribonucleic acid. *Phys.Chem.Chem.Phys.*, 2007, 9(13), 1616.
191. Kharidia, R.; Friedman, K.; Liang, J. F. Improved gene expression using low molecular weight peptides produced from protamine sulfate. *Biochemistry (Moscow)*, 2008, 73(10), 1162.
192. Alami, E.; Beinert, G.; Marie, P.; Zana, R. Alkanediyl-. alpha.,. omega.-bis (dimethylalkylammonium bromide) surfactants. 3. Behavior at the air-water interface. *Langmuir*, 1993, 9(6), 1465.
193. Chen, X.; Wang, J.; Shen, N.; Luo, Y.; Li, L.; Liu, M.; Thomas, R. K. Gemini surfactant/DNA complex monolayers at the air-water interface: effect of surfactant structure on the assembly, stability, and topography of monolayers. *Langmuir*, 2002, 18(16), 6222.
194. Jabbari, E. Targeted delivery with peptidomimetic conjugated self-assembled nanoparticles. *Pharmaceutical Research*, 2009, 26(3), 612.
195. Colombo, G.; Soto, P.; Gazit, E. Peptide self-assembly at the nanoscale: a challenging target for computational and experimental biotechnology. *Trends in Biotechnology*, 2007, 25(5), 211.
196. Mehnert, W.; Mäder, K. Solid lipid nanoparticles:: Production, characterization and applications. *Advanced Drug Delivery Reviews*, 2001, 47(2-3), 165.
197. Simberg, D.; Danino, D.; Talmon, Y.; Minsky, A.; Ferrari, M. E.; Wheeler, C. J.; Barenholz, Y. Phase behavior, DNA ordering, and size instability of cationic lipoplexes. *Journal of Biological Chemistry*, 2001, 276(50), 47453.

198. Braun, C. S.; Jas, G. S.; Choosakoonkriang, S.; Koe, G. S.; Smith, J. G.; Middaugh, C. R. The structure of DNA within cationic lipid/DNA complexes. *Biophysical Journal*, 2003, 84(2), 1114.
199. Yang, P.; Singh, J.; Wettig, S.; Foldvari, M.; Verrall, R. E.; Badea, I. Enhanced gene expression in epithelial cells transfected with amino acid-substituted gemini nanoparticles. *European Journal of Pharmaceutics and Biopharmaceutics*, 2010, 75(3), 311.
200. Tseng, W.; Haselton, F. R.; Giorgio, T. D. Transfection by Cationic Liposomes Using Simultaneous Single Cell Measurements of Plasmid Delivery and Transgene Expression. *Journal of Biological Chemistry*, 1997, 272(41), 25641.
201. Schuber, F. Influence of polyamines on membrane functions. *Biochemical Journal*, 1989, 260(1), 1.
202. Xu, Y.; Hui, S.; Frederik, P.; Szoka Jr., F. C. Physicochemical Characterization and Purification of Cationic Lipoplexes. *Biophysical Journal*, 1999, 77(1), 341.
203. Wiethoff, C. M.; Gill, M. L.; Koe, G. S.; Koe, J. G.; Middaugh, C. R. A fluorescence study of the structure and accessibility of plasmid DNA condensed with cationic gene delivery vehicles. *Journal of Pharmaceutical Sciences*, 2003, 92(6), 1272.
204. Badea, I. Gemini cationic surfactant-based delivery systems for non-invasive cutaneous gene therapy. PhD Thesis, University of Saskatchewan: Saskatoon, 2006.
205. Wheeler, C. J.; Sukhu, L.; Yang, G.; Tsai, Y.; Bustamente, C.; Felgner, P.; Norman, J.; Manthorpe, M. Converting an alcohol to an amine in a cationic lipid dramatically alters the co-lipid requirement, cellular transfection activity and the ultrastructure of DNA-cytofectin complexes. *Biochimica Et Biophysica Acta (BBA) - Biomembranes*, 1996, 1280(1), 1.
206. Bennett, M. J.; Aberle, A. M.; Balasubramaniam, R. P.; Malone, J. G.; Nantz, M. H.; Malone, R. W. Considerations for the Design of Improved Cationic Amphiphile-Based Transfection Reagents. *Journal of Liposome Research*, 1996, 6(3), 545.
207. Hong, S.; Leroueil, P. R.; Janus, E. K.; Peters, J. L.; Kober, M. M.; Islam, M. T.; Orr, B. G.; Baker Jr, J. R.; Holl, M. M. B. Interaction of polycationic polymers with supported lipid bilayers and cells: nanoscale hole formation and enhanced membrane permeability. *Bioconjugate Chem*, 2006, 17(3), 728.
208. Tribet, C.; Vial, F. Flexible macromolecules attached to lipid bilayers: impact on fluidity, curvature, permeability and stability of the membranes. *Soft Matter*, 2008, 4(1), 68.
209. Mecke, A.; Majoros, I. J.; Patri, A. K.; Baker Jr, J. R.; Holl, M. M. B.; Orr, B. G. Lipid bilayer disruption by polycationic polymers: The roles of size and chemical functional group. *Langmuir*, 2005, 21(23), 10348.

210. Boussif, O.; Lezoualc'h, F.; Zanta, M.; Mergny, M.; Scherman, D.; Demeneix, B.; Behr, J. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proceedings of the National Academy of Sciences of the United States of America*, 1995, 92(16), 7297.
211. Yager, E. J.; Dean, H. J.; Fuller, D. H. Prospects for developing an effective particle-mediated DNA vaccine against influenza. *Expert Review of Vaccines*, 2009, 8(9), 1205.
212. Gene therapy proves effective in treating severe heart failure; holds potential to drastically reduce healthcare costs for heart failure patients. Available at www.hfsa.org/pdf/HFSA_Gene_Therapy_Heart_Failure.pdf.
213. Mintzer, M. A.; Simanek, E. E. Nonviral vectors for gene delivery. *Chemical Reviews*, 2008, 109(2), 259.
214. Wang, L. H.; Rothberg, K. G.; Anderson, R. G. Mis-assembly of clathrin lattices on endosomes reveals a regulatory switch for coated pit formation. *The Journal of Cell Biology*, 1993, 123(5), 1107.
215. Larkin, J. M.; Brown, M. S.; Goldstein, J. L.; Anderson, R. G. W. Depletion of intracellular potassium arrests coated pit formation and receptor-mediated endocytosis in fibroblasts. *Cell*, 1983, 33(1), 273.
216. Matveev, S.; Li, X.; Everson, W.; Smart, E. J. The role of caveolae and caveolin in vesicle-dependent and vesicle-independent trafficking. *Advanced Drug Delivery Reviews*, 2001, 49(3), 237.
217. Orlandi, P. A.; Fishman, P. H. Filipin-dependent inhibition of cholera toxin: evidence for toxin internalization and activation through caveolae-like domains. *The Journal of Cell Biology*, 1998, 141(4), 905.
218. Parton, R. G.; Joggerst, B.; Simons, K. Regulated internalization of caveolae. *The Journal of Cell Biology*, 1994, 127(5), 1199.
219. Rodal, S. K.; Skretting, G.; Garred, O.; Vilhardt, F.; Van Deurs, B.; Sandvig, K. Extraction of cholesterol with methyl-beta-cyclodextrin perturbs formation of clathrin-coated endocytic vesicles. *Molecular Biology of the Cell*, 1999, 10(4), 961.
220. Thomsen, P.; Roepstorff, K.; Stahlhut, M.; van Deurs, B. Caveolae are highly immobile plasma membrane microdomains, which are not involved in constitutive endocytic trafficking. *Molecular Biology of the Cell*, 2002, 13(1), 238.
221. Subtil, A.; Gaidarov, I.; Kobylarz, K.; Lampson, M. A.; Keen, J. H.; McGraw, T. E. Acute cholesterol depletion inhibits clathrin-coated pit budding. *Proceedings of the National Academy of Sciences*, 1999, 96(12), 6775.

222. Araki, N.; Johnson, M. T.; Swanson, J. A. A role for phosphoinositide 3-kinase in the completion of macropinocytosis and phagocytosis by macrophages. *The Journal of Cell Biology*, 1996, 135(5), 1249.
223. Singh, J.; Yang, P.; Michel, D.; Verrall, R. E.; Foldvari, M.; Badea, I. Amino Acid-Substituted Gemini Surfactant-Based Nanoparticles as Safe and Versatile Gene Delivery Agents. *Current Drug Delivery*, 2011, 8(3), 299.
224. Arcaro, A.; Wymann, M. P. Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3, 4, 5-trisphosphate in neutrophil responses. *Biochemical Journal*, 1993, 296(Pt 2), 297.
225. Kilsdonk, E. P. C.; Yancey, P. G.; Stoudt, G. W.; Bangerter, F. W.; Johnson, W. J.; Phillips, M. C.; Rothblat, G. H. Cellular cholesterol efflux mediated by cyclodextrins. *Journal of Biological Chemistry*, 1995, 270(29), 17250.
226. Zuhorn, I. S.; Kalicharan, R.; Hoekstra, D. Lipoplex-mediated transfection of mammalian cells occurs through the cholesterol-dependent clathrin-mediated pathway of endocytosis. *Journal of Biological Chemistry*, 2002, 277(20), 18021.
227. Zhang, X. X.; Allen, P. G.; Grinstaff, M. Macropinocytosis is the major pathway responsible for DNA transfection in CHO cells by a charge-reversal amphiphile. *Molecular Pharmaceutics*, 2011, 8(3), 758.
228. Simões, S.; Slepishkin, V.; Pires, P.; Gaspar, R.; Pedroso de Lima, M. C.; Düzgüne, N. Human serum albumin enhances DNA transfection by lipoplexes and confers resistance to inhibition by serum. *Biochimica Et Biophysica Acta (BBA)-Biomembranes*, 2000, 1463(2), 459.
229. Vercauteren, D.; Vandenbroucke, R. E.; Jones, A. T.; Rejman, J.; Demeester, J.; De Smedt, S. C.; Sanders, N. N.; Braeckmans, K. The use of inhibitors to study endocytic pathways of gene carriers: optimization and pitfalls. *Molecular Therapy*, 2009, 18(3), 561.
230. Zabner, J.; Fasbender, A. J.; Moninger, T.; Poellinger, K. A.; Welsh, M. J. Cellular and molecular barriers to gene transfer by a cationic lipid. *Journal of Biological Chemistry*, 1995, 270(32), 18997.
231. Sonawane, N. D.; Szoka, F. C.; Verkman, A. S. Chloride accumulation and swelling in endosomes enhances DNA transfer by polyamine-DNA polyplexes. *Journal of Biological Chemistry*, 2003, 278(45), 44826.
232. Chen, Y.; Ghim, S.; Jenson, A.; Schlegel, R. Mutant canine oral papillomavirus L1 capsid proteins which form virus-like particles but lack native conformational epitopes. *Journal of General Virology*, 1998, 79(9), 2137.

233. Schreckenberger, C.; Sethupathi, P.; Kanjanahaluethai, A.; Müller, M.; Zhou, J.; Gissmann, L.; Qiao, L. Induction of an HPV 6bL1-specific mucosal IgA response by DNA immunization. *Vaccine*, 2000, 19(2-3), 227.
234. Srikrishna, S.; Cardozo, L. The vagina as a route for drug delivery: a review. *International Urogynecology Journal*, 2012, 24(4), 537.
235. Donkuru, M.; Wettig, S. D.; Verrall, R. E.; Badea, I.; Foldvari, M. Designing pH-sensitive gemini nanoparticles for non-viral gene delivery into keratinocytes. *Journal of Materials Chemistry*, 2012, 22(13), 6232.
236. Singh, J.; Michel, D.; Chitanda, J. M.; Verrall, R. E.; Badea, I. Evaluation of cellular uptake and intracellular trafficking as determining factors of gene expression for amino acid-substituted gemini surfactant-based DNA nanoparticles. *Journal of Nanobiotechnology*, 2012, 10(1), 7.
237. Mohammed-Saeid, W.; Buse, J.; Badea, I.; Verrall, R.; El-Aneed, A. Mass spectrometric analysis of amino acid/di-peptide modified gemini surfactants used as gene delivery agents: Establishment of a universal mass spectrometric fingerprint. *International Journal of Mass Spectrometry*, 2012, 309, 182.
238. Wettig, S. D.; Wang, C.; Verrall, R. E.; Foldvari, M. Thermodynamic and aggregation properties of aza- and imino-substituted gemini surfactants designed for gene delivery. *Physical Chemistry Chemical Physics*, 2007, 9(7), 871.
239. Kang, J.; Davaa, E.; Kim, Y.; Park, J. A new vaginal delivery system of amphotericin B: a dispersion of cationic liposomes in a thermosensitive gel. *Journal of Drug Targeting*, 2010, 18(8), 637.
240. Lenaerts, V.; Triqueneaux, C.; Quartern, M.; Rieg-Falson, F.; Couvreur, P. Temperature-dependent rheological behavior of Pluronic F-127 aqueous solutions. *International Journal of Pharmaceutics*, 1987, 39(1-2), 121.
241. Lehrman, S. Virus treatment questioned after gene therapy death. *Nature*, 1999, 401(6753), 517.
242. Mallipeddi, R.; Rohan, L. C. Nanoparticle-based vaginal drug delivery systems for HIV prevention. *Expert Opinion on Drug Delivery*, 2010, 7(1), 37.
243. Georgakoudi, I.; Jacobson, B. C.; Muller, M. G.; Sheets, E. E.; Badizadegan, K.; Carr-Locke, D. L.; Crum, C. P.; Boone, C. W.; Dasari, R. R.; Van Dam, J. NAD (P) H and collagen as in vivo quantitative fluorescent biomarkers of epithelial precancerous changes. *Cancer Research*, 2002, 62(3), 682.
244. Richards-Kortum, R.; Sevick-Muraca, E. Quantitative optical spectroscopy for tissue diagnosis. *Annual Review of Physical Chemistry*, 1996, 47(1), 555.

245. Winnard Jr., P. T.; Kluth, J. B.; Raman, V. Noninvasive optical tracking of red fluorescent protein-expressing cancer cells in a model of metastatic breast cancer. *Neoplasia*, 2006, 8(10), 796.
246. Chang, J. Y.; Oh, Y.; Choi, H.; Kim, Y. B.; Kim, C. Rheological evaluation of thermosensitive and mucoadhesive vaginal gels in physiological conditions. *International Journal of Pharmaceutics*, 2002, 241(1), 155.
247. Edsman, K.; Carlfors, J.; Petersson, R. Rheological evaluation of poloxamer as an in situ gel for ophthalmic use. *European Journal of Pharmaceutical Sciences*, 1998, 6(2), 105.
248. Gilbert, J. C.; Richardson, J. L.; Davies, M. C.; Palin, K. J.; Hadgraft, J. The effect of solutes and polymers on the gelation properties of pluronic F-127 solutions for controlled drug delivery. *Journal of Controlled Release*, 1987, 5(2), 113.
249. SCCP (Scientific Committee on Consumer Products), Opinion on diethyleneglycol monoethylether, 2008. Available at http://ec.europa.eu/health/ph_risk/committees/04_sccp/docs/sccp_o_161.pdf (Accessed on April 01, 2013).
250. Inactive Ingredient Search for Approved Drug Products. Available at <http://www.accessdata.fda.gov/scripts/cder/iig/index.cfm> (Accessed on April 22, 2013).
251. Ganem-Quintanar, A.; Lafforgue, C.; Falson-Rieg, F.; Buri, P. Evaluation of the transepidermal permeation of diethylene glycol monoethyl ether and skin water loss. *International Journal of Pharmaceutics*, 1997, 147(2), 165.
252. Proniuk, S.; Dixon, S. E.; Blanchard, J. Investigation of the utility of an in vitro release test for optimizing semisolid dosage forms. *Pharmaceutical Development and Technology*, 2001, 6(3), 469.
253. Escobar-Chávez, J. J.; Quintanar-Guerrero, D.; Ganem-Quintanar, A. In vivo skin permeation of sodium naproxen formulated in pluronic F-127 gels: effect of Azone® and Transcutol®. *Drug Development and Industrial Pharmacy*, 2005, 31(4-5), 447.
254. Li, H.; Yu, G. E.; Price, C.; Booth, C.; Hecht, E.; Hoffmann, H. Concentrated aqueous micellar solutions of diblock copoly (oxyethylene/oxybutylene) E41B8: a study of phase behavior. *Macromolecules*, 1997, 30(5), 1347.
255. Alexandridis, P.; Holzwarth, J. F.; Hatton, T. A. Micellization of poly (ethylene oxide)-poly (propylene oxide)-poly (ethylene oxide) triblock copolymers in aqueous solutions: thermodynamics of copolymer association. *Macromolecules*, 1994, 27(9), 2414.
256. Mortensen, K.; Pedersen, J. S. Structural study on the micelle formation of poly (ethylene oxide)-poly (propylene oxide)-poly (ethylene oxide) triblock copolymer in aqueous solution. *Macromolecules*, 1993, 26(4), 805.

257. Charrueau, C.; Tuleu, C.; Astre, V.; Grossiord, J.; Chaumeil, J. Poloxamer 407 as a thermogelling and adhesive polymer for rectal administration of short-chain fatty acids. *Drug Development and Industrial Pharmacy*, 2001, 27(4), 351.
258. Park, J.; Oh, Y.; Yoon, H.; Kim, J. M.; Kim, C. In situ gelling and mucoadhesive polymer vehicles for controlled intranasal delivery of plasmid DNA. *Journal of Biomedical Materials Research*, 2001, 59(1), 144.
259. Ginn, S. L.; Alexander, I. E.; Edelstein, M. L.; Abedi, M. R.; Wixon, J. Gene therapy clinical trials worldwide to 2012—an update. *The Journal of Gene Medicine*, 2013, 15(2), 65.
260. Miyata, K.; Nishiyama, N.; Kataoka, K. Rational design of smart supramolecular assemblies for gene delivery: chemical challenges in the creation of artificial viruses. *Chemical Society Reviews*, 2012, 41(7), 2562.
261. Ayuni, E. L.; Gazdhar, A.; Giraud, M. N.; Kadner, A.; Gugger, M.; Cecchini, M.; Caus, T.; Carrel, T. P.; Schmid, R. A.; Tevaearai, H. T. In vivo electroporation mediated gene delivery to the beating heart. *PloS One*, 2010, 5(12), e14467.
262. Best, S. R.; Peng, S.; Juang, C.; Hung, C.; Hannaman, D.; Saunders, J. R.; Wu, T.; Pai, S. I. Administration of HPV DNA vaccine via electroporation elicits the strongest CD8 T cell immune responses compared to intramuscular injection and intradermal gene gun delivery. *Vaccine*, 2009, 27(40), 5450.
263. Gonzalez, R.; Hutchins, L.; Nemunaitis, J.; Atkins, M.; Schwarzenberger, P. O. Phase 2 trial of Allovectin-7 in advanced metastatic melanoma. *Melanoma Research*, 2006, 16(6), 521.
264. Keasey, N.; Herse, Z.; Chang, S.; Liggitt, D. H.; Fairman, J.; Lay, M.; Claxton, D. F. A non-coding cationic lipid DNA complex produces lasting anti-leukemic effects. *Cancer Biology & Therapy*, 2010, 10(6), 625.
265. Konstan, M. W.; Davis, P. B.; Wagener, J. S.; Hilliard, K. A.; Stern, R. C.; Milgram, L. J.; Kowalczyk, T. H.; Hyatt, S. L.; Fink, T. L.; Gedeon, C. R. Compacted DNA nanoparticles administered to the nasal mucosa of cystic fibrosis subjects are safe and demonstrate partial to complete cystic fibrosis transmembrane regulator reconstitution. *Human Gene Therapy*, 2004, 15(12), 1255.
266. Lisziewicz, J.; Bakare, N.; Calarota, S. A.; Bánhegyi, D.; Szlávik, J.; Újhelyi, E.; Tőke, E. R.; Molnár, L.; Lisziewicz, Z.; Autran, B. Single DermaVir immunization: dose-dependent expansion of precursor/memory T cells against all HIV antigens in HIV-1 infected individuals. *PloS One*, 2012, 7(5), e35416.
267. Elouahabi, A.; Ruyschaert, J. Formation and intracellular trafficking of lipoplexes and polyplexes. *Molecular Therapy*, 2005, 11(3), 336.

268. Ruponen, M.; Ylä-Herttuala, S.; Urtti, A. Interactions of polymeric and liposomal gene delivery systems with extracellular glycosaminoglycans: physicochemical and transfection studies. *Biochimica Et Biophysica Acta (BBA)-Biomembranes*, 1999, 1415(2), 331.
269. Seow, W. Y.; Liang, K.; Kurisawa, M.; Hauser, C. A. Oxidation as a facile strategy to reduce the surface charge and toxicity of polyethyleneimine gene carriers. *Biomacromolecules*, 2013.
270. Epand, R. M.; Epand, R. F.; Ahmed, N.; Chen, R. Promotion of hexagonal phase formation and lipid mixing by fatty acids with varying degrees of unsaturation. *Chemistry and Physics of Lipids*, 1991, 57(1), 75.
271. Johnsson, M.; Engberts, J. B. Novel sugar-based gemini surfactants: aggregation properties in aqueous solution. *Journal of Physical Organic Chemistry*, 2004, 17(11), 934.
272. Bell, P. C.; Bergsma, M.; Dolbnya, I. P.; Bras, W.; Stuart, M. C.; Rowan, A. E.; Feiters, M. C.; Engberts, J. B. Transfection mediated by gemini surfactants: engineered escape from the endosomal compartment. *Journal of the American Chemical Society*, 2003, 125(6), 1551.
273. Le Gall, T.; Loizeau, D.; Picquet, E.; Carmoy, N.; Yaouanc, J.; Burel-Deschamps, L.; Delépine, P.; Giamarchi, P.; Jaffres, P.; Lehn, P. A novel cationic lipophosphoramidate with diunsaturated lipid chains: synthesis, physicochemical properties, and transfection activities. *Journal of Medicinal Chemistry*, 2010, 53(4), 1496.
274. Loizeau, D.; Le Gall, T.; Mahfoudhi, S.; Berchel, M.; Maroto, A.; Yaouanc, J.; Jaffrès, P.; Lehn, P.; Deschamps, L.; Montier, T.; Giamarchi, P. Physicochemical properties of cationic lipophosphoramidates with an arsonium head group and various lipid chains: A structure–activity approach. *Biophysical Chemistry*, 2013, 171(0), 46.
275. Bai, G.; Wang, J.; Wang, Y.; Yan, H.; Thomas, R. K. Thermodynamics of hydrophobic interaction of dissymmetric gemini surfactants in aqueous solutions. *The Journal of Physical Chemistry B*, 2002, 106(26), 6614.
276. Wang, H.; Wettig, S. D. Synthesis and aggregation properties of dissymmetric phytanyl-gemini surfactants for use as improved DNA transfection vectors. *Physical Chemistry Chemical Physics*, 2011, 13(2), 637.
277. Dauty, E.; Remy, J.; Blessing, T.; Behr, J. Dimerizable cationic detergents with a low cmc condense plasmid DNA into nanometric particles and transfect cells in culture. *Journal of the American Chemical Society*, 2001, 123(38), 9227.
278. Lin, Y.; Lin, H.; Tsou, Y.; Chitra, E.; Hsiao, K.; Shao, H.; Liu, C.; Sia, C.; Chong, P.; Chow, Y. Human SCARB2-mediated entry and endocytosis of EV71. *PloS One*, 2012, 7(1), e30507.

279. Lin, H.; Yang, Y.; Yu, S.; Hsiao, K.; Liu, C.; Sia, C.; Chow, Y. Caveolar Endocytosis is Required for Human PSGL-1-Mediated Enterovirus 71 Infection. *Journal of Virology*, 2013.
280. Daniels, T. R.; Bernabeu, E.; Rodríguez, J. A.; Patel, S.; Kozman, M.; Chiappetta, D. A.; Holler, E.; Ljubimova, J. Y.; Helguera, G.; Penichet, M. L. The transferrin receptor and the targeted delivery of therapeutic agents against cancer. *Biochimica Et Biophysica Acta (BBA)-General Subjects*, 2012, 1820(3), 291.
281. Badea, I.; Foldvari, M.; Verrall, R. E. DNA delivery with gemini cationic surfactants. WO Patent App. PCT/CA2004/001,870 (published on 06 May, 2005).
282. Kruger Kjaer, S.; Chackerian, B.; van den Brule, A. J. C.; Svare, E. I.; Paull, G.; Walbomers, J. M. M.; Schiller, J. T.; Bock, J. E.; Sherman, M. E.; Lowy, D. R.; Meijer, C. L. M. High-Risk Human Papillomavirus Is Sexually Transmitted: Evidence from a Follow-Up Study of Virgins Starting Sexual Activity (Intercourse). *Cancer Epidemiology Biomarkers Prevention*, 2001, 10(2), 101.
283. Neutra, M. R. Mucosal vaccines: The promise and the challenge. *Nature Reviews Immunology*, 2006, 6(2), 148.
284. Muñoz, N.; Castellsagué, X.; de González, A. B.; Gissmann, L. Chapter 1: HPV in the etiology of human cancer. *Vaccine*, 2006, 24(Supplement 3), S1.
285. Jung, W. W.; Chun, T.; Sul, D.; Hwang, K. W.; Kang, H. S.; Lee, D. J.; Han, I. K. Strategies against human papillomavirus infection and cervical cancer. *J Microbiol*, 2004, 42(4), 255.
286. Harper, D. M.; Franco, E. L.; Wheeler, C.; Ferris, D. G.; Jenkins, D.; Schuind, A.; Zahaf, T.; Innis, B.; Naud, P.; De Carvalho, N. S.; Roteli-Martins, C. M.; Teixeira, J.; Blatter, M. M.; Korn, A. P.; Quint, W.; Dubin, G. Efficacy of a bivalent L1 virus-like particle vaccine in prevention of infection with human papillomavirus types 16 and 18 in young women: a randomised controlled trial. *The Lancet*, 2004, 364(9447), 1757.
287. Kirnbauer, R.; Booy, F.; Cheng, N.; Lowy, D.; Schiller, J. Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. *Proceedings of the National Academy of Sciences*, 1992, 89(24), 12180.
- 288.288. Borysiewicz, L. K.; Fiander, A.; Nimako, M.; Man, S.; Wilkinson, G. W. G.; Westmoreland, D.; Evans, A. S.; Adams, M.; Stacey, S. N.; Bournsnel, M. E. G.; Rutherford, E.; Hickling, J. K.; Inglis, S. C. A recombinant vaccinia virus encoding human papillomavirus types 16 and 18, E6 and E7 proteins as immunotherapy for cervical cancer. *The Lancet*, 1996, 347(9014), 1523.
289. Shope, R. E.; Hurst, E. W. Infectious papillomatosis of rabbits: with a note on the histopathology. *The Journal of Experimental Medicine*, 1933, 58(5), 607.

290. Brandsma, J. L.; Yang, Z. H.; Barthold, S. W.; Johnson, E. A. Use of a rapid, efficient inoculation method to induce papillomas by cottontail rabbit papillomavirus DNA shows that the E7 gene is required. *Proceedings of the National Academy of Sciences of the United States of America*, 1991, 88(11), 4816.
291. Manchester, K. L. Use of UV methods for measurement of protein and nucleic acid concentrations. *BioTechniques*, 1996, 20(6), 968.
292. Fleige, S.; Pfaffl, M. W. RNA integrity and the effect on the real-time qRT-PCR performance. *Molecular Aspects of Medicine*, 2006, 27(2-3), 126.
293. Mueller, O.; Lightfoot, S.; Schroeder, A. RNA integrity number (RIN)—Standardization of RNA Quality Control. *Agilent Application Note, Publication*, 2004, 5989, 1.
294. Shaner, N. C.; Campbell, R. E.; Steinbach, P. A.; Giepmans, B. N. G.; Palmer, A. E.; Tsien, R. Y. Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. *Nature Biotechnology*, 2004, 22(12), 1567.