PHYSIOCHEMICAL STABILITY AND MASS SPECTROMETRIC ANALYSIS OF GEMINI SURFACTANT-BASED LIPOPLEXES

A Thesis Submitted to

the College of Graduate Studies and Research

in Partial Fulfillment of the Requirements

for the Degree of Master of Science

in the College of Pharmacy and Nutrition

University of Saskatchewan

Saskatoon, Saskatchewan

By

Waleed A. Mohammed Saeid

PERMISSION TO USE

In presenting this thesis/dissertation in partial fulfillment of the requirements for a postgraduate degree from the University of Saskatchewan, I agree that the Libraries of this University may make it freely available for inspection. I further agree that permission for copying of this thesis/dissertation in any manner, in whole or in part, for scholarly purposes may be granted by the professors who supervised my thesis/dissertation work (Drs. A. El-Aneed or I. Badea) or, in their absence, by the Head of the Department or the Dean of the College in which my thesis work was done. It is understood that any copying or publication or use of this thesis/dissertation or parts thereof for financial gain shall not be allowed without my written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis/dissertation.

Requests for permission to copy or to make other uses of materials in this thesis/dissertation in whole or part should be addressed to:

Dean of the College of Pharmacy and Nutrition

University of Saskatchewan

Saskatoon, Saskatchewan S7N 5C9

Canada

ABSTRACT

Cationic lipids have been comprehensively studied as non-viral vectors for gene therapy, focusing on improving the gene transfer efficiency and the safety profile. However, clinical applications of cationic lipid/DNA lipoplexes are restricted due to their low physical stability in aqueous formulations. One specific group of cationic lipids that showed efficient transfection activity is the gemini surfactants.

Two main objectives were determined in this work. The first was to evaluate the feasibility of lyophilization as a formulation technique for preparing gemini surfactant-based lipoplexes with long-term stability. The second objective was to establish a universal tandem mass spectrometric "fingerprint" of novel amino acid modified gemini surfactants as a pre-requirement for the identification and quantification of gemini surfactants in different pharmaceutical matrices.

In order to investigate the influence of lyophilization on the essential physiochemical properties and the *in vitro* transfection efficiency of gemini surfactant-lipoplexes, a diquaternary ammonium gemini surfactant (12-7NH-12) and plasmid DNA (pDNA) encoding for interferon-γ (IFNγ) were used to prepare pDNA/gemini surfactant [P/G] lipoplexes. Helper lipid DOPE [L] was incorporated in all formulations producing a [P/G/L] system. Several excipients were utilized as stabilizing agents. Lipoplexes formulated with the cryoprotectant were subjected to a lyophilization/rehydration cycle. Transfection activity was assessed by measuring the level of expressed IFNγ and cellular toxicity (MTT assay). The results showed that the physiochemical properties of gemini surfactant-based lipoplexes were dependent on the nature of the stabilizing agents used to prepare the lipoplexes. Disaccharide sugars, sucrose and trehalose, provided the most efficient cryoprotectant effect based on their ability to physically stabilize the lipoplexes during the lyophilization process. The transfection efficiency of the lyophilized lipoplexes

increased 2-3 fold compared to fresh formulations upon lyophilization. This effect can be attributed to the improvement of DNA compaction and changes in the lipoplex morphology due to the lyophilization/rehydration cycles.

Based on these results, we evaluated the ability of lyophilization to improve the stability of gemini surfactant-based lipoplexes. Four lyophilized formulations were stored at 25˚C for three months. The formulations were analyzed monthly for physical appearance, physiochemical properties (particle size and zeta potential, pDNA compaction, gemini surfactant:pDNA interaction) and *in vitro* transfection. The physiochemical properties of the lyophilized formulations were maintained throughout the three month study. All lyophilized formulations showed a loss of gene transfection activity after three months of storage. Nevertheless, no significant losses of transfection efficiency were observed for three formulations after two months storage at 25 ˚C. These findings suggest that lyophilization significantly improved the physiochemical stability of gemini surfactant-based lipoplexes compared to liquid formulations. As well, lyophilization improved the transfection efficiency of gemini surfactant-based lipoplexes. The loss of transfection activity upon storage is most probably due to the conformational changes in the supramolecular structure of the lipoplexes as a function of time and temperature, rather than to DNA degradation.

To establish a foundation for employing the mass spectrometric methods in the evaluation of the chemical stability of the gemini surfactant, we evaluated the tandem mass spectrometric (MS/MS) behavior of six amino acid/di-peptide modified gemini surfactants that were synthesized based on the precursor compound 12-7NH-12. This was accomplished by using a hybrid quadrupole orthogonal time-of-flight mass spectrometer (QqToF-MS) and a triple quadrupole linear ion trap mass spectrometer (QqQ-LIT MS) equipped with electrospray

ionization (ESI) source. The single stage QqToF-MS data obtained in the positive ion mode verified the molecular composition of all tested gemini surfactants. Tandem mass spectrometric (MS/MS) analysis showed common fragmentation behavior among all tested compounds, allowing for the establishment of a universal fragmentation pattern. The fragmentation pathway was confirmed by MS/MS/MS experiments utilizing a Q-TrapTM 4000 LC/MS/MS system and (MS/MS) analysis of the deuterated form of 12-7N(Glycine)-12 gemini surfactant. Unique product ions, originating from the loss of one or both head groups along with the attached tail region(s), confirmed the chemical structure of the tested compounds.

In conclusion, different lyophilization strategies and analytical methods have been established to develop and examine the physiochemical stability of gemini surfactant-based lipoplex. A tandem mass spectrometric fragmentation pathway was established to enable the identification and quantification of these compounds in pharmaceutical formulations.

ACKNOWLEDGEMENTS

"In the Name of Allâh (God), the Most Gracious, the Most Merciful"

All my praises and thanks be to Allâh

I would like to express my sincere thanks and appreciation to my supervisors, Dr. Aans El-Aneed and Dr. Ildiko Badea for their guidance, mentorship, kindness and support. I also express thanks to my advisory committee members Dr. Ronald Verrall and Dr. Azita Haddadi and committee chairs Dr. Ed Krol and Dr. Fred Rémillard for their guidance and advices.

I sincerely acknowledge the guidance and support I have received from Dr. Ismail Niazi (Dean of the College of Pharmacy, Taibah University, Saudi Arabia).

I would like to extend my thanks to Dr. Praveen Kumar, Ms. Ravinder Batta and Mr. Jason Solomon, Helix BioPharma Corp. (Saskatoon, SK) for the assistance with stability chambers.

I thank Dr. Nicholas H. Low and Mr. Yuanlong Cao, College of Agriculture and Bioresources, University of Saskatchewan, for assistance regarding the Karl Fisher titration.

I thank Mr. Ken Thoms, Saskatchewan Structural Sciences Centre (Saskatoon, SK) for his technical assistant with QSTAR system.

I am grateful to Ms. Deborah Michel, she taught me several technical skills and she always was there to help and advice.

I appreciate and thank all my colleagues for their friendship and support: Dr. Jackson Chitanda, Jagbir Singh, Joshua Buse, Mukasa Bagonluri, McDonald Donkuru, Randeep Kaur, Masoomeh Poorghorban and Hanan Awad

DEDICATION

This work is dedicated to:

My parents (*Maryam & Abdul-Aziz*), brothers and sisters (especially my brother *Fahad*) who were always supporting me emotionally and financially. Their love and care has been a great support for me during my study.

My wife (*Sarah Fatani*), my daughter (*Maryam*) and my son (*Albaraa*). This thesis would not have been possible without their love, care, support, and patience.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

APCI	Atmospheric pressure chemical ionization
API	Atmospheric pressure ionization
CD	Circular dichroism spectroscopy
CMC	Critical micelle concentration
COS-7	African green monkey kidney fibroblast
DC-Chol	3β-[*N*-(*N'*,*N'*-dimethylaminoethane)-carbamoyl]cholesterol
DDAB	Dioctadecyldimethylammonium bromide
DMEM	Dulbecco's Modified Eagles Medium
DMRIE	1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide
DOPE	1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane
DOTMA	N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay,
ESI	Electrospray ionization
ESI-MS^n	Multiple-stage tandem mass spectrometry
EtBr	Ethidium bromide
FDA	Food and Drug Administration
G/L	Gemini surfactant:DOPE
GFP	Green fluorescent protein
GMP	Good manufacturing practice
IFN-γ	Interferon gamma
MS/MS	Tandem mass spectrometry

MS	Mass spectrometry
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
P/G/L	pDNA:gemini surfactant:DOPE
P/G	pDNA:gemini surfactant
PAMAM	Polyamidoamine
PBS	Phosphate buffered saline
PDI	Polydispersity index
pDNA	plasmid DNA
PEG	Polyethylene glycol
PEI	Polyethylenimine
PLL	Poly-L-lysine
QqQ-LIT MS	Triple quadrupole linear ion trap mass spectrometer
Qq-ToF MS	Quadrupole time-of-flight mass spectrometer.
RH	Relative humidity
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate

Chapter 1

Literature Review

1.1. Introduction

Gene therapy is a promising therapeutic approach that has the potential to significantly improve human health. Achieving the ultimate goal of gene therapy depends on the design of efficient, safe and stable gene delivery systems. Viral and non-viral systems have been extensively investigated for gene delivery. Viral vectors (e.g. adenoviral and retroviral vectors) are the most effective gene delivery agents that have been tested in several clinical trials. However, they suffer from numerous toxicity-related drawbacks. On the other hand, non-viral chemically mediated vectors, such as cationic lipids, exhibit low toxicity and show no immunogenic activity compared to viral vectors. One specific group of cationic lipids that demonstrated efficient transfection activities *in vitro* and *in vivo* are cationic gemini surfactants. They are dimeric surfactants comprised of two hydrophobic tail regions which are covalently attached to cationic head groups (linked by spacer region) [Figure 1.1]. Two major disadvantages of cationic lipid-DNA complexes (lipoplexes) that limit their clinical applications are the low transfection efficiency and physical instability.

Over the last decades, numerous cationic lipids were synthesized and modified to overcome their low transfection activity. The transfection efficiency of lipoplex-based systems depends on the integrity of various components of the delivery system and their related physiochemical properties. The stability of non-viral gene delivery systems is complicated as it involves the structural integrity of the genetic material, the physical, chemical and conformational stability of the DNA-carrier complexes. Therefore, investigation of the physiochemical stability of the lipoplex vectors during the manufacturing and treatment is

required to understand the behavior of such complex systems. To date, lyophilized lipoplex formulations have shown the most promising stability. However, the influence of the lyophilization process on the supramolecular structure of lipoplex systems formulated with different cationic lipids is not fully elucidated.

The purpose of my research is to develop qualitative and quantitative analytical methods that can be used to evaluate the physiochemical stability of gemini surfactant/plasmid DNA lipoplexes used for gene delivery. As well, the feasibility of the lyophilization technique for preparation of stable gemini surfactant-based lipoplexes was evaluated. Mass spectrometry is employed to confirm the molecular structure of six amino acid/di-peptide modified gemini surfactants and to establish a universal fragmentation fingerprint that can be used to identify and quantify these compounds in different matrices (including pharmaceutical formulations). Also, different analytical methods are developed to evaluate the *in vitro* transfection activity, cellular toxicity and physiochemical stability of lyophilized gemini surfactants/plasmid DNA (pDNA) formulations during three months of stability studies. These methods include: enzyme-linked immunosorbent assay (ELISA), MTT assay, dynamic light scattering (DLS), zeta potential measurements, gel electrophoresis, circular dichroism spectroscopy and Karl Fischer titration.

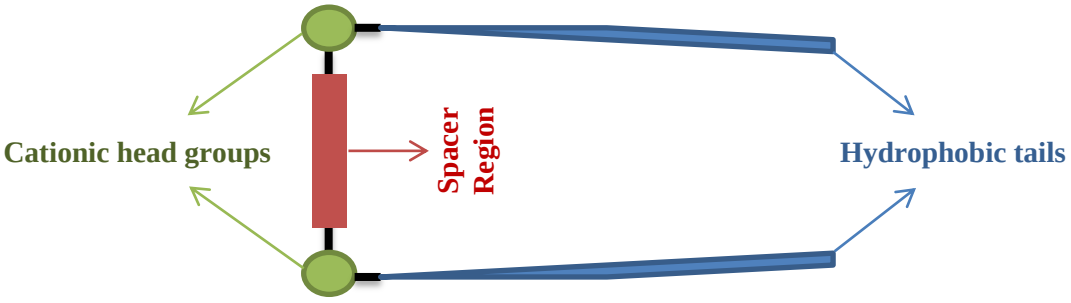


Figure 1.1: General structure of cationic gemini surfactants

1.2. Background

1.2.1. Gene therapy: Successes, setbacks and future

Significant advances in the biomedical and biotechnological sciences in the last few decades have contributed to a better understanding of the human biology. The completion of the sequencing of the human genome by the Human Genome Project has provided information regarding the role of genes in disease initiation and progression, which also expedites the development of gene therapy. Gene therapy can be defined as the introduction of exogenous nucleic acids into targeted cells for the purpose of preventing, terminating or reversing the progress of a pathological condition.[1] Nucleic acid based therapy is employed to manage a wide variety of acquired and genetic diseases in order to: 1) repair or substitute a defective gene, 2) stimulate humoral and cell-mediated immune responses to protein antigens – genetic immunization or 3) silence a defective gene expression at cellular mRNA level – gene knockdown.[1] Therefore, gene therapy introduces unique and promising prospects for the treatment of many diseases where traditional medicine and treatment methods lack efficiency.

Two main approaches have been exploited for gene transfer (transfection): *ex vivo* and *in vivo*.[2] In the *ex vivo* approach, the targeted cells are isolated from the patient, purified and treated outside the body. The genetically treated cells are re-infused into the patient's body. The *in vivo* method involves direct gene transfer to targeted cells. The first human clinical trial was performed by Rosenberg and his group, (at the National Cancer Institutes in Bethesda, MD, USA), in 1989. Their aim was to validate the safety and efficacy of using a virus-based gene transfer coding for resistance to neomycine into human tumor-infiltrating lymphocytes as a potential treatment for metastatic melanoma.[3] This groundbreaking study has initiated exploring the practicality of gene therapy as a novel medical technology to fight diseases at the genetic

level. Subsequently, a four-year clinical trial was conducted using retroviral-based gene transduction of adenosine deaminase gene into T-lymphocyte cells of two children with severe combined immune deficiency.[4] This trial was the first actual attempt to clinically evaluate gene therapy. Since then, over 1,785 clinical trials have been registered by the end of 2011 .[5] Around 65% of these clinical trials aimed to treat cancer. In addition, thousands of studies have been published regarding different types of transgene (genetic material used for gene therapy) and delivery systems.

The clinical momentum of gene therapy suffered a setback in 1999 due to the death of an 18-year old participant in a gene therapy pilot safety study that included 18 patients suffering from ornithine transcarboxylase deficiency.[6] The death was caused by severe systemic inflammatory response syndrome and multiple organ system failure triggered by the virus capsid used as gene delivery vector[6]. Due to this event, FDA halted all the gene therapy clinical trials at the University of Pennsylvania School of Medicine where the incident happened.[7] In 2000, a French team reported the successful treatment of ten children with severe X-linked combined immunodeficiency using a retrovirus–derived vector.[8] However, in 2002, two of the participant children suffered from severe leukemia-like symptoms due to the integration of the transgene into the chromosome of the treated cells.[9,10] These unanticipated outcomes raised a debate over the safety and future of manipulation of human cells at the gene level and led the USA and several European countries to suspend several clinical trials at that time to reevaluate the ethics and the safety procedures of human gene therapy. This action had a negative impact on the reduced number of human clinical trials between 2000 and 2003.

In spite of these setbacks, in 2003 the Chinese State Food and Drug Administration approved the world's first commercially available gene therapy medication Gendicine®

(Shenzhen Sibiono Genetech Co. Ltd, China) for the treatment of head and neck cancer.[11,12] However, due to safety concerns associated with viral-based gene therapy, several non-viral delivery systems have been developed and investigated extensively as safe alternative approaches.[13,14] Generally, the non-viral gene delivery systems are classified into 1) physically mediated and 2) chemically mediated gene delivery systems.

In the following section, I will discuss the different gene delivery systems (viral and non-viral) with focus on the cationic lipid based gene delivery vectors.

I.2.2. Gene delivery systems

1.2.2.1.Viral gene delivery systems

Viruses have the ability to transfer genetic materials to the host cells as infective agents. Scientists have capitalized on this property to develop gene delivery systems. New strains of nonpathogenic attenuated viruses have been used in numerous studies as viral vectors with significant transfection levels being achieved in different tissues, such as the lung, eye, kidney, muscle , and the ovary.[15-19] The most frequently used vectors in gene therapy which have reached advanced stages of clinical trials are viral vectors; around 65% of human clinical trials in gene therapy have used virus-based vectors.[5] The major advantage of the viral vectors compared to physical and chemical vectors is their high transfection efficacy. For instance, a study demonstrated that the transfection ability of adenoviral vectors in human monocyte-derived macrophages and African green monkey kidney fibroblast (COS-7 cells) was higher than that achieved by lipid-mediated vectors.[20] When COS-7 cells were infected with the adenoviral vectors, all cells expressed the transgene while only 30% of COS-7 cells were able to show gene expression after lipofection.[20] Furthermore, the ability to target certain cells or tissue and to control the gene expression are two advantages of viral vectors.[21-23] Targeting the viral vectors has been achieved either by genetically mutating the virus[24,25] or by modifying the virus with targeting ligands or antibodies.[26,27]

Despite the advantages of viral-based systems, there are several concerns about the safety of using viruses as gene delivery vectors. The major drawback is the reported cases of mortality and strong immune responses associated with the administration of viral gene therapy in clinical trials.[6,9] In addition, the long term effects of viral vectors in humans and the possibility of gene mutation are still unclear.[28] Besides the safety concerns, other shortcomings may limit the use of

viruses as gene delivery agents. For instance, the tendency of the viral vectors to lose their transfection activity during storage in conventional pharmaceutical formulations is considered to be a main stability issue.[29,30] However, the recent progresses in biotechnology and bioengineering technologies have improved the efficiency and stability of the viral vectors, keeping viral vectors at the forefront of gene delivery methods.[31-33]

1.2.2.2. Non-viral gene delivery systems

1.2.2.2.1. Naked DNA delivery

The simplest non-viral gene delivery method is the direct delivery of the genetic material (DNA) into the targeted cells. However, there are many obstacles that hinder this method. To achieve efficient gene expression, the DNA must 1) be stable in the biological system until it reaches the targeted cells, 2) be internalized by the cell and 3) enter the cell nucleus. DNA is easily destroyed by plasma and cellular nuclease enzymes and scavenger cells limiting its serum half-life to 10 minutes.[34,35] Additionally, the cell membrane has a dynamic lipophilic structure which restrains the uptake of large hydrophilic and charged molecules such as DNA. However, direct injection of naked DNA into the organ or targeted tissue of laboratory animals has led to successful gene expression in the liver, the muscle, the lung, the heart, the kidney and solid tumors.[36-41] Similarly, direct intravascular injection could deliver naked DNA *in vivo*.[42,43] Liang *et al* reported successful delivery of semi-systemic pDNA encoding full-length dystrophin through the intra-artery and tail vein of *mdx* mice and wild-type C57. [43] The purpose was to treat Duchenne muscular dystrophy, showing a significant restoration of dystrophin protein in all muscles of both hind limbs.[43] However, inefficient gene expression and the need for a large amount of genetic material are the drawbacks of naked DNA delivery method.[44] To improve the

cellular uptake and achieve higher gene expression levels, different physical methods have been employed to deliver naked genetic materials.

1.2.2.2.2. Physical gene delivery systems

In an attempt to increase cellular uptake of naked pDNA, different physical methods have been used to disturb the plasma membrane and facilitate the diffusion of genetic materials into cells. These methods have the potential to evade biological barriers, providing a significant level of transfection in comparison to the delivery of naked pDNA. Physical gene delivery methods include: particle bombardment (gene gun transfer), electroporation, ultrasound induced pores (sonophoresis), and magnetic field assisted transfection (magnetofection).[45-48]

Although numerous physical methods have been developed for gene delivery purposes, only a few have been successfully evaluated in clinical trials. Particle bombardment , a gene delivery method based on carrying the transgene coated on the surface of non-toxic inert particles into the targeted cells using a gene gun, has been evaluated for safety and efficacy in several clinical trials as a genetic vaccination method.[45,49,50] Electroporation, where electrical pulses are applied to enhance gene transfer, is another physical method that has been demonstrated to be the most feasible in gene transfer activity among other physical methods.[51,52] Almost all physical delivery methods suffer from major disadvantages, namely damage to cellular membrane and inefficient gene transfer.[53]

Chemically mediated gene delivery systems, on the other hand, are considered more promising gene delivery vectors due to numerous factors. They have low immunogenicity and cytotoxicity, can carry large genes, can be modified for cellular targeting, and are easy to synthesize on a large scale under good manufacturing practice (GMP) conditions.[54-57]

In the following section, I will discuss the chemically-mediated gene delivery systems focusing on cationic lipid based vectors that have shown the most promising gene delivery activity.

1.2.2.2.3. Chemical gene delivery systems

The ideal chemical gene vector should have the following properties: 1) the ability to protect the transgene (DNA) from the biological degradative environment until full delivery to the cell nucleus, 2) the ability to release the DNA once having reached the site of action, 3) minimum side effects without any immune response, 4) specificity to the targeted cell or tissue, and 5) the ability to maintain the stability of the vector.[58] Therefore, chemical gene delivery systems are considered a promising gene delivery vector in gene therapy. Diethylaminoethyl dextran (DEAE-dextran), and calcium phosphate co-precipitation were the first chemical carriers used to deliver nucleic acid to cells and achieve gene expression.[59,60] The two main classes currently used as chemical non-viral gene delivery systems are ***polymer-based*** and ***lipid-based*** vectors.

Polymer-based gene delivery

Cationic polymers are a group of polymeric compounds that either condense and protect the DNA or carry the genetic materials without condensing.[61] The DNA-polymer complex is known as a "polyplex". Polyethylenimine (PEI) was the first polymer utilized in gene therapy. It is a cationic polymer that can be in two forms: linear or branched.[62] PEI contains several amine groups which offer pH-dependent protonation (proton sponge effect) that trigger the DNA release upon uptake[63]. It has been established that the transfection efficiency of PEI is proportional to its molecular weight. However, high molecular weight polymers exhibit high cytotoxicity.[64]

Poly-L-lysines (PLL) are polymeric vectors that have been studied extensively for DNA transfection.[56] The presence of an ϵ-amino acid in the structure of the PLL facilitates the endosomal escape of the DNA due to the protonation at physiological pH.[65] High molecular

weight PLL polymers condense DNA effectively but they show high cytotoxicity. In addition, high molecular weight PLL-DNA complexes have the tendency to aggregate in biological systems leading to low gene expression.[66] To overcome such drawbacks, PLL was chemically modified by poly(ethylene glycol) reducing the formation of aggregates.[67,68] Other types of cationic polymers that have been utilized in gene therapy include polymethacrylate, carbohydrate-base polymers and linear poly(amino-amine), PAA, among others.[56,61,63]

A special class of polycationic non-viral vector is the dendrimer-based vectors.[69,70] Polyamidoamine (PAMAM) and phosphorus-containing dendrimers have been investigated as gene transfer vectors.[69,71,72] Dendrimers bind to the DNA electrostatically through the terminal amino groups and form DNA-dendrimer complexes. The DNA- dendrimer polyplexes exhibit good endosomal escape due to the availability of the internal tertiary amines groups that facilitate the release of the DNA after cellular internalization via the proton sponge mechanism.[73] The new generations of dendrimers provide structural flexibility due to hydrolytic degradation in aqueous medium.[74] This phenomenon triggered the swelling of the endosome and facilitated the release of DNA resulting in 50-fold improved transfection, compared to previous generations.[74] Other types of dendrimers have also been developed for gene therapy, including poly(propylenimine), poly(L-lysine) and carbosilane dendrimers.[56,61]

Lipid-based gene delivery

The introduction of a cationic lipid as DNA carrier was first reported in 1987 by Felgner and colleagues when they used liposomes formed from cationic lipid *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) and helper lipid 1,2-di-(9*Z*-octadecenoyl)-*sn*-glycero-3-phosphoethanolamine (DOPE) as a transfection agent.[75] This work has pioneered the use of cationic lipids in gene therapy leading to the commercial production of the first lipid based transfection agents (Lipofectin®).[76] The basic structure of all cationic lipids consists of polar head group(s) attached by linker (spacer) chain to hydrophobic groups (which may be single or double fatty acids, alkyl or cholesterol moieties).[77] At a specific concentration (i.e., critical micelle concentration, CMC), these agents self-assemble to form supramolecular structures, such as liposomes, micelles and cubic- or rod- like structures. The assembly is an important step in DNA binding and compaction process.[78] A large variety of compounds can be synthesized by chemically modifying the hydrophobic, hydrophilic as well as the spacer regions. Therefore, cationic lipids can be produced with different physiochemical properties and uses. DNA delivery efficiency of cationic lipids is attributed to the following factors: 1) the ability of cationic lipids to condense and encapsulate DNA forming a supramolecular complex known as a "lipoplex" through the electrostatic interaction of the polar head group of the lipid and the negatively charged phosphate groups of the nucleic acid, 2) cationic lipid/DNA complexes (lipoplexes) can be formulated to have an overall net positive charge that allows the association of lipoplex with the negatively charged cell membrane promoting cellular uptake, and 3) the fusogenic property of cationic lipids as a function of the hydrophobic alkyl tails promotes the escape of the entrapped DNA to the nucleus.[57,79] Based on these requirements, structural

manipulations with the basic components of cationic lipids have been applied aiming to improve transfection efficiency and reduce cytotoxicity.[77,80-82]

The polar head group(s) of cationic lipids play a major role in DNA condensation and compaction through neutralization of the negatively charged DNA phosphate backbone facilitating the formation of the lipoplexes and cellular uptake. The charge ratio of cationic lipids to anionic phosphate groups of DNA (+/- ratio) is an important factor that determines the transfection efficiency of the cationic lipid/DNA lipoplexes, as well as the cytotoxicity of the system. Based on the chemical structure of the cationic head group(s), cationic lipids are classified into the three main categories: quaternary ammonium salt lipids, lipopolyamines[83-86] and amidinium/guanidinium salt lipids.[87-90]

In the following section, I will discuss the quaternary ammonium salt lipids as this group of cationic lipids has shown the most promising transfection activity among other cationic lipid classes. Additionally, I will explore diquaternary ammonium gemini surfactants as a subgroup of cationic lipids, as it represents the group of compounds I used in my research work.

Quaternary ammonium salt lipids

Quaternary ammonium salt lipids are the oldest and the most extensively developed cationic lipids group used for gene delivery which was introduced by Felgner *et al*, (i.e. DOTMA).[75] DOTMA co-formulated with helper lipid DOPE (commercially known as Lipofectin^{TM}) was the gold standard for developing and designing cationic lipids in this group [Figure 1.2]. The replacement of the di-ether linkage in DOTMA with a more biodegradable di-ether linker produced 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) which showed a reduction in the cytotoxicity of the parent compound.[91] Several other compounds were synthesized based on the structure of DOTMA and DOTAP in order to investigate the effect of structural modification of the head group and the alkyl tail regions on the physiochemical properties and the transfection efficiency.[91-94] One of the most efficient compounds that was found in this group is 1,2-dimyristyloxypropyl-3-dimethyl-hydroxy ethyl ammonium bromide (DMRIE) which showed superior activity compared to DOTAP and its analogs in different transfection conditions; evaluated *in vitro* on COS-7 cells and pDNA encoding RSV-*β*-galactosidase. DOPE was used as helper lipid with all tested cationic lipids.[92]

(a) DOTMA[75]

(b) DOPE

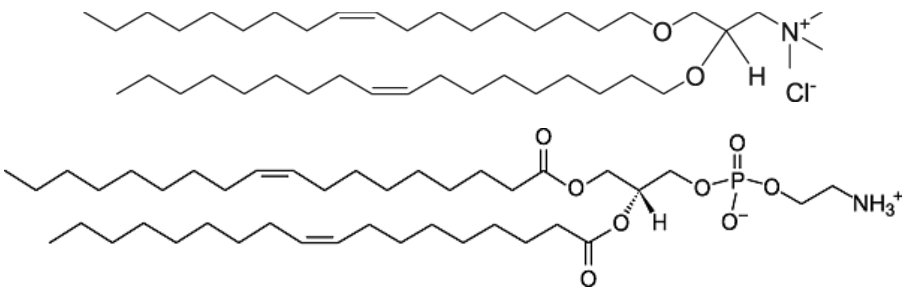
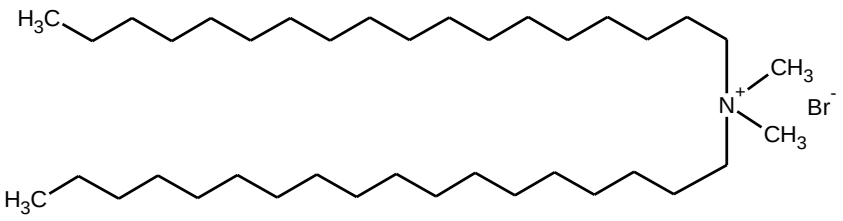


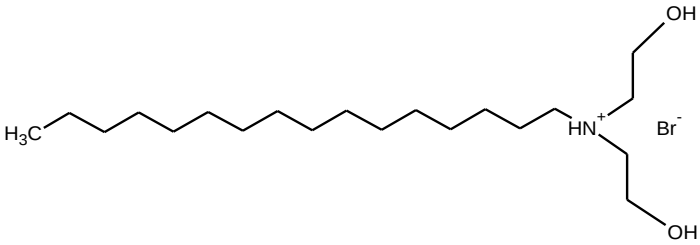
Figure 1.2: The chemical structure of (a) DOTMA, the first quaternary ammonium salt lipid used for gene delivery. (b) neutral lipid DOPE

Banerjee and co-workers synthesized a new class of non-glycerol quaternary ammonium cationic lipids where the quaternary nitrogen atom of the head group is directly linked to a 16-carbon atom hexadecyl tail(s) [Figure 1.3].[95] Additionally, two hydroxyethyl groups were attached to the polar head group aiming to enhance the cellular uptake of the lipoplexes. Two compounds were synthesized: N-n-hexadecyl-N,N-dihydroxyethylammonium bromide (HDEAB) bearing a single side chain, and N,N-di-n-hexadecyl-N,N-dihydroxyethylammonium bromide (DHDEAB) with a di-alkyl tail region.[95] HDEAB co-formulated with cholesterol showed to be the least efficient compared to dioctadecyldimethylammonium bromide (DDAB) (the parent compound without the hydroxyethyl groups in the polar head), DHDEAB, and Lipofectamine™. This observation suggested that the double chain alkyl tail is an essential element in lipoplex formation.[95] The transfection efficiency of DHDEAB, co-formulated with cholesterol on a 1:1 molar ratio, was assessed *in vitro* in green monkey fibroblasts cell lines (COS-1cells). DHDEAB showed a 2-3-fold increase in transfection efficiency compared to (DDAB).[95] This finding proved that the introduction of a hydroxyethyl moiety to the head group improved the transfection activity of the lipoplexes. The enhancement of the activity was partially due to the hydrogen-bonding interactions between the lipid head groups and the cell membrane. Based on these outcomes, the same research group developed four more compounds by attaching simple sugars (arabinose and xylose) to the polar head group as multiple hydroxyl moieties.[96] All four cationic lipids demonstrated high levels of *in vitro* gene expression with a superior performance for 1-deoxy-1-[dihexadecyl(methyl)-ammonio]-D-xylitol, which showed a doubling in transfection activity compared to the parent compound DHDEAB.[96]

DDAB



HDEAB



DHDEAB

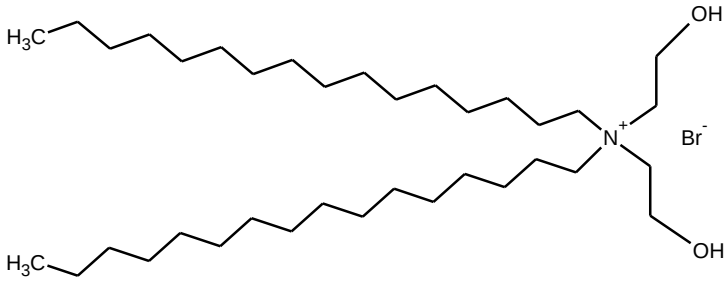


Figure 1.3: The chemical structure of quaternary ammonium salt lipids, without spacer region (non-glycerol) , one/two alkyl chain tail(s)

The first introduction of cationic surfactants as a transfection agent was in 1989 by Pinnaduwage *et al,* when they synthesized a group of quaternary ammonium detergents (dodecyl, tetradecyl-, or cetyl-trimethylammonium bromide) for DNA delivery.[97] However, these compounds showed lower gene expression activity compared to Lipofectin® and caused relatively high cellular toxicity even when these surfactants were used in combination with DOPE.[97] Another method that has been employed to produce quaternary ammonium cationic lipids that can be used in DNA delivery, is by mimicking the single head, double tailed lipids, through joining two single-tailed quaternary ammonium surfactants together via a spacer moiety producing diquaternary ammonium gemini surfactants.[98-103]

Diquaternary ammonium gemini surfactants

Gemini surfactants [Figure 1.1] are dimeric surfactants with a characteristically low surface tension making them suitable for use in material sciences as solubilizing and emulsifying agents.[104] In recent years, gemini surfactants have been investigated intensively as non-viral gene delivery carriers for both *in vitro* and *in vivo* transfection due their ability to compact DNA to form nano-sized lipoplexes, versatility in chemical structure, relatively low toxicity and inexpensive production.[102,103] Similarly to other cationic lipids, the transfection activity of gemini surfactants is influenced by the chemical nature of the head groups, length and saturation of the hydrophobic chains, and the chemical composition and length of the spacer.

In an effort to develop effective and safe gene delivery vectors, a series of diquaternary ammonium gemini surfactants were developed in our laboratory as non-viral gene delivery agents [Table 1.1].[105-109] These gemini surfactants were classified into three generations, based on modification within the molecular structure. The first generation gemini surfactants have the simplest structure which consists of two hydrophobic alkyl tail regions (*m*) attached to diquaternary ammonium head groups separated from each other through an alkyl spacer region (*s*) (i.e., structure of *m*-*s*-*m*).[105,110] A variety of gemini surfactants within this generation have been produced by changing the length of the spacer region and the alkyl chain tails. The gemini surfactants were able to compact pDNA forming nanoparticles with a particle size below 200 nm which is a requirement for successful endocytosis of the gemini surfactant/DNA complex.[110] *In vivo* transfection studies of these gemini surfactants showed the transfection activity to be dependent on the spacer length with the most efficient transfection observed with 12-3-12 and 16-3-16 gemini surfactants.[105] The cytotoxicity profile was considerably lower in comparison with the commercial Lipofectamine Plus gene delivery agent.[105]

Based on the above mentioned findings, a new generation (second generation) of gemini surfactants has been synthesized by inserting in the alkyl spacer region, nitrogen substituted moieties (e.g., –N– or –NH–).[106,111] The introduction of such functional groups created pH-sensitive gemini surfactants that facilitated fusion and endosomal escape of DNA from the transfection complex after cellular uptake.[106] A transfection study of nanoparticles constructed from the 12-7NH-12 gemini surfactant, pDNA and the helper lipid (DOPE) showed a 9-fold increase in transfection compared to complexes formed with 12-2-12 gemini surfactant.[106] In an attempt to enhance the efficiency of gemini surfactants, chemical derivatization of the 12-7NH-12 compound was performed through the coupling of various biocompatible amino acids moieties to the spacer region producing the third generation gemini surfactants.[108,109] Transfection efficiency of these novel amino acid substituted gemini surfactants was assessed in different epithelial cell lines.[109] The amino acid-substituted gemini surfactants (specifically, 12-7N(Glycine)-12) transfected all cell lines with a higher level of gene expression compared to the unsubstituted compound.[109] More recently, the cellular toxicity of these novel gemini surfactants has been evaluated revealing no change in the toxicity profile compared to 12-7NH-12, but a significant improvement when compared to Lipofectamine® Plus.[114]

More gemini surfactants were produced by the introduction of complexed moieties to polar head, spacer and tail regions. Cationic gemini surfactants with branched head groups, (e.g., polylysine-based gemini surfactants) showed considerable gene transfer activity.[112] The ability of a sugar based gemini surfactant-DNA complex to undergo a morphological change from lamellar to inverted hexagonal structures in low pH medium promoted the endosomal escape of the DNA, resulting in efficient transfection.[113,114]

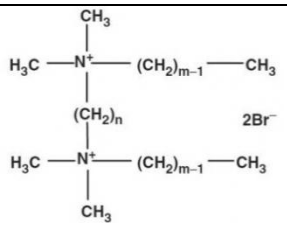
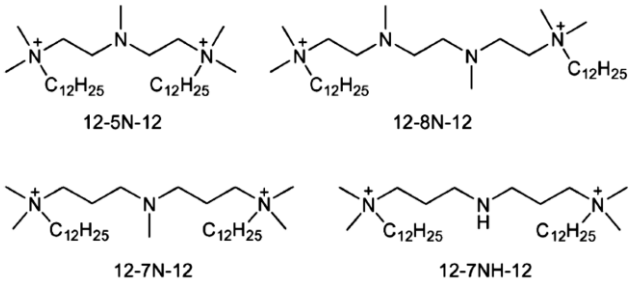
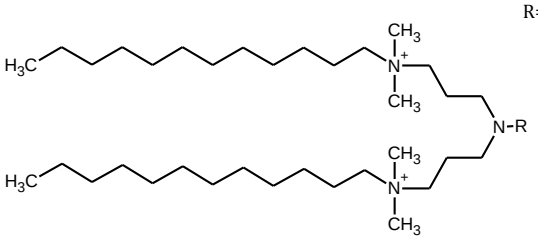
1. First generation gemini surfactant (*m-s-m*) [105,110]	
2. Second generation gemini surfactant (amine substituted spacer) [106,111]	
3. Third generation gemini surfactant (amino acid substituted spacer) [108,109]	 R= Glycine : 12-7N(Gly)-12 Lysine: 12-7N(Lys)-12 Histidine: 12-7N(His)-12 Glycyl-Lysine: 12-7N(Gly-Lys-12 Lysyl-Lysine: 12-7N(Lys-Lys)-12 Glycyl-Glycine: 12-7N(Gly-Gly)-12

Table 1.1: The general structure of three classes of diquaternary ammonium gemini surfactant based on chemical modifications.

Cationic lipid gene delivery vectors in clinical trials

The high level of transfection and low toxicity achieved in *in vivo* studies using lipoplexes for gene delivery have prompted the use of these unique systems in human clinical trials. By the end of 2011, lipoplex-based gene therapy has been employed in 110 clinical trials around the world (6 % of all approved trials) with a majority in phase I or II trials.[5] Cancer and cystic fibrosis are the primary interest areas in the development of lipofection agents. DC-Chol (3β-[*N*-(*N'*,*N'*-dimethylaminoethane)-carbamoyl]cholesterol) was the first cationic lipid that had been approved for human clinical trial for gene therapy of a lung disease, cystic fibrosis.[115] This study illustrated the concept and safety of using cationic lipid-based gene delivery. DC-Chol in combination with DOPE lipoplexes also were evaluated in several other clinical trials for breast and ovarian cancer, melanoma, head and neck cancer.[116-118] DMRIE was another cationic lipid that has been evaluated in several clinical trials for managing a wide range of cancers, namely melanoma, prostate cancer, renal cell carcinoma, head and neck cancer using different pDNA.[119-125] Recently, a large randomized controlled phase 3 clinical trial was completed that compared the efficacy of pDNA/DMRIE/DOPE lipoplexes encoding HLA-B7 and β-2 microglobulin genes versus dacarbazine (antineoplastic chemotherapy drug) in patients with stage III or stage IV melanoma.[126] The full results of the study have not yet been published.

With all of the success in lipoplex-based gene delivery, two major difficulties are still limiting their broad clinical applications; the low transfection efficiency compared to viral-based vectors and the instability of lipid-based gene delivery vectors in conventional pharmaceutical dosage forms.[127,128] As discussed in a previous section, large numbers of cationic lipids have been synthesized and modified to overcome the low transfection activity, with little attention to their stability from a pharmaceutical perspective. The transfection efficiency of lipoplexes

depends on the integrity of the delivery system components and their related physiochemical properties (particle size and surface charge ratio).[129-131] Stability of non-viral gene delivery systems is complicated as it involves 1) the conformational and chemical stability of genetic material, 2) the physical stability of DNA-carrier complexes and 3) the chemical stability of the carrier.[132-134]

In the following section I will discuss the factors influencing the stability of a cationic lipid-based gene delivery system in pharmaceutical formulations and the techniques used to optimize shelf-life stability of such systems.

1.2.3. Stability of cationic lipid-based gene delivery systems in pharmaceutical formulations

The chemical and physical stability of pharmaceutical formulations during manufacturing, shipping and storing is of critical importance to produce marketable therapeutic products. On one hand, for conventional pharmaceutical formulations, the main concern is the chemical stability of the active ingredient. The long-term stability can be assessed by monitoring the biological activity and degradation by-products using different analytical techniques.[135] On the other hand, the stability of multi-component drug delivery systems such as lipoplex-based vectors is a more complicated issue. The transfection efficiency of lipoplexes depends on both the chemical integrity of the components of the delivery system and conservation of their related physiochemical properties (e.g., particle size and surface charge ratio).[129-131] The stability of DNA-lipoplexes involves: 1) the chemical and conformational stability of pDNA, 2) the chemical stability of the carrier, and 3) the physical stability of the complexes.[132-134]

Plasmid DNA is an extra-chromosomal, circular double stranded DNA that encodes for protein of interest.[136] The use of pDNA, as a therapeutic agent, has increased in the last few decades, especially in gene therapy research and DNA vaccination.[137,138] pDNA exists in three different forms: supercoiled, open circular and linear. It has been established that the supercoiled form has the highest gene expression activity compared to the other forms.[139] However, like other genetic materials; pDNA is sensitive to environmental conditions and undergoes several degradation pathways. In aqueous medium, DNA is subjected to two degradation processes: 1) depurination/β-elimination, and 2) free radical oxidation leading to strand breakage.[140] The depurination/β-elimination process is the rate limiting degradation process and is catalyzed in acidic environment resulting in the formation of open-circular DNA. The depurinated DNA is consequently degraded into linear DNA if stored in a basic pH enviroment.[140] From this

perspective, two issues should be taken into consideration when incorporating DNA in pharmaceutical formulations: the pH profile of the final product and the presence of any free radicals such as reactive oxygen species (ROS) and trace metals. Evans *et al* evaluated the degradation pathways for pDNA via accelerated stability studies using different buffer systems, metal ion chelators and free ion scavengers.[141] They found that depurination/β-elimination was the rate limiting process if the plasmid was stored in demetalated buffer systems with EDTA and ethanol. A minimum conversion of the supercoiled DNA to open circular and linear forms was observed at pH values of 7.5 – 9.[141] While the chemical structure of DNA is well established, the stability of DNA in the different pharmaceutical formulations still requires more investigation.

As mentioned earlier, one of the major disadvantages of the lipoplex-based vectors is their poor physical stability in aqueous medium. This physical instability results from the inability to maintain optimal physiochemical properties of such systems (particle size and positive surface charge). In aqueous formulations, positively charged lipoplex particles tend to form micro-sized aggregates as a function of random collisions, Brownian motion, and gravity forces.[128,142-144] Consequently, disassociation of DNA from the lipoplexes may occur leading to the loss of the biological activity.[143] To evade such a stability issue, most of the studies that employed cationic lipids as non-viral carrier for gene delivery used freshly prepared lipoplexes for the transfection studies. Three different formulation strategies have been investigated to optimize the physiochemical stability of cationic lipid/DNA complexes: 1) liquid formulations, 2) frozen formulations, and 3) dehydrated formulations.[128]

To minimize the aggregation of lipoplexes in liquid formulations, different methods have been explored.[145-147] For instance, Hong *et al* employed two approaches to stabilize cationic lipid-DNA complexes in aqueous media.[145] In the first approach, a small amount of

poly(ethylene glycol)-phospholipid conjugate was incorporated to the DNA-lipid complexes to provide a steric stabilization to prevent particle aggregation. The second approach involved the condensation of the DNA with polyamines (spermidine) before the complexation with the cationic lipid. Both approaches were successful in maintaining the original transfection activity levels up to two months when stored at 4 ˚C.[145] However, almost all liquid formulations needed to be stored under special conditions (e.g., refrigeration) and only short term stability (3-8 months) was achieved.[145-147]

Freezing technique is a formulation strategy that has been investigated for producing lipoplexes with long term stability.[148-151] However, freezing is a physical stress that can negatively impact the physical stability of the lipoplexes and cause damage to DNA structure.[148,152,153] In addition, the requirement for maintaining the freezing condition during transportation and shipping increases the production cost. Considering the deficiencies of liquid and frozen formulations, more interest was focused on dehydrated formulations that showed the most promising shelf life stability.[154]

Dehydrated formulations provide several advantages including long-term physical and chemical stability of the dried products that can be stored at room temperature. In addition, the dehydrated formulations are highly resistant to stress and agitation occurring during transportation. The most common dehydration technique that has been widely investigated in non-viral gene delivery DNA vectors is freeze-drying (lyophilization).[133,155-158] However, lyophilization is a complicated process that includes many physical stresses. In the following section, I will discuss the use of lyophilization as a formulation technique for chemically-mediated gene delivery with long term stability.

For clarity of the following section, four concepts related to the freeze-drying technique need to be defined:

Glass transition temperature (*Tg*): temperature at which the amorphous material changes from a rigid glassy state to a liquid-like form,

Glass transition temperature of frozen component (*Tg*'): the temperature at which the amorphous material is transformed into the rigid glassy state,

Eutectic temperature (T_{eu}): the critical temperature at which the water and dissolved solutes crystallize,

Collapse temperature (T_c): the temperature at which the amorphous structure collapses.

1.2.4. Freeze-drying in chemically mediated gene delivery systems

Freeze-drying (lyophilization) is a widely employed technique for conferring long-term stability at ambient temperature to physically unstable bio-pharmaceuticals and liposomal drug delivery systems.[159] In general, freeze-drying is a dehydration technique in which the liquid formulation tranitions into the solid phase by freezing, then the frozen water is removed through sublimation under low pressure conditions.[160] In addition to long-term stability, lyophilized products are easily handled and transported. Freeze-drying in the pharmaceutical industry was used primarily for the production of parenteral drugs and bio-products (e.g., vaccines, proteins, and peptides). Lyophilization has also been investigated for stabilizing non-viral gene delivery systems.[133,155-158] Lyophilized non-viral delivery vectors showed promising results that can be utilized for the production of highly stable formulations with good manufacturing procedures.

The standard freeze-drying protocol includes two major steps: a freezing step and a drying step. Both steps must be optimized to ensure long-term stability of the formulations.[160] These two steps are considered physical stresses that have been reported to damage the components and the supramolecular structure of the non-viral vectors.[155,161] In addition, lipid phase transition in lyophilized liposomal formulations during dehydration-rehydration has been reported.[155,162] For the highest stability, the DNA-carrier complexes must retain the original physiochemical properties and complex morphology that govern the transfection activity during both steps of lyophilization protocol. The optimization of the freeze-drying protocol and the incorporation of certain stabilizers, known as cryo- or lyo-protectant agents, have been employed to improve the stability of the chemical carrier-based DNA formulations.[155,156]

In the following section I will discuss the major aspects that must be considered during each step of the freeze-drying of chemically mediated-DNA vectors, particularly lipoplex-based vectors.

1.2.4.1.Freezing step

In the freezing step, the liquid solution containing DNA-carrier complexes and other excipients is frozen to the solid state at a temperature below the freezing point of the sample. To obtain the desirable stable final product, several aspects should be considered in the freezing process: the components of the sample, the freezing rate and the use of cryoprotectant agents. Different methods of freezing have been employed to produce frozen formulations including 1) super-freezing by immersing the sample in liquid nitrogen, 2) placing the sample on precooled shelves (e.g., at -20, -40, -80 ˚C) or 3) placing the sample in a ramped cooling chamber.[160,163] During the freezing step, different solid phases are formed in the non-frozen portion depending on the components of the formulations: water ice crystals, crystalline solutes and amorphous phases. The increase in the concentration of the formulation component within the non-frozen part is known as freeze-concentrate or cryo-concentration effect.[160,163]

Two parameters must be monitored during the freezing cycle: *glass transition temperature* (T_g') for substances forming amorphous phase in frozen state (e.g., sugars) and *eutectic temperature* (T_{eu}) for substances forming crystalline phase (e.g., salts). The glass transition is a reversible transition of amorphous material in the liquid from solid-like structure (glassy structure) to fluid-like structure (rubbery or viscous structure).[164] During the freezing cycle, the temperature at which the amorphous material is transferred into the rigid glassy state is known as the glass transition temperature (T_g'). Generally, the presence of salts in the chemically mediated gene delivery systems can cause aggregation of the DNA, thus buffer salts are rarely used to

prepare such formulations. Therefore, during freeze-drying cationic carrier-DNA complexes, the T_g' is considered an essential property that has been found to influence the stability of the system during freezing. The glass transition temperature is dependent on the chemical composition and concentration of the formulation, and it can be measured via thermoanalytical methods (e.g, differential scanning calorimetry DSC).[164,165]

The rate of the freezing can affect significantly the biological activity of non-viral DNA complexes.[148,153] The phase separation and freeze-concentrate effects have been reported to cause aggregation of colloidal systems via several mechanisms: electrostatic interaction, liposomal fusion and particle collision.[155] In addition, the formation of ice crystals in the formulation have been reported to damage the integrity of the liposomal and DNA-carrier complexes.[148,163] A freeze-thaw study can be performed to assess the effect of the freezing on the physiochemical properties and biological activity of such systems.

In cationic based-DNA lipoplexes, significant alteration in the physiochemical properties and loss of gene transfection activity were reported after freeze-thaw studies in the absence of stabilizing agents.[148,166,167] For instance, Anchordoquy and co-workers found that transfection activity of cationic lipid-DNA complexes formed with three different cationic lipids (DMRIE, Lipofectamine and DOTAP:DOPE) showed significant reduction in *in vitro* transfection activity upon slow freezing-thawing cycle when no stabilizing agent (sucrose) was used.[148] The reduction in the transfection activity upon freezing was associated with significant increase in particle size. On the other hand, when super-freezing was used (by submerging sample vials in liquid nitrogen) and then the formulations were thawed at room temperature, insignificant loss of transfection activity of the lipoplexes was reported.[148] The loss of activity and the original physiochemical properties during the slow freezing cycle, in comparison to super-freezing, can

be rationalized by two explanations. Firstly, super-freezing generally results in the formation of fine water crystals which have been found to have little effect on the liposomal bilayer systems.[148,168,169] Conversely, large ice crystals are formed when the slow-freezing method is used which has harmful effects on the supramolecular structures.[148,168,169] The second explanation for the alteration of the properties of the lipoplexes is liposomal fusion. During the slow-freezing cycle, particle collision can be augmented in the non-freezing part of the sample encouraging liposomal fusion and particle aggregation and this effect develops as cryo-concentration increases.[148,155] It can be eliminated in the super-freezing cycle by reducing the time required for diffusion. Similar observations were reported during the freezing of polymer-based DNA polyplexes and solid lipid-DNA vectors.[158,170] Therefore, the use of freezing protectant agents (known as cryoprotectants) is essential to stabilize the DNA-complexes and retain the physiochemical properties of the systems and the transfection activity.

Several classes of excipients have been used for the preparation of lyophilized formulations as cryoprotectants: monosaccharaides (glucose), disaccharides (sucrose, trehalose), oligosaccharides (inulin), polymers (dextran, povidone, polyethylene glycol) and sugar alcohols (mannitol, glycerol, sorbitol).[150,171-175] Different mechanisms have been proposed to explain the protective action of the cryoprotectants in colloidal systems and proteins: preferential exclusion[176,177], vitrification[178,179], and particle isolation hypothesis.[166]

The *preferential exclusion hypothesis* was proposed to explain the protective effect of sugars on the macromolecular structures of proteins during the freezing process. Based on this hypothesis, the sugar molecules do not access the surface of the protein in the formulation, hence, preferentially are excluded from the surface and form a layer around the protein. During the freezing, these sugar layers protect the protein structure and prevent protein unfolding. The

applicability of preferential exclusion hypothesis as a protective mechanism for lipid-based DNA complexes is debatable since it assumes phase separation between the protein and the sugar layer which is not the case in liposomal based structure.[163] In addition, previous studies investigating the lyophilization of chemically mediated non-viral vectors reported that a high concentration of sugars is required to stabilize the vectors during the freezing.[148,166]

The *vitrification* (*glass formation*) *hypothesis* proposed that when the sample containing biologically active molecules and cryoprotectant agent is cooled to a temperature below the glass transition temperature (T_g'), the active molecules are entrapped in the cryoprotectant amorphous glass matrix, which inhibits the kinetic activity of the complexes.[178,179] The formation of a cryoprotectant glassy matrix, an effect of cryo-concentration, strictly immobilizes the drug complexes and prevents the particles from aggregating and fusion of the drug from the complexes. Furthermore, the amorphous glass matrix of the cryoprotectant agent minimizes the damage caused by the formation of ice crystals. The vitrification hypothesis is widely applied as a protective mechanism when biological drug molecules or liposomal drug delivery systems are lyophilized.[163,178,180-182] Several studies of lyophilized lipoplexes and polyplexes have explained the protective action of cryoprotectant sugars by the glass formation theory.[183,184] However, the vitrification hypothesis is not applicable to the cryoprotectant agents which do not form glassy matrices (e.g., mannitol, dextran). Allison *et al.* showed that the particle size and transfection activity of DMRIE-cholesterol:DNA lipoplexes was preserved after freezing the formulation with glucose as cryoprotectant agent at - 40 ˚C (T_g' for glucose is approximately - 43 ˚C).[166] In brief, the glass formation hypothesis is not the only protective mechanism in case of lyophilized lipoplexes and some sugars are able to preserve the essential physiochemical properties of lyophilized lipoplexes even if the formulation is frozen above the sugar's T_g'.

The *particle isolation hypothesis* proposes that the full cryoprotection effect can be achieved only at a crucial excipient:DNA weight ratio.[166] At this ratio and during the freezing, the increase in the concentration of cryoprotectant agent and other suspended particles in the unfrozen fraction of the formulation (i.e., freeze-concentration effect) leads to the isolation of DNA:carrier complexes in the cryoprotectant viscous matrix and prevents the diffusion of complexes and aggregation of particles. Thus, the vitrification of the stabilizing agent is not a requirement to achieve the cryoprotectant action.[155,166,185]

As mentioned earlier, the rate of freezing controls the size of the ice crystals formed in the frozen formulations. Super-cooling resulted in the formation of small ice crystals with small pores which can increase the time of drying and result in dried cake with high moisture content. On the other hand, when the formulation freezes at a slow rate large ice crystals are formed with larger pores accelerating the drying process, hence, this could augment particle aggregation.[155,163]

1.2.4.2. Drying Step

After the freezing step, the formulation is separated into two phases: ice crystals and the fraction of water containing the freeze-concentrated components.[155] The drying process includes two steps: the primary drying (sublimation) and the secondary drying (water desorption). In the primary drying cycle, more that 90% of the water content of the frozen formulation is removed by sublimation of water-ice crystals.[160] The sublimation process starts by reducing the pressure in the drying chamber to the level below the triple point of the formulation. The shelf temperature of the freeze-drier increases with time to drive the sublimation process by transferring heat to the vials. Several issues must be considered during the primary drying cycle to achieve stable and elegant dried product. The removal of ice crystals begins at the top of the frozen formulation by removing the ice and forming dried layers of the formulation. As a result,

the shape and dimensions of samples vials and depth of filling affect the time required for drying and the appearance of the lyophilized cake.[160] The temperature of the drying chamber, during the primary drying cycle, should be maintained below a critical temperature, known as the *collapse temperature (T_c).* The T_c of a product is almost similar to its T_g'. However, it has been reported that by reducing the water content from the frozen formulation during the drying process, T_c is increased.[186] The increase in sample temperature during the primary drying above the T_c causes the collapse of the porous matrix. As a result, the collapsed structure hinders the removal of moisture content by sublimation which can increase the required time for drying and alters the appearance of the final dried product. Therefore, monitoring the T_c and T_g' during the primary during cycle is a key factor for successful lyophilization.

Once all the ice crystals (free water) are removed from the frozen samples by sublimation, the secondary drying cycle takes place. The purpose of the second drying step (water desorption) is to remove bound water from the freeze-concentrated fraction, to increase the glass transition temperature T_g of the dried product and to achieve long shelf-life. By the end of this step, the moisture content of the dried product is reduced to a level below 2%.[160] The secondary drying cycle is accomplished by increasing the shelf temperature providing heat energy necessary to release the bound water. At this stage, since all the ice crystals are already removed, the product temperature can exceed the (T_c), but not (T_g). However, caution should be taken to avoid excessive heating that may cause product degradation.

As discussed previously, the drying process could cause a physical stress to the DNA-carrier complexes leading to changes in their physiochemical properties and loss of transfection activity. In fact, the removal of the hydration shell from non-viral vector complexes could cause more damage to the supramolecular morphology than the freezing step.[155,156] Dehydration-

rehydration studies are routinely conducted to evaluate the influence of the drying process on the cationic carrier-DNA vectors.[158,183,187] The use of lyoprotectants, which are usually polyhydroxy compounds such as sugars, is essential to preserve the structure of the biologically active compounds and liposomal structure during the dehydration.[159,163,177] In the freeze-drying processes of protein- and liposome-based structures, lyoprotection by polyhydroxy compounds is explained by two major hypotheses: *water replacement hypothesis* and *vitrification hypothesis*.[163,176]

The *water replacement hypothesis* proposes that lyoprotectant sugars are able to form hydrogen bonds with the protein and with the lipid phase of liposomes, replacing the water hydration shell, while stabilizing the structure of protein and liposomal membrane during the dehydration process.[188,189] The water replacement hypothesis has been used to explain the protective effect of sugars (especially disaccharide sugars) and the changes in the physiochemical properties of lyophilized cationic non-viral DNA complexes.[156-158,183] Several studies demonstrated that the entrapment of liposomal structures in the freeze-concentrated glass matrix of sugar during freezing (*vitrification hypothesis*) could prevent the phase transition of lipid resulting from the dehydration.[163] In addition to the aforementioned hypothesis, the *particle isolation theory* has recently emerged in the field of lyophilization of non-viral vectors. It has been utilized to explain the lyoprotective effect of agents that crystallized during freezing without forming hydrogen bonds with the cationic carrier such as dextran and polyethylene glycol polymer (PEG).[155,157]

However, the protective effect of lyoprotectants during lyophilization of multi-component systems such as cationic non-viral vectors is still not widely explored and more investigations are required to fully understand the effect of dehydration on the components of these systems.

1.2.4.3. Stability of lyophilized lipoplex vectors

Optimization of the freeze-drying parameters can significantly improve the stability of lipoplex-based vectors. In addition, successful lyophilization of lipoplexes leads to reproducible production of gene delivery systems that can be controlled and monitored under good manufacturing practice. The stability of lyophilized lipoplexes during storage was evaluated in numerous studies using different cationic lipids and excipients.[155,156,184,187,190,191] Several factors influence the stability of lyophilized lipoplexes: formulation composition, storage temperature, moisture content in the dried cake, and the presence of reactive oxygen species (ROS).[134,192,193] Li *et al*, reported that the physical properties and transfection activity of lyophilized cationic lipid-protamine-DNA (LPD) complexes (formulated using DOTAP/cholesterol and protamine liposomes in 10% sucrose) were not significantly altered when stored at room temperature for 8 weeks.[194] Similarly, Clement *et al* proposed a continuous-mixing followed by lyophilization technique for large-scale production of lipoplexes with long shelf-stability.[190] Following this technique, pDNA:DC-Chol/DOPE lipoplexes were able to maintain the original size and biological activity up to 18 months when stored at 4-8 ˚C.[190]

The most extensive stability evaluation for lyophilized lipoplexes was performed by Molina and co-workers.[184] In this study, the long-term stability of lyophilized lipoplexes constructed from pDNA:DOTAP/DOPE using different stabilizing agents (glucose, sucrose or trehalose) was evaluated. Lyophilized formulations were stored for two years at five storage temperatures -20, 4, 22, 40 and 60 ˚C. The physiochemical properties (particle size and zeta potential), DNA-lipid interaction (ethidium bromide accessibility), pDNA supercoiled content, moisture content and *in vitro* transfection activity of lyophilized formulations were analyzed at multiple sampling points. Additionally, the level of ROS was assessed in the lyophilized cake.

The results from the stability study showed progressive decrease in transfection activity at all storage conditions, including samples stored at – 20 ˚C.[184] The reduction in transfection activity was attributed to the continuing reduction of supercoiled content of pDNA and the changes in the conformational state of the lipoplexes, particularly when samples were stored at high temperatures (above 22 ˚C). The loss of transfection activity for samples stored at – 20 ˚C and 4 ˚C was partially due to the oxidative stress resulting from the formation of ROS in dried cake.[184] In fact, the same research group investigated the effect of ROS on the stability of individual components of the lyophilized lipoplexes (e.g., lipid, pDNA, and sugar).[133,134] The results suggested that ROS caused the degradation of the lyophilized lipid (DOTAP-DOPE) and had a less damaging effect on the free lyophilized pDNA. This damaging effect can be minimized by the incorporation of antioxidant agents (e.g., α-tocopherol) or metal chelator agents such as diethylenetriaminepentaacetic acid (DTPA) and by optimizing the drying process to reduce the moisture content in dried cake.[133,134]

To date, most of the studies that employed lyophilization techniques in cationic lipid based-vectors utilized commercially available cationic lipids (such as DOTAP, DC-Chol, DMRIE) which bear one singly-charged cationic group. In addition, no work monitored the degradation of the lipid phase component (cationic lipid or neutral lipid). It is an essential requirement for all drug authorities around the world to examine the chemical stability of all components in drug formulations and to identify potential degradation by-products.[195,196] Different analytical techniques can be utilized to characterize and to quantify the degradation by-products of pharmaceutical formulations during stability studies. Mass spectrometry is one of these techniques that shows a great ability in pharmaceutical analysis. In the following section, I will briefly discuss the applications of mass spectrometry in drug discovery and development.

1.2.5. Mass spectrometry in drug discovery and development

In the last three decades, there has been a significant development in the capabilities of mass spectrometry (MS) and its use as an analytical tool. Major developments in soft ionization techniques and high resolution mass analyzers have made MS a powerful technique in chemical analyses.[197] Unlike other usual physiochemical analytical techniques (namely, UV, IR and NMR), MS provides several advantages including, high-throughput analysis, high sensitivity and selectivity, and the capability of coupling with chromatographic techniques.[198-201] MS is an excellent tool to distinguish between different molecules with small variations in their molecular masses, especially when used in conjugation with a chromatographic technique.

Before the invention of atmospheric pressure ionization (API) sources, the coupling of MS to liquid chromatography (LC) was a very difficult and complicated process. The first interface was the moving belt interface, using electron impact (EI) and chemical ionization (CI).[202] The major shortcomings of the moving belt interface were that the analyte had to be thermostable and the cleaning of the belt was a difficult task. Other ionization methods have subsequently been developed including: thermospray[203], continuous flow fast atom bombardment (CF-FAB)[204] and particle beam interface.[205] The introduction of API-based ionization sources allowed for efficient combination of MS and liquid chromatography. The advantage of API sources, when coupled with LC, is that the high vacuum in MS is not interrupted. This is because the analyte ionizes outside the spectrometer at atmospheric pressure and only the resulting ions are introduced into the instrument. In addition, API-based sources are considered a "soft" ionization technique, in comparison to old interfaces, that can be used for thermolabile molecules without degradation. Currently, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are the most widely used ionization methods in pharmaceutical analysis [206]

Taking advantage of the rapid improvements of MS instrumentations and hyphenated MS technologies (e.g. LC-MS), MS has become widely utilized in qualitative and quantitative analyses in the drug development processes. Several MS-based strategies have been developed to accelerate the drug discovery and development processes. In fact, MS is broadly used in numerous pharmaceutical analyses, including high-throughput screening[207,208], studies of drug metabolites [209,210], pharmacokinetic studies[200,211], and in the identification of pharmaceutical impurities and degradation products.[212-214]

Mass spectrometry in high-throughput screening of drug molecules

Mass spectrometric techniques are routinely utilized for high-throughput screening of bioactive compounds for qualitative and quantitative analyses with high accuracy and precision.[215-218] For example, ESI-quadrupole time-of-flight tandem mass spectrometric (ESI-Qq-ToF MS/MS) analysis determined the exact molecular structure of Lipid A moieties isolated from mutant and wild-type *Aeromonas salmonicida* lipopolysaccharide, that has been evaluated for therapeutic activity in immune disease and as a potential anticancer agent.[217] Similarly, the use of ESI multiple-stage tandem mass spectrometry (ESI-MS^n) to analyze saponins, naturally occurring glycosides with a variety of biological activities (e.g., antimicrobial and anti-inflammatory), allowed for structural differentiation between several types of saponins.[218] Subsequently, a liquid chromatography (LC)-MS/MS method was also developed for quick and precise quantification of different saponins from plant extract.[218,219]

Mass spectrometry in pharmacokinetic and metabolomics studies

Qualitative and quantitative determination of drug metabolites and drug pharmacokinetic studies are another area in the drug development process where MS and hyphenated MS techniques are employed extensively.[220,221] MS is capable of providing detailed structural

information for metabolites which usually have a similar chemical structure as the original drug. Different LC/MS methods have been described for the determination and the monitoring of drug metabolites in different human fluids and tissues.[222-227] For instance, an HPLC-ESI-MS/MS method was developed to detect and identify the potential reactive metabolites of tamoxifen, an antiestrogen agent used in the treatment of breast cancer, in the plasma of breast cancer patients.[226,228] The LC/MS analysis of human Hep G2 cell line extract, after incubation with tamoxifen, showed five metabolites in the positive ESI mode. These metabolites, in addition to three others, were observed in plasma samples obtained from a patient that had been treated with tamoxifen for a long period (more than 6 months).[226] Tandem mass spectrometric (MS/MS) analysis of tamoxifen and its potential metabolites showed a common fragmentation pattern confirming the proposed structure of the metabolites and allowed for the identification of the metabolic pathway of tamoxifen.[228] Similarly, Hodel *et al* described a LC-MS/MS method for monitoring the human plasma level of 14 different antimalarial agents and some of their active metabolites using ESI-triple quadrupole mass spectrometry.[227] LC-MS/MS provides a simple, fast, sensitive and selective technique that can be used for pharmacokinetic studies of these drugs and to evaluate the treatment regime.

Mass spectrometry in drug impurity profiling

In addition to screening bio-active compounds and pharmacokinetic studies, MS techniques are widely utilized in drug impurity and degradation by-product profiling. In the pharmaceutical industry, profiling of unknown impurities, especially when present in excess of threshold limits, is very important for safety and to address shelf-stability concerns.[195,196] During the synthesis of new drug substances, the identification of the chemical structure of the intermediate and by-product impurities can help researchers identify the source of these

impurities and, as a result, to avoid or at least minimize the production of these impurities by changing the reaction conditions. In addition, impurity profiling can be used as a "fingerprint" for the quality and level of consistency of the manufacturing process.[229] MS is one of the most powerful tools in the analysis of drug impurities and degradation by-products that has been used as a qualitative and quantitative method.[230-236] For instance, during the development of Caspofungin, a semi-synthetic antifungal drug, an impurity was observed under HPLC conditions at 0.1% level.[234] Using triple quadrupole MS and ESI in positive ion mode, and the same HPLC mobile phase, four major peaks were observed in the full-scan spectrum; three peaks were attributed to the original drug molecules and a peak to the impurity. Using hydrogen/deuterium (H/D) exchange and the LC-MS method, the structure of the impurity was elucidated and confirmed; this helped the chemists to minimize the formation of this impurity by eliminating the oxidation source during the manufacturing process.[234] Similar to drug impurity profiling, MS and MS-LC methods were developed to monitor and identify the degradation products of drugs during stability studies. Shah *et al* used multiple stage MS analysis (MS^n) to establish the mass spectrometric fragmentation pathway of atorvastatin, a lipid-lowering medication.[235] The proposed fragmentation pattern was utilized to study the degradation of the drug under controlled stress (e.g., hydrolysis, oxidation and photolysis). By utilizing liquid chromatography/time-of-flight mass spectrometric (LC/ToF-MS) analyses, the structure of six degradation by-products was identified.[235] Thus, MS techniques provide a rapid and accurate tool to elucidate the structure of drug impurities and degradation products.

Chapter 2

Rationale, hypothesis and objective

In our research group, a series of cationic gemini surfactants were developed as chemical carriers for DNA delivery.[105,106,109] Although, significant improvements in gene expression activity and enhancement in the cellular toxicity profile were achieved, the instability of the pDNA/gemini surfactant lipoplexes in aqueous formulation remained an issue. Lyophilization showed promising results to improve the physical stability of lipoplex-based vectors.[133,155-158] However, the lyophilization process must be optimized to avoid damages, caused by the freezing and dehydration steps, to the structure of lipoplex and to ensure long-term stability.[155,157] Therefore, development and optimization of analytical methods are essential to characterize and monitor the structure and various components of lipoplex vectors.

In this study, I have focused on evaluating the ability of lyophilization to improve the stability of pDNA/gemini surfactant lipoplexes. In addition, I have investigated the influence of the lyophilization and storage conditions on the essential physiochemical properties of the lipoplexes and their *in vitro* transfection. This was achieved by developing different quantitative and qualitative methods.

To achieve the overall goal of this study, I proposed two hypotheses each with their respective objectives.

2.1. Formulation strategies to optimize the physiochemical stability of gemini surfactant-based lipoplexes

2.1.1. Research hypothesis:

Optimization of the formulation compositions and the lyophilization processes of the gemini surfactant/DNA lipoplexes will lead to long-term physiochemical stability of the pharmaceutical preparations while maintaining transfection efficiency.

2.1.2. Objective:

To develop quantitative and qualitative methods that will be used to evaluate the feasibility of lyophilization as a formulation technique for preparing gemini surfactant-based lipoplexes with long term stability.

2.1.3. Specific objectives:

To develop qualitative and quantitative analytical methods for:

- Assessing the influence of different excipients and lyophilization strategies on the physiochemical properties and the transfection activity of the lipoplex formulations
- Assessing the influence of storage conditions on the physiochemical properties and the transfection activity of the lyophilized lipoplex formulations

2.2. Mass spectrometric analysis of cationic gemini surfactant

2.2.1. Research hypothesis:

Tandem mass spectrometric (MS/MS) analysis of cationic diquaternary ammonium gemini surfactants is a suitable method for the assessment of the stability and the identification of possible degradation by-products of gemini surfactant-based DNA formulations

2.2.2. Objective:

To evaluate the suitability of mass spectrometric and tandem mass spectrometric (MS/MS) techniques in the identification and characterization of amino acid/di-peptide gemini surfactants.

2.2.3. Specific objectives:

- To confirm the molecular structure of amino acid/di-peptide substituted gemini surfactants using electrospray-Time-of-Flight mass spectrometer.
- To establish a universal tandem mass spectrometric fragmentation pattern of the amino acid/di-peptide substituted gemini surfactants.
- To establish a quantification method using MS techniques.

References

1. Friedmann T. The development of Human Gene Therapy: Cold Spring Harbor Laboratory Pr; 1999.

2. Pfeifer A, Verma IM. Gene therapy: promises and problems. Annual Review of Genomics and Human Genetics 2001;2:177-211.

3. Rosenberg SA, Aebersold P, Cornetta K, *et al*. Gene transfer into humans—immunotherapy of patients with advanced melanoma, using tumor-infiltrating lymphocytes modified by retroviral gene transduction. New England Journal of Medicine 1990;323:570-8.

4. Blaese RM, Culver KW, Miller AD, *et al*. T lymphocyte-directed gene therapy for ADA− SCID: initial trial results after 4 years. Science 1995;270:475-80.

5. Gene Therapy Clinical Trials Worldwide John Wiley and Sons Ltd., 2012. (Accessed April, 25, 2012, at http://www.wiley.com//legacy/wileychi/genmed/clinical/.)

6. Raper SE, Chirmule N, Lee FS, *et al*. Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. Molecular Genetics and Metabolism 2003;80:148-58.

7. Edelstein ML, Abedi MR, Wixon J. Gene therapy clinical trials worldwide to 2007—an update. The Journal of Gene Medicine 2007;9:833-42.

8. Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, *et al*. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. Science 2000;288:669-72.

9. Hacein-Bey-Abina S, von Kalle C, Schmidt M, *et al*. A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. New England Journal of Medicine 2003;348:255-6.

10. Hacein-Bey-Abina S, Von Kalle C, Schmidt M, *et al*. LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Science 2003;302:415-9.

11. Pearson S, Jia H, Kandachi K. China approves first gene therapy. Nature Biotechnology 2004;22:3-4.

12. Garber K. China approves world's first oncolytic virus therapy for cancer treatment. Journal of the National Cancer Institute 2006;98:298-300.

13. Niidome T, Huang L. Gene therapy progress and prospects: nonviral vectors. Gene Therapy 2002;9:1647-52.

14. Wells D. Gene therapy progress and prospects: electroporation and other physical methods. Gene Therapy 2004;11:1363-9.

15. Schuler M, Rochlitz C, Horowitz JA, *et al*. A phase I study of adenovirus-mediated wild-type p53 gene transfer in patients with advanced non-small cell lung cancer. Human Gene Therapy 1998;9:2075-82.

16. Haviv YS, Blackwell JL, Kanerva A, *et al*. Adenoviral gene therapy for renal cancer requires retargeting to alternative cellular receptors. Cancer Research 2002;62:4273.

17. Chévez-Barrios P, Chintagumpala M, Mieler W, *et al*. Response of retinoblastoma with vitreous tumor seeding to adenovirus-mediated delivery of thymidine kinase followed by ganciclovir. Journal of Clinical Oncology 2005;23:7927-35.

18. Foster K, Foster H, Dickson J. Gene therapy progress and prospects: Duchenne muscular dystrophy. Gene Therapy 2006;13:1677-85.

19. Mahasreshti PJ, Navarro JG, Kataram M, *et al*. Adenovirus-mediated soluble FLT-1 gene therapy for ovarian carcinoma. Clinical Cancer Research 2001;7:2057-66.

20. Huan B, Van Atta R, Cheng P, Wood M, Zychlinsky E, Albagli D. Comparison of Lipid-Mediated and Adenoviral Gene Transfer in Human Monocyte-Derived Macro-phages and COS-7 Cells. Histochem Cell Biol 1999;108:325-33.

21. St George J. Gene therapy progress and prospects: adenoviral vectors. Gene Therapy 2003;10:1135-41.

22. Gafni Y, Pelled G, Zilberman Y, *et al*. Gene therapy platform for bone regeneration using an exogenously regulated, AAV-2-based gene expression system. Molecular Therapy 2004;9:587-95.

23. Stieger K, Le Meur G, Lasne F, *et al*. Long-term doxycycline-regulated transgene expression in the retina of nonhuman primates following subretinal injection of recombinant AAV vectors. Molecular Therapy 2006;13:967-75.

24. Magnusson MK, Hong SS, Boulanger P, Lindholm L. Genetic retargeting of adenovirus: novel strategy employing "deknobbing" of the fiber. Journal of Virology 2001;75:7280-9.

25. Noureddini SC, Curiel DT. Genetic targeting strategies for adenovirus. Molecular Pharmaceutics 2005;2:341-7.

26. Mei L, Jin X, Song C, Wang M, Levy R. Immobilization of gene vectors on polyurethane surfaces using a monoclonal antibody for localized gene delivery. The Journal of Gene Medicine 2006;8:690-8.

27. Levy R, Song C, Tallapragada S, *et al*. Localized adenovirus gene delivery using antiviral IgG complexation. Gene Therapy 2001;8:659.

28. Favre D, Provost N, Blouin V, *et al*. Immediate and long-term safety of recombinant adeno-associated virus injection into the nonhuman primate muscle. Molecular Therapy 2001;4:559-66.

29. Nyberg-Hoffman C, Aguilar-Cordova E. Instability of adenoviral vectors during transport and its implication for clinical studies. Nature Medicine 1999;5:955-57.

30. Croyle M, Cheng X, Wilson J. Development of formulations that enhance physical stability of viral vectors for gene therapy. Gene Therapy 2001;8:1281-90.

31. Maheshri N, Koerber JT, Kaspar BK, Schaffer DV. Directed evolution of adeno-associated virus yields enhanced gene delivery vectors. Nature Biotechnology 2006;24:198-204.

32. Allocca M, Doria M, Petrillo M, *et al*. Serotype-dependent packaging of large genes in adeno-associated viral vectors results in effective gene delivery in mice. The Journal of Clinical Investigation 2008;118:1955-64.

33. Heilbronn R, Weger S. Viral vectors for gene transfer: current status of gene therapeutics. Drug Delivery 2010:143-70.

34. 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;12:825-30.

35. Lew D, Parker SE, Latimer T, *et al*. Cancer gene therapy using plasmid DNA: pharmacokinetic study of DNA following injection in mice. Human Gene Therapy 1995;6:553-64.

36. Maruyama H, Higuchi N, Nishikawa Y, *et al*. High-level expression of naked DNA delivered to rat liver via tail vein injection. The Journal of Gene Medicine 2002;4:333-41.

37. Hartikka J, Sawdey M, Cornefert-Jensen F, *et al*. An improved plasmid DNA expression vector for direct injection into skeletal muscle. Human Gene Therapy 1996;7:1205-17.

38. Tsan M, White JE, Shepard B. Lung-specific direct in vivo gene transfer with recombinant plasmid DNA. American Journal of Physiology-Lung Cellular and Molecular Physiology 1995;268:L1052-L6.

39. Lin H, Parmacek M, Morle G, Bolling S, Leiden J. Expression of recombinant genes in myocardium in vivo after direct injection of DNA. Circulation 1990;82:2217-21.

40. Maruyama H, Higuchi N, Nishikawa Y, *et al*. Kidney-targeted naked DNA transfer by retrograde renal vein injection in rats. Human Gene Therapy 2002;13:455-68.

41. Nomura T, Yasuda K, Yamada T, *et al*. Gene expression and antitumor effects following direct interferon (IFN)- , gene transfer with naked plasmid DNA and DC-chol liposome complexes in mice. Gene Therapy Basingstoke 1999;6:121-9.

42. Hagstrom J. Plasmid-based gene delivery to target tissues in vivo: the intravascular approach. Current Opinion in Molecular Therapeutics 2003;5:338-44.

43. Liang K, Nishikawa M, Liu F, Sun B, Ye Q, Huang L. Restoration of dystrophin expression in mdx mice by intravascular injection of naked DNA containing full-length dystrophin cDNA. Gene Therapy 2004;11:901-8.

44. Herweijer H, Wolff J. Progress and prospects: naked DNA gene transfer and therapy. Gene Therapy 2003;10:453-8.

45. Mahvi DM, Sondel P, Yang N, *et al*. Phase I/IB Study of Immunization with Autologous Tumor Cells Transfected with the GM-CSF Gene by Particle-Mediated Transfer in Patients with Melanoma or Sarcoma University of Wisconsin, Madison, Wisconsin. Human Gene Therapy 1997;8:875-91.

46. Gehl J. Electroporation: theory and methods, perspectives for drug delivery, gene therapy and research. Acta Physiologica Scandinavica 2003;177:437-47.

47. Nishida K, Doita M, Takada T, *et al*. Sustained transgene expression in intervertebral disc cells in vivo mediated by microbubble-enhanced ultrasound gene therapy. Spine 2006;31:1415-19.

48. Plank C, Schillinger U, Scherer F, *et al*. The magnetofection method: using magnetic force to enhance gene delivery. Biological Chemistry 2005;384:737-47.

49. Mahvi D, Shi FS, Yang NS, *et al*. Immunization by particle-mediated transfer of the granulocyte-macrophage colony-stimulating factor gene into autologous tumor cells in melanoma or sarcoma patients: report of a phase I/IB study. Human Gene Therapy 2002;13:1711-21.

50. Jones S, Evans K, McElwaine-Johnn H, *et al*. DNA vaccination protects against an influenza challenge in a double-blind randomised placebo-controlled phase 1b clinical trial. Vaccine 2009;27:2506-12.

51. Daud AI, DeConti RC, Andrews S, *et al*. Phase I trial of interleukin-12 plasmid electroporation in patients with metastatic melanoma. Journal of Clinical Oncology 2008;26:5896-903.

52. C Heller L, Heller R. Electroporation gene therapy preclinical and clinical trials for melanoma. Current Gene Therapy 2010;10:312-7.

53. Mehier-Humbert S, Guy RH. Physical methods for gene transfer: improving the kinetics of gene delivery into cells. Advanced Drug Delivery Reviews 2005;57:733-53.

54. Guo X, Szoka Jr FC. Chemical approaches to triggerable lipid vesicles for drug and gene delivery. Accounts of chemical research 2003;36:335-41.

55. Li S, Huang L. Gene therapy progress and prospects: non-viral gene therapy by systemic delivery. Gene therapy 2006;13:1313-9.

56. Schaffert D, Wagner E. Gene therapy progress and prospects: synthetic polymer-based systems. Gene therapy 2008;15:1131-8.

57. Martin B, Sainlos M, Aissaoui A, *et al*. The design of cationic lipids for gene delivery. Current Pharmaceutical Design 2005;11:375-94.

58. Davis ME. Non-viral gene delivery systems. Current Opinion in Biotechnology 2002;13:128-31.

59. Graham F, Van der Eb A. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 1973;52:456-67.

60. Lopata MA, Cleveland DW, Sollner-Webb B. High level transient expression of a chloramphenicol acetyl transferase gene by DEAE-dextran mediated DNA transfection coupled with a dimethyl sulfoxide or glycerol shock treatment. Nucleic Acids Research 1984;12:5707-17.

61. Park TG, Jeong JH, Kim SW. Current status of polymeric gene delivery systems. Advanced Drug Delivery Reviews 2006;58:467-86.

62. Boussif O, Lezoualc'h F, Zanta MA, *et al*. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. Proceedings of the National Academy of Sciences 1995;92:7297.

63. Pack DW, Hoffman AS, Pun S, Stayton PS. Design and development of polymers for gene delivery. Nature Reviews Drug Discovery 2005;4:581-93.

64. Putnam D, Gentry CA, Pack DW, Langer R. Polymer-based gene delivery with low cytotoxicity by a unique balance of side-chain termini. Proceedings of the National Academy of Sciences 2001;98:1200.

65. Benns JM, Choi JS, Mahato RI, Park JS, Kim SW. pH-sensitive cationic polymer gene delivery vehicle: N-Ac-poly (L-histidine)-graft-poly (L-lysine) comb shaped polymer. Bioconjugate Chemistry 2000;11:637-45.

66. Dash P, Read M, Barrett L, Wolfert M, Seymour L. Factors affecting blood clearance and in vivo distribution of polyelectrolyte complexes for gene delivery. Gene Therapy 1999;6:643.

67. Männistö M, Vanderkerken S, Toncheva V, *et al*. Structure–activity relationships of poly (l-lysines): effects of pegylation and molecular shape on physicochemical and biological properties in gene delivery. Journal of Controlled Release 2002;83:169-82.

68. Kwoh DY, Coffin CC, Lollo CP, *et al*. Stabilization of poly-L-lysine/DNA polyplexes for in vivo gene delivery to the liver. Biochimica et Biophysica Acta (BBA) Gene Structure and Expression 1999;1444:171-90.

69. Dufès C, Uchegbu IF, Schätzlein AG. Dendrimers in gene delivery. Advanced Drug Delivery Reviews 2005;57:2177-202.

70. Gillies ER, Frechet JMJ. Dendrimers and dendritic polymers in drug delivery. Drug Discovery Today 2005;10:35-43.

71. Dennig J, Duncan E. Gene transfer into eukaryotic cells using activated polyamidoamine dendrimers. Reviews in Molecular Biotechnology 2002;90:339-47.

72. Majoros IJ, Myc A, Thomas T, Mehta CB, Baker Jr JR. PAMAM dendrimer-based multifunctional conjugate for cancer therapy: synthesis, characterization, and functionality. Biomacromolecules 2006;7:572-9.

73. Kihara F, Arima H, Tsutsumi T, Hirayama F, Uekama K. Effects of structure of polyamidoamine dendrimer on gene transfer efficiency of the dendrimer conjugate with α-cyclodextrin. Bioconjugate Chemistry 2002;13:1211-9.

74. Nam HY, Hahn HJ, Nam K, *et al*. Evaluation of generations 2, 3 and 4 arginine modified PAMAM dendrimers for gene delivery. International Journal of Pharmaceutics 2008;363:199-205.

75. Felgner PL, Gadek TR, Holm M, *et al*. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. Proceedings of the National Academy of Sciences 1987;84:7413.

76. Felgner PL, Ringold G. Cationic liposome-mediated transfection. Nature 1989;337:387-8.

77. Ilies M, Seitz WA, Balaban AT. Cationic lipids in gene delivery: principles, vector design and therapeutical applications. Current Pharmaceutical Design 2002;8:2441-73.

78. Hofland H, Shephard L, Sullivan SM. Formation of stable cationic lipid/DNA complexes for gene transfer. Proceedings of the National Academy of Sciences 1996;93:7305.

79. Pedroso de Lima MC, Simões S, Pires P, Faneca H, Düzgünes N. Cationic lipid-DNA complexes in gene delivery: from biophysics to biological applications. Advanced Drug Delivery Reviews 2001;47:277-94.

80. Hoekstra D, Rejman J, Wasungu L, Shi F, Zuhorn I. Gene delivery by cationic lipids: in and out of an endosome. Biochemical Society Transactions 2007;35:68-71.

81. Mevel M, Yaouanc JJ, Laurent P, *et al*. Cationic Lipids Based on Phosphonate and Phosphoramidate Chemistry: Synthesis and Application to Gene Therapy. Phosphorus, Sulfur, and Silicon 2008;183:460-8.

82. Donkuru MD, Badea I, Wettig S, Verrall R, Elsabahy M, Foldvari M. Advancing nonviral gene delivery: lipid-and surfactant-based nanoparticle design strategies. Nanomedicine 2010;5:1103-27.

83. Behr JP, Demeneix B, Loeffler JP, Perez-Mutul J. Efficient gene transfer into mammalian primary endocrine cells with lipopolyamine-coated DNA. Proceedings of the National Academy of Sciences 1989;86:6982.

84. Loeffler JP, Barthel F, Feltz P, Behr J, Sassone-Corsi P, Feltz A. Lipopolyamine-Mediated Transfection Allows Gene Expression Studies in Primary Neuronal Cells. Journal of Neurochemistry 1990;54:1812-5.

85. Behr J, Loeffler J. Lipopolyamines, their preparation and use. In: EP Patent 0,394,111; 1997.

86. Remy JS, Kichler A, Mordvinov V, Schuber F, Behr JP. Targeted gene transfer into hepatoma cells with lipopolyamine-condensed DNA particles presenting galactose ligands: a stage toward artificial viruses. Proceedings of the National Academy of Sciences 1995;92:1744-48.

87. Ruysschaert JM, Elouahabi A, Willeaume V, *et al*. A novel cationic amphiphile for transfection of mammalian cells. Biochemical and Biophysical Research Communications 1994;203:1622-8.

88. Vigneron JP, Oudrhiri N, Fauquet M, *et al*. Guanidinium-cholesterol cationic lipids: efficient vectors for the transfection of eukaryotic cells. Proceedings of the National Academy of Sciences 1996;93:9682-6.

89. Oudrhiri N, Vigneron JP, Peuchmaur M, Leclerc T, Lehn JM, Lehn P. Gene transfer by guanidinium-cholesterol cationic lipids into airway epithelial cells in vitro and in vivo. Proceedings of the National Academy of Sciences 1997;94:1651-6.

90. Luton D, Oudrhiri N, de Lagausie P, *et al*. Gene transfection into fetal sheep airways in utero using guanidinium-cholesterol cationic lipids. The Journal of Gene Medicine 2004;6:328-36.

91. Leventis R, Silvius JR. Interactions of mammalian cells with lipid dispersions containing novel metabolizable cationic amphiphiles. Biochimica et Biophysica Acta (BBA)-Biomembranes 1990;1023:124-32.

92. Felgner JH, Kumar R, Sridhar C, *et al*. Enhanced gene delivery and mechanism studies with a novel series of cationic lipid formulations. Journal of Biological Chemistry 1994;269:2550-61.

93. Felgner PL. Improvements in cationic liposomes for in vivo gene transfer. Human Gene Therapy 1996;7:1791-3.

94. Stephan DJ, Yang ZY, San H, *et al*. A new cationic liposome DNA complex enhances the efficiency of arterial gene transfer in vivo. Human Gene Therapy 1996;7:1803-12.

95. Banerjee R, Das PK, Srilakshmi GV, Chaudhuri A, Rao NM. Novel Series of Non-Glycerol-Based Cationic Transfection Lipids for Use in Liposomal Gene Delivery 1. Journal of Medicinal Chemistry 1999;42:4292-9.

96. Banerjee R, Mahidhar YV, Chaudhuri A, Gopal V, Rao NM. Design, synthesis, and transfection biology of novel cationic glycolipids for use in liposomal gene delivery. Journal of Medicinal Chemistry 2001;44:4176-85.

97. Pinnaduwage P, Schmitt L, Huang L. Use of a quaternary ammonium detergent in liposome mediated DNA transfection of mouse L-cells. Biochimica et Biophysica Acta (BBA)-Biomembranes 1989;985:33-7.

98. Menger FM, Littau C. Gemini-surfactants: synthesis and properties. Journal of the American Chemical Society 1991;113:1451-2.

99. Menger F, Littau C. Gemini surfactants: a new class of self-assembling molecules. Journal of the American Chemical Society 1993;115:10083-90.

100. Karaborni S, Esselink K, Hilbers P, *et al*. Simulating the self-assembly of gemini (dimeric) surfactants. Science 1994;266:254-6.

101. Hait S, Moulik S. Gemini surfactants: a distinct class of self-assembling molecules. CURRENT SCIENCE-BANGALORE- 2002;82:1101-11.

102. Kirby AJ, Camilleri P, Engberts JBFN, *et al*. Gemini surfactants: new synthetic vectors for gene transfection. Angewandte Chemie International Edition 2003;42:1448-57.

103. Wettig SD, Verrall RE, Foldvari M. Gemini surfactants: a new family of building blocks for non-viral gene delivery systems. Current Gene Therapy 2008;8:9-23.

104. Shukla D, Tyagi V. Cationic gemini surfactants: a review. Journal of oleo science 2006;55:381-90.

105. Badea I, Verrall R, Baca-Estrada M, *et al*. In vivo cutaneous interferon-γ gene delivery using novel dicationic (gemini) surfactant–plasmid complexes. The Journal of Gene Medicine 2005;7:1200-14.

106. Wettig SD, Badea I, Donkuru MD, Verrall RE, Foldvari M. Structural and transfection properties of amine-substituted gemini surfactant-based nanoparticles. The Journal of Gene Medicine 2007;9:649-58.

107. Wang C, Li X, Wettig SD, Badea I, Foldvari M, Verrall RE. Investigation of complexes formed by interaction of cationic gemini surfactants with deoxyribonucleic acid. Physical Chemistry Chemical Physics 2007;9:1616-28.

108. Singh J, Yang P, Michel D, E Verrall R, Foldvari M, Badea I. Amino Acid-Substituted Gemini Surfactant-Based Nanoparticles as Safe and Versatile Gene Delivery Agents. Current Drug Delivery 2011;8:299-306.

109. Yang P, Singh J, Wettig S, Foldvari M, Verrall RE, Badea I. Enhanced gene expression in epithelial cells transfected with amino acid-substituted gemini nanoparticles. European Journal of Pharmaceutics and Biopharmaceutics 2010;75:311-20.

110. 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;1:165-76.

111. Wettig SD, Wang C, Verrall RE, Foldvari M. Thermodynamic and aggregation properties of aza-and imino-substituted gemini surfactants designed for gene delivery. Physical Chemistry Chemical Physics 2006;9:871-7.

112. Ronsin G, Perrin C, Guédat P, Kremer A, Camilleri P, Kirby AJ. Novel spermine-based cationic gemini surfactants for gene delivery. Chemical Communications 2001:2234-5.

113. Fielden ML, Perrin C, Kremer A, *et al*. 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;268:1269-79.

114. Wasungu L, Scarzello M, van Dam G, *et al*. Transfection mediated by pH-sensitive sugar-based gemini surfactants; potential for in vivo gene therapy applications. Journal of Molecular Medicine 2006;84:774-84.

115. Gill D, Southern K, Mofford K, *et al*. A placebo-controlled study of liposome-mediated gene transfer to the nasal epithelium of patients with cystic fibrosis. Gene Therapy 1997;4:199-209.

116. Yoo GH, Hung MC, Lopez-Berestein G, *et al*. Phase I trial of intratumoral liposome E1A gene therapy in patients with recurrent breast and head and neck cancer. Clinical Cancer Research 2001;7:1237-45.

117. Hortobagyi GN, Ueno NT, Xia W, *et al*. Cationic liposome-mediated E1A gene transfer to human breast and ovarian cancer cells and its biologic effects: a phase I clinical trial. Journal of Clinical Oncology 2001;19:3422-33.

118. Madhusudan S, Tamir A, Bates N, *et al*. A multicenter phase I gene therapy clinical trial involving intraperitoneal administration of E1A-lipid complex in patients with recurrent epithelial ovarian cancer overexpressing HER-2/neu oncogene. Clinical Cancer Research 2004;10:2986-96.

119. Galanis E, Hersh E, Stopeck A, *et al*. Immunotherapy of advanced malignancy by direct gene transfer of an interleukin-2 DNA/DMRIE/DOPE lipid complex: phase I/II experience. Journal of Clinical Oncology 1999;17:3313-23.

120. Hoffman DMJ, Figlin RA. Intratumoral interleukin 2 for renal-cell carcinoma by direct gene transfer of a plasmid DNA/DMRIE/DOPE lipid complex. World Journal of Urology 2000;18:152-6.

121. Belldegrun A, Tso CL, Zisman A, *et al*. Interleukin 2 gene therapy for prostate cancer: phase I clinical trial and basic biology. Human Gene Therapy 2001;12:883-92.

122. Bergen M, Chen R, Gonzalez R. Efficacy and safety of HLA-B7/β-2 microglobulin plasmid DNA/lipid complex (Allovectin-7®) in patients with metastatic melanoma. Expert Opinion on Biological Therapy 2003;3:377-84.

123. Galanis E, Burch PA, Richardson RL, *et al*. Intratumoral administration of a 1, 2-dimyristyloxypropyl-3-dimethylhydroxyethyl ammonium bromide/dioleoylphosphatidylethanolamine formulation of the human interleukin-2 gene in the treatment of metastatic renal cell carcinoma. Cancer 2004;101:2557-66.

124. Gonzalez R, Hutchins L, Nemunaitis J, Atkins M, Schwarzenberger PO. Phase 2 trial of Allovectin-7 in advanced metastatic melanoma. Melanoma Research 2006;16:521-6.

125. Bedikian AY, Richards J, Kharkevitch D, Atkins MB, Whitman E, Gonzalez R. A phase 2 study of high-dose Allovectin-7 in patients with advanced metastatic melanoma. Melanoma Research 2010;20:218-26.

126. Soares HP, Lutzky J. Velimogene aliplasmid. Expert Opinion on Biological Therapy 2010;10:841-51.

127. de Lima M, Neves S, Filipe A, Duzgunes N, Simoes S. Cationic liposomes for gene delivery: from biophysics to biological applications. Current Medicinal Chemistry 2003;10:1221-31.

128. Anchordoquy TJ, Koe GS. Physical stability of nonviral plasmid-based therapeutics. Journal of Pharmaceutical Sciences 2000;89:289-96.

129. Barteau B, Chèvre R, Letrou-Bonneval E, Labas R, Lambert O, Pitard B. Physicochemical parameters of non-viral vectors that govern transfection efficiency. Current Gene Therapy 2008;8:313-23.

130. Almofti MR, Harashima H, Shinohara Y, Almofti A, Li W, Kiwada H. Lipoplex size determines lipofection efficiency with or without serum. Molecular Membrane Biology 2003;20:35-43.

131. Ross P, Hui S. Lipoplex size is a major determinant of in vitro lipofection efficiency. Gene Therapy 1999;6:651-9.

132. Walther W, Stein U, Voss C, Schmidt T, Schleef M, Schlag PM. Stability analysis for long-term storage of naked DNA: impact on nonviral in vivo gene transfer. Analytical Biochemistry 2003;318:230-5.

133. Molina MDC, Anchordoquy TJ. Formulation strategies to minimize oxidative damage in lyophilized lipid/DNA complexes during storage. Journal of Pharmaceutical Sciences 2008;97:5089-105.

134. Anchordoquy TJ. Degradation of lyophilized lipid/DNA complexes during storage: the role of lipid and reactive oxygen species. Biochimica et Biophysica Acta (BBA)-Biomembranes 2008;1778:2119-26.

135. Cartwright AC. Drug Substance and Drug Product Stability. In: International Pharmaceutical Product Registration:269-89.

136. Stadler J, Lemmens R, Nyhammar T. Plasmid DNA purification. The Journal of Gene Medicine 2004;6:S54-S66.

137. Prazeres DMF, Ferreira GNM, Monteiro GA, Cooney CL, Cabral J. Large-scale production of pharmaceutical-grade plasmid DNA for gene therapy: problems and bottlenecks. Trends in Biotechnology 1999;17:169-74.

138. Barry MA, Johnston SA. Biological features of genetic immunization. Vaccine 1997;15:788-91.

139. Remaut K, Sanders NN, Fayazpour F, Demeester J, De Smedt SC. Influence of plasmid DNA topology on the transfection properties of DOTAP/DOPE lipoplexes. Journal of Controlled Release 2006;115:335-43.

140. Lindahl T. Instability and decay of the primary structure of DNA. Nature 1993;362:709-15.

141. Evans RK, Xu Z, Bohannon KE, Wang B, Bruner MW, Volkin DB. Evaluation of degradation pathways for plasmid DNA in pharmaceutical formulations via accelerated stability studies. Journal of Pharmaceutical Sciences 2000;89:76-87.

142. Antimisiaris SG, Kallinteri P, Fatouros DG. Liposomes and drug delivery. In: Pharmaceutical Sciences Encyclopedia: Drug Discovery, Development, and Manufacturing 2008.

143. Lai E, van Zanten JH. Evidence of lipoplex dissociation in liquid formulations. Journal of Pharmaceutical Sciences 2002;91:1225-32.

144. Simberg D, Danino D, Talmon Y, *et al*. Phase behavior, DNA ordering, and size instability of cationic lipoplexes Relevance to optimal transfection activity. Journal of Biological Chemistry 2001;276:47453-9.

145. Hong K, Zheng W, Baker A, Papahadjopoulos D. Stabilization of cationic liposome-plasmid DNA complexes by polyamines and poly (ethylene glycol)-phospholipid conjugates for efficient in vivo gene delivery. FEBS letters 1997;400:233-7.

146. Wheeler J, Palmer L, Ossanlou M, *et al*. Stabilized plasmid-lipid particles: construction and characterization. Gene therapy 1999;6:271-81.

147. Harvie P, Wong FMP, Bally MB. Use of poly (ethylene glycol)–lipid conjugates to regulate the surface attributes and transfection activity of lipid–DNA particles. Journal of Pharmaceutical Sciences 2000;89:652-63.

148. Anchordoquy TJ, Girouard LG, Carpenter JF, Kroll DJ. Stability of lipid/DNA complexes during agitation and freeze–thawing. Journal of Pharmaceutical Sciences 1998;87:1046-51.

149. Zelphati O, Nguyen C, Ferrari M, Felgner J, Tsai Y, Felgner P. Stable and monodisperse lipoplex formulations for gene delivery. Gene therapy 1998;5:1272-82.

150. Hinrichs W, Manceņido F, Sanders N, *et al*. The choice of a suitable oligosaccharide to prevent aggregation of PEGylated nanoparticles during freeze thawing and freeze drying. International Journal of Pharmaceutics 2006;311:237-44.

151. Armstrong TKC, Girouard LG, Anchordoquy TJ. Effects of PEGylation on the preservation of cationic lipid/DNA complexes during freeze-thawing and lyophilization. Journal of Pharmaceutical Sciences 2002;91:2549-58.

152. Shikama K. Effect of freezing and thawing on the stability of double helix of DNA. 1965.

153. Aso Y, Yoshioka S. Effect of freezing rate on physical stability of lyophilized cationic liposomes. Chemical and Pharmaceutical Bulletin 2005;53:301-4.

154. Anchordoquy TJ, Allison SD, Girouard LG, Carson TK. Physical stabilization of DNA-based therapeutics. Drug discovery today 2001;6:463-70.

155. Allison SD, Anchordoquy TJ. Lyophilization of nonviral gene delivery systems. Methods in Molecular Medicine 2001;65:225-52.

156. Anchordoquy TJ, Carpenter JF, Kroll DJ. Maintenance of transfection rates and physical characterization of lipid/DNA complexes after freeze-drying and rehydration. Archives of Biochemistry and Biophysics 1997;348:199-206.

157. Allison SD, Anchordoquy TJ. Mechanisms of protection of cationic lipid-DNA complexes during lyophilization. Journal of Pharmaceutical Sciences 2000;89:682-91.

158. del Pozo-Rodríguez A, Solinís M, Gascón A, Pedraz J. Short-and long-term stability study of lyophilized solid lipid nanoparticles for gene therapy. European Journal of Pharmaceutics and Biopharmaceutics 2009;71:181-9.

159. Franks F. Freeze-drying of bioproducts: putting principles into practice. European Journal of Pharmaceutics and Biopharmaceutics 1998;45:221-9.

160. Akers MJ. Chapter 20: Freeze-dry (lyophilization) processing. In: Sterile Drug Products: Formulation, Packaging, Manufacturing, and Quality. First ed: Informa Healthcare; 2010:296-312.

161. Poxon SW, Hughes JA. The effect of lyophilization on plasmid DNA activity. Pharmaceutical Development and Technology 2000;5:115-22.

162. Montanari J, Roncaglia D, Lado L, Morilla M, Romero E. Avoiding failed reconstitution of ultradeformable liposomes upon dehydration. International Journal of Pharmaceutics 2009;372:184-90.

163. Chen C, Han D, Cai C, Tang X. An overview of liposome lyophilization and its future potential. Journal of Controlled Release 2010;142:299-311.

164. Sperling LH. Introduction to physical polymer science. USA: Wiley Online Library; 2006:349-426.

165. Her LM, Nail SL. Measurement of glass transition temperatures of freeze-concentrated solutes by differential scanning calorimetry. Pharmaceutical Research 1994;11:54-9.

166. Allison SD, Molina MC, Anchordoquy TJ. Stabilization of lipid/DNA complexes during the freezing step of the lyophilization process: the particle isolation hypothesis. Biochimica et Biophysica Acta (BBA)-Biomembranes 2000;1468:127-38.

167. Armstrong TK, Anchordoquy TJ. Immobilization of nonviral vectors during the freezing step of lyophilization. Journal of Pharmaceutical Sciences 2004;93:2698-709.

168. Nakagawa K, Hottot A, Vessot S, Andrieu J. Influence of controlled nucleation by ultrasounds on ice morphology of frozen formulations for pharmaceutical proteins freeze-drying. Chemical Engineering and Processing: Process Intensification 2006;45:783-91.

169. Hottot A, Vessot S, Andrieu J. Freeze drying of pharmaceuticals in vials: Influence of freezing protocol and sample configuration on ice morphology and freeze-dried cake texture. Chemical Engineering and Processing: Process Intensification 2007;46:666-74.

170. Howard MD, Lu X, Jay M, Dziubla TD. Optimization of the lyophilization process for long-term stability of solid-lipid nanoparticles. Drug Development and Industrial Pharmacy 2012:1-10.

171. Kuo JS, Hwang R. Preparation of DNA dry powder for non-viral gene delivery by spray-freeze drying: effect of protective agents (polyethyleneimine and sugars) on the stability of DNA. Journal of Pharmacy and Pharmacology 2004;56:27-33.

172. Vighi E, Ruozi B, Montanari M, Battini R, Leo E. Re-dispersible cationic solid lipid nanoparticles (SLNs) freeze-dried without cryoprotectors: characterization and ability to bind the pEGFP-plasmid. European Journal of Pharmaceutics and Biopharmaceutics 2007;67:320-8.

173. Chacon M, Molpeceres J, Berges L, Guzman M, Aberturas M. Stability and freeze-drying of cyclosporine loaded poly (, lactide-glycolide) carriers. European Journal of Pharmaceutical Sciences 1999;8:99-107.

174. Abdelwahed W, Degobert G, Fessi H. A pilot study of freeze drying of poly (epsilon-caprolactone) nanocapsules stabilized by poly (vinyl alcohol): formulation and process optimization. International Journal of Pharmaceutics 2006;309:178-88.

175. Saez A, Guzman M, Molpeceres J, Aberturas M. Freeze-drying of polycaprolactone and poly (-lactic-glycolic) nanoparticles induce minor particle size changes affecting the oral pharmacokinetics of loaded drugs. European Journal of Pharmaceutics and Biopharmaceutics 2000;50:379-87.

176. Arakawa T, Timasheff SN. Stabilization of protein structure by sugars. Biochemistry 1982;21:6536-44.

177. Randolph TW. Phase separation of excipients during lyophilization: effects on protein stability. Journal of Pharmaceutical Sciences 1997;86:1198-203.

178. Crowe JH, Carpenter JF, Crowe LM. The role of vitrification in anhydrobiosis. Annual Review of Physiology 1998;60:73-103.

179. Wolfe J, Bryant G. Freezing, drying, and/or vitrification of membrane-solute-water systems. Cryobiology 1999;39:103-29.

180. Roughton BC, Topp E, Camarda KV. Use of glass transitions in carbohydrate excipient design for lyophilized protein formulations. Computers & Chemical Engineering 2011.

181. Levine H, Slade L. Principles of "cryostabilization" technology from structure/property relationships of carbohydrate/water systems–a review. Cryo-letters 1988;9:21-63.

182. Ohtake S, Schebor C, Palecek SP, de Pablo JJ. Phase behavior of freeze-dried phospholipid–cholesterol mixtures stabilized with trehalose. Biochimica et Biophysica Acta (BBA)-Biomembranes 2005;1713:57-64.

183. Kasper JC, Schaffert D, Ogris M, Wagner E, Friess W. Development of a lyophilized plasmid/LPEI polyplex formulation with long-term stability A step closer from promising technology to application. Journal of Controlled Release 2011.

184. Molina, M. d.C., Armstrong TK, Zhang Y, Patel MM, Lentz YK, Anchordoquy TJ. The stability of lyophilized lipid/DNA complexes during prolonged storage. Journal of Pharmaceutical Sciences 2004;93:2259-73.

185. Crowe JH, Leslie SB, Crowe LM. Is vitrification sufficient to preserve liposomes during freeze-drying? Cryobiology 1994;31:355-66.

186. Bonelli P, Schebor C, Cukierman Anal, Buera MP, Chirife J. Residual Moisture Content as Related to Collapse of Freeze-dried Sugar Matrices. Journal of Food Science 1997;62:693-5.

187. Maitani Y, Aso Y, Yamada A, Yoshioka S. Effect of sugars on storage stability of lyophilized liposome/DNA complexes with high transfection efficiency. International Journal of Pharmaceutics 2008;356:69-75.

188. Crowe J, Crowe L, Carpenter J. Preserving dry biomaterials: the water replacement hypothesis, part 1. BIOPHARM-EUGENE 1993;6:28-37.

189. Crowe J, Crowe L, Carpenter J. Preserving dry biomaterials: The water replacement hypothesis, part 2. BIOPHARM-EUGENE 1993;6:40-3.

190. Clement J, Kiefer K, Kimpfler A, Garidel P, Peschka-Süss R. Large-scale production of lipoplexes with long shelf-life. European Journal of Pharmaceutics and Biopharmaceutics 2005;59:35-43.

191. Yadava P, Gibbs M, Castro C, Hughes JA. Effect of Lyophilization and Freeze-thawing on the Stability of siRNA-liposome Complexes. AAPS PharmSciTech 2008;9:335-41.

192. Yu J, Anchordoquy TJ. Effects of moisture content on the storage stability of dried lipoplex formulations. Journal of Pharmaceutical Sciences 2009;98:3278-89.

193. Allison SD, Anchordoquy TJ. Maintenance of nonviral vector particle size during the freezing step of the lyophilization process is insufficient for preservation of activity: insight from other structural indicators. Journal of Pharmaceutical Sciences 2001;90:1445-55.

194. Li B, Li S, Tan Y, *et al*. Lyophilization of cationic lipid–protamine–DNA (LPD) complexes. Journal of Pharmaceutical Sciences 2000;89:355-64.

195. Branch SK. Guidelines from the international conference on harmonisation (ICH). Journal of Pharmaceutical and Biomedical Analysis 2005;38:798-805.

196. Alsante KM, Ando A, Brown R, *et al*. The role of degradant profiling in active pharmaceutical ingredients and drug products. Advanced Drug Delivery Reviews 2007;59:29-37.

197. El-Aneed A, Cohen A, Banoub J. Mass spectrometry, review of the basics: Electrospray, MALDI, and commonly used mass analyzers. Applied Spectroscopy Reviews 2009;44:210-30.

198. Hopfgartner G, Bourgogne E. Quantitative high-throughput analysis of drugs in biological matrices by mass spectrometry. Mass Spectrometry Reviews 2003;22:195-214.

199. Chen H, Talaty NN, Takáts Z, Cooks RG. Desorption electrospray ionization mass spectrometry for high-throughput analysis of pharmaceutical samples in the ambient environment. Analytical Chemistry 2005;77:6915-27.

200. Feng WY, Chan KK, Covey JM. Electrospray LC-MS/MS quantitation, stability, and preliminary pharmacokinetics of bradykinin antagonist polypeptide B201 (NSC 710295) in the mouse. Journal of Pharmaceutical and Biomedical Analysis 2002;28:601-12.

201. Shockcor JP, Unger SE, Wilson ID, Foxall PJD, Nicholson JK, Lindon JC. Combined HPLC, NMR spectroscopy, and ion-trap mass spectrometry with application to the detection and characterization of xenobiotic and endogenous metabolites in human urine. Analytical Chemistry 1996;68:4431-5.

202. Games DE, Hirter P, Kuhnz W, Lewis E, Weerasinghe N, Westwood SA. Studies of combined liquid chromatography-mass spectrometry with a moving-belt interface. Journal of Chromatography A 1981;203:131-8.

203. Blakley C, Vestal M. Thermospray interface for liquid chromatography/mass spectrometry. Analytical Chemistry 1983;55:750-4.

204. Caprioli RM. Continuous-flow fast atom bombardment mass spectrometry. Analytical Chemistry 1990;62:477-85.

205. Cappiello A, Bruner F. Micro flow rate particle beam interface for capillary liquid chromatography/mass spectrometry. Analytical Chemistry 1993;65:1281-7.

206. Ermer J, Vogel M. Applications of hyphenated LC-MS techniques in pharmaceutical analysis. Biomedical Chromatography 2000;14:373-83.

207. Zhao YZ, van Breemen RB, Nikolic D, *et al*. Screening solution-phase combinatorial libraries using pulsed ultrafiltration/electrospray mass spectrometry. Journal of Medicinal Chemistry 1997;40:4006-12.

208. Chu YH, Dunayevskiy YM, Kirby DP, Vouros P, Karger BL. Affinity capillary electrophoresis-mass spectrometry for screening combinatorial libraries. Journal of the American Chemical Society 1996;118:7827-35.

209. Zhang N, Fountain ST, Bi H, Rossi DT. Quantification and rapid metabolite identification in drug discovery using API time-of-flight LC/MS. Analytical Chemistry 2000;72:800-6.

210. Yu X, Cui D, Davis MR. Identification of in vitro metabolites of indinavir by "intelligent automated LC-MS/MS"(INTAMS) utilizing triple quadrupole tandem mass spectrometry. Journal of the American Society for Mass Spectrometry 1999;10:175-83.

211. Ma Y, Li P, Chen D, Fang T, Li H, Su W. LC/MS/MS quantitation assay for pharmacokinetics of naringenin and double peaks phenomenon in rats plasma. International Journal of Pharmaceutics 2006;307:292-9.

212. Lee H, Shen S, Grinberg N. Identification and control of impurities for drug substance development using LC/MS and GC/MS. Journal of Liquid Chromatography & Related Technologies 2008;31:2235-52.

213. Ermer J. The use of hyphenated LC-MS technique for characterisation of impurity profiles during drug development. Journal of Pharmaceutical and Biomedical Analysis 1998;18:707-14.

214. Wu Y. The use of liquid chromatography–mass spectrometry for the identification of drug degradation products in pharmaceutical formulations. Biomedical Chromatography 2000;14:384-96.

215. Gehrig PM, Hunziker PE, Zahariev S, Pongor S. Fragmentation pathways of N^G methylated and unmodified arginine residues in peptides studied by ESI-MS/MS and MALDI-MS. Journal of the American Society for Mass Spectrometry 2004;15:142-9.

216. Ye Y, Cao LF, Niu MY, Liao XC, Zhao YF. ESI-MS fragmentation pathways of *N*-methylpyrrole polyamide/peptide conjugates. International Journal of Mass Spectrometry 2006;253:141-5.

217. El-Aneed A, Banoub J. Elucidation of the molecular structure of lipid A isolated from both a rough mutant and a wild strain of Aeromonas salmonicida lipopolysaccharides using electrospray ionization quadrupole time-of-flight tandem mass spectrometry. Rapid Communications in Mass Spectrometry 2005;19:1683-95.

218. Cui M, Song F, Zhou Y, Liu Z, Liu S. Rapid identification of saponins in plant extracts by electrospray ionization multi-stage tandem mass spectrometry and liquid chromatography/tandem mass spectrometry. Rapid Communications in Mass Spectrometry 2000;14:1280-6.

219. Jin M, Yang Y, Su B, Ren Q. Rapid quantification and characterization of soyasaponins by high-performance liquid chromatography coupled with electrospray mass spectrometry. Journal of Chromatography A 2006;1108:31-7.

220. Korfmacher WA. Using mass spectrometry for drug metabolism studies: CRC Press; 2009.

221. Baillie TA. Advances in the application of mass spectrometry to studies of drug metabolism, pharmacokinetics and toxicology. International Journal of Mass Spectrometry and Ion Processes 1992;118:289-314.

222. Kostiainen R, Kotiaho T, Kuuranne T, Auriola S. Liquid chromatography/atmospheric pressure ionization–mass spectrometry in drug metabolism studies. Journal of Mass Spectrometry 2003;38:357-72.

223. Hopfgartner G, Husser C, Zell M. Rapid screening and characterization of drug metabolites using a new quadrupole–linear ion trap mass spectrometer. Journal of Mass Spectrometry 2003;38:138-50.

224. Holčapek M, Kolářová L, Nobilis M. High-performance liquid chromatography–tandem mass spectrometry in the identification and determination of phase I and phase II drug metabolites. Analytical and BioAnalytical Chemistry 2008;391:59-78.

225. Ceglarek U, Leichtle A, Brügel M, *et al*. Challenges and developments in tandem mass spectrometry based clinical metabolomics. Molecular and Cellular Endocrinology 2009;301:266-71.

226. Poon G, Walter B, Lønning P, Horton M, McCague R. Identification of tamoxifen metabolites in human Hep G2 cell line, human liver homogenate, and patients on long-term therapy for breast cancer. Drug Metabolism and Disposition 1995;23:377-82.

227. Hodel E, Zanolari B, Mercier T, *et al*. A single LC-tandem mass spectrometry method for the simultaneous determination of 14 antimalarial drugs and their metabolites in human plasma. Journal of Chromatography B 2009;877:867-86.

228. Lim C, Yuan ZX, Jones R, White I, Smith L. Identification and mechanism of formation of potentially genotoxic metabolites of tamoxifen: study by LC-MS/MS. Journal of Pharmaceutical and Biomedical Analysis 1997;15:1335-42.

229. Görög S, Babjak M, Balogh G, *et al*. Drug impurity profiling strategies. Talanta 1997;44:1517-26.

230. Haskins NJ, Eckers C, Organ AJ, Dunk MF, Winger BE. The use of electrospray ionization with Fourier transform ion cyclotron resonance mass spectrometry in the analysis of trace impurities in a drug substance. Rapid Communications in Mass Spectrometry 1995;9:1027-30.

231. Volk KJ, Hill SE, H Kerns E, Lee MS. Profiling degradants of paclitaxel using liquid chromatography-mass spectrometry and liquid chromatography-tandem mass spectrometry substructural techniques. Journal of Chromatography B: Biomedical Sciences and Applications 1997;696:99-115.

232. Visky D, Jimidar I, Van Ael W, Vennekens T, Redlich D, De Smet M. Capillary electrophoresis-mass spectrometry in impurity profiling of pharmaceutical products. Electrophoresis 2005;26:1541-9.

233. Galmier MJ, Bouchon B, Madelmont JC, Mercier F, Pilotaz F, Lartigue C. Identification of degradation products of diclofenac by electrospray ion trap mass spectrometry. Journal of Pharmaceutical and Biomedical Analysis 2005;38:790-6.

234. Novak T, Helmy R, Santos I. Liquid chromatography-mass spectrometry using the hydrogen/deuterium exchange reaction as a tool for impurity identification in pharmaceutical process development. Journal of Chromatography B 2005;825:161-8.

235. Shah RP, Kumar V, Singh S. Liquid chromatography/mass spectrometric studies on atorvastatin and its stress degradation products. Rapid Communications in Mass Spectrometry 2008;22:613-22.

236. Reddy GVR, Kumar AP, Reddy BV, Kumar P, Gauttam HD. Identification of degradation products in Aripiprazole tablets by LC-QToF mass spectrometry. European Journal of Chemistry 2010;1:20-7.

Chapter 3

Lyophilization of gemini surfactant-based lipoplexes: influence of stabilizing agents on the long term stability – pilot study

Waleed Mohammed-Saeid[1], Ronald Verrall[2], Anas El-Aneed[1], Ildiko Badea[1*]

[1] Drug Design and Discovery Research Group, College of Pharmacy and Nutrition, University of Saskatchewan

[2] Department of Chemistry, University of Saskatchewan

* Correspondence: ildiko.badea@usask.ca
Drug Design and Discovery Research Group, College of Pharmacy and Nutrition,
University of Saskatchewan, 110 Science Place, Saskatoon, S7N 5C9,
Canada

3.1. Abstract

Purpose

Clinical applications of cationic lipid/DNA lipoplexes are restricted by their low physical stability in aqueous formulations. In this work, we describe the effects of different cryoprotectant agents on the physiochemical properties (particle size and surface charge density) and *in vitro* transfection of gemini surfactant-based lipoplexes. Additionally, we investigated the ability of these agents to maintain the physical stability and biological activity of the lipoplexes during the freeze-drying cycle.

Methods

Plasmid DNA, diquaternary ammonium gemini surfactant and helper lipid DOPE were used to prepare [P/G/L] lipoplexes. A series of 35 formulations were prepared using different classes of cryoprotectant agent (sucrose, trehalose, lactose, polysorbate 80, PEG 1450, PEG 8000, glycerin, and their combinations) The influences of these agents on the physiochemical properties (particle size and zeta potential) were evaluated. Lipoplexes formulated with the cryoprotectant were subjected to lyophilization/rehydration evaluations. The appearance of the lyophilized cake and the clarity of the formulations after rehydration were evaluated. The lyophilized formulations were evaluated for transfection activity (ELISA) and cellular toxicity (MTT assay). The stability of the formulations was evaluated in a three-month stability study at two storage temperatures (25 ˚C and 40 ˚C).

Results

The physiochemical properties of gemini surfactant-based lipoplexes were altered based on the stabilizing agents used to prepare the lipoplexes. Disaccharide sugars sucrose and trehalose and their combination with glycerin provided the most efficient cryoprotectant effect based on the

ability to physically stabilize the lipoplexes during the lyophilization process. The transfection efficiency of most formulations was lost after lyophilization and rehydration. On the other hand, the transfection activity of lyophilized lipoplexes prepared in 10% trehalose significantly increased upon lyophilization.

Conclusion

A wide variety of excipients were evaluated as cryoprotective agents to enhance the physical stability of [P/G/L] lipoplexes during the freeze-drying cycle. Disaccharide sugars sucrose and trehalose were the most efficient cryoprotectant agents to maintain the physiochemical characteristics of [P/G/L] lipoplexes after lyophilization and rehydration. However, stabilizing the particle size and positive zeta potential was not sufficient to preserve the transfection activity during freeze-drying as the activity dropped significantly in most of the formulations upon lyophilization/rehydration cycle. Interestingly, the use of trehalose as a cryoprotectant caused a significant enhancement in gene expression after freeze drying. In this respect, optimization of the lyophilization process and the use of appropriate stabilizing agents could improve the long-term stability of gemini surfactant-based lipoplexes.

3.2. Introduction

Chemically mediated gene delivery systems have been proposed as a safe and versatile alternative to viral vectors for gene therapy.[1,2] Among chemically mediated-vectors, cationic lipid based systems have demonstrated the highest gene expression activity with relatively low toxicity both *in vivo* and *in vitro*.[3,4] The promising results have prompted the use of these unique systems in human clinical trials. By the end of 2011, lipoplex-based gene therapy have been employed in 110 clinical trials around the world (6 % of all approved trials) with a majority in phase I or II trials.[5]

The basic structure of all cationic lipids consists of polar head group(s) attached by linker (spacer) chain to hydrophobic groups (which may be single or double fatty acids, alkyl or cholesterol moieties).[4] The transfection efficiency of cationic lipids depends upon the following properties: 1) the ability of the cationic lipid to condense and encapsulate DNA by electrostatic interaction, forming a supramolecular complex known as a lipoplex with certain size and morphology, 2) cationic lipid/DNA lipoplexes must have an overall net positive charge that allows the association of the lipoplex with the negatively charged cell membrane promoting cellular uptake, and 3) the fusogenic property of cationic lipids as a function of the hydrophobic alkyl tails promotes the escape of the entrapped DNA to the nucleus.[6,7]

One specific group of cationic lipids that has demonstrated efficient transfection activity is the gemini surfactant family.[8,9] Gemini surfactants [Figure 3.1] are dimeric surfactants with characteristically low surface tension activity primarily used for material sciences.[10,11] In recent years, gemini surfactants have been investigated extensively as non-viral gene delivery carriers for both *in vitro* and *in vivo* applications.[12-14]

Notwithstanding their successful applications as gene delivery systems, two major difficulties are still limiting their broad clinical use: 1) the low transfection efficiency compared to viral-based vectors and 2) the instability of lipid-based gene delivery vectors in aqueous pharmaceutical dosage forms.[15,16] Extensive work has been conducted toward improving the transfection efficiency of cationic lipoplex-based vectors by modifying the chemical structure of the cationic lipid.[17-19] However, little concern has been given to the physical and chemical stability from a pharmaceutical standpoint. The stability of a non-viral gene delivery system is complicated as it involves the physical stability of DNA-carrier complexes, conformational structure of the genetic material and the chemical stability of the carrier.[20-22]

Lyophilization (freeze-drying) has been employed as a practical technique to produce non-viral vectors with long-term stability.[23-25] However, the lyophilization process includes three stress steps that could destabilize the lipoplexes: freezing, drying (dehydration) and rehydration.[26] The optimization of the freeze-drying protocol and incorporation of certain stabilizing agents, known as cryo- or lyo-protectant agents, have proven to improve the stability of cationic lipid-based DNA formulations.[26,27] Different classes of stabilizer have been used for the preparation of lyophilized non-viral gene delivery systems: monosaccharaides (glucose), disaccharides (sucrose, trehalose), oligosaccharides (inulin) and polymers (dextran, povidone, polyethylene glycol).[28-30] Different mechanisms have been proposed to explain the protective action of the cryoprotectants in colloidal systems and proteins: preferential exclusion, vitrification, and particle isolation hypothesis.[31-35]

It has been reported that freeze-drying cycles induced changes in the physiochemical properties (particle size and surface charge density) of lipoplex-mediated gene delivery vectors even when a cryoprotectant agent was used.[36-39] The alteration of physiochemical properties of

lipoplexes is usually associated with changes in transfection efficiency. Although, many studies have been carried out to explore the effects of stabilizing agents and freeze-drying cycles on the physiochemical properties and transfection activity of cationic lipid-DNA vectors, all of them have employed mono-cationic lipids (e.g., DOTAP, DC-Chol, DMRIE).[23,37,39,40] From this perspective, the aims of this work were 1) to evaluate the effects of several stabilizing agents on the physiochemical properties (i.e., particle size and surface charge density) of di-cationic gemini surfactant-based lipoplexes 2) to investigate the ability of these agents to physically stabilize the lipoplexes during freeze-drying cycle and 3) to observe the influence of the freeze-drying cycles on the properties and transfection activity of lipoplexes.

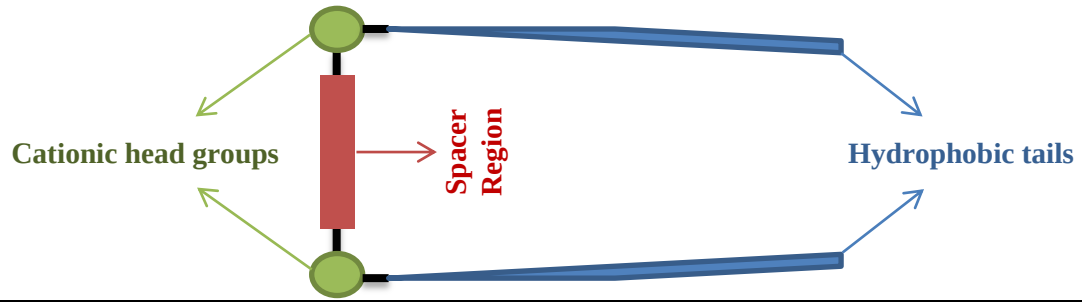


Figure 3.1: General structure of cationic gemini surfactant.

3.3. Materials and Methods

3.3.1. Materials

Two plasmids were used in this work. The pG.td.Tomato, encoding for tomato red protein, was obtained from Clontech Laboratories, Inc. (Mountain View, CA, USA). This plasmid was used for formulation development and physiochemical characterization purposes. The plasmid (pGThCMV.IFN-GFP)[12], encoding for murine interferon gamma (IFN-γ) and green fluorescent protein (GFP), was used for *in vitro* transfection evaluations. Plasmids were amplified and purified using QIAGEN Plasmid Giga Kit (Mississauga, ON, Canada) following the manufacturer's protocols. The synthesis and characterization of the gemini surfactants used in this study have been previously described.[14,41] Aqueous solutions of 3 mM gemini surfactant were used to prepare lipoplexes. Helper lipid 1,2 dioleyl-*sn*-glycero-phosphatidylethanolamine (DOPE) (Avanti Polar Lipids, Alabaster, AL) was co-formulated in all formulations. Stabilizer excipients (analytical grade) sucrose and trehalose were obtained from Sigma Aldrich (Oakville, ON, Canada), lactose, glucose, polysorbate 80 (tween 80) and glycerin from Spectrum Chemical (Gardena, CA, USA) and polyethylene glycol (PEG) from Union Carbide Corporation (Houston, TX, USA). All excipients were used without further purification. Chemical solvents (GS grade) were obtained from EMD Chemicals Inc. (Gibbstown, NJ, USA).

3.3.2. Preparation of lipoplexes

Lipoplexes were formulated using a pDNA to gemini surfactant charge ratio of 1:10 in the presence of DOPE as co-lipid creating plasmid/gemini surfactant/lipid lipoplexes [P/G/L]. DOPE lipid vesicles were prepared using a sonication technique as described previously.[12] The DOPE film was dispersed in the specific stabilizing solution at 1 mM DOPE final concentration and filtered through Acrodisc® 0.45 μm syringe filters (Pall Gelman, Ann Arbor, MI). The

stabilizing solutions were prepared by dissolving the stabilizing agent [Table 3.1] in nuclease-free ultrapure water (Gibco, Invitrogen Corporation, Grand Island, NY, USA) on a weight/weight percentage (w/w%) and the pH was adjusted with NaOH solution to 9.The plasmid/gemini surfactant [P/G] lipoplexes were prepared by mixing an aliquot of 200 µg pDNA aqueous solution with an appropriate amount of 3 mM gemini surfactant solution to obtain the 1:10 charge ratio and incubated at room temperature for 20 minutes. The [P/G/L] systems were prepared by mixing [P/G] lipoplexes with the DOPE vesicles at gemini surfactant to DOPE molar ratio of 1:10 and incubated at room temperature for 20 minutes.

Table 3.1: Examples of stabilizing solutions used for preparing DOPE lipid and the role of each ingredient

ID	**Solution used to re-suspend DOPE film (% w/w)**	**Role of ingredient**
1	9.25 % Sucrose	Sucrose: isotonic agent, cryoprotectant
2	10% Trehalose	Trehalose: cryoprotectant sugar
3	10% Glucose	Glucose: cryoprotectant sugar
4	10% Lactose	Lactose: cryoprotectant sugar
5	5% PEG 1450	PEG 1450: cryoprotectant polymer
6	5% PEG 8000	PEG 8000: cryoprotectant polymer
7	1% Tween 80	Tween 80: surfactant polymer
8	9.25% Sucrose + 1% Glycerin	Sucrose: cryoprotectant sugar Glycerin: antifreeze cryoprotectant
9	9.25% Sucrose + 0.5% Glycerin	Sucrose: cryoprotectant sugar Glycerin: antifreeze cryoprotectant
10	10% Trehalose+ 1% Glycerin	Trehalose: cryoprotectant sugar Glycerin: antifreeze cryoprotectant
11	10% Trehalose+ 0.5% Glycerin	Trehalose: cryoprotectant sugar Glycerin: antifreeze cryoprotectant

3.3.3. Extraction of pDNA from lipoplexes

Several methods were evaluated to extract pDNA from the [P/G/L] and [P/G] systems. A brief description of each method is shown in Table 3.2. Gel electrophoresis was used to validate the efficiency of the method to extract pDNA from the lipoplexes as follows: the extracted portion (containing 0.4 µg pDNA) was tested in 1% agarose gel stained with ethidium bromide-EtBr (0.01%) using a Bio-Rad PowerPac HC electrophoresis apparatus (Biorad, Mississagua, ON, Canada) in tris-acetate-EDTA (TAE) buffer at 100 V for 45 minutes. EtBr was visualized by UV fluorescence using an AlphaImager™ (Alpha Innotech, San Leandro, CA, USA).

Table 3.2: Summary of methods used for extraction of pDNA from freshly prepared [P/G/L] and [P/G] lipoplexes.

Method ID	Description
M-1[42]	1. Freshly prepared [P/G/L] or [P/G] systems containing 15 µg DNA were mixed with sodium dodecyl sulfate (SDS) solution to a final concentration of SDS of 25 mM 2. The resulting solution was incubated in water bath at 75˚C for 15 min then allowed to cool to room temperature 3. Aliquots of solution containing 0.4 µg were used in gel electrophoresis.
M-2	1. The same steps 1 and 2 from method M-1 were performed 2. The SDS solution containing the pDNA was mixed with isopropyl alcohol (0.7 volumes), and centrifuged at 14,000 rpm at 4˚C for 15 min 3. The supernatant was decanted and the precipitated pDNA pellet was washed with 70% ethanol and centrifuged at 14,000 rpm at 4˚C for 15 min 4. The supernatant was decanted, and the pellet was reconstituted in nuclease-free ultrapure water (40 µL) 5. Aliquots of solution containing 0.4 µg were used in gel electrophoresis
M-3	1. Similar to M-2 but the samples were incubated at room temperature for 30 min with continuous mixing instead of thermal treatment at 75˚C for 15 min
M-4	1. 1 mL of freshly prepared [P/G/L] system containing 15 µg DNA was mixed with an equal volume of 1% Triton X-1 solution (Spectrum Chemical, Gardena, CA, USA) and incubated at room temperature for 15 min 2. The resulting solution was mixed with isopropyl alcohol (0.7 volumes), centrifuged at 14,000 rpm and 4˚C for 15 min 3. The supernatant was decanted and the precipitated pDNA pellet was washed with 70% ethanol and centrifuged at 14,000 rpm and 4˚C for 15 min 4. The supernatant was decanted, the pellet was reconstituted in nuclease-free ultrapure water (40 µL) 5. Aliquots of solution containing 0.4 µg were used in gel electrophoresis
M-5	1. pDNA was isolated and extracted following the QIAGEN® plasmid purification method 2. An a liquot of extracted solution containing 0.4 µg was used in gel electrophoresis

3.3.4. Lyophilization of the formulations

Volumes of 3 mL of different [P/G/L] formulations containing a total of 22.2 µg pDNA were transferred to 15 mL polypropylene copolymer centrifuge tubes (VWR International, Mississauga, ON, Canada). The top of the tubes were covered with delicate-task wipes (Kimtech Science® Kimwipes®, VWR International, Mississauga, ON, Canada) and stored at – 80 ˚C overnight. Tubes containing the formulations were then placed in the freeze dryer (Lyph-Lock, 6 liter bench freeze-dryer, Labconco, Kansas City, MO, USA). Samples were lyophilized for 48 hours. After the freeze-drying cycle, the tubes were flushed with nitrogen gas (Praxair Canada Inc., Mississauga, ON, Canada) and capped.

Four lyophilized formulations (F_S, F_{SG}, F_T, and F_{TG}) were selected for the pilot stability study to evaluate the efficiency of three stabilizing agents (sucrose, trehalose and glycerin) to preserve the physiochemical properties and biological activity of lyophilized lipoplexes [Table 3.3]. In addition, formulation F_S', containing only the pDNA/gemini [P/G] surfactant lipoplexes, was lyophilized without including the DOPE vesicles or any stabilizing agent.

Table 3.3: Preparation methods for the formulations used in the pilot accelerated stability study

Formulation	**Stabilizing solution**
F_S	[P/G/L] system was prepared in 9.25% sucrose, then was lyophilized for 48 hours
F_{SG}	[P/G/L] system was prepared in 9.25% sucrose + 1% glycerin, then was lyophilized for 48 hours
F_T	[P/G/L] system was prepared in 10% trehalose, then was lyophilized for 48 hours
F_{TG}	[P/G/L] system was prepared in 10% trehalose + 1% glycerin, then was lyophilized for 48 hours
F_S'	[P/G] lipoplex was prepared and lyophilized for 48 hours, the lyophilized [P/G] lipoplex was rehydrated in DOPE vesicles prepared in 9.25% sucrose

3.3.5. Rehydration of the lyophilized formulations

Lyophilized formulations containing the [P/G/L] system and the excipient were rehydrated to a final volume of 3 mL with ultrapure water (Gibco, Invitrogen Corporation, Grand Island, NY, USA) and incubated for 30 minutes at room temperature prior to the physiochemical and biological evaluation. Lyophilized formulation containing the [P/G] lipoplex (i.e., formulation $F_{S'}$) was rehydrated, firstly, in 222 µL of ultrapure water and incubated for 30 minutes at room temperature. After 30 minutes, freshly prepared DOPE vesicles in 9.25% sucrose were added to the rehydrated lipoplexes to a final volume of 3 mL. Appropriate volumes of rehydrated formulation, containing 0.2 µg pDNA, were used for *in vitro* transfection evaluation and physiochemical characterization measurements.

3.3.6. Stability study

For the accelerated stability study, the lyophilized formulations were stored in stability chambers at two storage conditions: 1) 25 ˚C and 75% relative humidity (RH) (Sanyo growth cabinet MLR-350, Sanyo, Osaka, Japan) and 2) 40 ˚C and 75% RH (Caron environmental test chambers 6010, Caron, Marietta, OH, USA) for three months. Sampling points were determined at one week, and one, two and three month storage periods. Formulations were prepared and analyzed in triplicate (n=3).

3.3.7. Size and ζ-potential measurements

Fresh and rehydrated formulations (800 µL) were transferred into a special cuvette (DTS1061, Malvern Instruments, Worcestershire, UK) for size distribution and zeta-potential measurements using a Zetasizer Nano ZS instrument (Malvern Instruments, Worcestershire, UK). Each sample was measured four times, and the results were expressed as the average ±

standard deviation (SD) of three samples (n=3) with a corresponding polydispersity index (PDI) value.

3.3.8. Cell culture and *in vitro* transfection

COS-7 African green monkey kidney fibroblasts cell line (ATCC, CRL-1651) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% antibiotic and incubated at 37 ˚C with 5% CO_2. On the day before transfection, the cells were seeded in 96-well tissue culture plates (Falcon, BD Mississauga, ON, Canada) at a density of 1.5×10^4 cells/well. One hour prior to transfection, the supplemented DMEM was replaced with DMEM. The cells were transfected with 0.2 µg pGThCMV.IFN-GFP plasmid/well in quadruplicate. Lipofectamine Plus reagent (Invitrogen Life Technologies) was used as a positive control according to the manufacturer's protocol with 0.2 µg pDNA/well in quadruplicate. The 96-well tissue culture plates were then incubated at 37 ˚C in CO_2 for five hours. The transfection agents were removed and replaced with supplemented DMEM. Supernatants containing the secreted IFN-γ were collected at 24, 48 and 72 hours and replaced with fresh supplemented DMEM. The collected supernatants were stored at -80 ˚C.

3.3.9. Cell toxicity assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to evaluate the effect of lyophilization and storage conditions on the cellular toxicity of [P/G/L] systems in COS-7 cell line. A sterile solution of 4 mg/mL of MTT (Invitrogen Corporation, Grand Island, NY, USA) in PBS buffer was prepared. The COS-7 cell lines were seeded on 96-well plate and transfected with fresh and lyophilized formulations (as described above). After 72 hour, the cell lines were evaluated for the cell toxicity. The supplemented DMEM was removed from the well and replaced with 0.45 mg/mL MTT in supplemented DMEM and incubated at 37˚

C in CO_2 for 3 hours. The supernatant was removed, and each well washed with PBS. The formed purple formazan crystal was dissolved in dimethyl sulfoxide (spectroscopy grade, Sigma-Aldrich, Oakville, ON, Canada). Plates were incubated for 10 minutes at 37˚C. Absorbance was measured at 550 nm using a BioTek microplate reader (Bio-Tek Instruments, VT, USA). The cellular toxicity is expressed as a percentage of the non-transfected control cells ± SD.

3.3.10. Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was performed using flat bottom 96-well plates (Immulon 2, Greiner Labortechnik, Frickenhausen, Germany) following the BD Pharmingen protocol and as described earlier[12]. The concentration of expressed IFNγ was calculated from a standard IFNγ curve using recombinant mouse IFN-γ standard (BD Pharmingen, BD Biosciences).

3.3.11. Statistical analysis

Statistical analyses were performed using SPSS software (Version 17.0). Results expressed as the average of $n \geq 3 \pm SD$. One way analysis of variance (ANOVA) and Pearson's correlation were used for statistical analyses. Significant differences were considered at $p<0.05$ level.

3.4. Results and Discussion

3.4.1. Effects of the stabilizing agents on the physiochemical properties of gemini surfactant- lipoplexes after lyophilization/rehydration cycle

It has been established that the physiochemical properties of lipoplex-based gene delivery vectors (namely particle size and positive surface charge) are essential factors that govern the cellular uptake and consequently the transfection activity of such systems.[17,43,44] The lyophilization process includes three stress steps that have been reported to destabilize the lipoplexes: freezing, drying (dehydration) and rehydration.[26,45] Our data showed that, [P/G/L] lipoplexes prepared without using any protectant agents [Formulation 0, Table 3.4], upon lyophilization, aggregated into large particles (average particle size of 642±247 nm with broad range of particle distribution as expressed by PDI value of 0.708) having negative zeta potential values. In this section we report on the ability of the added excipients to preserve the primary physiochemical characteristics of the lipoplexes. A series of 35 formulations were developed using the stabilizing agents as solutions for the [P/G/L] systems. In all formulations, the pDNA was complexed with the gemini surfactant to form [P/G] lipoplexes first and the DOPE vesicles prepared in the stabilizing solution were incorporated afterward. The lipoplexes were formulated at 1:10 plasmid to gemini surfactants charge ratio, as established earlier.[12] Stabilizing agents used in this work were: monosaccharides (glucose), disaccharides (sucrose, trehalose, lactose), polymers (polyethylene glycol), surfactant polymer (polysorbate 80) and simple polyol (glycerin). These agents have been widely employed as a cryoprotectant agent during the lyophilization of liposomal structures and lipoplex-based gene delivery systems.[28-30,46-48]

The influence of the freeze drying process on the physiochemical properties (particle size and zeta potential) of selected formulations prepared using different stabilizing agents was evaluated [Table 3.4].

Table 3.4.: The components of selected formulations and the influence of lyophilization on the physiochemical properties (size distribution and zeta potential).

ID#	Stabilizing agents solution used to prepare DOPE film	Fresh Formulation		Lyophilized Formulation	
		Particle Size (PDI) Nm	Zeta potential mV	Particle Size (PDI) nm	Zeta potential mV
0	PBS (no stabilizing agent)	145±1 (0.11)	- 1±3	642±247 (0.71)	-11±3
1	9.25 % Sucrose	76.1±0.6 (0.19)	30±2	125±2 (0.18)	29±1
2	9.25 % Sucrose + 0.5%Glycerin	81±2 (0.19)	32±2	99±2 (0.20)	38±1
3	9.25 %Sucrose + 1%Glycerin	79 ±2 (0.19)	30±2	96±3 (0.21)	37±3
4	10 % Trehalose	97±3 (0.19)	24±1	162±9 (0.18)	24.3±0.6
5	10 %Trehalose + 0.5%Glycerin	107±2 (0.22)	28±2	109±3 (0.17)	35±3
6	10 % Trehalose + 1%Glycerin	109±3 (0.17)	17±2	126±3 (0.19)	33±1
7	9.25 % Sucrose + 0.5% Tween 80	34.6±0.7 (0.49)	3±3	100±0.5 (0.52)	2±4
8	1% Tween 80	54±1 (0.25)	- 6±4	192±47 (0.56)	- 1.9±0.2
9	5% PEG 1450	88.1±0.5 (0.29)	- 16±7	301±5 (0.43)	-23±7
10*	9.25 % Sucrose	80±0.1 (0.23)	31±1	131.6 ± 5.47 (0.23)	24.0 ± 1.0

Values are shown as the average of 4 measurements; (PDI) is indicated for size distribution measurements. Formulations (0-9) prepared with pDNA:12-7NH-12:DOPE [P/G/L] lipoplexes in the indicated stabilizing solution. ***** In formulation (10); pDNA:12-7NH-12 lipoplexes [P/G] lyophilized and rehydrated with DOPE suspension.

The clarity of the fresh formulations, (i.e., turbidity) was assessed visually. Formulations showing sedimentation or large visible particles were discarded. Particle size distribution and zeta potential of the clear formulations were measured using the dynamic light scattering (DLS) technique. Fresh formulations used in lyophilization/rehydration evaluation were selected based on two criteria: positive zeta potential and particle size < 200 nm (with PDI< 0.4). After lyophilization, the physical state of lyophilized formulations was evaluated again (physical appearance of the lyophilized cakes). In addition, the appearance of rehydrated formulations was evaluated for clarity.

The results showed that the size and zeta potential of the lipoplexes varied based on the excipient that was used to prepare the formulations [Table 3.4]. Not all stabilizers were efficient in preserving the physiochemical characteristics of the fresh corresponded formulations.

Sugars are widely used as cryoprotectants and stabilizing agents for lyophilized lipid-based gene delivery systems.[25,28,36] Disaccharide sugars sucrose and trehalose were the most efficient cryoprotectant agents in terms of maintaining the particle size (resulting particle size was in the range of 70 -200 nm) and positive zeta potential values of the lipoplexes after lyophilization. These observations were similar to previous assessments that evaluated these agents during the lyophilization of synthetic gene delivery vectors.[37,38,49] A moderate increase in the particle size was observed with both sugars after lyophilization [Formulations 1 and 4, Table 3.4]. Lyophilization of lipoplexes with trehalose and sucrose produced a powder-like lyophilized cake. Lyophilization with lactose as a stabilizing agent showed similar cake structure but the original size and zeta potential value were not preserved. Fresh lipoplexes prepared with monosaccharide sugar (glucose) showed a particle size of 70± 1 nm and a negative zeta potential value (-24± 2 mV) with collapsed cake. Lactose and glucose were eliminated from further investigations for

two reasons: 1) both sugars failed to preserve the essential physiochemical properties of the [P/G/L] systems after lyophilization and 2) they could chemically destabilize the lyophilized lipoplexes during storage (both are reducing sugars).[23,50,51]

The addition of glycerin as anti-freeze bulking agent at 0.5 and 1% w/w concentrations to disaccharide sugar solutions (i.e, sucrose and trehalose) had no significant effects on the physiochemical properties compared to formulations containing only the sugar. However, glycerin was able to minimize the increase in particle size after the lyophilization [Formulations 2,3,5 and 6, Table 3.4]. The use of polymeric cryoprotectant agents (polyethylene glycol PEG 450, PEG 8000) caused aggregation of the lipoplexes (particle sizes of 300-400 nm) and shifted the zeta potential to negative values. The addition of Tween 80 as surfactant polymer to sucrose solution [Formulation 7, Table 3.4] caused significant reduction in particle size (more than 50% reduction) compared to lipoplexes formulated in sucrose only and a slightly positive zeta potential was observed. Upon lyophilization, the particle size significantly increased (almost a 2 fold increase) with no change in zeta potential compared to fresh formulation.

Helper lipid DOPE is frequently used to enhance the transfection activity of cationic lipid mediated gene delivery. The synergistic effect of DOPE is attributed to its ability to undergo polymorphic phase transition that facilitates cell membrane fusion and enhances pDNA endosomal escape.[52,53] To provide this action, it is essential that DOPE vesicles be formed under special conditions with specific morphology. It has been reported that the dehydration-rehydration process could cause lipid phase transition in lyophilized liposomal formulations.[26,54] Therefore, we lyophilized pDNA:gemini surfactant lipoplexes without the incorporation of DOPE or any stabilizing agents to investigate the effects of lyophilization cycles on the [P/G] system. After the freeze-drying cycle, the lyophilized [P/G] lipoplexes were rehydrated with

DOPE vesicles prepared in 9.25% sucrose solution [Formulations 10 Table 3.4]. There were no significant changes in particle size or zeta potential compared to fresh lipoplexes (Formulation 1).

Based on these findings, we selected sucrose and trehalose and these sugars in combination with glycerin as stabilizing agents to investigate their influence on the biological activity of the gemini surfactant lipoplexes before and after lyophilization. Table 3.5 shows the composition of seven formulations that were evaluated.

Table 3.5: Lyophilized formulations used for biological activity (transfection activity and cytotoxicity).

ID#	**pDNA:gemini surfactant (-/+) charge ratio**	**System lyophilized**	**Stabilizing agents solution used to prepare DOPE film**
F1	pDNA:12-7NH-12 1:10	[P/G/L]	9.25 % Sucrose
F2	pDNA:12-7NH-12 1:10	[P/G/L]	9.25 % Sucrose + 0.5% Glycerin
F3	pDNA:12-7NH-12 1:10	[P/G/L]	9.25 %Sucrose + 1% Glycerin
F4	pDNA:12-7NH-12 1:10	[P/G/L]	10 % Trehalose
F5	pDNA:12-7NH-12 1:10	[P/G/L]	10 %Trehalose + 0.5% Glycerin
F6	pDNA:12-7NH-12 1:10	[P/G/L]	10 % Trehalose + 1% Glycerin
F7*	pDNA:12-7N(Glycine)-12 1:10	[P/G]	9.25 % Sucrose

*In formulation (F7), [P/G] system was lyophilized without the incorporation of DOPE or stabilizing agent and then and rehydrated with DOPE suspension prepared in the indicated sugar solution.

3.4.2. Effect of stabilizing agents and lyophilization process on the biological activity of gemini surfactant-lipoplexes

The effect of disaccharide sugars sucrose and trehalose alone, and in combination with glycerin on the transfection activity and cytotoxicity were evaluated [Figure 3.2, white bars].

Fresh formulation F1, [P/G/L] system formulated using 12-7NH-12 gemini surfactant in 9.25% sucrose, is the standard formulation that was comprehensively characterized in our previous work.[41,55] *In vitro* transfection of fresh formulation F1 showed 2.5±0.8 ng of IFNγ/1.5×10^4 COS-7 cells was expressed after 48 hours of treatment. The addition of glycerin to the sucrose solution [Formulations F2 and F3 in Table 3.5] caused significant reduction in transfection activity (approximately 80%) at both concentrations [Figure 3.2]. Similarly, the replacement of sucrose with trehalose [Formulation F4] severely hampered the transfection activity, and a more than 85% reduction in the IFNγ levels was observed compared with the fresh F1. However, the introduction of glycerin as stabilizing agent to trehalose solution [F5 and F6] enhanced the transfection activity compared to fresh formulation prepared with 10% trehalose. Formulation F5 prepared in 10% trehalose and 0.5% glycerin efficiently transfected COS-7 cells and 1.3±0.4 ng IFNγ/1.5×10^4 COS-7 cells was expressed. However, the increase of glycerin concentration to 1% [F6] significantly reduced the transfection activity to 0.32±0.23 ng IFNγ/1.5×10^4 COS-7 cells. There was no correlation between the changes in physiochemical properties (size and zeta potential) and transfection activity.

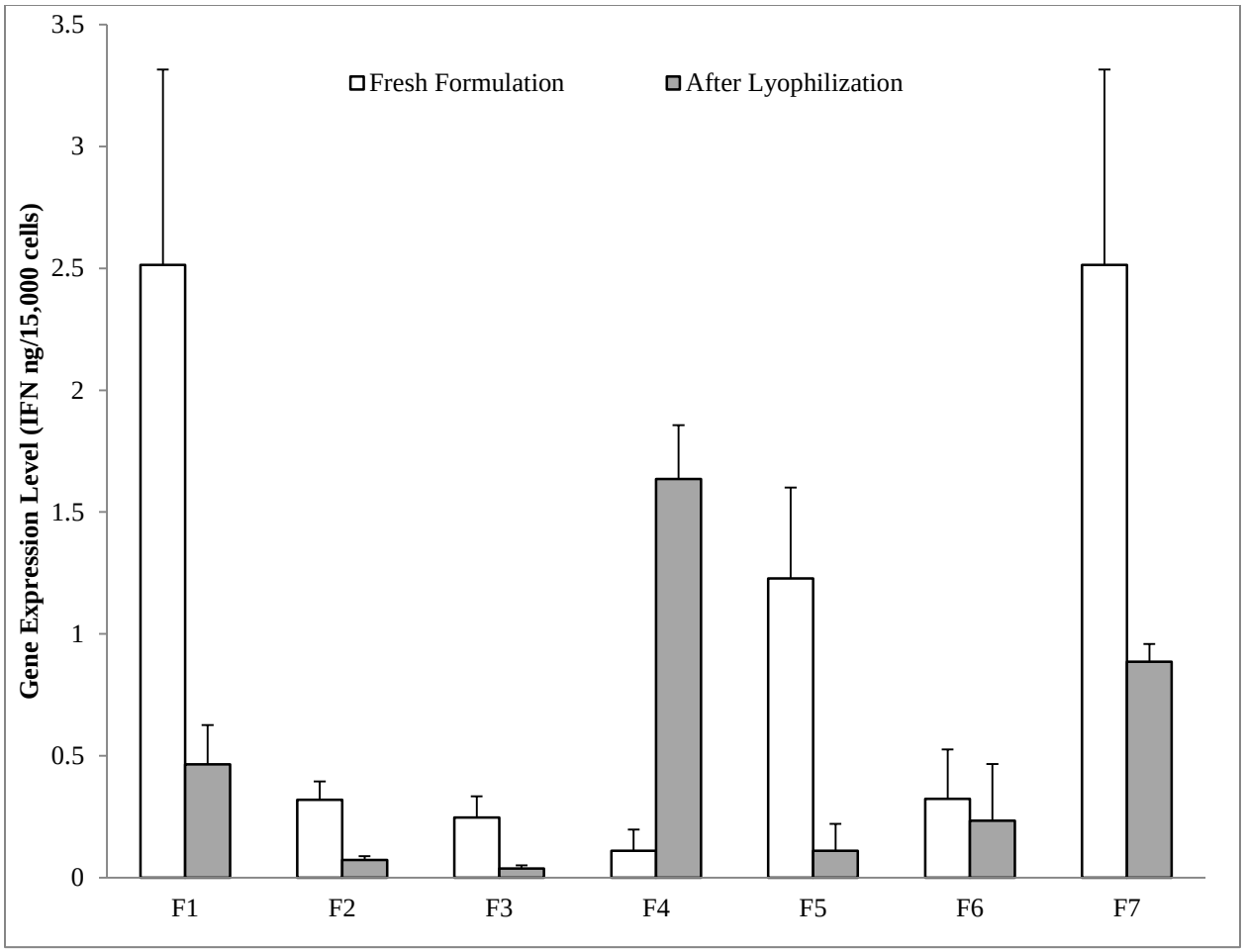


Figure 3.2: The influence of stabilizing agents (white bars) and lyophilization process (gray bars) on the *in-vitro* transfection activity (ELISA-IFNγ). Results are average of four measurements (n=4), error bars ± SD.

The effect of stabilizing agents on cellular toxicity was also evaluated by using the MTT assay [Figure 3.3., white bars]. No significant changes in cytotoxicity were observed between lipoplexes formulated with sucrose and trehalose [F1 and F4], when used alone (cell viability of 64±1.4% and 59±7.5%, respectively) [Figure 3.3]. The addition of glycerin to sucrose or trehalose improved the cell viability compared with lipoplexes formulated with sugar alone. The highest cell viability was observed when 0.5% glycerin was added to 10% trehalose [Formulation F5, 77±1.7% cell viability, Figure 3.3]. This formulation showed 15% enhancement in cell viability compared to formulation F4 using 10% trehalose.

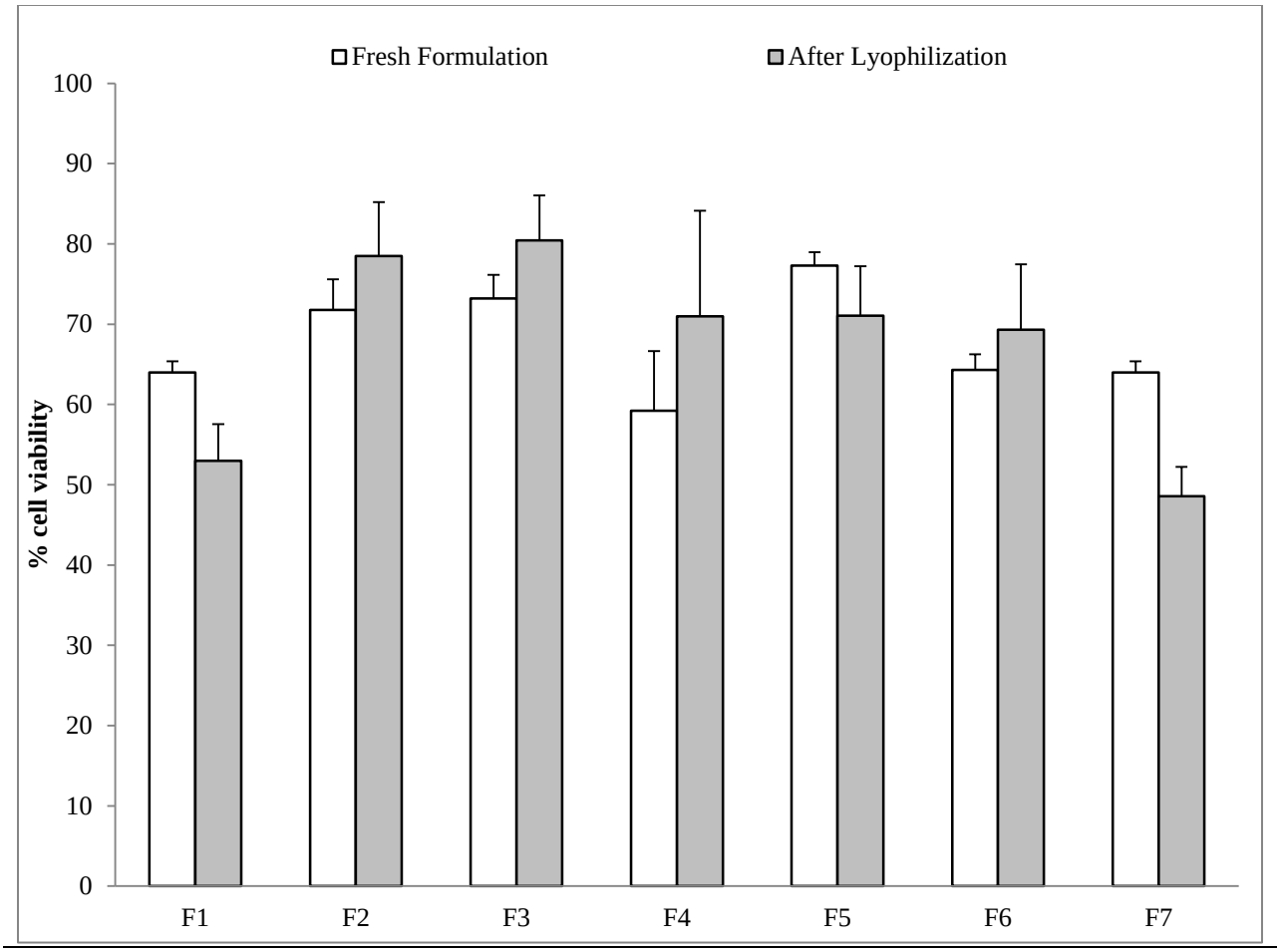


Figure 3.3: The influence of stabilizing agents (white bars) and lyophilization process (gray bars) on the cellular toxicity (MTT assay). Results are expressed as percentage of un-treated cells (100%). Results are average of four measurements (n=4), error bars ± SD.

We performed a lyophilization/rehydration study on the seven formulations described in Table 3.5 to investigate the effect of the lyophilization process on the biological activity [Figure 3.2, gray bars]. Upon lyophilization/rehydration, four formulations showed more than 70% loss in transfection activity in comparison with the corresponding fresh formulations [Formulations F1, F2, F3 and F5, Figure 3.2.]. Among these formulations, only F1, standard formulation in 9.25% sucrose, was able to express an adequate level of IFNγ after the lyophilization/rehydration cycle (0.47±0.16 ng IFNγ/1.5×10^4 COS-7). It should be noted that fresh formulation F1 showed the highest gene expression activity among all fresh formulations evaluated in this study as discussed early. Formulation F6 (in 10% trehalose + 1% glycerin) showed the least loss in transfection activity after lyophilization process (approximately 25% loss compared with fresh F6).

One of the most interesting findings in this study was the influence of lyophilization on the transfection activity of [P/G/L] lipoplexes formulated in 10% trehalose [Formulation F4, Figure 3.2]. A significant increase in transfection efficiency (10 fold increase) was observed upon lyophilization/rehydration of formulation F4 (1.6±0.22 ng IFNγ/1.5×10^4 COS-7).

Lyophilized [P/G] lipoplexes without the incorporation of helper lipid DOPE or any stabilizing agents [formulation F7, Table 3.5, Figure 3.2] were able to preserve approximately 40% of the activity of the original formulation F1. Compared with lyophilized [P/G/L] in 9.25% sucrose (lyophilized F1), lyophilization of [P/G] without the stabilizing sugar, (lyophilized F7) was more efficient in maintaining the transfection activity as the level of expressed IFN was 465±161 ng and 886±72 ng, respectively. Therefore, it may be concluded that the loss of transfection activity in case of the lyophilization of [P/G/L] systems can be attributed to the conformational changes in the supramolecular structure of the lipid phase of the lipoplexes and

to the possibility of the loss of pDNA morphology. In fact, previous work showed that the electrostatic interaction of the hydroxyethylated cholesterol-based cationic lipid with pDNA was able to protect the pDNA during the freeze drying cycle when no cryoprotectant agent was used.[56] Therefore, the incorporation of stabilizing agent during the freeze-drying cycles is more important in preserving the conformational structure of the lipoplex systems than the pDNA content.[40] To prove that, we evaluated different methods to extract the pDNA from the [P/G/L] and [P/G] systems aiming to investigate the effect of the lyophilization process on pDNA content [Table 3.2.]. Unfortunately, all the methods failed to isolate the pDNA from the [P/G/L] systems. pDNA from [P/G] lipoplexes was detected only with methods M-2 and M-3. We believe that the presence of DOPE vesicles in high concentrations in the [P/G/L] system hindered the release of the pDNA.

In addition to the transfection activity, the effect of freeze-drying on the cellular toxicity of gemini surfactant-based lipoplexes [Figure 3.3., gray bars] was evaluated. Upon lyophilization/rehydration cycles slight improvement in cell viabilities were observed with most formulations. Lyophilization of standard formulation (F1) caused approximately 15% reduction in cell viability compared with the fresh F1. Similarly, 25% reduction in cell viability was observed when lyophilized [P/G] lipoplexes without stabilizing agent was used as transfection vector (compared to fresh F1).

Based on findings illustrated in previous sections, we selected five formulations to evaluate the influence of lyophilization parameters and storage temperature on the physical and biological activity of lyophilized gemini surfactant-based lipoplexes [Table 3.3].

3.4.3. Stability of lyophilized lipoplexes

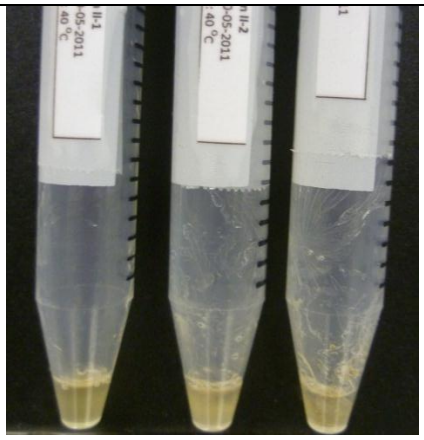
We evaluated the effect of the storage temperature and aging on the physiochemical properties (particle size and zeta potential) and transfection activities of lyophilized gemini surfactant-based lipoplex by conducting a three-month stability study at two storage conditions (25 ˚C/75% RH and 40 ˚C/75% RH).

The stability of lyophilized lipoplex-based vectors depends on several factors. The optimization of freeze drying cycles (i.e., freezing and drying cycles) and the incorporation of proper stabilizing agents are essential to maintain the physiochemical properties and transfection activity of lipoplex systems.[26,37,49] In addition, the long-term stability of the lyophilized lipoplexes can be influenced by: formulation composition, storage temperature, moisture content in the lyophilized cake, and the presence of reactive oxygen species (ROS).[22,37,42]

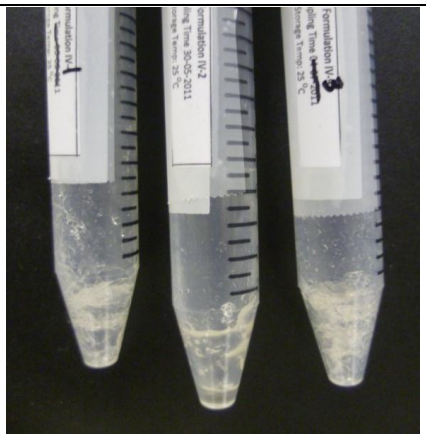
Unfortunately, the inability of the freeze dryer to handle large amounts of the formulations resulted in lyophilized products with high levels of moisture content. This caused the collapse of the dried cake of lyophilized lipoplexes formulated with a sugar component (sucrose and trehalose) after one week of storage of at 25 ˚C [A and B, Figure 3.4]. The lyophilized cake of formulations containing 1% glycerin (F_{SG} and F_{TG}) showed a gummy texture that was unable to rehydrate easily [C and D, Figure 3.4]. This can be attributed to the fact that glycerin inhibits the growth of ice crystals which leads to the formation of small pores in frozen lipoplexes during the freezing cycle.[57] This effect could increase the time required for the drying process and lead to the formation of dried cake with a high moisture content.[58,59]

[A] Lyophilized (F_S)

[B] Lyophilized (F_T)



[C] Lyophilized (F_{SG})



[D] Lyophilized (F_{TG})

Figure 3.4: The appearance of lyophilized cake of four lyophilized formulations after one week of storage at 25 ˚C.

Despite the inefficiency of the lyophilization, we continued the physiochemical and biological assessments during the three months stability study. The purpose of continuing the study was to evaluate the feasibility of the developed analytical techniques to identify the changes in the physiochemical properties (particle size and zeta potential) and the transfection efficiency of the lyophilized formulations during the stability study. Therefore, the results from the stability study were insignificant to be discussed.

3.5. Conclusion

Gemini surfactant-based [P/G/L] lipoplexes were prepared with different stabilizing agents and the physiochemical properties of [P/G/L] systems before and after freeze-drying cycles were evaluated. These properties varied based on the stabilizing agents used to prepare the lipoplexes. Sucrose and trehalose alone and in combination with glycerin were able to maintain the particle size and positive zeta potential upon lyophilization/rehydration. However, even with preserving the physiochemical properties, the transfection activity of lyophilized lipoplexes was severely hampered compared with freshly prepared lipoplexes. The only exception was trehalose used at 10% concentration as a /stabilizing agent; the transfection activity of lyophilized lipoplexes was significantly higher than the corresponding fresh formulation. The findings from this study provide information about the effects of stabilizing agents and the lyophilization process on the physical stability of gemini surfactant-based lipoplexes that can be used for further investigation in order to optimize long-term stability of lyophilized [P/G/L] systems.

Acknowledgment

The authors acknowledge the Helix BioPharma Corporation for the use of stability chambers. This work was funded in part by a grant from the Natural Science and Engineering Research Council of Canada [NSERC] and a grant from Saskatchewan Health Research Foundation [SHRF].

References

1. Thomas M, Klibanov A. Non-viral gene therapy: polycation-mediated DNA delivery. Applied Microbiology and Biotechnology 2003;62:27-34.

2. Brown MD, Schätzlein AG, Uchegbu IF. Gene delivery with synthetic (non viral) carriers. International Journal of Pharmaceutics 2001;229:1-21.

3. Hirko A, Tang F, Hughes JA. Cationic lipid vectors for plasmid DNA delivery. Current Medicinal Chemistry 2003;10:1185-93.

4. Ilies M, Seitz WA, Balaban AT. Cationic lipids in gene delivery: principles, vector design and therapeutical applications. Current Pharmaceutical Design 2002;8:2441-73.

5. Gene Therapy Clinical Trials Worldwide John Wiley and Sons Ltd., 2012. (Accessed April, 25, 2012, at http://www.wiley.com//legacy/wileychi/genmed/clinical/.)

6. Pedroso de Lima MC, Simões S, Pires P, Faneca H, Düzgünes N. Cationic lipid-DNA complexes in gene delivery: from biophysics to biological applications. Advanced Drug Delivery Reviews 2001;47:277-94.

7. Martin B, Sainlos M, Aissaoui A, et al. The design of cationic lipids for gene delivery. Current Pharmaceutical Design 2005;11:375-94.

8. Kirby AJ, Camilleri P, Engberts JBFN, et al. Gemini surfactants: new synthetic vectors for gene transfection. Angewandte Chemie International Edition 2003;42:1448-57.

9. Wettig SD, Verrall RE, Foldvari M. Gemini surfactants: a new family of building blocks for non-viral gene delivery systems. Current Gene Therapy 2008;8:9-23.

10. Menger FM, Littau C. Gemini-surfactants: synthesis and properties. Journal of the American Chemical Society 1991;113:1451-2.

11. Menger F, Littau C. Gemini surfactants: a new class of self-assembling molecules. Journal of the American Chemical Society 1993;115:10083-90.

12. Badea I, Verrall R, Baca-Estrada M, et al. In vivo cutaneous interferon-γ gene delivery using novel dicationic (gemini) surfactant–plasmid complexes. The Journal of Gene Medicine 2005;7:1200-14.

13. Donkuru MD, Wettig SD, Verrall RE, Badea I, Foldvari M. Designing pH-sensitive gemini nanoparticles for non-viral gene delivery into keratinocytes. Journal of Materials Chemistry 2012;22:6232-44.

14. Yang P, Singh J, Wettig S, Foldvari M, Verrall RE, Badea I. Enhanced gene expression in epithelial cells transfected with amino acid-substituted gemini nanoparticles. European Journal of Pharmaceutics and Biopharmaceutics 2010;75:311-20.

15. de Lima M, Neves S, Filipe A, Duzgunes N, Simoes S. Cationic liposomes for gene delivery: from biophysics to biological applications. Current Medicinal Chemistry 2003;10:1221-31.

16. Anchordoquy TJ, Koe GS. Physical stability of nonviral plasmid-based therapeutics. Journal of Pharmaceutical Sciences 2000;89:289-96.

17. Barteau B, Chèvre R, Letrou-Bonneval E, Labas R, Lambert O, Pitard B. Physicochemical parameters of non-viral vectors that govern transfection efficiency. Current Gene Therapy 2008;8:313-23.

18. Almofti MR, Harashima H, Shinohara Y, Almofti A, Li W, Kiwada H. Lipoplex size determines lipofection efficiency with or without serum. Molecular Membrane Biology 2003;20:35-43.

19. Ross P, Hui S. Lipoplex size is a major determinant of in vitro lipofection efficiency. Gene Therapy 1999;6:651-9.

20. Walther W, Stein U, Voss C, Schmidt T, Schleef M, Schlag PM. Stability analysis for long-term storage of naked DNA: impact on nonviral in vivo gene transfer. Analytical Biochemistry 2003;318:230-5.

21. Molina MDC, Anchordoquy TJ. Formulation strategies to minimize oxidative damage in lyophilized lipid/DNA complexes during storage. Journal of Pharmaceutical Sciences 2008;97:5089-105.

22. Anchordoquy TJ. Degradation of lyophilized lipid/DNA complexes during storage: the role of lipid and reactive oxygen species. Biochimica et Biophysica Acta (BBA)-Biomembranes 2008;1778:2119-26.

23. Molina, M. d.C., Armstrong, T. K., Zhang, Y., Patel, M. M., Lentz, Y. K., Anchordoquy, T. J. The stability of lyophilized lipid/DNA complexes during prolonged storage. Journal of Pharmaceutical Sciences 2004;93:2259-73.

24. del Pozo-Rodríguez A, Solinís M, Gascón A, Pedraz J. Short-and long-term stability study of lyophilized solid lipid nanoparticles for gene therapy. European Journal of Pharmaceutics and Biopharmaceutics 2009;71:181-9.

25. Kasper JC, Schaffert D, Ogris M, Wagner E, Friess W. Development of a lyophilized plasmid/LPEI polyplex formulation with long-term stability--A step closer from promising technology to application. Journal of Controlled Release 2011;151:246-55.

26. Allison SD, Anchordoquy TJ. Lyophilization of nonviral gene delivery systems. Methods in Molecular Medicine 2001;65:225-52.

27. Anchordoquy TJ, Carpenter JF, Kroll DJ. Maintenance of transfection rates and physical characterization of lipid/DNA complexes after freeze-drying and rehydration. Archives of Biochemistry and Biophysics 1997;348:199-206.

28. Kuo JS, Hwang R. Preparation of DNA dry powder for non-viral gene delivery by spray-freeze drying: effect of protective agents (polyethyleneimine and sugars) on the stability of DNA. Journal of Pharmacy and Pharmacology 2004;56:27-33.

29. Hinrichs W, Manceņido F, Sanders N, et al. The choice of a suitable oligosaccharide to prevent aggregation of PEGylated nanoparticles during freeze thawing and freeze drying. International Journal of Pharmaceutics 2006;311:237-44.

30. Vighi E, Ruozi B, Montanari M, Battini R, Leo E. Re-dispersible cationic solid lipid nanoparticles (SLNs) freeze-dried without cryoprotectors: characterization and ability to bind the pEGFP-plasmid. European Journal of Pharmaceutics and Biopharmaceutics 2007;67:320-8.

31. Arakawa T, Timasheff SN. Stabilization of protein structure by sugars. Biochemistry 1982;21:6536-44.

32. Randolph TW. Phase separation of excipients during lyophilization: effects on protein stability. Journal of Pharmaceutical Sciences 1997;86:1198-203.

33. Crowe JH, Carpenter JF, Crowe LM. The role of vitrification in anhydrobiosis. Annual Review of Physiology 1998;60:73-103.

34. Wolfe J, Bryant G. Freezing, drying, and/or vitrification of membrane-solute-water systems. Cryobiology 1999;39:103-29.

35. Allison SD, Molina MC, Anchordoquy TJ. Stabilization of lipid/DNA complexes during the freezing step of the lyophilization process: the particle isolation hypothesis. Biochimica et Biophysica Acta (BBA)-Biomembranes 2000;1468:127-38.

36. Allison SD, Anchordoquy TJ. Mechanisms of protection of cationic lipid-DNA complexes during lyophilization. Journal of Pharmaceutical Sciences 2000;89:682-91.

37. Allison SD, Anchordoquy TJ. Maintenance of nonviral vector particle size during the freezing step of the lyophilization process is insufficient for preservation of activity: insight from other structural indicators. Journal of Pharmaceutical Sciences 2001;90:1445-55.

38. Yadava P, Gibbs M, Castro C, Hughes JA. Effect of Lyophilization and Freeze-thawing on the Stability of siRNA-liposome Complexes. AAPS PharmSciTech 2008;9:335-41.

39. Cortesi R, Esposito E, Nastruzzi C. Effect of DNA complexation and freeze-drying on the physicochemical characteristics of cationic liposomes. Antisense and Nucleic Acid Drug Development 2000;10:205-15.

40. Maitani Y, Aso Y, Yamada A, Yoshioka S. Effect of sugars on storage stability of lyophilized liposome/DNA complexes with high transfection efficiency. International Journal of Pharmaceutics 2008;356:69-75.

41. Wettig SD, Wang C, Verrall RE, Foldvari M. Thermodynamic and aggregation properties of aza-and imino-substituted gemini surfactants designed for gene delivery. Physical Chemistry Chemical Physics 2006;9:871-7.

42. Yu J, Anchordoquy TJ. Effects of moisture content on the storage stability of dried lipoplex formulations. Journal of Pharmaceutical Sciences 2009;98:3278-89.

43. Kneuer C, Ehrhardt C, Bakowsky H, et al. The influence of physicochemical parameters on the efficacy of non-viral DNA transfection complexes: a comparative study. Journal of Nanoscience and Nanotechnology, 6 2006;9:2776-82.

44. Rejman J, Oberle V, Zuhorn IS, Hoekstra D. Size-dependent internalization of particles via the pathways of clathrin-and caveolae-mediated endocytosis. Biochemical Journal 2004;377:159.

45. Poxon SW, Hughes JA. The effect of lyophilization on plasmid DNA activity. Pharmaceutical Development and Technology 2000;5:115-22.

46. Chacon M, Molpeceres J, Berges L, Guzman M, Aberturas M. Stability and freeze-drying of cyclosporine loaded poly (, lactide-glycolide) carriers. European Journal of Pharmaceutical Sciences 1999;8:99-107.

47. Abdelwahed W, Degobert G, Fessi H. A pilot study of freeze drying of poly (epsilon-caprolactone) nanocapsules stabilized by poly (vinyl alcohol): formulation and process optimization. International Journal of Pharmaceutics 2006;309:178-88.

48. Saez A, Guzman M, Molpeceres J, Aberturas M. Freeze-drying of polycaprolactone and poly (-lactic-glycolic) nanoparticles induce minor particle size changes affecting the oral pharmacokinetics of loaded drugs. European Journal of Pharmaceutics and Biopharmaceutics 2000;50:379-87.

49. Armstrong TK, Anchordoquy TJ. Immobilization of nonviral vectors during the freezing step of lyophilization. Journal of Pharmaceutical Sciences 2004;93:2698-709.

50. Li B, Li S, Tan Y, et al. Lyophilization of cationic lipid–protamine–DNA (LPD) complexes. Journal of Pharmaceutical Sciences 2000;89:355-64.

51. Li S, Patapoff TW, Overcashier D, Hsu C, Nguyen TH, Borchardt RT. Effects of reducing sugars on the chemical stability of human relaxin in the lyophilized state. Journal of Pharmaceutical Sciences 1996;85:873-7.

52. Koltover I, Salditt T, Rädler JO, Safinya CR. An inverted hexagonal phase of cationic liposome-DNA complexes related to DNA release and delivery. Science 1998;281:78-81.

53. Hirsch-Lerner D, Zhang M, Eliyahu H, Ferrari ME, Wheeler CJ, Barenholz Y. Effect of "helper lipid" on lipoplex electrostatics. Biochimica et Biophysica Acta (BBA)-Biomembranes 2005;1714:71-84.

54. Montanari J, Roncaglia D, Lado L, Morilla M, Romero E. Avoiding failed reconstitution of ultradeformable liposomes upon dehydration. International Journal of Pharmaceutics 2009;372:184-90.

55. Wettig SD, Badea I, Donkuru MD, Verrall RE, Foldvari M. Structural and transfection properties of amine-substituted gemini surfactant-based nanoparticles. The Journal of Gene Medicine 2007;9:649-58.

56. Percot A, Briane D, Coudert R, et al. A hydroxyethylated cholesterol-based cationic lipid for DNA delivery: effect of conditioning. International Journal of Pharmaceutics 2004;278:143-63.

57. Hey J, MacFarlane D. Crystallization of ice in aqueous solutions of glycerol and dimethyl sulfoxide 2: ice crystal growth kinetics. Cryobiology 1998;37:119-30.

58. Chen C, Han D, Cai C, Tang X. An overview of liposome lyophilization and its future potential. Journal of Controlled Release 2010;142:299-311.

59. Akers MJ. Chapter 20: Freeze-dry (lyophilization) processing. In: Sterile Drug Products: Formulation, Packaging, Manufacturing, and Quality. First ed: Informa Healthcare; 2010:296-312.

Chapter 4

Development of lyophilized gemini surfactant-based gene delivery systems: Influence of lyophilization on the structure, activity and stability of the lipoplexes[§]

Waleed Mohammed-Saeid[1], Deborah Michel[1], Anas El-Aneed[1], Ronald Verrall[2], Nicholas H. Low[3], Ildiko Badea[1]*

[1] Drug Design and Discovery Research Group, College of Pharmacy and Nutrition, University of Saskatchewan

[2] Department of Chemistry, University of Saskatchewan

[3] Department of Food and Bioproduct Sciences, University of Saskatchewan

[§] This chapter was submitted as a research manuscript to the Journal of Pharmacy and Pharmaceutical Sciences on August 3rd, 2012.

* Correspondence: ildiko.badea@usask.ca
Drug Design and Discovery Research Group, College of Pharmacy and Nutrition,
University of Saskatchewan, 110 Science Place, Saskatoon, S7N 5C9,
Canada

4.1. Abstract

Purpose

Cationic lipid-based gemini surfactants have been extensively studied as non-viral vectors for gene therapy. Clinical applications of cationic lipid/DNA lipoplexes are restricted by their instability in aqueous formulations. In this work, we investigated the influence of lyophilization on the essential physiochemical properties and *in vitro* transfection of gemini surfactant-lipoplexes. Additionally, we evaluated the feasibility of lyophilization as a technique for preparing gemini surfactant-lipoplexes with long term stability.

Methods

A diquaternary ammonium gemini surfactant [12-7NH-12] and plasmid DNA encoding for interferon-γ were used to prepare gemini surfactant/pDNA [P/G] lipoplexes. Helper lipid DOPE [L] was incorporated in all formulation producing a [P/G/L] system. Sucrose and trehalose were utilized as stabilizing agents. To evaluate the ability of lyophilization to improve the stability of gemini surfactant-based lipoplexes, four lyophilized formulations were stored at 25˚C for three months. The formulations were analyzed at three different time-points for physical appearance, physiochemical properties (particle size and zeta potential, gemini surfactant pDNA interaction) and *in vitro* transfection.

Results

The results showed that both sucrose and trehalose provided the anticipated stabilizing effect. The transfection efficiency of the lipoplexes increased 2-3 fold compared to fresh formulations upon lyophilization. This effect can be attributed to the improvement of DNA compaction and changes in the lipoplex morphology due to the lyophilization/rehydration cycles. The physiochemical properties of the lyophilized formulations were maintained throughout the three month study at 25 ˚C. All lyophilized formulations showed a significant loss of gene transfection activity after three months of storage.

Nevertheless, no significant losses of transfection efficiency were observed for three formulations after two months storage at 25 ˚C.

Conclusion

Lyophilization significantly improved the physiochemical stability of gemini surfactant-based lipoplexes compared to liquid formulations. As well, lyophilization improved the transfection efficiency of gemini surfactant-based lipoplexes. The loss of transfection activity upon storage is most probably due to the conformational changes in the supramolecular structure of the lipoplexes as a function of time and temperature rather than to DNA degradation.

4.2. Introduction

Gene therapy is a promising therapeutic approach that has the potential to improve, significantly, human health.[1] Successful gene therapy depends on the design of efficient, safe and stable gene delivery systems. Chemically mediated non-viral vectors, such as cationic lipids, exhibit low immunogenicity compared to viral vectors.[2,3] One specific group of cationic lipids that has demonstrated efficient transfection activity is the gemini surfactants [Figure 4.1].[4,5] They are dimeric surfactants primarily used in material sciences because of their characteristic low surface tension.[6,7] In recent years, gemini surfactants have been investigated extensively as a non-viral gene delivery carriers for both *in vitro* and *in vivo* applications. These agents have versatile chemical structure, can be produced easily on a laboratory scale, are able to compact DNA to nano-sized lipoplexes, and show relatively low toxicity compared to monomeric surfactants.[4,8,9]

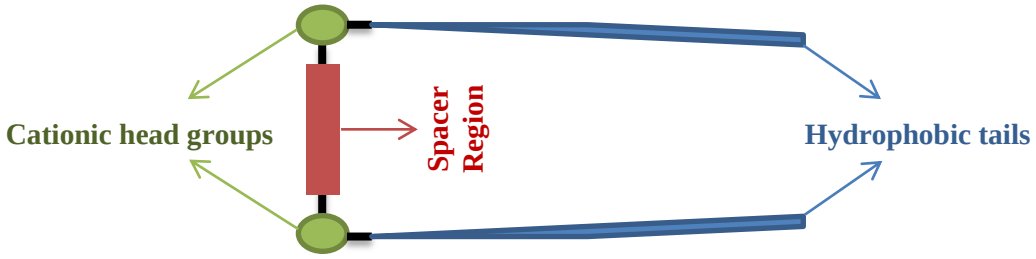


Figure 4.1: General structure of cationic gemini surfactants

The low transfection efficiency and the instability of lipid-based gene delivery vectors in liquid pharmaceutical dosage forms are two major deficiencies that limit their wide clinical application.[10,11] Over the last decade, a large number of cationic lipids have been synthesized and modified to overcome their low transfection activity, but little concern has been given to the stability of lipoplexes from a pharmaceutical perspective. The transfection efficiency of lipoplexes depends not only on the stability and integrity of all components of the delivery system but also on the maintenance of their related physiochemical properties (particle size and surface charge ratio).[12-14] The stability of a non-viral gene

delivery system depends upon the conformational integrity of the genetic material, the chemical stability of the carrier and the physiochemical stability of the DNA-carrier complexes.[15-17] In aqueous formulations, lipoplexes tend to aggregate and form large particles. This phenomenon can also lead to the dissociation of DNA from the lipoplexes and loss of the biological activity due to enzymatic degradation of the unprotected genetic material.[18] To evade the stability issue, most of the studies that employ cationic lipids as a non-viral carrier for gene delivery use freshly prepared lipoplexes. Three different formulation methods have been explored to optimize the physical stability of cationic lipid/DNA complexes: liquid, frozen, and dehydrated.[19] To maintain the stability of the liquid and frozen formulations, special storage conditions and formulation strategies are needed that limit large scale production of the lipoplexes using good manufacturing procedures.[20-23] Lyophilized (freeze-dried) formulations demonstrated the most efficient stability among these three formulation techniques.[24]

Lyophilization has been employed widely for the production of highly stable protein-based pharmaceutical products.[25,26] Recently, lyophilization was also investigated as a practical technique to produce non-viral vectors with long-term stability.[27-29] However, lyophilization is a complicated process that includes freezing and drying stresses which can damage the DNA structure and cause aggregation of lipoplexes.[24] The damage to the DNA integrity and lipoplex structure during the freezing step can result from the increased concentration of the suspended materials (cryoconcentration effect) as the liquid freezes, leading to formation of larger aggregates in the unfrozen part. In addition, the formation of ice crystals or the crystallization of solutes in the formulation have been reported to damage the lipoplex integrity.[22,30] The removal of the unbound water and ice from the frozen formulations during the drying step can affect the lipoplexes as the condition shifts from a fully hydrated environment to a drier state.[24,30] In addition, phase transition of lipid membrane in lyophilized liposomal formulations during dehydration-rehydration has been reported.[24] The optimization of the freeze-drying protocol and

incorporation of certain stabilizers, known as cryo- or lyo-protectant agents, have been shown to improve the stability of the lipid-based DNA formulations.[24,31] Different classes of stabilizing agent have been used for the preparation of lyophilized non-viral gene delivery systems: monosaccharaides (glucose), disaccharides (sucrose, trehalose), oligosaccharides (inulin) and polymers (dextran, povidone, polyethylene glycol).[23,32,33] It has been reported that several aspects govern the ability of the lyophilization process to stabilize and preserve the activity of cationic lipoplexes: lyophilization protocol, type and amount of stabilizing agent, nature of the cationic lipid, DNA to cationic lipid charge ratio and incorporation of helper lipid.[19,28,29]

Although several studies have investigated the influence of lyophilization on the cationic lipid-DNA vectors, most of these studies utilized singly charged cationic lipids (e.g., DOTAP, DC-Chol, DMRIE).[27,34,35] To the best of our knowledge, the effect of lyophilization on lipoplexes formed with multiply charged cationic lipids and high concentration of the helper lipid DOPE has not been addressed. The aim of this work was to evaluate the feasibility of lyophilization to stabilize gemini surfactant-based lipoplexes over long periods of storage at room temperature. The influence of the lyophilization process and stabilizing agents on the physiochemical properties, DNA compaction and *in vitro* transfection activity were investigated and the results are reported herein.

4.3. Materials and Methods

4.3.1.Materials

The construction of the plasmid (pGThCMV.IFN-GFP), encoding for murine interferon gamma (IFN-γ) and green fluorescent protein (GFP), was described previously.[4] Plasmid DNA was isolated and purified using QIAGEN Plasmid Giga Kit (Mississauga, ON, Canada) as prescribed in the manufacturer's protocols. The synthesis and characterization of the gemini surfactants used in this study have been previously described.[36] Aqueous solutions of 3 mM gemini surfactant were used to prepare plasmid DNA/gemini surfactant lipoplexes. Helper lipid 1,2 dioleyl-*sn*-glycero-phosphatidylethanolamine (DOPE) (Avanti Polar Lipids, Alabaster, AL) was co-formulated in all formulations. Stabilizer agents (analytical grade) sucrose and trehalose were obtained from Sigma Aldrich (Oakville, ON, Canada). All excipients were used without further purification.

4.3.2.Preparation of lipoplexes

The plasmid/gemini surfactant [P/G] complexes were prepared by mixing an aqueous solution of pDNA with an appropriate amount of 3 mM gemini surfactant solution at 1:10 charge ratio and incubated at room temperature for 20 minutes. Lipoplexes were formulated in the presence of DOPE as helper lipid creating plasmid/gemini surfactant/lipid lipoplexes [P/G/L]. These [P/G/L] lipoplex systems were prepared by mixing [P/G] lipoplexes with the DOPE vesicles at gemini surfactant to DOPE weight ratio of 1:10 and incubated at room temperature for 20 minutes. The stabilizing solutions were prepared by dissolving the sugar in nuclease-free ultrapure water (Gibco, Invitrogen Corporation, Grand Island, NY, USA) on weight/weight (w/w) percentage basis and the pH was adjusted with NaOH solution to 9. These solutions were used to redisperse the DOPE, as described previously [4], at a final DOPE concentration of 1 mM and filtered through Acrodisc® 0.45 μm syringe filters (Pall Gelman, Ann Arbor, MI).

Gemini surfactant/DOPE [G/L] vesicles were prepared at a gemini surfactant to DOPE weight ratio 1:10. A stock solution of 12-7NH-12 gemini surfactant was prepared in anhydrous ethanol and used to prepare a [G/L] film. Stabilizing solutions were used to re-disperse the [G/L] film and then filtered through Acrodisc® 0.45 µm syringe filters. An aliquot of plasmid solution and the G/L dispersion were mixed to obtain lipoplexes at a plasmid to gemini surfactant charge ratio of 1:10.

Four formulations were prepared for the three-month stability study. Two were prepared as described earlier by complexing the DNA with the gemini surfactant first, then mixing with the DOPE vesicles dispersed in stabilizing solutions (S: sucrose, T: trehalose) [Method A, Table 4.1]. The lipoplexes were then lyophilized. For the other two formulations, P-[G/L-S]$_{lyp}$ and P-[G/L-T]$_{lyp}$, only the [G/L] component was lyophilized. Then fresh DNA solution was added to the reconstituted formulations for all assays. Table 4.1 summarizes the preparation methods of these formulations. In all analyses, triplicate batches of each formulation were evaluated.

Table 4.1: Preparation methods for the formulations used in this study

Formulation	**Preparation method**	**Description**
[P/G/L-S]$_{lyp}$	A	P/G/L lipoplexes prepared with DOPE vesicles dispersed in 9.25% sucrose solution were lyophilized for 48h
[P/G/L-T]$_{lyp}$		P/G/L lipoplexes prepared with DOPE vesicles dispersed in 10% trehalose solution were lyophilized for 48h
P-[G/L-S]$_{lyp}$	B	G/L vesicles prepared in 9.25% sucrose were lyophilized for 48h and the plasmid solution was added to the lipid vesicles after reconstitution to prepare the lipoplexes
P-[G/L-T]$_{lyp}$		G/L vesicles prepared in 10% trehalose were lyophilized for 48h and the plasmid solution was added to the lipid vesicles after reconstitution to prepare the lipoplexes

4.3.3.Lyophilization of the formulations

A volume of 2 mL of freshly prepared formulations P/G/L-S and P/G/L-T, containing a total of 7.4 µg/mL pDNA and G/L-S and G/L-T without pDNA, were transferred to 5-mL flat bottom low extractable borosilicate USP Type I lyophilization serum vials (Wheaton Industries Inc, Millville, NJ, USA). The vials were partially closed with three-legged lyophilization stoppers and stored at -80 ˚C for 2 h. After freezing, the formulation vials were transferred to a Labconco® Freezone Plus 6 L cascade freeze dryer (Labconco, Kansas City, MO, USA) at -80 ˚C and 0.03 mBar pressure and lyophilized for 48 h. The vials were removed from the freeze dryer, flushed with nitrogen gas, and the vial stoppers were fully closed and sealed with a crimp aluminum cap. All formulations were prepared under aseptic conditions.

4.3.4.Stability study

For the stability study, the lyophilized formulations were stored in a stability chamber at 25 ºC and 75% relative humidity (RH) (Sanyo growth cabinet MLR-350, Sanyo, Osaka, Japan) for three months. Samples were tested at one, two and three month storage periods. Formulations were prepared and analyzed in triplicate (n=3).

4.3.5.Rehydration of the lyophilized formulations

Lyophilized formulations containing the pDNA, $[P/G/L\text{-}S]_{lyp}$ and $[P/G/L\text{-}T]_{lyp}$, were rehydrated to a final volume of 2 mL with ultrapure water (Gibco, Invitrogen Corporation, Grand Island, NY, USA). Formulations $[G/L\text{-}S]_{lyp}$ and $[G/L\text{-}T]_{lyp}$, without pDNA, were rehydrated using pDNA solution in UltraPure water to a final pDNA concentration of 7.4 µg/mL (1:10 plasmid to gemini charge ratio) and incubated for 30 minutes at room temperature, generating the P-$[G/L\text{-}S]_{lyp}$ and P-$[G/L\text{-}T]_{lyp}$ formulations. All rehydrated formulations, containing 0.2 µg pDNA, were used for *in-vitro* transfection evaluation.

4.3.6. Determination of moisture content

Lyophilized formulations at the time of preparation and after 3 months of storage were evaluated for moisture content using a Karl Fisher Titrator (Automat Model 633; Metrohm, Herisau, Switzerland). The lyophilized formulations (approximately 45±5 mg of lyophilized cake) were dissolved in HPLC grade methanol (Fisher Scientific, Edmonton, AB, Canada) previously blanked with pyridine-free Karl Fischer reagent (BDH, Edmonton, AB, Canada) and titrated with the same reagent. Ten microliters of purified water (Milli-Q™ Water System, Milford, MA, USA) was used to standardize the Karl Fischer reagent. A 20 second delay was used to ensure end point stabilization. Formulations were analyzed in triplicate (n=3)..

4.3.7. Size and ζ-potential measurements

Fresh and rehydrated formulations were transferred into a cuvette (DTS1061, Malvern Instruments, Worcestershire, UK) for size distribution and zeta-potential measurements using a Zetasizer Nano ZS instrument (Malvern Instruments, Worcestershire, UK). Each sample was measured four times, and the results were expressed as the average ± standard deviation (SD) of three samples (n=3) with a corresponding polydispersity index (PDI) value.

4.3.8. Ethidium bromide binding

Fresh and rehydrated samples containing 0.5 µg pDNA were tested in 1% agarose gel stained with ethidium bromide (EtBr) (0.01%) using Bio-Rad PowerPac HC electrophoresis apparatus (Biorad, Mississagua, ON, Canada) in tris-acetate-EDTA (TAE) buffer at 100 V for 45 minutes. EtBr was visualized by UV fluorescence using an AlphaImager™ (Alpha Innotech, San Leandro, CA, USA).

4.3.9.Circular dichroism spectroscopy

Fresh, lyophilized, and stored formulations (3 months), prepared/reconstituted to a 15 µg/mL pDNA concentration, were evaluated by using circular dichroism (CD) spectroscopy. CD spectra were

obtained by using a Pi-star-180 instrument (Applied Photophysics, Leatherehead, UK) with 2 nm slit at 37 ˚C under a N_2 atmosphere.

4.3.10. Cell culture and *in vitro* transfection

COS-7 African green monkey kidney fibroblasts cell line (ATCC, CRL-1651) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% antibiotic and incubated at 37 ˚C with 5% CO_2. On the day before transfection, the cells were seeded in 96-well tissue culture plates (Falcon, BD Mississauga, ON, Canada) at a density of 1.5×10^4 cells/well. One hour prior to transfection, the supplemented DMEM was replaced with DMEM. The cells were transfected with 0.2 µg pGThCMV.IFN-GFP plasmid/well in quadruplicate. Lipofectamine Plus reagent (Invitrogen Life Technologies) was used as a positive control according to the manufacturer's protocol with 0.2 µg pDNA/well in quadruplicate. The 96-well tissue culture plates were then incubated at 37 °C in CO_2 for five hours. The transfection agents were removed and replaced with supplemented DMEM. Supernatants containing the secreted IFN-γ were collected at 24, 48 and 72 h and replaced with fresh supplemented DMEM. The collected supernatants were stored at -80 ˚C.

4.3.11. Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was performed using flat bottom 96-well plates (Immulon 2, Greiner Labortechnik, Frickenhausen, Germany) following the BD Pharmingen protocol and as described earlier.[4] The concentration of expressed IFNγ was calculated from a standard IFNγ curve using recombinant mouse IFN-γ standard (BD Pharmingen, BD Biosciences).

4.3.12. Statistical analysis

Statistical analyses were performed using SPSS software (Version 17.0). Results expressed as the average of $n \geq 3 \pm SD$. One way analysis of variance (ANOVA, Dunnett's test) and Pearson's correlation were used for statistical analyses. Significant differences were considered at $p<0.05$ level.

4.4. Results

4.4.1. Characterization of fresh formulations

Sugars are widely used as cryoprotectants and stabilizing agents for lyophilized lipid-based gene delivery systems.[29,32,37] Based on preliminary formulation studies (results not shown), sucrose and trehalose were selected for this work. In addition, two different formulation approaches were developed in an aim to maintain the essential physiochemical properties of the P/G/L delivery system (i.e., particle size and zeta potential) upon lyophilization. For the first method [Method A, Table 4.1], the pDNA was complexed with the gemini surfactant to form P/G lipoplexes first, and the DOPE vesicles were incorporated afterward. [P/G/L-S] lipoplexes prepared by this method, formulated in 9.25% sucrose, were comprehensively characterized in our previous work.[36,38] The lipoplexes had a particle size of 124±1.9 nm and zeta potential of +29±4.8 mV [Table 4.2-A]. However, when 10% trehalose was used in the formulation [P/G/L-T] instead of sucrose, a major drop in both size and zeta potential (average size of 81.7±0.6 nm, zeta potential of +21.4±2.5) were observed [Table 4.2-A].

In addition to the previously established lipoplex preparation method [Method A], another formulation method was described in this work [Method B, Table 4.1]. In the P-[G/L-S] formulation, sucrose solution (9.25%) was used to prepare the G/L vesicles. While the composition of this formulation was the same as [P/G/L-S], this preparation method caused a significant increase in both particle size (by approximately 60 nm) and zeta potential (16 mV) [Table 4.2-A]. Similarly, trehalose was used as cryoprotectant to prepare formulation P-[G/L-T] following Method B [Table 4.1]. Both particle size and zeta potential showed in excess of 65% increase compared to the chemically identical formulation prepared by Method A [P/G/L-T].

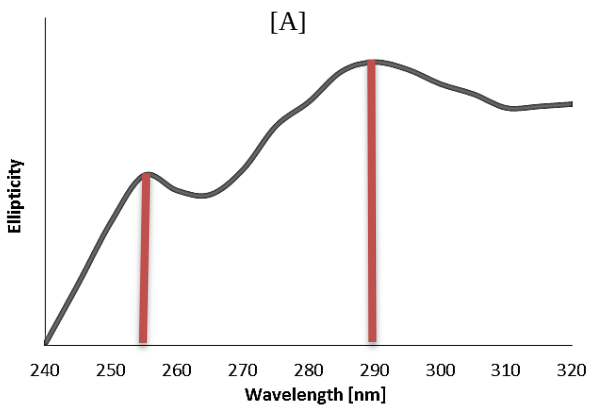
Table 4.2: The influence of lyophilization process on the physiochemical properties (particle size, zeta potential and pH of lipoplexes

Formulation	[A] Fresh (time zero)			[B] After lyophilization		
	Size (nm) (PDI)	Zeta potential (mV)	pH	Size (PDI)	Zeta potential (mV)	pH
[P/G/L-S]	124.3±1.9 (0.220±0.007)	29.0±4.8	5.8±0.08	126.8±1.8 (0.237±0.023)	36.4±5.9	5.7±0.15
[P/G/L-T]	81.7±0.6 (0.221±0.007)	21.4±2.5	7.4±0.17	100.8±1.3 (0.298±0.009)	23.3±2.3	7.0±0.07
P-[G/L-S]	183.6±2.7 (0.393±0.012)	45.3±1.8	6.0±0.10	194.3±5.6 (0.377±0.012)	47.7±5.2	5.8±0.08
P-[G/L-T]	158.7±2.7 (0.289±0.016)	35.8±1.5	6.8±0.17	199.7±4.0 (0.250±0.016)	49.7±4.0	6.8±0.17

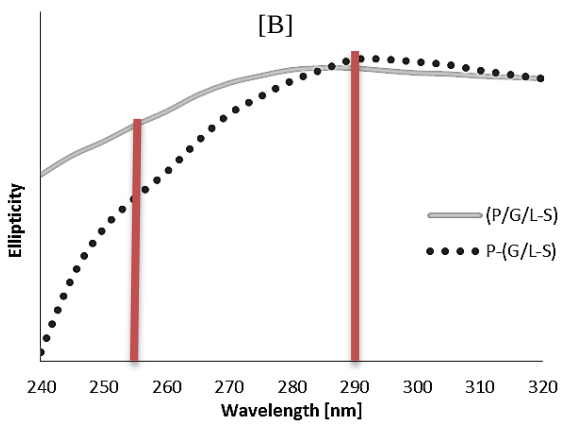
Values are shown as the average of three measurements of each formulation at [A] zero time (fresh) and [B] just after lyophilization cycle ± standard deviation.

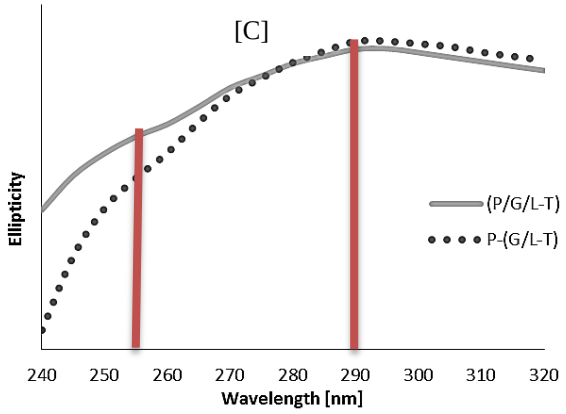
Circular dichroism (CD) measurements showed that all fresh formulations induced changes in the native structure of the DNA, as observed in the alterations in the CD spectra [Figure 4.2]. The spectrum of free pDNA showed two positive peaks at 255 nm and 290 nm and a negative tail in the region of 240-250 nm [Figure 4.2-A]. Upon complexation of the pDNA with the fresh formulation [P/G/L-S], a blue-shift was observed for the positive peak at 290 nm and a depression of the 255 nm peak [Figure 4.2-B]. Conversely, the [P/G/L-T] formulation caused a red-shift of the positive peak at 290 nm and a flattening of the 255 nm peak [Figure 4.2-C]. The complexation of pDNA with the [G/L] system caused a red-shift of the 290 nm peak with a negative tail for the area below 270 nm in both P-[G/L-S] and P-[G/L-T] formulations.

Fresh pDNA

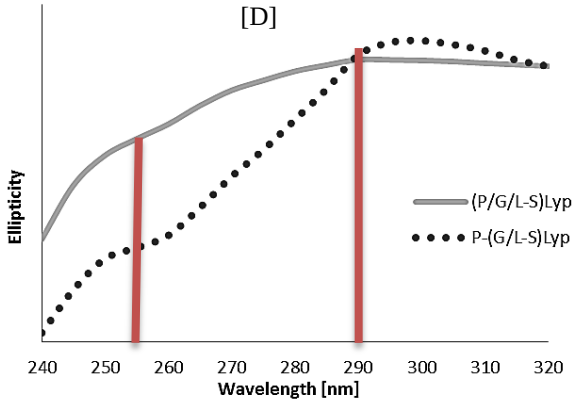


Fresh Formulations





Lyophilized Formulations



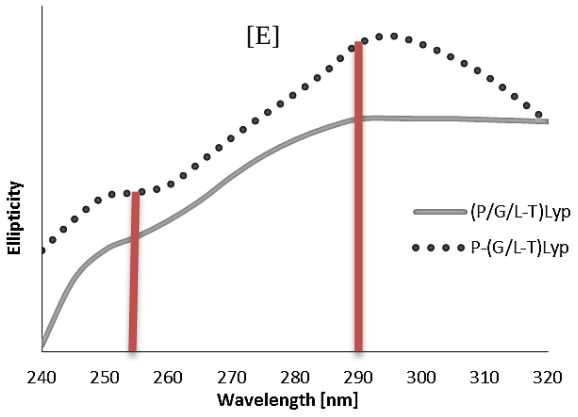


Figure 4.2: Circular dichroism [CD] of [A] free pDNA, [B,C] fresh formulations and [D,E] the lyophilized formulations. Values are average of three measurements [n=3].

The influence of different sugars and preparation methods on the *in-vitro* transfection activity of fresh formulations was also investigated [Figure 4.3, white bars]. All fresh formulations showed significant levels of gene expression compared to non-transfected COS-7 cells and cells treated with [G/L] system. The [P/G/L-S] formulation showed the highest gene expression activity with 8.2±2.6 ng of IFNγ/1.5×10^4 COS-7 cells after 72 hour of the transfection. The lowest gene expression among all fresh formulations was observed for the [P/G/L-T] formulation, which showed 2.3±1.9 ng IFNγ/1.5×10^4 COS-7 cells. Formulation P-[G/L-S] [Method B] showed significantly lower gene expression activity compared with the corresponding [P/G/L-S] formulation. However, no significant difference between the transfection efficiency of the P-[G/L-T] and formulation [P/G/L-S] was observed.

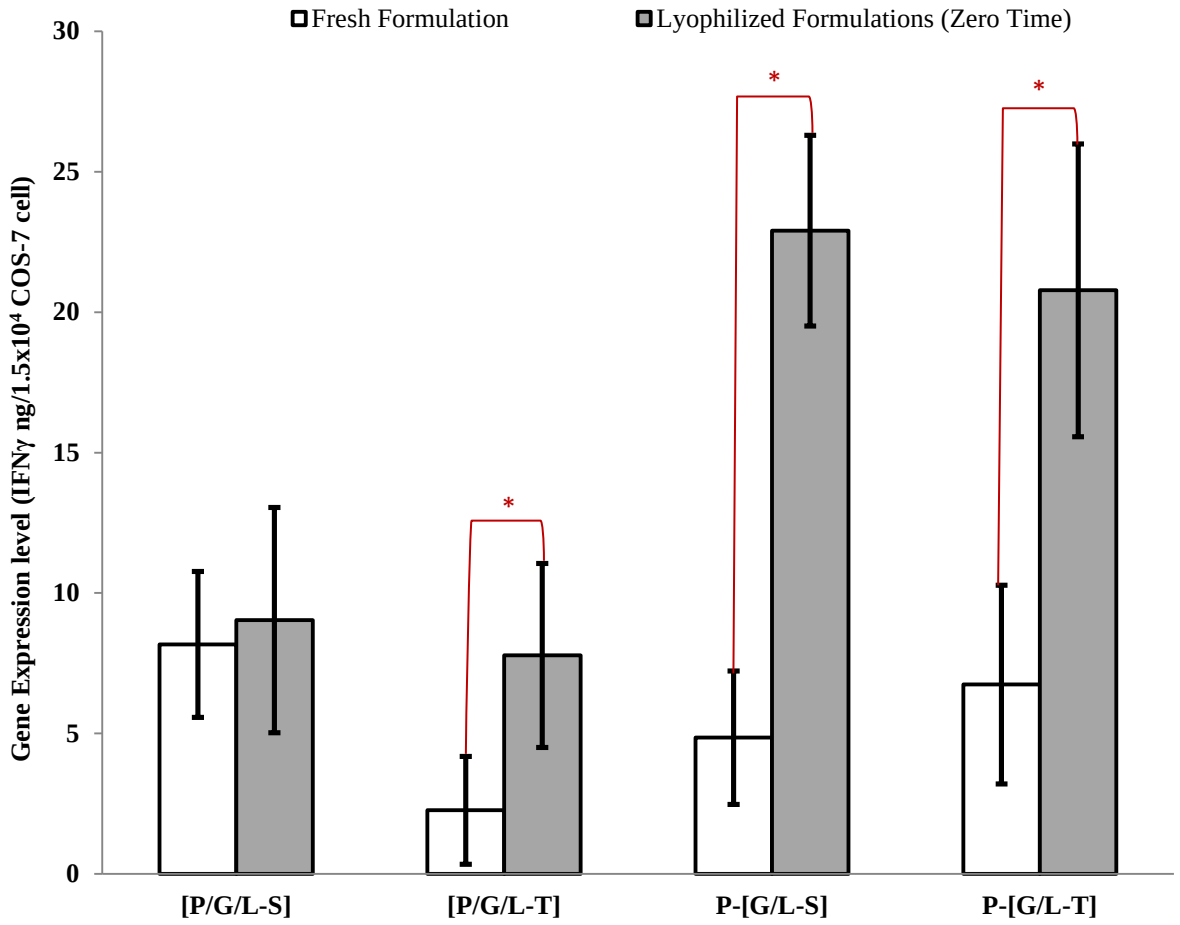


Figure 4.3: Gene expression activity of lipoplex (ELISA-IFNγ) after 72 h. White columns represent fresh formulations. Grey columns represent the influence of lyophilization [lyophilized formulations]. Results are average of three samples of each formulation (n=3), error bars ± SD. * Indicates significant at $p < 0.05$.

4.4.2.Influence of lyophilization/rehydration processes

4.4.2.1. Particle size and zeta potential

The lyophilized formulations $[P/G/L\text{-}S]_{lyp}$ and $[P/G/L\text{-}T]_{lyp}$ showed particle sizes less than 130 nm whereas the particle size values of the formulations $P\text{-}[G/L\text{-}S]_{lyp}$ and $P\text{-}[G/L\text{-}T]_{lyp}$ were approximately 200 nm [Table 4.2-B]. The lyophilization process caused a significant increase ($p < 0.05$) in the particle size of all formulations (11 to 41 nm) except for the [P/G/L-S] formulation (2 nm increase was observed). The zeta potential [Table 4.2-B] increased in all formulations upon lyophilization (2 to 13 mV).

4.4.2.2. DNA compaction

Similar to fresh formulations, lyophilized formulations altered the CD spectra of pDNA. The CD spectra of $[P/G/L\text{-}S]_{lyp}$ and $[P/G/L\text{-}T]_{lyp}$ showed an increase in the positive ellipticity of the 290 nm peak and a flat positive area above 290 nm. In addition, a blue-shift of the peak at 255 nm with a depression to negative values was observed [Figure 4.2, D and E, solid line].

The lyophilized [G/L] system in the case of formulations P-$[G/L\text{-}S]_{lyp}$ and P-$[G/L\text{-}T]_{lyp}$ caused a red-shift of the 290 nm peak to 295 nm and blue-shift of the 255 nm with a significant depression to negative values [Figure 4.2, D and E, dotted line].

4.4.2.3. Ethidium bromide binding

The influence of lyophilization on the interaction between the pDNA and gemini surfactant 12-7NH-12 was assessed by using ethidium bromide binding assay and gel electrophoresis [Figure 4.4]. The gel image shows that the pDNA was completely retarded in all freshly prepared [P/G/L] and P-[G/L] systems incorporating both sucrose and trehalose cryoprotectants, indicating that it was totally shielded by the gemini surfactant [Figure 4.4-B]. The lyophilization process had no effect on the pDNA/gemini surfactant interaction as no pDNA migration was observed in any of the lyophilized formulations [Figure 4.4-C].

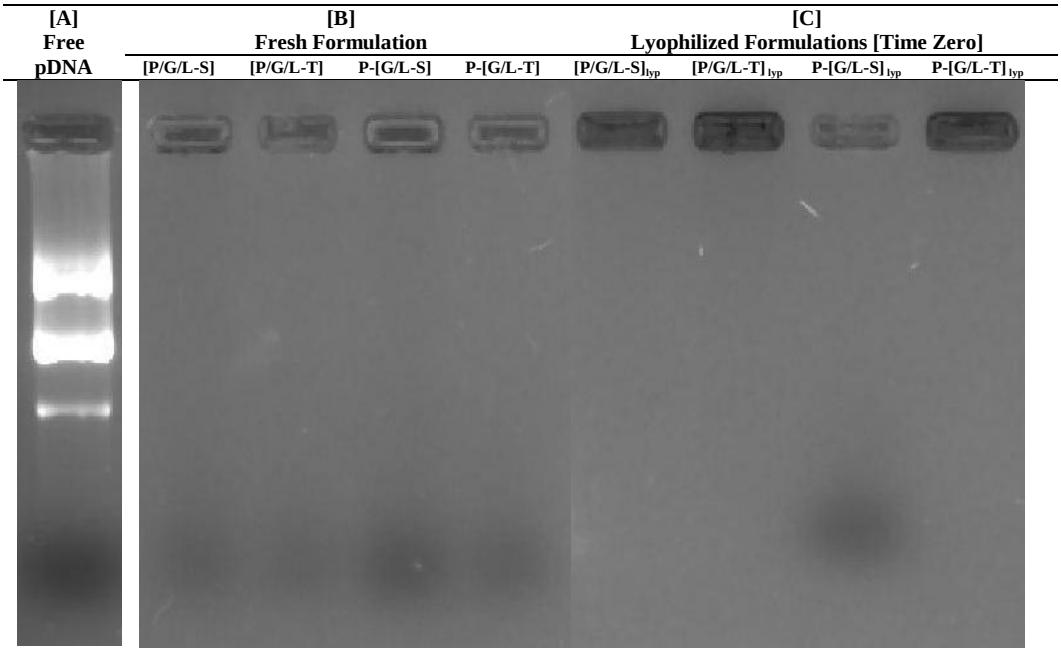


Figure 4.4: Ethidium bromide binding assay using agarose gel electrophoresis [A] free pDNA 0.5 µg, [B] fresh formulations showed total binding of the pDNA to the gemini surfactant with no pDNA band being observed in all four formulations, [C] lyophilized formulations, no pDNA band was observed in all formulations upon lyophilization proving that the lyophilization process did not affect the pDNA-gemini surfactant binding.

4.4.2.4. *In vitro* transfection activity

Lyophilized formulations were evaluated for their *in-vitro* transfection in COS-7 cell line to investigate the influence of the lyophilization process on the gene expression activity [Figure 4.3, gray bars]. Interestingly, freeze-drying significantly improved the transfection activity of three formulations ($p < 0.05$) in comparison to corresponding fresh formulations. The P-[G/L-S]$_{lyp}$ formulation showed the most significant improvement in transfection activity (approximately 3.5 fold). Similarly, the [P/G/L-T]$_{lyp}$ and P-[G/L-T]$_{lyp}$ formulations exhibited a significant increase (2.5 fold). The [P/G/L-S]$_{lyp}$ formulation showed no significant change in transfection activity after lyophilization.

4.4.3. Stability study

4.4.3.1. Particle size and zeta potential

The lyophilized cake of all formulations retained a free powdery appearance throughout the stability study [Figure 4.5]. The rehydrated formulations at all sampling times were clear dispersions with no visible particles.

Particle size and zeta potential values, of the lyophilized formulations, were measured during the stability study, and compared to the corresponding fresh non-lyophilized formulations (time zero) [Figure 4.6].

The particle size and PDI of the formulations preserved with sucrose, $[P/G/L\text{-}S]_{lyp}$, revealed no significant changes during the three-month study compared with the fresh lipoplexes [P/G/L-S] at time zero. Conversely, the particle size and PDI values of the lyophilized $[P/G/L\text{-}T]_{lyp}$ formulation, stabilized with trehalose, increased with time and displayed a significant size increase of approximately 20% within the first month of storage ($p < 0.05$).

The values of the particle size of formulations P-$[G/L\text{-}S]_{lyp}$ and P-$[G/L\text{-}T]_{lyp}$ displayed some fluctuation during the study. For instance, in comparison to the fresh P-[G/L-S] at time zero, the particle size of lyophilized P-$[G/L\text{-}S]_{lyp}$ showed a significant decrease within the first two months of storage ($p < 0.05$). The particle size decreased significantly ($p < 0.05$) from 183±2.7 nm at time zero to 113±3.9 nm at the second sampling point. After 3 months, an increase in particle size was observed (129 nm) in comparison to the value at the 2-month time. Nevertheless, the particle size remained within the range of 100-200 nm at all sampling points.

The influence of storage on the zeta potential of lyophilized formulations was monitored through the three-month stability study [Figure 4.6-B]. In all lyophilized formulations, a positive zeta potential was maintained during storage. Formulations $[P/G/L\text{-}S]_{lyp}$ showed significant ($p <$

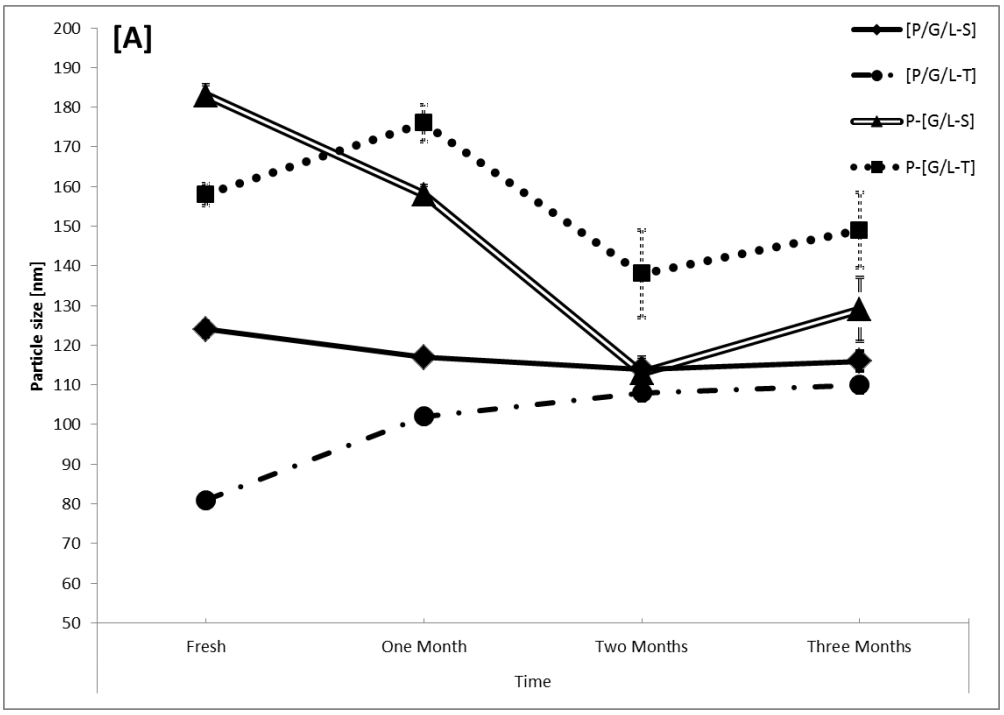
0.05) increase in zeta potential values upon storage with a maximum increase observed at the three month sampling point (46±5.1 mV). The zeta potential for formulations $[P/G/L\text{-}T]_{lyp}$ remained steady during storage (ranging from 20 to 24 mV), whereas, the $P\text{-}[G/L\text{-}S]_{lyp}$ formulation fluctuated slightly during storage. Finally, the zeta potential of lyophilized lipoplexes of $P\text{-}[G/L\text{-}T]_{lyp}$ showed a significant increase (20%, $p < 0.05$) after one month of storage followed by a significant, continuous decrease with time to the 3-month value of 20.8+6.6 mV. Overall, the zeta potential of all formulations remained positive throughout the storage period.

$[P/G/L\text{-}S]_{lyp}$ $[P/G/L\text{-}T]_{lyp}$ $P\text{-}[G/L\text{-}S]_{lyp}$ $P\text{-}[G/L\text{-}T]_{lyp}$

[A] Lyophilized formulations just after the freeze-drying cycle

[B] Lyophilized formulations after three months of storage at 25 ˚C

Figure 4.5: the appearance of lyophilized cake of four formulations [A] just after the freeze drying and [B] the influence of time after three months of storage at 25 ˚C.



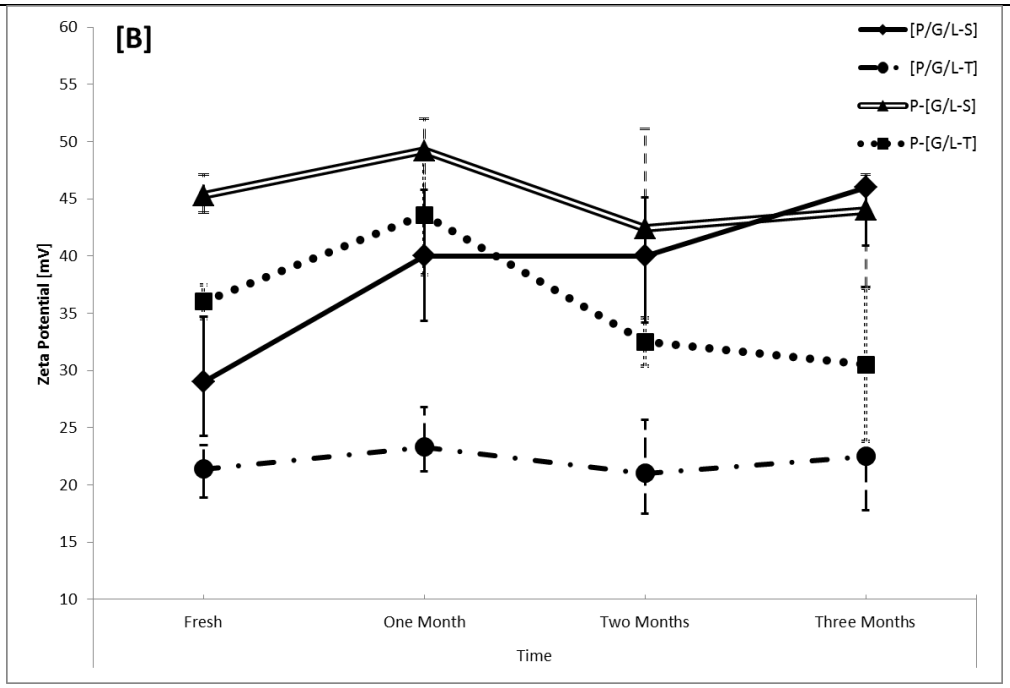


Figure 4.6: The influence of time on [A] the particle size and [B] zeta potential stored at 25 ˚C. Results are average of three samples of each formulation (n=3), error bar ± SD.

4.4.3.2. Ethidium bromide binding

The pDNA band was not observed in all lyophilized formulations after three months of storage at 25 °C/75% RH. This observation provided evidence that the complete interaction of the pDNA with the cationic gemini surfactant was maintained throughout the study [Figure 4.7-B].

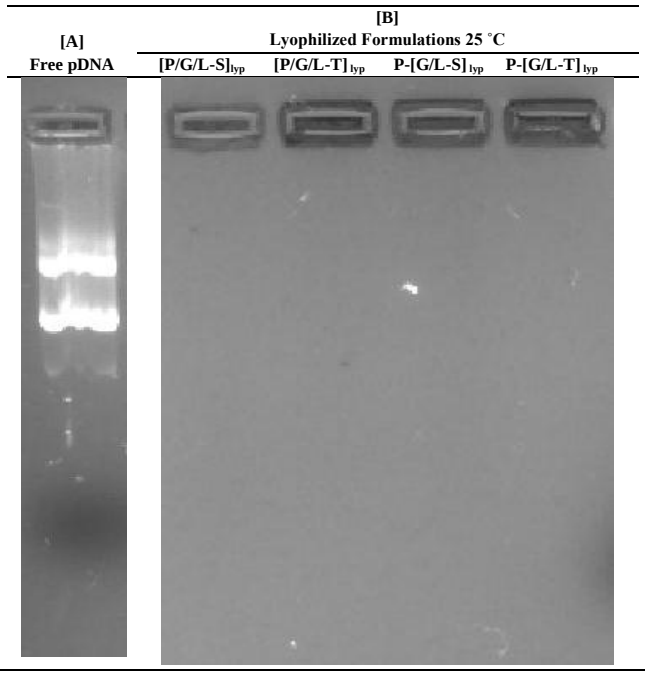


Figure 4.7: Ethidium bromide binding assay using agarose gel electrophoresis [A] free pDNA 0.5 µg, [B] lyophilized formulations stored at 25 ˚C for three months.

4.4.3.3. Moisture content

The moisture content of lyophilized formulations was determined to assess the efficiency of the freeze-drying process and to evaluate the effect of storage on the moisture content. Following the freeze-drying cycle, the moisture content in all formulations was less than 2% (w/w). After three months of storage at 25 ˚C, the moisture content in the lyophilized cake increased by 55-170% compared to the values reported just after the lyophilization [Table 4.3].

Table 4.3: Moisture content of lyophilized formulations (%w/w)

Formulation	**Before storage**	**After storage**
$[P/G/L\text{-}S]_{lyp}$	1.8±0.2	2.8±0.2
$[P/G/L\text{-}T]_{lyp}$	1.5±0.3	3.1±0.5
$P\text{-}[G/L\text{-}S]_{lyp}$	1.3±0.2	3.5±0.2
$P\text{-}[G/L\text{-}T]_{lyp}$	1.5±0.3	3.0±0.1

Values are shown as the average of three measurements of each formulation at each sampling point ± standard deviation.

4.4.3.4. *In vitro* transfection activity

Upon storage at 25 ˚C, formulations P-[G/L-S]$_{lyp}$ and P-[G/L-T]$_{lyp}$ were able to preserve the transfection levels of their corresponding fresh non-lyophilized formulations for up to two months of storage [Figure 4.8].

After three months, formulations [P/G/L-S]$_{lyp}$, P-[G/L-S]$_{lyp}$ and P-[G/L-T]$_{lyp}$ showed a significant decrease in transfection activity with maximum reduction reported for formulations P-[G/L-S]$_{lyp}$ and P-[G/L-T]$_{lyp}$ (> 70% and 80% loss in activity, respectively, Figure 4.8). The transfection activity of [P/G/L-S]$_{lyp}$ was reduced by approximately 40% compared to the original activity of non-lyophilized [P/G/L-S]. Formulation [P/G/L-T]$_{lyp}$ was the most stable formulation in terms of retaining its starting transfection activity, with no significant change observed throughout the three month period of study.

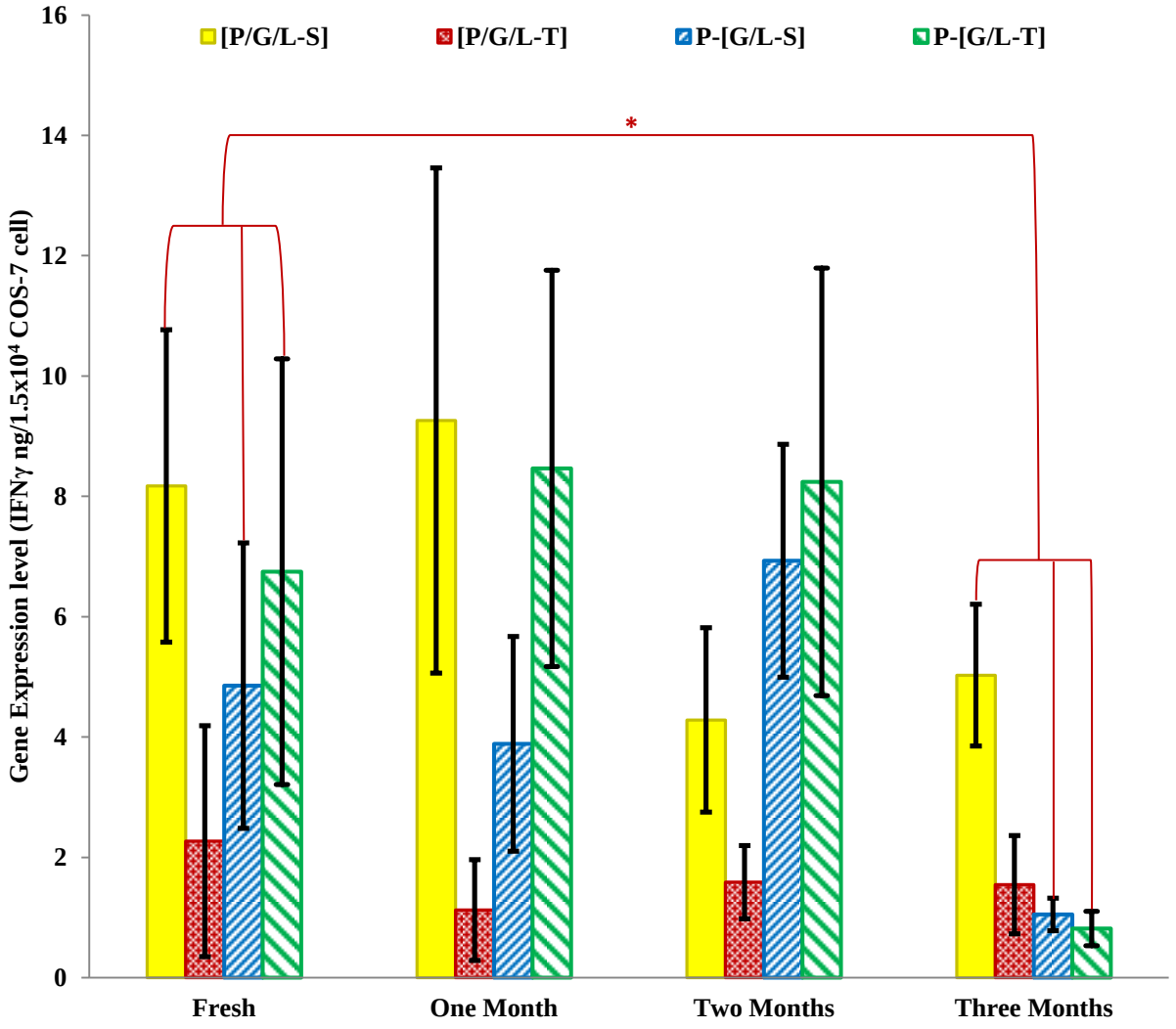


Figure 4.8: *In vitro* transfection activity of the lyophilized formulations stored at 25 ˚C for three months (ELISA-IFNγ). Results are the average of three samples of each formulation (n=3), error bars ± SD. * Indicates significant at $p < 0.05$ level.

4.5. Discussion

The purpose of this work was to evaluate freeze-drying as a technique to improve the stability of pDNA/gemini surfactant lipoplexes and to investigate the influence of the lyophilization and storage conditions on the essential physiochemical properties and *in-vitro* transfection activity of the lipoplexes. We have developed in recent years a series of cationic gemini surfactants as a chemical carrier for DNA delivery.[4,9,38,39] Although a significant improvement in gene expression activity and improvement in the cellular safety profile have been achieved, the instability of the pDNA/gemini surfactant lipoplexes in aqueous formulations remained a concern. Due to several physical and chemical factors, freshly prepared formulations showed loss of transfection activity after one week of storage at room temperature (results not shown). The physical instability of the lipoplexes is a result of changes in physiochemical properties such as particle size and positive zeta potential. When stored at room temperature, these positively charged particles tend to form micro-sized aggregates as a function of random collisions, Brownian motion, and gravity forces.[18,40] These changes can cause the loss of the supra-molecular structure of the lipoplexes leading to the leakage of the pDNA from the lipoplex and loss of its biological activity.[18,40] The chemical stability of the different components of the lipoplexes (i.e., pDNA, gemini surfactant, DOPE) depends on the storage environment, namely pH of the formulation, temperature and the presence of metal contaminants.[15-17,27,41] All these factors can lead to loss of the integrity of the lipoplexes and reduction of the gene delivery efficiency. Thus we investigated different formulation strategies to optimize the physiochemical stability of the lipoplexes and evaluate whether lyophilization could preserve their structural integrity and transfection efficiency.

As mentioned previously, the lyophilization process includes three stress steps that can destabilize the lipoplexes: freezing, drying (dehydration) and rehydration. Aggregation of the lipoplexes and a shift to negative zeta potential upon lyophilization/rehydration were observed when formulations were prepared without cryoprotectant agents (results not shown). For that reason, we evaluated several cryo-/lyo-protectants to determine whether they could stabilize and protect the lipoplexes during the freezing and lyophilization processes. The physiochemical properties that were examined included the particle size and zeta potential of the lipoplexes as several previous studies had reported a strong relation between the physiochemical properties of lipoplexes and cellular uptake and consequently, transfection activity.[12,42,43] In the pilot evaluation of the cryoprotectant activity of different agents (data not shown), we assessed a number of stabilizing agents as a function of concentration and different combinations. These agents included monosaccharaides (glucose), disaccharides (sucrose, trehalose, lactose), polymers (polyethylene glycol) and simple polyol (glycerin). Disaccharide sugars, sucrose and trehalose, effectively maintained the particle size and the positive surface charge of the lipoplexes after lyophilization, similar to previous assessments that evaluated these agents during the lyophilization of chemically based gene delivery vectors.[29,34,44,45] Based on these findings, sucrose and trehalose were selected as lyo-/cryo-protectant agents to investigate the factors affecting the long-term stability of lyophilized gemini surfactant based lipoplexes.

4.5.1. Characterization of fresh formulations

The physiochemical and biological properties of four different lyophilized formulations were evaluated for stability at room temperature (25 ˚C). Fresh formulation [P/G/L-S] prepared in 9.25% sucrose, our standard formulation, showed the highest gene expression activity among all fresh formulations evaluated in this work. The replacement of sucrose by 10% trehalose in the

formulation [P/G/L-T] caused significant changes in physiochemical properties, as the particle size and zeta potential both decreased. Additionally, CD results showed that the [P/G/L-T] altered the native structure of pDNA in a different manner compared to standard formulation [P/G/L-S]. As a result, the transfection activity was severely hampered resulting in a low level of IFNγ expression (60% reduction compared with [P/G/L-S]). The reduction in the particle size can be attributed to the pH-active imino group of the 12-7NH-12 gemini surfactant as the pH value of [P/G/L-T] formulation increased to 7.4 as compared to fresh [P/G/L-S] formulation (pH 5.8) [Table 4.2-A].[38] Given these results, we hypothesize that trehalose produces strongly compacted lipoplexes that hinders the release of the pDNA after the cellular uptake, thus causing a lower level of gene expression.

Formulations P-[G/L-S] and P-[G/L-T] were prepared initially to investigate the effect of the lyophilization process and storage conditions on the gemini surfactant/DOPE [G/L] lipid structure. They have the same chemical composition as formulations [P/G/L-S] and [P/G/L-T], respectively.

However, elimination of the pDNA from the lyophilized complex could permit the determination of whether any alteration in transfection activity, upon lyophilization and storage, is a result of DNA degradation or due to changes in the [G/L] polymorphic structure. The modification of the preparation method led to changes in the physiochemical properties and transfection activity. Fresh formulations P-[G/L-S] and P-[G/L-T] formed lipoplexes with larger particle size and greater positive zeta potential values compared to formulations [P/G/L-S] and [P/G/L-T], prepared by method A. In the standard formulation method [Method A, Table 4.1], the primary lipoplexes are formed by the electrostatic interaction between the gemini surfactant and pDNA producing the [P/G] lipoplexes. After the formation of the P/G lipoplexes, addition of

DOPE vesicles can induce polymorphic changes in the lipoplex structure by producing a lipid bilayer packed in lamellar, cubic or inverse hexagonal morphologies.[8,38] The excess of the gemini surfactant (10 to 1 positive to negative charge ratio) provides the positive surface charge for the P/G/L system. On the other hand, the first step in the preparation of P-[G/L] systems [Method B, Table 4.1] involves the formation of [G/L] vesicles by the incorporation of the gemini surfactant into the DOPE film. Therefore, it is assumed that different polymorphic structures were induced in which the gemini surfactant molecules with DOPE form a bilayer lipid membrane and the positively charged gemini surfactant molecules are distributed in both layers. The addition of pDNA to the [G/L] bilayer system caused the formation of lipoplexes with larger particles that are more positively charged than the [P/G/L] systems. The [G/L] lipid systems were able to completely interact with the pDNA as no pDNA bands were observed in ethidium bromide gel-electrophoresis [Figure 4.4-B] and this interaction caused changes in the pDNA structure as the CD spectra of pDNA was also shown to be altered. It is important to note that, CD spectra obtained from fresh P-[G/L-S] and P-[G/L-T] formulations were nearly identical but differed significantly from the spectra of the fresh [P/G/L-S] and [P/G/L-T] formulations [Figure 4.2-B,C].

These modifications in physiochemical properties caused significant changes in the biological activity as observed by the levels of gene expression. In the case of P-[G/L-S] formulation, the transfection activity was considerably reduced (40%) in comparison to [P/G/L-S]. Conversely, formulation P-[G/L-T] showed a substantial improvement in gene expression level compared to [P/G/L-T]. Since not all the gemini surfactant molecules in [G/L] vesicles were available to completely interact with the pDNA, the lipoplexes formed in the P-[G/L] formulations resulted in less compacted pDNA. Therefore, the lower gene expression activity of

the P-[G/L-S] systems could be related to the loose DNA-compaction that could cause premature release of the DNA before nuclear internalization. On the other hand, the subordinate pDNA compaction caused by [G/L-T] improved the transfection activity of P-[G/L-T] lipoplexes in comparison to highly compacted [P/G/L-T] lipoplexes.

4.5.2. Influence of lyophilization on the lipoplexes properties

A major focus of this work was to examine the effect of the lyophilization process on the transfection efficiency of the lipoplexes. A significant improvement in gene expression activity was observed for three formulations upon freeze-drying; $[P/G/L\text{-}T]_{lyp}$, $P\text{-}[G/L\text{-}S]_{lyp}$ and $P\text{-}[G/L\text{-}T]_{lyp}$ [Figure 4.3]. The $P\text{-}[G/L\text{-}S]_{lyp}$ and $P\text{-}[G/L\text{-}T]_{lyp}$ formulations showed more than a 40% increase in gene expression levels compared to the activity of standard fresh formulation [P/G/L-S].

The results indicated that both particle size and zeta potential increased after lyophilization in all formulations [Table 4.2-B]. Additionally, the lyophilization altered the CD spectrum of pDNA in a different manner compared to the CD spectra obtained from fresh formulations [Figure 4.2, D and E]. We believe that these modifications were caused by changes in the lipid phase arrangement as a result of the freezing and dehydration cycles of the lyophilization protocol. In fact, previous studies reported relatively similar increases in transfection activity of cationic lipid/DNA complexes after freezing or lyophilization stresses. For instance, cationic hydroxyethylated cholesterol (DMHAPC-Chol) co-formulated with helper lipid DOPE as a system for gene delivery showed a four to five-fold increase in gene expression activity after lyophilization compared to the fresh non-lyophilized liposomes.[46] The improvement in gene activity profile was correlated to the degree of hydration of the phosphate head groups of DOPE leading to polymorphic phase change as observed by NMR spectra of hydrated and dehydrated

lipid systems.[46] Similarly, a three-fold increase in transfection activity of the DOTAP:DOPE system was observed after a freeze-thaw cycle compared to freshly prepared lipoplexes in the absence of the cryoprotectant.[22] However, lyophilization did not increase the transfection efficiency of the lipoplexes prepared with sucrose as cryoprotectant, the activity remained at the level of the fresh formulation.[22] The improvement in gene expression activity was only reported in lipoplexes co-formulated with DOPE and the increase of transfection activity of frozen formulation was justified by possible structural alteration of DNA/lipid complexes induced by the freezing stress.[22] It should be noted that in both studies, no cryo- or lyo-protectant agents were employed during the freezing/drying cycles.

In the present study, improvements of transfection activity were only observed for three lyophilized formulations and only when disaccharide sugars (sucrose or trehalose) were used. The improvement can be attributed to the alteration in the supramolecular structure of the lipoplexes induced by the disaccharide sugars during the freeze-drying cycle. A number of studies have proposed different hypotheses that explain the protective mechanisms of lyo-/cryo-protectant agents during the freeze-drying process of biopharmaceutical products. These hypotheses include: preferential exclusion, vitrification, particle isolation and water replacement hypotheses.[47-51] The water replacement hypothesis is a well-established hypothesis proposing that lyoprotectant sugars are able to form hydrogen bonds with the lipid phase of liposomes and replace the surrounding water molecules leading to stabilization of the structure of lipid membrane during the dehydration phase. [50,51]

Based on the water replacement hypothesis, we believe that the disaccharide sugars (sucrose and trehalose) form a hydrogen bond with the C=O and P=O moieties of the DOPE head group lipid molecules, more favorably than with the quaternary ammonium head group or

the nitrogen atom of the spacer region of the gemini surfactant.[52,53] In addition, DOPE is more abundant on the outer surface of the lipoplexes compared to 12-7NH-12 molecules (1 to 10 molar ratio). This interaction facilitates the preservation of the original supramolecular structure of the fully hydrated system during the drying step. Additionally, the effect of replacing water molecules by sugar can explain the increase of the particle size upon lyophilization. Consequently , after rehydration of lyophilized formulations, we assume an alteration of the surface properties of $[G/L]_{lyp}$ systems occurred due to partial rehydration of the DOPE molecules imposing the formation of an inverted hexagonal phase rather than the cubic or lamellar phases.[54,55] Previous studies indicated that the [P/G/L] systems exist in a mix of polymorphic phases (i.e., lamellar, cubic and hexagonal).[8] It was established that the inverted hexagonal phase is responsible for high transfection in *in-vitro* transfection.[56,57] Thus, upon rehydration, the P/G/L system might assume more hexagonal structure rather than a polymorphic assembly. We plan to investigate the assembly of the rehydrated formulations in the future by small and wide angle x-ray scattering.

Another potential explanation for the increase in the transfection activity might be due to the stress resulting from the freezing step (i.e., cryoconcentration effect) and the interaction of sugar molecules with the [G/L] lipid phase. It was established that this effect could cause fusion of the lipid bilayer membrane.[58,59] Based on this, it is possible that during the freezing cycle, free 12-7NH-12 vesicles (or free molecules) present in the fresh formulations, particularly in the case of P-$[G/L\text{-}S]_{lyp}$ and P-$[G/L\text{-}T]_{lyp}$, were incorporated in the G/L vesicles. Upon rehydration and the addition of pDNA, the lipoplexes might have formed at an higher apparent +/– charge ratio compared to fresh P-[G/L-S] and P-[G/L-T].[22] In fact, this mechanism can explain the

increase in zeta potential values that are observed after the lyophilization/rehydration cycle [Table 4.2-B].

4.5.3. Stability study

We evaluated the stability of four lyophilized formulations stored at 25 ˚C for three months to investigate the ability of lyophilization to improve the shelf stability of gemini surfactant lipoplexes. It was found that the ability of the formulations to preserve the transfection activity was dependent on the formulation method and the nature of the protectant sugar.

During the stability study, the lyophilized cake in all formulations maintained the original solid aspect and no shrinking or collapse of the lyophilized cakes was observed [Figure 4. 5-B]. Additionally, the lyophilized products were reconstituted to form clear dispersions with no aggregation or large particles. All lyophilized formulations retained particle size within the original size range (100-200 nm) and positive zeta potential values throughout the three months [Figure 4.6]. The lyophilized formulations containing the pDNA (i.e., $[P/G/L\text{-}S]_{lyp}$ and $[P/G/L\text{-}T]_{lyp}$) were able to preserve adequate levels of gene expression up to three months of storage. Formulation $[P/G/L\text{-}S]_{lyp}$ maintained the same transfection activity as the fresh formulation (time zero) for one month of storage and approximately 60% of original activity after three months of storage [Figure 4.8]. Formulation $[P/G/L\text{-}T]_{lyp}$,with trehalose as freeze-drying protectant agent, was able to preserve almost 70% of the gene expression activity of the fresh [P/G/L-T] formulation at the end of the study [Figure 4.8]. We believe that the partial loss of the transfection activity of $[P/G/L\text{-}S]_{lyp}$ and $[P/G/L\text{-}T]_{lyp}$ formulations is due to conformational changes in the lipoplex structure, particularly in the presence of sucrose cryoprotectant [Figure 4.6]

While formulations P-[G/L-S]$_{lyp}$ and P-[G/L-T]$_{lyp}$ stored at 25 ˚C were able to maintain their transfection activity for two months, during the third month both formulations lost more than 60% of their original transfection activity. As there was no difference between these formulations and the [P/G/L-S]$_{lyp}$ and [P/G/L-T]$_{lyp}$ formulations lyophilized and stored with the pDNA, we believe that the loss of the activity after three months of storage is a result of the loss of the [G/L] bilayer arrangement resulting from the freeze-drying cycle rather than DNA degradation.

The moisture content of lyophilized formulations showed an increase with time for most of the formulations upon storage [Table 4.3]. However, no correlations were observed between the changes in the moisture content and the physiochemical properties and biological activity of the lyophilized formulations during the stability study at both conditions. In fact, Yu and co-workers, found that there is no correlation between the biological activity of lyophilized DC-Cholesterol:DOPE-based lipoplexes and the level of moisture content when samples were stored for three months at room temperature.[60]

4.6. Conclusion

We evaluated the practicality of lyophilization to formulate cationic gemini surfactant-based lipoplexes with long-term stability at room temperature. Both trehalose and sucrose were useful as lyoprotectant agents to stabilize the physiochemical properties of lipoplexes during the freeze-drying. Substantial enhancements in transfection efficiencies of gemini surfactant/DNA lipoplexes after lyophilization of G/L systems were observed. These observations were attributed to more balanced compaction of the DNA and the possible formation of inverted hexagonal phase lipoplexes. Lyophilization appears to be acceptable as a formulation technique to prepare highly efficient gemini surfactant-based lipoplexes. The stability study at 25 ˚C showed that the lyophilized $[G/L]_{lyp}$ lipoplexes formulated with sucrose and trehalose can be stored at room temperature for up to two months without significant changes in physiochemical properties or gene expression activity. The loss of transfection activity upon storage is most probably due to the conformational changes in the supramolecular structure of the lipid phase that result during the lyophilization process.

Detailed structural characterizations of the lyophilized gemini surfactant:DOPE complexes and [P/G/L] lipoplexes are essential for further optimizations of the formulation strategies and to improve the lyophilization technique to achieve long-term stability. This can be achieved by using advance techniques like small and wide angle x ray scattering. Recently we developed a new series of cationic gemini surfactants with amino acid and small peptide moieties attached in the spacer region, and have observed an improvement in the biological activity and cellular toxicity.[9,61] In addition, we have synthesized a novel gemini surfactant modified with a targeting peptide. We plan to evaluate the effect of lyophilization on the stability of these new vectors and investigate the stability of the carrier during the storage using mass spectrometry.

Acknowledgment

The authors acknowledge the Helix BioPharma Corporation for the use of stability chambers. This work was funded in part by a grant from the Natural Science and Engineering Research Council of Canada [NSERC] and a grant from Saskatchewan Health Research Foundation [SHRF].

References

1. Friedmann T. The development of human gene therapy: Cold Spring Harbor Laboratory Pr; 1999.

2. Thomas CE, Ehrhardt A, Kay MA. Progress and problems with the use of viral vectors for gene therapy. Nature Reviews Genetics 2003;4:346-58.

3. Ilies M, Seitz WA, Balaban AT. Cationic lipids in gene delivery: principles, vector design and therapeutical applications. Current Pharmaceutical Design 2002;8:2441-73.

4. Badea I, Verrall R, Baca-Estrada M, et al. In vivo cutaneous interferon-γ gene delivery using novel dicationic (gemini) surfactant–plasmid complexes. The Journal of Gene Medicine 2005;7:1200-14.

5. Singh J, Yang P, Michel D, E Verrall R, Foldvari M, Badea I. Amino Acid-Substituted Gemini Surfactant-Based Nanoparticles as Safe and Versatile Gene Delivery Agents. Current Drug Delivery 2011;8:299-306.

6. Menger FM, Littau C. Gemini-surfactants: synthesis and properties. Journal of the American Chemical Society 1991;113:1451-2.

7. Menger F, Littau C. Gemini surfactants: a new class of self-assembling molecules. Journal of the American Chemical Society 1993;115:10083-90.

8. Donkuru MD, Wettig SD, Verrall RE, Badea I, Foldvari M. Designing pH-sensitive gemini nanoparticles for non-viral gene delivery into keratinocytes. Journal of Materials Chemistry 2012;22:6232-44.

9. Yang P, Singh J, Wettig S, Foldvari M, Verrall RE, Badea I. Enhanced gene expression in epithelial cells transfected with amino acid-substituted gemini nanoparticles. European Journal of Pharmaceutics and Biopharmaceutics 2010;75:311-20.

10. Elsabahy M, Nazarali A, Foldvari M. Non-viral nucleic acid delivery: key challenges and future directions. Current Drug Delivery 2011;8:235-44.

11. de Lima M, Neves S, Filipe A, Duzgunes N, Simoes S. Cationic liposomes for gene delivery: from biophysics to biological applications. Current Medicinal Chemistry 2003;10:1221-31.

12. Barteau B, Chèvre R, Letrou-Bonneval E, Labas R, Lambert O, Pitard B. Physicochemical parameters of non-viral vectors that govern transfection efficiency. Current Gene Therapy 2008;8:313-23.

13. Almofti MR, Harashima H, Shinohara Y, Almofti A, Li W, Kiwada H. Lipoplex size determines lipofection efficiency with or without serum. Molecular Membrane Biology 2003;20:35-43.

14. Ross P, Hui S. Lipoplex size is a major determinant of in vitro lipofection efficiency. Gene Therapy 1999;6:651.

15. Walther W, Stein U, Voss C, Schmidt T, Schleef M, Schlag PM. Stability analysis for long-term storage of naked DNA: impact on nonviral in vivo gene transfer. Analytical Biochemistry 2003;318:230-5.

16. Molina MDC, Anchordoquy TJ. Formulation strategies to minimize oxidative damage in lyophilized lipid/DNA complexes during storage. Journal of Pharmaceutical Sciences 2008;97:5089-105.

17. Anchordoquy TJ. Degradation of lyophilized lipid/DNA complexes during storage: the role of lipid and reactive oxygen species. Biochimica et Biophysica Acta (BBA)-Biomembranes 2008;1778:2119-26.

18. Lai E, van Zanten JH. Evidence of lipoplex dissociation in liquid formulations. Journal of Pharmaceutical Sciences 2002;91:1225-32.

19. Anchordoquy TJ, Koe GS. Physical stability of nonviral plasmid-based therapeutics. Journal of Pharmaceutical Sciences 2000;89:289-96.

20. Hong K, Zheng W, Baker A, Papahadjopoulos D. Stabilization of cationic liposome-plasmid DNA complexes by polyamines and poly (ethylene glycol)-phospholipid conjugates for efficient in vivo gene delivery. FEBS Letters 1997;400:233-7.

21. Wheeler J, Palmer L, Ossanlou M, et al. Stabilized plasmid-lipid particles: construction and characterization. Gene Therapy 1999;6:271-81.

22. Anchordoquy TJ, Girouard LG, Carpenter JF, Kroll DJ. Stability of lipid/DNA complexes during agitation and freeze–thawing. Journal of Pharmaceutical Sciences 1998;87:1046-51.

23. Hinrichs W, Manceņido F, Sanders N, et al. The choice of a suitable oligosaccharide to prevent aggregation of PEGylated nanoparticles during freeze thawing and freeze drying. International Journal of Pharmaceutics 2006;311:237-44.

24. Allison SD, Anchordoquy TJ. Lyophilization of nonviral gene delivery systems. Methods in Molecular Medicine 2001;65:225-52.

25. Wang W. Lyophilization and development of solid protein pharmaceuticals. International Journal of Pharmaceutics 2000;203:1-60.

26. Franks F. Freeze-drying of bioproducts: putting principles into practice. European Journal of Pharmaceutics and Biopharmaceutics 1998;45:221-9.

27. Molina, M. d.C., Armstrong, T. K., Zhang, Y., Patel, M. M., Lentz, Y. K., Anchordoquy, T. J. The stability of lyophilized lipid/DNA complexes during prolonged storage. Journal of Pharmaceutical Sciences 2004;93:2259-73.

28. del Pozo-Rodríguez A, Solinís M, Gascón A, Pedraz J. Short-and long-term stability study of lyophilized solid lipid nanoparticles for gene therapy. European Journal of Pharmaceutics and Biopharmaceutics 2009;71:181-9.

29. Kasper JC, Schaffert D, Ogris M, Wagner E, Friess W. Development of a lyophilized plasmid/LPEI polyplex formulation with long-term stability A step closer from promising technology to application. Journal of Controlled Release 2011;151:246-55.

30. Chen C, Han D, Cai C, Tang X. An overview of liposome lyophilization and its future potential. Journal of Controlled Release 2010;142:299-311.

31. Anchordoquy TJ, Carpenter JF, Kroll DJ. Maintenance of transfection rates and physical characterization of lipid/DNA complexes after freeze-drying and rehydration. Archives of Biochemistry and Biophysics 1997;348:199-206.

32. Kuo JS, Hwang R. Preparation of DNA dry powder for non-viral gene delivery by spray-freeze drying: effect of protective agents (polyethyleneimine and sugars) on the stability of DNA. Journal of Pharmacy and Pharmacology 2004;56:27-33.

33. Vighi E, Ruozi B, Montanari M, Battini R, Leo E. Re-dispersible cationic solid lipid nanoparticles (SLNs) freeze-dried without cryoprotectors: characterization and ability to bind the pEGFP-plasmid. European Journal of Pharmaceutics and Biopharmaceutics 2007;67:320-8.

34. Allison SD, Anchordoquy TJ. Maintenance of nonviral vector particle size during the freezing step of the lyophilization process is insufficient for preservation of activity: insight from other structural indicators. Journal of Pharmaceutical Sciences 2001;90:1445-55.

35. Maitani Y, Aso Y, Yamada A, Yoshioka S. Effect of sugars on storage stability of lyophilized liposome/DNA complexes with high transfection efficiency. International Journal of Pharmaceutics 2008;356:69-75.

36. Wettig SD, Wang C, Verrall RE, Foldvari M. Thermodynamic and aggregation properties of aza-and imino-substituted gemini surfactants designed for gene delivery. Physical Chemistery Chemical Physics 2006;9:871-7.

37. Allison SD, Anchordoquy TJ. Mechanisms of protection of cationic lipid-DNA complexes during lyophilization. Journal of Pharmaceutical Sciences 2000;89:682-91.

38. Wettig SD, Badea I, Donkuru MD, Verrall RE, Foldvari M. Structural and transfection properties of amine-substituted gemini surfactant-based nanoparticles. The Journal of Gene Medicine 2007;9:649-58.

39. Singh J, Michel D, Chitanda JM, Verrall RE, 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;10:7.

40. Simberg D, Danino D, Talmon Y, et al. Phase behavior, DNA ordering, and size instability of cationic lipoplexes Relevance to optimal transfection activity. Journal of Biological Chemistry 2001;276:47453-9.

41. Poxon SW, Hughes JA. The effect of lyophilization on plasmid DNA activity. Pharmaceutical Development and Technology 2000;5:115-22.

42. Kneuer C, Ehrhardt C, Bakowsky H, et al. The influence of physicochemical parameters on the efficacy of non-viral DNA transfection complexes: a comparative study. Journal of Nanoscience and Nanotechnology, 6 2006;9:2776-82.

43. Rejman J, Oberle V, Zuhorn IS, Hoekstra D. Size-dependent internalization of particles via the pathways of clathrin-and caveolae-mediated endocytosis. Biochemical Journal 2004;377:159-69.

44. Brus C, Kleemann E, Aigner A, Czubayko F, Kissel T. Stabilization of oligonucleotide–polyethylenimine complexes by freeze-drying: physicochemical and biological characterization. Journal of Controlled Release 2004;95:119-31.

45. Yadava P, Gibbs M, Castro C, Hughes JA. Effect of Lyophilization and Freeze-thawing on the Stability of siRNA-liposome Complexes. AAPS PharmSciTech 2008;9:335-41.

46. Percot A, Briane D, Coudert R, et al. A hydroxyethylated cholesterol-based cationic lipid for DNA delivery: effect of conditioning. International Journal of Pharmaceutics 2004;278:143-63.

47. Randolph TW. Phase separation of excipients during lyophilization: effects on protein stability. Journal of Pharmaceutical Sciences 1997;86:1198-203.

48. Wolfe J, Bryant G. Freezing, drying, and/or vitrification of membrane-solute-water systems. Cryobiology 1999;39:103-29.

49. Allison SD, Molina MC, Anchordoquy TJ. Stabilization of lipid/DNA complexes during the freezing step of the lyophilization process: the particle isolation hypothesis. Biochimica et Biophysica Acta (BBA)-Biomembranes 2000;1468:127-38.

50. Crowe J, Crowe L, Carpenter J. Preserving dry biomaterials: the water replacement hypothesis, part 1. BIOPHARM-EUGENE 1993;6:28-37.

51. Crowe J, Crowe L, Carpenter J. Preserving dry biomaterials: The water replacement hypothesis, part 2. BIOPHARM-EUGENE 1993;6:40-3.

52. Cacela C, Hincha DK. Low amounts of sucrose are sufficient to depress the phase transition temperature of dry phosphatidylcholine, but not for lyoprotection of liposomes. Biophysical Journal 2006;90:2831-42.

53. Popova AV, Hincha DK. Effects of cholesterol on dry bilayers: interactions between phosphatidylcholine unsaturation and glycolipid or free sugar. Biophysical Journal 2007;93:1204-14.

54. Tsonev L, Tihova M, Brain A, Yu ZW, Quinn P. The effect of the cryoprotective sugar, trehalose on the phase behaviour of mixed dispersions of dioleoyl derivatives of phosphatidylethanolamine and phosphatidylcholine. Liquid Crystals 1994;17:717-28.

55. Webb MS, Irving TC, Steponkus PL. Effects of plant sterols on the hydration and phase behavior of DOPE/DOPC mixtures. Biochimica et Biophysica Acta (BBA)-Biomembranes 1995;1239:226-38.

56. Ma B, Zhang S, Jiang H, Zhao B, Lv H. Lipoplex morphologies and their influences on transfection efficiency in gene delivery. Journal of Controlled Release 2007;123:184-94.

57. Kai E, Slack NL, Ayesha A, et al. Cationic lipid-DNA complexes for gene therapy: understanding the relationship between complex structure and gene delivery pathways at the molecular level. Current Medicinal Chemistry 2004;11:133-49.

58. Strauss G, Schurtenberger P, Hauser H. The interaction of saccharides with lipid bilayer vesicles: Stabilization during freeze-thawing and freeze-drying. Biochimica et Biophysica Acta (BBA)-Biomembranes 1986;858:169-80.

59. Crowe JH, Crowe LM, Carpenter JF, Wistrom CA. Stabilization of dry phospholipid bilayers and proteins by sugars. Biochemical Journal 1987;242:1.

60. Yu J, Anchordoquy TJ. Effects of moisture content on the storage stability of dried lipoplex formulations. Journal of Pharmaceutical Sciences 2009;98:3278-89.

61. 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;309:182-91.

Chapter 5

Mass Spectrometric analysis of amino acid/di-peptide modified gemini surfactants used as gene delivery agents: Establishment of a universal mass spectrometric fingerprint[§]

Waleed Mohammed-Saeid[a,c], Joshua Buse[a], Ildiko Badea[a], Ronald Verrall[c], Anas El-Aneed[a,*]

[a] Drug Design and Discovery Research Group, College of Pharmacy & Nutrition, University of Saskatchewan, Saskatoon, SK, Canada

[b] Department of Chemistry, University of Saskatchewan, Saskatoon, SK, Canada

[c] College of Pharmacy, Taibah University, Saudi Arabia

[§] This chapter was published on the International Journal of Mass Spectrometry 2012;309:182-191.

[*] Corresponding author:

Tel.: +1 (306) 966-2013

E-mail address: anas.el-aneed@usask.ca (A. El-Aneed)

5.1. Abstract

Lipid based gemini surfactant nanoparticles have been extensively studied as non-viral vectors for gene therapy. Novel amino acid substituted gemini surfactants have been recently developed with a molecular structure consisting of two positively charged quaternary ammonium head groups, symmetrical saturated dodecyl tails, and a spacer region containing a secondary amine group. Various amino acids were attached to the amine functional group. The purpose of this work was to confirm the molecular structure of six novel amino acid substituted gemini surfactants and to establish a universal fragmentation (MS/MS) pattern of the tested compounds (i.e., fingerprint). This was accomplished by using a hybrid quadrupole orthogonal time-of-flight mass spectrometer (QqToF-MS) and a triple quadrupole linear ion trap mass spectrometer (QqQ-LIT MS) equipped with electrospray ionization (ESI) source. The single stage QqToF-MS data obtained in the positive ion mode verified the molecular composition of all tested gemini surfactants. Tandem mass spectrometric (MS/MS) analysis showed common fragmentation behavior among all tested compounds, allowing for the establishment of a universal fragmentation pattern. The fragmentation pathway was confirmed by MS/MS/MS experiments utilizing a Q-Trap^{TM} 4000 LC/MS/MS system. Unique product ions, originating from the loss of one or both head groups along with the attached tail region(s), confirmed the chemical structure of the tested compounds. The established MS/MS fingerprint will be used for qualitative purposes as well as the development of future multiple reaction monitoring (MRM) HPLC-MS/MS quantification methods.

5.2. Introduction

Nucleic acids (DNA and RNA) have been widely investigated as therapeutic agents for the treatment of hereditary and acquired diseases in a promising medical approach known as gene therapy.[1-9] However, the great potential of gene therapy will not be fully achieved until the issue of improved gene delivery is properly addressed. Gene delivery vectors can be categorized as *viral* or *non-viral.* Viral vectors (adenovirus and retrovirus vectors) are the most effective gene delivery agents and have been utilized in several clinical trials.[7-9] However, they suffer from numerous toxicity-related drawbacks including mortality and morbidity.[10] In addition, the severe immune response caused by the viral capsid and the limited loading capacity of viral vectors significantly limit their therapeutic applications.[6,11] Conversely, non-viral vectors such as cationic lipids have exhibited low toxicity and no immunogenic activity.[12,13] Cationic lipids are able to condense genetic materials, through electrostatic interaction with the phosphate backbone of nucleic acid, to a nano-sized complex (lipoplex).[14]

One specific group of cationic lipids that have demonstrated efficient transfection activities *in-vitro* and *in-vivo* are the gemini surfactants. They are dimeric surfactants comprised of two hydrophobic tail regions, each of which is covalently attached to a cationic head group linked to each other by a spacer region [Figure 1].[15-17] A wide range of gemini surfactants can be produced through chemical modifications within the head, spacer or tail regions. These modifications are intended to enhance the transfection efficiency of the lipoplex while reducing cytotoxicity.[18-20] For instance, the inclusion of a secondary amine functional group in the spacer region of 1,9-bis(dodecyl)-1,1,9,9-tetramethyl-5-imino-1,9-nonanediammonium dibromide gemini surfactant, resulted in a nine-fold increase in transfection efficiency in various cell lines compared to non-substituted gemini surfactants.[19] This increase was attributed to the pH-dependent morphological

changes to the DNA-gemini complex, facilitating the cytoplasmic escape of the DNA. Additional structural modifications include the covalent attachment of biocompatible and biodegradable amino acids (glycine, lysine) and dipeptides (glycyl-lysine, lysyl-lysine) to the amino group in the spacer region, enhancing transfection efficiency in epithelial cells while maintaining a low cytotoxicity profile.[20,21]

The transfection efficiency of lipoplex depends on the integrity of the various components of the delivery system and their related physiochemical properties. Therefore, investigation of the physiochemical stability of the lipoplex during the manufacturing process and, furthermore its biological fate after treatment, is essential to understand and evaluate the behavior of such complex systems. To date, most research in non-viral gene delivery has focused on the development of efficient delivery systems and less work has been done to investigate the chemical stability and biological fate of the vector. To achieve the last goal, proper analytical methods should be developed for both qualitative and quantitative applications. Mass spectrometry (MS) and tandem mass spectrometry (MS/MS) are ideally suited to achieve this goal.

Tandem mass spectrometric MS/MS studies of bioactive materials are routinely utilized for quantitative and qualitative analyses with high accuracy and precision.[22-25] For example, ESI-Qq-ToF MS/MS analysis determined the exact molecular structure of Lipid A moieties isolated from mutant and wild-type *Aeromonas salmonicida* lipopolysaccharide.[24] Similarly, the analysis of saponins, naturally occurring glycosides with varieties of biological activities, using electrospray ionization multiple-stage tandem mass spectrometry (ESI-MS^n) allowed for structural differentiation between several types of saponins.[25] Subsequently, a liquid chromatography (LC)-

MS/MS method was also developed for quick and precise identification of different saponins from plant extract.

Recently, we confirmed the molecular structure of ten un-substituted diquaternary ammonium gemini surfactants belonging to two different structural families G12-***s*** and G18:1-***s*** (where 12 and 18:1 correspond to the length and saturation of the alkyl tail, [-***s***] corresponds to the length of spacer region) using an electrospray ionization (ESI) quadrupole time-of-flight (Qq-ToF) mass spectrometer.[26] The Q-ToF (MS/MS) analysis showed significant similarities in the fragmentation pattern for all tested geminin surfactants. In addition, we expanded our studies for MS/MS behavior of non-substituted diquaternary ammonium gemini surfactants by including 29 compounds categorized into four distinct families based upon the molecular composition of the spacer region and the length of the tail group.[27] The similarities in the fragmentation behavior assisted us to establish a universal fragmentation pathway for these novel compounds. In this study, we determined the tandem mass spectrometric behavior of novel amino acid/peptide modified diquaternary ammonium gemini surfactants, specially designed for gene delivery. Mass spectrometric analysis was performed by positive ESI on Time-of-Flight (Q-ToF) and triple quadrupole linear ion trap (QqQ-LIT) mass spectrometers. The suggested fragmentation patterns (i.e., fingerprints) of all compounds were confirmed by means of MS/MS/MS experiments.

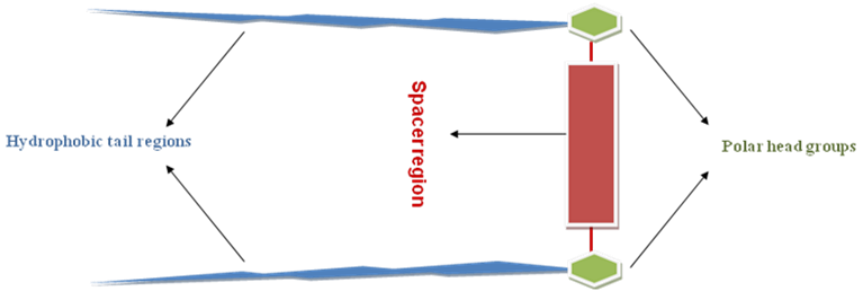


Figure 5.1: General structure of cationic gemini surfactants.

5.3. Experimental

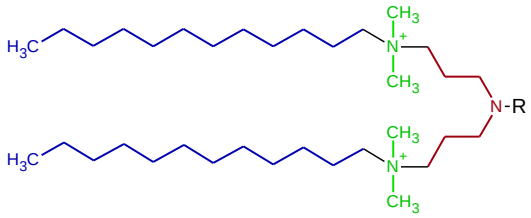
5.3.1. Gemini surfactant

Six novel mono-amino acid/dipeptide-substituted gemini surfactants were provided by Dr. Ronald E. Verrall's research group (Department of Chemistry, University of Saskatchewan). The synthesis of these gemini surfactants and their efficiency in gene transfer were recently reported.[20,21] Tested compounds were given the designation of 12-7N(R)-12, where (12) is the number of carbon atoms in the tail region, (7) is the length of the amine substituted spacer region and (R) represents the amino acid(s) substituent:

R= Glycine, Lysine, Histidine, Glycyl-Lysine, Lysyl-Lysine, Glycyl-Glycine

The general molecular structure of these gemini surfactants is shown in Figure 5.2.

Stock solutions of 3mM gemini surfactant were prepared in methanol/water 50:50 and 0.1 % formic acid and stored at -20 ˚C. Samples for the MS experiment were further diluted 1000x prior to injection using the same solvent.



R= Glycine : 12-7N(Gly)-12
Lysine: 12-7N(Lys)-12
Histidine: 12-7N(His)-12
Glycyl-Lysine: 12-7N(Gly-Lys)-12
Lysyl-Lysine: 12-7N(Lys-Lys)-12
Glycyl-Glycine: 12-7N(Gly-Gly)-12

Figure 5.2: General structure of amino acid/di-peptide gemini surfactant 12-7N(R)-12 where (R) corresponds to the amino acid/di-peptide substituent.

5.3.2. Electrospray-Quadrupole Orthogonal Time-of-Flight Mass spectrometry (ESI-QqToF MS)

Gemini surfactants were analyzed in the positive ion mode by using an API QSTAR XL MS/MS hybrid QqToF tandem mass spectrometer equipped with an ESI source (Applied Biosystems Inc., CA, USA). The instrument parameters were optimized as follows: declustering potential 35 V and focusing potential of 290 V. Sample solutions were infused into the source chamber (Turbo Ionspray source) by using an integrated Harvard syringe pump (Harvard Apparatus, MA, USA) at a rate of 10 µL/min with the following parameters: spray chamber temperature 80 ˚C – 100 ˚C, needle voltage 5500 V. Nitrogen was used as the drying gas and ESI nebulizing gas. Internal calibration was used to ensure high mass accuracies and to minimize errors in mass measurements. Similar to our recent work[26], we used doubly charged standards given that the tested gemini surfactants are doubly charged species. These include [Glu1]-Fibrinopeptide B, Human(peptide EGVNDEEGFFSAR, m/z 785.4821), (BaChem Bioscience Inc., PA, USA), and the previously characterized diquaternary ammonium gemini surfactant N,N-bis(dimethyldodecyl)-1,2-ethanediammonium dibromide m/z 234.2685.[28-30] Mass spectra acquisitions were analyzed using the Analyst software.

Tandem mass spectrometric analysis was obtained by collision-activated dissociation (CAD) using nitrogen as collision gas. The collision energy (CE) values were optimized to allow for a dissociation of the gemini surfactant while ensuring the abundance of the precursor ion (ranging from 27-33 eV).

5.3.3. Triple Quadrupole Linear Ion Trap Mass spectrometry (QqQ-LIT MS)

The suggested fragmentation pathways were confirmed by performing MS/MS/MS experiments using a Q-Trap 4000 LC/MS/MS system (Applied Biosystems, Foster City, CA,

USA) a hybrid triple quadrupole linear ion trap mass spectrometer (QqQ-LIT) equipped with a "Turbo V Ion Spray" ESI source. The QqQ-LIT system provides valuable structural information because of its ability to trap ions in the LIT analyzer and, subsequently, to perform MS/MS/MS experiments.[31,32]

The MS/MS/MS analysis of the precursor ion and selected product ions of the tested gemini surfactants were acquired in the MS/MS/MS mode. Stock samples were diluted 1000x and infused directly into the ionization source by using a model 11 Plus syringe pump (Harvard Apparatus, MA, USA) at a flow rate of 50 µL/min. The declustering potential (DP) was set in the range of 40-100 V (compound dependent) and collision energy (CE) was optimized to obtain the greatest abundance of product ions. Excitation energy (AF2), the energy used to fragment the second precursor ion, was set at 100 mV.

5.4. Results and Discussion

5.4.1. Single stage QqToF analysis

For all tested mono-amino acid and di-peptide gemini surfactants, abundant doubly charged $[M]^{2+}$ species were observed during the full scan ESI- QqToF-MS analysis providing evidence for the presence of the diquaternary ammonium head groups. In addition, the exact mass for the tested compounds were assessed; mass accuracies were less than 10 ppm mass error using internal calibration [Table 5.1].

5.4.2. Tandem mass spectrometric Analysis

The variation within the substituent of the amine group of the spacer region (i.e., amino acid/di-peptide substituents) resulted in the production of gemini surfactant-specific product ions upon collision-activated dissociation (CAD) positive ESI-QqToF MS/MS analyses. The formation of these compound-specific product ions follows a similar fragmentation pattern for all mono-amino acid and dipeptide gemini surfactants, which originates from the loss of one or both quaternary ammonium head group(s) along with the attached tail region(s). This allowed for the authentication of the molecular structure, confirming the attachment of the amino acid/di-peptide moieties to the amine group of the spacer region.

The following sections include a detailed discussion of the fragmentation patterns of 12-7N(Glycyl-Lysine)-12, illustrative of a di-peptide substituted gemini surfactants with the most complex MS/MS spectra among all tested compounds. In addition, the MS/MS behavior of 12-7N(Glycine)-12, illustrative of mono-amino acid substituted gemini surfactants, will be discussed briefly to highlight the fragmentation with mono-amino acid substituted compounds.

Table 5.2 displays the product ions of all gemini surfactants studied herein with their corresponding molecular formula and the theoretical *m/z* values.

Table 5.1: Mass Accuracies obtained from single stage ESI-QqToF MS using internal calibration.

Gemini Surfactants	**Molecular Formula**	**Mono-isotopic Mass**	**Theoretical m/z**	**Observed m/z**	**Mass Accuracy (ppm)**
12-7N(Glycine)-12	$C_{36}H_{78}N_4O$	582.6164	291.3082	291.3094	4.1193
12-7N(Lysine)-12	$C_{40}H_{87}N_5O$	653.6899	326.8449	326.8462	3.9774
12-7N(His)-12	$C_{40}H_{82}N_6O$	662.6539	331.3269	331.3293	7.2436
12-7N(Gly-Lys)-12	$C_{42}H_{90}N_6O_2$	710.7114	355.3557	355.3571	3.9397
12-7N(Lys-Lys)-12	$C_{49}H_{99}N_7O_2$	781.7849	390.8924	390.8926	0.5116
12-7N(Gly-Gly)-12	$C_{38}H_{81}N_5O_2$	639.6379	319.8189	319.8185	1.2507

Table 5.2: MS/MS product ion designations and corresponding theoretical mass-to-charge (*m/z*) values for all gemini surfactants evaluated.

Gemini Surfactants		**12-7N(Glycine)-12**	**12-7N(Lysine)-12**	**12-7N(Histidine)-12**	**12-7N(Gly- Lys)-12**	**12-7N(Lys-Lys)-12**	**12-7N(Gly-Gly)-12**
Molecular Formula		**$C_{36}H_{78}N_4O$**	**$C_{40}H_{87}N_5O$**	**$C_{40}H_{82}N_6O$**	**$C_{42}H_{90}N_6O_2$**	**$C_{49}H_{99}N_7O_2$**	**$C_{38}H_{81}N_5O_2$**
Precursor ion [M]$^{2+}$		291.3082	326.84495	331.32695	355.3555	390.89245	319.81895
Product ions		***m/z***	***m/z***	***m/z***	***m/z***	***m/z***	***m/z***
1	[M-$C_{12}H_{24}$] $^{2+}$	207.21	242.75	247.23	271.26	306.79	235.72
2	[M-($C_{12}H_{24}$)-(C_2H_7N)] $^{2+}$	184.68	220.22	224.70	248.73	284.26	213.19
3	[M-$C_{14}H_{32}N$-(NH_3)] $^{2+}$		211.70	216.19	240.22	275.75	204.68
4	[M-$C_{14}H_{32}N$-(NH_3)-(C_2H_4)] $^{2+}$		197.69		226.20	261.74	
5	[M-$C_{14}H_{32}N$-(NH_3)-(C_2H_4)-(C_3H_5N)] $^{2+}$		170.17		198.68		
6	[$C_{21}H_{44}N_2O$]$^{2+}$	170.17	170.17	170.17	170.17	170.17	170.17
7	[(ION 6)-(CHO^+)]$^+$	311.34	311.34	311.34	311.34	311.34	311.34
8^{++}	[M-2($C_{12}H_{24}N$) -(C_2H_7N)]$^{2+}$	100.59	136.12	140.69	164.63	200.17	129.10
8$^+$	[(ION 8^{++})-(H^+)]$^+$	200.17	271.24	280.21	238.27	399.34	257.19
9^{++}	[M-2($C_{12}H_{24}N$) -2(C_2H_7N)]$^{2+}$	78.06	113.59	118.08	142.11	177.64	106.57
9+	[(ION 9^{++})-(H^+)]$^+$	155.11	226.19	235.15	283.21	354.28	212.14
10^{++}	[M-2($C_{12}H_{24}N$) -2(C_2H_7N) -(NH_3)]$^{2+}$		105.15		133.59	169.13	98.06
10$^+$	[(ION 10^{++})-(H^+)]$^+$		209.16		266.18	337.25	195.10
11	[$C_{14}H_{32}N$] $^+$	214.25	214.25	214.25	214.25	214.25	214.25
B_1	(N-Terminal ion from peptide bond cleavage)				155.11	226.19	155.11
Y_1	(C-Terminal ion from peptide bond cleavage)				129.10	129.10	58.02

5.4.2.1. MS/MS fragmentation pathway of the di-peptide substituted gemini surfactants

The di-peptide gemini surfactants include three novel gemini surfactants in which a di-peptide substituent is attached to the amine group of the spacer region: 12-7N(Glycyl-Lysine)-12,12-7N(Lysyl-Lysine)-12and 12-7N(Glycyl-Glycine)-12. Figure 5.3 shows the ESI-QqToF MS/MS spectrum of 12-7N(Glycyl-Lysine)-12, as a representative for this group, and the proposed fragmentation pathway. The MS/MS spectra of and the corresponded fragmentation pathways of 12-7N(Lysyl-Lysine)-12 and 12-7N(Glycyl-Lysine)-12 are shown in Appendices 5.1 and 5.2, respectively.

The fragmentation pathway of 12-7N(Glycyl-Lysine)-12 (Figure 5.3a) starts with the formation of the minor diagnostic doubly charged product ion designated as $[M\text{-}C_{12}H_{24}]^{2+}$ at *m/z* 271.26 **(ion 1, Figure 5.3b)** which is formed from the neutral loss of the aliphatic tail region of 168.18 Da. It can be speculated that due to the possible close proximity of the two positively charged head groups, this ion is not stable and will fragment instantly; hence, being a substantially minor ion. In fact, ion (**1**) was observed in the MS/MS analysis of all tested compounds as a minor fragment ion. Ion **(1)** fragments to the major doubly charged product ion observed at *m/z* 248.73 **(ion 2)** through the neutral loss of N-methylmethanamine (i.e., head group). It is expected that the second charge within **ion 2** is localized within the di-peptide (Glycyl-Lysine) terminal, possibly distant from the quaternary nitrogen; hence, enhancing their stability and abundance in comparison to **(ion 1)**.

Subsequently, product ion **(2)** fragments via three different mechanisms into three diagnostic product ions **(ions 3, 8^{++} and 9^{+}, Figure 5.3b).** Product ion **(3)** at *m/z* 240.22 is formed by losing a (NH_3) moiety, while product ion **(8^{++})** is generated by the neutral loss of the second tail region at *m/z* 164.63. Additionally, product ion **(9^{+})** at *m/z* 283.21 is formed through

the complementary loss of the singly charged ion of the tail region with the attached head group *m/z* 214.25 **(11)**.

The product ion **(3),** designated as $[M\text{-}C_{14}H_{32}N\text{-}(NH_3)]^{+2}$, is the predominant product ion observed in the MS/MS spectrum of 12-7N(Gly-Lys)-12 and can undergo two main fragmentation process. Firstly, the loss of a (NH_3) from ion **(3)** yields the product ion **(3')** at *m/z* 231.70. The second mechanism involves the loss of the ethene moiety from product ion **(3)** producing a doubly charged product ion at *m/z* 226.20 **(ion 4).** This product ion is further fragmented to several product ions resulting from various elimination processes within the dipeptide residue. The neutral loss of (C_3H_5N) from ion (**4**) produces a doubly charged fragment ion at *m/z* 198.68 **(ion 5)**. Subsequently, the loss of a carbon monoxide moiety from ion (**5**) produces a unique product ion , **(ion 5')** at *m/z* 184.68, with a glycine residue within the spacer region. Furthermore, product ion **(6)** is formed by the neutral loss of a methyleneimine (methanimine) moiety ($-CH_2=NH$) from the head group, producing a doubly charged fragment ion at *m/z* 170.17 **(ion 6).** The subsequent loss of oxomethylium (HCO^+) from this ion produces a singly charged product ion observed at *m/z* 311.34 **(ion 7)**. Oxomethylium is a well identified loss in MS analysis that can occur in two isomeric forms; HCO^+ and HOC^+.[33,34] Fragment ions **(6)** and **(7)** are common ions observed in the MS/MS analysis of all tested compounds.

The second fragmentation mechanism for **ion 2** involves the formation of product ion **(8)** which was observed as both; singly **(8^+)** and doubly **(8^{++})** charged ions at *m/z* 328.27 and *m/z* 164.63, respectively. The neutral loss of the second head group from product ions **(8^+/8^{++})** produces the diagnostic product ions **(9^+/9^{++})** at *m/z* 283.21 and *m/z* 142.11. The Q-ToF MS/MS analysis of the precursor ion and the QqQ-LIT MS/MS/MS spectrum of ion **(9^+)** [Table 5.3] indicates that this ion produces three main elimination products. The loss of (NH_3) from the di-

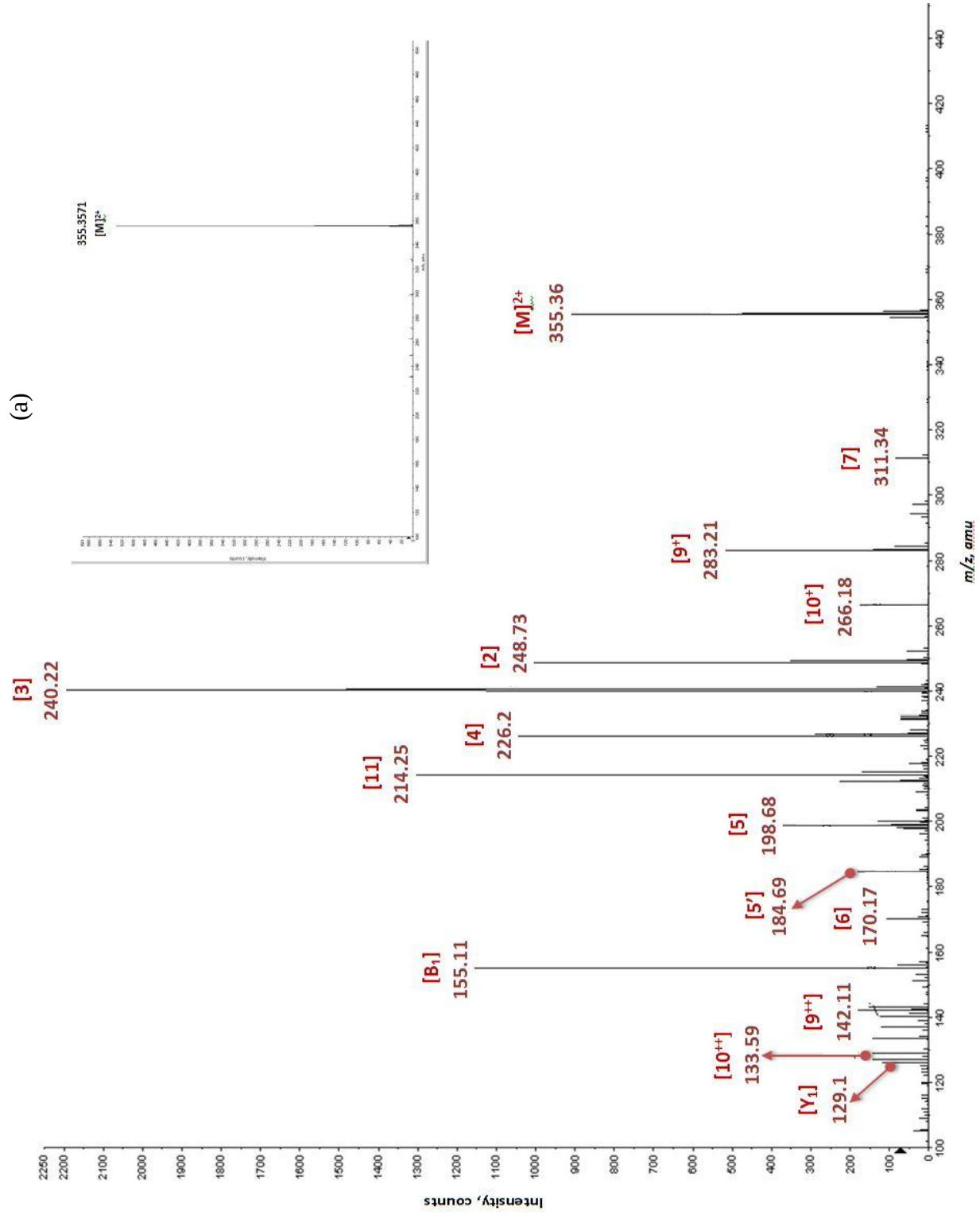
peptide residue of product ion **(9)** leads to the formation of product ion **(10)** which was observed as doubly charged ion **(10^{++})** at *m/z* 133.59 and singly charged ion **(10^{+})** at *m/z* 266.18. The formation of singly and doubly charged product ions can be explained by the presence of multiple nitrogen centers in the spacer region of the di-peptide gemini surfactants that can easily capture a proton from other species within the collision cell.

Unique breakage of the peptide bond between the glycine and lysine amino acids produced two complementary fragment ions; ion **(B_1)** that corresponds to the N-terminal ion (glycine residual ion) at *m/z* 155.11 and ion **(Y_1)** corresponding to the C-terminal ion (lysine residual ion) at *m/z* 129.10. Designations for these ions follow the Roepstorff nomenclature for mass spectrometry of peptides.[35] It should be noted that ion **(B_1)** can also originate from ions **(3)** and **(4)** upon the loss of the tail region, which is supported by MS/MS/MS results [Table 5.3].

MS/MS/MS analysis was informative and assisted in the confirmation of the proposed fragmentation mechanism. For instance, the MS/MS/MS spectrum of product ion **(4)** shows fragment ions at *m/z* 283.21 and 266.18, ions **(4')** and **(4'')**, respectively [Figure 5.3b, Table 5.4]. These product ions have the same *m/z* values as product ions **(9^{+})** and **(10^{+})** which are fragments of ion **(8^{+})**. This can be explained by the fact that two isomers having the same *m/z* values were formed for each product ion originating by different fragmentation mechanisms [Figure 5.3b]. MS/MS/MS analysis allowed for the differentiation of these structural isomers.

12-7N(Lysyl-Lysine)-12 and 12-7N(Glycyl-Glycine)-12 compounds followed the same fragmentation pathway as the glycyl-lysine substituted gemini surfactant [Table 5.2].

Figure 5.3: (a) The ESI-QqToF MS/MS spectra of 12-7N(Glycyl-Lysine)-12 as a representative example of di-peptide gemini surfactants (Full MS spectrum in the box), (b) the MS/MS fragmentation pattern showing the most distinctive product ions, other non-diagnostic product ions are not included.



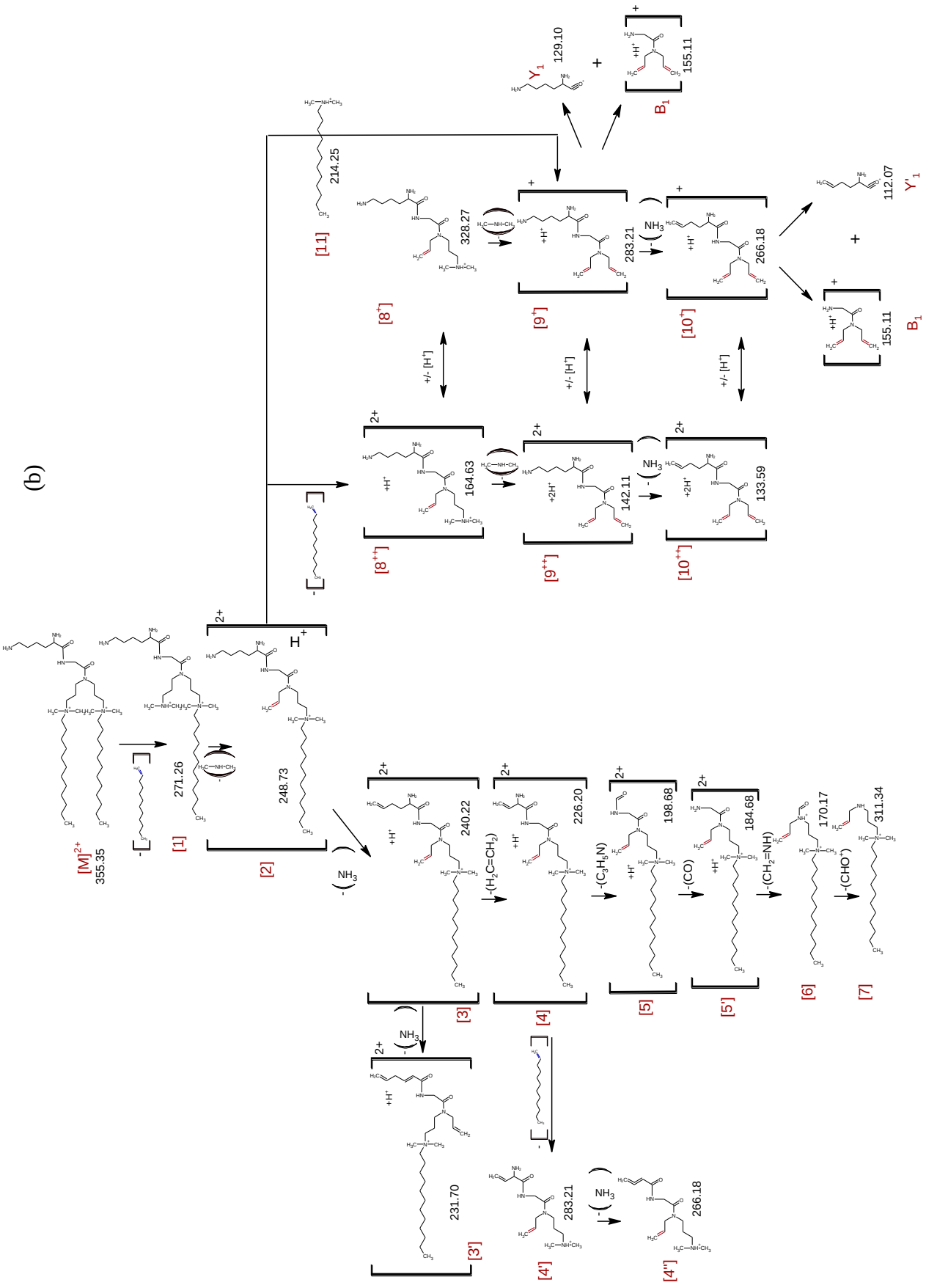

(b)
[M]2+
355.35
[1]
271.26
[2]
248.73
H+
NH3
[3]
240.22
-(H2C=CH2)
[4]
226.20
-(C3H5N)
[5]
198.68
-(CO)
[5']
184.68
-(CH2=NH)
[6]
170.17
-(CHO•)
[7]
311.34
[3']
231.70
[4']
283.21
NH3
[4'']
266.18
[11]
214.25
[8++]
164.63
[9++]
142.11
NH3
[10++]
133.59
+/- [H+]
[8+]
328.27
[9+]
283.21
NH3
[10+]
266.18
Y1
129.10
B1
155.11
Y'1
112.07
B1
155.11

Table 5.3: Summary of MS/MS/MS experiment for 12-7N(Gly-Lys)-12, using QqQ-LIT

MS/MS fragment ions of 12-7N(Gly-Lys)-12	**MS/MS/MS fragment ions**
248.73 [2]	**240.22 [3]** → **226.20[4]**, **198.68 [5]**, **170.17 [6]**, **311.34 [7]**, **214.25 [11]**, **155.11 [B_1]**
	226.20 [4] → **184.68 [5']**, **170.17 [6]**, **214.25 [11]**, **283.21 [4']**, **266.18 [4'']**
	328.27 [8^+] → **283.21 [9^+]**, **266.18 [10^+]**, **155.11 [B_1]**
	283.21 [9^+] → **266.18 [10^+]**, **155.11 [B_1]**, **129.10 [Y_1]**
	266.18 [10^+] → **155.11 [B_1]**, **112.07 [Y'_1]**
	311.34 [7]
	214.25 [11]
	155.11 [B_1]
	129.10 [Y_1]

5.4.2.2. MS/MS fragmentation pathway of the mono-amino acid gemini surfactants

Mono-amino acid gemini surfactants included three novel compounds in which a single amino acid is attached to the amine group of the spacer region: 12-7N(Glycine)-12 ,12-7N(Lysine)-12 and 12-7N(Histidine)-12. Figure 5.4 shows the ESI-QqToF MS/MS spectrum of 12-7N(Gly)-12, and the corresponding fragmentation pathway. The MS/MS spectra of and corresponding fragmentation pathway of 12-7N(Lysine)-12 and 12-7N(Histidine)-12 are shown in Appendices 5.3 and 5.4, respectively.

Similar to the fragmentation pathway of the di-peptide substituted gemini surfactants, the fragmentation pathway of 12-7N(Glycine)-12 begins with the production of the characteristic minor doubly charged product ion $[M\text{-}C_{12}H_{24}N]^{2+}$ at *m/z* 207.21 **(ion 1, Figure 5.4b)** formed through the neutral loss of one hydrophobic tail region as explained earlier. This fragment ion further fragments to the major doubly charged product ion observed at *m/z* 184.68 **(ion 2)** through the neutral loss of N-methylmethanamine (i.e., head group).

Consequently, product ion **(2)** can undergo three fragmentation pathways producing fragment ions **(6)**, **(8^{++})** and **(9^{+})** [Figure 5.4b]**.** The formation of commonly observed product ions **(6)** and **(7)** was mentioned previously in the discussion of di-peptide substituted gemini surfactants. The loss of neutral methylene (CH_2) moiety from the spacer region of product ion **(7)** produces a singly charged fragment ion at *m/z* 297.32 **(ion 7')**.

As indicated earlier, product ion (**2)** also yields the formation of product ion **(8)** through the neutral loss of the second twelve carbon atom tail region. This ion can exist as a doubly charged species **(ion 8^{++})** at *m/z* 100.59 or singly charged ion **(ion 8^{+})** at *m/z* 200.17. A neutral loss of the remaining head group (i.e., CH_3NHCH_3) from product ion **(8^{+}/8^{++})** results in the formation of ion **(9)** which also exists as a singly charged **(ion 9^{+})** *m/z* 155.11and doubly charged

(ion 9^{++}) *m/z* 78.06 (not shown in the spectrum). Product ion **(9)** further fragments via two fragmentation mechanisms. In the first mechanism, product ion **(9a)** is formed through the same mechanism as fragment ion **(6)**; i.e., via the neutral loss of the methanimine moiety producing product ion **(9a)** at *m/z* 126.09. The second mechanism involves neutral loss of ethyne (acetylene) moiety forming fragment ion **(9b)** at *m/z* 129.10.

Finally, as explained earlier in the case of the 12-7N(Gly-Lys)-12 compound, product ion **(2)** can also fragment through a third pathway and form product ion **(9^{+}).** This mechanism can occur through the loss of the remaining tail region with the attached head group from product ion **(2)** as a singly charged protonated ion designated as $[C_{14}H_{32}N]^{+}$ observed at *m/z* 214.25 (**ion 11**).

Similar to the 12-7N(Gly-Lys)-12 compound, the proposed fragmentation pathway for the 12-7N(Gly)-12 was confirmed via MS/MS/MS analysis using QqQ-LIT MS1. In addition, a deuterated form of glycine substituted gemini surfactant has been synthesized to be used as internal standard for the purpose of developing a quantitative multiple-reaction-monitoring LC-MS/MS method. This deuterated compound retains two deuterated tail regions of dodeceyl-d_{25} and has the designation 12_{D25}-7N(Glycine)-12_{D25}. The MS/MS analysis of this compound confirms the proposed fragmentation pathway by showing an increase in the *m/z* values of products ions **(2)**, **(6)**, **(7),** and **(11)** corresponding to the presence of deuterium in the structure [Table 5.4]. On the other hand, fragment ions bearing no tail regions were identical (in terms of structure and *m/z* values) to those observed on the MS/MS analyses of non- deuterated compound.

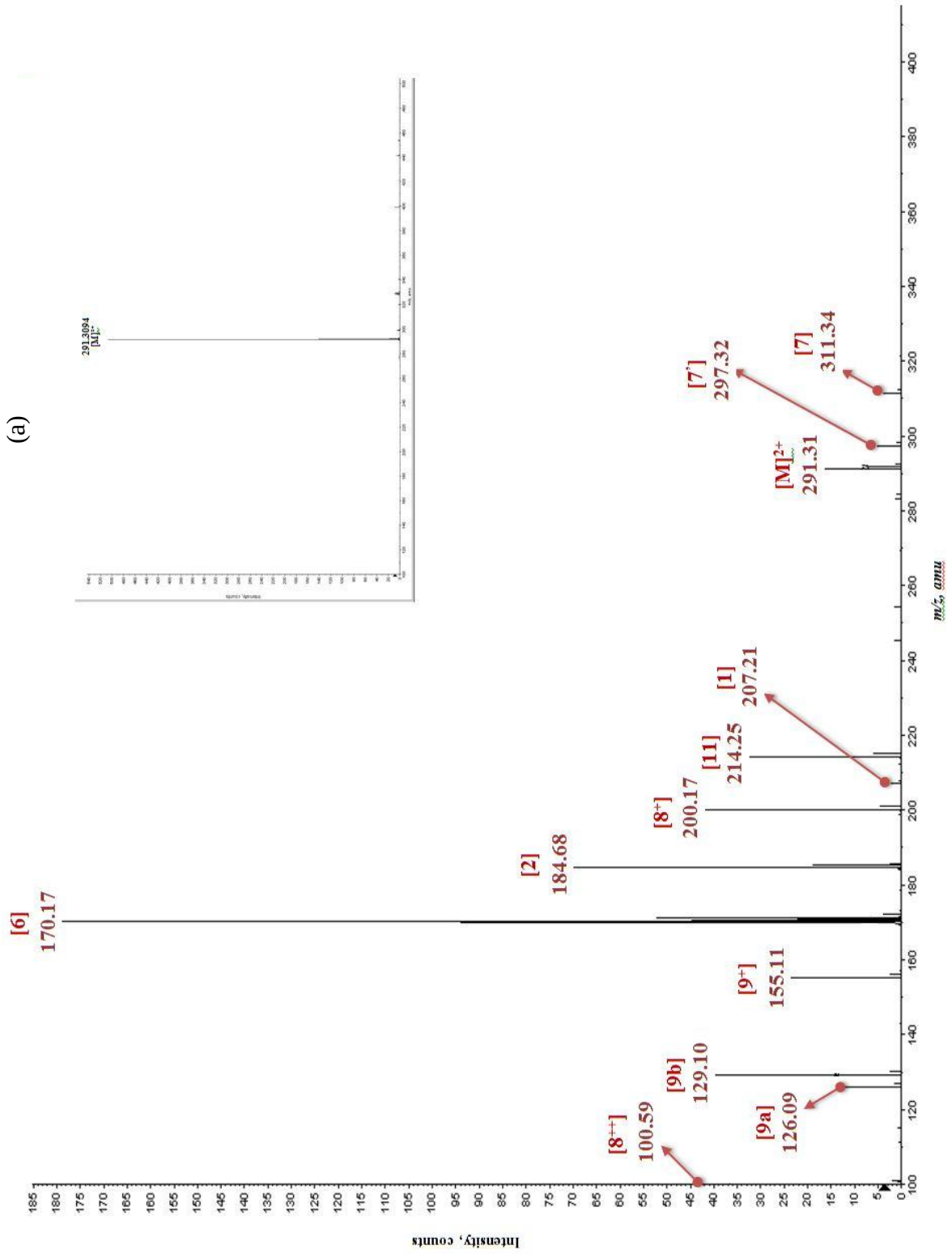
Both histidine and lysine-substituted gemini surfactants followed the same fragmentation pattern as 12-7N(Glycine)-12 with minor variations resulting from the differences in the molecular structure of the amino acid substituents. For instance fragment ions **(3), (4), (5)** and

(10^{+}/10^{++}) [Table 5.2] were not observed in the MS/MS spectrum of 12-7N(Glycine)-12 compound. The formation of these product ions required the loss of the (NH_3) moiety from the terminal amino acid which is not applicable in the case of the glycine amino acid substitution. For the same reason, we could not observe product ions **(4), (5)** and **(10^{+}/10^{++})** in the MS/MS analysis of 12-7N(His)-12. However**,** these product ions were detected in the MS/MS analysis of lysine-substituted gemini surfactant due to the presence of two amine groups in the structure of lysine.

In addition to this difference, the 12-7N(His)-12 gemini surfactant showed an unique fragmentation mechanism resulting from the presence of the heterocyclic imidazole ring: product ion **(3)** is formed by the neutral loss of the (NH_3) moiety from histidine, producing a doubly charged fragment ion at *m/z* 216.19 **(ion 3, Table 5.2, Figure 5.5)**. Distinct from the fragmentation pattern of glycine and lysine substituted gemini surfactants, this product ion can undergo two fragmentation pathways that involve hydrogen relocalization. The first pathway produces the commonly observed product ion **(6)** at *m/z* 170.17 **(Table 5.2, Figure 5.5).** The second pathway results in the formation of two complementary product ions that were designated as **(3a)** and **(3b)** resulting from the cleavage between the carbon atom of the carbonyl group and the adjacent carbon atom of histidine within product ion **(3).** Ion **(3b)** observed at *m/z* 339.57 is the singly charged form of ion **(6)**. However, the production of ions **(6)** and **(3b)** occurs concurrently by two different mechanisms as shown in Figure 5.5. Ion **(6)** is produced by relocalizing the second charge from the imidazole ring to the secondary amine group of the spacer region (proton transfer), while the singly charged ion **(3b)** bears a tertiary amine. It is worth to mention that product ion **(3b)** was observed only in the MS/MS analysis of 12-7N(His)-12 compound, which confirms the proposed fragmentation mechanism. This proposition is

supported by the formation of a complementary ion **(3a)** that was observed at *m/z* 93.1 which was detected during scanning for product ions below *m/z* 100 (data not shown).

Figure 5.4: (a) The ESI-QqToF MS/MS spectra of 12-7N(Glycine)-12 as a representative example of mono amino acid gemini surfactants (Full MS spectrum in the box), (b) the MS/MS fragmentation pattern showing the most distinctive product ions, other non-diagnostic product ions are not included,



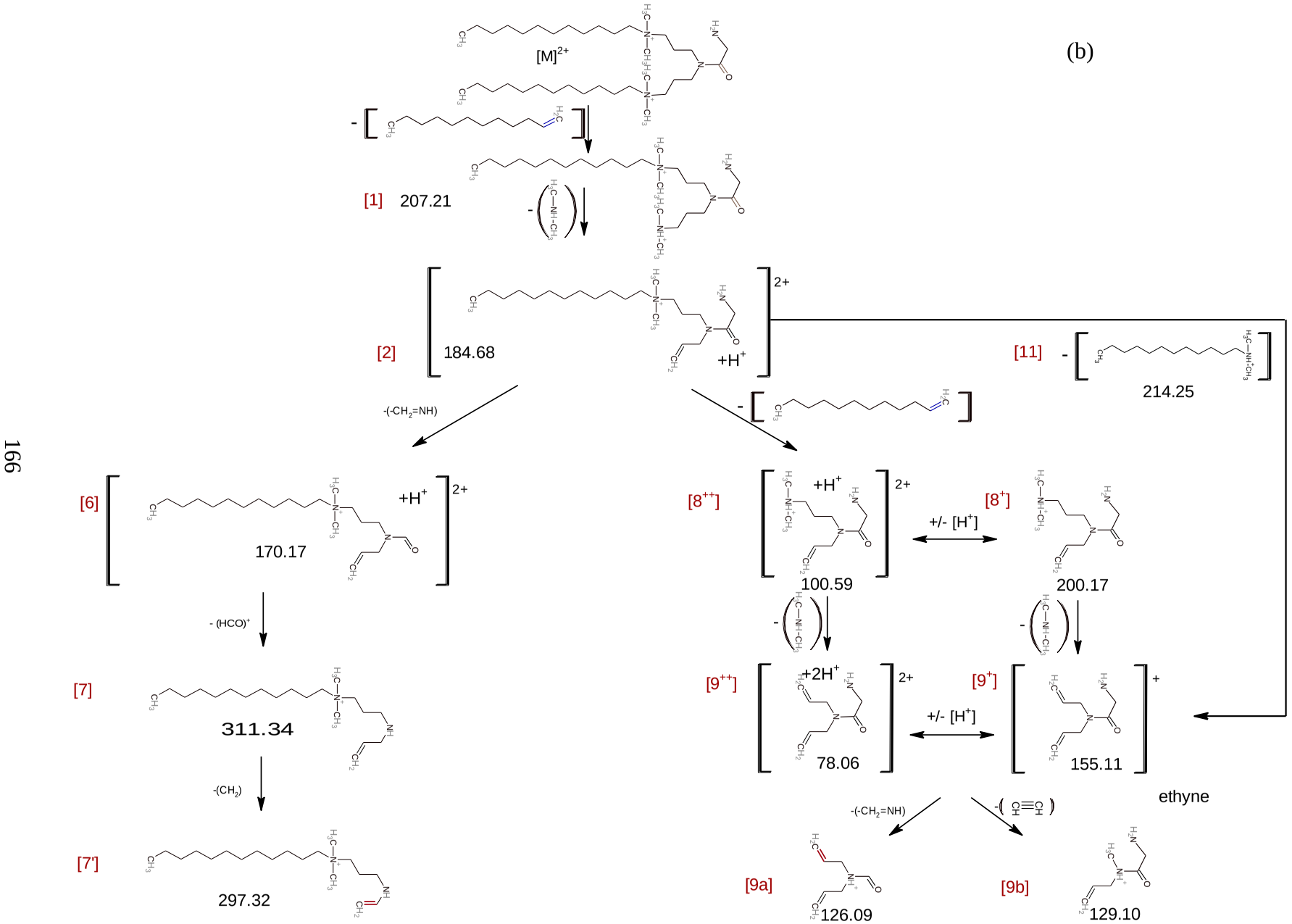
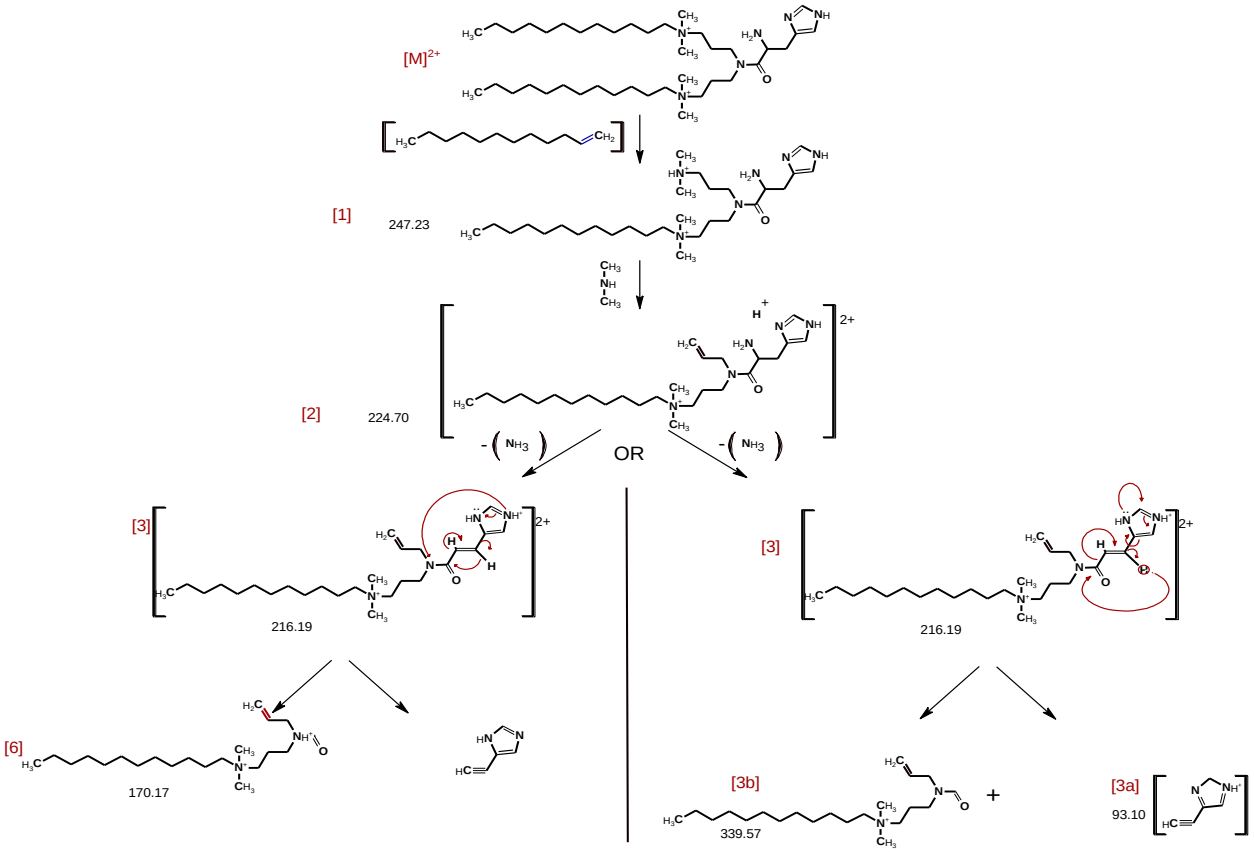

(b)
[M]2+
[1] 207.21
[2] 184.68
+H+
2+
-(-CH2=NH)
[11]
214.25
[6]
170.17
- (HCO)+
[7]
311.34
-(CH2)
[7']
297.32
[8++]
100.59
+/- [H+]
[8+]
200.17
[9++]
78.06
+2H+
[9+]
155.11
ethyne
-(-CH2=NH)
[9a]
126.09
[9b]
129.10

Table 5.4: The difference in *m/z* values between 12-7N(Glycine)-12 and its deuterated form 12_{D25}-7N(Glycine)-12_{D25} confirm the proposed fragmentation pathway.

Product ion	**12-7N(Glycine)-12**	**12_{D25}-7N(Glycine)-12_{D25}**	***m/z* Difference**
Ion 2	184.68	197.26	12.58
Ion 6	170.17	182.75	12.58
Ion 7	311.34	336.49	25.15
Ion 11	214.25	239.41	25.15

Figure 5.5: Fragmentation mechanisms of product ion **(3)** of 12-7N(Histidine)-12



5.4.2.3. Universal MS/MS Fragmentation Pattern

Similarities in the MS/MS fragmentation behavior of the novel mono-amino acid/di-peptide substituted gemini surfactants allowed for the establishment of a universal MS/MS fragmentation pattern. Formation of product ions observed in the universal MS/MS fragmentation **[Figure 5.6]** starts with the homolytic cleavage of (-C-N-) bond between the twelve carbon atom tail region and the quaternary ammonium head group producing a minor doubly charged product ion $[M\text{-}C_{12}H_{24}]^{2+}$ **(ion 1, Figure 5.6).** It is noteworthy that we were unable to conduct a MS/MS/MS experiment with this ion since it was always observed with very low intensity (except in the case of 12-7N(Gly)-12). Product ion **(1)** then produces product ion **(2)** and two pathways were proposed**.** The first mechanism was explained by the neutral elimination of the head group ($-C_2H_7N$) from ion **(1)** forming a diagnostic doubly charged ion $[M\text{-}(C_{12}H_{24})\text{-}(C_2H_7N]^{2+}$ **(Pathway A, ion 2**). On the other hand, the second mechanism includes a neutral loss of hydrophobic tail region with the attached head group from the precursor ion $[M]^{2+}$ **(Pathway B, ion 2**). Product ion **(2)** is a predominant product ion in the MS/MS spectra of all gemini surfactants evaluated herein.

The product ion **(2)** in ESI-QqToF MS/MS conditions undergoes three main fragmentation processes. The elimination of (NH_3) forms the doubly charged fragment ion **(3)** $[M\text{-}C_{14}H_{32}N\text{-}(NH_3)]^{2+}$**.** This fragment ion is subjected to several fragmentation processes, producing different product ions which are shown in Figure 5.6 and have been discussed with specific examples in Figures 5.3b and 5.4b.

The second elimination process results from the heterolytic cleavage between the second tail region and the attached head group yielding the fragment ion **(8^{++}).** The subsequent loss of the ammonium head group from ion **(8^{++})** gives the product ion **(9^{++})** designated as [M-

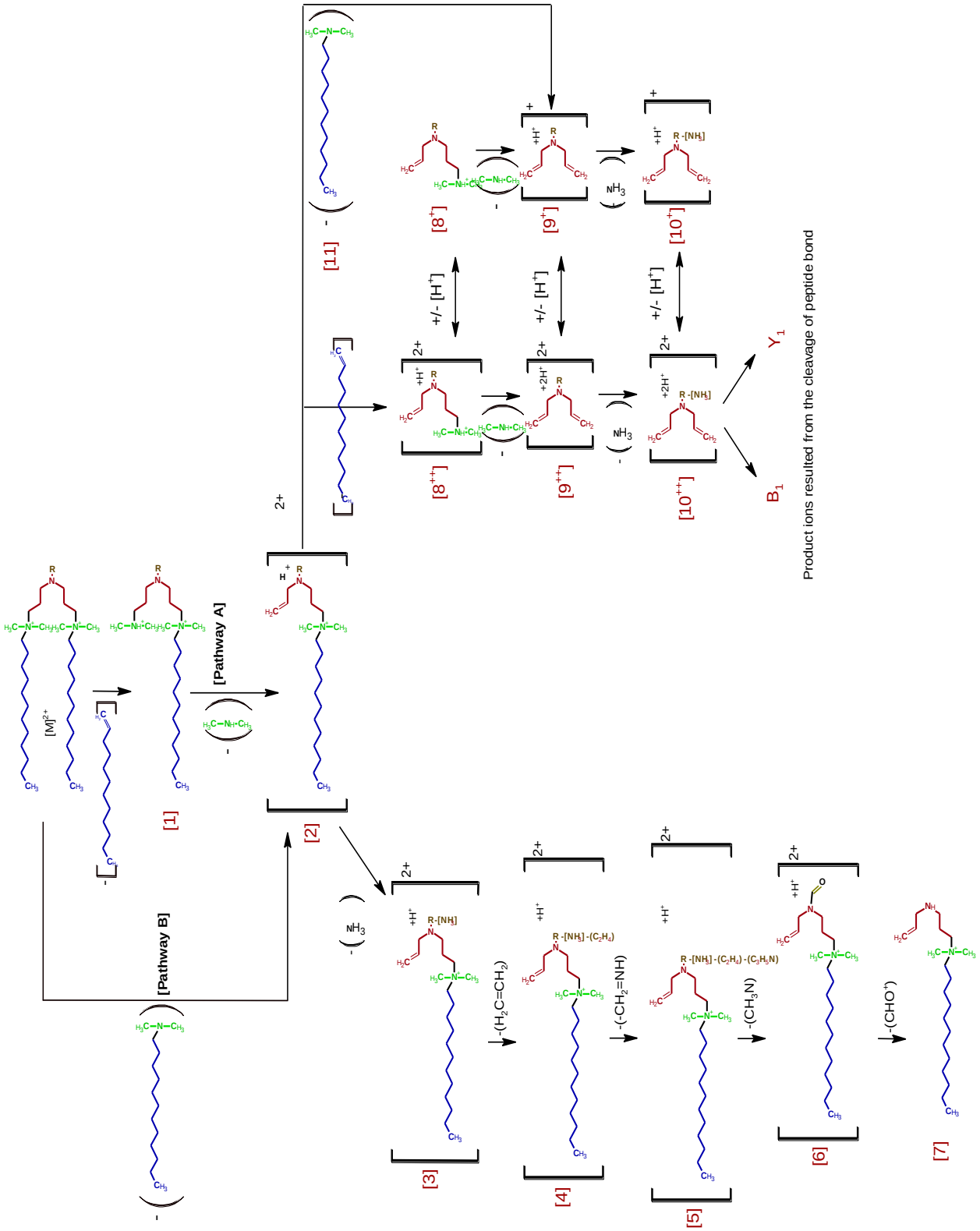
$2(C_{12}H_{24}N$ -$2(C_2H_8N))]^{2+}$. Product ion **(9^{++})** is further fragmented to ion **(10^{++})** via the neutral loss of (NH_3).

In the case of the di-peptide gemini surfactants, peptide bond cleavage occurs in the product ion (**10^{++}**) producing two complementary fragment ions **(B_1)** and (**Y_1**).

Finally, in the third mechanism product ion **(9^{+})** is formed directly from product ion **(2)** through the loss of the remaining tail region with the attached head group as a singly charged species of *m/z* 214.25 **(product ion 11).** Products ions **(8^{++}, 9^{++}, 10^{++})** were always observed as minor peaks supporting the argument that the predominant singly charged form **(8^{+}, 9^{+}, 10^{+})** is more stable.

Several remarkable differences are observed in the MS/MS analyses of these novel amino acid substituted gemini surfactants in comparison with our recent study evaluating the ESI-QqToF MS/MS behavior of the non-substituted diquaternary ammonium gemini surfactants[25]. For instance, product ion **(2)** is diagnostic for the amino acid/di-peptide substituted gemini surfactants since the formation of this ion was not observed in the analysis of non-substituted compounds[26-27]. This can be explained by the fact that the first generation diquaternary ammonium gemini surfactants do not have an amine group within the spacer region which can easily be charged. This ion was the source of all other fragments as shown in Figures 5.3a and 5.4a. In addition, all diagnostic product ions formed through fragmentation within the spacer region are observed only in substituted gemini surfactants evaluated in this work.

Figure 5.6: Universal MS/MS Fragmentation Pattern for 12-7N[Amino acid(s)]-12 gemini surfactants.



5.5. Conclusion

In this study, the molecular structure of six novel mono-amino acid/di-peptide diquaternary ammonium gemini surfactants was confirmed using ESI-QqToF MS with internal calibration. The tandem mass spectrometric analysis (QqTof-MS/MS) showed similarities in the fragmentation patterns of all tested compounds. This allowed us to establish a universal MS/MS fragmentation pathway which was confirmed through performing MS/MS/MS experiments. In addition, we performed the MS/MS analysis for a deuterated 12_{D25}-7N(Glycine)-12_{D25}which bears deuterated tail region. It was observed that fragment ions identical to those observed when analyzing non-deuterated 12-7(Glycine)-12, were generated, differing merely by the ions bearing the deuterated tail region(s) [Table 5.4]. This confirms the proposed universal fragmentation pathway shown in Figure 5.6. The deuterated compound was synthesized as an internal standard that will be used during the development of HPLC-MS/MS quantification methods.

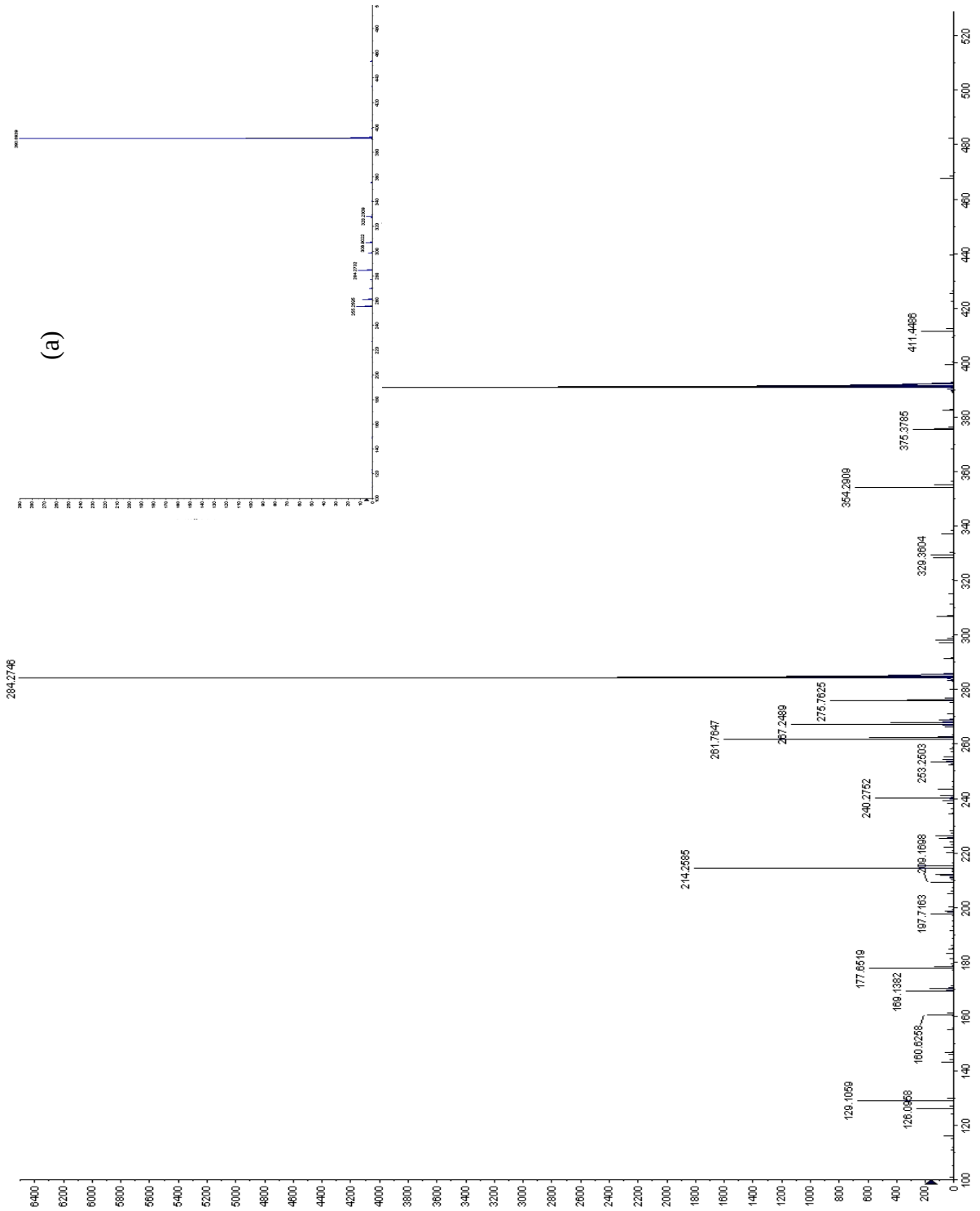
In summary, eleven common product ions were observed in the MS/MS analysis of almost all tested gemini surfactants. Two abundant diagnostic product ions observed in all tested gemini surfactants resulted from the loss of one tail region with attached head group (ion 2) or both tails and heads (ion 9). The proposed fragmentation pathway can be used as a "fingerprint" for rapid and accurate identification of these compounds in different biological or pharmaceutical matrices. In addition, by utilizing the MS/MS fragmentation pattern, we are currently developing a multiple reaction monitoring (MRM) HPLC-MS/MS method for the purpose of quantitation of these novel non-viral gene delivery agents.

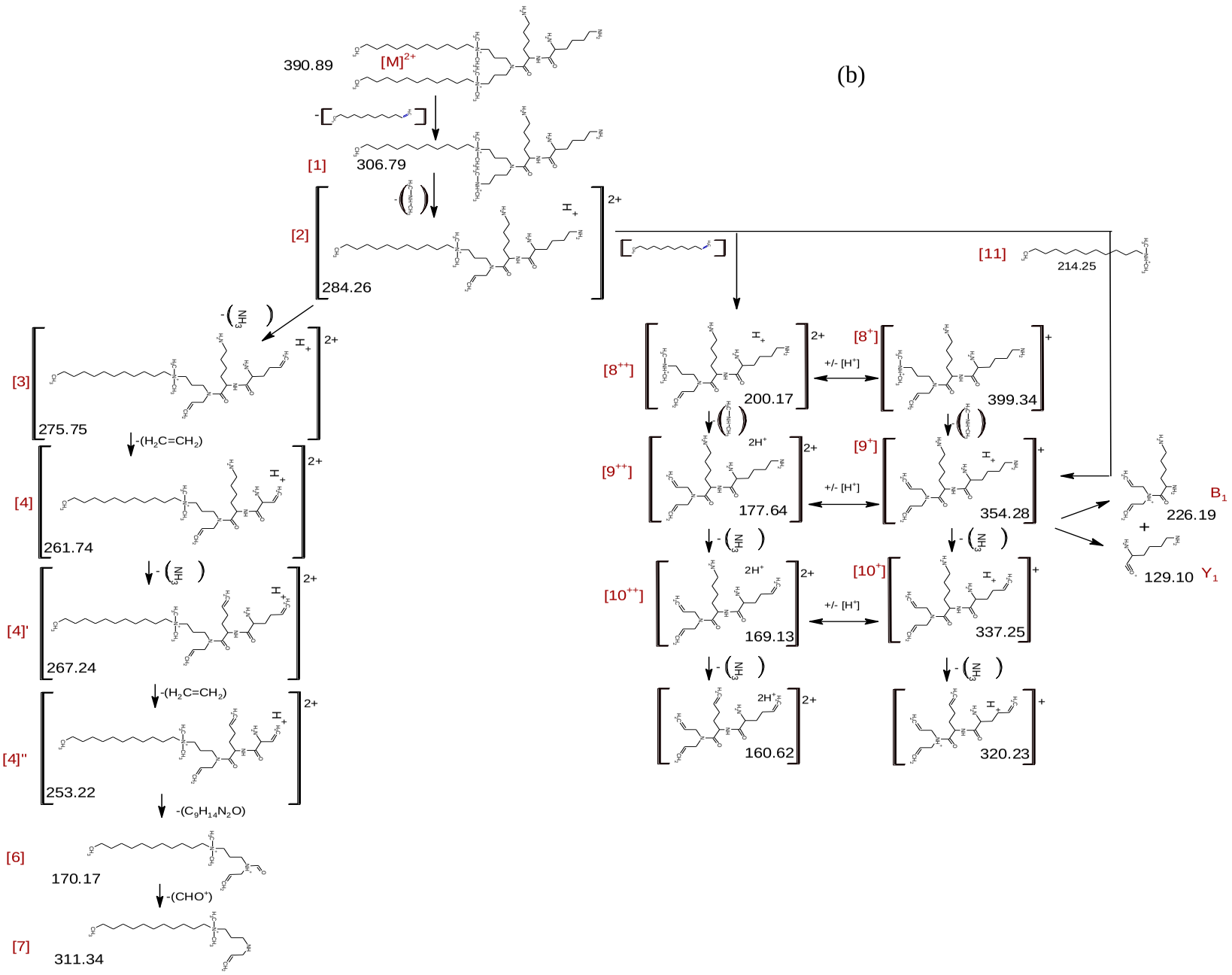
Acknowledgements

The authors acknowledge the Saskatchewan Structural Sciences Centre (SSSC) for the use of QSTAR system and Mr. Ken Thoms for his technical assistance. The authors acknowledge funding from the Natural Science and Engineering Research Council of Canada (NSERC) through NSERC Discovery Grant.

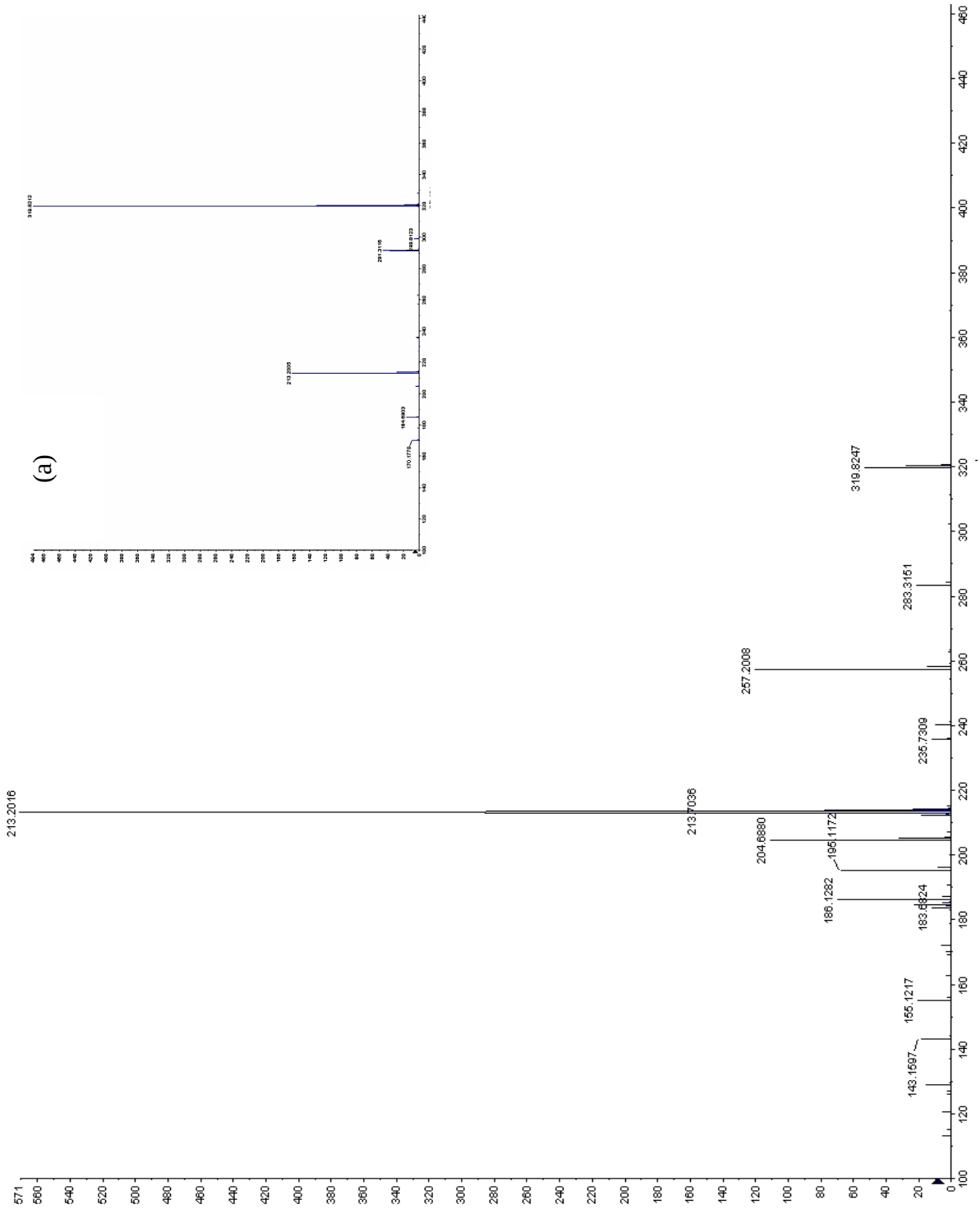
Appendices

Appendix 5.1: (a) The ESI-QqToF MS/MS spectra of 12-7N(Lysyl-Lysine)-12 a di-peptide gemini surfactants (Full MS spectrum in the box), (b) the MS/MS fragmentation pattern showing the most distinctive product ions, other non-diagnostic product ions are not included.

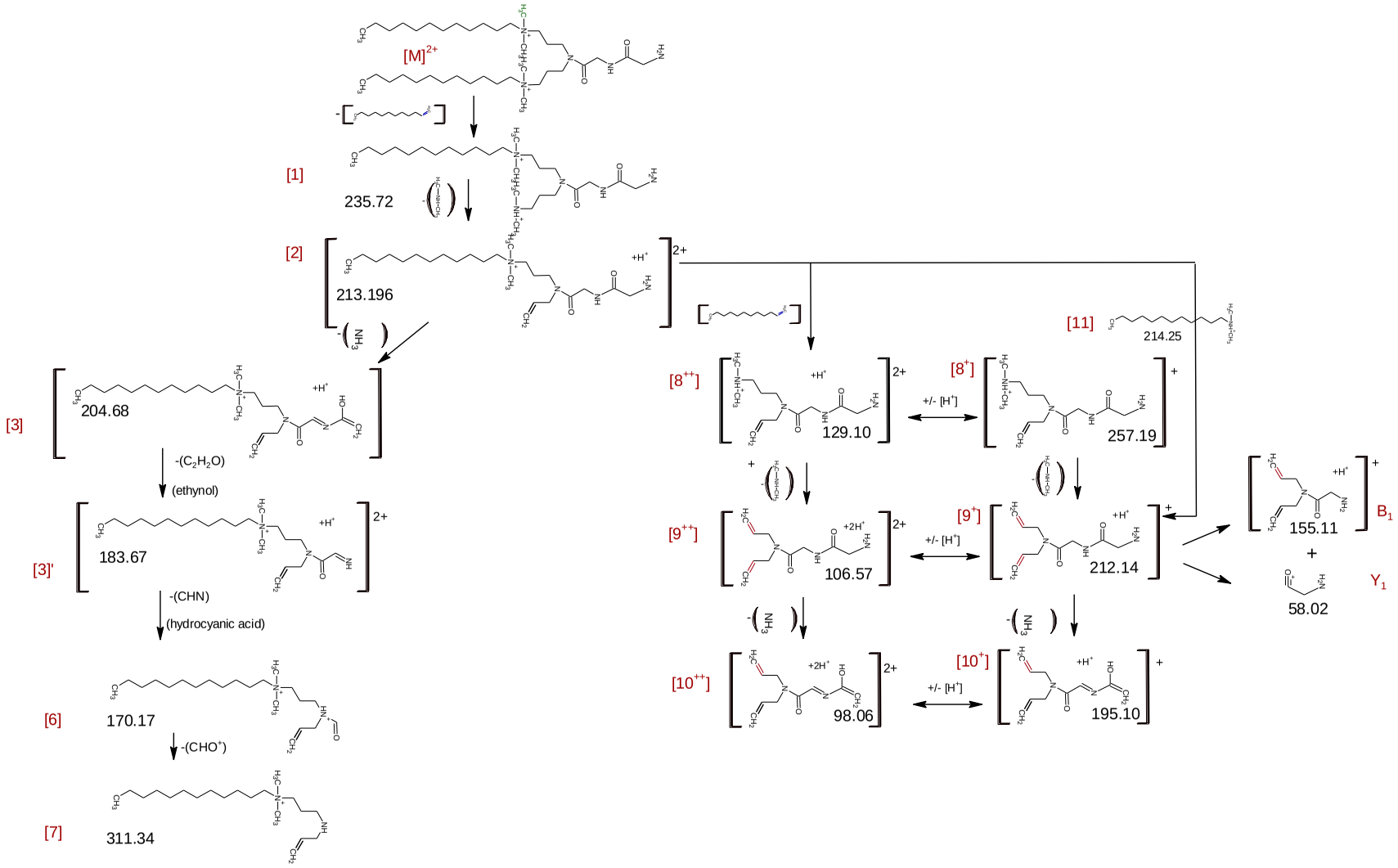



(b)
390.89
[M]2+
[1]
306.79
[2]
284.26
[3]
275.75
-(H2C=CH2)
[4]
261.74
[4]'
267.24
-(H2C=CH2)
[4]''
253.22
-(C9H14N2O)
[6]
170.17
-(CHO•)
[7]
311.34
[11]
214.25
[8++]
200.17
+/- [H+]
[8+]
399.34
[9++]
177.64
[9+]
354.28
B1
226.19
+
129.10
Y1
[10++]
169.13
[10+]
337.25
160.62
320.23

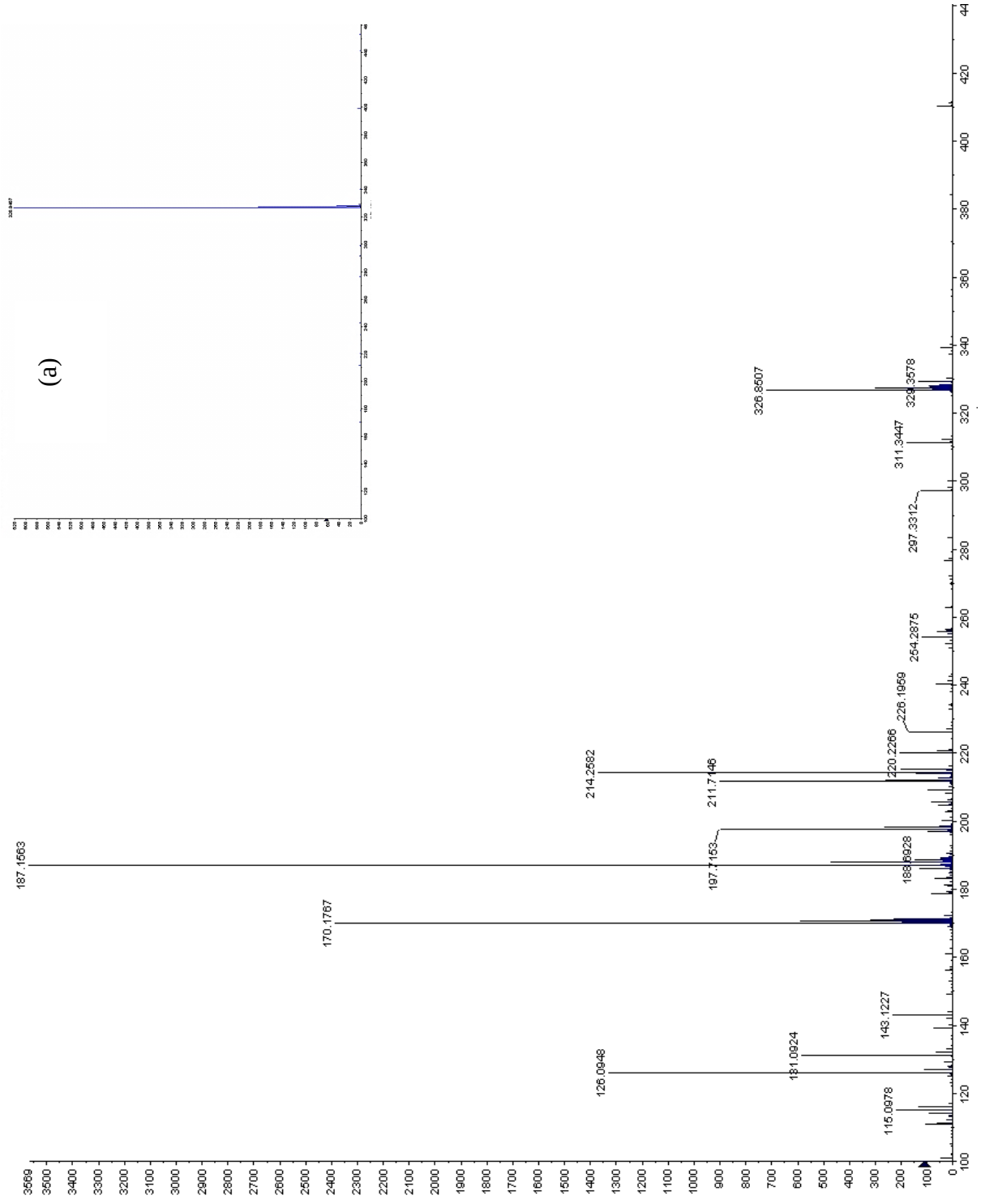
Appendix 5.2: (a) The ESI-QqToF MS/MS spectra of 12-7N(Glycyl-Glycine)-12 a di-peptide gemini surfactants (Full MS spectrum in the box), (b) the MS/MS fragmentation pattern showing the most distinctive product ions, other non-diagnostic product ions are not included.

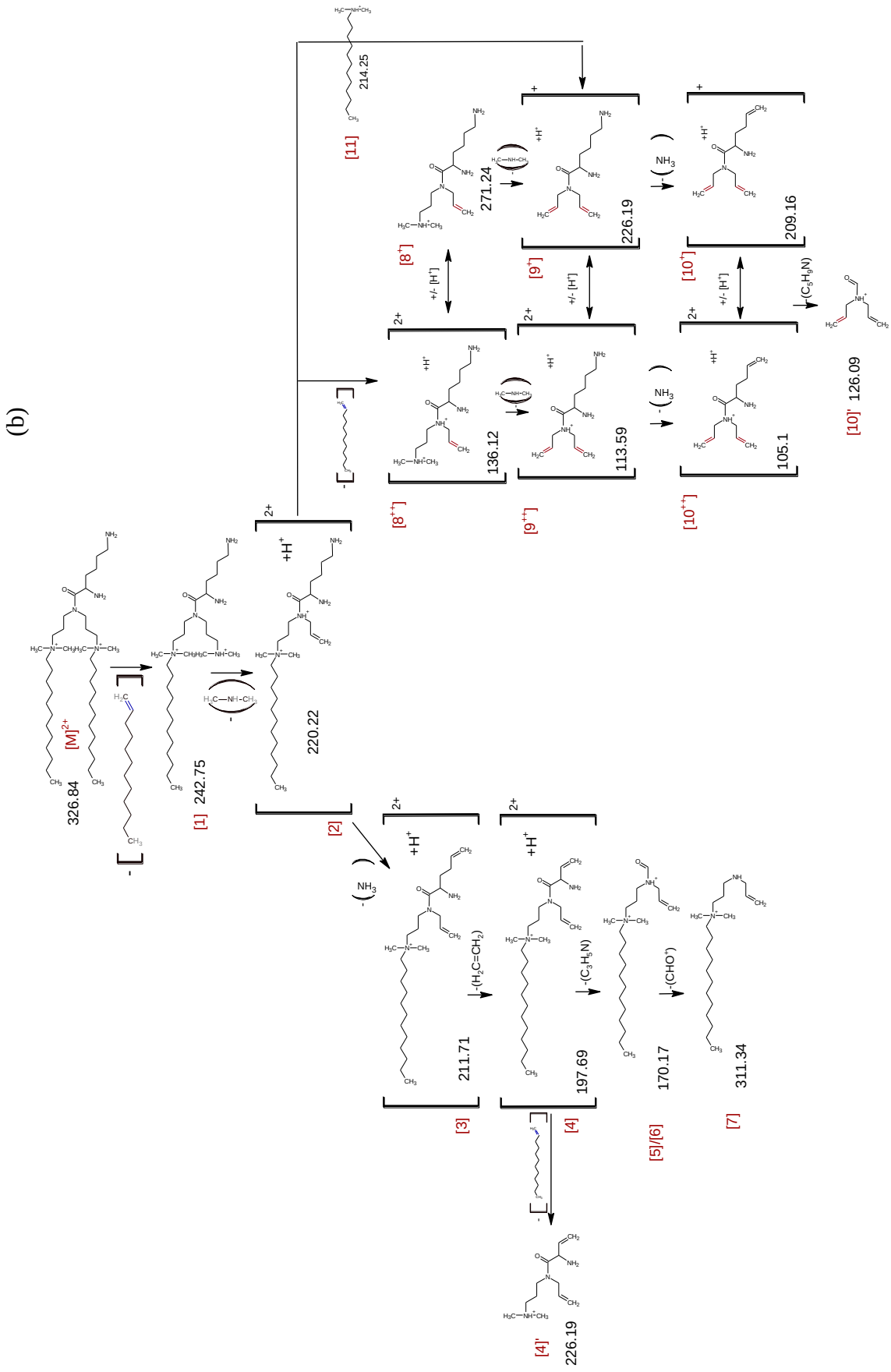


(b)

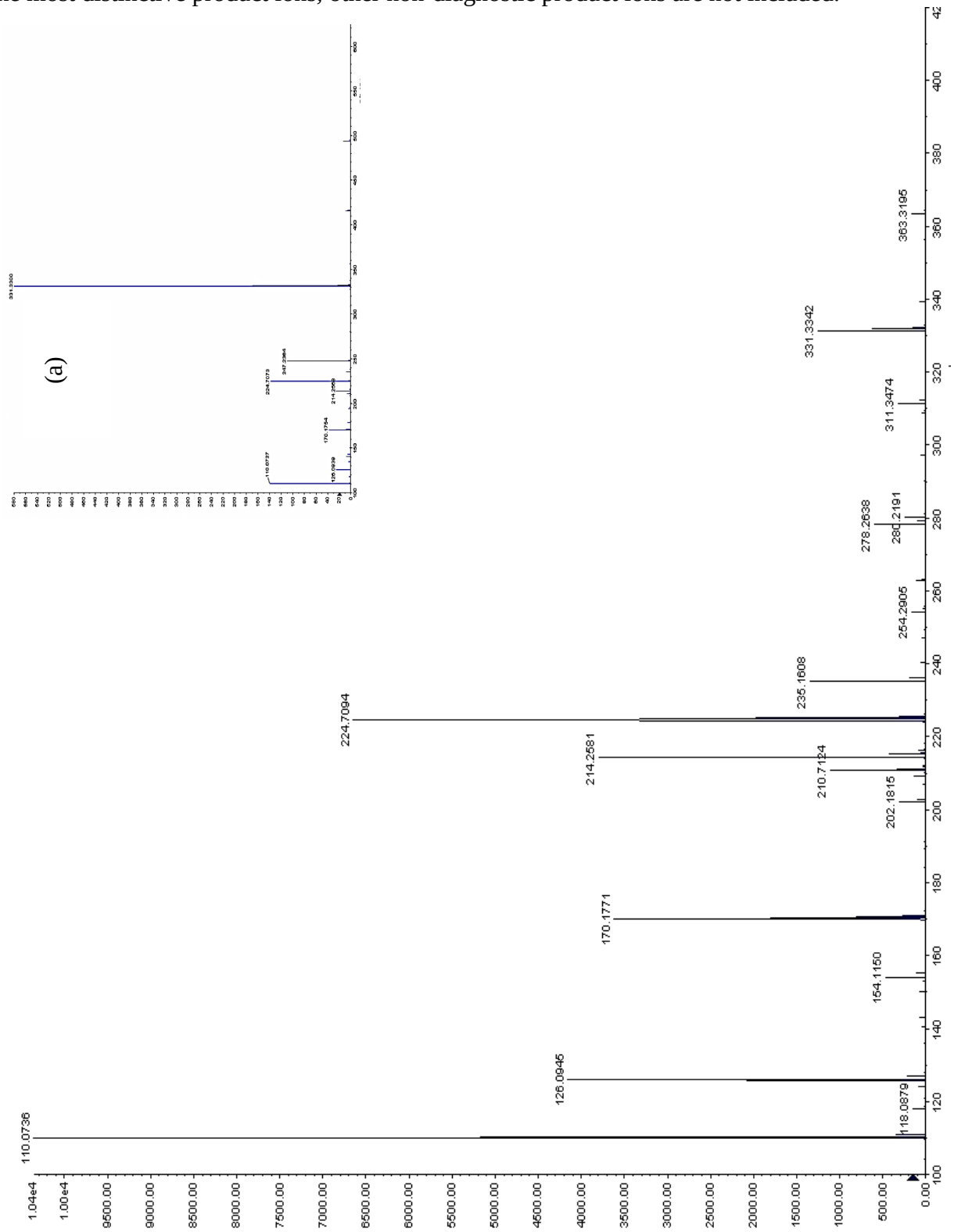

[M]2+
[1]
235.72
[2]
213.196
2+
+H+
-(NH3)
[3]
204.68
-(C2H2O)
(ethynol)
[3]'
183.67
-(CHN)
(hydrocyanic acid)
[6]
170.17
-(CHO+)
[7]
311.34
[11]
214.25
[8++]
129.10
+/- [H+]
[8+]
257.19
[9++]
106.57
[9+]
212.14
[10++]
98.06
[10+]
195.10
B1
155.11
+
Y1
58.02

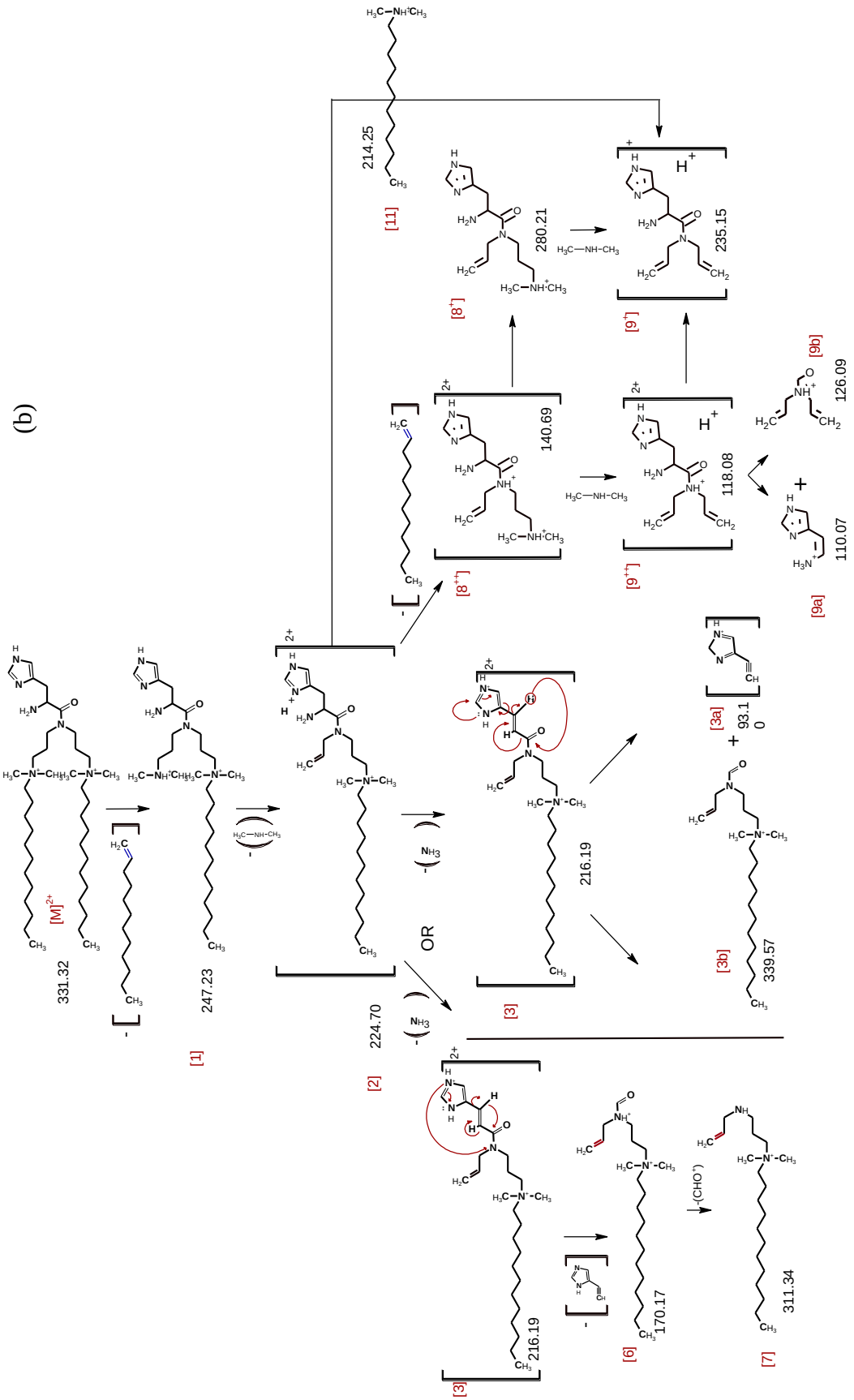
Appendix 5.3: (a) The ESI-QqToF MS/MS spectra of 12-7N(Lysine)-12 a mono-amino acid gemini surfactants (Full MS spectrum in the box), (b) the MS/MS fragmentation pattern showing the most distinctive product ions, other non-diagnostic product ions are not included.




(b)
326.84
[M]2+
[1]
242.75
220.22
[2]
214.25
[11]
[8++]
136.12
[8+]
271.24
[9++]
113.59
[9+]
226.19
[10++]
105.1
[10+]
209.16
[10']
126.09
NH3
+/- [H+]
-(C5H9N)
[3]
211.71
[4]
197.69
[4']
226.19
-(H2C=CH2)
-(C3H5N)
-(CHO•)
[5]/[6]
170.17
[7]
311.34

Appendix 5.4: (a) The ESI-QqToF MS/MS spectra of 12-7N(Histidine)-12 a mono-amino acid gemini surfactants (Full MS spectrum in the box), (b) the MS/MS fragmentation pattern showing the most distinctive product ions, other non-diagnostic product ions are not included.




(b)
[M]2+
331.32
[1]
247.23
[2]
224.70
OR
[3]
216.19
[3a]
93.1
0
[3b]
339.57
[6]
170.17
[7]
311.34
[8++]
140.69
[8+]
280.21
[9++]
118.08
[9+]
235.15
[9a]
110.07
[9b]
126.09
[11]
214.25
-(CHO+)

References

1. Bjorklund T, Kordower JH. Gene therapy for Parkinson's disease. Movement Disorders 2010;25:S161-S73.

2. Kohn DB, Candotti F. Gene therapy fulfilling its promise. New England Journal of Medicine 2009;360:518-21.

3. Qasim W, Gaspar H, Thrasher A. Progress and prospects: gene therapy for inherited immunodeficiencies. Gene Therapy 2009;16:1285-91.

4. Shirakawa T. Clinical trial design for adenoviral gene therapy products. Drug News Perspect 2009;22:140-5.

5. Williams DA. Gene therapy continues to mature and to face challenges. Molecular therapy: the journal of the American Society of Gene Therapy 2009;17:1305.

6. Daya S, Berns KI. Gene therapy using adeno-associated virus vectors. Clinical Microbiology Reviews 2008;21:583-93.

7. Mueller C, Flotte TR. Clinical gene therapy using recombinant adeno-associated virus vectors. Gene Therapy 2008;15:858-63.

8. Kimball KJ, Numnum TM, Rocconi RP, Alvarez RD. Gene therapy for ovarian cancer. Current Oncology Reports 2006;8:441-7.

9. Edelstein ML, Abedi MR, Wixon J, Edelstein RM. Gene therapy clinical trials worldwide 1989–2004 an overview. The Journal of Gene Medicine 2004;6:597-602.

10. Raper SE, Chirmule N, Lee FS, et al. Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. Molecular Genetics and Metabolism 2003;80:148-58.

11. Grieger JC, Samulski RJ. Packaging capacity of adeno-associated virus serotypes: impact of larger genomes on infectivity and postentry steps. Journal of Virology 2005;79:9933-44.

12. Mintzer MA, Simanek EE. Nonviral vectors for gene delivery. Chemical Reviews 2009;109:259.

13. Kiefer K, Clement J, Garidel P, Peschka-Süss R. Transfection efficiency and cytotoxicity of nonviral gene transfer reagents in human smooth muscle and endothelial cells. Pharmaceutical Research 2004;21:1009-17.

14. Wasungu L, Hoekstra D. Cationic lipids, lipoplexes and intracellular delivery of genes. Journal of Controlled Release 2006;116:255-64.

15. Menger FM, Keiper JS. Gemini surfactants. Angewandte Chemie International Edition 2000;39:1906-20.

16. Hait S, Moulik S. Gemini surfactants: a distinct class of self-assembling molecules. CURRENT SCIENCE-BANGALORE- 2002;82:1101-11.

17. Kirby AJ, Camilleri P, Engberts JBFN, et al. Gemini surfactants: new synthetic vectors for gene transfection. Angewandte Chemie International Edition 2003;42:1448-57.

18. Wasungu L, Scarzello M, van Dam G, et al. Transfection mediated by pH-sensitive sugar-based gemini surfactants; potential for in vivo gene therapy applications. Journal of Molecular Medicine 2006;84:774-84.

19. Wettig SD, Verrall RE, Foldvari M. Gemini surfactants: a new family of building blocks for non-viral gene delivery systems. Current Gene Therapy 2008;8:9-23.

20. Yang P, Singh J, Wettig S, Foldvari M, Verrall RE, Badea I. Enhanced gene expression in epithelial cells transfected with amino acid-substituted gemini nanoparticles. European Journal of Pharmaceutics and Biopharmaceutics 2010;75:311-20.

21. Singh J, Yang P, Michel D, E Verrall R, Foldvari M, Badea I. Amino Acid-Substituted Gemini Surfactant-Based Nanoparticles as Safe and Versatile Gene Delivery Agents. Current Drug Delivery 2011;8:299-306.

22. Gehrig PM, Hunziker PE, Zahariev S, Pongor S. Fragmentation pathways of NG-methylated and unmodified arginine residues in peptides studied by ESI-MS/MS and MALDI-MS. Journal of the American Society for Mass Spectrometry 2004;15:142-9.

23. Ye Y, Cao LF, Niu MY, Liao XC, Zhao YF. ESI-MS fragmentation pathways of N-methylpyrrole polyamide/peptide conjugates. International Journal of Mass Spectrometry 2006;253:141-5.

24. El-Aneed A, Banoub J. Elucidation of the molecular structure of lipid A isolated from both a rough mutant and a wild strain of Aeromonas salmonicida lipopolysaccharides using electrospray ionization quadrupole time-of-flight tandem mass spectrometry. Rapid Communications in Mass Spectrometry 2005;19:1683-95.

25. Cui M, Song F, Zhou Y, Liu Z, Liu S. Rapid identification of saponins in plant extracts by electrospray ionization multi-stage tandem mass spectrometry and liquid chromatography/tandem mass spectrometry. Rapid Communications in Mass Spectrometry 2000;14:1280-6.

26. Buse J, Badea I, Verrall RE, El-Aneed A. Tandem Mass Spectrometric Analysis of the Novel Gemini Surfactant Nanoparticle Families G12-s and G18: 1-s. Spectroscopy Letters 2010;43:447-57.

27. Buse J, Badea I, Verrall RE, El-Aneed A. Tandem mass spectrometric analysis of novel diquaternary ammonium gemini surfactants and their bromide adducts in electrospray-positive ion mode ionization. Journal of Mass Spectrometry 2011;46:1060-70.

28. Wettig S, Verrall R. Thermodynamic studies of aqueous msm gemini surfactant systems. Journal of Colloid and Interface Science 2001;235:310-6.

29. Jenkins KM. Thermodynamic studies of bis(quaternaryammonium) and (N-alkyl-) ionic surfactants. M.Sc. thesis. Saskatoon, SK, Canada: University of Saskatchewan; 2000.

30. Wettig SD. Studies of the interaction of gemini surfactnats with polymers and triblock copolymers. Ph.D. thesis. Saskatoon, SK, Canada: University of Saskatchewan; 2000.

31. Hager JW. A new linear ion trap mass spectrometer. Rapid Communications in Mass Spectrometry 2002;16:512-26.

32. Hopfgartner G, Varesio E, Tschäppät V, Grivet C, Bourgogne E, Leuthold LA. Triple quadrupole linear ion trap mass spectrometer for the analysis of small molecules and macromolecules. Journal of Mass Spectrometry 2004;39:845-55.

33. Illies A, Jarrold M, Bowers M. Experimental investigation of gaseous ionic structures of interstellar importance: HCO+ and HOC+. Journal of the American Chemical Society 1983;105:2562-5.

34. Bohme D, Goodings J, Ng CW. In situ chemical ionization as a probe for neutral constituents upstream in a methane-oxygen flame. International Journal of Mass Spectrometry and Ion Physics 1977;24:335-54.

35. P. R, J. F. Nomenclature for Mass Spectrometry of Peptides, Letter to the editors. Journal of Biomedical Mass Spectrometry 1984;11:601-3.

Chapter 6

Overall conclusions

Ideal cationic lipid-based lipoplexes should conform to the following criteria 1) high transfection activity and low cytotoxicity, 2) ability of targeting specific sites and 3) ability to be produced as a pharmaceutical dosage form with acceptable shelf stability[1,2]. The cationic gemini surfactant family provides a wide variety of carriers that can be modified to achieve optimum gene transfection activity with the ability to be customized chemically for targeting purposes. In our drug delivery research group in the College of Pharmacy and Nutrition, in collaboration with Dr.Verrall's research group in the Department of Chemistry, a series of quaternary ammonium gemini surfactants have been synthesized and characterized for the purpose of using as non-viral gene delivery agents.[3-6] Significant enhancements in gene expression activity (*in vitro* and *in vivo)* were achieved by rational modifications in the chemical structure of the gemini surfactants. Comprehensive physiochemical characterization and evaluation of the biological activity were implemented to optimize the transfection efficiency of the synthesized compounds.[3,7-10] However, in-depth analytical methods for the identification and quantification of the gemini surfactants in mixtures have not been developed and the physical and chemical stability of the gemini surfactant-based lipoplexes have not been addressed or investigated.

In the present work, two research streams were identified and explored aiming to improve the long term stability of gemini surfactant-based lipoplexes. The first stream was the investigation of the influence of different formulation strategies combined with lyophilization on the physiochemical stability of gemini surfactant-based lipoplexes. The second component was the mass spectrometric analysis of six amino acid-modified gemini surfactants that can be utilized for the evaluation of the chemical stability of the DNA carrier.

6.1. Lyophilization of gemini surfactant-based lipoplexes

6.1.1. Formulation development and pilot evaluation of stabilizing agents

I evaluated the feasibility of lyophilization as a technique for preparing gemini surfactant-based lipoplexes with long term stability. In the first stage I investigated the ability of the lyophilization to preserve the essential physiochemical properties and transfection activity of the lipoplex systems. The formulation development stage revealed that the lyophilization of plasmid/gemini surfactant/DOPE [P/G/L] lipoplexes without any stabilizing agents caused a complete loss of the optimal physiochemical properties of the lipoplexes (particle size and zeta potential) indicating the necessity of using stabilizing agents for lyophilization. Among all stabilizing agents (cryo-/lyo-protectants) evaluated in this work, disaccharide sugars sucrose and trehalose and glycerin (a polyol agent) were shown to be capable of preserving the properties of the lipoplexes and their biological activity.

The electrostatic interaction between the pDNA and gemini surfactant was able to protect the pDNA and (P/G) lipoplex structure against the stress resulting from the freeze-drying cycle even when no stabilizing agent was used. This finding suggests that the damage caused to the [P/G/L] lipoplexes by the lyophilization process was due to the changes in the supramolecular assembly resulting from the presence of DOPE in the formulation. The pilot stability study showed that the presence of glycerine in the formulation prevented the complete dehydration of the lyophilized formulation resulting in a lyophilized cake with high moisture content. As a result, a complete loss of gene activity was observed after one week of storage at both 25 ˚C and 40 ˚C.

During the formulation development stage, I had faced some limitations and challenges. The major limitation was the ineffectiveness of the freeze dryer (Lyph-Lock, 6 liter bench

freeze-dryer, Labconco, Kansas City, MO) that could not produce a fully dehydrated product. Consequently, the lyophilized formulations for the pilot stability study showed high moisture content that negatively impacted the physical and biological stability of the formulations. Therefore, the results from this stability study were relatively inconclusive and were not discussed in details. Nevertheless, I showed that preservation of the original physical characteristics (particle size and the positive zeta potential) during lyophilization and storage is not enough to maintain the transfection activity. The major challenge in this stage was the lack of a suitable method for extracting the pDNA from the [P/G/L] lipoplexes to quantify the pDNA content after freeze-drying cycle and during the stability study. I investigated several methods for this purpose but all were unsuccessful.

6.1.2. Stability study

Based on the results obtained from the formulation developments stage, I selected sucrose and trehalose as stabilizing agents and designed a three month stability study to evaluate the long term stability of the lyophilized gemini surfactant-based lipoplexes. I investigated two preparation methods [Table 4.1]. In the first method, two [P/G/L] systems were prepared in the stabilizing agent solution (sucrose or trehalose) by complexing the plasmid with the gemini surfactant first, then combining with the DOPE dispersion, followed by the lyophilisation of the [P/G/L] lipoplexes. I also developed the second method to overcome the challenge associated with the extraction and quantification of pDNA content. In this case, the [G/L] system in the sugar solution was prepared and lyophilized without including the pDNA in the formulation. Fresh pDNA was incorporated into the formulation during the rehydration step.

The influence of the freeze-drying on the physiochemical properties of the lipoplexes such as particle size, zeta potential, interaction between pDNA and gemini surfactant and pDNA

compaction were investigated. The physiochemical characterization of lyophilized lipoplexes revealed that the freeze-drying process caused an increase in particle size and zeta potential values [Table 4.2-B]. These changes in the physiochemical properties of the lipoplexes had no effect on the ability of the gemini surfactant to electrostatically interact with pDNA and provide complete protection as showed by the ethidium bromide/gel electrophoresis [Figure 4.4-C]. However, the CD analysis showed that all lyophilized lipoplexes altered the native structure of the pDNA in a different manner compared to their corresponding fresh lipoplexes [Figure 4.2].

The influence of the lyophilization process on the *in vitro* transfection activity of gemini surfactant-based lipoplexes revealed an unexpected finding: it caused a significant increase in gene expression of three formulations just after lyophilization/rehydration cycle. The two P-$[G/L]_{lyp}$ systems showed the highest increase in transfection activity (about 3-fold increase) in comparison with the standard fresh formulation (P/G/L-S) [Figure 4.3]. Considering the changes in the physiochemical properties, I proposed two hypotheses to explain the increase in gene expression upon freeze-drying. In the first instance, I believe that the lyophilization process induced polymorphic changes in the [G/L] structure forcing the formation of an inverted hexagonal phase that is responsible for high gene expression activity. In the second explanation, I proposed that during the freezing cycle free gemini surfactant molecules or vesicles in the formulation were incorporated in the [G/L] phase, as a result of cryoconcentration effect. This effect caused the formation of P-[G/L] lipoplexes with an apparent higher +/– charge ratio which might improve the cellular uptake and consequently transfection activity.

I conducted a three month stability study at 25 ˚C/75 RH to evaluate the ability of lyophilized formulations to maintain the stability of the gemini surfactant-based lipoplexes. Lyophilized formulations stored at 25 ˚C showed relatively good stability [Figure 4.8]. The

formulations lyophilized with the pDNA (i.e., $[P/G/L\text{-}S]_{lyp}$ and $[P/G/L\text{-}T]_{lyp}$) were able to maintain more than 60% of original transfection activity at the last sampling point. The P-$[G/L]_{lyp}$ formulations retained their full transfection activity for two months. However, the transfection activity of both formulations dropped after three months of storage. The loss of the transfection activity of the $[P/G/L]_{lyp}$ formulations can be rationalized by two mechanisms. The first mechanism involves the degradation of pDNA and the loss of supercoiled form as a result of oxidative stress exerted by the presence of free radicals or reactive oxygen species as mediators. In the second mechanism presumes that the primary polymorphic morphology of the $[P/G/L]_{lyp}$ lipoplexes was lost during the storage by reorganization or chemical degradation of lipid phase (DOPE/gemini surfactant).

In the case of P-$[G/L]_{lyp}$ formulations, the only mechanism that could explain the loss of transfection activity can be described by the loss of the supramolecular structure during the storage, since the pDNA was added freshly at each sampling point. I proposed that the $[G/L]_{lyp}$ underwent a polymorphic phase transition during the storage at 25 ˚C resulting in the loss of inverted hexagonal rearrangement formed during the lyophilization/rehydration.

It should be noted that the physiochemical characterization of lyophilized formulations during the stability study revealed that there was no statistical correlation between the changes in physicochemical properties (particle size and zeta potential) and the changes in transfection activity. However, the particle size was maintained below 200 nm without any significant changes in particle size distribution as indicated by the unchanged PDI values [Table 4.2-B]. In addition, positive zeta potential was observed for all formulation during the stability study.

In general, I achieved significant improvement in the stability of gemini surfactant based-lipoplexes by employing lyophilization and both sucrose and trehalose performed well as

stabilizing agents. I developed a formulation method that can be utilized to prepare lipoplexes with high gene expression activity. However, more structural characterizations are required to understand the structural changes induced during the freeze-drying process. In addition, further investigation of the factors affecting the long-term stability of lyophilized gemini surfactant-based lipoplexes is essential to optimize the formulation and lyophilization methods.

6.2. Mass spectrometric analysis of amino acid modified gemini surfactants

Tandem mass spectrometric analysis was performed to establish a foundation for qualitative analysis of the gemini surfactant, detection of possible degradation by-products, and for pharmacokinetic and metabolomics studies. This was accomplished by utilizing a hybrid quadrupole orthogonal time-of-flight mass spectrometer (QqToF-MS) and a triple quadrupole – linear ion trap mass spectrometer both equipped with an electrospray ionization (ESI) source.

In this respect, the single stage QqToF-MS with internal calibration was used to confirm the molecular structure of six amino acid/di-peptide modified gemini surfactants with high mass accuracy (less than 10 ppm) [Table 5.1]. In addition, the tandem mass spectrometric (MS/MS) results contributed to establishing a universal (MS/MS) fragmentation pathway for all six compounds [Table 5.2, Figure 5.6]. Eleven common fragment ions were observed in all tested compounds. Two compound-specific diagnostic fragment ions were observed in all tested gemini surfactants originating from the loss of; one tail+head region [ion 2, Figure 5.6] or both tail+head regions [ion 9, Figure 5.6]. All fragment ions formed through two main pathways. Those in the first pathway resulted from the neutral loss of a tail region as (dodec-1-ene) moiety followed by the neutral loss of the attached head group as (*N*-methylmethanamine) moiety [Pathway A, Figure 5.6]. In the second pathway, the fragment ions originated from the complementary ion loss of one (tail+head) region as singly charged ion (*N*,*N*-dimethyldodecan-1-aminium, ion 11)

[Pathway B, Figure 5.6]. The fragmentation pathway was confirmed by two means: by performing multiple stage mass spectrometric analysis (MS/MS/MS) and by (MS/MS) analysis of a deuterated form of the 12-7N(Glycine)-12 (i.e., 12_{D25}-7N(Glycine)-12_{D25}) [Table 5.4]. The established fragmentation pathway can be used as a "fingerprint" for:

- rapid and accurate detection of these compounds in biological matrices or pharmaceutical formulations,
- the development of a multiple reaction monitoring (MS/MS) quantification method,
- detection of possible degradation by-products during an accelerated stability study and
- pharmacokinetic studies.

6.3. Future research directions

The present work was the first attempt to evaluate and improve the long-term stability of the gemini surfactant-based lipoplexes designed and developed by our research group. To further advance the research that was discussed in this work, different objectives can be investigated in the future. The following directions are relevant to my work and need to be addressed.

6.3.1. Comprehensive characterization of lyophilized gemini surfactant lipoplexes

Novel cationic gemini surfactants are being developed in our research group to enhance the transfection activity and achieve specific targeting by the lipoplexes. The chemical structure of the new series compounds is more complicated than the gemini surfactant used in the present work. The data presented in this work showed that lyophilization can be used as a formulation technique to enhance the transfection activity. Therefore, detailed structural characterization is essential to investigate the actual influence of the formulation methods and freeze-drying cycles (freezing and drying) on the polymorphic phase behaviour of the lipoplex system. This can be achieved by employing synchrotron-based X-ray diffraction techniques such as small-angle X-ray scattering (SAXS) or wide-angle X-ray scattering (WAXS). In addition, electron microscopy techniques such as freeze-fracture electron microscopy and scanning electron microscopy could be used to examine the lipid phase morphology. Determination of glass transition temperature is also essential to understand the influence of sugars on the stability of the lyophilized formulations. The glass transition temperature can be measured via differential scanning calorimetric (DSC) technique.

6.3.2. Optimization of formulation and lyophilization technique

The full characterization of lyophilized formulations can assist further optimization of the formulation methods and freeze-drying parameters that could enhance the transfection activity and the stability of the lyophilized gemini surfactant-based lipoplexes. The optimization of preparative methods includes the removal of any possible oxidative stress mediators that could be found in the starting materials. In addition, the formulation improvement could include the replacement of the helper lipid DOPE by other lipids such as cholesterol. My results indicated that the higher concentration of DOPE lipid could be a reason for the loss of the transfection activity of the lyophilized lipoplexes during the storage.

The optimization of the freeze-drying could include the investigation of the influence of other freezing methods such as super-freezing by immersion in liquid nitrogen or a ramped freezing cycle. In addition, freeze-thawing studies must be conducted to understand the exact effect of the freezing cycle on the physiochemical properties and the biological activity of lipoplexes. The determination of the glass transition temperature of freeze-concentrate component (*Tg*') could be useful in obtaining the optimum sugar to lipoplex weight ratio that provides the maximum cryo-protective effect.[11,12] The improvement of the drying cycles through monitoring the glass transition temperature of lyophilized cake (*Tg*) and moisture content could significantly improve the long-term stability of lyophilized formulations.

6.3.3. Mass spectrometric-based quantification method

The chemical stability of the lipid phase components of the lyophilized lipoplexes is a concern that to our knowledge has not been explored. The investigation of the stability of the gemini surfactant and helper lipid DOPE is important for the long-term stability of lipoplexes. Additionally, the determination of possible degradation by-products and the fate of the lipid

component upon uptake are critical during the development of lipoplex-based pharmaceutical products for clinical trials. In our research group, mass spectrometric and hyphenated mass spectrometric techniques (i.e., LC-MS) are employed for chemical characterization and quantification of drug delivery systems.[13-15] MS-based techniques provide fast, sensitive, accurate and reliable results that can be utilized for stability study.[16-19] My MS/MS results can be used to build a MS-base quantification method to investigate the influence of the lyophilization process and storage conditions on the stability of gemini surfactant and DOPE molecules.

References

1. Elsabahy M, Nazarali A, Foldvari M. Non-viral nucleic acid delivery: key challenges and future directions. Current Drug Delivery 2011;8:235-44.

2. de Lima M, Neves S, Filipe A, Duzgunes N, Simoes S. Cationic liposomes for gene delivery: from biophysics to biological applications. Current Medicinal Chemistry 2003;10:1221-31.

3. Badea I, Verrall R, Baca-Estrada M, et al. In vivo cutaneous interferon-γ gene delivery using novel dicationic (gemini) surfactant–plasmid complexes. The Journal of Gene Medicine 2005;7:1200-14.

4. 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;1:165-76.

5. Wettig SD, Badea I, Donkuru MD, Verrall RE, Foldvari M. Structural and transfection properties of amine-substituted gemini surfactant-based nanoparticles. The Journal of Gene Medicine 2007;9:649-58.

6. Yang P, Singh J, Wettig S, Foldvari M, Verrall RE, Badea I. Enhanced gene expression in epithelial cells transfected with amino acid-substituted gemini nanoparticles. European Journal of Pharmaceutics and Biopharmaceutics 2010;75:311-20.

7. Wettig SD, Wang C, Verrall RE, Foldvari M. Thermodynamic and aggregation properties of aza-and imino-substituted gemini surfactants designed for gene delivery. Physical Chemistry Chemical Physics 2006;9:871-7.

8. Wang C, Li X, Wettig SD, Badea I, Foldvari M, Verrall RE. Investigation of complexes formed by interaction of cationic gemini surfactants with deoxyribonucleic acid. Physical Chemistry Chemical Physics 2007;9:1616-28.

9. Donkuru MD. Non-viral gene delivery with pH-sensitive gemini nanoparticles: synthesis of gemini surfactant building blocks, characterization and in vitro screening of transfection efficiency and toxicity: M.Sc. thesis. Saskatoon, SK, Canada: University of Saskatchewan; 2008.

10. Singh J, Michel D, Chitanda JM, Verrall RE, 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;10:7.

11. Allison SD, Anchordoquy TJ. Maintenance of nonviral vector particle size during the freezing step of the lyophilization process is insufficient for preservation of activity: insight from other structural indicators. Journal of Pharmaceutical Sciences 2001;90:1445-55.

12. Allison SD, Molina MC, Anchordoquy TJ. Stabilization of lipid/DNA complexes during the freezing step of the lyophilization process: the particle isolation hypothesis. Biochimica et Biophysica Acta (BBA)-Biomembranes 2000;1468:127-38.

13. Buse J, Badea I, Verrall RE, El-Aneed A. Tandem Mass Spectrometric Analysis of the Novel Gemini Surfactant Nanoparticle Families G12-s and G18: 1-s. Spectroscopy Letters 2010;43:447-57.

14. Buse J, Badea I, Verrall RE, El-Aneed A. Tandem mass spectrometric analysis of novel diquaternary ammonium gemini surfactants and their bromide adducts in electrospray-positive ion mode ionization. Journal of Mass Spectrometry 2011;46:1060-70.

15. Michel D, Chitanda JM, Balogh R, et al. Design and evaluation of cyclodextrin-based delivery systems to incorporate poorly soluble curcumin analogs for the treatment of melanoma. European Journal of Pharmaceutics and Biopharmaceutics 2012;3:548-56.

16. Hopfgartner G, Bourgogne E. Quantitative high-throughput analysis of drugs in biological matrices by mass spectrometry. Mass Spectrometry Reviews 2003;22:195-214.

17. Chen H, Talaty NN, Takáts Z, Cooks RG. Desorption electrospray ionization mass spectrometry for high-throughput analysis of pharmaceutical samples in the ambient environment. Analytical Chemistry 2005;77:6915-27.

18. Feng WY, Chan KK, Covey JM. Electrospray LC-MS/MS quantitation, stability, and preliminary pharmacokinetics of bradykinin antagonist polypeptide B201 (NSC 710295) in the mouse. Journal of Pharmaceutical and Biomedical Analysis 2002;28:601-12.

19. Shockcor JP, Unger SE, Wilson ID, Foxall PJD, Nicholson JK, Lindon JC. Combined HPLC, NMR spectroscopy, and ion-trap mass spectrometry with application to the detection and characterization of xenobiotic and endogenous metabolites in human urine. Analytical Chemistry 1996;68:4431-5.