Cationic Lipid and Peptide Synthesis for Co-delivery and Gene Therapy

Master's Thesis by:

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Abstract

Gene therapy is an intracellular delivery of genetic materials into specific cells to generate a therapeutic effect by correcting an existing abnormality or providing the cells with a new function. A major obstacle has been the delivery of genes to the appropriate cell affected by the disorder, without triggering unwanted side effects like cytotoxicity or unsettling the immune balance. Liposomes as non-viral gene delivery vectors have been to some extent successful in avoiding these side-effects. This study has employed novel liposomal gene delivery vehicles by incorporating short-chained (C_{11}) cationic saturated and unsaturated dimethylamine (SDMA and USDMA) lipids, cationic innate defense regulatory (IDR) peptides, IDR-1018 and IDR-HH2 and neutral helper lipids, cholesterol (Chol) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), in their formulations. These 3-component liposomes (peptide/lipid/co-lipid) were made to investigate their effects on delivering DNA in terms of cytotoxicity and transfection efficiency. This was done through formulating liposomes with DNA, forming lipoplexes, in four different molar charge ratios, (+/-) 1.5:1, 3:1, 5:1 and 10:1. The so-called lipoplexes were characterized using particle sizing and DNA-binding assays and then transfected into Chinese hamster ovarian cells (CHO-K1) with the aid of Lipofectamine 2000™. Their relative transfection efficiency and cytotoxicity were evaluated using β-galactosidase assay and MTS assay, respectively. The comparisons of USDMA versus SDMA, IDR-1018 versus IDR-HH2 and Chol versus DOPE were evaluated. The mean values of the results were calculated and the relative *p*-values from the student's t-tests were measured to observe any significant differences between these constituents.

Although the results from the gel retardation assay did not appear to be successful in showing any clear trends between bindings of the DNA to the proteins, the hydrodynamic diameter measurements showed reasonable size variations, between 200-900 nm, at <1 Polydispersity, which are relatively optimal for lipoplexes prepared without extrusion. The results showed that all of the lipoplexes were transfected into the cells. The lipoplexes at molar charge ratios 3:1 and 5:1 showed higher transfection efficiencies than the rest. The results from the student's t-test revealed that USDMA performed better than SDMA, Chol performed better than DOPE and the two peptides equally as good, in the relative transfection efficiencies. The cytotoxicity measurements showed a concentration dependent cytotoxicity. The results indicated a high cell viability at low molar charge ratios (1.5:1), with all of them exceeding ≥ 115% cell viability. The *p*-values showed no overall significant difference between the cytotoxicity of the two

peptides, the two lipids and the two co-lipids. The transfection efficiency as well as the cell viability results from the hybrid of cationic peptides and cationic lipids to form a liposome for non-viral DNA delivery vector showed better outcomes compared to the results obtained from using the same cationic lipids (alone) as the DNA delivery vectors. This suggests that incorporation of cationic peptides in the vector could potentially have a positive impact on gene therapy. Further analysis and investigations are, however, necessary to prove these results.

Acknowledgement

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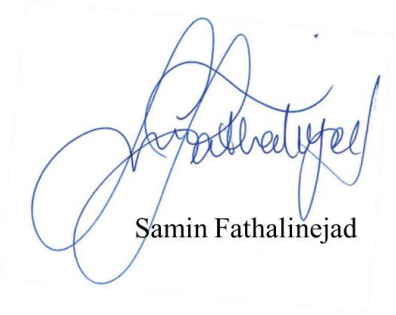


Table of Contents

List of Abbreviations

Overview of abbreviations used throughout this thesis.

Abbreviations	
AIDS	Acquired Immune Deficiency Syndrome
BCA	Bicinchoninic acid
β-GAL	B-Galactosidase
BSA	Bovine Serum Albumin
$CDCl_3$	Deuterated Chloroform
CHO-K1	Chinese Hamster Ovarian cells - mutant
Chol	Cholesterol
CPP	Cell Penetrating Peptide
DAD	Diode Array Detection
DCC	N,N-dicyclohexylcarbodiimide
DCM	Dichloromethane
DMA	Dimethylamine
DMAP	4-(dimethylamino)pyridine
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
DOPE	Dioleoylphosphatidyl-ethanolamine
EDTA	Ethylenediaminetetraacetic Acid
ESI	Electron Spray Ionization
FBS	Fetal bovine serum
FLC	Flash Liquid Chromatography
HBTU	2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HCl	Hydrochloric acid
HDP	Host Defence Peptide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High Pressure Liquid Chromatography
IDR	Innate Defence regulators
IUPAC	The International Union of Pure and Applied Chemistry
LC-MS	Liquid Chromatography- Mass Spectroscopy
LDH	Lactate dehydrogenase
M.W.	Molecular Weight

MALDI	Matrix Assisted Laser Desorption Ionization
MeOH	Methanol
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3 carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NADH	Nicotinamide Adenine Dinucleotide - Hydrogen
NMM	N-Methylmorpholine
NMP	N-Methyl-2-pyrrolidone
NMR	Nuclear Magnetic Resonance
OPTI-MEM	Minimum Essential Medium
PC	Phosphatidylcholine
*p*DNA	Plasmid DNA
PE	Phosphoethanolamine
RLU	Relative Light Unit
RPMI	Roswell Park Memorial Institute
SDMA	Saturated Dimethylamine
SPPS	Solid Phase Peptide Synthesis
TGS RAM	TentaGel Rink Amide
TMS	Tetramethylsilane
USDMA	Unsaturated Dimethylamine
UV	Ultra Violet
WHO	World Health Organisation

List of Figures

List of Schemes

List of Tables

1. Introduction

There are currently more than 4000 diseases that are caused by genetic mutations (WHO). These include different forms of cancer, AIDS, Alzheimer's disease, Parkinson's disease, cystic fibrosis, amyotrophic lateral sclerosis (Lou Gehrig's disease) and many more. It is this number that has kept the scientific world anxious to find a cure to these diseases since the beginning of their discovery. The pursuit of developing techniques, with which specific genetic mutations and disorders can be addressed, has led to many breakthroughs in the chemical and biological industries, one of which is the introduction of Gene Therapy. Gene therapy involves the in-cell delivery of a healthy gene into the site of mutation.[1] This methods approach to curing genetic diseases is through a target specific delivery of the relevant DNA and other genetic material (e.g. siRNA) to the mutation site, and consequently performing therapeutic stimulations, where it either silences or enhances gene expression.[2],[3]

The science of in-cell delivery of genes to correct defective genes or to selectively silence gene expression has been the center of attention since the late 1960's. Various types of vectors, aiding the transfer of specific genes, have been introduced throughout the years with viral vectors being the first suggested method.[4] In this case, a virus was used to ferry the useful gene into the cells. Viruses are thought to be a good delivery system for gene therapy because they are able to reside on specific tissues, invade cells and make viral proteins.[5] To date they are the most efficient mechanism of delivering a gene into the cell.[6] However, often they can merely be injected into a person only once or twice before the immune response they aggravate poses an immunological response and destroys the viral vector and blocks the production of the useful protein.[7]

In the late 1990's there was an accident involving a loss of a life in the viral delivery of a gene in a clinical trial and ever since the use of viral vectors for clinical use has been limited.[8] This is why researchers are putting an enormous effort into developing non-viral methods that are as efficient as viruses, yet will deliver the drug (gene) to the target cell without triggering carcinogenesis or a dangerous immune response.

1.1. Non-viral Drug Delivery Vectors

A number of investigations have established that genes can be delivered effectively without using viruses. [6],[9]–[16] In the race to develop a reliable drug delivery method, scientists have been working on a wide variety of vectors (Figure 1.1). Most non-viral vectors fly under the radar of the immune system and are cheaper and easier to manufacture than viral approaches. These carrier vectors range from cationic/anionic lipids to peptides, polymers, dendrimers (three-dimensional, branched, well-organized nanoscopic macromolecules) and other physical methods.[17]

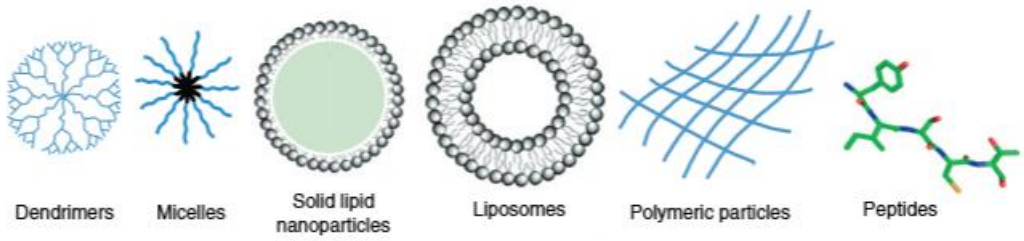


Figure 1.1. Structural representations of different non-viral drug delivery vectors.

A tremendous amount of work and investigations into these methods have shown that drug delivery systems should commonly possess a few features so that they can be considered effective. These features include having a high cell-penetrating affinity, target specificity, bio-degradability, drug-release efficacy and low toxicity.[18],[19] Studies of each one of the methods shown above have revealed considerable advantages as well as disadvantages regarding the in-cell delivery of drug molecules, with some being more successful in attaining the desired characteristics, than others. The delivery of genetic molecules using liposomes and peptides have long been introduced and investigated and the theoretical background to how these vectors can act as gene delivery vehicles are elaborated below.

1.1.1. Lipid-based vectors

Charged lipids are a novel example of non-viral vectors that have gained a lot of popularity amongst the scientific world today. Being negatively charged, DNA is not quite cell penetrating by itself and is in need of a cell delivery vehicle.[20],[21] It is

because of this that for so many years, cationic lipid vectors in the form of liposomes have been synthesized and combined with anionic nucleic acids to facilitate transfection into the cells for therapeutic purposes.

In this approach, as shown in Figure 1.2, the charged liposome coats the therapeutic DNA through ionic and hydrophobic interactions and forms a lipid-DNA complex known as a lipoplex.[22],[23]

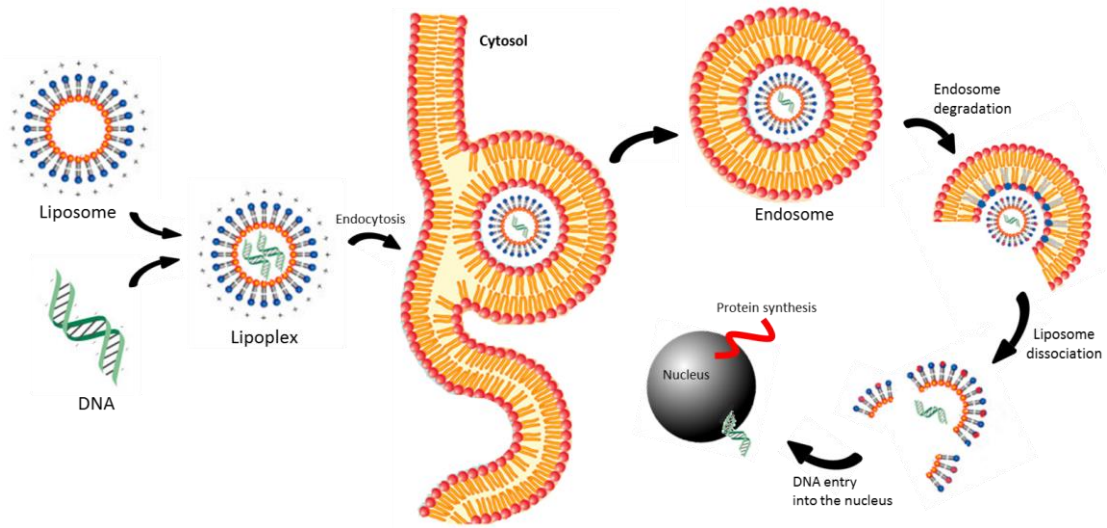


Figure 1.2. The mechanism of in-cell delivery of lipoplexes through endocytosis. © Samin Fathalinejad**.**

The lipoplex will bind to the cell surface through electrostatic interactions between charged liposome and the charged cell surface. The complex enters the cell by endocytosis, leading to the formation of an endosome. As the formed endosome enters the cytosol, the pH of its lumen decreases. This results in the degradation of the lipid bilayer of the lipoplex and in the integration of lipoplex lipids with the membrane of the endosome. This integration causes a destabilization of the endosomal membrane which dissociates and releases the DNA cargo in the cytosol of the cell. Cytoplasmic transport carries the DNA cargo near the nucleus region. The DNA cargo enters the nucleus and comes in contact with the cell's genetic material via one of two mechanisms. 1. Passive DNA entry during mitosis where the nuclear membrane dissociates temporarily or 2. Via the nuclear pores by active transport. Once the DNA enters the nucleus, it may code for the protein needed to correct the defective genes or to silence them. [24]

Synthetic vectors, such as cationic lipids, offer an alternative to viral transfection. Lipid-based DNA delivery vectors have unique characteristics, such as the ability to incorporate hydrophilic and hydrophobic drugs, low toxicity, no activation of immune system, and targeted delivery of bioactive compounds to the site of action. But the rapid degradation of lipid based vectors (liposomes) due to the reticuloendothelial system and the inability to achieve sustained drug delivery over a prolonged period of time are the two drawbacks of these delivery systems, compared to the viral ones.[25],[26] Improvement can be achieved by a careful design of the structures of the lipids and/or by adding other cell-friendly (low toxic) moieties into the mixture of the liposomes.

1.1.1.1. Lipid Structures

The typical structure of a cationic lipid vector includes a positively charged polar (hydrophilic) head-group, for DNA binding, connected to a hydrophobic domain (tail) via a spacer unit (typically an ester) and a glycerol backbone (Figure 1.3). [14] One of the simplest type of hydrophobic domain consists of two flexible (non-rigid) saturated or unsaturated hydrocarbon chains, typically 6 to 12 carbon atoms-long.

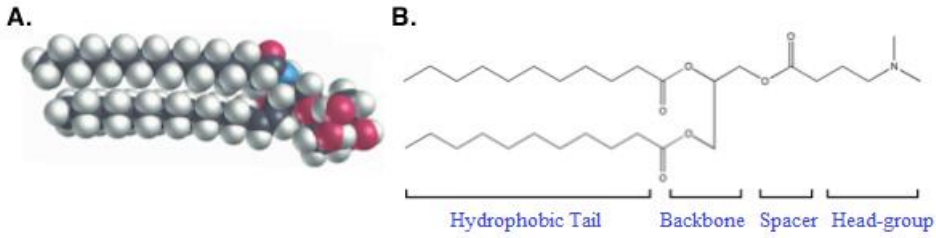


Figure 1.3. (A) space-filling model and (B) the structural formula of a typical lipid containing a Dimethylaminobutyrate head group and spacer as well as a glycerol backbone and a saturated hydrophobic domain.

Many researchers such as *Goldring and Pungente*[14],[16] have carried out gene therapy studies using cationic lipids. These scientists, among others, have collectively synthesized different types of lipids with special attention given to the design of the charged head-groups and the length and the shape of the hydrophobic domain (tails), which has a considerable effect on their functionality.

They have introduced modifications in the hydrophilic head-groups as well as the hydrophobic hydrocarbon tails. Some examples of these modifications include:

insertion of macrocyclic moieties as the polar head-groups resulting in many different charge numbers, insertion of carbon-carbon bonds (crosslinking) between the hydrophobic tails and hence creating cyclic non-polar ends, increase or decrease the length of the tails as well as their degree of saturation. They have also been able to draw conclusions on these different lipids regarding their behaviour once entering the cells, for example that the transfection efficiency of short (C_7) cyclic hydrophobic tails in lipid analogues give rise to a slightly better transfection performance in comparison to their longer (C_{11}) acyclic analogues.[16]

Other scientists have shown that incorporation of acyclic moieties in the design of the hydrophobic tails lipid structures are in favour of the cell penetration properties of the liposome.[16],[27],[28] *Heyes et al. 2005*[29] showed that existence of one or two double bonds per hydrocarbon tail is very optimal for cell penetration. They, further, investigated the positions of the double bonds and their effect on transfection efficiency, study of which is intriguing but beyond the scope of this report.

The spacer region also called the linker, (see figure 1.3B) is located between the polar head-group and the glycerol backbone of a lipid, creating a distance between the two. The modification of this part of the lipid may lead to different outcomes ranging from the chemical and enzymatic stability as well as the range of hydrophilicity, cellular toxicity and morphology of lipids.[30] Some researchers have investigated lipid particles containing ester linkers and they have observed good stability of the ester-containing lipids *in vivo* in comparison to what was previously reported by *Semple et al. 2010*.[31] Additionally, the ester linkers are less prone to toxicity as opposed to the other alternatives (e.g ether groups). [14],[16],[30]

The polarity of a lipid has a direct effect on its mechanism of action. [6] A careful design of the head-groups will bring about a better transfection efficiency as well as better liposomal formulation rate. Dimethylamine (DMA) head groups have been used as the benchmark for simple head groups.[16] In this study, DMA lipids have been used due to their simplicity in design and synthesis. They contain one positive charge and they make for a good low-charged delivery carriers for the encapsulation of anionic cargos. [14]

1.1.2. Peptide-based vectors

Alongside the research towards developing liposomes for transferring biological molecules into the cells (e.g. DNA and proteins), new strategies that involve other vectors have also been investigated. Peptides are one of the examples of an alternative to the viral vectors. Peptides are naturally occurring short chains (<30) of amino acid monomers linked by peptide (amide) bonds. There are many different types of peptides with different biological characteristics and functions such as antimicrobial peptides (AMPs) and host defence peptides (HDPs). Peptides are extensively used in the pharmaceutical laboratories to better understand (used as drug simulators) and develop new drugs.[32] Their structure can be modified to facilitate cell entry on their own, without the need of a delivery vector.[33] This has resulted in the discovery and design of curative peptides that can enter the cells through many processes such as endocytosis or direct penetration. Cell penetrating peptides (CPPs) and innate defence regulators (IDRs) are amongst these powerful discoveries.[32],[34],[35]

CPPs have served as vehicles for delivery of different molecules and particles into the cells since their discovery. The efficiency and the non-invasive nature of peptide-mediated cellular transduction, through CPPs, promised a great tool for biomedical researches. They have also been served as good tools for delivery of nucleic acids. [36]–[39] IDRs can loosely be defined as an artificial class of HDPs.[40] HDPs are part of the innate immune response and are able to kill many major immune threats.[41] IDR peptides enhance the efficiency of immune response making them an attractive anti-infective drug.[35] Although, IDR peptides are not yet categorised as a subclass of the CPPs, they do in general possess some cell penetrating affinities, as do most other peptides.[32],[38] Similar to cationic lipids, peptides are also easy to make and flexible for additional modifications.[42] Within the study of IDR peptides, IDR-1018 (figure 1.4) and IDR-HH2 have gained a lot of recognition due to their ability to significantly promote chemokine (cytokines that respond to chemotoxins) production and their anti-infective and anti-inflammatory activities. [35],[43],[44]

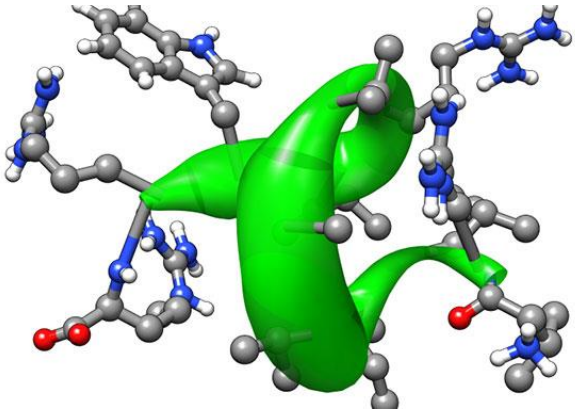


Figure 1.4. The 3D structure of the peptide IDR-1018 is presented.

Peptides, in general, are selective and efficient signalling or regulatory molecules. They exert a variety of biological roles and provoke a variety of physiological effects. Peptide suitability as therapeutics has arisen from their high selectivity, excellent safety and low immunogenicity, as well as efficacy profiles in humans. [45] The flexibility and the small molecular weight of the peptides help the target accessibility as well as, to some minor extent, the cell permeability.[38],[46],[47] The human body rapidly absorbs peptides which makes peptides decent candidates for therapeutic applications. [48]

IDR-Peptides and other members of the HDP family, nevertheless, are non-specific in the cells they target. That is why, in theory, they must be used in conjunction with other cell targeting molecules (additional short chained peptides like CPPs) to increase transfection efficiency.[49] In theory this is very optimal, however, the long amino acid chains that it forms will hinder the cell penetration affinity and that's why short length peptides are recommended for this purpose. One consequence of having short peptides is their charge deficiency and hence their imbalance charge ratios in comparison to the heavily charged cargos such as DNA.[50] This then requires a large number of peptides relative to DNA to achieve a balance or excess of positive charge. Due to these issues, a combination of IDR peptides with other cell-targeting molecules such as cationic lipids could potentially benefit delivery of anionic cargos into the cell, by benefiting from the advantages of both moieties.

1.2. Lipid/peptide hybrid as a gene delivery vector

The immunomodulating properties of IDR peptides could be a great complement to the liposomal genetic delivery methods. It could potentially help create the most ideal genetic delivery vehicle where the cell viability as well as transfection efficiency can reach its highest limit yet. This statement is, as of now, a mere speculation as there are no literatures backing it up. Lipid liposomes have been proven to be toxic to the cells at high concentrations. [16],[30],[44],[51],[52] Addition of IDR peptides could potentially decrease this toxicity. In this system, DNA is pre-condensed with several synthetic polypeptides, followed by lipid wrapping using cationic lipids (Figure 1.5). This model has been adapted from the work of *Li and co-workers* who added peptides (CPPs) into cationic lipid liposomal complexes to deliver genetic material into cells.

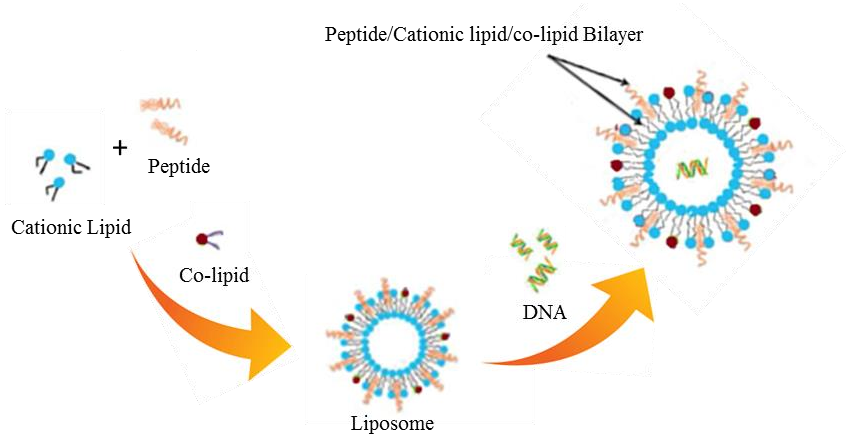


Figure 1.5. Systematic representation of forming DNA-carrying liposomes with IDR-peptides and synthetic cationic lipids as well as co-lipids incorporated in the bilayer. (Modified from [53])

Although there is little to no literature available on how the IDRs behave once combined with the cationic lipids to mediate DNA delivery, there are a few papers that support the idea. *Pisa et al. 2015*[37] proved that the hybrids of lipids with peptide polymers improve gene delivery. *Wadhwani et al. 2012*[38] has also verified that AMPs can exhibit an improved cell viability and maintain their antimicrobial trait once combined with synthetic lipids. Furthermore, *Li et al. 2016*[53] have shown that liposomes combined with CPPs embedded in the bilayer structure, have higher activity *in vivo* as well as *in vitro*. Additional efforts are needed to determine whether lipid/protein hybrid systems are superior to lipids alone for gene delivery. All the

different types of peptides (e.g. IDR peptides) would need to be examined to see which work better for this purpose. This has served as a motivation to find out what happens when IDR peptides are formulated with cationic lipids to form gene delivery vectors.

1.3. Liposomal Drug Delivery pathway

Herein, the theoretical background of all aspects leading to the liposomal drug delivery, such as liposome formation as well as the lipoplex formulation and in-cell delivery system, is described.

1.3.1. Liposomes

In chemistry, lipids are defined as a group of naturally occurring molecules that include fats, sterols, the fat-soluble vitamins, phospholipids and many others. There are many functions that are assigned to lipids such as signalling, energy storage and being the structural components of cell membranes. Lipids can be broadly defined as amphiphilic small molecules, which means that they have distinct polar and nonpolar regions. It is this nature of the lipids that allows them to form defined structures such as vesicles, liposomes (Figure 1.6) and membranes in aqueous solutions. [54]

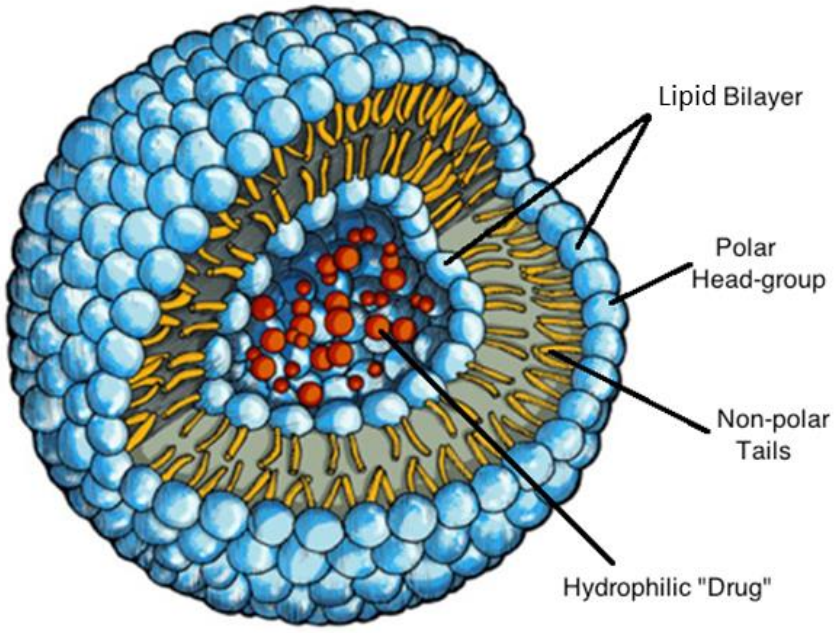


Figure 1.6. A typical structure of a unilamellar (composed of one lipid bilayer) liposome containing a hydrophilic drug. (Modified from Zümbuhl and Müller, 2016)

Liposomes are made up of a lipid bilayer with an aqueous environment around them. These structures form spontaneously when lipids are dispersed in aqueous solution. They are believed to be a promising delivery system for both hydrophobic and hydrophilic drugs since the hydrophobic chemicals interact with the bilayer and the hydrophilic drugs interact with the polar surface or can be dissolved in the aqueous environment in the inside of the liposome. They can be used to administer nutrients, genes and other pharmaceutical drugs into the cells. [53,54] In theory, the lipid bilayer can fuse with other lipid bilayers such as the cell membrane and deliver the liposomal content within. [42],[57] The ability of the lipids to form liposomes that can encapsulate biologically active molecules, and act as vectors for DNA delivery, has primarily formed the core of this study. The gene delivery efficiency of liposomes is dependent on the size, structure, the amount of the liposome, the charge ratio between DNA and the liposome, presence of helper lipid and cell type.[26]

Depending on the required function, a combination of different lipids, can be formulated to make optimal liposomes. As an example, cationic lipids (such as the DMA lipids mentioned earlier) in combination with membrane stabilizing neutral lipids (also known as co-lipids) can form good liposome candidates for delivering DNA into the cells.[30],[51] The helper lipids, here referred to as co-lipids, are neutral and/or zwitterionic lipids that are used in combination with charged lipids in the formation of liposomes to enhance the delivery affinity. Dioleoylphosphatidyl-ethanolamine (DOPE) and cholesterol (Chol) are the two examples represented here. They are both, individually, incorporated in the recipe for liposome formation due to their fusogenic, modulator of membrane and lipoplex stability properties. Their addition decreases the concentration of cationic lipid and promotes fusion with the cell membrane and therefore facilitates gene delivery into the cytosol.[58]–[60]

Although helpful, there have been reports that these co-lipids don’t increase the transfection efficiency. [61] Some argue that they increase the molecular weight of the delivery vehicle and could hinder the cell penetration affinity. Many researches have differentiated the characteristics of Chol and DOPE separately to observe their differences in response to complexation with different designs of synthetic

lipids.[13],[14],[16],[28],[30],[62],[63] According to these studies, the responses differ greatly where, for example, some show a better stability and higher transfection affinity with Chol, while others claim that DOPE is less rigid so it helps the DNA-release into the cytosol.[62] Hence the results regarding the different co-lipids are contradictory. A possible reason for that could be the different behaviour of these co-lipids when they are in complex with different types (structurally) of lipids.

1.3.2. Lipoplex (liposome-DNA complex) Formulation

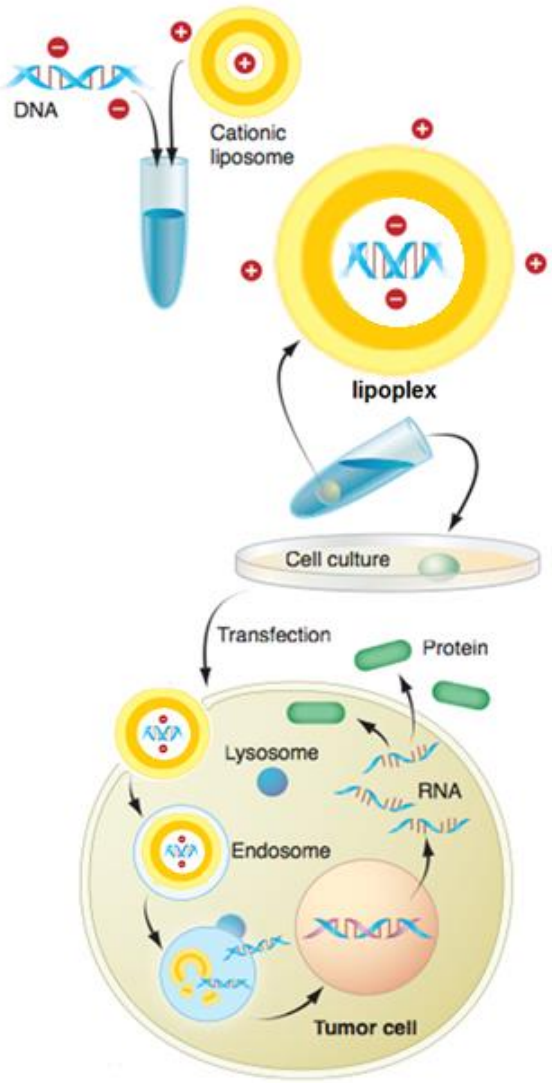


Figure 1.7. Gene therapy using lipoplexes. DNA is complexed with cationic liposomes consisting of a cationic lipid, a peptide and a neutral helper co-lipid (yellow circle). This allows uptake of the DNA into the cell by endosomes. DNA is freed and enters the nucleus (orange), where the therapeutic gene is transcribed into corrected proteins (green). Modified from [9]

Figure 1.7 shows gene therapy using liposomes complexed with DNA, forming lipoplexes, and transfected into the cells. In the sections above, we described how liposomes are formed and how their structural and general composition could have an effect on the way they are transfected into the cells and deliver the genetic cargo. Now we need to talk about what happens when they are in complex with the nucleic acids. Once these lipid liposomes are formed with the help of the cationic lipids, co-lipids, cationic peptides or any other components in the recipe (all together or individually), they'll have to be formulated with the polar genetic material in different molar charge ratios. The concept of molar charge ratio is a very important concept that it has to be fully understood.

As it was stated before, the DNA is highly anionic, where one molecule of DNA contains about 6000 negative charges. The liposome molecule, depending on its constituents, could possess a vast variety of positive charges (from +1 upwards), but it cannot be remotely close to what DNA has. It is not, therefore, possible to combine the liposomes and the genetic material (i.e. form a lipoplex) in molar ratios, as they will not be able to balance each other out. This is where the concept of molar charge ratio comes into the picture. This means that the liposomes and the DNA cargo will have to mix in such a way that there is enough positive charge to encapsulate the negative charge. Rationally, the ratio of positive charge will have to be bigger than the negative charge to be able to hold the cargo and successfully deliver it into the cells. [16] [62]

Previous studies have investigated different molar charge ratios of the cationic vector to the anionic genetic material and have reached numerous conclusions regarding what ratios are commonly better for transfection and have positive influences on the different parameters of obtaining a successful delivery (e.g. release affinity, cell penetration, cell viability, etc.). Molar charge ratios (+/-) of 3:1 and 5:1 have been studied before and they have obtained better results, in terms of high transfection efficiency and low cytotoxicity, compared to higher (10:1) or lower (0.5:1, 1.5:1) ratios. This could be due to the fact that at low molar charge ratios, the lipoplex is not stable enough to hold the DNA and liposomes together and at high molar charge ratios, liposomes bind too tightly

to the DNA cargo and stimulate release problems. These researchers have also investigated that lipoplexes with high concentration of cationic lipids are toxic to the cells. This, thus, emphasizes on the fact that finding the right molar charge ratios for the lipoplexes are just as important as finding the right constituents for the delivery vectors (i.e. liposomes). [14],[16],[30],[51],[63]

1.4. Aims and Objectives

An introduction to the lipid-based and the peptide-based non-viral gene delivery vehicles was given in the sections above. Both classes of vectors have made great contributions to the progress of gene therapy by having considerable advantages compared to other non-viral delivery vehicles. Such advantages are them being biodegradable, less toxic and have better transfection efficiency. While several studies have been made to investigate the potential of using either peptide-based or lipid-based vectors for gene therapy, little research has been done on mixed lipid and peptide-based vectors. Most of the available documentation focuses on the effect of combining CPPs and cationic lipids to generate gene delivery vehicles. There are however, to the best of our knowledge, not many researchers who have looked at mixed IDR peptides and cationic lipids vectors for gene therapy. This has therefore prompted us to investigate this relatively unexplored area.

The aim of this study is to investigate the effect of IDR peptide/cationic lipid liposomes as novel non-viral DNA delivery vehicles. We would also like to probe into different structural variations between the cationic peptides, cationic lipids and co-lipids and different lipoplex formulations. The objectives for achieving these goals are divided into three categories stated as follows:

1. Select and synthesize cationic lipids as well as cationic peptides.
2. Formulate the lipoplexes into different molar charge ratios and characterize them with respect to their physical and chemical properties.
3. Perform biological assays to evaluate their relative transfection efficiency and cell viability.

For this study we decided to synthesise acyclic lipids. Reports from *Semple and co-workers*[31], show that lipids with simple hydrocarbon tails highly favour cell delivery. They also hint at the fact that simpler (structurally not complicated) and smaller (short chained) lipids enhance gene delivery. Therefore, the cationic lipids selected to be synthesized herein are both comprised of a mono-charged DMA head-group and two C_{11} saturated or unsaturated hydrocarbon tails (figure 1.8).

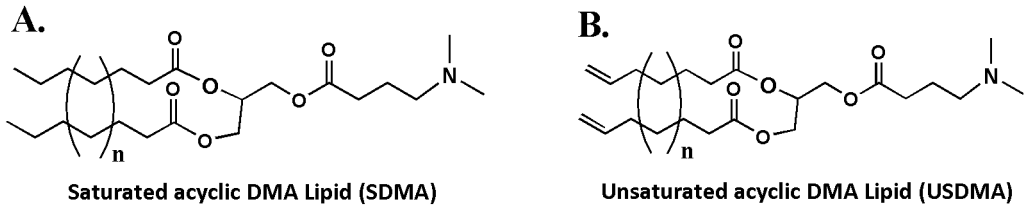


Figure 1.8. Saturated (A) and Unsaturated (B), DMA lipids synthesized in this study (n=5).

Concerning the cationic IDR peptides, the previously described IDR-1018 (charge +4, VRLIVAVRIWRR-NH_2) and IDR-HH2 (charge +5, VQLRIRVAVIRA-NH_2) were chosen for synthesis and further evaluation in this study (structural amino acid sequence of each peptide is given in Appendix I). They both possess very similar characteristics such as having the same length of amino acids (12-mer) and are immunomodulatory as they inherit characteristics from the HDPs.[44] Their implementation into commercial products, however, is often hindered due to their characteristic physicochemical and biological instabilities.[43] The formation of a peptide-DNA complex with the lipids could in fact offer better protection from the degradative conditions in the cells.[64]

After synthesizing the selected lipids as well as the peptides, they are co-formulated together with the two helper co-lipids, Chol and DOPE figure1.9), at fixed molar ratios (peptide/lipid/co-lipid; 3:3:2) to form liposomes. These cationic liposomes are then combined with the negatively-charged DNA, to form lipoplexes, at 4 different molar charge ratios (+:-), 1.5:1, 3:1, 5:1 and 10:1. The investigation of formulations of the mentioned molar charge ratios has been done before using cationic lipids and neutral co-lipids, without peptides, as the delivery vehicle.[16],[30] It seems reasonable to investigate the effect of the peptide/lipid hybrid on gene delivery, using the exact same molar ratios for comparison. The produced liposomes and lipoplexes are characterized by particle sizing and DNA/protein binding assays.

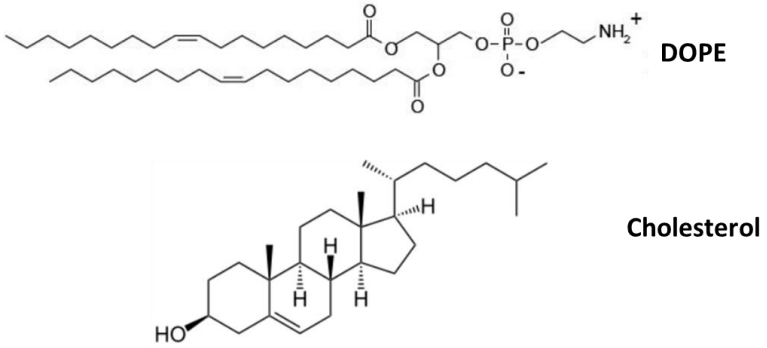


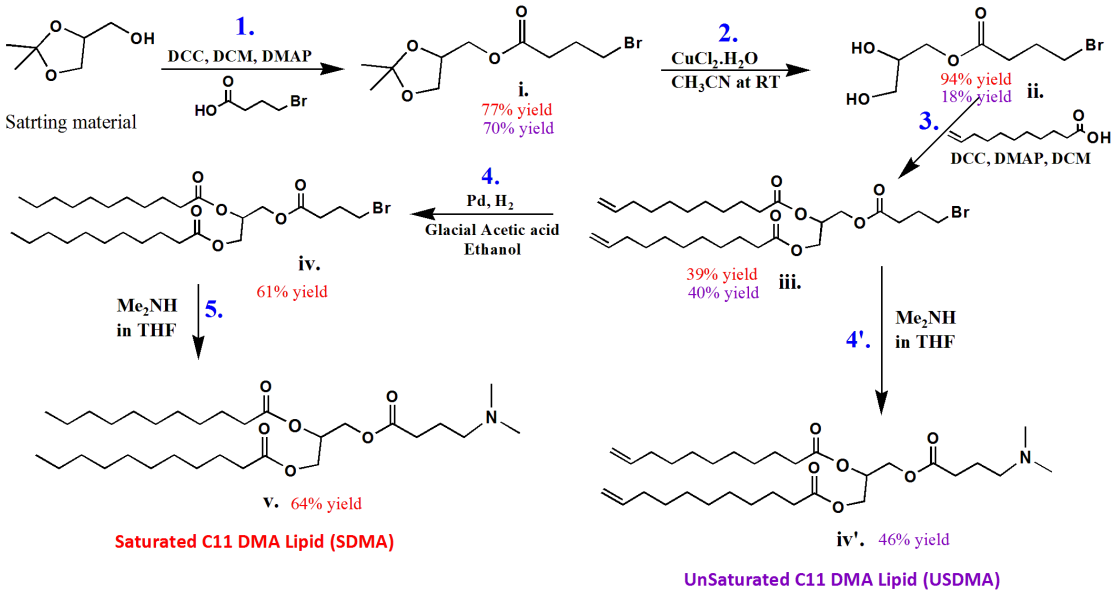
Figure 1.9. Structural representation of the two co-lipids DOPE and Cholesterol applied in this study.

The lipoplexes will then be transfected into Chinese hamster ovarian cells (CHO-K1) which will allow us to investigate the third and the final objective to achieve our goal in this study. This is done by assessing the effects these peptide/lipid lipoplexes have on the cells and delivery of DNA into the nucleus, in terms of cell viability and transfection efficiency. In the end, the results will be compared in the following way: USDMA versus SDMA, IDR-1018 versus IDR-HH2 and Chol versus DOPE. This is done to conclude on which combination(s) has the highest impact on both cell viability as well as transfection efficiency.

2. Results

2.1. Synthesis of cationic lipids

Two different acyclic lipids were synthesized for this study (see figure 1.8). The hydrophobic regions of both lipids were composed of two identical C_{11} unbranched hydrocarbon chains that were fully saturated (undecanoic) in one lipid and terminally unsaturated (10-undecenoic) in the other. Ester groups were chosen as the linkers between the hydrocarbon chains and the glycerol backbone in the structural design because they are less prone to toxicity as opposed to their alternatives (e.g. ether groups). [65] A singly-charged dimethylamine (DMA) group was chosen as the polar head group for both of the lipids, because of its good reproducibility according to previous studies. [14],[16] A C_4 saturated, unbranched ester group (butyric) was used as a spacer between the dimethylamine head and the glycerol backbone in both lipids. Notice then, that the only difference between the two cationic lipids was the degree of unsaturation of the hydrophobic tails. The two lipids will thus be referred to as saturated DMA lipid (SDMA) and unsaturated DMA lipid (USDMA) throughout this report.



Scheme 2.1. The systematic 5-step SDMA and 4-step USDMA lipid synthesis pathway. Red shows the yields for the SDMA and purple is for the USDMA.

The lipid synthesis comprised of 5 steps for the SDMA and 4 steps for USDMA as it is illustrated in reaction scheme 2.1. For the SDMA synthesis, an ester bond between 4-bromobutyric acid (spacer) and the free hydroxyl group of solketal (a protected form of glycerol that would form the backbone of the lipid) was first formed by Steglich esterification to yield compound **i**. The reaction was carried out in dichloromethane (DCM), N,N-dicyclohexylcarbodiimide (DCC) as the carboxylic acid activator and 4-dimethylaminopyridine (DMAP) as the catalyst at 0 °C. This reaction gave a total yield of 77% of **i**. The acetal deprotection of **i** was carried out in acetonitrile using copper(II) chloride dihydrate as the acid reagent and gave the diol **ii** in 94% yield, which was expected as the reaction of the dimethyldioxolane with DCM is spontaneous and normally we would expect high yields.[65] The hydrocarbon tails were then attached to the two free hydroxyls of the glycerol backbone by Steglich esterification between **ii** and 10-undecenoic acid in DCM using DCC and DMAP. Tryglyceride **iii** was obtained from this reaction in 39% yield. It is worth mentioning here that the initial crude yield of this reaction was about 76%; however, a few technical complications occurred during the loading of the material onto the column that it became necessary to wash the entire column with 100% ethyl acetate to recover the crude product. This resulted in a substantial decrease in the yield of the crude reaction material to 60%. Upon further purification, the final yield of compound **iii** was 39%. Apart from the technical errors occurred during this step of the synthesis, the abundance of the reaction starting material could be among the reasons why the yield of the purified product considerably decreased.

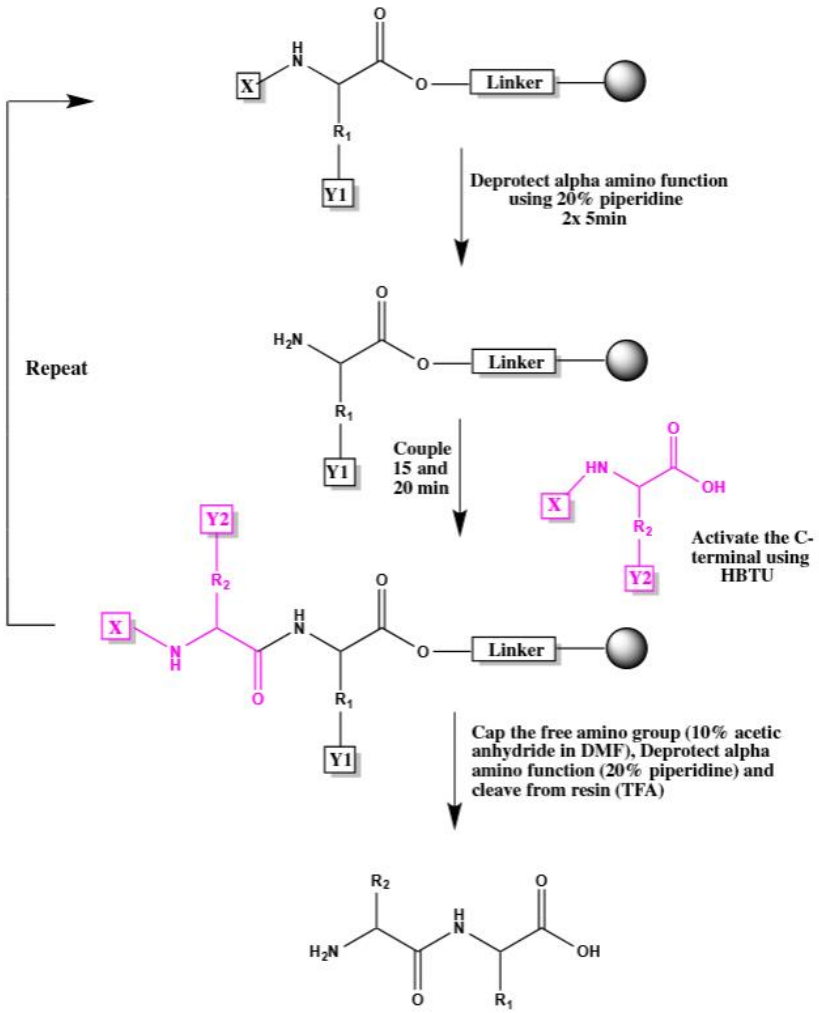
In the following step, the two 10-undecenoate groups of compound **iii**, were hydrogenated using hydrogen gas with palladium on carbon as a catalyst to give compound iv in 61% yield. The hydrogenation step was carried out with extreme caution to prevent the hydrogenolysis of the C-Br bond in the spacer moiety, which may occur if the reaction is allowed to proceed for longer than 24h. This, fortunately, did not happen during this synthesis (neither for the USDMA synthesis). On the other hand, the reaction needs sufficient time to properly hydrogenate the alkene units, so this step should be carefully dealt with. As the last step of the synthesis, the DMA head-group was introduced by amination of **iv** by dimethylamine in THF. The reaction was

allowed to stir at room temperature for 72 hours to yield 78.5 mg or 64% of the final product, **v**, after purification. A portion of the final product was treated with ice cold 2M HCl in dimethyl ether to protonate the DMA head-group so that it could be used further in the study.

The synthesis steps 1-3 were repeated a second time for the synthesis of USDMA. In this case product iii was directly aminated with dimethylamine to yield USDMA. The total yields, in percentage, of the reaction products were as follows: **i**=70%, **ii**=18%, **iii**=40%, **iv'**= 46%. The low yields of the reaction products, like the results from SDMA, are as a result of presence of many unreacted materials in the reaction mixture as well as the poor purification skills employed.

2.2. Synthesis of cationic peptides

IDR peptides were investigated and amongst this family, two of the well-studied Peptides, IDR-1018 and IDR-HH2, were chosen to be synthesized using F-moc solid phase peptide synthesis (SPPS) (Scheme 2.2).



Scheme 2.2. General scheme of F-moc solid phase peptide synthesis. X = F-moc, α amino function protecting group and Y= sidechain protecting group (e.g. tBu).

The C-terminal of an amino acid residue is attached to an insoluble support (TG S RAM) via its carboxyl group. Any functional groups in the amino acid side chains must be masked with compatible protecting groups that are not affected by the reaction conditions employed during peptide chain assembly. Once the first amino acid is bound to the resin, it is capped using 10% acetic anhydride in DMF. This step is done to make sure that the free (unreacted) amino group of the amino acid is not interfering in the

peptide synthesis further in the steps. After the capping step, the temporary protecting group (F-moc) of the alpha-amino function is removed, using 20% piperidine. The carboxyl group of the second amino acid is activated before addition to the first amino acid, using a standard activating agent, HBTU. To convert carboxylic acids into activated form, their hydroxyl group is replaced by an electron withdrawing substituent (such as 1-oxybenzotriazolyl in this case) to enhance the electrophilicity at the carboxyl carbon. This facilitates the nucleophilic attack by the amino group of the first amino acid.[66]

The activated carboxyl group of the amino acid will form an amide bond with the de-protected amino group of the first amino acid bound to the resin. Once the free amino groups are capped and the excess reagents are removed by washing (with DMF), the protecting group of the N-terminus of the 2nd amino acid is removed to couple the resulting dipeptide to a 3rd amino acid. This procedure is repeated until the desired peptide sequence is assembled. In the final step the side chain protecting groups of the peptide sequence are removed (Y in scheme 2.2, an example of side chain protecting groups include tert-Butyl group (tBu)) and the peptide is cleaved from the resin using TFA.

IDR-1018 (molecular weight: 1535.89 g/mole) and IDR-HH2 (molecular weight: 1394.76 g/mole) were synthesized exactly as explained above. For a 0.1 mmol scale synthesis we used around 0.5 g of the resin. This value was calculated based on the capacity of the TG S RAM resin used (0.23 mmol/g). According to these numbers the exact amounts of all reagents and solvents were calculated. The crude yield of the IDR-1018 peptide product was ≈ 43% and the crude yield of IDR-HH2 was ≈ 35%. The crude products were purified using prep-HPLC and the weight of the pure products was found to be 24 mg (16% yield) for IDR-1018 and 27 mg (20% yield) for IDR-HH2. The HPLC chromatograms and the MS spectra of the final pure synthesis products are presented in appendix II.

2.3. Lipoplex formulation

The lipoplex formation is highly dependent on the ionic charge of the components in question. Molar charge ratios are extremely relevant in determining the transfection efficacies of a lipoplex and are particularly important when we are transfecting DNA as a cargo into the cells because DNA molecules carry a very large negative charge and therefore a high number of molar equivalents of cationic lipids and peptides are needed to balance it and obtain a stable and efficient lipoplex. The net charge of both the SDMA and USDMA is +1, the net charges of the peptides IDR-1018 and IDR-HH2 at pH ≈ 8.5 are +4 and +5 respectively and there are 6600 negative charges per DNA strand. Therefore, once the liposomes are formed we consider the molar charge ratio between the cationic components and DNA, rather than the number of molecules.

Each lipid was co-formulated with one of the peptides in a 1:1 molar ratio, along with one of the two neutral co-lipids 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) or cholesterol (Chol) in an overall cationic lipid to cationic peptide to neutral co-lipid molar ratio of 3:3:2. DOPE is used for its reported fusogenic properties which are very important for the endosomal penetration and Chol is thought to be a rigid backbone that promotes liposome stability. [27],[62],[67]

A total of 8 liposomal formulations, which included one of the two cationic lipids, one of the two co-lipids and one of the two peptides were prepared, and then each was combined with plasmid DNA (4.13E+06 g/mol) in four different molar charge ratios (+/-) i.e.1.5:1, 3:1, 5:1 and 10:1. The 32 (8x4) different lipoplexes thus obtained were then characterized in respect to their physicochemical properties as well as their transduction efficiency and cytotoxicity.

2.4. Characterization

2.4.1. Gel retardation assay

In order to determine whether the DNA has bound to each liposome, a gel retardation assay was carried out. This is a common affinity electrophoresis technique used to study protein-DNA interactions. The speed at which different molecules (and combinations

thereof) move through the gel is determined by their size and charge as well as their shape. Normally the gel-binding results should have a concentration dependent trend where there is full binding of the DNA at the highest lipoplex concentration (i.e. 10:1). [16],[65] The results from the 32 lipoplex formulations run through 1% agarose gel impregnated with the DNA stain ethidium bromide is shown in Figure 2.1 below.

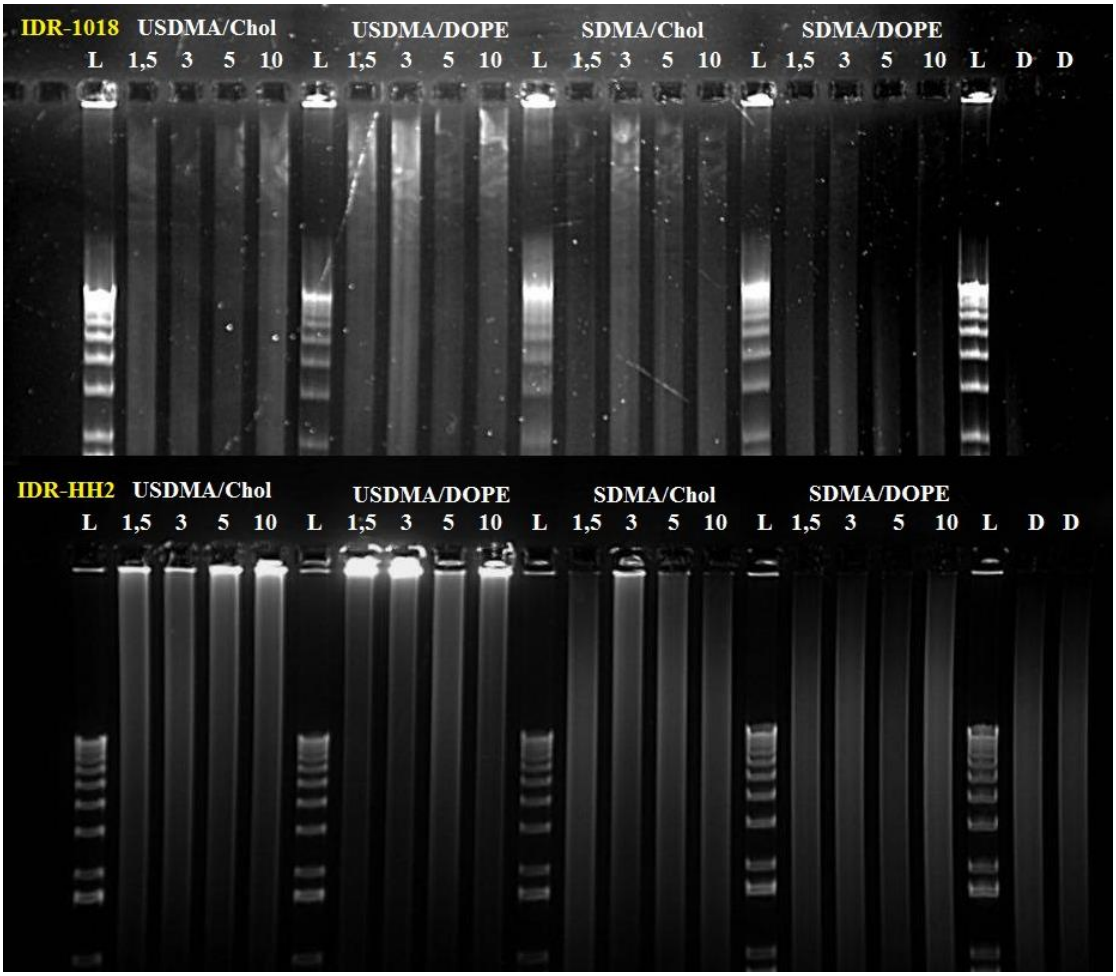


Figure 2.1. The Gel retardation results for the two peptides IDR-1018 (top) and IDR-HH2 (bottom). All the designated Lipoplex formulations were compared with the negative control DNA ladder (L, 1Kb+) and the *p*DNA as positive control (D).

The results of the gel retardation assay were not as expected and were not same as the ones published before.[16] All the formulations show the same general outcome and have the same issues. The gels appear to show retention (i.e. full binding) of the complex at the top of some of the wells but not in all. These results are inconsistent (see the IDR-HH2 gel) with regards to observing any trends with the concentration of the lipoplexes.

It seems that the IDR-HH2/USDMA/Chol formulation showed an increased binding as the charge ratio increased. However, with the IDR-HH2/USDMA/DOPE formulation the binding decreased from charge ratio 3 to 5 and then again increased to charge ratio 10. The column assigned to show the *p*DNA showed bound DNA at the top but streaked down the gel, instead of showing a clear band (or bands if the *p*DNA is nicked) down the gel

The streaking that appears on all lanes and the DNA control columns would suggest that the DNA is degrading under the conditions of the gel assay or that the DNA stock was degraded. This could be due to a contamination of the lipoplex preparations or of the DNA stock possibly with a nuclease enzyme. The results of the following experiments, however, appear to exclude that the DNA stock was degraded, so the most likely explanation seem to be related to the gel electrophoresis itself (perhaps the buffer used). The gel retardation assay did not yield any conclusive result and should have been repeated to ensure the validity of the results obtained in the following experiments. The reason why the lipoplexes assayed here were used in downstream experiments is that the gel retardation assay was performed after the cells had been treated with the lipoplexes.

2.4.2. Particle sizing

An important physical property that affects the performance of lipoplexes is the particle size. Therefore, as part of the characterization of the liposomes and lipoplexes created in this study, a particle size measurement was carried out. The results from the particle sizing analysis, using dynamic light scattering, are presented in table 2.1 below.

Along with the hydrodynamic diameter (in nm) of the lipoplexes and liposomes, the polydispersity index (PDI) of the synthesized complexes was also measured. The term polydispersity is used to describe the degree of heterogeneity of sizes of the particles present in a sample. Lower PDI values indicate that the sample is composed of particles of more uniform size. The particle sizes of lipoplexes should normally be within the range of 100-500 nm to be ideal for cellular delivery.[16] The majority of the data obtained here are within that range with few exceeding this limit. The PDI measured

are also within the optimal range of <1. There is, however, no apparent correlation between the diameter of the particles and the PDI values. There are also no correlations found between the size and the different liposomes and/or lipoplex charge ratios.

Table 2.1. Showing the hydrodynamic diameter (nm) of the liposome/lipoplex molecules as well as their polydispersity indexes

Liposomes/lipoplexes		A: IDR-1018		B: IDR-HH2	
		Z-Ave, d_H (nm)	**PDI**	**Z-Ave, d_H (nm)**	**PDI**
USDMA/Chol	**Liposome**	672,1	0,5	541,3	0,5
	1,5	389,4	0,5	445,8	0,6
	3	609,3	0,8	500,2	0,6
	5	513,3	0,8	562,2	0,7
	10	622,3	0,3	388,1	0,4
USDMA/DOPE	**Liposome**	530,5	0,5	419,8	0,6
	1,5	511,2	0,5	287,2	0,5
	3	390,1	0,7	258,3	0,7
	5	499,1	0,8	440,2	0,5
	10	408,9	0,8	429,1	0,3
SDMA/Chol	**Liposome**	517,8	0,6	385,2	0,7
	1,5	288,1	0,8	297,3	0,8
	3	767,4	0,2	719,2	0,5
	5	531,1	0,9	434,4	0,6
	10	239,3	0,3	483,4	0,6
SDMA/DOPE	**Liposome**	499,6	0,6	530,1	0,5
	1,5	465,1	0,4	601,1	0,7
	3	889,5	0,3	688,2	0,5
	5	561,1	0,6	399,2	0,4
	10	324,2	0,8	306,2	0,5

2.5. Relative Transfection Efficiency

Evaluation of the relative liposome-mediated *in vitro p*DNA delivery efficiencies of the lipoplex formulations was performed on CHO-K1 cells using the beta-glo assay system (Promega) to quantify the activity of β-galactosidase protein coded by the DNA cargo of the lipoplexes. The data were normalized as a relative percentage of the commercial transfection reagent Lipofectamine 2000^{TM}, which was used as the primary positive

control and arbitrarily assigned 100% relative transfection efficiency. This is done to see which combinations give better results in terms of getting into the cells and releasing the DNA that codes for the proteins. And also see, within the two examples of the three constituents (lipid, peptide, co-lipid) of our liposomes, which example performs better. A most common way of measuring that, is to conduct a β-galactosidase (β-gal) assay. The lipoplexes were prepared using plasmid DNA containing a gene coding for the β-galactosidase enzyme. In the event of a successful transfection, the DNA cargo would enter the cytosol of the cell and then its nucleus in which active transcription of the genes on it contained would take place. Consequently, the activity of β-galactosidase observed in the cells would be proportional to the transfection efficiency. This enzyme will cleave 6-*O*-β-galactopyranosyl-luciferin to release luciferin. This will then be catalysed by the enzyme luciferase and emit light. The light that is emitted can thus be measured quantitatively which corresponds to the amount of DNA transfected into the cells.

Comparisons between the two cationic lipids (USDMA and SDMA), two cationic peptides (IDR-1018 and IDR-HH2) and the two co-lipids (DOPE and Chol) were made and the overall results are shown in Figure 2.2. Two negative controls were also assayed, namely the cells incubated with the media alone in the absence of lipoplexes and with DNA alone Naked DNA is not cell penetrable all by itself and, thus, there should be little to no transfection observed. In this case 16.5% DNA transfection was observed. All of the 32 lipoplexes showed transfection efficiency values above 30%, except for one (1.5:1 SDMA/HH2/DOPE).

The mean values and the student's t-test *p*-values were calculated for different formulations. Figure 2.3 shows the means values for the transfection efficiencies and the significant differences in paired student's t-test *p*-values results, expressed as $p<0.05$ (*first degree of significance) and $p<0.01$ (**second degree of significance).

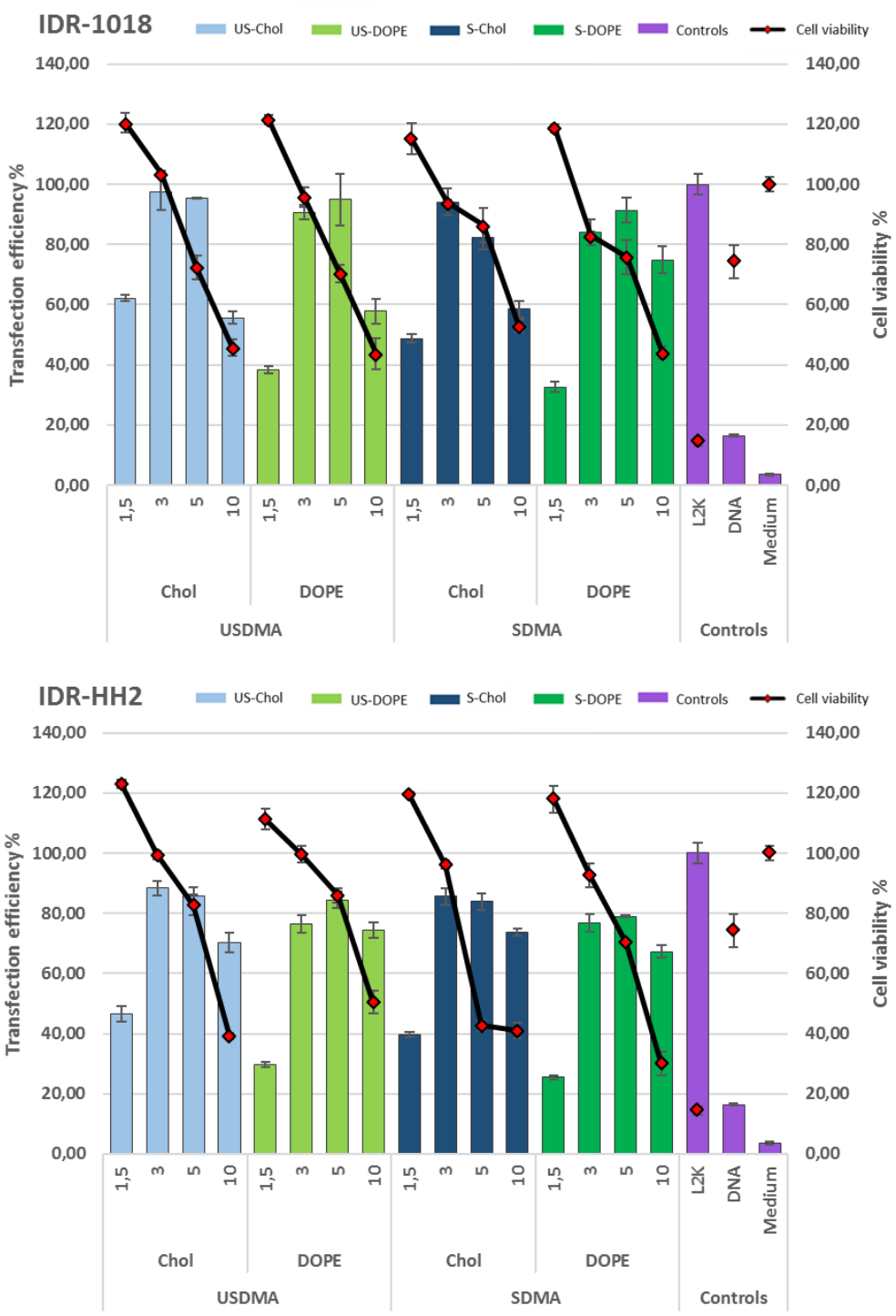


Figure 2.2. The relative percentages of transfection efficiency (bars) as well as the cell viability (♦) of different combinations of liposomes in complex with increasing liposome (+)**:**DNA (-) molar charge ratios of 1.5:1, 3:1, 5:1 and 10:1. The results for the transfection efficiency are normalised against the transfection medium, Lipofectamine 2000 and the results for the cell viability are normalised against the cells being alone in the media. The data is divided into two groups of A. IDR-1018 and B. IDR-HH2. The plasmid DNA was also used as the third control.

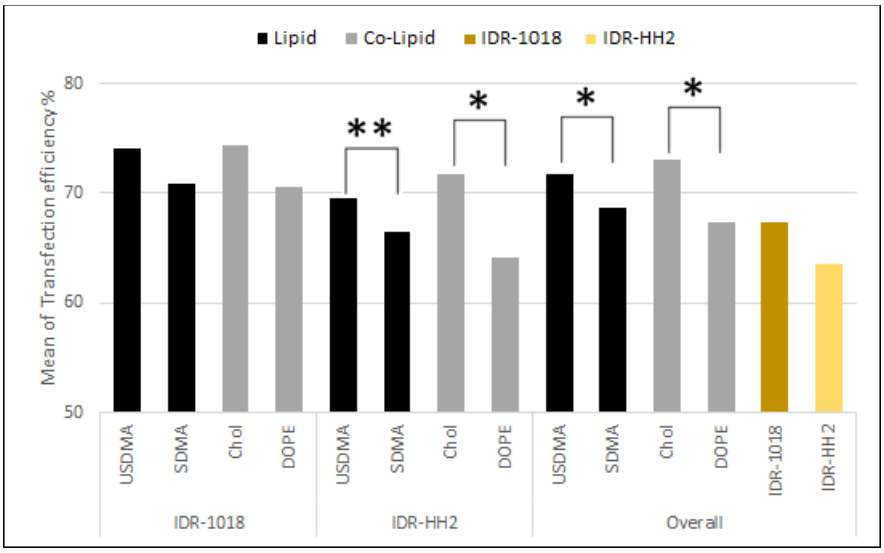


Figure 2.3. The total calculated means of the transfection efficiency data, based on the different formulations done in this study. The data is separated into two groups of formulations with the two different peptides, IDR-1018 and IDR-HH2. The overall means calculated for each of the variables are also represented above. significant differences in paired student's t-test (*p*-values) are expressed as *: $p<0.05$ and **: $p<0.01$.

The transfection efficiency data of the 32 lipoplexes was divided into two groups of IDR-1018 and IDR-HH2 peptides, and then analysed between the two cationic lipids (Figure 2.2 A and B). The relative transfection efficiency of the 8 liposomes in relation to their molar charge ratio showed the standard bell-shaped trend which is commonly observed.[14],[16],[27] This means that almost all of the formulations have higher transfection efficiency at the molar charge ratios (+/-) of 3:1 and 5:1, with all of them showing a mean transfection efficiency between 75% and 98% compared to Lipofectamine 2000.

2.5.1. Impact of the structural differences of the cationic lipids

The relative transfection efficiencies of the different lipoplexes were analysed for possible correlations with respect to the structural differences in the hydrophobic region of the cationic lipids. The two lipids differ only in the degree of saturation in their hydrocarbon tails, with USDMA having a single terminal double bond in each of the two tails.

Overall (figure 2.3), the formulations containing USDMA and SDMA, showed a *p*-value of 0.049 which has marginally passed the first degree of significance with USDMA showing higher transfection efficiency than SDMA. For the specific formulations, the lipid USDMA showed a higher transfection efficiency as opposed to its counterpart, SDMA, in combination with IDR-HH2 (p=0.025), whereas the formulations of these cationic lipids with IDR-1018 did not show any significant difference (p=0.19).

2.5.2. Impact of the two cationic peptides, IDR-1018 and IDR-HH2

The IDR peptides were added to the lipids to see whether they can obtain a better transfection efficiency as well as a lower cytotoxicity. The differences between the two peptides were examined in the hope of attaining an ultimate overall better result for one of them in comparison to the other. Visually, by looking at the graphs in figure 2.2, the mean transfection efficiencies of the lipoplexes containing IDR-1018 appear to be, in all but one case, higher than those with IDR-HH2. However, by looking at the mean values (figure 2.3), there is not an overall significant difference observed between the transfection efficiency of the two peptides (p=0.055).

2.5.3. Impact of the two co-lipids, Cholesterol and DOPE.

Along with the trends and relationships observed between the transfection efficiency of some combinations of the cationic lipids and the cationic peptides, the role of the co-lipids in the lipoplex formulations was also probed. Overall, the lipoplexes combined with Chol showed a higher transfection efficiency in comparison to the ones formulated with DOPE. Visually by looking at figure 2.2, all the formulations using Chol as co-lipid had higher transfection efficiencies with the results being a little less prominent for the SDMA/Chol, in comparison to SDMA/DOPE, in combination with the peptide IDR-1018. The student's t-test also showed that there was an overall one degree of significant difference between the two co-lipids, in total for all formulations (p=0.045), with Chol having a higher transfection efficiency. The most significant difference is observed when we combine the co-lipids with IDR-HH2 (p=0.017), as opposed to IDR-1018 (p=0.22).

2.6. Cytotoxicity measurements

Cytotoxicity is the ability of a certain compound to kill living cells. The ability to measure accurately the cytotoxicity of an agent can be a great tool in identifying toxic molecules and has helped the pharmaceutical industry to a vast extent. While cytotoxicity can be quantified in numerous different ways, it is commonly measured by assessing cell viability after treatment with the agent through the use of dyes that can only be metabolized by living cells (such as tetrazole dyes, as in this case).[68]

In this study, the relative toxicity of the lipoplexes was measured as a percentage of the survived cells after treatment with the lipoplexes compared to untreated cells by performing the MTS assay. The assay is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) into a formazan dye by the action of cellular oxidoreductase enzymes. [65] Since cells rapidly lose the ability to reduce MTS after death, the amount of dye formed by a cell culture following a period of incubation with MTS is proportional to the number of viable cells in the culture.

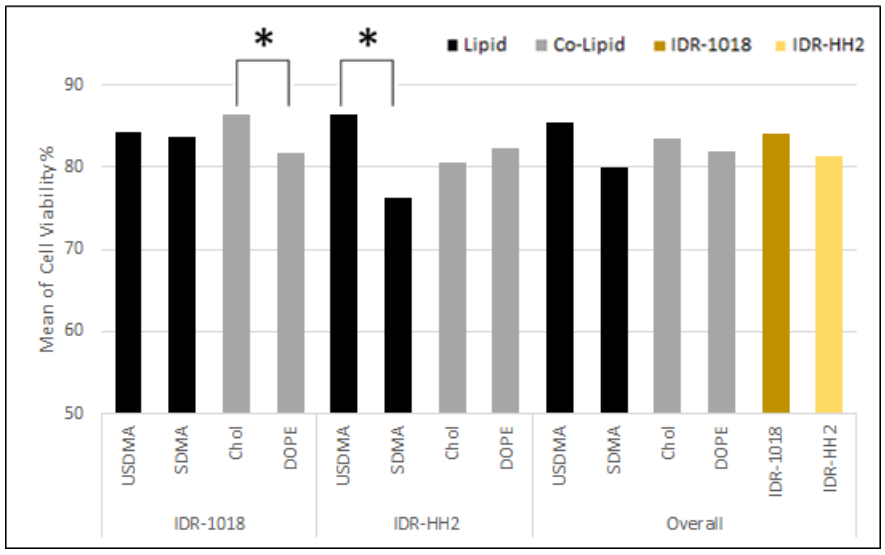


Figure 2.4. The total calculated means of the cell viability data, based on the different formulations used in this study. The overall means calculated for each of the variables are also represented above. significant differences in paired student's t-test (*p*-values) are expressed as *: $p<0.05$ and **: $p<0.01$.

The impact of each of the lipoplexes at different molar charge ratios on cell viability was analysed by organizing the data into 3 different groups based on the lipids, peptides and co-lipids used. All the comparisons are made in reference to data presented in figure 2.2, which represents the percentage of cell viability (♦). The cytotoxicity data was analysed and organized and the relative means were calculated and they were plotted in figure 2.4.

2.6.1. Impact of the cationic peptides, IDR-1018 and IDR-HH2

In order to draw a conclusion on which peptide shows an overall lower level of toxicity, the cytotoxicity assays were, like transfection efficiency assays, all divided into two groups of IDR-1018 and IDR-HH2. Since the peptides are considered to be therapeutic (for example promote cell survival and cell proliferation), the relative viability of the cells treated with lipoplex formulations of low molar charge ratio surpassed the bench-mark of 100% viability with some exceeding 120% at the molar charge ratio (+/-) of 1.5:1. All of the lipoplexes showed a similar "descending" trend as the molar charge ratio within the group increased, which was expected. This is because higher amounts of cationic lipid are considered toxic to the cells thus the cell viability is expected to decrease. [14,16,29,50]

Although the graphs in figure 2.2 would appear to suggest that IDR-1018 was less cytotoxic than IDR-HH2, the mean difference between the two peptides was not significant (p=0.22).

2.6.2. Impact of the structural differences of the cationic lipids

By looking at the cytotoxicity results from figure 2.2, we cannot draw a concrete conclusion as to which cationic lipid possesses the least toxicity value. The statistical p-values obtained from the student's t-test (figure 2.4) did not show any overall significant differences between the cytotoxicity of the two cationic lipids either. There was, nevertheless, a single degree of significant difference observed when the mean results were differentiated between the two peptides. Lipid/IDR-HH2 showed a p-value of 0.047 with USDMA having a higher cell viability percentage than SDMA in complex with that peptide.

2.6.3. Impact of the two co-lipids, Cholesterol and DOPE.

The co-lipids were co-formulated with the cationic lipids and the peptides, because they were believed to add stability and rigidity to the complexes as well as to have fusogenic properties which are important for the cell penetration potency and degradability of the liposomes.[62] Chol and DOPE were the two co-lipids employed in this study and previous work suggested that generally Chol-containing lipoplexes would have a lower cytotoxicity than DOPE-containing ones.[16] The student's t-test results, however, did not show any significant differences between the two co-lipids overall. But it showed a single degree of mean difference when the results where differentiated between the peptides. One level of significant difference (p=0.022) was observed for the IDR-1018/co-lipid formulations with Chol showing a higher cell viability than DOPE.

3. Discussion

The aim of this study was to develop and evaluate new non-viral gene delivery vehicles. As a continuation of the previous researches done in the field of liposomal DNA delivery, this study too chose to employ liposomes as the main element of the delivery vectors. In order to assemble a novel and efficient gene delivery vector, extended research was carried out to carefully select the constituents for synthesis. Cationic lipids are one of the main components of DNA-carrying liposomes and therefore two cationic lipids were carefully selected based on the difficulty and reproducibility of their synthesis as well as on their reported performance with respect to transfection. These lipids both have a DMA polar head-group with a single positive charge and two C_{11} hydrocarbon tails. The two differ in the degree of saturation in the two hydrocarbon tails, with one of them having a double bond in each of the tails (designated Saturated and Un-Saturated DMA cationic lipids). This allowed us to compare the differences in cell viability and transfection efficiency with regards to the differences in the degree of saturation.

High toxicity of these cationic lipids to the cells, at high concentrations, have been reported before. [14],[16],[30] In order to optimize the gene delivery liposome vectors, it was decided that the lipids should be in complex with IDR, 12-amino acid containing peptides. These peptides, like lipids, were chosen based on their simplicity of synthesis and their unique characteristics such as having immunomodulation tendencies. Amongst the IDR-peptides, IDR-1018 and IDR-HH2 are well-known, thus, they were chosen, as the second constituent, to be synthesized and added to our cationic lipids.

As the synthesis was a major part of this study many precautionary steps were taken into consideration in order to save time, energy and valuable resources. This study, especially, ran into a few problems during the peptide synthesis, which resulted into repeating some of the steps several times. The entire process of SPPS is labour intensive. Therefore, many precautions need to be taken into account for perfect execution of each step in order to avoid repeating the entire process again. The most common mistake happens at the amino acid coupling step. A non-complete de-

protection of the N-terminal of the bound amino acid or a poor activation of the C-terminal of the introducing amino acid could be among the reasons for an inconclusive coupling reaction.[69] It is, therefore, very important to perform a few tests (such as Kaiser test) at the end of each coupling step to ensure the success in the attachment. The Kaiser test was applied in our studies to confirm the absence of free amino acids which validates the attachment of each amino acid to the previous one.

Purification steps are also amongst the most laborious and most important steps in synthesis for assuring a fruitful outcome. In the present study, the cationic lipids were purified using manual FLC. Even though the yields of the final purified product were not as high as it was hoped they would be, we were still able to isolate enough product to carry out our investigations. The low recoveries after each purification step may primarily be the result of technical errors such as in the choice of the mobile phase or the slope of the gradient used, which might result in the retention of our product in the stationary phase (silica) of the column.

The process of SPPS is, on the other hand, primarily limited by the yield of each elongation reaction, since the loss of product during the washing of the solid support is minimal. The important consideration is to generate an extremely high yield in each coupling step and that is why amino acids were added in large excess at each step.[69] As an example if each coupling step were to have 99% yield (which is a little bit unrealistic when we manually do the SPPS), a 12-amino acid peptide would be synthesized in 88% final yield (assuming 100% yield in each de-protection). Protecting the side-chains of the amino acids eliminates the frequency of unwanted side-reactions, but the formation of some reaction by-products is inevitable. These by-products could end up in our crude product and that could be among the reasons why we obtained a low yield of the crude peptide product (16% and 20% for IDR-1018 and IDR-HH2 respectively). In order to purify the crude compound, the crude product needed to be dissolved in a solvent and then run through Prep-HPLC to be pure. This step is very crucial as some of the impurities in the final product hinder the ability of the powder to dissolve in a preferred solvent (e.g. water) or it only allows solubility in marginal quantities.[70] There was a lot of trial and error steps in the dissolving strategies carried

out at this stage. Sonication was applied after each trial to enhance the solubility. In the end, 10% ACN in water (plus massive sonication) was optimal enough to carry out the purification steps.

As the third and the last constituent of this synthetic vector, certain neutral helper lipids, referred to as co-lipids, were chosen to be added to the mix. They are believed to be complementing the combination with their added stability as well as membrane fusogenity features. The two co-lipids Chol and DOPE were chosen to be the two co-lipids used in this study due to their pervious success with mixing with the cationic lipids to form liposomes.[14],[16],[59],[67]

After achieving the first objective of the whole study, which was to synthesize the constituents of the DNA delivery vectors (except for the provided co-lipids), it was time to prepare them for further analysis. In order to do that, we first needed to combine the right amount of each constituent with each other and form liposomes. The two cationic lipids, two cationic peptides and the two neutral co-lipids were all formulated with each other in a fixed 3:3:2 molar ratios, respectively, to form a total of eight liposome formulations. Each one of these formulations were combined with the plasmid DNA cargo, in a buffered system, at molar charge ratios (+/-) of 1,5:1, 3:1, 5:1 and 10:1 to form lipoplexes. Different combinations are believed to have significant influence on cargo encapsulation affinity, cell penetration ability, cargo release affinity, cell viability and transfection efficiency. The most ideal transfection conditions are those with the highest levels of DNA expression as well as having a minimal treatment-associated toxicity.

The prepared liposomes and lipoplexes were characterized using particle sizing and DNA-binding gel assays. Particle size is very important for the delivery of complexes as it influences the transfection efficiency.[71] The particle sizing range of the liposomes and lipoplexes in this study were around 200-900 nm which are very optimal in comparison to the previous studies (reported range of 200-2000 nm).[16] PDI range of around 0.1-0.9 was observed. This broad range is common for liposomes prepared without extrusion.[65] High mean diameters, when observed in conjunction with high PDI values, could result from the fusion of liposomes/lipoplexes together or

electrostatic adhesion of neighbouring complexes or particle aggregation. [72] Such liposome preparations of large particle size (> 1000 nm) can hinder the delivery of the cargo *in vitro* whereas they probably would not be a problem for other administrative routes (e.g. intraperitoneal injection or inhalation).[73] Although the mean particle size of the lipoplexes differed between the various formulations, no meaningful trend was observed between the size, PDIs, and constituents of the liposomes/lipoplexes. We did not observe any correlations between particle size and transfection efficiency either. Since the lipoplex preparations appeared to match the expected size ranges, no further optimization of the particle size range of the delivery vectors was done, though it seems quite interesting for future researches.

The other method used to characterize the synthesized delivery vectors was to assess the DNA binding affinity of the lipoplexes using gel retardation assay. Gel assay is important in a sense that it gives us an insight into whether the DNA is bound to the proteins or not and which liposome:DNA molar charge ratios have had the highest binding. Too little binding could result in lipoplex dissociation as the electrostatic forces between the positive and negative are not too strong to hold everything together. Too high binding could result in liposomal aggregation and too tight a bond between the positive and negative, stimulates problems with cargo release. [27],[74]

In this study, a simple DNA-binding assay was carried out using 1% agarose gel loaded with all the 32 lipoplexes. The expected result of a gel retardation assay is to see an increased binding of the DNA to the lipoplex at higher molar charge ratios (+/-), which is visualized in the gel as a high MW band retained in the well in which the lipoplex sample was loaded.[65] The results obtained from this assay, however, were not conclusive. Although some lipoplex formulations containing IDR-HH2 appeared to show full binding of DNA to the liposomes, in general no clear trend was observed between the different formulations. We can see full bindings at the top of some of the lanes and then smears down the gel. This could be due to many reasons, such as having degradative gel conditions, too high loading of the DNA, too concentrated DNA, contamination of the plasmid DNA with nucleases or other proteins, etc. The most obvious choice would be that the DNA was degraded under the gel electrophoresis

conditions. Sometimes, if the concentration of salts is too high, the buffers used for running the electrophoresis can increase the temperature of the gel and degrade the DNA. Similarly, an old gel electrophoresis buffer may have got too acidic, causing the degradation of the loaded DNA. However, the fact that the DNA ladder actually showed very clear bands unlike the band designated for the *p*DNA, points all the problems towards the plasmid DNA stock and/or the way it was handled and loaded on to the gel.

Another possible explanation of the results may be that the stock of plasmid DNA used to make the lipoplexes had been degraded. However, this hypothesis contradicts the results of the evaluation assays, particularly the relative transfection assay which showed a level of β-galactosidase activity in the cells exposed to the lipoplex preparations that would not be explainable if the plasmid DNA had been degraded. It is, however, worth mentioning here that since the result of the gel retardation assay was inconclusive, it was impossible to confirm that the prepared lipoplexes really carried the desired intact DNA cargo. Consequently, the validity of the evaluation assays performed using those lipoplexes may be questioned. The fact that we have obtained poor results from this step of the study is a serious issue and it is suggested that in the future studies, we pay more attention to the loading concentration of the DNA and the conditions in which the assay is done. There is no denying that, if there had been sufficient time left for this Master's thesis, a repetition of the gel assay, as well as of the other assays, using fresh made liposomes and lipoplexes, would have had to be done in order to validate all the present results.

This study sought to determine how different constituents of the synthesized liposomal DNA delivery vectors (USDMA vs SDMA, IDR-1018 vs IDR-HH2, Chol vs DOPE, and molar charge ratio) affected the performance of the vector in terms of its relative transfection efficiency and its cytotoxicity. All of the transfection efficiency results, for combining the liposomes with DNA at 4 different charge ratios, showed the signature bell curve[16] distribution where the results for the molar charge ratios (+/-) of 3:1 and 5:1 where substantially higher than the remaining two ratios. One of the reasons why this type of distribution is observed is that, at low charge ratios (e.g. 1.5:1), there is not a lot of cationic moiety to encapsulate the anionic ones. This, thus, promotes

destabilization and membrane permeability impediment. Whereas, at high charge concentrations (e.g. 10:1), the liposome will have a tighter grip to the DNA and therefore stimulate release problems, which hinders the DNA cargo to enter the nucleus and code for the encoded protein.[16],[30] The cytotoxicity results also all showed an ascending trend with the lipoplexes formulated to have a molar charge ratio (+/-) of 1.5:1 showing the highest cell survival and those having a molar charge ratio of 10:1 showing the lowest cell survival. This is in accordance with most other reports investigating these cationic lipids.

It has been previously proven that lipids with one unit of unsaturation within each hydrocarbon chain in their hydrophobic domain transfect more efficiently, compared to their saturated counterparts. [75][76] These studies are in alignment with the results obtained from our study. There was an overall one level of significant difference observed in the transfection efficiency between the USDMA and the SDMA (p=0.049), with USDMA performing better than the latter. This could be due to the fact that the introduction of a double bond especially at the end of the alkyl chains increases the membrane fluidity as well as the liposome stability and rigidity. [77] The difference was especially prominent when the two cationic lipids were formulated with the peptide IDR-HH2 (p=0.025).

Jubeli and co-workers, had discovered that the unsaturated lipids exhibit a lower cytotoxicity compared to their saturated counterparts.[16] The same pattern was not observed here in our study. The overall mean results from all the formulations incorporating the cationic lipids showed that the lipoplexes with USDMA in their structure performed slightly better than SDMA (p=0.049). One level of significant difference (p=0.047) was observed when USDMA was in complex with IDR-HH2 having a higher transfection efficiency than IDR-HH2/SDMA. The reasons why unsaturated lipids appear to be marginally less toxic than saturated ones is not well understood, though we can speculate that the unsaturated lipids may have the tendency to decompose faster in the cytosol. Further biological tests need to be carried out in order to verify this statement.

Our calculated mean results showed that, there is no significant difference observed in the cell viability between the formulations with IDR-1018 and IDR-HH2. Given the fact that we could not find any previous studies that have carried out the same investigations using the same artificial peptides, we can't compare our results to any other or postulate that these attained results were expected or not. This means that the obtained results in the present study can stamp a benchmark for a start of new investigations toward incorporating synthetic, therapeutic peptides inside the gene delivery liposome vectors.

One purposes of adding the cationic peptides to the liposomal complex was to see if the cell viability and the relative transfection efficiency improves, with respect to the therapeutic characteristic of the peptides. *Jubeli and co-workers* investigated the cell viability of employing the same cationic lipids as we used in this study. The examined the same formulations, with respect to cytotoxicity and transfection efficiency, using the same liposomes (minus the peptides) at the same molar ratios. The cytotoxicity results obtained by our study has shown an overall increase in the cell viability as 6 out of 8 formulations with the molar charge ratio of 10:1 (the reported most toxic ratio) had a value above 40%, in contrast to the results obtained by these scientists (all values except for one have less than 30% cell viability).[16] The immunomodulating properties of the peptides could have brought about this increase in cell survival and promotion of cell proliferation. Another hypothesis could be that they prevent the cargo DNA from being degraded or the β-galactosidase gene from being silenced in the cell.

The positive charge on the cationic lipids that compose a lipoplex is required for binding to the negatively charged nucleic acid and it also facilitates transfer into the cell.[78] However the neutral helper lipids are required to achieve effective delivery. Its presence in the formulation aids efficient transfection by further promoting the fusion of the cationic lipoplex with cellular membranes through preferential cell binding (electrostatic interactions).[79] This promotes membrane destabilizing effects and later facilitates endosomal escape of the genetic cargo allowing for efficient transfection to occur.[80] The inclusion of helper lipids within the lipoplex formulation has also been linked with lower cytotoxicity and enhanced protection of the genetic cargo from

enzymatic degradation within the living organism.[80],[81] This protective nature is especially true of Chol, a commonly employed neutral helper lipid, which protects the lipoplex through the likely formation of domains, which in turn reduces the interaction of proteins within serum onto the lipoplex protecting it from disintegration.[82]

By only looking at figure 2.2, the formulations employing Chol as co-lipid visually showed slightly higher transfection efficiency as well as cell viability in comparison to DOPE. These results are in harmony with the previous reports stating that the hybrid of Chol and cationic lipids are more fruitful than the DOPE/cationic lipid option. This is especially more noticeable in the reports for cell viability assessments where the results for DOPE are 20% lower than Chol.[16] However, by looking at the overall mean values we can see that this pronounced difference is not seen in our results at all, as the mean calculations show no significant difference in cell survival between the two co-lipids. This could probably be due to the inclusion of therapeutic peptides.

4. Conclusion

In this study, we synthesized and formulated 8 new liposomal DNA delivery vectors by incorporating artificial cationic IDR peptides in the complex. These cationic liposomes were mixed with plasmid DNA, in 4 different charge ratios, and the obtained lipoplexes were transfected into CHO-K1 cells. The relative cytotoxicity and transfection efficiency of the lipoplexes were measured against the applied controls, L2K and RPMI medium. There was transfection observed with all of the 32 lipoplexes with the molar charge ratios of 3:1 and 5:1 performing better than the rest. The lipoplexes composed of unsaturated hydrophobic domain, displayed better transfection in comparison to their counterpart. In addition, the cholesterol-containing formulations exhibited greater transfection efficiency than those with DOPE. There was no overall significant difference observed between the transfection efficiency of the two cationic peptides. All lipoplex formulations displayed concentration dependent cytotoxicity against the CHO-K1 cells studied with most of the lipoplexes at the charge ratio of 1.5:1 reaching 120% cell viability. There was no overall significant difference observed between the two cationic lipids, the two cationic peptides and the two co-lipids, in terms of cytotoxicity.

Collectively, the results of this study revealed that addition of cationic IDR peptides to the lipid liposomes helped increase the cell viability and, to some degree, the transfection efficiency, although none of the lipoplexes exceeded the efficiency of the positive control, Lipofectamine 2000. As it was repeatedly stated, some of the aspects from this study have not been investigated before; therefore, we cannot conclude that what we have obtained are definitive. There needs to be a further research done to see if the impact of the IDR peptides is truly the reason for obtaining better transfection and cell viability results than previous researches. Additional efforts are needed to optimize the formulations. Extending the library of the lipids employed in the complex should also be amongst the future plans of this study. This would pave the way for obtaining the best possible non-viral vector for delivering DNA into the mammalian cells.

In conclusion, our results, as a whole, showed slightly higher transfection efficiency and cell viability when compared to the results of the previous studies. This suggests that our novel acyclic cationic lipid and cationic peptide hybrid are effective gene transfer vectors, suitable for further investigations in the study of non-viral gene delivery.

5. Experimental

5.1. Lipid synthesis: Saturated and Unsaturated lipids

5.1.1. General details

All lipids, that were used in this study, were synthesized by the researcher herself according to the protocol that was provided *by Jubeli et al. 2015*[16]. Saturated and unsaturated acyclic lipid series that included a C_{11} acyl chain and a Dimethylamine (DMA) head-group, were selected to be synthesized.

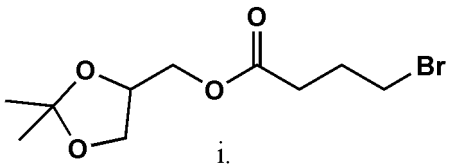
Proton NMR spectra were recorded on a Bruker (400 MHz), as dilute solutions in deuterated chloroform and deuterated methanol. Chemical shifts are referenced to residual protonated solvent (δ_H = 7.26 for $CDCl_3$ and δ_H = 3.35 for methanol-d_4) are quoted in parts per million (ppm). The multiplicity of signal is designated by one of the following abbreviations: s = singlet; d = doublet; t = triplet; q= quartet; m = multiplet. All coupling constants, *J*, are reported in Hertz and quoted to the nearest 0.1 Hz.

All of the steps in the synthesis of lipids were monitored by thin layer chromatography using silica gel 60 F_{254} pre-coated aluminium plates, which were developed using a potassium permanganate solution. Flash liquid chromatography (FLC) was performed on Sigma-Aldrich silica gel 60 as the stationary phase and the solvents employed were of analytical grade. Unless stated otherwise, all of the commercially available reagents were used as received. When necessary, some of the organic solvents were dried prior to use. Dichloromethane was distilled from calcium hydride. The products were concentrated on rotary evaporator under pressure. Where necessary, reactions requiring anhydrous conditions were performed under an atmosphere of nitrogen using oven dried apparatus.

5.1.2. Method details

5.1.2.1. Synthesis of SDMA

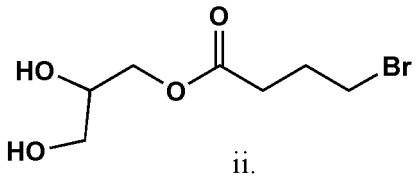
(2,2-Dimethyl-1,3-dioxolan-4-yl)methyl 4-bromobutanoate (i)



i.

4-bromobutyric acid (2.206 g, 13.2 mmol 1.1 eq. in dichloromethane (DCM), 125 ml, 0.1 M), DCC (N,N-dicyclohexylcarbodiimide) (3.72 g, 18 mmol, 1.5 eq.), DMAP (4-(dimethylamino)pyridine) (444 mg, 3.6 mmol, 0.3 eq.) was stirred under nitrogen gas at 0 °C for 30 minutes. Solketal (1.5 ml, 1 eq.) was added to the reaction and it was allowed to warm to room temperature, stirred overnight and then filtered through Celite and washed with DCM (3x 20 ml). The crude compound was concentrated and then purified using flash liquid chromatography (FLC) on silica using 30% ethylacetate in petroleum ether as eluent to give the ester i., (2.63 g, 77%) as a pale yellow oil. δ_H (400 MHz/$CDCl_3$), 1.39 (3H, s, CH_3), 1.43 (3H, s, CH_3), 2.20 (2H, quint., *J* 6.7 CH_2CH_2Br), 2.57 (2H, t, *J* 7.1, $OOCCH_2$), 3.47 (2H, t, *J* 6.4, CH_2Br), 3.73 (1H, dd, *J* 6.2 and 8.5, OCHHCH), 4.10 (2H, m, OCHHCHCHHOOC), 4.20 (2H, dd, OCHHCHCHHOOC), 4.32 (1H, quint, *J* 4.5 CHCHHOOC).

4-bromo-butyric acid 2,3-dihydroxy-propyl ester (ii)

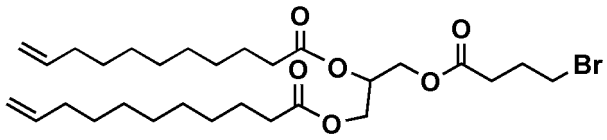


ii.

The acetal **i** (2.46 g, 8.75 mmol, 1 eq.) was treated with copper (II) chloride dihydride (2.248 g, 13.12 mmol, 1.5 eq.) in acetonitrile (89 ml, 0.1M) and stirred at room temperature for 24 hours. The reaction mixture was concentrated and then diluted with diethyl ether (≈ 200 ml), sodium hydrogen carbonate (in water ≈100 ml) and brine (100

ml). The organic layer (**ii**) was dried using magnesium sulphate (1 table spoon) and then the product **ii** was used as a crude for the next procedure (1.97 g, 94%). δ_H (400 MHz/$CDCl_3$), 1.36-1.42 (1H, s, SM) 2.20 (2H, qui, *J* 6.6 CH_2CH_2Br), 2.56 (2H, t, *J* 7.3, $OOCCH_2$), 2.65-3.25 (2H, br. s, 2x OH), 3.49 (2H, t, *J* 6.3, CH_2Br), 3.60 (1H, dd, *J* 6.2 and 11.5, HOCHH), 3.70 (1H, d, *J* 3.5, HOCHH), 3.92 (1H, t, HOCH), 4.18 (1H, m, *J* 6.1, CHHOOC), 4.20 (1H, m, *J* 5.1, CHHOOC), 4.34 (1H, m, SM), 4.92 (1H, t, unknown).

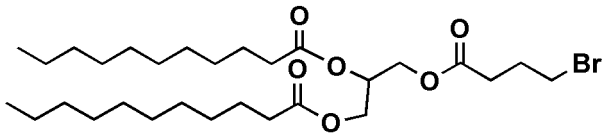
Undec-10-enoic acid 1-(4-bromo-butyryloxymethyl)-2-undecenoyloxy-ethyl ester



iii.

10-undecenoic acid (3.31g, 17.97mmol, 2.2 eq.), DCC (5.08g, 24.51mmol, 3eq.), dry DCM (33 ml, 0.25M) and DMAP (0.404g, 3.27mmol, 0.4eq.) were combined together and were stirred at 0 ^{o}C for 30 minutes before the crude diol (**ii**) (1.97 g, 8.17mmol, 1eq.) was then added to the reaction mixture (diluted in 30 ml DCM). The reaction mixture was left to stir overnight at room temperature under nitrogen gas. The reaction material was filtered through Celite and washed with DCM (≈ 60 ml). This was then concentrated to obtain the crude film (60 % yield) which was further purified by FLC (using 20% ethylacetate in petroleum ether as eluent) to give the triester **iii** (1.81g, 39% yield. δ_H (400 MHz/$CDCl_3$), 1.20-1.35 (20H, m, 10x CH_2), 1.57-1.67 (4H, m, 2x CH_2), 1.70-1.75 (1H, quin, unknown) 1.91-1.95 (1H, m, unknown), 2.01-2.08 (4H, dd, 2x H_2C=$CHCH_2$), 2.13-2.20 (2H, qui, *J* 7.1, CH_2CH_2Br), 2.31-2.35 (2H, td, *J* 7.4, CH_2COO), 2.51-2.55 (2H, t, *J* 7.2, $OOCCH_2$) 3.46 (2H, t, *J* 6.5, CH_2Br), 4.15 (2H, ddd, *J* 3.6, 5.8, and 11.5, 2x OCHHCH), 4.30 (2H, ddd, *J* 4.3, 8.8 and 11.8, 2x OCHHCH), 5.03-4.90 (4H, m, H_2C=CH), 5.30 (1H, tt, *J* 4.0 and 5.5, COOCH), 5.92 (2H, ddt, *J* 16.5, 9.1 and 6.5, 2x H_2C=CH).

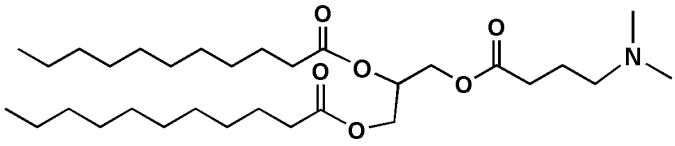
Undecanoic acid 1-(4-bromo-butyryloxymethyl)-2-undecenoyloxy-ethyl ester



iv.

The di-ene (**iii**) (1.81 g, 3.15 mmol, 1 eq.) was treated with palladium on carbon (0.1 g, 0.945 mmol, 0.3 eq.) in glacial acetic acid (10.5 ml, 0.3 M) and ethanol (105 ml, 0.03 M). The reaction mixture was stirred under nitrogen overnight. The reaction was filtered through Celite (using ≈100 ml DCM as eluent) and the filtrate was treated with sodium hydrogen carbonate (100 ml) and dried over magnesium sulphate. The reaction mixture was then left to evaporate and thus obtain the crude product which was further purified by FLC (10-20% ethyl acetate in petroleum ether) to give the triester **iv** (1.11 g, 61% yield). δ_H (400 MHz/$CDCl_3$), 0.83-0.89 (6H, m, 2x CH_3), 1.20-1.36 (28H, m, 14x CH_2), 1.57-1.64 (4H, m, 2x CH_2), 2.05 (1H, s, unknown), 2.18 (2H, qui, *J* 6.7, CH_2CH_2Br), 2.31 (2H, t, *J* 7.5, 2x CH_2COO), 2.52 (2H, t, *J* 7.3, $OOCCH_2$), 3.49 (2H, t, *J* 6.4, CH_2Br), 4.13 (2H, m, 2x OCHHCH), 4.30 (2H, ddd, *J* 4.3, 8.6 and 11.8, 2x OCHHCH), 5.30 (1H, tt, *J* 4.2 and 5.7, COOCH). (*Unknown=not assigned to any known functional groups)

Undecanoic acid 1-(4-dimethylamino-butyryloxymethyl)-2-undecenoyloxy-ethylester

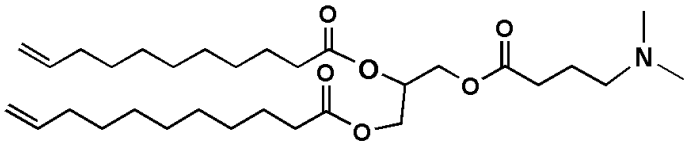


v.

A solution of dimethylamine in THF (1.12 ml, 2.24 mmol, 2M, 10 eq.) and the alkyl bromide **iv** (129.4 mg, 0.224 mmol, 1 eq.) were stirred in a sealed flask at room temperature for 72 hours. The reaction mixture was concentrated and then purified by FLC (using 2-10% MeOH in pentane) and the final product, **v**, was isolated (78.5mg, 64% yield). δ_H (400 MHz, $CDCl_3$) 0.86-0.89 ppm (6H, m, 2x CH_3), 1.23-1.33 ppm (28H, m, 14x CH_2), 1.52-1.65 ppm (4H, m, 2x CH_2), 1.81 ppm (2H, quint, *J* 7.1, $CH_2CH_2N(CH_3)_2$), 2.23 ppm (6H, s, $N(CH_3)_2$), 2.25-2.29 ppm (2H, m, $CH_2N(CH_3)_2$),

2.30 (2H, t, *J* 7.5 CH_2COO)), 2.37 ppm (2H, t, *J* 7.4, CH_2COO), 2.37 ppm (2H, t, *J* 7.5 CH_2COO), 4.15 ppm (2H, dd, *J* 5.5 and 11.1 CH_2OOCH), 4.29 ppm (2H, dd, *J* 5.5 and 7.3, $COOCH_2$), 5.25 ppm (1H, t, *J* 5.8, CH_2COO). Ice cold HCl in ether (2.5ml, 2M) was used to form the salts of the mixture into the desired lipid (20.7mg, 26% yield).

Undec-10-enoic acid 1-(4-dimethylamino-butyryloxymethyl)-2-undecenoyloxy-ethylester



iv'.

The process of **i-iii** was repeated for the synthesis of USDMA. In the final step, the dimethylamine (0.8 ml, 1.6mmol, 2M, 10 eq.) and the reaction product, **iii** (0.092g, 0.16mmol, 1eq.), were added together and was let to stir for 72 hours at room temperature. The reaction mixture was concentrated and then purified by FLC (using 2-10% MeOH in DCM), **iv'** (0.0538g, 63% yield). δ_H (400 MHz, $CDCl_3$) 1.25-1.40 ppm (20H, m, 10x CH_2), 1.50-1.53 ppm (4H, m, 2x CH_2), 1.60-1.72 ppm (2H, m, *J* 7.0, $CH_2CH_2N(CH_3)_2$), 2.0-2.05 ppm (4H, m, 2x H_2C=$CHCH_2$), 2.22 ppm (6H, s, $N(CH_3)_2$), 2.27-2.30 ppm (2H, m, $CH_2N(CH_3)_2$), 2.31-2.32 ppm (2x 2H, t, CH_2COO)), 2.37 ppm (2H, t, *J* 7.1, CH_2COO), 4.18 ppm (2H, dd, *J* 5.5, CH_2OOC), 4.30 ppm (2H, dd, *J* 5.0 and 10.8, $COOCH_2$), 4.90-5.01 ppm (4H, m, 2x H_2C=CH), 5.3 ppm (1H, tt, *J* 5.0 and 7.4, COOCH), 5.70 (2H, ddt, *J* 14.0 and 10.5 and 7.5, 2x H_2C=CH). Ice cold HCl in ether (2.5ml, 2M) was used to form the salts of the mixture which were the desired polar lipid (20.7mg, 39% yield).

5.2. Solid phase synthesis of cationic peptides

5.2.1. General details

The amino acids that were used here in this study were all Fmoc/tBu protected amino acids. The C-terminal of the amino acid is bound to a TFA labile resin. The resins are often named after the linker structures that are conjugated on the resin. Rink Amide Resin (RAM) (cas 431041-83-7) or TG S RAM (S-30023.0025; 0.23mmol/g), is

typically used. This study has used the TGS RAM; 0.23mmol/g as the resin, purchased from Novabiochem A/S.

Loading value (mmol/g) is essential and refers to the amount of functional groups (mmol) per gram of resin. In order to have 100 µmol of the product on each resin beads, around 0.5 gram of the above-mentioned resin was used. High loading is cost effective with respect to ratio between resin and coupling solution, but can cause peptide aggregation and other undesired intermolecular reactions.[83] Note that loading from 0.4-0.7 mmol/g is ideal. Mesh size of the resin was (100-200 or 200-400) equivalent to particle size 74-149 µm vs 37-74 µm, respectively. Larger size, lower the aggregation issues, but it can create isolated pockets resulting in lower purity, if not sufficient agitation is applied.

The Fmoc protecting group is removed by an alkaline solution of 20% piperidine in DMF. A cocktail of amino acid derivative, a C-terminal activating agent (HBTU as an example), a base (NMM) and a regent (NMP) is made and added to the resin. The activating cocktail provide an in situ benzotriazolyl esters that are ready to couple with the N-termini of the resin-bound amino acid. Kaiser test was done after each amino acid coupling reaction. This test is a qualitative test for the presence or absence of free primary amino groups, and it can be a useful indication about the completeness of a coupling step. The test is based on the reaction of ninhydrin with primary amines, which gives a characteristic dark blue colour. A few drop of Phenol in water and potassium cyanide working as analyte is used to help ninhydrin to perform its analysis. The cleavage of the peptides from the resin is done via a 97.5% TFA solution in water.

An Äkta-Pure preparative High Performance Liquid Chromatography, equiped with Diod Array Detection (DAD), was used to purify the peptides synthesized by SPPS. The separation was done using 2 types of buffers (buffer A , 0.1% Trifluoroacetic acid (TFA) in MilliQ water and buffer B, 0.1% TFA in acetonitrile) and a GRACE C18 column (19.5 mL Column Volume). The gradient was kept constant at 100% A for 2.5 column volumes before the sample was injected into the system. Upon sample injection (500 µl – *lower the injection volume, better the fragmentation) from the manual sampler to the column, the gradient was changed to 5% B and it was gradually elevated

to 50% B over 10 column volumes at a flow rate of 5 ml/min. The column was then flushed with 100 % B to clear out any possible non-polar residues that are stuck on the column.

The UV absorption of the effluent was continuously measured at 214 and 280 nm. The wavelength of 214 nm is normally used for detection of proteins and 280 nm is the classic signal for aromatic compounds (aromatic amino acids in this case). The retention time of the relevant peaks appearing at 280 nm for the peptides IDR-1018 and IDR-HH2 were around 4.54 and 6.35 column volumes, respectively. The samples were collected in a 2 ml fractions in a scouting mode and the aloquotes that gave any signals at the abovementioned wavelengths were selected for further identifications using mass spectroscopy.

An Agilent 6400 series triple Quadrupole LC-MS equipped with an electrospray ionization interface (ESI) coupled to high pressure liquid chromatographer (HPLC), was used to identify the purified proteins (IDR-HH2 and IDR-1018) synthesized in this study. The fractionation of 2µl aliquot of the peptides were loaded on a ZORBAX 300SB, 4.6 x 150 mm, 5 µm column. Buffers A , 0.1% Trifluoroacetic acid (TFA) in MilliQ water and B, 0.1% TFA in acetonitrile, were used as the mobile phase. The HPLC chromatograms and MS spectra of purified compounds are provided in Appendix 1.

The complete and detailed protocol that was used to synthesize the peptides is given below. This protocol is a modification of protocols introduced by *Amblard et al. 2006*[84], *Pooga et al. 2005*[85] and *Tokmina-Roszyk et al. 2013*[86]. Note that the entire process is done under nitrogen gas using standard laboratory glassware.

5.2.2. Method details

The computation of all the regents and the amino acids used in this synthesis are given in the tables 1 to 3 below.

Table 5.1: showing the Reagents in the SPPS and their corresponding amounts used

Reagents	Volume used (ml)	Prepared amount
Activator (HBTU)	30	6.4g+28 ml DMF
Base (NMM)	6.25	5.7 ml
NMP	3.15	3,15
DCM	13.6	13,6
Cap Mixture 10% in DMF (acetic anhydride)	15.5	15,5
Piperidine 20% in DMF	28.5	2.4 ml + 9.6 ml DMF

Table 5.2: Showing the Amino acids used in the SPPS of IDR-1018 and their used amounts

Amino Acids	Code	Weight (mg)	Solv. (ml)	Volume used (ml)
Ala	A	254.5	2.96	3.27
Arg	R	1632.5	8.11	10.06
Ile	I	489	4.95	5.53
Leu	L	289	2.92	3.27
Trp	W	430	2.75	3.27
Val	V	662	7	7.8

Table 5.3: Showing the amino acids used in SPPS of IDR-HH2 and their used amounts

Amino Acids	Code	Weight (mg)	Solv. (ml)	Volume used (ml)
Ala	A	430,5	5,02	3,27
Arg	R	1265	6,28	10,06
Gln	Q	499	2,67	5,53
Ile	I	489	4,95	3,27
Leu	L	289	2,92	3,27
Val	V	662	7	7,8

To 0.5g of the TGS RAM resin, 3 ml of DMF was added and was allowed to fizzle under nitrogen gas for 30 minutes. The DMF reagent was filtered off using the automated suction and to the resin the deprotection agent, 20% piperidine (1.8 ml) was added. The reaction was let to couple for 5 minutes before it was filtered of and the procedure was repeated for a second time. Before addition of the amino acid cocktail, the resin was washed using DMF (3ml, 3x). The amino acid cocktail contained of the activator agent HBTU (850 µl), the base NMM (250 µl), NMP (10 µl) and the respective amino acid solution (in DMF) (1050µl). The reaction was let to react under nitrogen gas for 15 minutes before it was washed with DCM (3ml, 1x). The second

coupling reaction using the same amino acid cocktail was carried out for 20 minutes and the amino acid was capped using the capping mixture (10% acetic anhydride in DMF). The resin was then again washed with DMF (3ml, 3x) and the resin was ready to couple with the next amino acid using the same procedure explained above.

The procedure is repeated for the entire amino acid sequence. Since the amino acids are fused together from the de-protected C-terminal of one to the N- terminal of the other, we start from the last amino acid and worked our way up to the first one. After the amino acids are coupled with each other, the resin was washed with DMF (3ml, 3x) and DCM (3ml, 2x). The peptide was then cleaved from the resin using 97.5% TFA in water in 4 x 30 minute intervals, under vigorous shaking. The cleaved peptides were dried and precipitated using nitrogen gas and ice cold diethyl ether. After the diethyl ether-treated compound was concentrated using a rotary evaporator, the peptide was diluted in mili-Q water and freeze dried to obtain the crude powder (IDR-1018 crude yield ≈ 66 mg (43%) and IDR-HH2 crude yield ≈ 48 mg (35%)). The final product was purified using preparative-HPLC (IDR-1018 yield ≈ 24 mg (16%) and IDR-HH2 yield ≈ 27 mg (20%)) and the purified compound was detected using LC-MS. The m/z values observed were 292.2, 378.1, 429.5, 818.3, 1036.9, 1536.0 and 2040.6 for IDR-1018 and 309.9, 387.1, 814.3 and 1395.1 for IDR-HH2. The signals at 1536.0 and 1395.1 belong to the molecular masses of both peptides IDR-1018 and IDR-HH2 respectively and it serves as a confirmation of their existence.

5.3. Formulations

5.3.1. Ethanolic stock solutions

Stock solutions of all of the lipids, peptides and the commercial co-lipids were made by dissolving a known amount of each component in DCM and were concentrated using a rotary evaporator for 60-75 minutes. The thin film obtained through evaporation of DCM was then dissolved in anhydrous ethanol to achieve a total stock concentration of 1 mM for the lipids and the peptides and 0.667 mM for the commercial co-lipids. The prepared 6 stocks were stored in -80 °C until the liposome preparations.

5.3.2. Liposome formulations

A known amount of the ethanolic stocks of each ones of the synthesized peptides (IDR-1018, M.W.:1535 and IDR-HH2, M.W.:1394), the synthesized lipids (USDMA, M.W.:537 and SDMA, M.W.:541), and the co-lipids (Chol, M.W.:386 and DOPE, M.W.:744) were mixed together in a fixed molar ratio of 3:3:2 (1mM:1mM:0.667mM), respectively. Each of the formulations (a total of 8 for all the lipid/peptide/co-lipid combinations) were concentrated to create a thin film using a rotary evaporator and they were then dissolved in a known amount of autoclaved water to give a total liposome stock concentration of 2 mM. All liposomes stocks were stored in -18°C until the lipoplexes were formed. Note that storage of these liposome formulations in a -18°C is not recommended at all. The liposome stocks should have been stored at +4°C.

5.3.3. Lipoplex preparations (liposome/DNA complexes)

Before the lipoplex preparations, the hydrated 2 mM liposome stocks were thawed at room temperature and sonicated for 30 minutes. A target lipoplex concentration of 0.243 mM, 0.486 mM, 0.81 mM and 1.62 mM, corresponding to the (+/-) molar charge ratios of 1.5:1, 3:1, 5:1 and 10:1, respectively, were prepared from the liposome stocks made previously. Then 72 µl of each liposome stock was added to 72 µl of a 50 ng/µl DNA stock solution. The mixture appeared to be cloudy at first, but upon sufficient vortexing and sonication, they mixed together. Forty-eight µl of the total 144 µl lipoplex solution was taken out at this stage to use for the gel retardation assay and the remaining 96 µl was dissolved in 204 µl of OPTI-MEM buffer. This means that we had 300 µl of lipoplex for each charge ratio for transfection and cytotoxicity assessments (each in triplicate).

5.4. Cell culture

Chinese hamster ovary (CHO) cells, were provided by Statens Serum Institut, Copenhagen, Denmark. The cells were continuously maintained in culture medium consisted of Roswell Park Memorial Institute's media (RPMI), supplemented with glutamine, 25mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1%

penicillin-streptomycin and 10% heat inactivated fetal bovine serum (FBS). CHO-K1 cells were cultured in T75 cell culture flasks in a humidified 5% CO_2 incubator at $37^{O}C$. Sub cultivation of the cells was done by removing the old media through suction using a Pasteur pipette. The adherent cells were carefully washed (x3) with 10 ml phosphate buffered saline (PBS) without Ca^{2+} or Mg^{2+}. After removing the PBS solution, 1 ml of trypsin-EDTA was added to the cells and the flask was incubated at 37°C (+5% CO_2) for 5 minutes, until the cells were completely detached from the surface. The detached cells were re-suspended in media and approximately 1.0 x 10^6 cells were transferred into a new T75 flask, which contained a fresh culture media and subsequently incubated at $37^{o}C$ in a humidified 5% CO_2 incubator.

5.4.1. Transfection

The prepared CHO-K1 cells, grown in RPMI media supplemented with 10% FBS and 1% penicillin/streptomycin, were seeded onto 2 opaque and 2 transparent 96-well plates at the density of 10^4 cells per well. The plates were incubated for 24 hours at $37^{o}C$ in the presence of 5% CO_2 atmosphere until the cells had reached an estimated 80-90% confluence. The cells were then washed 1x with PBS and then 45 µl of each lipoplex stock solutions (4 charge ratios x 8 liposomes =32 lipoplex stocks in total) was added to each well in triplicate. Note that three sets of controls, lipofectamine 2000, DNA alone with the media and the cells alone in the media, were also added in triplicate to each of the plates. The plates were then incubated for 4 hours, before they were washed again with 1x PBS and then fresh media was added to each well and then incubated for an additional 44 hours. The relative transfection efficiency as well as the cytotoxicity assays were done following the incubation of the plates.

5.5. Characterization

5.5.1. Gel retardation assay

For a better understanding of the binding interactions between the liposomes and the DNA at different molar charge ratios, gel retardation assay was carried out. 5 µl of the DNA loading dye (6x) was added to 48 µl of each of the remaining lipoplex stock

solutions and sufficiently mixed by pipetting. A 1% agarose gel impregnated with ethidium bromide was prepared and 18 µl of each of the 32 lipoplex samples were loaded on to the gel wells along with 5µl of $1Kb^{+}$ DNA ladder and run for 1 hour in 1xTBE (Tris-borate-EDTA) buffer at 105V. The migration of the DNA, complexed with the liposomes, is hampered by the electronic field, depending on their binding affinities in accordance to the charge concentrations. The gel was then viewed using a High performance UV Trans-illuminator.

5.5.2. Liposome and Lipoplex sizing

The hydrodynamic diameter, d_H, of liposomes and lipoplexes, after they were dissolved in OPTI-MEM buffer to be added to the wells, was measured by dual angle Dynamic Light Scattering using a Malvern Zetasizer nano ZS at a detection angle of 90° at room temperature.

5.6. Evaluation

5.6.1. β-Galactosidase assay

After the 48-hour incubation of the opaque 96-well plates, the relative transfection efficiencies of the lipoplexes were determined using the β-galactosidase (β-gal) assay (using the Beta-Glo® Assay System, Promega protocol). The wells were washed using 1x PBS, then 50µl of Dulbecco's Modified Eagle's medium (DMEM: phenol red-free media) was added to each well. Fifty microliters of Beta-Glo™ working solution was, subsequently added to the wells, which was prepared according to the protocol, through mixing and pipetting. The plates were then incubated at $37^{\circ}C$ in a humidified 5% CO_2 incubator for approximately 45-60 minutes (until a faint yellow colour is observed) and the relative light unites (RLU) were then read through Promega Glomax™ 96 microplate luminometer at 420 nm. RLU reflects on the β-gal activity which is produced by the luminescence of luciferin.

5.6.2 BCA assay (total protein count)

The principle of the Bicinchoninic acid (BCA) assay relies on the formation of Cu^{2+}-protein complex under alkaline conditions, followed by reduction of the Cu^{2+} to Cu^{1+}. The amount of reduction is proportional to the protein present. In the present study, the total protein count was measured using Pierce® BCA Protein Assay protocol, after the measurements for the MTS assay was done on the transparent 96-well plates. Once the wells were washed using 1x PBS, 10 µl of lysis buffer was added to each well and the plates were incubated at room temperature for 30 minutes. Two hundred microliters of the BCA working reagent which was prepared according to the protocol was then added to the wells and mixed gently by pipetting and the plates were again incubated at room temperature for an additional 60 minutes. The BCA readings were obtained by using an ELISA, synergy HT Biotech plate reader at 562 nm. A calibration curve is needed to calculate the total cellular protein count in µg. This is obtained from a Bovine Serum Albumin standard solution prepared at different concentrations (provided with the Pierce® BCA assay kit).

5.6.3 MTS assay

The biocompatibility of the lipid-peptide complexes was evaluated by quantification of cell viability through MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3 carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay. MTS assay is a calorimetric assay for determining cell viability and cellular proliferation of an infected cell line. It measures the mitochondrial activity and it can be used to determine the metabolism in viable cells. The metabolism in viable cells produces reducing components like NADH. These reducing components pass their electrons to the electron transport receptors that can then reduce the tetrazolium product, MTS, into an aqueous, soluble formazan product. At death, cell rapidly loses its ability to reduce the tetrazolium products.

The MTS assay carried out in this study was done according to the Cell-titer 96® Aqueous One Solution Cell Proliferation Assay. The 2 transparent 96-well plates, after the lipoplex transfection, were washed with 2x PBS. To each well, 100 µl of the DMEM

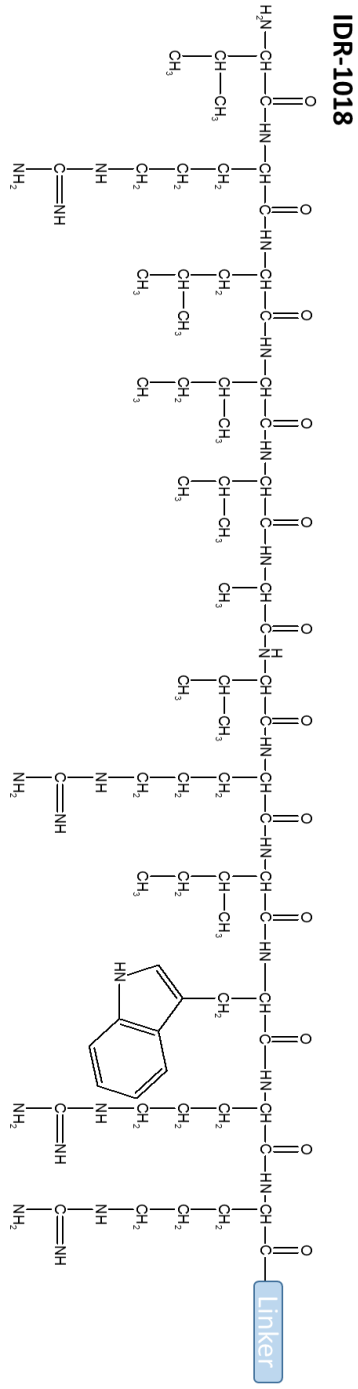
media was added, along with 20µl of the cell-tier 96® (Promega) One Solution reagent. The plates were tilted and mixed gently and incubated at 37^{o}C in a humidified CO_2 incubator, for 4 hours. The solution is blue (purplish) and the relative absorbance was recorded at 490 nm on an ELISA, synergy HT Biotech plate reader. The production of the colour Formazan product is directly proportional to the number of the viable cells in a culture (darker the colour, the more viable cells the wells contain).

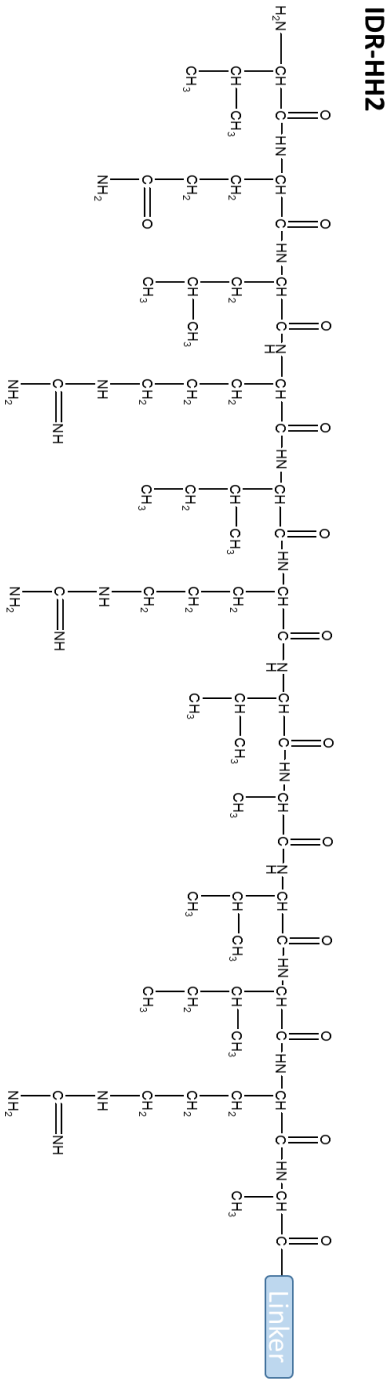
5.7. Statistical Analysis

A paired student t-test was used to evaluate the impact of each lipid in combination with the two peptides and the co-lipids on the DNA transfection efficiency and the cell viability. The comparisons were as follows; USDMA versus SDMA, IDR-1018 versus IDR-HH2, Chol versus DOPE. And then within each peptide, the comparisons were made between each of the Cationic lipids and the co-lipids. Values of $p < 0.05$ were considered statistically significant and they were categorized into two levels of significant differences with $p < 0.05$ being the first level (*) and the values $p < 0.01$ were the in the second level (**).

Appendix I

The structural representation of the IDR-1018 and IDR-HH2 synthesized in this study.

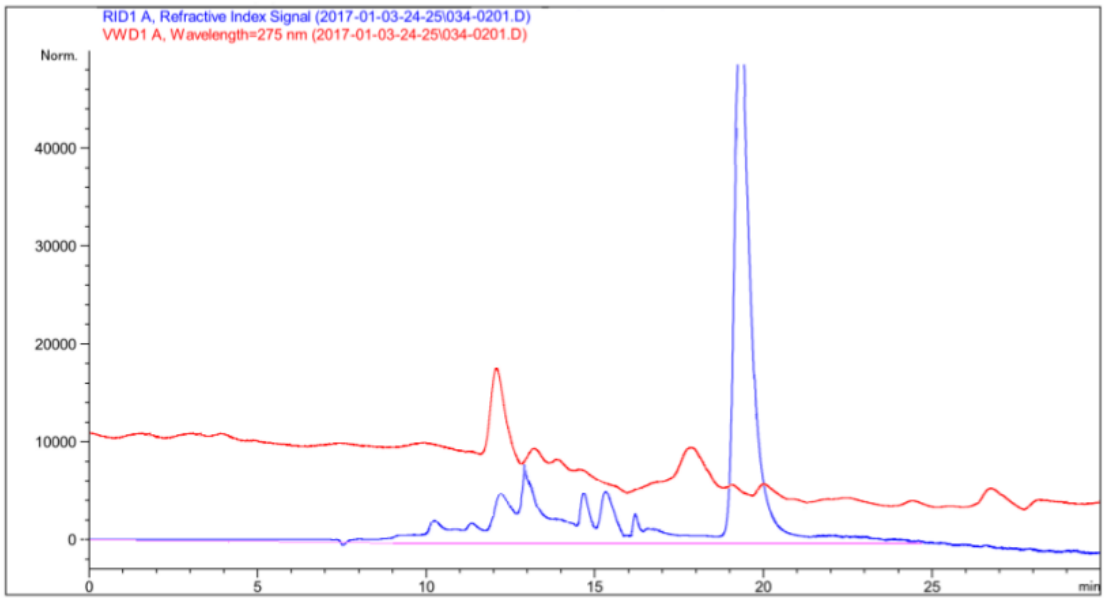




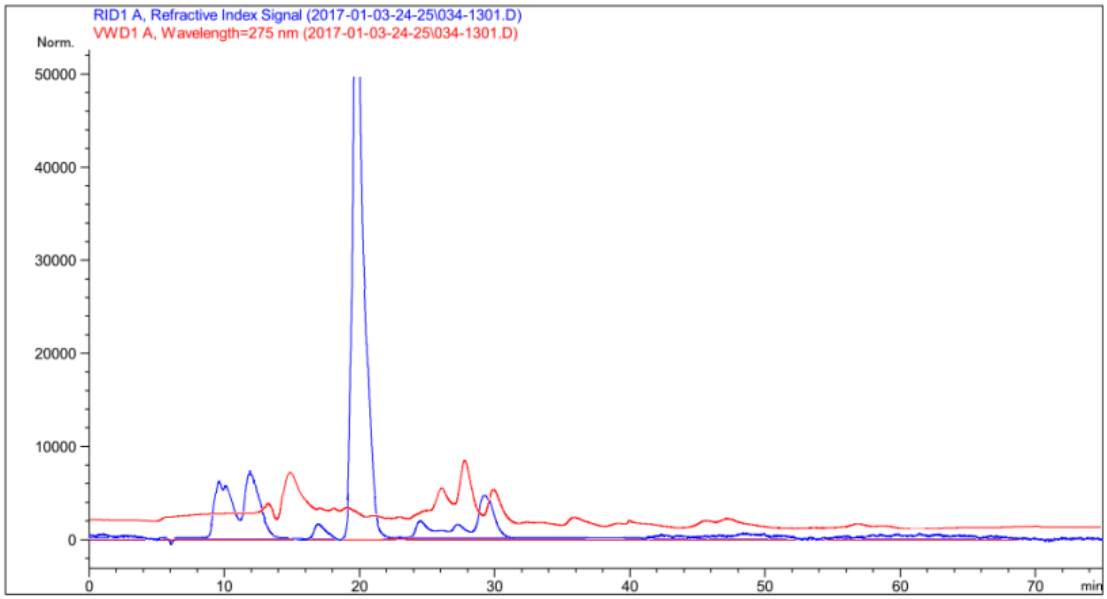
Appendix II

The HPLC chromatograms of the purified peptides.

IDR-1018

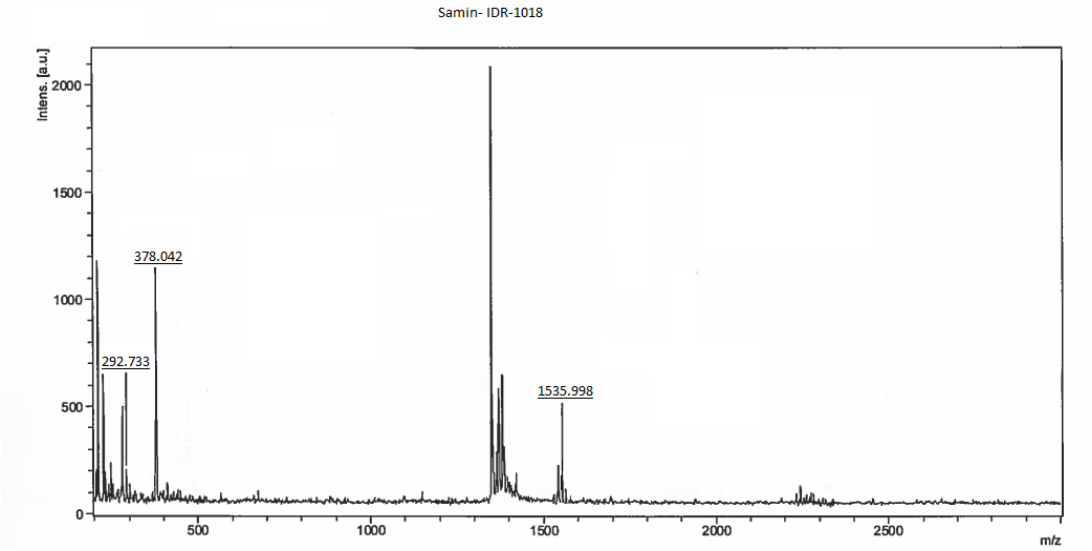


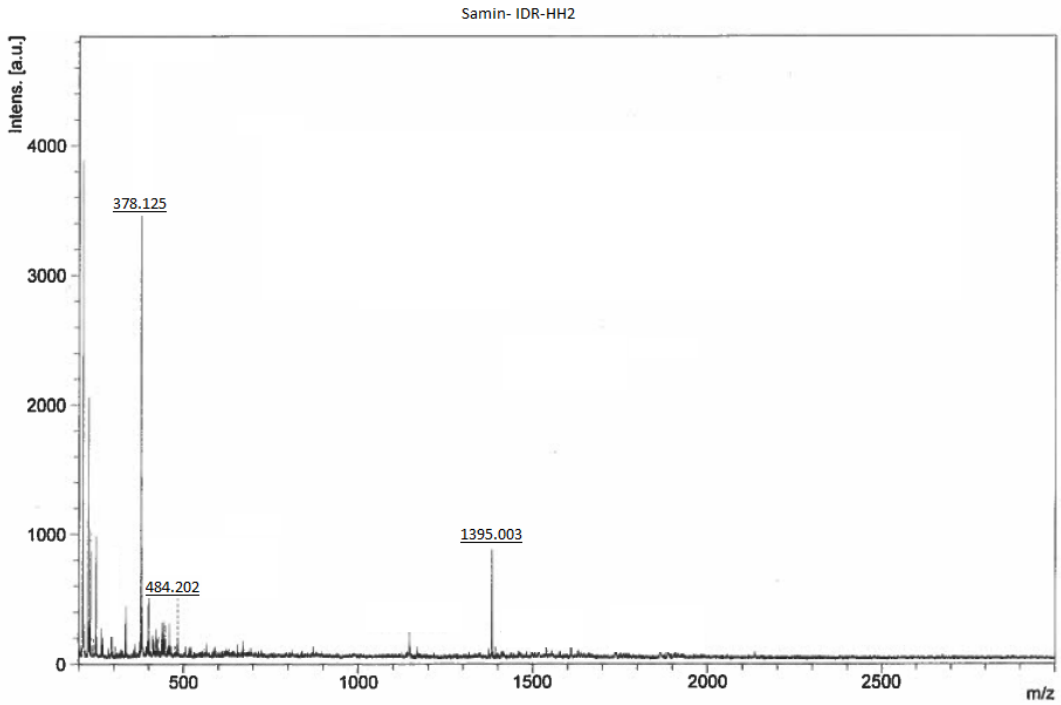
IDR-HH2



*The peptide isolate was carefully extracted from the crude by selecting the right fractions to collect. Please note that the chromatograms show that the peptides are not a 100% clean. The presence of marginal impurities is ultimately inevitable. Multiple purification steps are recommended for obtaining a complete pure compound. But for now these obtained peptides were optimal enough to carry out or research.

The MS spectra of the large peak observed in the HPLC chromatograms for both of the IDR peptides.





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