Gene Delivery

Combination of Cationic Lipids and Peptide Neurotensin for Lipofection

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ABSTRACT

Gene therapy has potential for treating a great number of genetic disorders e.g. different types of cancers, cystic fibrosis etc. However current gene therapy is limited by inefficient gene delivery vehicles. Non-viral delivery needs to be optimised in terms of higher transfection efficiency and lower cytotoxicity. Specific cell targeting is also of great interest. Specific cell targeting could be achieved by exploiting receptor mediated endocytosis, in which the non-viral vector is decorated with either receptor or ligand that interacts with either receptor or ligand expressed on target cell surface. A potential ligand candidate is neurotensin. Neurotensin interacts with a neurotensin receptor that is highly expressed on the membrane in certain types of cancer cells. This study investigates the potential of using cationic lipids in combination with co-lipid and peptide to achieve competitive transfection efficiency and lower cytotoxicity.

Two different cationic lipids and the active fragment of cationic neurotensin were synthesised and subsequently formulated together with co-lipid dioleoyl-phosphatidylethanolamine (DOPE). When these are formulated together it is unknown how the peptide is arranged in the lipoplex. Conjugating the peptide with a lipid could anchor the peptide in the lipid membrane. Therefore, a neurotensin-lipid conjugate was synthesised and tested in parallel with other formulations to assess whether the position of the peptide is of great importance. Formulations were based on a molar ratio of 2:3 DOPE:cationic component, with the cationic component being either lipid, full length neurotensin, a fragment of neurotensin or combinations of these. Furthermore, the formulations were prepared at different concentration, yielding different molar charge ratios when combined with a fixed amount of plasmid DNA. Plasmid DNA binding was assessed with electrophoretic agarose gels, showing no binding, gradual binding or complete binding for all formulations at all charge ratios. The ability to protect plasmid DNA was also assessed with electrophoretic agarose gels, after treating complexes with Dnase I. Transfection efficiency and cytotoxicity (cell viability) was evaluated *in vitro* using MTS, BCA and Celtiter assays, with untreated cells and cells treated with naked *p*DNA as negative controls and the commercially available Lipofectamine2000 as positive control. Results showed potential for some of the formulation, especially the formulations combining peptide and lipid, which achieved competitive transfection and greater cell viability compared to Lipofectamine2000.

RESUME

Genterapi har potentiale til at behandle et stort antal genetiske sygdomme, fx. forskellige typer af kræft, cystisk fibrose etc. Nuværende genterapi er begrænset af ineffektive måder at transportere DNA ind cellen. Ikke-viral levering skal optimeres for at opnå højere transfektionseffektivitet og lavere cytotoksisitet. Derudover kunne specifik celle selektivitet også være en optimering, som kunne opnås ved at anvende receptor medieret endocytose. Neurotensin er en potential ligand, som associere med receptor, der er særligt udtryk i visse typer af cancerceller. Dette studie undersøger muligheden for at anvende kationiske lipider i kombination med co-lipid og peptid for at opnå konkurrencedygtig transfektionseffektivitet og lavere cytotoksicitet. Når disse formuleres sammen, er det ukendt, hvordan peptidet er arrangeret i lipoplekset. Derfor vil et neurotensin-lipid konjugat blive testet parallelt for at undersøge om peptidets position har stor betydning for transfektion. Konjugeringen forankrer peptidet i lipoplex-lipidmembranen.

To forskellige kationiske lipider og det aktive fragment af kationisk neurotensin vil blive syntetiseret og formuleret sammen med co-lipid DOPE. Endvidere vil et lipopeptid blive syntetiseret ved konjugering af lipid og neurotensin fragment. Formuleringer er baseret på et molforhold på 3:2 DOPE: kationisk komponent, hvor den kationiske komponent kunne være enten lipid, neurotensin, neurotensin fragment, eller en kombination af disse. Formuleringerne var desuden fremstillet med forskellige koncentrationer, og giver derfor forskellige ladningsratioer, når disse kombineres med en bestemt koncentration af *p*DNA. pDNA-binding blev vurderet med elektroforetisk agarose gel, hvilket viste fuldstændig binding, gradvis binding eller ingen binding for alle formuleringer ved alle ladningsforhold. Transfektionseffektivitet og cytotoksicitet (celle-levedygtighed) blev evalueret *in vitro* med MTS, BCA og Celtiter assays, med ubehandlede celler og celler behandlet med ikke formuleret plasmid DNA som negative kontroller og Lipofectamin2000 som positiv kontrol. Resultater viste potentiale for nogle af formuleringerne, især formuleringer der kombinere peptid og lipid, da disse havde en konkurrencedygtig transfektion sammenlignet med Lipofectamine2000 og en større celle levedygtighed.

PREFACE & AKNOWLEDGEMENT

This study is an integrated 60 ECTS point master's thesis, written in molecular biology and chemistry at Roskilde University. The thesis was handed in the 01.06.18. The study is concerned with gene delivery using lipofection in combinations with peptides.

I would like to thank Kirsten Olesen for general help and guidance in the laboratory. Thanks to Jacob Krake for help with HPLC-MS. Thanks to Natalia Molchanova for advice regarding peptide chemistry. A thanks to Jeanne Paustian for laying the ground work for this master thesis. A special thanks to Kristian Jensen Pedersen, Houssein Elsalhi and Morten Lund, for supporting me and keeping my spirits high.

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CONTENT

LIST OF ABBREVIATIONS

AA	Amino Acid
ACN	Acetonitrile
$CDCl_3$	Deuterated Trichloromethane
CHO-k1	Chinese Hamster ovarian cells
CR	Charge Ratio
DCC	Dicyclohexylcarbidiimide
DCM	Dichloromethane
DIC	Diisopropylcarbodiimide
DMAP	4-dimethylaminopyridine
DMEM	Dulbecco's Modified Eagle Medium
DMF	*N*,*N*-Dimethylformamide
DOGS	Dioctadecylamidoglycylspermine
DOPE	Dioleoylphosphatidylethanolamine
DORIE	1,2-Dioleyloxypropyl-3-dimethyl-hydroxyethyalm - 3-dimethyl-hydroxyethylammonium bromide
DOSPA	2,3-Dioleyloxy-*N*-[2-(sperminecarboxamido)ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate
DOTAP	1,2-Dioleoyloxy-3-(trimethylammonio)propane
DOTMA	1,2-di-O-Octadecenyl-3-trimethylammoniumpropane
FBS	Fetal Bovine Serum
FLC	Flash Liquid Chromatography
FMOC	Fluorenylmethyloxycarbonyl chloride
GAP-DLRIE	(+/-)-*N*-(3-Aminopropyl)-*N*,*N*-dimethyl-2,3-bis(dodecyloxy-1-propanaminium) bromide
GPCR	G-Protein Coupled Receptor
HBTU	3-[Bis(dimethylamino)methyliumyl]-3H-benzotriazol-1-oxide hexafluorophosphate
HOBt	Benzotriazol-1-ol
LUV	Large Unilamellar Vesicles
L2K	Lipofectamine2000
MLV	Multilamellar Large Vesicles

MTS	3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NMM	*N*-Methylmorpholine
NMP	*N*-Methylpyrrolidone
NT	Neurotensin
NT^{8-13}	Neurotensin Fragment from AA 8-13
NTS1	Neurotensin Receptor 1
NTS2	Neurotensin Receptor 2
NTS3	Neurotensin Receptor 3
PBF	Pentamethyldihydrobenzofuran-5-sulfonyl chloride
*p*DNA	Plasmid DNA
RPC	Reverse Phase Chromatography
RPMI	Roswell Park Memorial Institute (medium)
Rt	Room Temperature
SAR	Structure-Activity Relationship
SDS	Sodium Dodecyl Sulphate
S_N2	Substitution Nucleophilic Bi-molecular
SUV	Small Unilamellar Vesicles
sst_x	Somatostatin Membrane Receptors (x = 1, 2A, 2B, 3, 4, 5)
SPPS	Solid Phase Peptide Synthesis
TFA	Trifluoro Acetic Acid
TIPS	Triisopropylsilane
TLC	Thin Layer Chromatography

LIST OF FIGURES

INTRODUCTION

Mutated genes are related to a great number of human diseases such as various types of cancer, Alzheimer's disease and cystic fibrosis. Genetic disorders can be treated with gene therapy i.e. the delivery of nucleic acids into cells to achieve a therapeutic effect (Karmali and Chaudhuri, 2007). Transgene expression could potentially restore the role of mutated genes. The first successful attempt of transferring genes was done in 1990 by Rosenberg *et al*. In this experiment viral-mediated gene delivery was used to introduce a reporter gene into human tumour-infiltrating lymphocytes and interleukin-2 prior to injection into human (Rosenberg *et al.*, 1990). Until 2016, more than 2000 gene delivery systems had been tested in clinical trials (*Vectors used in Gene Therapy Clinical Trials*, 26.05.18).

Current gene therapy is limited by inefficient gene delivery systems. Delivery vehicles are often divided in two categories: viral and non-viral. Each have advantages and disadvantages. Viral vectors have an inherent high transfection efficiency, but also a high risk of immunogenicity, propagation and insertional mutagenesis (Byk *et al.*, 1998; Heyes *et al.*, 2002). Non-viral gene carriers can be made up from a variety of molecules; lipids, dendrimers, polymers and block-copolymers, all of which can entrap and carry nucleic acids within the particulate compartment. Liposomes are the most extensively studied group as potential gene delivery systems (Heyes *et al.*, 2002). Liposomes are a promising candidate as they are biologically inert and biocompatible (Torchilin, 2009). In 1965 Alec Bangham *et al*. where amongst the first to report on liposome formations and liposome properties (Bangham, Standish and Watkins, 1965). In 1972 Gregoriadis and Ryman reported on animal experiments in which they delivered protein albumin and β-fructofuranosidase in liposomes into rat liver and spleen cells, effectively establishing liposomes ability to entrap and deliver cargo (Gregoriadis and Ryman, 1972). In 1987 P. L. Felgner *et al.* synthesised the cationic ammonium salt lipid *N*-[1-(2,3,-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) and succeeded in transfecting simian and murine cell lines *in vitro* (Felgner *et al.*, 1987). Nowadays, DOTMA formulated with 1,2-dioleoyl-sn-glycero-3-phosphatidyl-ethanol-amine (DOPE) is commercially known as Lipofectin (Felgner *et al.*, 1994).

Since these discoveries, modifications to alter the properties of liposomes have been suggested to overcome some of the issues using liposomes. The ideal delivery system

should be non-toxic and capable of containing and protecting nucleic acids from nucleases. The delivery system should be able to interact with, or adhere to, and penetrate the cellular membrane to ensure the nucleic acids arrive at the nucleus and permit biological activity by transgenic expression (Labas *et al.*, 2010).

Cationic liposomes have a certain degree of unspecific targeting as the liposomes adheres more to negatively charged surfaces, such as the surface of cancer cells (Chen *et al.*, 2016). Further more passive targeting for tumour cells is observed due to enhanced endothelial permeability and retention (EPR) in tumour cells (Marcucci and Lefoulon, 2004; Falciani *et al.*, 2011). Using active specific targeting could potentially increase specificity and accumulate higher concentrations of DNA in target cells. Specific targeting could be achieved be exploiting receptor-mediated endocytosis, by using a receptor-ligand system. Peptides has been suggested as potential ligands (Myers *et al.*, 2009).

Peptides are short, linear polymers of amino acids, linked with amide bonds. The distinction between peptides and proteins are increasingly unclear, however peptides are classically defined as being less than 50 amino acids in length and does not form larger complexes with other peptides, cofactors, RNA etc (Sewald and Jakubke, 2008). Since the discovery of Glutathione in the 1920s, hundreds of peptides have been discovered, isolated, synthesised and investigated. Peptides are omnipresent, and plays important roles in a huge variety of cellular activities. They have been found in the central and periphery nervous system, as autocrine, paracrine and endocrine hormones and as neurotransmitters. Furthermore, some peptides have been shown to have antimicrobial activities (Jenssen, Hamill and Hancock, 2006). Some peptides elicit their response through specific receptors. The peptide neurotensin has specific membrane receptors that are highly expressed in certain types of cancer cells, making neurotensin a potential candidate for active targeting of cancer cells (Falciani *et al.*, 2013).

Peptides can be formulated with liposomes, but it is unknown how peptides organise in liposomes and lipoplexes. However physical linkage of a peptide to a lipid, could anchor this conjugate in the liposomal membrane, with the peptide positioned outside (or inside) of the liposome. This study aims to investigate the effects on lipofection of formulating liposomes with peptides and formulating liposomes with a lipopeptide conjugate.

Lipofection

Due to structural and amphiphilic properties, cationic lipids spontaneously form liposomes in polar solvents, such as water (Bangham, Standish and Watkins, 1965). Furthermore, liposomes can accommodate polynucleotides. The complex formed by combining a liposome and polynucleotide is called a lipid-DNA complex or lipoplex. The lipoplex can enter mammalian cells and thereby transport DNA or RNA into cells (**Figure 1**). The mechanistic detail behind cellular uptake is still to be elucidated, however it is widely accepted that uptake happens through endocytosis (Farhood, Serbina and Huang, 1995; Wrobel and Collins, 1995; Xu and Szoka, 1996). Subsequent endosomal escape is crucial for DNA to reach the nucleus intact, as DNA is degraded in endo-lysosomes (Lechardeur, Verkman and Lukacs, 2005). Endosomal escape is facilitated by endosomal destabilisation and disruption, which is induced by lipid mixing of endosomal lipids and lipoplex lipids. (Xu and Szoka, 1996). Finally, once the DNA escapes the endosome, it must reach the nucleus to be expressed.

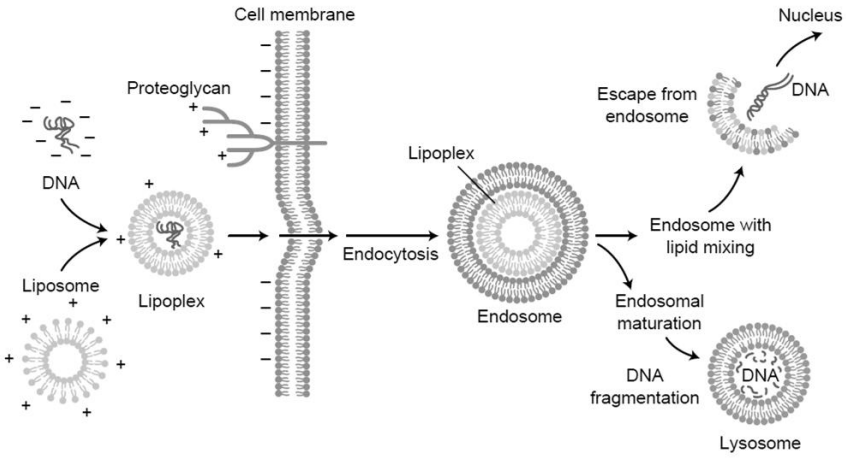


Figure 1. Schematic illustration of Lipofection. The figure shows how combined liposome and DNA, lipoplex, enters the cell via endocytosis. After endocytosis the endosome disintegrate, releasing DNA to cytosol, and from here DNA can get to the nucleus and be expressed. If the endosome is not disrupted it will mature and fuse with lysosome. Is this case, DNA will be degraded. Modified from (Parker *et al.*, 2003).

The efficacy of a lipoplex is often measured by transfection and cell viability assays, measuring the activity of a reporter plasmid (Felgner *et al.*, 1987; Behr *et al.*, 1989; Remy

et al., 1994; Aberle *et al.*, 1998; Byk *et al.*, 1998). The efficacy of a lipoplex is governed by the size and structure of the lipoplex, the molar ratio of lipid and co-lipid, molar charge ratio of liposome to DNA, together with the choice of co-lipid and design of cationic lipid.

Liposomes and Lipoplexes Morphology

Liposomes can range in size from a diameter of 0.025 μm to 2.5 μm (Sharma and Sharma, 1997). Liposomes vary structurally, and form multilamellar vesicle (MLV) or unilamellar vesicles. Unilamellar vesicles are divided into small (SUV) and large (LUV) unilamellar vesicles (Sharma and Sharma, 1997)(**Figure 2**). Once liposomes have formed, the addition of DNA results in spontaneous formation of lipoplexes. The cationic lipids of a liposome enable condensation with DNA, forming a lipoplex through electrostatic interactions with the negatively charged phosphate group in the DNA backbone (Byk *et al.*, 1998). Lipoplex morphology has an impact on transfection efficiencies (Ma *et al.*, 2007). Different structures of lipoplex have been reported, such as lamellar and inverted hexagonal morphologies. The lamellar model includes DNA that is sandwiched in the compartments between lipid bilayers (Miller, 1998) Rod like structures have also been observed in the so-called hexagonal system (Obata, Suzuki and Takeoka, 2008). The structure depends on a range of different factors including the cationic lipid structure, charge ratio, molar ratio etc. Another morphologic parameter is the lipoplex size. Larger lipoplex are considered more efficient *in vitro* when internalised through endocytosis. Larger lipoplexes have a larger surface area that can adhere with the cell membrane, which will increase endocytosis (Decuzzi and Ferrari, 2007). However, larger lipoplexes have also been observed to aggregate.

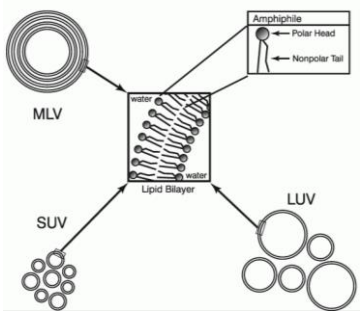


Figure 2. Liposome Structures. Modified from
("Liposome Preparations," 26.05.18)

Formulations and Charge ratios

Due to mixing of negatively charged DNA and positively charged liposome, lipoplexes are often investigated in terms of charge ratio. Lipoplexes formulated with a charge ratio (+/-) > 1 have an overall positive charge, and the lipoplex will therefore adhere to negatively charged surfaces. Positively charged lipoplexes form colloidal systems with DNA intercalated between lipids. Lipoplexes with neutral charge (+/- = 1) aggregates due to reduced electrostatic repulsion and are therefore not suitable for gene delivery. Formulations with a molar charge ratio (+/-) < 1 forms lipoplexes, but have a low transfection efficiency (Pitard *et al.*, 1997). The optimal charge ratio is system dependent and varies for different formulations, cationic lipids and co-lipids. However, in general high charge ratios leads to a decrease in transfection efficiencies (Labas *et al.*, 2010). It is hypothesised that the increased positive charge interacts too strongly with DNA and does not release DNA once the lipoplex is inside the cell (Byk *et al.*, 1998). High charge ratios also induce cytotoxicity, which could be due to higher cationic lipid concentrations (Goldring *et al.*, 2012; Jubeli *et al.*, 2015). On the other hand, the charge ratio should be large enough to facilitate DNA binding and protection, but not too high to significantly impact on cell viability.

Co-lipids or helper lipids are often included in liposome formulations to enhance transfection. One commonly used co-lipid is the neutral lipid dioleylphosphatidylethanolamine (DOPE, **Figure 3**). DOPE forms micelles at pH>9, but can transition into an inverted hexagonal structure at lower pH. The inverted hexagonal structure is believed to induce lipid mixing between a lipoplex and endosomal lipids, destabilising endosome and thereby facilitate endosomal escape of genetic material (Farhood, Serbina and Huang, 1995; Mochizuki *et al.*, 2013).

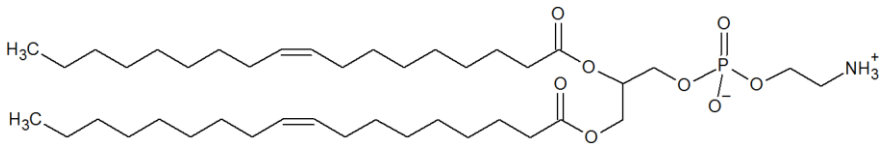


Figure 3. Structure of zwitterionic co-Lipid DOPE.

Structural Design of Cationic Lipids

Cationic lipids have been extensively investigated and evaluated as part of gene delivery systems in the form of lipoplexes (Akbarzadeh *et al.*, 2013). The organisation of lipids depends on the structure of the specific lipids, which thus have impact on the transfection efficiency and cytotoxicity. Cationic lipids are composed of a cationic headgroup moiety, a hydrophobic tail region and a linker (or spacer) connecting the two other groups (Byk *et al.*, 1998) This general structure results in amphiphilic properties needed for liposome formation. Through different studies, the general structure has been maintained, but with variations of one or more of the individual parts. Some of the features that have been investigated are the structure, length, units of unsaturation and symmetry of the hydrocarbon tails, the length and structure of the linker, the type, number of charges and structure of the headgroup (Felgner *et al.*, 1987; Behr *et al.*, 1989; Leventis and Silvius, 1989; Byk *et al.*, 1998; Balazs and Godbey, 2011). However, the effects of these changes and designs are still the centre of great debate, as different studies report contradictory results, and are often not directly comparable.

The cationic headgroup contains one or more of quaternary ammonium salt, primary, secondary or tertiary amine group, amidinium salt, guanidinium or amino acid (Bielke and Erbacher, 2010; Labas *et al.*, 2010). A great number of studies relating the structure of the headgroup to the impact on transfection efficiency and cell viability has been conducted (Felgner *et al.*, 1987; Behr *et al.*, 1989; Leventis and Silvius, 1989; Byk *et al.*, 1998).

The linker unit connects the hydrophobic and hydrophilic moieties and commonly constitutes ether, ester or amide bonds (Labas *et al.*, 2010). Ether bonds are less labile, but also less biodegradable and therefore can be more cytotoxic than esters (Karmali and Chaudhuri, 2007).

The hydrophobic region often constitutes a steroid or two hydrocarbon moieties. The most studied are hydrocarbon tails of C_7 to C_{18}, sometimes with different degrees of unsaturation and asymmetry (Tros de Ilarduya, Sun and Düzgüneş, 2010).

In 1987 Felgner *et al*. synthesised DOTMA (**Figure 4**). DOTMA has a quaternary ammonium headgroup, a glycerol linker with ether bonds to a dioleoyl (18:1) hydrophobic moiety (Felgner *et al.*, 1987). The design of DOTMA has given inspiration to

other designs, which vary different structural features in the pursuit of yielding higher transfection efficiency and cell viability. In a later study by the same group, variations of the headgroup, that substituted one methyl group on the ammonium unit with a hydroxyalkyl group were assessed. A variety of structures were studied, which varied the number of carbon atoms separating the ammonium and the hydroxyl group. Transfection efficiency evaluations found 1,2-dioleyloxypropyl-3-dimethyl-hydroxyethyalm-3-dimethyl-hydroxyethyl-ammonium bromide (DORIE), which possessed 2 CH_2 spacer groups was most efficient (Felgner *et al.*, 1994). This study also investigated the impact of hydrocarbon length of the hydrophobic moiety. Transfection assays found transfection efficiency to be inversely proportional to hydrocarbon length (C14 > C18:1 > C16 > C18), but also that units of unsaturation could increase transfection efficiency. The fact that the transfection efficiency is inversely proportional with chain length is observed in several studies, however, this trend is limited to a certain range of hydrocarbon lengths, as C8 chains have proven inefficient (Remy *et al.*, 1994; Kim *et al.*, 2004; Jubeli *et al.*, 2015). The fact that shorter chains (within a certain range) and units of unsaturation improves transfection could be explained by lipid fluidity. Shorter and unsaturated lipids are more fluid, and therefore have better fusogenic properties, that facilitates mixing between endosomal lipids and cationic lipids, which lead to endosomal disruption (Heyes *et al.*, 2002).

Another cationic lipid that has been widely studied is 2,3-dioleyloxy-*N*-[2-(sperminecarboxamido)ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate (DOSPA, **Figure 4**), which is a derivative of DOTMA. DOSPA has a spermine group, which is linked through an amide bond to a primary amine, two carbon atoms from the quaternary ammonium group. DOSPA, formulated in a 3:1 ratio with DOPE, is commercially available as Lipofectamine (Balazs and Godbey, 2011). Spermine is known to bind to the major groove of DNA (Jain, Zon and Sundaralingam, 1989), allowing more effective packing of DNA.

Similarly, dioctadecylamidoglycylspermine (DOGS) also possesses a spermine headgroup, but has a saturated dioleoyl hydrophobic moiety, an amide linkage between these groups, and the structure lacks the quaternary ammonium (Behr *et al.*, 1989). DOGS is commercially available as Transfectam (Balazs and Godbey, 2011). DOGS exhibited

high transfection towards melanotroph cells, and also showed no considerable cytotoxicity, even after 48 hrs of incubation time.

Byk *et al*. synthesised a homologous series of lipids, all with spermidine derivatives as headgroup moiety, and with varying geometries. The geometry, or relative orientation and linkage of the headgroup also affects transfection efficiency, as it was observed that a linear headgroup have higher transfection efficiency than T-shaped, globular or branched headgroups (Byk *et al.*, 1998).

1,2-dioleoyloxy-3-(trimethylammonio)propane (DOTAP) also takes inspiration from DOTMA, and only varies from this in the linker region, connecting the hydrophobic and hydrophilic moiety with ester rather than ether bonds (Leventis and Silvius, 1989). Ester bonds are more labile than ether bonds and could potentially be hydrolysed in the acidic environment of the endosome after internalisation. This prevents the cationic lipid for integration in the cell membrane, where it is believed to disturb normal cell function by changing the net charge of the cell membrane. DOTAP, co-formulated in a 1:1 ratio with DOPE, was found to have 2-4 fold higher transfection than 1:1 DOTMA:DOPE in mouse fibroblast, which could be an expression for lower cytotoxicity.

It has been suggested that headgroup combinations of quaternary ammonium and polyamines enhance transfection efficiency (Bielke and Erbacher, 2010). (+/-)-*N*-(3-aminopropyl)-*N*,*N*-dimethyl-2,3-bis(dodecyloxy-1-propanaminium) bromide (GAP-DLRIE) takes inspiration from DOTMA and is an example of combining a primary and quaternary amine as headgroup functionality (Wheeler *et al.*, 1996). Formulated with DOPE, GAP-DLRIE obtained more than a 100-fold expression of *p*DNA compared to naked *p*DNA in mouse lung.

Amino acids and amino acid derivatives has also been investigated as headgroup component. Arginine and lysine has proven effective as functional headgroup moieties for transfection efficiencies (Heyes *et al.*, 2002; Adami *et al.*, 2011).

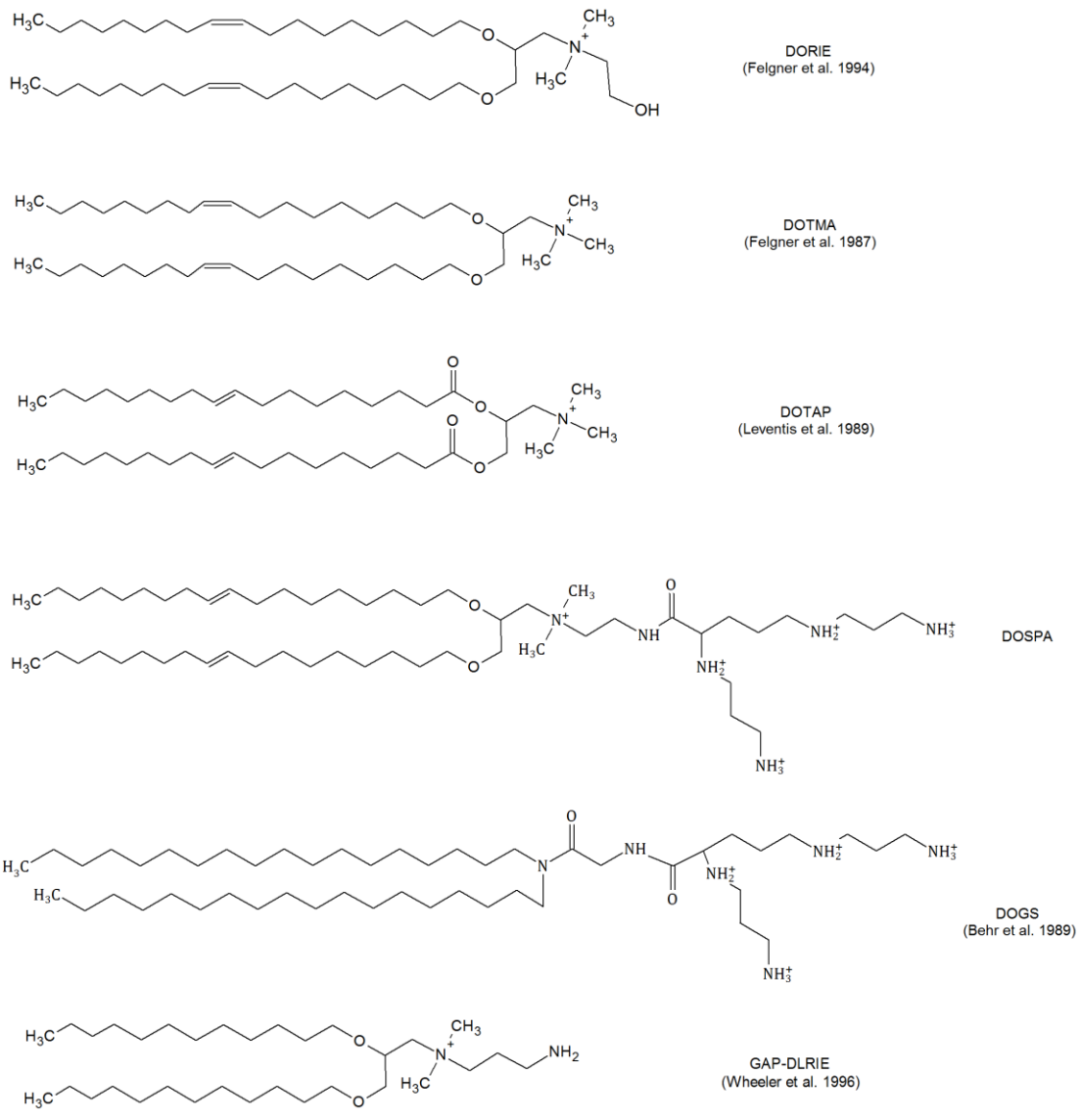


Figure 4. A selection of some well-studied lipids.
These lipids represent some of the structural features that have been investigated.

Peptides

Peptides has been suggested as potential ligands for cell targeting, exploiting receptor-ligand interactions. Different peptides are known to have different receptors. Specific receptors are expressed differently in different kinds of cells and could therefore be exploited to target these cells. An example is the hormone peptide Somatostatin, which regulates growth hormone secretion from the neuroendocrine system. Somatostatin membrane receptors (sst_1, sst_{2A}, sst_{2B}, sst_3, sst_4, sst_5) are overly expressed in

neuroendocrine tumours and radiolabelled somatostatin have therefore been used in diagnostics as a biomarker for neuroendocrine tumours (Reubi, 2003).

Another approach to use peptides in lipofection, is not by exploiting receptor-mediated endocytosis, but rather fusogenic properties of the peptides themselves. An example worth mentioning is the use of peptide GALA. GALA (WEAALAEALAEALAEHLAEALAEAL EALAA) is a synthetic, 30 amino acid peptide designed for membrane fusion at pH < 5 (Subbarao *et al.*, 1987). Transfection efficiency and cell viability was evaluated for complex formulations with DOTAP, DOPE, *p*DNA and fusogenic peptide GALA towards HeLa cells (Simões *et al.*, 1998). Transfection efficiency was assessed via luciferase activity, and formulations with GALA showed almost 4 times higher activity compared to controls. It is hypothesised that GALA is capable of facilitating higher gene expression by endosomal destabilisation by inducing mixing with endosomal lipids and cationic lipids from the lipoplex (Simões *et al.*, 1998).

There is a current search for other peptide-receptor systems relevant for tumour targeting. Neurotensin is a promising candidate, though more research is required. Neurotensin is a tridecapeptide with the sequence QLYENKPRRPYIL (Falciani *et al.*, 2011) and was originally isolated from calf hypothalamus. The 8^{th} to 13^{th} amino acid segment of the C-terminus (RRPYIL) plays an important role in receptor binding (Tate *et al.*, 2012). Neurotensin has a dual function as neurotransmitter or neuromodulator in the nervous system, and as a hormone in the peripheral nervous system (Vincent *et al.*, 1999). Neurotensin shows binding with three different membrane receptors (NTS1, NTS2 and NTS3) at the C-terminus of its structure. NTS1 and NTS2 both belong to the G-Protein Coupled Receptors (GPCR) superfamily, whereas NTS3 is structurally different from the former two and shares more homology with sortilin (Vincent *et al.*, 1999).

NTS1 is found in the membrane of neurons in different parts of the brain and spinal cord of healthy individuals and in certain types of tumour cells; meningioma, exocrine pancreatic tumour, small cell lung cancer, medullary thyroid carcinoma and Ewing's Sarcoma (Reubi, 2003). NTS2 and NTS3 is also found in brain tissue. NTS3 is furthermore found in heart, skeletal muscles, thyroid, placenta and testis (Vincent *et al.*, 1999). NTS1 exhibits stronger affinity for neurotensin than NTS2 and NTS3 (Myers *et al.*, 2009). Both NTS1 and NTS2 internalise from the outer membrane upon binding with neurotensin

(Mazella *et al.*, 1991). If a lipoplex was bound to the receptor through neurotensin, the whole complex could be internalised through this mechanism.

To achieve ligand-receptor binding the peptide should be well exposed to the exterior of the lipoplex and in the appropriate conformation on the liposome surface (Falciani *et al.*, 2011). The position of peptide could be optimised by a physical linkage to a lipid, effectively anchoring the peptide in the lipid-membrane. The conjugation with lipid, known as lipidation, is observed in Nature, and often observed in connection to membrane anchoring and cell signalling. The linkage between the lipid moiety and the peptide/protein is often a thioester associating a fatty acid with cysteine-S. Another kind of linkage is through an amide bond between a fatty acid and the *N*-terminal of the peptide/protein (Palomo, 2014).

In a study from 2011 by Falciani *et al.*, liposomes functionalised with branched neurotensin fragment was used to deliver doxorubicin into HT29 human colon adenocarcinoma and TE671 human rhabdomyosarcoma (Falciani *et al.*, 2011). HT29 express the receptors NTR1 and NTR3, whereas TE671 only has the receptor NTR1. According to their findings the functionalised, drug loaded liposomes led to a higher internal concentration of doxorubicin than unfunctionalized drug loaded liposomes. Cytotoxicity was improved for drug loaded liposomes (regardless of functionalisation) compared to the free drug.

OBJECTIVE

This study aims to investigate the transfection efficiency and cytotoxicity of two different lipids, co-formulated with the peptide neurotensin or a neurotensin fragment (NT^{8-13}) at different molar and charge ratios. These results will be compared to the transfection efficiency and cytotoxicity of a neurotensin fragment-lipid conjugate, synthesised as a part of this work.

The design of the lipids takes inspiration from earlier work conducted in the laboratory by Master's student Jeanne Paustian. In this previous work 5 different lipids were investigated, co-formulated with DOPE or cholesterol and three different peptides. Based on these results it was decided to further investigate lipid **1**, and synthesise a novel lipid **2** (**Figure 5**). Lipid **2** is the saturated version of lipid **1**. Furthermore, formulations including neurotensin showed promising result and because of the receptor-ligand potential of neurotensin, this was decided to be further investigated. As the arrangement of peptide in lipoplex is unknown, it was decided to covalently link neurotensin to a lipid, to anchor it in the liposome membrane, and evaluate the effects of this. As only the NT^{8-13} is important for receptor interaction, it was decided to conjugate this with a lipid.

Lipid **3** was designed as a precursor for the synthesis of lipopeptide **4**. All three lipids have a glycerol linker which is attached through esters to each lipid tail and to the headgroup. The headgroup for lipids **1** and **2** contain a quaternary ammonium structure with two methyl groups and a 1,3-propanediol group. Lipid **3** possesses a quaternary ammonium group, and a carboxylic acid, which can participate in amide bond formation with the *N*-terminal of NT^{8-13}.

We planned to synthesise lipid **1**, **2** and **3** using protocols established by the Goldring group and then confirm structures with spectroscopic data such as NMR and MS. We furthermore planned to synthesise NT^{8-13} fragment and confirm its structure with HPLC-MS. The lipopeptide **4** will be synthesised and verified with HPLC-MS.

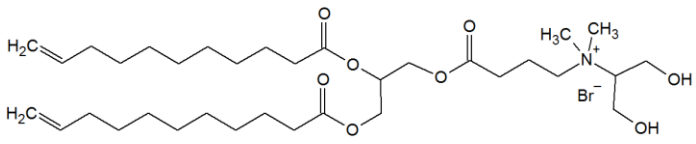
All formulations will be formulated with co-lipid DOPE, in a molar ratio of 2:3 DOPE:cationic component, with varying cationic lipid:peptide ratios. This is done to investigate the optimal molar ratio of lipid to peptide to achieve efficient transfection and good cell viability.

Lipoplex size have been emphasized to play a role for transfection efficiency and will therefore be measured using dynamic light scatter (DLS). However, this is outside the scope of this study, and no attempt was made to yield lipoplexes of equal size.

Liposomes should be able to bind and protect *p*DNA as lipoplexes to achieve good transfection. Therefore, binding and protection of *p*DNA will be investigated using agarose electrophoresis.

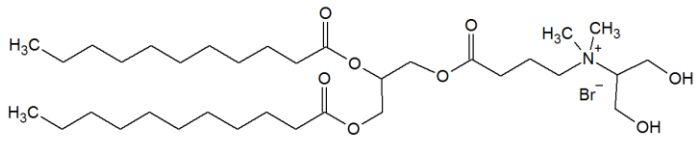
Transfection efficiencies will be evaluated using a β-galactosidase assay and using epithelial Chinese Hamster Ovarian cells (CHO-K1) cell line. *p*DNA used in this study, encodes for β-galactosidase, which is expressed as a result of successful gene delivery. β-galactosidase expression is measured as hydrolysation of substrate yielding luciferin, which upon reaction with luciferase generates light (Promega, 2011). Cytotoxicity is also evaluated with CHO-K1 using an assay in which reduction of tetrazolium compound gives coloured formazan product.

CHO-K1 cells are used as they are easy to transfect and often used as a model system for transfection assays. CHO-K1 cells have a high protein production, a short doubling time, and are in general easy to work with. Furthermore, tBlastn transcriptome analysis confirms NTR1 [Homo Sapiens] (Acces No. AAR07901) mRNA in CHO-K1 cells showing 87% identity with human NTR1 (EGW11057) (Xu *et al.*, 2011). However, investigation concerning the receptor is not conducted in this study.



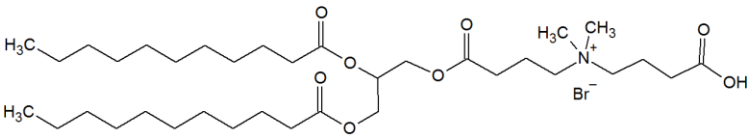
Cationic Lipid 1

$M(C_{34}H_{62}NO_8Br) = 692.78$ g/mol



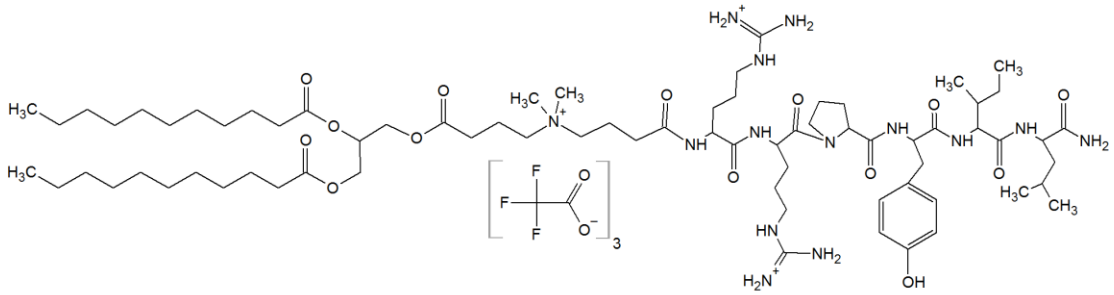
Cationic Lipid 2

$M(C_{34}H_{66}NO_8Br) = 696.79$ g/mol



Cationic Lipid 3

$M(C_{35}H_{66}NO_8Br) = 708.80$ g/mol



Lipopeptide 4

$M(C_{79}H_{131}F_9N_{14}O_{20}) = 1767.95$ g/mol

*Figure 5. Structure of lipids to be synthesised in this study. Cationic lipid **2** and **3**, and lipopeptide **4** are all novel. Cationic lipid **1** and **2** both have a 2-dimethylamino-1,3-propanediol headgroup, a glycerol linker that connects the hydrophobic tails with the headgroup through ester linkages, and two undecanoic acid derived hydrophobic moieties. The precursor cationic lipid **3** has the same structure as lipid **2** except for the headgroup, which constitutes a carboxylic acid.*

RESULTS

Synthesis of Lipid 1 and 2

Two cationic lipids were designed, synthesised and purified, for further biological assessment. The synthesis of lipid **1** and **2** employed nucleophilic substitution of a tertiary amine with an alkyl bromine via a S_N2 reaction (**Figure 6**). Over-substitution was not possible in this case since a tertiary amine was used as the nucleophile. Bromo-lipid **5** was reacted with 2-dimethylamino-1,3-propanediol to yield lipid **1**. In a separate set of steps, bromo-lipid **5** was treated with hydrogen gas and catalytic amounts of palladium on carbon to give the saturated bromo-lipid **6**, which was then reacted under same condition as lipid **1** to yield lipid **2**. Loss of bromide due to over-reduction of bromo-lipid **5** was evident from proton NMR spectrum. This resulted in formation of 16.4% of the by-product. Yields for both lipid **1** and lipid **2** were poor (5.2% and 8.2%, respectively).

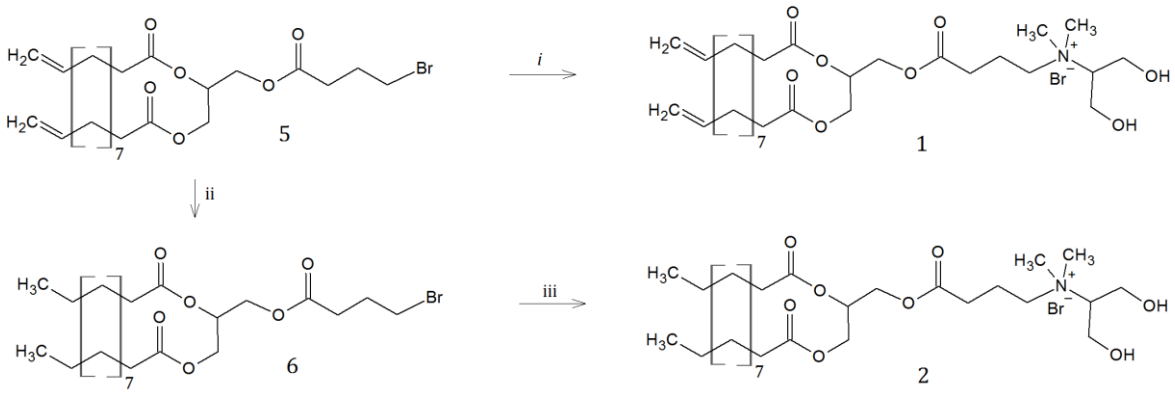


Figure 6. Reaction scheme for the synthesis of lipid 1 and lipid 2. Reagents and conditions: (i) 2-dimethyl-1,3.propanediol, 90°C, under pressure, 67 hrs. (ii) Pd/C 10% w/w, CH_2COOH and EtOH, H_2 atmosphere, rt, 5 hrs. (iii) same as (i).

Synthesis of Precursor Lipid 3

Lipid **3** was designed and synthesised as a precursor for the lipopeptide **4**. Steglich esterification (Steglich, 1990) was used to couple bromobutyric acid **7** with benzylalcohol, using coupling reagent DCC and catalytic amounts of DMAP, to yield benzyl 4-bromobutanoate **8** (**Figure 7**). This was reacted with dimethylamine through a S_N2

reaction to give the tertiary amine **9**. Next, reaction of the lipid bromide **5** with the amine **9** gave the benzyl ester lipid **10**. Hydrogenolysis of **10** gave the precursor lipid **3**. As a consequence of this reaction the double bonds in lipid **10** were reduced (99.2% yield).

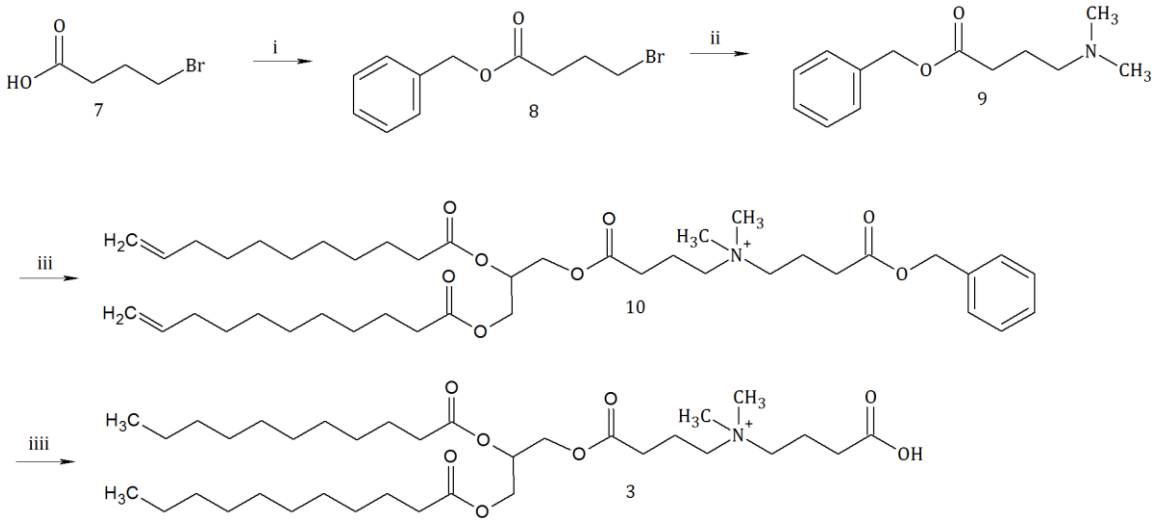


*Figure 7. Reaction scheme for the synthesis of lipid **3**. Reagents and conditions: (i) Dry DCM, DCC and DMAP (0˚C), benzylalkohol, rt, 18 hrs. (ii) NMe_2 (2M in THF), rt, 88 hrs. (iii) ipid **5**, 90˚C, under pressure, 48 hrs. (iiii) Pd/C 10% w/w, CH_2COOH and EtOH, H_2 atmosphere, rt, 5.5 hrs.*

Synthesis of NT^{8-13} fragment

A six residue fragment of neurotensin has been demonstrated to be involved in the neurotensin receptor interaction (White *et al.*, 2012). Synthesis of this fragment, NT^{8-13}, was achieved using the standard protocol for solid phase Fmoc-based peptide synthesis (SPPS). The basic principle of SPPS, based on Merrifield's approach (Merrifield, 1963) is shown in **Figure 8**. The solid phase approach has many advantages and is now the most widely used for peptide synthesis (Chan and White, 2000). In this method, peptide is attach to an insoluble resin, which makes separation of solvents and solubilised reagents from peptide more convenient. However, by-products from uncomplete reactions accumulates on the resin and can be difficult to separate after peptide is cleave from the resin (Amblard *et al.*, 2005).

Rink amide resin was used as the solid phase support in this synthesis. The rink amide linker can be coupled with carboxylic acid of an amino acid forming an acid-labile bond. The α-amine of the amino acid needs protection as to inhibit coupling to the carboxyl group of other amino acids in solution. Fluorenylmethyloxycarbonyl chloride (Fmoc) can offer protection to the α-amine, and is a base-labile bond. Reactive sidechains are also protected to inhibit reaction at these moieties. Sidechains are often protected with acid labile bonds, so to be cleaved with trifluoroacetic acid (TFA), when the linker to the resin is cleaved as well, yielding the peptide in one cleavage step. In the NT^{8-13} sequence both R-groups were protected with Pentamethyldihydrobenzofuran-5-sulfonyl chloride (Pbf).

3-[Bis(dimethylamino)methyliumyl]-3H-benzotriazol-1-oxide hexafluorophosphate (HBTU) and a base was used as coupling reagents. Coupling reagents are needed to activate the carboxy group of the incoming amino acid. Especially the first coupling step, loading the *C*-terminal amino acid to the linker resin, is crucial, as this step determines the yield of the whole reaction. After each coupling step, unreacted amines were acetylated to prevent further reaction. The NT^{8-13} fragment was yielded with an amidated *C*-terminal as a consequence of the synthesise method (80.3% yield).

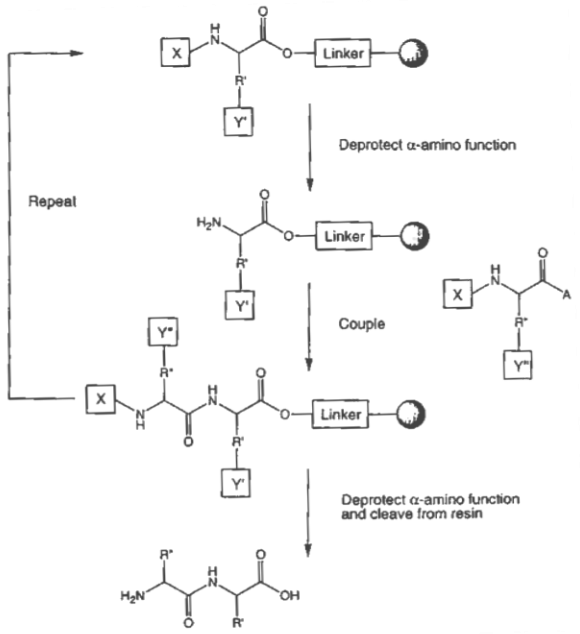


Figure 8. Basic principle of solid phase peptide synthesis. The α-amine in the growing peptide is initially protected and is deprotected before amide bond formation with the carbonyl C of the next AA. This is repeated to reach full length AA sequence, and the linker is cleaved to yield peptide. (Chan and White, 2000)

Synthesis of Lipopeptide 4

To have more control over the positioning of the peptide in the lipoplex, we chose to conjugate NT^{8-13} to the headgroup of lipid **3**. Conjugation between lipid **3** and NT^{8-13} was achieved through the formation of an amide bond between the carboxylic acid group of **3** and the *N*-terminal of the peptide. The solid phase approach, and the same conditions as for amino acid linkage was used. First, the Fmoc protecting group was removed from the α-amine of the peptide with piperidine, while peptide was still linked to the resin and included Pbf protected arginine groups. Two different reactions, using two coupling reagents, Diisopropylcarbodiimide (DIC) with Benzotriazol-1-ol, and HBTU with N-methylmorpholine (NMM) were performed. HBTU/NMM is a classic coupling cocktail for amino acid coupling, whereas DIC/HOBt is often used for *N*-terminal modifications. The reaction using DIC/HOBt as the coupling solution reached completion after 12 hours and the reaction using HBTU/NMM reached completion after 24 hours as confirmed with

qualitative Kaiser test. The Kaiser test contains ninhydrin, which forms a blue compound upon reaction with free amines, thus it was used to monitor completion of the reaction.

The conjugate was cleaved using the same conditions as for normal peptide cleavage. The yield of the crude product was 87% for the reaction with HBTU/NMM and 92% for the reaction with DIC/HOBt. Monoisotopic weight of the product was verified with HPLC-MS (Appendix 1) and RPC analytical HPLC was done on C8 column to test the purity. However, purification was not feasible since we were not able to find a suitable preparative column. Therefore, due to time limitations crude product was used for further experiments.

Formulations

Formulation were generally prepared to achieve a molar concentration ratio of 2:3 DOPE:cationic component, with the cationic component being either cationic lipid, peptide or both in different molar concentration ratios. Formulations were made to be consistent with and complement formulations prepared by Jeanne Paustian and previous co-workers. In this previous work, molar concentration ratios were set at 2:3 DOPE:cationic component, with a 1:1, 1:0 or 0:1 cationic lipid:peptide ratio. In this study, the same formulation types were prepared in addition to 2:1 and 1:2 cationic lipid:peptide ratios. In practice, most formulations appeared slightly cloudy upon hydration, but turned clear after sonication or dilution. All formulations were combined with *p*DNA to give molar charge ratios (+/-) of 1.5:1, 3:1, 5:1 and 10:1. Because NT^{8-13} has a +3 charge, whereas neurotensin has a +1 charge, less cationic lipid was needed to meet the molar charge ratios listed above. Therefore, two additional formulations with NT^{8-13}, which were based on molar concentrations of corresponding formulations with NT, were also made.

Particle size

DLS measurements were performed the day after hydration and complexation with *p*DNA, and particles were observed in most formulation especially at charge ratio 10. The liposomes prepared in this work were found to have a diameter in the range between 416-3099 nm. The results had a polydispersity index (PDI) around 0.5. The lipoplexes were found to have sizes in the range between 425-6986 nm and PDI values of 0.214-1. PDI values indicate the distribution of liposome and lipoplex sizes (APPENDIX 2). Due to

the high transfection efficiencies they exhibited, some values for liposomes and lipoplexes are shown in table 1. However, it should be made very clear that no correlation between particle size and transfection efficiency was observed.

Table 1. Selection of liposome and lipoplex sizes.

	Liposome			Lipoplex		
Formulation	[+]	Z (nm)	PDI	[+/-]	Z (nm)	PDI
2:1.5:1.5	81.0	1004	0.507	5	6153	1
D:**2**:NT	162	1005	0.62	10	6986	1
2:1.5:1.5 D:**2**:NT^{8-13}	162	416	0.356	10	625.3	0.369
2:3 D:**2**	48.6	971.1	0.447	3	2817	0.425
2:1:2	81.0	895.7	0.584	5	8187	0.419
D:2:NT	162	821	0.622	10	2485	1
2:2:1	81.0	804.9	0.606	5	8164	0.344
D:**1**:NT	162	817.2	0.769	10	2314	1
2:1:2 D:**2**:NT^{8-13}	162	480.3	0.589	10	3049	0.424

*p*DNA Binding

Complexation between a liposome and *p*DNA is necessary for gene delivery. If *p*DNA is not complexed with liposome it cannot be transported into the cell. However, if the complexation is too strong, endosomal escape it not feasible either. Therefore, binding strength should be somewhere in between. *p*DNA binding for all formulations was evaluated using electrophoretic gel assays. Ethidium bromide was added to the agarose gel for visualisation of *p*DNA. Three general outcomes were observed; complete binding, gradual binding or no binding (**Figure 9**).

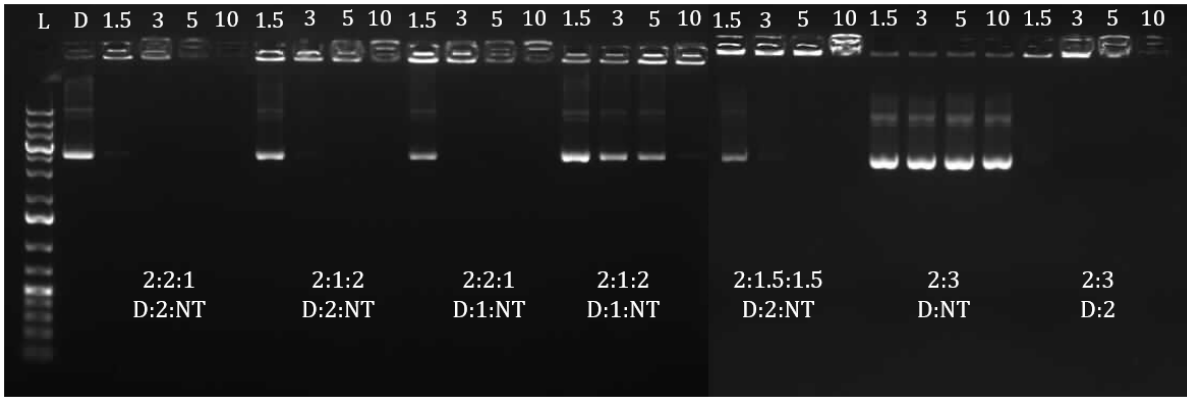
Complete binding was observed at all charge ratios for lipid **2** formulated without peptide, and for formulation 2:2:1 D:**2**:NT. This was observed as *p*DNA retention in the top of gel, although visualisation in the top of the gel was not obvious for all charge ratios. When *p*DNA is completely bound in lipoplex it is immobilised due to neutralisation of negative charge. No binding was observed at any charge ratios for formulations with peptides neurotensin or NT^{8-13} fragment formulated with DOPE and without cationic

lipid. This indicates that the peptides did not effectively bind to *p*DNA, and *p*DNA was therefore not retained. No binding was also observed for 2:1.5:1.5 D:**2**:**4**.

Formulations combining lipid and peptide showed gradual binding with increased charge ratios. Formulations 2:1:2 D:**1**:NT and D:**2**:NT^{8-13}, together with the formulation containing lipopeptide **4** showed gradual binding for charge ratio 1.5, 3 and 5 and complete binding at charge ratio 10. Formulation 2:1.5:1.5 D:**2**:NT^{8-13} showed gradual binding with complete binding after charge ratio 5. The remaining formulations all showed gradual binding, with complete binding at charge ratio 3 and higher charge ratios. Formulation 2:2:1 D:**2**:NT was assessed twice, showing complete binding (**Figure 9.A**) and no binding, with low intensity (**Figure 9.C**). The same was observed for formulation 2:2:1 D:**2**:NT^{8-13} (**Figure 9.B and C**).

In general, poor transfection efficiency is expected from formulations exhibiting complete or no binding, whereas formulations exhibiting gradual binding might be more promising. Therefore, formulations combining lipid and peptide were expected to perform well, but this is not always the case.

A.



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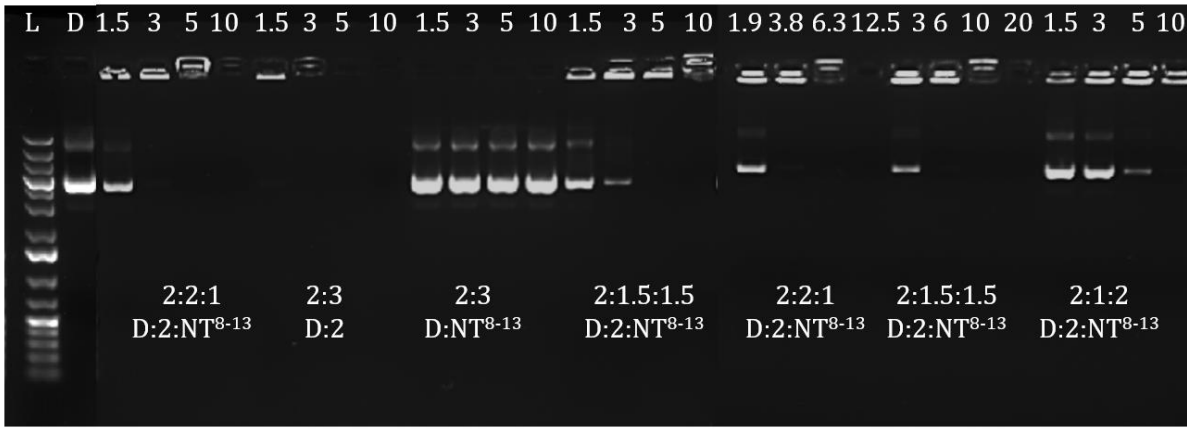

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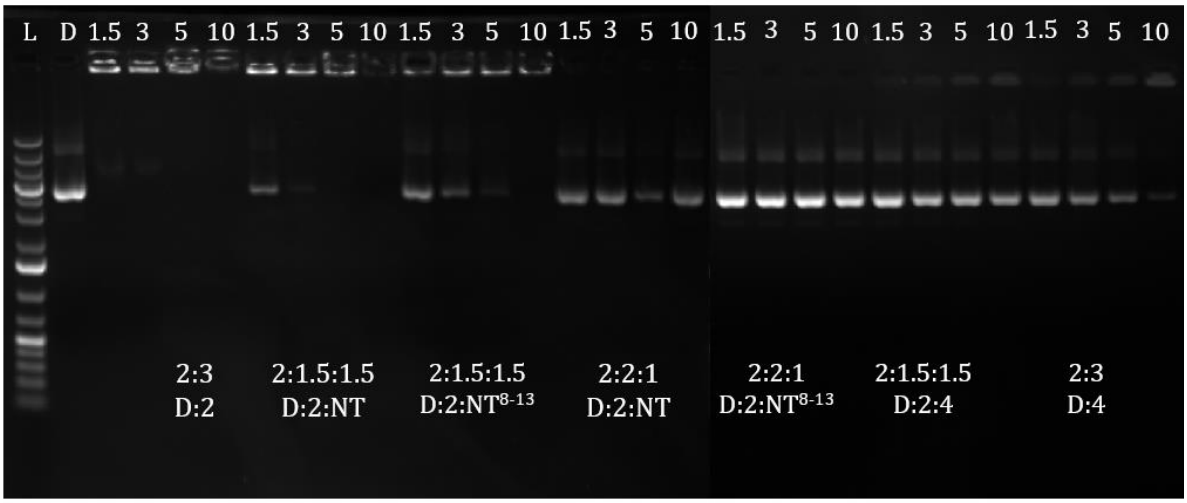


Figure 9. p*DNA Binding. The numbers in the top are charge ratios. The explanation in the bottom of the gel refer to molar ratios of different components. The two first lanes are DNA ladder (L) and naked* p*DNA (D).*

*p*DNA Protection

Optimal lipoplex formulations should protect *p*DNA from degradative enzymes. Protection was assessed in parallel with the binding experiments, and also employed electrophoretic gel assays. Lipoplexes were treated with DNase I and subsequently treated with 5% Sodium Dodecyl Sulphate (SDS) to disrupt lipoplex structure prior to running the gels. Two different outcomes were observed for all formulations (**Figure 10**). Either no protection or partial protection, which in some cases were gradually increasing with charge ratio. Protection is observed as visible *p*DNA bands, since the protected *p*DNA has not been degraded by DNase I. If no bands are observed, the *p*DNA has been degraded into nucleosides and due to small fragment size, these run quickly through the gel, and cannot be visualised with ethidium bromide. In general, formulations that exhibited no binding, did not exhibit protection either. This was observed for formulations with neurotensin or NT^{8-13}, without cationic lipid, which exhibited no *p*DNA protection. This was also observed for both formulations including lipopeptide **4** (**Figure 10.C**).

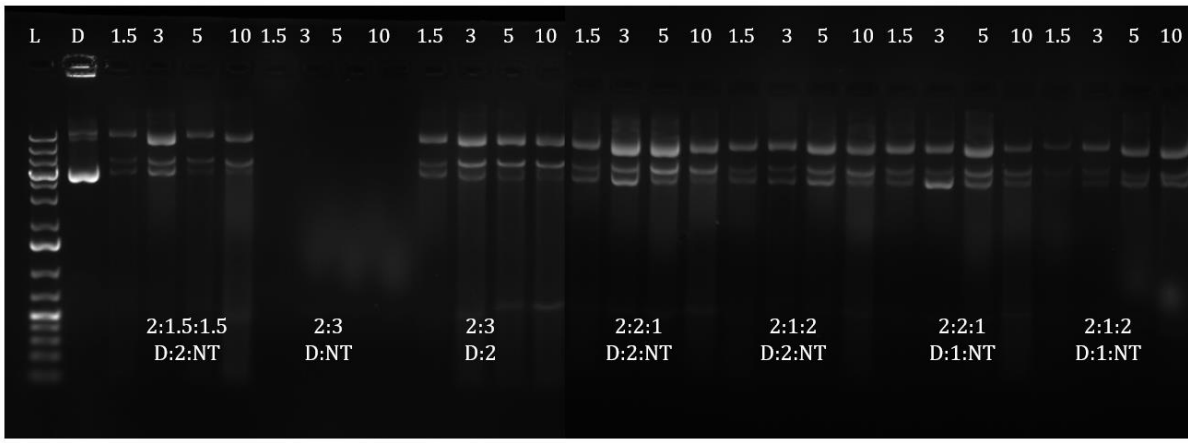
Formulations with more peptide than lipid all exhibited increasing protection with increasing charge ratio (2:1:2 D:**1**:NT, D:**2**:NT and D:**2**:NT^{8-13}). Formulations with more lipid than peptide had the highest *p*DNA intensity at medium charge ratios. Formulation 2:1.5:1.5 D:**2**:NT showed maximum intensity for the *p*DNA band at charge ratio 3. This was also observed for 2:3 D:**2**. Maximum intensity was observed at charge ratio 3 and 5 for 2:2:1 D:**2**:NT and D:**1**:NT, and 2:1.5:1.5 D:**2**:NT.

Formulation 2:1.5:1.5 D:**2**:NT^{8-13}, with charge ratios 3, 6, 10 and 20, showed decreasing intensity with increasing charge ratio (**Figure 10.B**). However, if the higher complexes are not destabilised by SDS, the *p*DNA would not be released, and the result would be similar to results showing no protection.

Some formulations were assayed twice, however, different results were observed. This was the case for formulations 2:2:1 D:**2**:NT and D:**2**:NT^{8-13}. In one gel the formulations exhibited protection (**Figure 10.A and B**), but both formulations exhibited no protection in the last gel assay (**Figure 10.C**).

In general, protection was regarded as necessary for delivery of intact *p*DNA *in vivo*. It was expected that formulations with poor protection, as these also exhibit poor binding, would exhibit poor transfection.

A.



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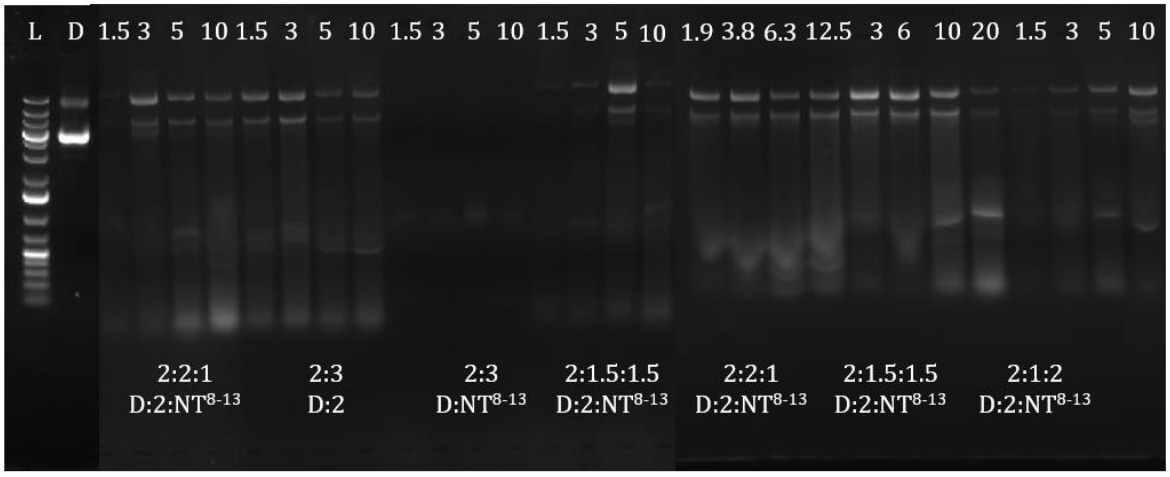

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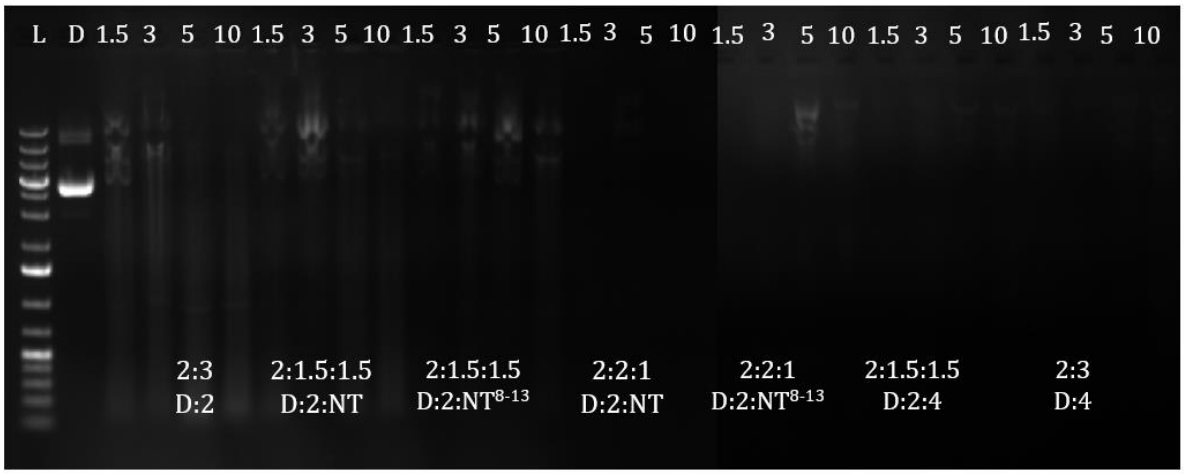


Figure 10. p*DNA protection. The numbers in the top are charge ratios. The explanation in the bottom of the gel refer to molar ratios of different components. The two first lanes are DNA ladder (L) and naked* p*DNA (D).*

Relative Transfection Efficiency

The lipoplexes ability to effectively deliver DNA into cells was compared using a beta-glo assay, measuring relative light unit (RLU). The results were normalised to RLU values of Lipofectamine2000, which was also used as a positive control (**Figure 11**). Cells treated with naked *p*DNA and untreated cells were used as negative controls. The experiments were obtained on different days with different batches of cells, and therefore the different graphs should be compared with caution. This was evident when comparing formulation 2:3 D:**2** which was included in all assays, but showed different outcomes.

No transfection was observed for formulations with neurotensin or NT^{8-13} formulated without cationic lipid. This agrees with the expectation as no binding, nor protection was observed. Formulations with lipid **2,** without peptide, was included in all assays. In general, these formulations exhibited maximum transfection at lower charge ratios. For assays 11.A and 11.B formulations with lipid **2** showed different, but comparable transfection efficiencies between 60% and 75% for the two lower charge ratios. At higher charge ratios the transfection efficiency was considerably lower. This could correspond with observed binding data. Formulations without peptide exhibited strong binding at all charge ratios. In **figure 11.C**, no transfection was observed for charge ratio 1.5 and 10, and transfection efficiency was 54% for charge ratio 3 and 41% for charge ratio 5.

Formulations with equimolar concentrations of cationic lipid and peptides achieved the highest transfection of all formulations tested (2:1.5:1.5 D:**2**:NT and D:**2**:NT^{8-13}, **Figure 11.A and B** respectively). The formulations with neurotensin has a transfection efficiency of 97% at charge ratio 5. The same formulations with neurotensin fragment has a maximum transfection efficiency of 87% at charge ratio 10. The same two formulations were repeated, however with no or considerably lower transfections around 20% (**Figure 11.C**).

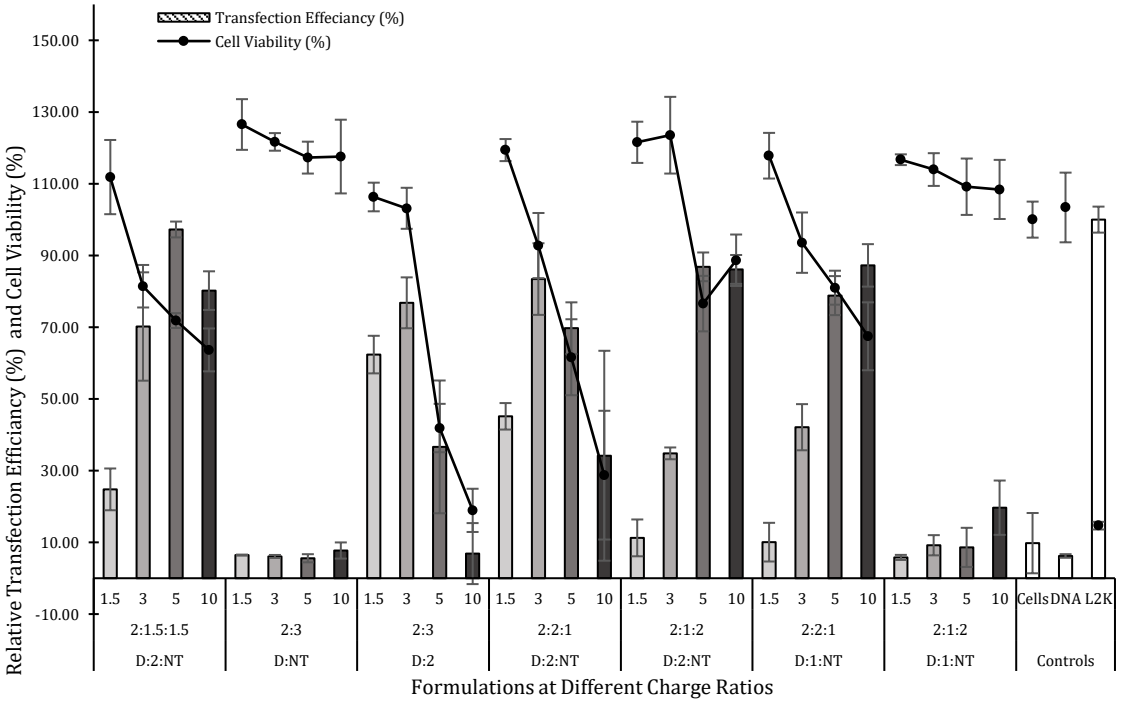
In general, when more peptide than cationic lipid **2** was used, optimal transfection efficiency was observed at higher charge ratios (2:1:2 D:**2**:NT and D:**2**:NT^{8-13} **Figure 11.A and B** respectively). When using more lipid **2** than peptide, optimal transfection was to some extent observed at lower charge ratios (2:2:1 D:**2**:NT and D:**2**:NT^{8-13}). This was also observed for the two formulations prepared based on molar concentrations. These two formulations, with neurotensin fragment, were prepared using the same concentrations

as the corresponding formulations with neurotensin. Consequently, these have different molar charge ratios (**figure 11.B**). The formulation with 2:1.5:1.5 D:**2**:NT^{8-13} has maximum transfection at charge ratio 6 at 76% and 2:2:1 D:**2**:NT^{8-13} has maximum transfection at charge ratio 3.8 at ~75%.

Formulations with lipid **1** exhibited optimal transfection at higher charge ratios, but showed almost no transfection when formulated at a molar ratio of 2:1:2 D:**1**:NT. When the same components were formulated at a molar ratio of 2:2:1 highest transfection of 74% at charge ratio 10 was observed.

The last assay showed considerably lower or no transfection for all formulations, except for lipofectamine2000 (**figure 11.C**). The first six formulations from the left are repeated from the two earlier experiments. The 2:2:1 formulations with neurotensin or NT^{8-13} showed no transfection in contrast the first two assays (**figure 11.A and 11.B**). The remaining formulations, which was repeated in this assay, also showed considerably lower transfection than in the two previous assays. The two formulations with lipopeptide **4** showed no or almost no transfection.

A.



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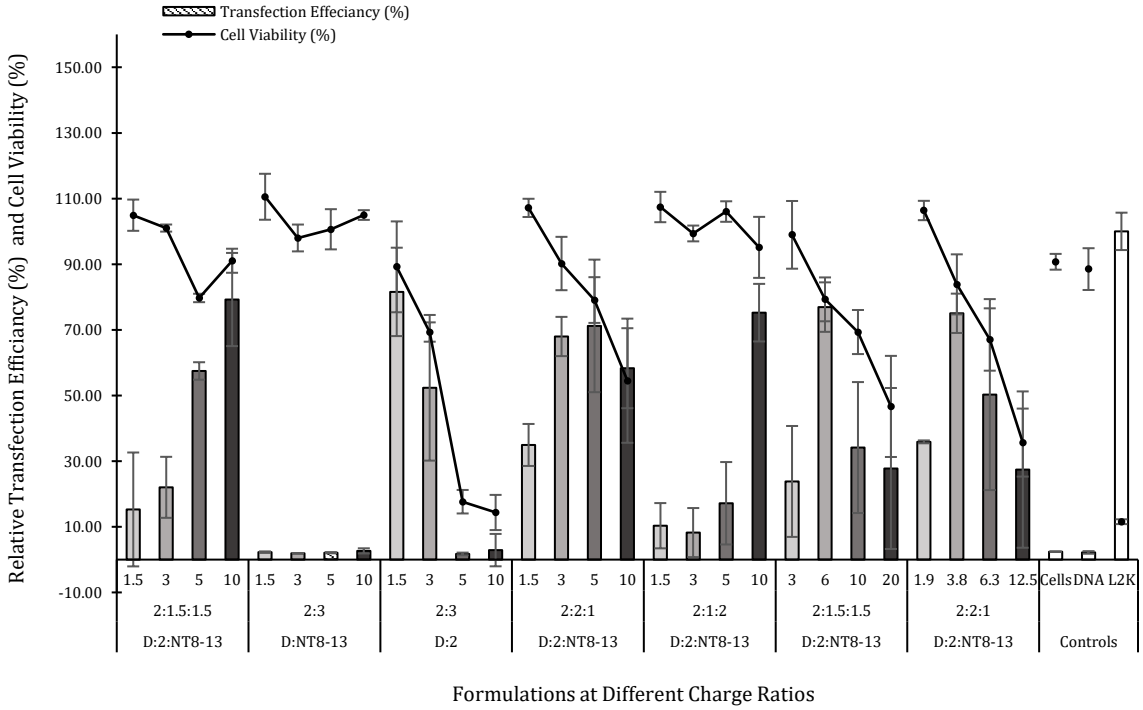

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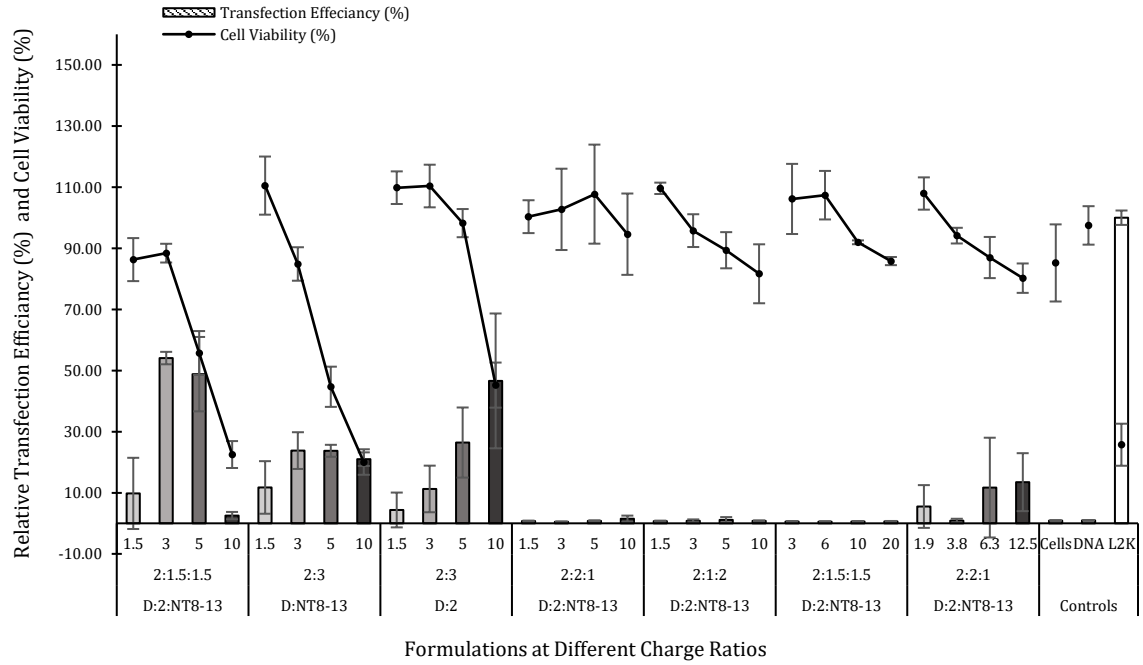

Figure 11. Relative transfection efficiencies (bars) and relative cell viabilities (graphs) for all formulations at 4 different charge ratios, which were all done in triplicates.

Relative Cell Viability

Besides transfection, cell viability is a very important factor. If a formulation shows great transfection, but is toxic to the cells, it is not useful for gene delivery. Cell viability was measured in parallel with transfection, on a different type of plate, using a MTS assay. Absorbance was normalised to untreated cells (**Figure 11**). Comparisons between 11.A, 11.B and 11.C should be made with caution, as the experiments were not performed on the same day and on different batches of cells. Therefore, cells may act differently, which was indeed demonstrated by BCA assays, which showed different protein contents from one experiment to another (results not shown). A general trend was that cell viability decreased with increasing charge ratio. This was especially observed when transfection was observed and especially for formulations with a higher molar ratio of cationic lipid.

Formulations with neurotensin or NT^{8-13}, without cationic lipid, showed a relatively high (~100%-120%) cell viability for all charge ratios. No binding, protection or transfection was observed for these formulations, which indicate that these formulations did not form complexes and entered the cell. Furthermore, the presence of components of these formulations outside the cells are not toxic.

Formulations with lipid **2**, without peptide, had the lowest cell viability at 18% (**figure 11.A**) or 14% (**figure 11.B**) except for control Lipofectamine2000, which also had a cell viability of 14%. This indicates that high concentrations of lipid might be toxic to cells.

In general, formulations containing more cationic lipid than peptide exhibited cell viabilities dependent on charge ratio. Cell viabilities of formulations with equimolar amounts of peptide and cationic lipid also exhibited this dependency to some extend. Formulations with more peptide than cationic lipid **2** did not exhibit the same dependency.

The formulations 2:1.5:1.5 D:**2**:NT^{8-13} and 2:1:2 D:**2**:NT showed an increased cell viability at charge ratio 10, of 91% and 89%, respectively. These formulations exhibited high transfection, but without decrease in cell viability.

Some formulations were repeated, but showed different results (**Figure 11.C**). Here, all formulation that showed transfection also exhibited a steep decrease in cell viability with increasing transfection efficiency.

DISCUSSION

Several considerations were necessary when designing the lipopeptide **4**. It was decided to use only the NT^{8-13} fragment instead of full length neurotensin. The reasoning behind this was that only the amino acids 8-13 are important for receptor binding. The linkage and hydrophobic moiety was also considered. The first idea was to link two fatty acid tails directly on a lysine moiety, which had been added to the *N*-terminal of the peptide. However, a certain resemblance with lipid **1** and **2** was desired for comparison of transfection efficiency and cell viability. Two different designs for precursor lipid **3** were attempted (**Figure 5**). The first design was based on a glycerolipid with an alcohol moiety as the headgroup, which could be functionalised to a carboxylic acid by reaction with succinic anhydride. However, the product and starting materials were inseparable. This could cause an issue as the alcohol moiety on the starting material could potentially undergo esterification by reaction with the carboxylic acid moiety of the product under the same conditions used for amide bond formation with the *N*-terminal of NT^{8-13}. The second design, lipid **3**, could easily be separated from by-products and starting material and was used for further synthesis.

Lipopeptide **4** was synthesised by amide bond formation between lipid **3** and NT^{8-13}. Before the synthesis was carried out, the reaction conditions needed consideration. First consideration was whether to carry out the reaction in solution or using the solid phase approach. The reaction was done as a solid phase synthesis, but could have been carried out in solution. Solution synthesis would have required different orthogonal protection of the guanidine groups of arginine. On the other hand, cleavage conditions required for solid phase synthesis would not have been a concern. When coupling to NT^{8-13} the coupling and cleaving conditions could interfere with the ester groups on lipid **3**. During coupling reaction, NMM was used, and during cleavage TFA was used. Both of these reagents could potentially catalyse hydrolysis of the ester groups on lipid **3**.

Two different coupling reagents were tried out. HBTU with NMM, and DIC with HOBt. HBTU/NMM is a classic coupling solution for normal peptide synthesis. The DIC/HOBt combination is often used for *N*-terminal modifications. Both reactions had high crude yields, however HBTU/NMM reached completion slower than DIC/HOBt. Purification of the lipopeptide **4** (afforded with HBTU/NMM) was attempted, but not completed due to time limitation and the fact that no preparative C8 column was available. The

chromatogram obtained with an analytical C8 column showed base separation for most peak and could therefore indicate that purification could be achieved. However, not all peaks showed base separation, so this depends on which peak is the product peak. It could be assumed that the last peak could be the lipopeptide **4** as this peak would arise from the most hydrophobic compound, which could be the hydrophobic carbon chains attached to lipopeptide **4** (APPENDIX I: Structural Analysis Data).

The molar and charge concentrations are of great importance for transfection and cell viability assays. We aimed to use a 2:3 molar concentration ratio of DOPE:cationic component, while varying the cationic lipid:peptide ratios between formulations. The ratios were adjusted to give a 2 mM concentration based on positive charge, except for the two formulations that were made based on molar concentrations only. Formulations were made by preparing ethanolic stock solutions of all components, mixing the components in chosen ratios, removing ethanol with a rotary evaporator and hydrating the resulting thin films with sterile H_2O, yielding liposomes of different sizes as evident by DLS. Upon adding sterile H_2O, some formulations were cloudy, but turned clear after sonication. Cloudy solutions, i.e. solution in which lipoplexes aggregate, are not suitable for gene delivery. Different sizes were expected as no attempt was made to form specific sizes or separate complexes based on sizes. Due to a large distribution of particle sizes it is difficult to make any correlations between transfection efficiency and particle size. Particle size should however be considered for future studies as lipoplex size could have great importance for transfection. Particle sizing were performed 24 hours after initial hydration and for most of the formulations aggregates and larger particles were observed. This was not observed on the day of formulation.

Complexation between *p*DNA and liposome was studied using agarose gel electrophoresis. Three different outcomes could be observed for the different molar and charge ratios: complete binding, no binding or gradual binding. No binding was observed for formulations with peptides neurotensin or NT^{8-13} without cationic lipid. As the peptides are unable to bind *p*DNA, they must be unable to carry *p*DNA into the cell, which was confirmed by transfection assays. It has been hypothesised that it is electrostatic forces driving the complexation between *p*DNA and liposome, however other factors may account for the observed results. The peptide NT^{8-13} has three times more positive charge per molecule compared to the cationic lipid, but does not complex with *p*DNA. Perhaps

peptides were unable to form liposomes on their own, and therefore could not form suitable complexes with *p*DNA. This emphasizes that the special amphiphilic properties of lipids are necessary for *p*DNA binding.

Lipid **2** formulated without peptide exhibited complete binding for all chosen charge ratios. In some cases, *p*DNA was not observed in the gel nor in the wells for some of the higher charge ratios. Lipid could potentially pack *p*DNA efficiently enough as to make *p*DNA unavailable for the intercalating fluorescent tag, ethidium bromide. The strong association between lipid and *p*DNA could be a concern if the complexation was so strong that *p*DNA would not be able to dissociate from the complex, which is necessary for subsequent expression. Transfection was only observed at the lower charge ratios. This could indicate that *p*DNA indeed could not escape the endosome or be released from the lipoplex at higher charge ratios. However, low cell viability was also observed for the larger charge ratios, and the low transfection could be a consequence of this.

A compromise between complete binding and no binding is needed for good transfection, hence gradual binding was more promising. Combinations of peptide and cationic lipid all exhibited gradual binding. Therefore, these were expected to yield competitive transfection efficiencies.

The first five formulations tested in the last binding gel (**Figure 9.C**) was repeats from the two first binding gels (**Figure 9.A and B**). The first three result were consistent with earlier results, however the middle two shows considerable less binding than earlier experiments. These, and the formulation with lipopeptide **4** and cationic lipid **2,** were prepared from another ethanolic stock solution of cationic lipid **2** than the first three formulations. After preparing the formulations, this stock solution was tested with NMR, which showed no signal. This could indicate that there had been less material in the flask, and the weight had been measured wrong, which would result in formulations with less lipid. If this is the case, less or no transfection would occur, which was indeed observed.

Another agarose electrophoresis experiment was performed to assess the ability of the formulations to protect *p*DNA from degrading enzymes. Prior to running the gel, each lipoplex was treated with DNase I. The resulting protection gels showed some correlations with the binding. When a formulation showed no binding, it did not offer *p*DNA protection either. However, opposite the binding, gradual increasing protection

with charge ratio was not observed for all formulations. Lipid **2** with DOPE, together with the formulation 2:1.5:1.5 D:**2**:NT, showed maximum protection at charge ratio 3. This could be attributed to incomplete disruption of lipoplex at higher charge ratios by the 5% SDS used stop DNase I. Smear was observed on the degradation gel, which could indicate that only partial protection was achieved. However, this could also be an effect of SDS.

All formulations in the last assay, tested on the last gel, showed no or almost no protection. This is in accordance with the binding gel, which showed no or almost no binding. Furthermore, the *p*DNA strands for this gel were not clear for any formulations and therefore nothing conclusive can be extracted from these results (**Figure 10.C**).

Transfection efficiency was measured based on the activity of enzyme β-galactosidase. It should be kept in mind that this is not a direct measure of the ability of a lipoplex to enter the cell. Transfection efficiency and cell viability was measured in parallel on two different 96 well plates. Lipofectamine2000 was used as a positive control and for normalisation of RLU values. Cells treated with naked *p*DNA and untreated cells were used as negative controls.

The peptides neurotensin and NT^{8-13} formulated without cationic lipid, proved inefficient as gene carriers. This was evident from these formulations inability to bind and protect *p*DNA, and especially their lack of transfection efficiency. However, these formulations did not exhibit cytotoxicity either. Cationic lipid **2** formulated without peptide exhibited both competitive transfection and cell viability at lower charge ratio, but neither at higher charge ratios. The fact that peptides were not toxic at high charge ratios, but cationic lipid **2** was, indicates that toxicity is not dependent on charge ratio, but rather on cationic lipid concentration. The low transfection at higher charge ratios for lipid **2** could merely be a consequence of this. Previous studies also report cytotoxicity at high doses for some lipids (Behr *et al.*, 1989; Goldring *et al.*, 2012). The lipid concentration can be lowered by exchanging some of the lipid molecules with neurotensin or NT^{8-13}. Dependent on the formulations, this can be done without considerable loss of transfection and improved cell viability.

When comparing the transfection efficiency of different formulations, it was evident that formulation matters. An interesting feature was the different formulations with more, less or equal amounts of neurotensin or NT^{8-13}, and cationic lipid had different optimal

charge ratios. When more peptide than cationic lipid was used, higher charge ratios were optimal for transfection, compared to using no or less neurotensin or NT^{8-13}. The formulations with neurotensin and NT^{8-13} contained less lipid, but without loss of transfection efficiency. Furthermore, formulations with neurotensin or NT^{8-13} in general exhibited higher cell viabilities. In some cases, these showed more than 100% cell viability. This was observed for formulations with neurotensin or NT^{8-13} only, but high cell viability was also observed for formulations with more or equal amounts of neurotensin or NT^{8-13} compared to cationic lipid. The formulations 2:1.5:1.5 D:**2**:NT and D:**2**:NT^{8-13}, and 2:1:2 D:**2**:NT and D:**2**:NT^{8-13} are especially interesting as they did not exhibit an unambiguous decrease in cell viability with increasing charge ratio and achieved high transfection efficiencies. This indicates that these formulations contain enough lipid to mediate transfection, but not enough to induce cytotoxicity.

Most formulations were made to meet a comparable molar charge ratio of 1.5, 3, 5 and 10. However since NT^{8-13} has three times more charge than neurotensin, these formulations would need less cationic lipid to reach the charge ratio. Therefore 2 formulations were made based on molar concentrations. In these formulations, no consideration was given to the final charge concentration, but was exclusively made with the same concentrations for NT^{8-13}, lipid, and DOPE as the corresponding neurotensin, lipid and DOPE formulations. This resulted in higher optimal charge ratios, with comparable transfection efficiency and cell viability for the 2:2:1 D:**2**:NT and D:**2**:NT^{8-13} formulations, except at charge ratio 12.5 which had a significantly lower transfection. This could indicate that the amount of lipid, peptide or both plays an important role, at charge ratios around 1.5-6.8, but at higher charge ratios (10-12.5) the importance of charge ratios is not negligible. The molar formulation 2:1.5:1.5 D:**2**:NT^{8-13} with charge ratios of 3, 6, 10 and 20 has transfection max at charge ratio 6, but an increase at charge ratio 20.

Lipofectamine2000 had the highest transfection of the results, but also had a low cell viability. Interestingly, when looking at formulations without peptide, these had both low transfection and cell viability at charge ratio 10. This could indicate that lipid **2** gets into the cell, but that *p*DNA fails to escape the endosome and therefore is not expressed.

The lipopeptide **4** used in bioassays was crude, hence the concentrations employed are not the actual concentrations used. When formulated with lipid **2** the formulation showed

no *p*DNA binding, protection, nor transfection. However, cell viability was relatively high. When using lipopeptide **4** without cationic lipid, a gradual, increasing *p*DNA binding was observed with increasing charge ratio. Transfection at charge ratio 10 was 13 %, and was the only charge ratio that exhibited any transfection, with a corresponding cell viability of 87%. However, these results are mostly inconclusive, as the results from this assay in general deviated from previous results. The cells were usually treated 24 hours subsequent to seeding. For this assay the cells had only reached ~50% confluence after 24 hours and were left for additional 6 hours to reach ~80% confluence before treatment with lipoplex. These cells should probably have been discarded, but as they were only in passage 11 it was decided to use them regardless. Furthermore, BCA results was approximately half compared to the other assays.

Lipopeptide **4** and NT^{8-13} were both constructed with an amidated *C*-terminus. The carboxyl end has been emphasised as an important moiety for receptor binding (Tate *et al.*, 2012). The amidated *C*-terminus could potentially lower binding properties, and thereby lower transfection. This was not an issue for neurotensin, as this was purchased from Bachem without amidation. The transfection efficiency for formulations based on NT^{8-13} were in general slightly lower than for those based on neurotensin, but whether this is due to the truncated *N*-terminus, or the amidated *C*-terminus is too speculative at this stage.

The bioassays need to be optimised as cell count is not measured before or after an experiment. An attempt was made to account for the number of cells/well, using BCA. However, this was not done on the same plate as the transfection assays, and was therefore not used to normalise transfection. It was evident from the BCA assays that cell count could vary a lot from day to day, and some measurement is therefore needed to account for this. This was especially evident for the last assay performed and illustrated in figure 11.C, which had much lower or no transfection at all, even for formulations that had shown promising results in previous assays. More experiments need to be conducted to investigate this difference, and to optimise reproducibility. Furthermore, some measure of cell count should be performed prior to treatment to test if an equal number of cells is treated.

CONCLUSION

This study set out to investigate the effect of using combinations of cationic lipid and peptide as gene carriers. Furthermore, the effect of physically conjugating peptide fragment to lipid prior to transfection was of great interest as this could influence receptor binding and lipoplex uptake.

In respect to physically conjugating lipid and peptide, the effects are inconclusive in this study, and further investigation is needed.

Lipid **1** showed mixed results dependent on the formulation. When formulated in molar ratio 2:2:1 D:**1**:NT competitive transfection and cell viability was observed and optimal charge ratio was 10. However when formulating 2:1:2 D:**1**:NT no transfection was observed. Cationic lipid formulated without peptide, was cytotoxic to cells at high doses. Peptides, formulated without lipids, were not able to carry *p*DNA into the cell. However, combinations of peptides and lipids showed promising results in respect to cell viability, which was higher, and with competitive transfection efficiencies compared to Lipofectamine2000. This was especially observed for the formulations 2:1.5:1.5 D:**2**:NT and D:**2**:$NT^{8\text{-}13}$, and 2:1:2 D:**2**:NT and D:**2**:$NT^{8\text{-}13}$. Even though more investigation is needed, these formulations are promising and indicates that peptides can indeed be exchanges for cationic lipid to achieve higher cell viability, without loss of transfection efficiency.

FUTURE PERSPECTIVES

A lot more investigation is needed to elucidate the mechanisms of lipofection and especially targeted lipofection. The structure-activity relationship between the structural features of the cationic lipids, co-lipids and peptides also need further investigation to elucidate the potential effects of this.

The third transfection and cell viability assay needs to be repeated, with purified lipopeptide **4**, and good cells, to get a better perception of the efficacy of lipopeptide **4**. Furthermore, it could be interesting to yield a neurotensin and lipopeptide **4** with a carboxyl *C*- terminal, and compare if receptor binding would be more efficient for these.

Furthermore, the assays used in this study needs optimisation in respect of cell count, but also the number of lipoplex particles could be accounted for, and how these particles are arranged. Are neurotensin and NT^{8-13} in fact associated with lipoplex? And are charge equally distributed amongst the particles in solution?

This work could be continued in a endless number of directions. An interesting direction could be to make an experimental set-up using a cancer cell line, expressing NTS1. Silenced or knock-out NTS1 could be achieved and transfection efficacy and cell viability could be investigated in parallel with wild-type cells in order to elucidate whether the receptor plays a role in cellular uptake or not.

EXPERIMENTAL

pMCV-lacZ reporter vector gene (*p*DNA) containing the gene for E. Coli enzyme Beta-galactosidase, with eukaryotic translation initiation signals, was provided by Josefine Olsson and Marietta Gugerel. *p*DNA and lipoplex was diluted in optimum buffer (ThermoFischer Scientific Gibco Life Technologies).

Proton NMR spectra were recorded on a Bruker Avance-III HD (400 MHz) Spectrometer. All samples were measured in $CDCl_3$ (if nothing else is stated) and chemical shifts are referenced to residual solvent $CHCl_3$ ($\delta_H = 7.26$) and quoted in ppm. Multiplicity of peaks was designated s:singlet, d:doublet, t:triplet, q:quartet and m:multiplet.

Thin layer chromatography (TLC) was used to monitor reactions, using aluminium plates coated with Merck silica gel 60 F_{254} and visualised with UV and/or Potassium Permanganate. Flash chromatograph on Sigma Aldrich Silica gel 60 or Sigma Aldrich Dowex 50 WX4-50 was used to purify cationic lipids and precursors.

2-dimethylamino-1,3-propanediol

2-Amino-1,3-propanediol (Serinol) (3.57 g, 3.22 mmol, 1 eq) was added to formic acid (98 % w/w in H_2O, 9.2 mL) and formaldehyde (37% w/w in H_2O, 7.6 mL) at 0˚C. The reaction mixture was heated to 80˚C and stirred under reflux for 24 hrs. The reaction mixture was cooled to rt, and then concentrated *in vacuo*. The crude residue was purified by flash column chromatography on silica using 25% v/v methanol in dichloromethane (DCM) as eluent. Fractions containing product were combined, concentrated *in vaccuo*, and then purified using Dowex 50 WX4-50 resin and eluted with 11 % v/v methanol in 32 % v/v ammonia in H_20 to afford the dimethylamine as a colourless oil. δ_H (400 MHz, $CDCl_3$) 3.67 (4H, d, J 6.0, 2xC*H*$_2$), 2.63 (1H, m, NC*H*), 2.39 (8H, m, 2xC*H*$_3$ and 2xO*H*); δ_C (100 MHz, $CDCl_3$) 65.7, 60.2, 41.5.

4-(2,3-Bis(undec-10-enoyloxy)propoxy)-*N*-(1,3-dihydroxypropan-2-yl)-*N*,*N*-dimethyl-4-oxobutan-1-aminium bromide (Lipid 1)

2-dimethylamino-1,2-propanediol (21.4 mg, 179.9 mmol, 1 eq) was added to the bromo-lipid **5** (94.6 mg, 164.9 mmol, 1 eq.) in a 5 mL round bottom flask. The reaction mixture was heated at 90 ˚C in a sandbath for 67 hrs in a sealed flask. The crude residue was purified by flash column chromatography on silica, using 10% v/v methanol in DCM as eluent to give lipid **1** (5.9mg, 5%) as a colourless solid. δ_H (400 MHz, $CDCl_3$) 5.08 (2H, ddt,

J 6.8, 10.2 and 17.0, 2xCH$_2$=C*H*), 5.29-5.23 (1H, m, OC*H*), 5.01-4.90 (4H, m, 2xC*H*$_2$=CH), 4.85 (2H, s, 2xO*H*), 433-4.28 (2H, m, 2xOC*H*H), 4.25-4.19 (4H, m, 2xC*H*$_2$OH), 4.18-4.11 (2H, m, 2xOCH*H*), 3.80-3.76 (1H, m, NCH), 3.73-3.68 (2H, m, NCH$_2$), 3.35 (6H, s, N(CH$_3$)$_2$), 2.55-2.51 (2H, m, C*H*$_2$COO), 2.34-2.29 (4H, m, 2xC*H*$_2$COO), 2.18-2.11 (2H, m C*H*$_2$CH$_2$COO), 2.06-2.01 (4H, m, 2xC*H*$_2$CHCH$_2$), 1.64-1.56 (4H, m, 2xC*H*$_2$), 1.40-1.24 (20H, m, 2x(CH$_2$)$_5$); δ_C (100 MHz, CDCl$_3$) 173.4, 173.1, 171.8, 139.2, 114.2, 73.3, 68.7, 64.3, 63.0, 62.1, 57.9, 51.2, 34.2, 34.1, 33.8, 30.1, 29.33, 29.31, 29.15, 29.11, 29.08, 28.9, 24.9, 24.85, 18.2.

3-((4-Bromobutanoyl)oxy)propane-1,2-diyl diundecanoate (6, Figure 6)

Glacial acetic acid (2.32 mL, 0.3M) and palladium on carbon (23.53 mg, 0.221 mmol, 1 eq) were added to a solution of the diene **5** (399.3 mg, 0.696 mmol, 3.1 eq) in ethanol (23 mL, 0.03M) and then stirred under a hydrogen atmosphere pressure at rt for 5 hrs. The reaction mixture was filtered through celite, and then rinsed with ethanol (20 mL). The filtrate was concentrated *in vacuo* and the crude residue was purified by flash column chromatography on silica with 5% ethyl acetate in petroleum ether as eluent to give a mixture of bromo lipid **6** and over reduced by-product (433.22 mg, 108%) as a clear oil. δ_H (400 MHz, CDCl$_3$) 5.29-5.23 (1H, m, OC*H*), 4.30 (2H, td, *J* 4.3 and 11.6 2xOC*H*H), 4.17-4.6 (2H, m, OCH*H*), 3.45 (2H, t, *J* 6.4, C*H*$_2$Br), 2.52 (2H, t, *J* 7.5, C*H*$_2$COO), 2.31 (2H, t, *J* 7.5, C*H*$_2$COO), 2.30 (2H, t, *J* 7.5, C*H*$_2$COO), 2.16 (2H, quin, *J* 6.8 C*H*$_2$CH$_2$Br) 1.67-1.50 (4H, m, 2xCH$_2$), 1.32-1.20 (28H, m, 2x(CH$_2$)$_7$), 0.86 (6H, t, J 7.09, CH$_3$).

4-(2,3-Bis(undecanoyloxy)propoxy)-N-(1,3-dihydroxypropan-2-yl)-N,N-dimethyl-4-oxobutan-1-aminium bromide (Lipid 2)

2-Dimethylamino-1,3-propanediol (68.7 mg, 0.577 mmol, 1 eq) was added to the saturated bromo-lipid **6** (432.2 mg, 0.748 mmol, 1 eq) and then heated at 90 ˚C for 67 hrs. The product was purified twice by flash column chromatography on silica with 5% ethyl acetate in petroleum ether followed by 10% methanol in DCM as eluent to give the diol **2** (33.1 mg, 8.2%) as a grey solid. δ_H (400 MHz, CDCl$_3$) 5.31-5.25 (1H, m, OC*H*), 4.83 (2H, s, 2xO*H*), 4.35-4.28 (2H, m, 2xOC*H*H), 4.25-4.21 (4H, m, 2xC*H*$_2$OH), 4.18-4.12 (2H, m, 2xOCH*H*), 3.79-3.75 (1H, m, NCH), 3.73-3.69 (2H, m, NCH$_2$), 3.36 (6H, s, N(CH$_3$)$_2$), 2.55-2.50 (2H, m, C*H*$_2$COO), 2.35-2.29 (4H, m, 2xC*H*$_2$COO), 2.18-2.11 (2H, m C*H*$_2$CH$_2$COO), 2.00-1.94 (4H, m, 2xC*H*$_2$CH=CH$_2$), 1.65-1.57 (4H, m, 2xC*H*$_2$), 1.32-1.26 (24H, m, 2x(CH$_2$)$_6$), 0.88 (6H, t, *J* 6.9, CH$_2$C*H*$_3$).

Benzyl 4-bromobutanoate (8, *Figure 7*)

DCC (1669.1 mg, 8.089 mmol, 1.39 eq,) and DMAP (199.9 mg, 1.096 mmol, 0.19 eq) were sequentially added to a solution of 4-bromobutyric acid (1008.4 mg, 6.038 mmol, 1.04 eq.) in dry DCM (60 mL) at 0 ˚C, and stirred for 30 min. Benzylalkohol (0.6 mL) was added and the solution was stirred at rt for 18 hrs, under N_2 atmosphere. The crude residue was purified twice by flash column chromatography on silica using 20% ethyl acetate in petroleum ether as eluent to give the bromobutanoate **8** (615.9 mg, 43.6%). δ_H (400 MHz, $CDCl_3$) 7.40-7.31 (5H, m, Ar*H*$_5$), 5.14 (2H, s, ArC*H*$_2$), 3.46 (2H, t, *J* 6.5, C*H*$_2$Br), 2.56 (2H, t, *J* 7.2, C*H*$_2$COO), 2.20 (2H, quin, *J* 6.8, C*H*$_2$CH$_2$Br).

Benzyl 4-(dimethylamino)butanoate (9, *Figure 7*)

Dimethylamine (2M in THF, 16.7 eq, 20 mL) was added to benzyl 4-bromobutanoate **8** (0.616 mg, 2.40 mmol, 1 eq) and stirred at rt for 88 hrs in a sealed flask. The crude residue was decanted, concentrated *in vaccuo* and purified by flash column chromatography using 20% v/v methanol in DCM as eluent. Fractions containing product was pooled and concentrated *in vaccuo*, and washed with a saturated $NaHCO_3$ solution in H_2O in a separating funnel, and the lower phase dried with $MgSO_4$. The solution was filtered and concentrated to yield the dimethylaminobutanoate **9** (472.1 mg, 89%) as an orange viscous oil. δ_H (400 MHz, $CDCl_3$) 7.36-7.29 (5H, m, Ar*H*$_5$), 5.11 (2H, s, ArC*H*$_2$), 4.76 (6H, s, N(C*H*$_3$)$_2$), 2.39 (2H, t, *J* 7.5, C*H*$_2$COO), 2.27 (2H, t, *J* 7.3, C*H*$_2$N), 1.80 (2H, quin, *J* 7.4, C*H*$_2$CH$_2$N).

4-(Benzyloxy)-N-(4-(2,3-bis(undec-10-enoyloxy)propoxy)-4-oxobutyl)-N,N-dimethyl-4-oxobutan-1-aminium bromide (10, *Figure 7*)

Benzyl-4-(dimethylamino)-butanoate **9** (472.1 mg, 2.133 mmol, 1 eq) was added to lipid bromide **5** (1223.1 mg, 2.132 mmol, 0.99 eq). The reaction mixture was heated at 90 ˚C for 48 hrs. The crude solution was reduced and purified by flash column chromatography with 25% methanol in DCM as eluent to give the benzyl lipid bromide **10** (1653.9 mg, 68.3%) as a yellow oil. δ_H (400 MHz, $CDCl_3$) 7.39-7.33 (5H, m, Ar*H*$_5$), 5.80 (2H, ddt, *J* 6.7, 10.2 and 17.1, 2xCH$_2$=C*H*), 5.30-5.25 (1H, m, OC*H*), 5.13 (2H, s, C*H*$_2$Ar), 5.02-4.91 (4H, m, 2xC*H*$_2$=CH), 4.34-4.27 (2H, m, 2xOCH*H*), 4.17-4.10 (2H, m, 2xOC*H*H), 3.69-3.64 (4H, m, 2xNC*H*$_2$), 3.40 (6H, s, N(CH$_3$)$_2$), 2.60-2.50 (4H, m, 2xC*H*$_2$COO), 2.35-2.29 (4H, m, 2xC*H*$_2$COO), 2.15-2.00 (8H, m, 2xC*H*$_2$CH$_2$COO and CH$_2$CHC*H*$_2$) 1.63-1.57 (4H, m, 2xC*H*$_2$), 1.39-1.29 (20H, m, 2(C*H*$_2$)$_5$); δ_C (100 MHz, $CDCl_3$) 173.4, 173.3, 173.0, 171.8, 171.7, 139.1,

135.4, 128.7, 128.5, 128.43, 128.40, 114.1, 68.6, 66.9, 66.8, 63.1, 62.7, 62.6, 61.9, 61.6, 57.1, 51.4, 43.1, 34.2, 34.0, 33.8, 30.9, 30.0, 29.8, 29.31, 29.29, 29.2, 29.09, 29.06, 28.9, 24.9, 24.8, 18.0, 17.8.

4-((4-(2,3-bis(undec-10-enoyloxy)propoxy)-4-oxobutyl)dimethylammonio)butanoate (Lipid 3)

Absolute ethanol (99.9% 0.03M 67 mL) and glacial acetic acid (0.3M 6.7 mL) was sequentially added to benzyl lipid bromide **10** (1653.9 mg, 2.08 mmol, 1 eq.). Pd/C 10% w/w (0.9 mg, 0.0085 mmol, 1 eq.) was carefully added and the flask blown with N_2. The reaction was stirred at rt for 6 hrs, under a hydrogen atmosphere. The reaction mixture was filtered over celite and the filtrate was reduced *in vacuo* to give the product (1146.2 mg, 73.3%) as an orange tinted solid. δ_H(400 MHz, CD_3OD) 5.5-5.46 (1H, m, OC*H*), 4.59-4.53 (2H, m, 2xOC*H*H), 4.39-4.33 (2H, m, 2xOCH*H*), 3.58-3.54 (4H, m, $N(CH_2)_2$), 3.49 (2H, quin, NCH_2CH_2COO, *J* 1.64), 3.31 (6H, s, $N(CH_3)_2$), 2.71-2.59 (4H, m, 2xCH_2COO), 2.53-2.49 (4H, m, 2xCH_2COO), 2.30-2.19 (4H, m, 2xCH_2CH_2COO), 1.83-1.74 (4H, m, 2xCH_2), 1.52-1.47 (24H, 2x$(CH_2)_6$), 1.09-1.06 (6H, m, 2xCH_3); δ_C (100 MHz, CD_3OD) 173.4, 173.1, 171.8, 69.1, 63.1, 62.6, 62.5, 61.9, 50.1, 33.6, 33.4, 33.4, 31.6, 29.4, 29.3, 29.27, 29.24, 29.1, 28.8, 28.76, 24.64, 24.61, 22.3, 17.9, 17.5, 13.1.

NT^{8-13} fragment

Peptide fragment was synthesised on Intavis Peptide Synthesiser using standard solid phase Fmoc based chemistry. 3x20.0 mg Rink Amide Resin was swelled in 2 mL DMF for 30 min. The swelled resin was transferred to a microcolumn and inserted into Intavis. All necessary amino acids were weighed out and dissolved in DMF according to Intavis protocol. Proline was dissolved in 4-Methylmopholine (NMM). All necessary solvent was added according to Intavis protocol.

Microcolumns were drained and dried for 10 min. 500 µL cleavage mixture (95% v/v Trifluoroacetic acid, 2.5% v/v H_2O and 2,5% v/v TIPS) was added and incubated at rt, shaking for 30 min. This was repeated 3 times. Cleavage mix was collected solvent was evaporated with N_2 gas. The crude was dissolved in 5 mL 20% v/v acetonitrile in H_2O and freeze dried. The crude was purified on Äkta Pure GE Healthcare Life Sciences or Copenhagen University by Natalia Molchanova, using reverse phase chromatography (RPC) on a Gemini® C18 column, 110Å, 5 µm, 4.6x250 mm. and verified with analytical

HPLC and Maldi-TOF or HPLC-MS. All amino acids used was bought from Sigma Aldrich or Novabiochem (33 mg, 80.3%)

Lipopeptide 4

NT^{8-13} fragment was synthesised as above, but not cleaved from resin. HBTU (105.5 mg, 0.278 mmol, 4.5 eq) in dimethylformamide (DMF), NMM in DMF (134 µL 45%), NMP (12 µL), and Lipid **3** in DMF (172.1 mg, 0.243 mmol, 3.9 eq, 486 µL) was mixed and added to the resin. The reaction was shaked for 12 hrs at rt. Kaiser test showed unreacted amines. HBTU (52.2 mg, 0.139 mmol, 2.25 eq) and NMM in DMF (45% 67 µL) was added, and the reaction was shaked at rt for additional 12 hrs. Kaiser test showed no free amines. The resin was washed with 3x200µL DMF, 3x200 µL methanol and 3x200 µL DCM, dried and cleaved from the resin using cleavage solution (95% v/v TFA, 2.5% v/v water and 2.5% v/v triisopropylsilane, 3 mL, 2x1.5 hrs). Solution was collected and solvent evaporated with N_2. The crude was solubilised in acetonitrile in water (10%) and freeze-dried O.N. (95.2 mg, 87 %). The crude was tested with HPLC-MS (UltiMate™ 3000 RSLCnano System from Thermo Scientific in series with LTQ Orbitrap Velos) to verify product formation by monoisotopic weight.

Lipopeptide **4** was also synthesised using by adding HOBt (38.2 mg, 0.249 mmol, 4.99 eq) and DIC (31.5 mg, 0.250 mmol, 4.99 eq) to lipid **3** (162.3 mg, 0.229 mmol, 4.58 eq). The reaction was carried out in 1.5 mL DMF and shaked at rt for 12 hrs. Kaiser test revealed no free amines. (81.6 mg, 92.4%). The crude was verified by measuring monoisotopic weight with HPLC-MS.

Purification of lipopeptide **4** was attempted on Äkta Pure GE Healthcare Life Sciences using reverse phase chromatography (RPC) on a Waters Spherisorb® C8 Column, 80Å, 5 µm, 4.6x150 mm (Flowrate 1 mL/min, gradient 20→95% ACN in H_2O, both with 5% TFA).

Liposome Formulations

1 mM ethanolic stock solution was made for each cationic lipid, NT^{8-13} fragment and lipopeptide **4**. 1 mM neurotensin (Bachem) stock solution was prepared in methanol in DCM (10%). 0.67 mM ethanolic stock solutions of DOPE (Avanti Polar Lipids LOT 181PE-319) and Cholesterol (Avanti Polar Lipids LOT CH-82) was prepared. Different formulations were prepared from the stock solutions based on either charge ratios or moles (APPENDIX 3: Concentration (µM) treated to Cells). Solvents were removed *in vacuo*

from the formulations to create a thin film, which was hydrated with sterile H_2O and incubated at rt for 15 min.

Preparations of Lipoplexes

The liposome formulations were diluted in sterile H_2O to 4 concentrations based on charge of 1.62 mM, 0.81 mM, 0.486 mM and 0.243 mM (or 2.03 mM, 1.01 mM, 0.61 mM, 0.30 mM for formulation 2:2:1 D:**2**:NT^{8-13} or 3.24mM, 1.62 mM, 0.97 mM, 0.49 mM for for formulation 2:1.5:1.5 D:**2**:NT^{8-13}). 100 µL of each was added to 100 µL 50 ng/µL *p*DNA in Gibco™ Opti-MEM™ I Reduced Serum Media buffer and incubated at rt. This results in 4 molar charge ratios for each formulation.

Liposome and Lipoplex Sizing

The hydrodynamic diameter (d_H) of 50-60 µL of lipoplex or liposome was measured with dynamic light scattering (DLS) using Malvern Panalytic Zetasizer Nano-S.

Cell Culture

CHO-K1 (Sigma Aldrich) cells were grown in T75 flask with RPMI medium (+ 25 mM HEPES, + L-Glutamine ThermoFischer Scientific Gibco™ Life Technologies. LOT 1868628) 10% FBS (ThermoFischer Scientific Gibco™ Life Technologies) and 1% Penicillin-streptomycin (Sigma Life Sciences. LOT 037M4877V), and incubated at 37 ˚C with 5% CO_2 atmosphere. Cells were only used in passage 8-20. Cells were split when reaching ~80% confluence using 10% trypsin (Sigma Life Sciences. LOT SLBH5909).

DNA binding and Protection

Gel assays were all done in a 1% agarose (Sigma Aldrich Life Technologies) in 1xTAE buffer (Invitrogen Life Technologies) in MQ water with 0.004% Ethidium Bromide using 105 V, 228 mA for 1 hour. Gels were run with 7.5 µL DNA ladder and 2 µL 6xloading buffer for 20 µL sample. 18 µL sample-loading buffer mixture was loaded onto gel. For the degradation gel assay the samples were incubated with 1 µL DNase I (Sigma Aldrich) for 1 hour at 37˚C followed by 30 min. incubation with 4 µL 5% SDS.

Lipoplex treatment of CHO-K1 cells

CHO-K1 cells were seeded in clear and opaque 96 well plates (Nunclon™ Delta Surface, Thermo Scientific. Roskilde Denmark. LOT 149768 (opaque), LOT 154496 (white) or Costar®, Corning Incorporated. Corning, NY 14831, USA. LOT 03917009) 24-30 hours

prior to transfection and should be ~70-80% confluent. Cells were washed with 100 µL PBS (Dulbecco's Phosphate Buffered Saline, - magnesium, - calcium. Sigma Aldrich Life Sciences. LOT RNBG3063) and transfected with 45 µL liposome formulation in triplicates and at 4 different charge ratios (+/-). Lipofectamine2000 (ThermoFischer Scientific) was used as a positive control. The negative controls were untreated cells and cells treated with naked *p*DNA. Cells were incubated at 37 ˚C, 5% CO_2 atmosphere for 4 hrs and subsequently washed with 100 µL PBS. 100 µL RPMI medium was added and cells were left for incubation at 37 ˚C, 5% CO_2 atmosphere for 44 hrs.

Beta-Glo® Assay

Cells were washed with 100 µL PBS and 50 µL Dulbecco's Modified Eagle Medium (DMEM) Phenol red free (ThermoFischer Scientific Gibco Life Technologies) was added to each well. Beta-Glo® Assay system (Promega Corporation, 2800 Woods Hollow Road, Madison, USA, LOT 0000264991) was prepared according to the manufactures protocol and 50 µL was added to each well. The plate was incubated at rt for 1 hr. and luminescence were measured with GloMax® 96 microplate luminometer (Promega), using the standard program Beta-Glo.

Cell Proliferation Assay

Cells were washed with 100 µL PBS. 50 µL DMEM phenol red free and 10 µL CellTiter 96® AQ_{ueous} One Solution (Promega Corporation, 2800 Woods Hollow Road, Madison, USA. LOT 0000244639) solution was added sequentially. The plate was gently shaken, and incubated at 37 ˚C for 1 hr. Absorbance was measured at 490 nm on plate reader Bio-TEK® Synergy HT.

BCA Protein Assay

Pierce™ BCA Protein Assay Kit (LOT SB245405) was used to measure protein content. After cell viability assay was performed, the cells were washed with 100 µL PBS. 10 µL 1X passive lysis buffer was added to each well and the plate was incubated at rt for 30 min. BCA working solution was prepared according to manufactures protocol and 200 µL was added to each well. The plate was covered and incubated at rt for 1 hr. Absorbance was measured at 562 nm. Absorbance was correlated to protein content using a bovine serum albumin standard curve.

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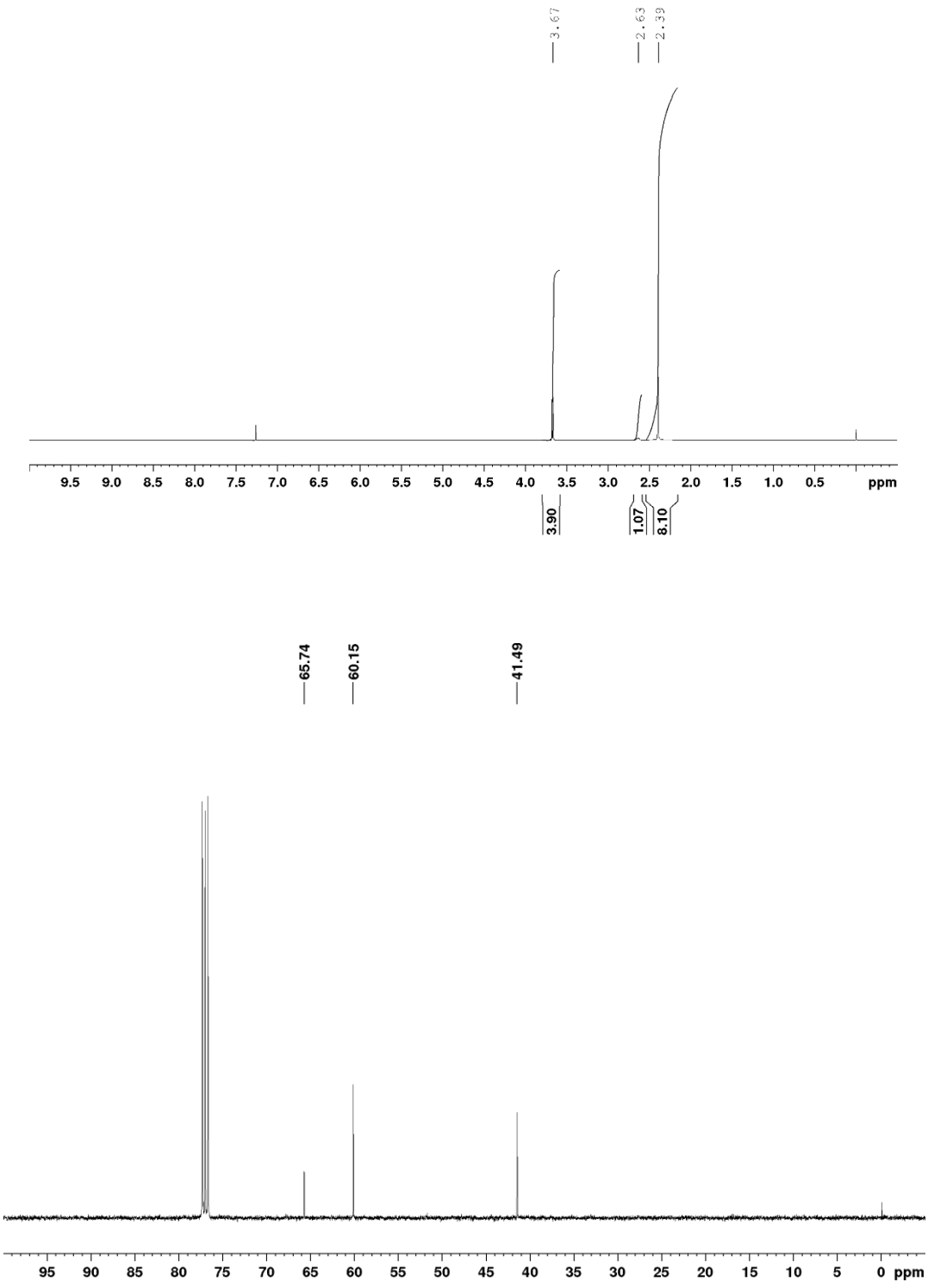
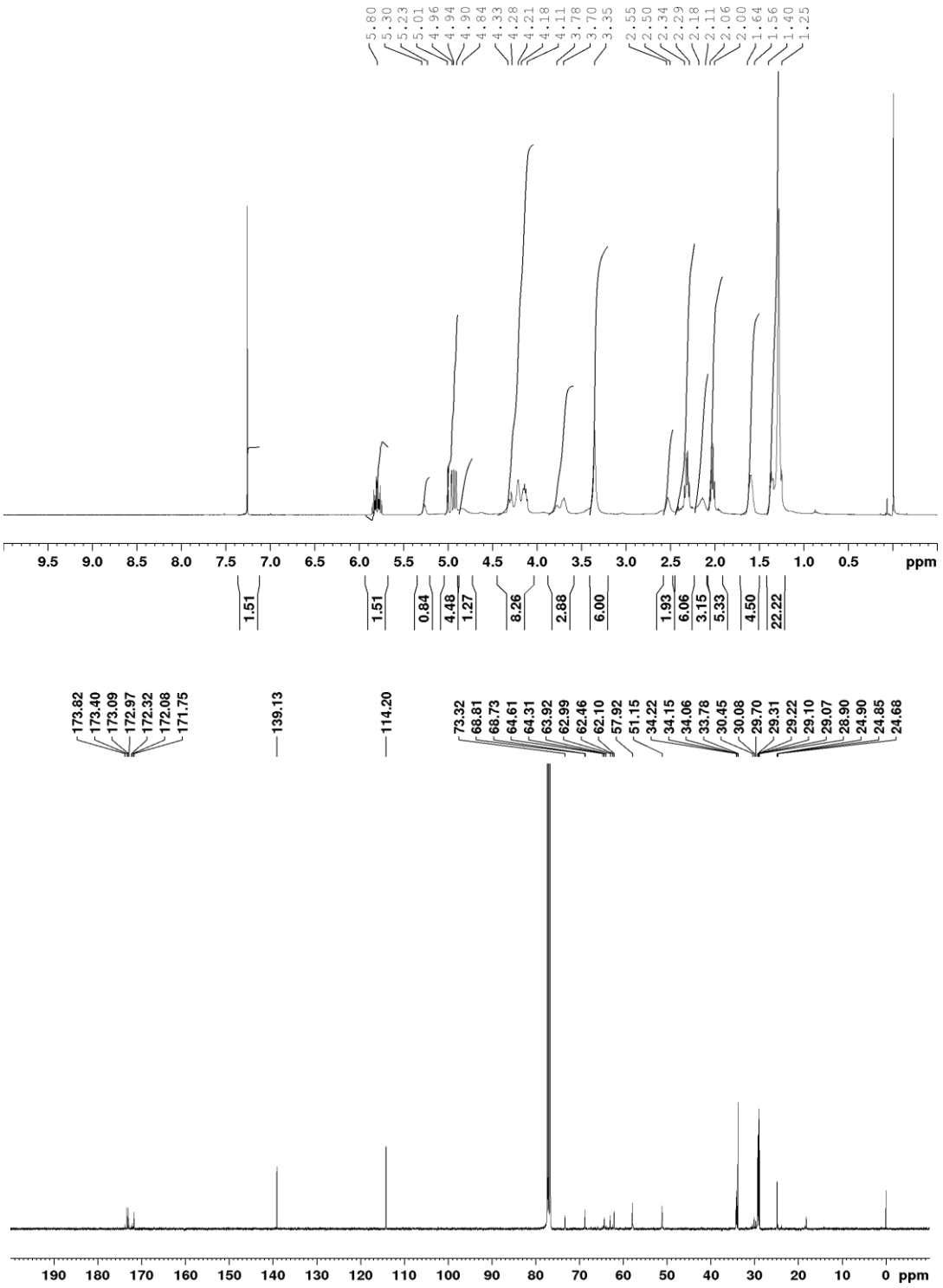
APPENDIX I: Structural Analysis Data

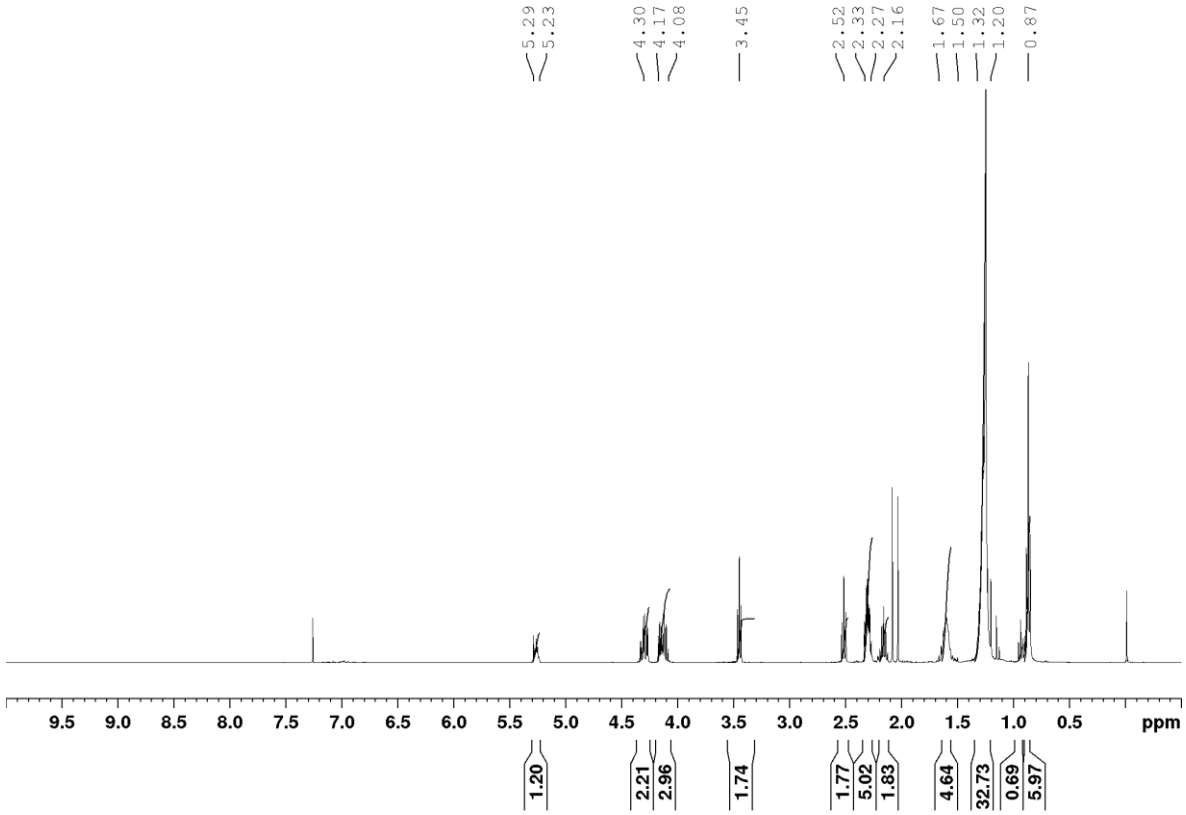
2-dimethylamino-1,3-propanediol



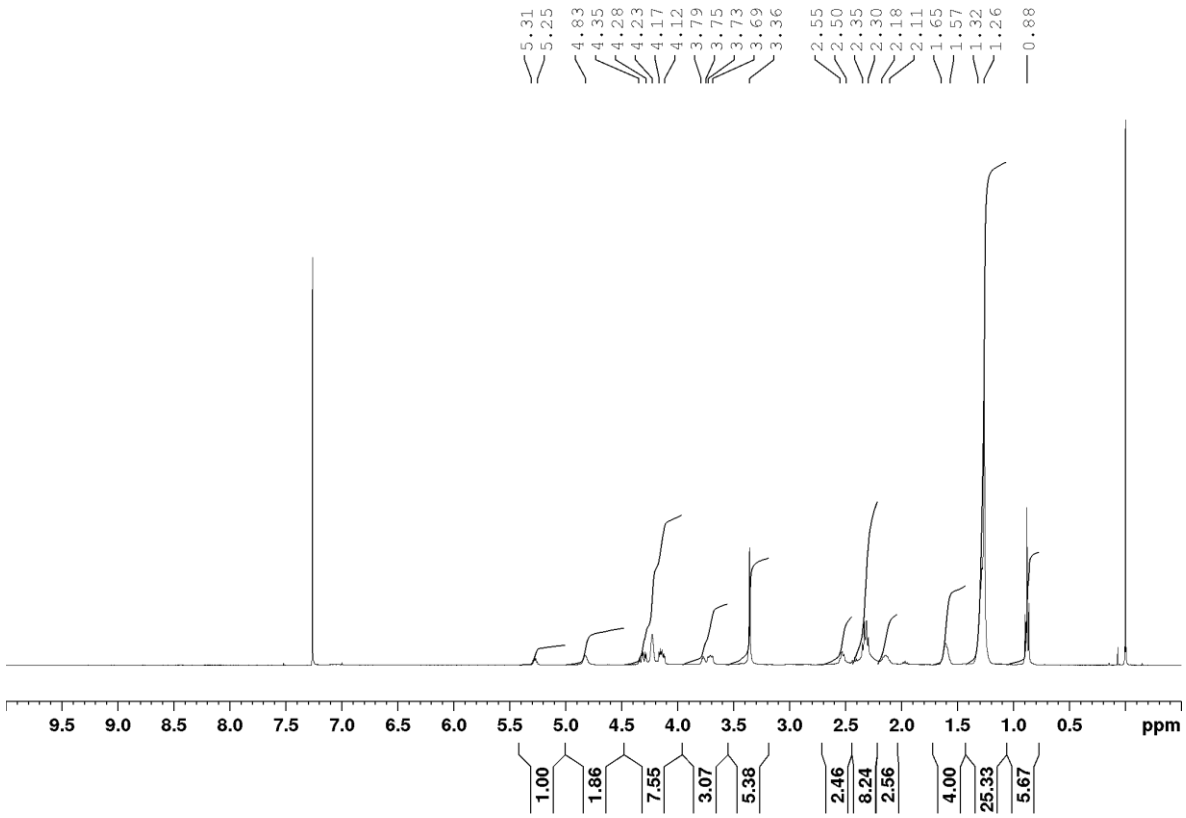
4-(2,3-Bis(undec-10-enoyloxy)propoxy)-N-(1,3-dihydroxypropan-2-yl)-N,N-dimethyl-4-oxobutan-1-aminium bromide (Lipid 1)



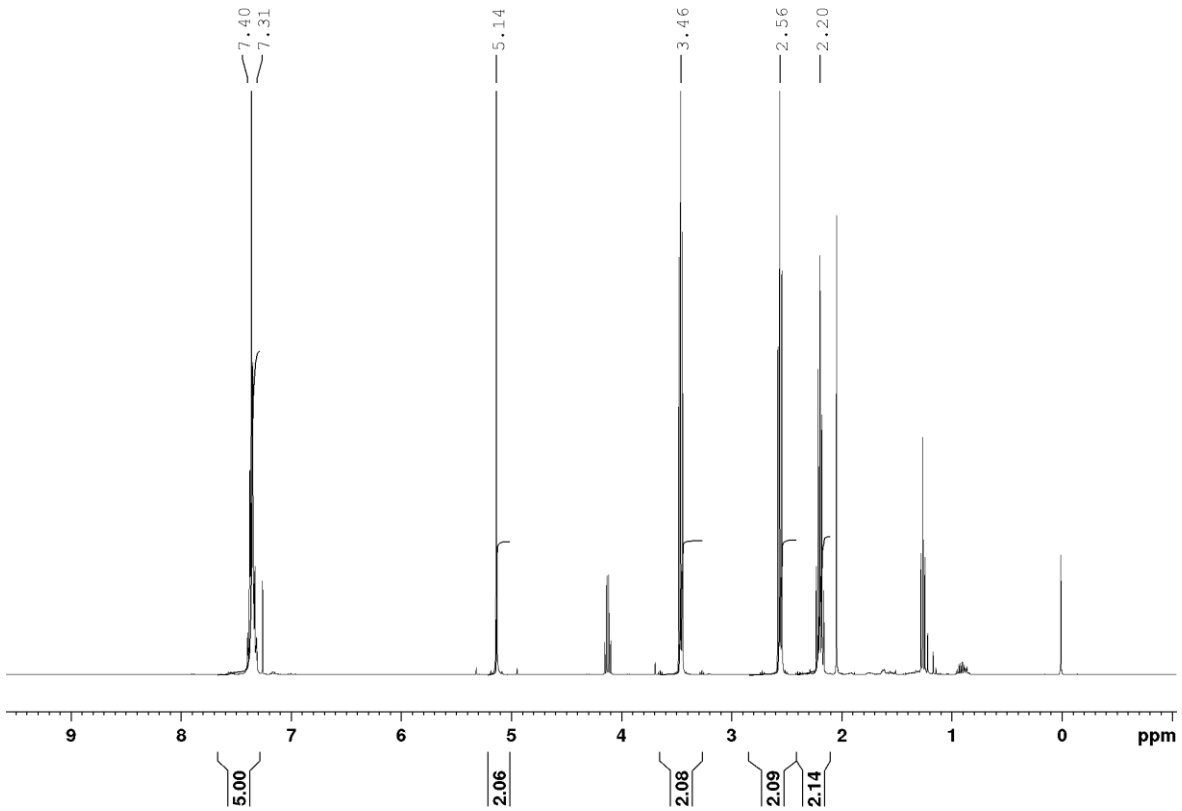
3-((4-Bromobutanoyl)oxy)propane-1,2-diyl diundecanoate (6, Figure 6)



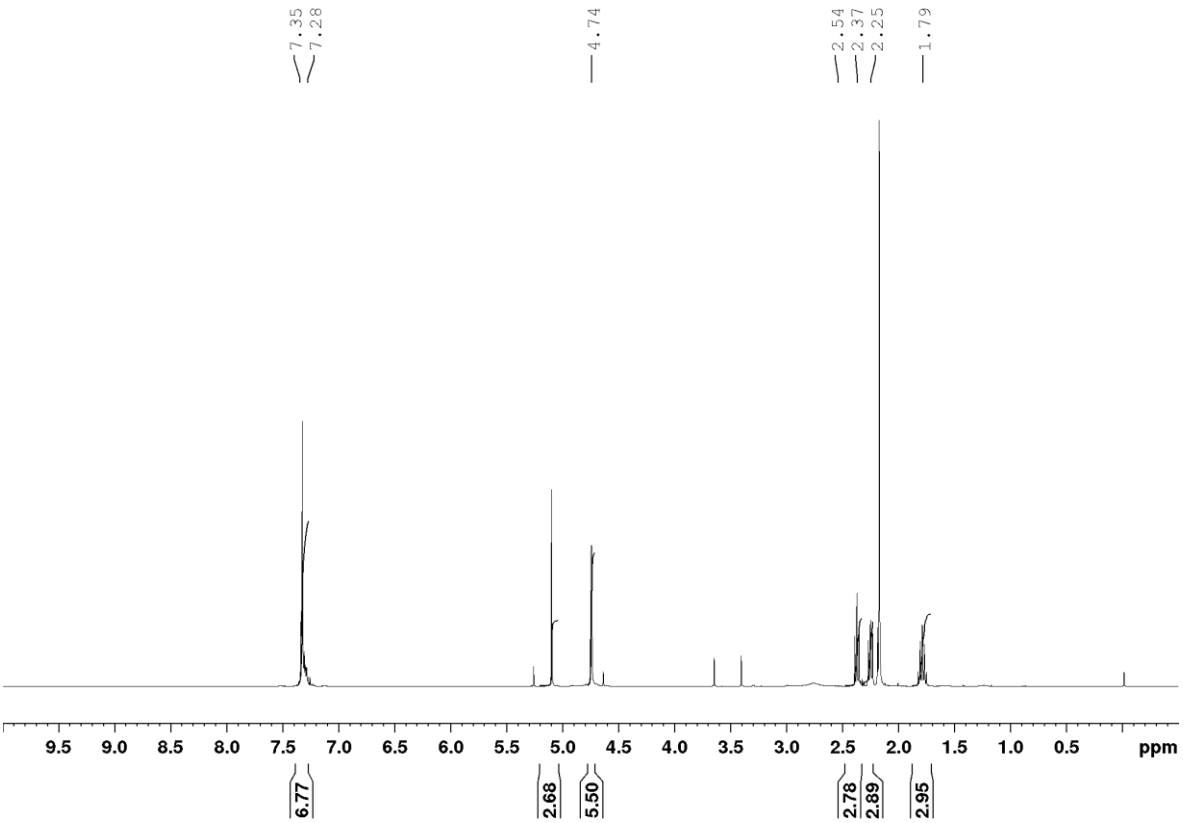
{4-[2,3-bis(undecanoyloxy)propoxy]-4-oxobutyl}(1,3-dihydroxypropan-2-yl)dimethylazanium bromide



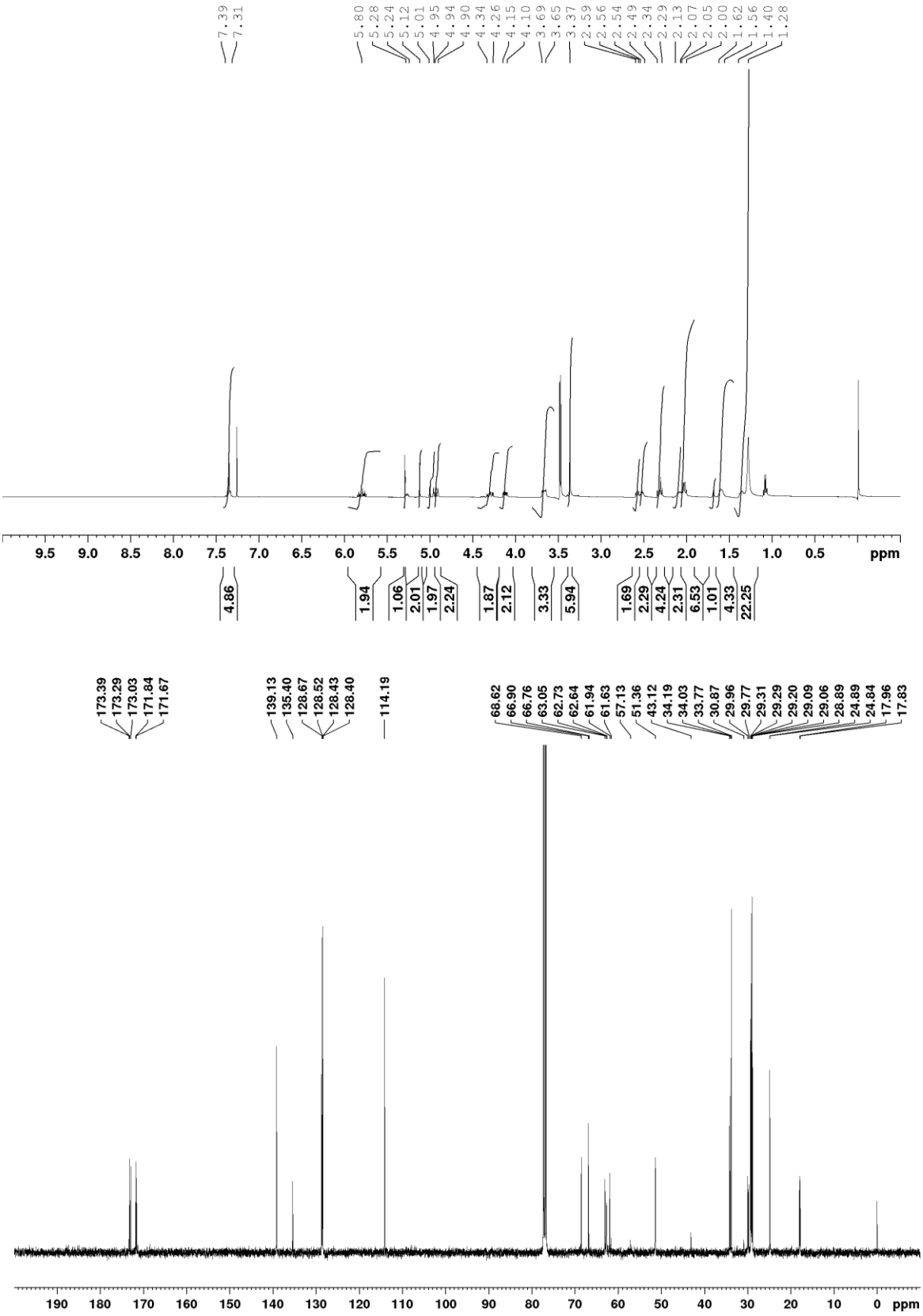
Benzyl 4-bromobutanoate (8, *Figure 7*)



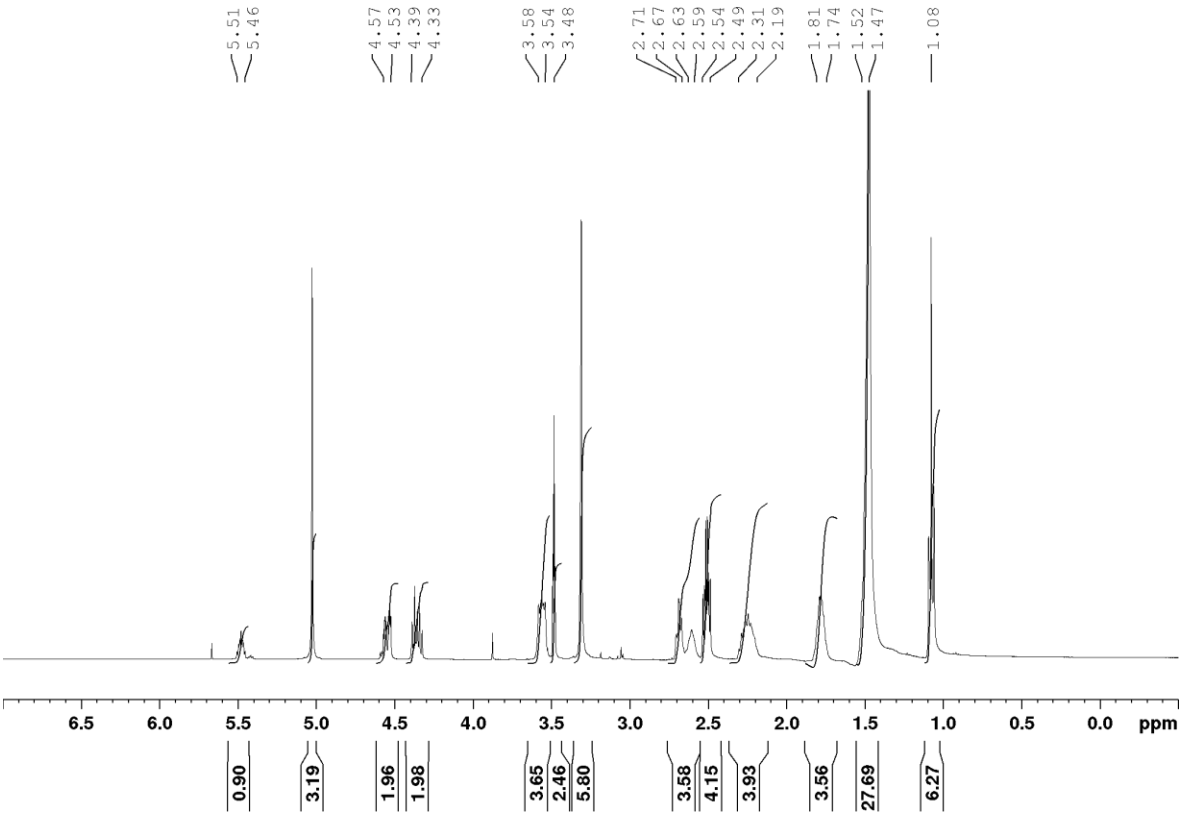
Benzyl 4-(dimethylamino)butanoate (9, *Figure 7*)

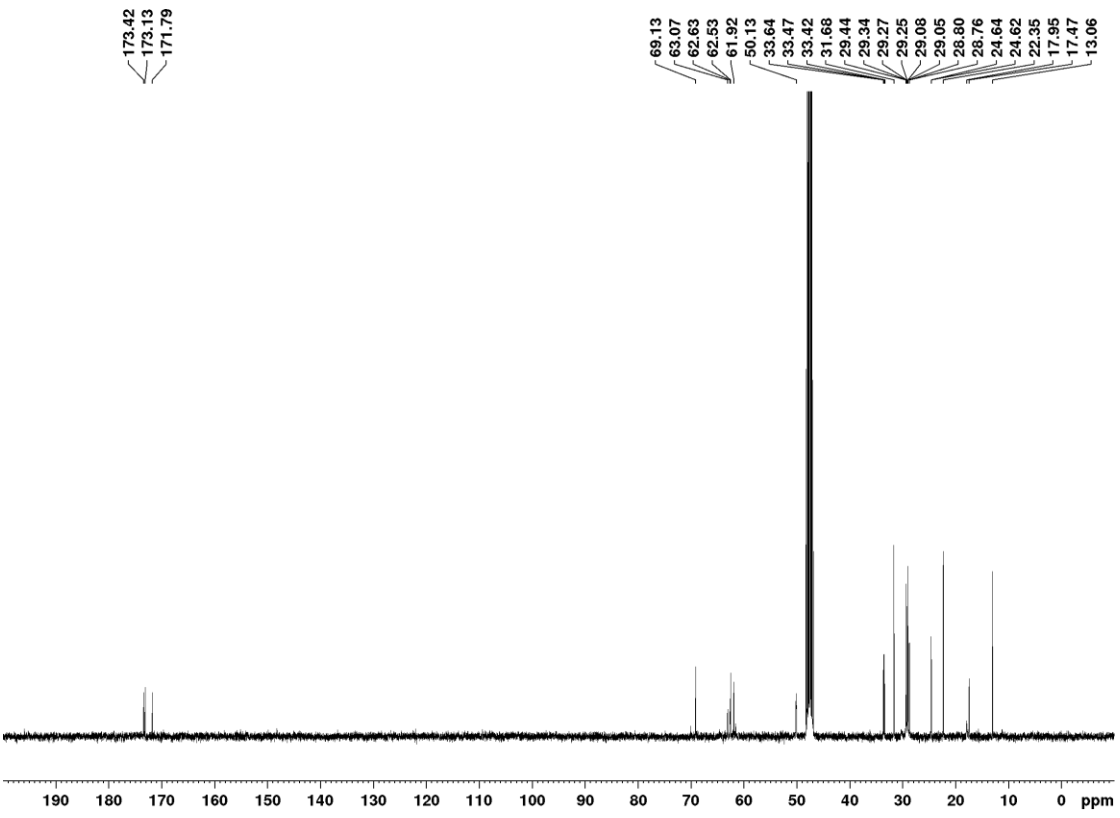


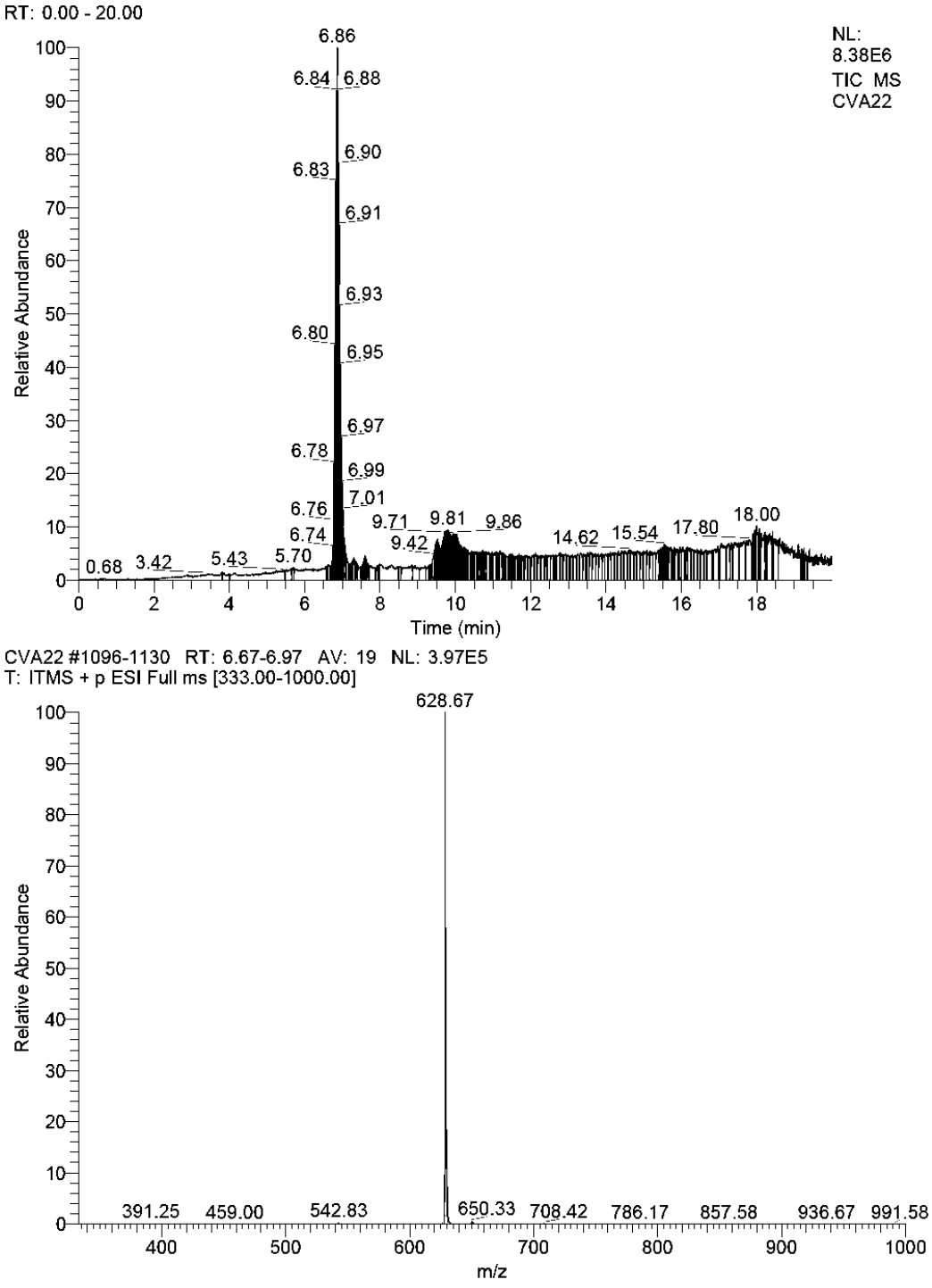
4-(Benzyloxy)-N-(4-(2,3-bis(undec-10-enoyloxy)propoxy)-4-oxobutyl)-N,N-dimethyl-4-oxobutan-1-aminium bromide (10, *Figure 7*)



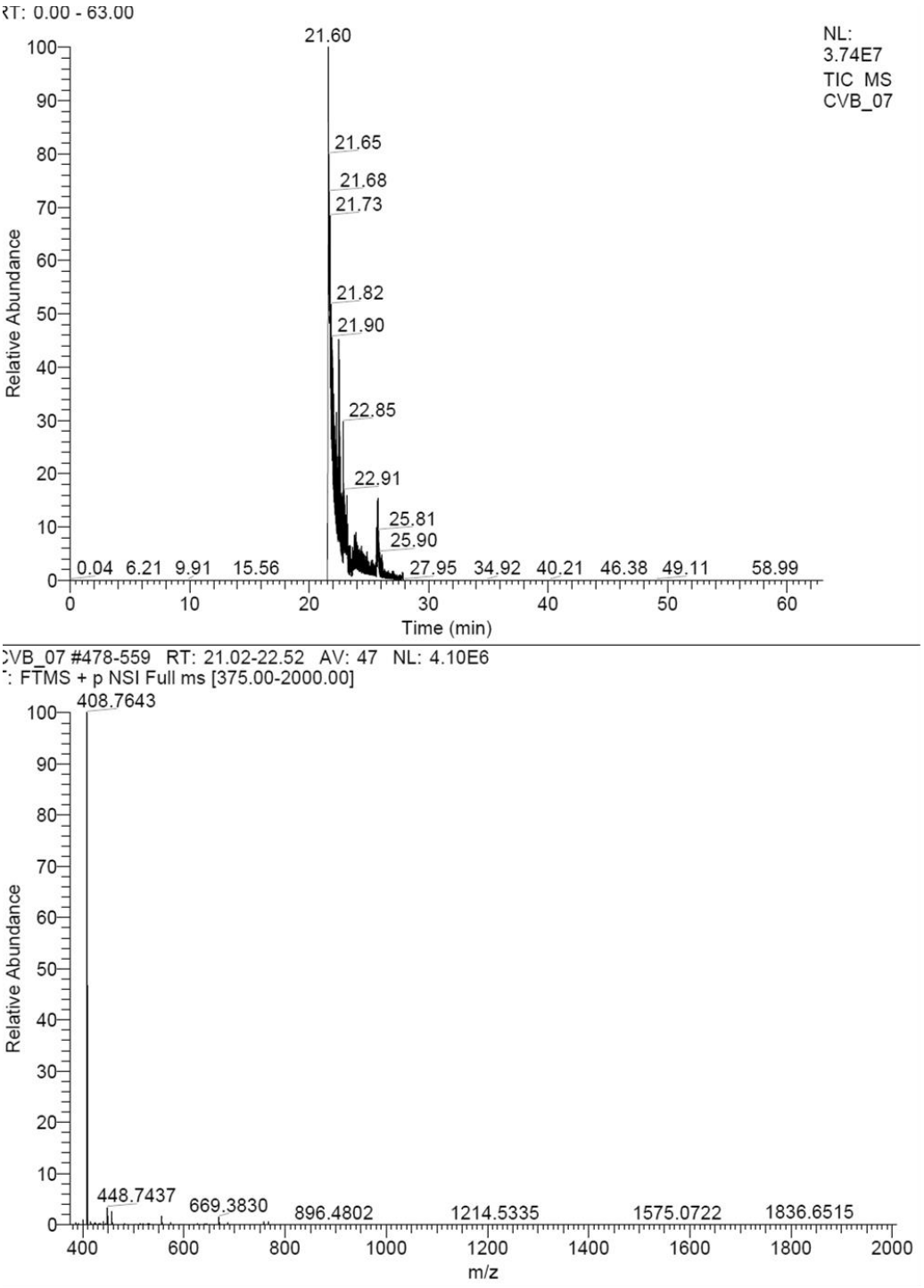
4-((4-(2,3-bis(undec-10-enoyloxy)propoxy)-4-oxobutyl)dimethylammonio)butanoate (Lipid 3)



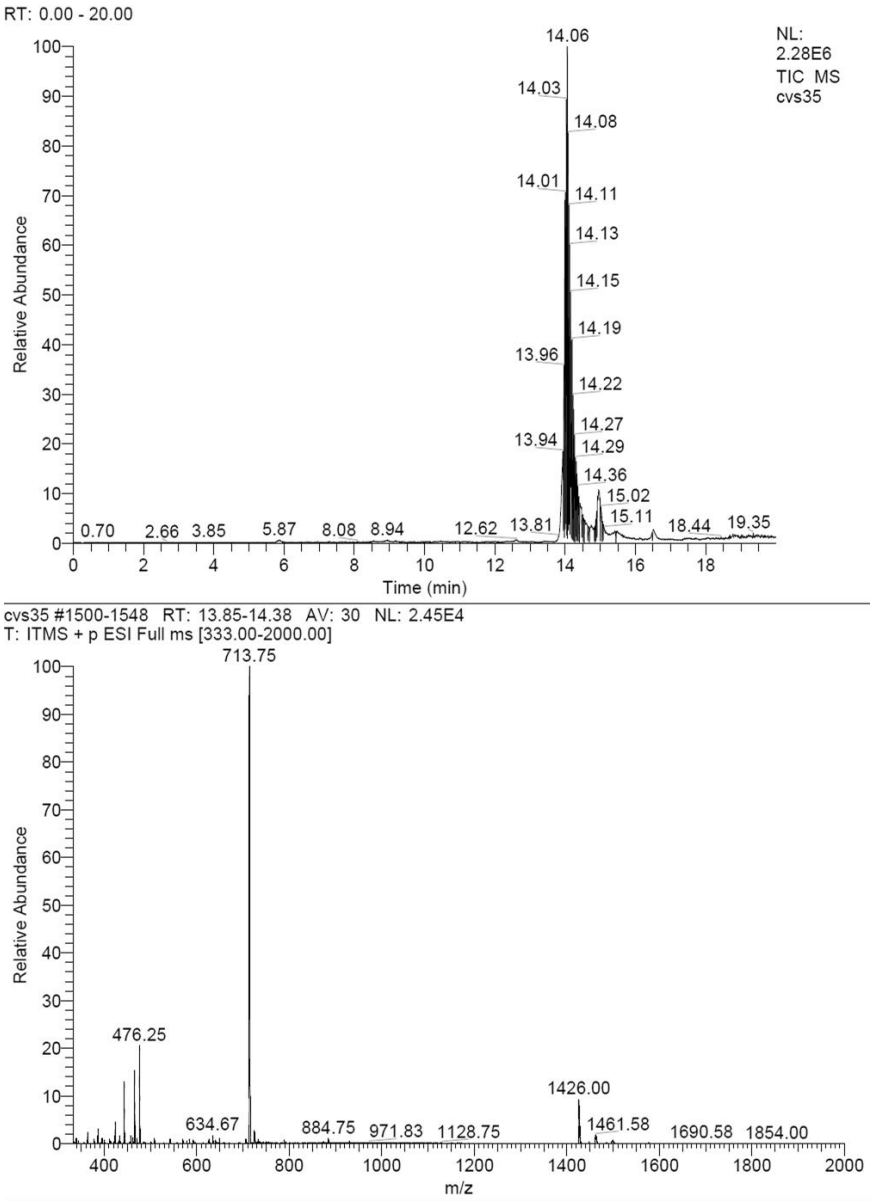



RT: 0.00 - 20.00
NL:
8.38E6
TIC MS
CVA22
Relative Abundance
Time (min)
6.86
6.84
6.88
6.83
6.90
6.91
6.93
6.80
6.95
6.97
6.78
6.99
7.01
6.76
6.74
9.71
9.81
9.86
9.42
14.62
15.54
17.80
18.00
0.68
3.42
5.43
5.70
CVA22 #1096-1130 RT: 6.67-6.97 AV: 19 NL: 3.97E5
T: ITMS + p ESI Full ms [333.00-1000.00]
628.67
391.25
459.00
542.83
650.33
708.42
786.17
857.58
936.67
991.58
m/z

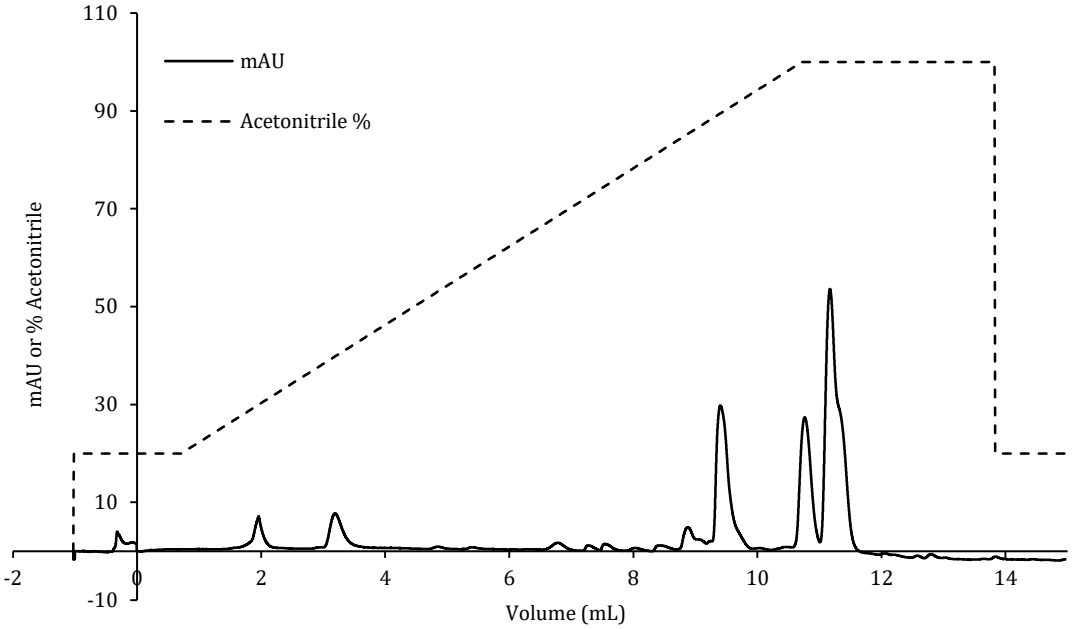
Neurotensin Fragment NT$^{8-13}$



Lipopeptide 4



Chromatogram: Lipopeptide **4**



APPENDIX 2: Paticle Sizing Data

	Liposome			Lipoplex		
Formulations	[+]	Z (nm)	PDI	[+/-]	Z (nm)	PDI
2:1.5:1.5	24.3	1167	0.547	1.5	1071	0.673
D:**2**:NT	48.6	1163	0.572	3	3345	0.332
	81.0	1004	0.507	5	6153	1
	162.0	1005	0.62	10	6986	1
2:3	24.3	1174	0.827	1.5	1888	0.912
D:NT	48.6	3099	0.612	3	642.3	0.607
	81.0	2290	0.967	5	629.8	0.97
	162.0	2691	0.786	10	803.4	0.486
2:3	24.3	1026	0.725	1.5	876.3	0.695
D:**2**	48.6	971.1	0.447	3	2817	0.425
	81.0	679.9	0.498	5	4309	0.586
	162.0	565.7	0.576	10	2043	0.488
2:2:1	24.3	1226	0.759	1.5	1462	0.681
D:**2**:NT	48.6	1250	0.489	3	3234	0.281
	81.0	1259	0.469	5	2256	0.948
	162.0	1181	0.454	10	2477	0.535
2:1:2	24.3	1111	0.619	1.5	804	0.771
D:**2**:NT	48.6	1040	0.481	3	1398	0.282
	81.0	895.7	0.584	5	8187	0.419
	162.0	821	0.622	10	2485	1
2:2:1	24.3	759.4	0.747	1.5	1105	0.802
D:**1**:NT	48.6	979.7	0.993	3	5005	0.39
	81.0	804.9	0.606	5	8164	0.344
	162.0	817.2	0.769	10	2314	1
2:1:2	24.3	1169	0.701	1.5	777.9	0.984
D:**1**:NT	48.6	1451	0.464	3	908.3	0.327
	81.0	1421	0.558	5	1202	0.25
	162.0	1784	0.661	10	1596	0.403
2:1.5:1.5	24.3	829.3	0.668	1.5	5513	0.779
D:**2**:NT[8-13]	48.6			3	3846	0.494
	81.0			5	1188	0.54
	162.0	416	0.356	10	625.3	0.369
2:3	24.3	1779	0.569	1.5	417.2	0.411
D:NT[8-13]	48.6			3	650.6	0.523
	81.0			5	1229	0.705
	162.0	402.7	1	10	425.4	0.848
2:3	24.3	1006	0.769	1.5	3589	0.729
D:**2**	48.6			3	4586	0.259
	81.0			5	3326	0.582
	162.0	484.8	0.475	10	3341	0.917
2:2:1	24.3	1075	0.528	1.5	799.9	0.818
D:**2**:NT[8-13]	48.6			3	2651	0.904

	81.0			5	3975	0.589
	162.0	585.9	0.746	10	7476	1
2:1:2	24.3	986.7	0.471	1.5	680.6	0.452
D:**2**:NT[8-13]	48.6			3	761.2	0.431
	81.0			5	1248	0.792
	162.0	480.3	0.589	10	3049	0.424
2:1.5:1.5	48.6	1019	0.515	3	1171	0.437
D:**2**:NT[8-13]	97.2			6	5798	0.307
	162			10	5621	0.652
	324	564.7	0.961	20	3788	0.473
2:2:1	30.375	1456	0.567	1.9	971.6	0.214
D:**2**:NT[8-13]	60.75			3.8	4126	0.835
	101.25			6.3	2897	0.93
	202.5	686.7	0.621	12.5	4681	0.451
2:3	24.3	163	0.477	1.5	5712	0.867
D:**2**	48.6	157.5	0.47	3	6028	0.59
	81.0	162.5	0.441	5	3282	0.978
	162.0	146.7	0.429	10	1377	1
2:1.5:1.5	24.3	1012	0.462	1.5	747	0.311
D:**2**:NT	48.6	1010	0.48	3	2579	0.859
	81.0	985	0.527	5	6458	0.68
	162.0	860.1	0.571	10	3613	0.545
2:1.5:1.5	24.3			1.5	1206	0.586
D:**2**:NT[8-13]	48.6	939	0.51	3	799.7	0.361
	81.0	808.3	0.597	5	4160	0.46
	162.0	533.2	0.619	10	6245	0.36
2:2:1	24.3	511.1	0.47	1.5	548.8	0.516
D:**2**:NT	48.6	524.5	0.495	3	548.8	0.516
	81.0	535	0.531	5	940.3	0.54
	162.0	561.6	0.561	10	1913	0.972
2:2:1	24.3	1368	0.294	1.5	329.4	0.577
D:**2**:NT[8-13]	48.6	1691	0.685	3	288.9	0.384
	81.0	1758	0.319	5	382.6	0.471
	162.0	1422	0.599	10	566	0.563
2:1.5:1.5	24.3	350	0.237	1.5	304.4	0.456
D:**2**:**4**	48.6	409.4	0.26	3	361.7	0.387
	81.0	417.2	0.245	5	364.8	0.263
	162.0	467.7	0.27	10	394.7	0.248
2:3	24.3	1183	0.626	1.5	221.7	0.511
D:**4**	48.6	1274	0.711	3	361.6	0.472
	81.0	1277	0.752	5	640	0.434
	162.0	975.8	0.651	10	922.2	0.318

APPENDIX 3: Concentration (μM) treated to Cells

Formulations	+/-	Lipid **1**	Lipid **2**	NT	NT^{8-13}	Lipopeptide **4**	DOPE
2:1.5:1.5	1.5		12.0	12.0			16.1
D:**2**:NT	3		24.0	24.0			32.2
	5		40.0	40.0			53.6
	10		129.6	129.6			173.7
2:3	1.5			24.0			16.1
D:NT	3			48.0			32.2
	5			80.0			53.6
	10			259.2			173.7
2:3	1.5		24.0				16.1
D:**2**	3		48.0				32.2
	5		80.0				53.6
	10		259.2				173.7
2:2:1	1.5		16.0	8.0			16.1
D:**2**:NT	3		31.9	16.0			32.2
	5		53.2	26.6			53.6
	10		172.4	86.2			173.7
2:1:2	1.5		8.0	16.0			16.1
D:**2**:NT	3		16.0	31.9			32.2
	5		26.6	53.2			53.6
	10		86.2	172.4			173.7
2:2:1	1.5	16.0		8.0			16.1
D:**1**:NT	3	31.9		16.0			32.2
	5	53.2		26.6			53.6
	10	172.4		86.2			173.7
2:1:2	1.5	8.0		16.0			16.1
D:**1**:NT	3	16.0		31.9			32.2
	5	26.6		53.2			53.6
	10	86.2		172.4			173.7
2:1.5:1.5	1.5		6.0		6.0		8.0
D:**2**:NT^{8-13}	3		12.0		12.0		16.1
	5		20.0		20.0		26.8
	10		64.8		64.8		86.8
2:3	1.5				8.0		5.3
D:NT^{8-13}	3				16.0		10.7
	5				26.6		17.8
	10				86.2		57.7
2:3	1.5		24.0				16.1
D:**2**	3		48.0				32.2
	5		80.0				53.6
	10		259.2				173.7
2:2:1	1.5		9.6		4.8		9.6
D:**2**:NT^{8-13}	3		19.2		9.6		19.3

	5		32.0		16.0		32.2
	10		103.7		51.8		104.2
2:1:2	1.5		3.4		6.8		6.9
D:**2**:NT[8-13]	3		6.8		13.7		13.7
	5		11.4		22.8		22.9
	10		36.9		73.9		74.2
2:1.5:1.5	3		12.0		12.0		16.1
D:**2**:NT[8-13]	6		24.0		24.0		32.2
	10		40.0		40.0		53.6
	20		129.6		129.6		173.7
2:2:1	1.9		12.0		6.0		12.1
D:**2**:NT[8-13]	3.8		24.0		12.0		24.1
	6.3		40.0		20.0		40.2
	12.5		129.6		64.8		130.2
2:3	1.5		24.0				16.1
D:**2**	3		48.0				32.2
	5		80.0				53.6
	10		259.2				173.7
2:1.5:1.5	1.5		12.0	12.0			16.1
D:**2**:NT	3		24.0	24.0			32.2
	5		40.0	40.0			53.6
	10		129.6	129.6			173.7
2:1.5:1.5	1.5		6.0		6.0		8.0
D:**2**:NT[8-13]	3		12.0		12.0		16.1
	5		20.0		20.0		26.8
	10		64.8		64.8		86.8
2:2:1	1.5		16.0	8.0			16.1
D:**2**:NT	3		31.9	16.0			32.2
	5		53.2	26.6			53.6
	10		172.4	86.2			173.7
2:2:1	1.5		9.6		5.0		9.6
D:**2**:NT[8-13]	3		19.2		10.0		19.3
	5		32.0		16.6		32.2
	10		103.7		53.8		104.2
2:1.5:1.5	1.5		6.8			6.8	9.2
D:**2**:**4**	3		13.7			13.7	18.3
	5		22.8			22.8	30.6
	10		73.9			73.9	99.0
2:3	1.5					9.6	6.4
D:**4**	3					19.2	12.9
	5					32.0	21.4
	10					103.7	69.5