Synthesis of Cationic Lipids

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Abstract

Gene therapy mediated through viral or non-viral carrier vectors has gained a lot of attention over the past several years. Specifically, cationic lipids used as non-viral vectors are currently being widely investigated as they present a safer alternative to viral vectors. They are relatively easy to synthesize and modify. Researchers have been synthesizing various cationic lipids, making alterations (changes?) to their structures and trying to find optimal molecules to carry DNA inside cells.

The aim of this project was to synthesize three cationic lipids with different headgroups in a series of reactions. This was done successfully, resulting in lipids **8**, **10** and **12** with dimethylamine, alcohol and diol headgroups respectively. These lipids could also undergo further modifications that could make them more stable and efficient transport vectors. However, only further analysis and experiments would show the efficiency of the lipids we were able to synthesize.

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Introduction

Genes, passed down from parents to their child, are what defines us. They contain the information necessary to synthesize functional proteins. If a problem appears in our genes, resulting in the synthesis of wrong or non-functional proteins, it is called a genetic disorder. This can either be inherited from parents or can be acquired with time. There is a method called gene therapy that has been widely researched in past years that could help treat genetic disorders by "fixing" the damaged genes inside the cells. This method is based on vectors that could carry a healthy copy of the damaged gene inside target cells that would then be transcribed into the right proteins. [1] The design of effective carrier vectors has therefore been gaining a lot of attention. Among the two classic groups, viral vectors have shown high transfection efficiency, but present problems with toxicity and immunogenicity. Because of this, non-viral vectors seem more appealing. [2] Even though they are less effective especially *in vivo*, they are relatively easy to produce and modify chemically and are safer. [3] Amongst these are peptides, polymers, dendrimers and cationic lipids. [4] The latter have especially shown success in forming complexes called lipoplexes with DNA, that can enter the cell, protect the DNA from degradation and release it inside. Many researchers have been synthesizing different structures of cationic lipids, in order to improve and optimize them and their binding to DNA. Based on these facts, our study is oriented towards the synthesis of three different cationic lipids, with a focus on the lipids' headgroups. Each of the three lipids synthesized contains two unsaturated hydrocarbon chains, a glycerol-based backbone and a different headgroup. We choose to synthesize the first lipid with a dimethylamine headgroup, the second with an addition of an alcohol and the third with an addition of a diol.

Background

Gene Therapy

Gene therapy represents an experimental technique that uses genes to treat or prevent disease. This could prove to be very useful for the treatment of inherited disorders, acquired diseases such as cancer, viral infections such as AIDS or certain autoimmune disorders. Gene therapy allows the delivery of specific genes inside cells thus targeting the problem at its genetic origin, representing an alternative to drugs and surgery. [5]

This technique can work in several ways. A healthy gene can be inserted inside a targeted cell and replace a gene that has mutated. Certain genes can be either activated or inactivated, depending on whether they promote or prevent disease. New genes could be introduced in cells that would help fight diseases. Also, disease cells could be made more evident to the immune system that would not otherwise recognise them. [6]

The two major methods used for the delivery of DNA into cells are the use of viral and non-viral vectors. The use of recombinant virus vectors is widely used as it has shown a high transfection efficiency, but the use of non-viral vectors has certain advantages, such as the fact that they are not targeted by the immune system.[2] The latter is therefore being widely researched and new delivery reagents are being found.

Viral Methods

There are two main ways to introduce DNA into recipient cells: viral and non-viral methods. Both methods are effective and have their strengths, but also weaknesses that require further modifications to make suitable vectors for gene transfer and delivery.

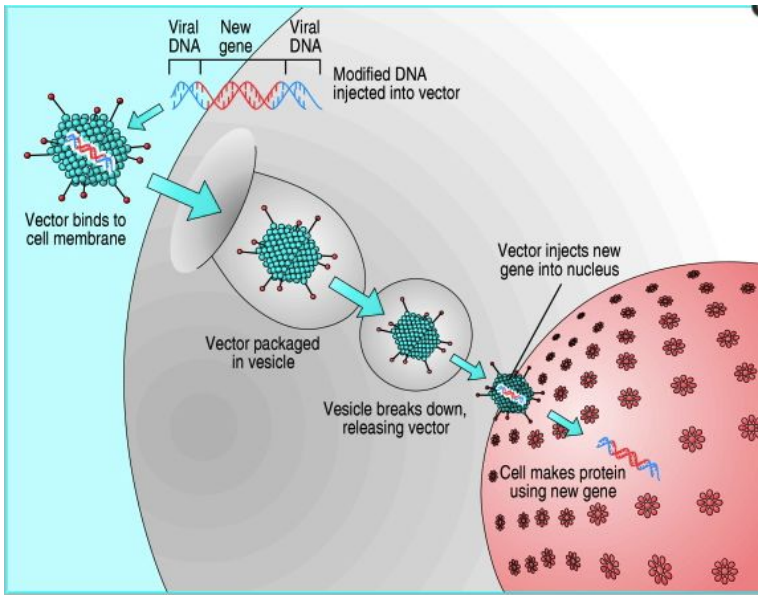


Figure 1. Gene transport mediated by a viral vector. [23]

Viral systems usually consist of the removal of genes coding for viral proteins from the viral genome, maintaining sequences required for viral replication within host cells. The virus is modified in a way that it contains packed therapeutic DNA, delivers it to the host cell and releases it (Figure 1). There are several classes of viruses that are mostly used in research, these include:

- **Retroviruses** are small RNA viruses that infect target cells through interaction between viral envelope protein and cell surface. The ability of the retrovirus to integrate DNA to the host genome allows the modification to be stable throughout the life of the cell.
- **Adenoviruses** has an ability to infect non dividing cells both in culture and *in vivo* resulting in high gene expression. However, the gene products of adenovirus synthesis stimulates an immune response and cells lose the inserted gene in 1-2 weeks after insertion. Therefore, it is not suitable for gene therapy in disorders that require long term gene expression.
- **Adeno-associated virus (AAV)** is a virus that needs a helper virus such as adenovirus or *Herpes Simplex* virus for replication. This virus is known to have no pathology by infection therefore it could be a great gene transfer vector. Although, it's application for research for human gene therapy needs to be better determined.

- ***Herpes simplex* virus (HSV)** is a linear double stranded DNA virus that can infect non dividing cells and is able to carry large DNA molecules to host cells. On the other hand, HSV based vectors are known to have high toxicity but by modifying the virus this could be avoided. [20]

As using viral methods happens to have many disadvantages such as virus toxicity or immune responses, non-viral gene therapy was introduced. However, viral methods are seen to be more efficient in carrying larger amounts of DNA, as non-viral method did not give such a good yield transporting therapeutic DNA because it was more difficult to go through the cell membrane.[7] Therefore, the main focus for many researchers was to find a way to avoid the difficulties by designing compounds that bind therapeutic DNA and transport it easily to the host cell.

Non-Viral Methods

There are three main types of molecule used to deliver DNA by non-viral methods. These include: dendrimer vectors, polymeric vectors or cationic lipids (Figure 2).

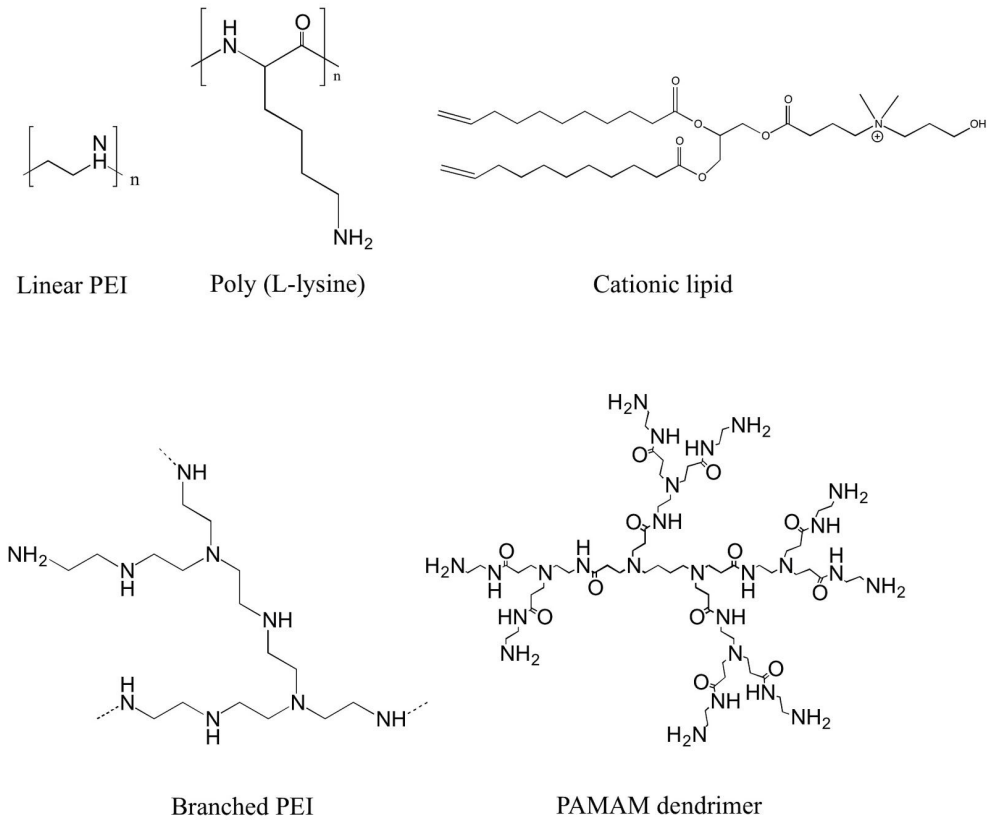


Figure 2. The variety of non-viral transport vectors used for gene therapy.

1) Polymeric Vectors

Polyethylamine (PEI) based vectors are one of the most used polymer vectors for gene delivery. PEI condenses DNA into positively charged particles (polyplexes) which bind to the negatively charged cell surface and enters the cell by endocytosis. The transfection efficiency was seen to be due to its ability to escape endosomes by protonating structural amines that create an influx of ions which lowers osmotic pressure resulting in cell burst and DNA release. [21] The structure exists in two forms: branched and linear structure. The branched structure of PEI contains nitrogen at every third atom which results in high charge density that contributes in better interaction with DNA. The studies have also shown that transfection efficiency is increased when the molecular weight of the vector is increased. However, higher molecular weight results in higher cytotoxicity and therefore it was concluded that the optimal size of PEI was 5-25 kDa. Studies also found linear PEI to have better transfection efficiency and lower toxicity than branched PEI [8].

Poly(L-lysine) (PLL) is another polymer that was one of the first polymer vectors used for gene delivery. The transfection principle of PLL is based on interaction between primary amine groups in PLL and negatively charged phosphate groups of DNA. This interaction forms particles called polyplexes which, like PEI, enter cells by endocytosis and release therapeutic DNA. Although, unmodified PLL does not contain any lysosomal disruption agent, has a low transfection efficiency and high cytotoxicity [4]. However, there are many options how to modify PLL and make it a more suitable transfection agent. One example is PLL coated with hydrophilic polymer PEG, which minimizes the interaction with serum components and increases circulation time [9]. Another possible modification is joining PLL with various compounds such as sugars, folate, antibodies or dendrimers to obtain better transfection efficiency by improving endosomal escape, better polyplex formation, lower toxicity, etc. [22]

2) Dendrimers

Dendrimers are ellipsoidal or globular shaped synthetic hyperbranched macromolecules consisting of an initiator core, interior layers made of repetitive units that are connected to the

core and an exterior attached to the outside [10]. Their exclusive structure provides lots of combinations and modifications, therefore high number of functional groups are available that makes it suitable for gene/drug delivery to target cells.

There are four main classes of dendrimers:

- **MAP-dendrimers** (Multiple Antigen Peptide) are based on polylysine skeleton.
- **PPI-dendrimers** (Poly(Propylenelmine)) are poly-alkylamines having primary amines as end-groups.
- **PAMAM-dendrimers** (Poly(Amido)Amine) are built by polyamide branches with tertiary amines as focal points.
- **Frechet-type dendrimers** are one of the newest type of dendrimers based on polybenzyl ether branched skeleton.

PAMAM and PPI dendrimers are most widely used in pharmaceutical and biomedical engineering. Both PAMAM and PPI have open ellipsoidal shape structure with inner pockets which are used to encapsulate hydrophobic drugs. However, these two dendrimers have many limitations such as cytotoxicity and inefficient escape from endosomes or lysosomes to release DNA into the host cell. Therefore, these structures have to be optimised in order to be suitable for delivery. Studies have shown that addition of lipophilic groups to PPI, substitution of PPI primary amines with guanidine or quaternization of primary amines by methyl groups increased transfection efficiency. While studies focusing on PAMAM dendrimer vectors have shown that substitution with arginine groups, PEGylation or addition of hydrophobic chains increased cellular uptake and drug loading capacity with only a slight or no increase in toxicity. When a dendrimer is optimised and is suitable to be used for gene delivery, it can form compact complexes with DNA and form a dendrimer-DNA complex. These complexes are taken up in cells by endocytosis where DNA is released. [11]

3) Cationic Lipids

Lipids are amphiphilic molecules which means they have both a hydrophobic or non-polar part and a hydrophilic or polar head group, as well a backbone linking those two domains. Due to the dual aspect of these organic molecules, they form closed bilayer shells – liposomes – in order to shield their hydrophilic part from the surrounding aqueous environment. According to the charge of their head group, lipids can be cationic, anionic or

neutral. Cationic lipids are currently the center of attention as their positively charged head groups can form complexes with DNA, a lipoplex, together with a helper lipid, making them favourable for gene therapy. Furthermore, they protect DNA from the attack of DNases and because of their membranous nature they can interact with the negatively charged membrane surfaces and deliver the DNA inside the cells. These lipoplexes act as vectors that can introduce DNA into cells without causing an immune response in the body. [12] There are two main structure morphologies of lipoplexes. The lamellar structure has the DNA "sandwiched" between lipid bilayers and the hexagonal structure has DNA rods that are surrounded by a lipid monolayer arranged into a hexagonal lattice (Figure 3). A recent study showed the lipoplex structure changes after contact with the cell membrane from a lamellar structure to an inverted hexagonal one. [13]

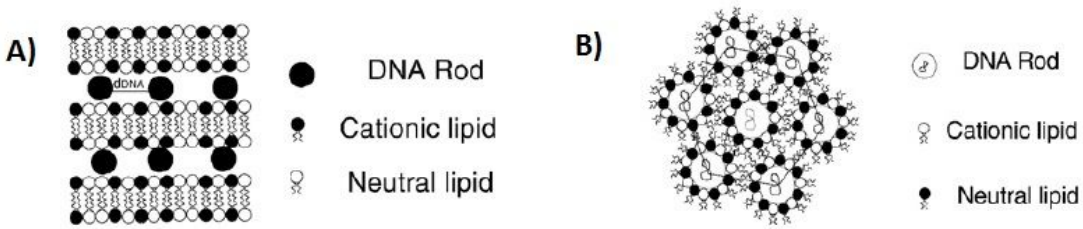


Figure 3. The two main lipoplex structures. A) Lamellar structure. B) Hexagonal structure. [13]

As mentioned, the structure of cationic lipids can be separated into three main parts: a hydrophobic domain, a backbone and a headgroup. Generally, the lipid **hydrophobic domain** consists of two alkyl chains, either saturated or unsaturated, or a steroid. Some studies have shown that the type and length of these chains can affect the transfection efficiency. However, it is hard to say for example, whether the chain should be longer or shorter because there are studies to support both structures, but it is clear the overall structure of the lipid determines the efficiency of transfection. Few other studies showed that the use of two chains with different lengths can improve the transfection efficiency probably by helping endosomal escape. [4] The most widely researched alkyl chains are either saturated or monounsaturated with chains varying from 5 to 25 carbons. "It is known that the membrane fluidity increased

when an unsaturated structure was introduced into the hydrophobic chain of the lipid, by disrupting membrane packaging and facilitating DNA escape inside the cells." [14] Therefore, it seems that in terms of the transfection efficiency, using unsaturated alkyl chains is more favourable as they help promote lipid bilayer fluidity. [14]

In some studies, where the hydrophobic moiety was cholesterol or a cholesterol derivative, it seemed to give good physical stability for lipoplex formation, due to the rigidity of the cholesterol ring system. [14] In another study, Goldring *et al*. investigated macrocyclic hydrophobic domains, based on the fact that macrocyclic lipids of archaebacteria can form liposomes showing higher stability and resistance to higher temperatures, oxidative stress, change of pH and to the action of proteins. Lipids with macrocyclic structures could possibly provide better DNA protection and form more tightly packed liposomes. They studied four different molecules, two with an acyclic hydrophobic domain (one saturated and one unsaturated) and two with the corresponding cyclic domain. These lipids were combined with 1,2-dimyristoyl-sn-glycero-3-ethylphosphocholine (EPC) and either 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) or cholesterol as a co-lipid. [15] Later, they continued their research with an expanded library of related cationic lipids where they included longer chains in the hydrophobic domain. Their results showed that the macrocyclic formulations of lipid analogues performed equally or better than the acyclic formulations. Furthermore, lipoplexes containing cholesterol as co-lipid showed better transfection efficiency in general as well as better cell viability compared to the combinations of lipids with DOPE. [18]

The **backbone** of cationic lipids is most often glycerol-based. [16] A bond, also called the linker, then connects the backbone of the lipid with its cationic headgroup and hydrophobic chain, and is usually an ester or ether, but can also be another common linker such as an amide or carbamate. [4] Cationic lipids with ether-like linkers have shown to give better transfection efficiency, but because they are more chemically stable they also show higher toxicity than, for example ester bonds that are biodegradable.[17]

Many gene therapy studies investigated the **headgroup** of cationic lipids, trying different variations to optimize binding to the DNA and its transport. This hydrophilic domain is

positively charged due to protonation of the headgroup or the presence of a permanent charge, allowing the binding to negatively charged DNA. Lipid headgroups can either be monovalent or multivalent according to the amount of positive charges – either one or several. [14] It has been shown that the characteristics of the headgroup, such as its size or charge, can have an effect on the transfection efficiency, which is often more significant than the nature of the hydrophobic chains. Some examples of studied types of lipid headgroups are: ammonium, primary/secondary/tertiary amines, amino acids, peptides, cyclic or heterocyclic structures, etc. [16]

As an example, in experiments conducted by both Clément, Floch *et al.* and Stekar *et al.*, the ammonium cation was replaced by phosphonium or arsenium groups in phosphonolipids as well as in edelfosine and miltefosine respectively. Both results showed significantly decreased toxicity. [4] Furthermore, another example are cationic lipids with amino acid based headgroups, such as serine or alanine. Springer and co-workers obtained the highest transfection efficiency with lipids based on lysine and arginine derivatives. Other studies incorporate a cyclic structure as the headgroup of cationic lipids. Chaudhuri *et al.* synthesized glycolipids with either a cyclic or an open D-galactose headgroup with the positive charge carried on the linker nitrogen atom. [5]

In one study, two different headgroups – dimethylamine (DMA) and trimethylamine (TMA) – were analyzed together with other domains of cationic lipids. In the experiment, the separate effect of the two headgroups was analyzed on the lipoplex formulation. Two different co-lipids were also used: cholesterol and DOPE. The results showed that the combination of DMA with cholesterol outperformed the combination of TMA with cholesterol in the transfection efficiency. However, both DMA and TMA combined with DOPE did not show any significant difference in transfection efficiency. It was also observed that the cell viability was better for lipids with the TMA headgroup, regardless of the co-lipid used. [18]

For lipids with multivalent headgroups, where there is more than one positive charge, there is a greater surface charge density in the liposomes. Therefore, these would be expected to bind

and transport DNA more efficiently. Possible multivalent headgroup structures are polyamines. For example, spermine or spermidine that are both known to be able to bind the minor groove of B-DNA. Some new investigations focused on the effect of the length as well as charge distribution of these polyamines. “Addition of amino groups separated by methylene portions to the end of a linear polyamine chain did not automatically improve gene transfer by a series of polyamine-steroid conjugates, regardless of the extra protonation sites.” [19] This would be due to an increase of folded conformations because of more flexibility which could alternatively lead to unfavourable interactions with DNA. [19]

Lipoplex Formation, DNA Transport and Release

Lipoplexes are self-assembled structures, made of condensed and packed DNA bound to lipids. The binding of DNA to the cationic lipids is mediated through electrostatic interactions between the positive lipid headgroup and the negatively charged DNA backbone. Once the lipoplex is formed, it can interact with the negatively charged cell membrane. The lipoplex enters the cell through endocytosis. [3] Once inside the cell, the lipoplex destabilizes the endosomal envelope and in a process called flip-flop the lipids of the endosomal membrane are rearranged. They then fuse with the lipids from the lipoplex which results in the release of DNA inside the cell. [4]

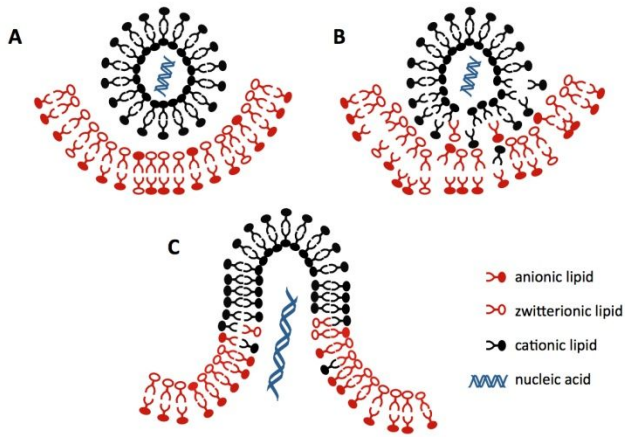


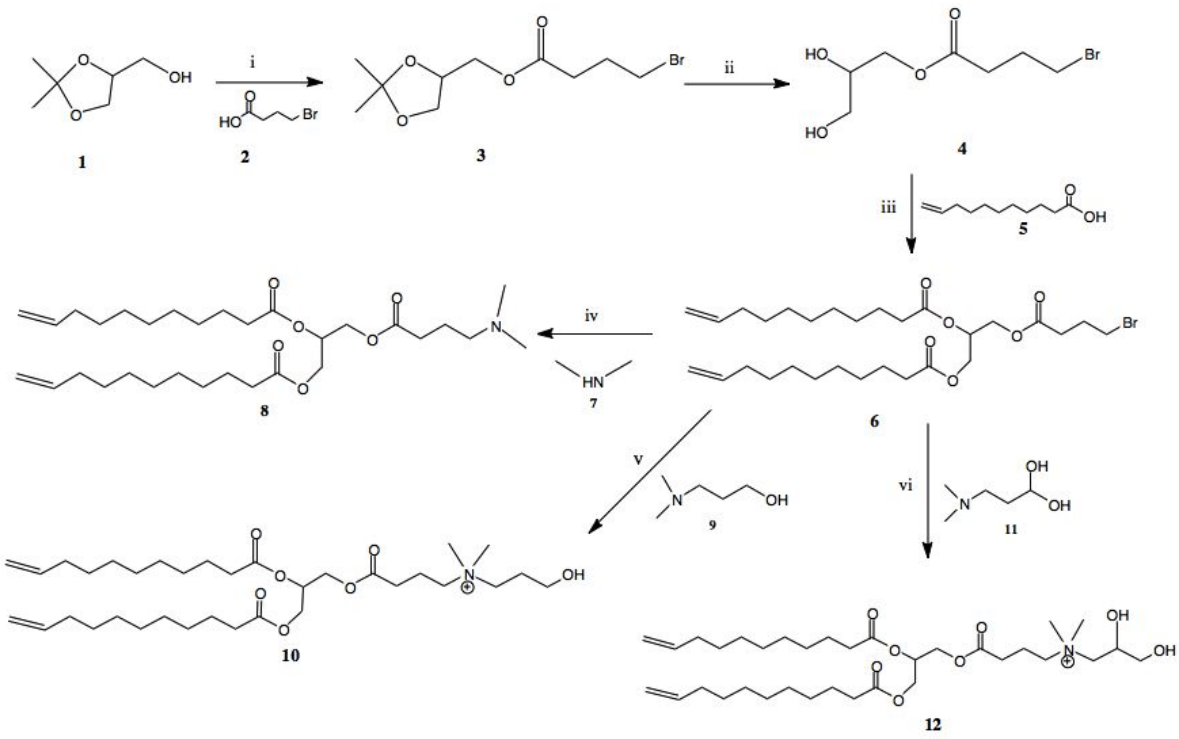
Figure 4. The lipids from the lipoplex combine with the endosomal lipids, allowing the DNA inside the cell. [24]

The overall stability of the lipoplex depends on the nature of the lipids that it is comprised of and it consequently affects transfection efficiency. For example, the bigger the difference between the cross-sectional area of the lipid's headgroup and the hydrophobic domain, the more unstable the lipoplex formed will be. This increases the probability of interaction with the endosomal vesicles. Therefore, the relative instability of lipoplexes is presumed to improve the transfection efficiency. [19]

Often, there are co-lipids or helper lipids used in the assembly of lipoplex in order to optimize the stability of the structure and the transfection efficiency. For instance, a very commonly used neutral co-lipid is DOPE that is thought to promote the change from a lamellar to a hexagonal structure of the lipoplex after the uptake inside the cell that is less stable and therefore facilitates the mixing of the lipids with the endosomal phospholipids. It might also affect the binding of the lipids to the DNA, making it looser and therefore easier to release. [19] [3]

Results

Based on a previous studies conducted by Goldring, W. *et al*., our main goal was to synthesize three cationic lipids similar in structure to previous experiments. In several reactions, we synthesized the three lipids with different headgroups, starting with a dimethylamine (DMA) headgroup and successively adding one and two alcohol molecules (Scheme 1).



Scheme 1. Synthesis of cationic lipids. Reagents and conditions: i) 4-bromobutyric acid, DCC, DMAP, DCM, rt, 72%. ii) $CuCl_2$ in MeCN, rt, 100%. iii)10-undecenoic acid DCC, DMAP, DCM, rt, 39%. iv) 2 M Me_2NH in THF, rt, 87%. v)3-dimethylamino-1-propanol, 90°C, 65%. vi) 3-dimethylamino-1,2-propanediol, 90°C, 60%.

In the first step to synthesize the diene **6** used for further synthesis of the three lipids, solketal **1** was coupled to 4-bromobutyric acid **2**, using DCC, DMAP and DCM , in order to obtain an ester **3** that would form the basis for the backbone and headgroup linker of our lipids (Figure 5). This was followed by a deprotection reaction of the acetal **3** with copper(II) chloride in acetonitrile which opened up the cyclic acetal to reveal the diol **4** to which hydrophobic chains could be attached.

The diol **4** was further used for the diesterification reaction with 10-undecanoic acid (**5**) resulting in the diene bromide **6** (Figure 6). Two of the acid chains were required in this reaction which is why 2.2 equivalents of the carboxylic acid was added to the reaction. The yield of 37% was a bit low but satisfying, the loss of the compound could have happened during the flash column chromatography. The proton NMR spectrum confirmed that the desired product was obtained, which was used for the individual synthesis of the three lipids with different cationic headgroups.

Once the hydrocarbon chains were assembled, we proceeded with the substitution of the bromide **6** with a dimethylamine headgroup. This was done by combining the diene bromide **6** with dimethylamine (**7**) in THF. However, following initial purification of the crude residue by flash column chromatography we were unable to isolate a pure product. Therefore, the flash column chromatography was repeated using the same conditions. We were able to combine 3 fractions containing the product in 87% yield. The obtained sample however still contained some impurities that we could not eliminate. The NMR spectra (Figure 7) showed approximate structure of our wanted lipid **8** product, however one peak corresponding 2 hydrogens was missing in the area of 1.2- 2.5ppm, which could indicate that the reaction was not stable and that compound did not fully obtain expected structure.

The diene bromide **6** was further used for a second reaction where it was combined with 3-dimethylamino-1-propanol (**9**). Once purified, the second lipid **10** was obtained in 65% yield. The NMR spectrum (Figure 8) showed that the bromide was successfully replaced by the amino group of 3-dimethylamino-1-propanol.

In a third reaction, 3-dimethylamino-1,2-propanediol (**11**) was added to the diene bromide **6** resulting in the third lipid **12** in a satisfying 60% yield (Figure 9). In this reaction, the scale of the reaction, and thus weight of the diene bromide **6** was doubled compared to the two previous reactions but the amine to bromide ratio was maintained at 1:1. This was done to get a higher amount of product in order to have enough for further reactions.

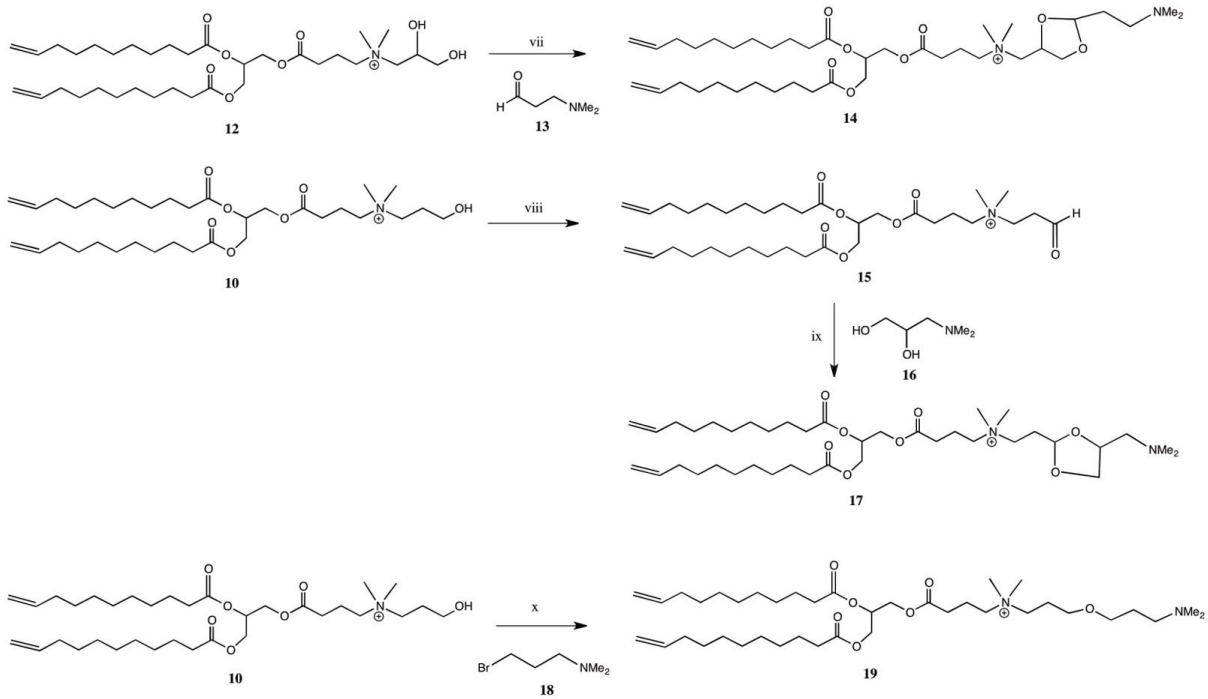
Conclusion

From the series of experimental reactions conducted during this project, we were able to successfully synthesize three cationic lipids, each containing a different headgroup structure. Further investigation is required to determine how efficient these individual lipids would be to complex with DNA and form stable lipoplexes, capable of carrying and delivering DNA inside target cells.

The final structure of the three lipids we synthesized leaves open the possibility to further modification. For example, in future experiments, the two alcohols in lipid **12** could be used to form a cyclic structure in the headgroup that could theoretically make the molecule more stable. Also, the single alcohol in lipid **10** could be used to add a chain onto the headgroup, elongating the whole molecule.

Perspectives

Further modifications of the synthesized lipids could be done in order to make them more suitable transfection agents. There are several options how the compounds could be changed but the plan for the future will be to focus on creating a cyclic structure in the headgroup of the lipid or to elongate it. Those modifications would be applied in order to make the structures more stable, to avoid degradation and to improve the lipid binding with DNA. The Scheme 2 below represents further possible adjustments that could be performed in Goldring's lab.



Scheme 2. Modification of cationic lipids. Reagents and conditions: vii) Toluene, $TolSO_3H$, 110°C. viii) DMP, DCM. ix) 3-dimethylamino-1,2-propanediol, $TolSO_3H$, Toluene. x) 3-bromo-N,N-dimethylpropan-1-amine, NaH.

The lipid **12** could be used for the first modification combining it with 3-(dimethylamino)propanal (**13**) using toluene and $TolSO_3H$ at 110°C. This would form a ring structure as the diol would be combined with the aldehyde.

The next modification could be done using lipid **10** in two steps. The first step would be to use DMP and DCM to convert the alcohol group to an aldehyde group. Then, this new compound (**15**) would be combined with 3-dimethylamino-1,2-propanediol (**16**) which would form a ring joining the newly synthesized headgroup with a diol.

Another possible modification would be to elongate the structure of lipid **10** by combining it, for example, with 3-bromo-N,N-dimethylpropan-1-amine (**18**).

The synthesis of a lipid in itself does not allow us to evaluate how efficient the lipid will be at binding DNA and delivering it to target cells. Further experiments are necessary, for the lipids we synthesized as well as for any future lipids, in order to conclude anything on their efficiency and stability.. Further testing can include the examination of DNA and lipid binding, lipoplex formation, lipoplex morphology, degradation assay, transfection efficiency and cytotoxicity.

Experimental procedures

General methods

Infrared spectra were recorded on Perkin Elmer RX I FT-IR spectrometer as solid between two sodium chloride plates. Proton NMR spectra were recorded on Bruker Ascend SB, Avance-III HD spectrometer as dilute solutions in $CDCl_3$. Chemical shifts are referenced to residual protonated solvent ($\delta H = 7.26$ for $CDCl_3$) and are quoted in parts per million (ppm). The multiplicity of a signal is designated by one of the following abbreviations: s = singlet; d = doublet; t = triplet; q = quartet; qt= quintet, m = multiplet. All coupling constants, J, are reported in Hertz (Hz) and quoted to the nearest 0.1 Hz. Carbon-13 NMR spectra were recorded on Bruker Ascend SB, Avance-III HD spectrometer as dilute solutions in $CDCl_3$ on a broad band decoupled mode. Chemical shifts are referenced to residual protonated solvent ($\delta C = 77.0$ for $CDCl_3$) and are quoted in parts per million (ppm). All reactions were monitored by thin layer chromatography (TLC) using Merck silica gel 60 F254 precoated aluminum plates, which were developed with basic potassium permanganate. Flash chromatography was performed on Sigma-Aldrich silica gel 60.

The synthesis of three different lipids with three different headgroups was performed in six different chemical reactions, described below. For compound numbers refer to Scheme 1.

2,3-dihydroxypropyl 4-bromobutanoate (4)

N,N'-Dicyclohexylcarbodiimide (DCC) (1.69 g, 8.19 mmol, 1.5 equivalents) and *N*,*N*-dimethylaminopyridine (DMAP) (0.21 g, 1.72 mmol, 0.3 eq) were sequentially added to a solution of 4-bromobutyric acid **(2)** (1.01 g, 6.05 mmol, 1.1 eq) in dichloromethane (DCM) (110 mL, 0.05 M) at 0 °C and stirred for 30 min. A solution of DL-1,2-isopropylideneglycerol **(1)** (solketal) (0.68 mL, 5.44 mmol, 1 eq) in DCM (220mL, 0.075 M) was added to the reaction mixture, which was then stirred at room temperature for 18 hours. The reaction mixture was filtered through celite, washed with DCM (250 mL) and then concentrated in vacuo. The crude residue was purified by flash column chromatography on silica gel using 20% ethyl acetate in petroleum ether as eluent to give the ester (**3)** (1.23 g,

72% yield). ν_{max} 2988, 2935, 2121, 1740, 1454, 1439, 1371, 1312, 1204 and 841; δH (MHz, $CDCl_3$) 4.3 (1H, ddd, J 4.65, 6.22 and 12.47), 4.21 (1H, dd, J 4.65 and 11.54), 4.15-4.08 (2H, m), 3.75 (1H, dd, J 6.14 and 8.53), 3.45 (2H, t, J 6.44), 2.57 (2H, t, J 7.14), 2.2 (2H, q, J 6.48), 1.42 (3H, s), 1.38 (3H, s). δC (MHz, $CDCl_3$) 172.4, 109.9, 73.6, 66.3, 64.9, 32.6, 32.3, 27.7, 26.7, 25.4.

Copper(II) chloride dihydrate (1.13 g, 6.63 mmol, 1.5 eq) was added to a solution of the previously obtained ester **(3)** (1.23 g, 4.37 mmol, 1 eq) in acetonitrile (87 mL, 0.05 M) and stirred at room temperature for 18 hours. The reaction mixture was concentrated in vacuo, diluted with diethyl ether (100 mL), and successively washed with a saturated aqueous solution of sodium hydrogen carbonate (100 mL) and transferred to a separating funnel. The organic layer was separated from the water and the process was repeated several times and the organic layer collected in fractions. The fractions containing the diol **(4)** product were selected and concentrated in vacuo into a colourless oil. All this oil was used for the next reaction.

3-((4-bromobutanoyl)oxy)propane-1,2-diyl bis(undec-10-enoate) (6)

DCC (3.98 g, 19.3 mmol, 3 eq) and DMAP (0.40 g, 3.21 mmol, 0.5 eq) were sequentially added to a solution of 10-undecenoic acid (**5**) (2.60 g, 14.1 mmol, 2.2 eq) in DCM (70 mL, 0.5 M) at 0 °C and stirred for 30 min. A solution of previously made diol **(4)** (1.06 g, 4.40 mmol, 1.0 eq) in DCM (60 mL, 0.5 M) was added gradually to the reaction mixture through a cannula under nitrogen pressure, which was then left to stir at room temperature for 3 days. The reaction mixture was filtered through celite, washed with DCM (200 mL) and then concentrated in vacuo. The crude residue was purified by flash column chromatography through silica gel using 10-20% ethyl acetate in petroleum ether to give the diene bromide (**6**) (0.99 g, 39%) as a colourless oil. ν_{max} 3085, 2927, 2855, 1742, 1641, 1458, 1439, 1415, 1369, 1166 and 909; δH (MHz, $CDCl_3$) 5.89-5.77 (2H, m, 2xCH), 5.3-5.21 (1H, m), 5.15-4.9 (4H, m, 2xCH2), 4.31 (2H, td, J 4.25 and 11.77), 4.2-4.1 (2H, m), 3.45 (2H, ddd, J 4.98, 6.05 and 11.82), 2.5 (2H, t, J 6.41), 2.31 (4H, ddd, J 3.87, 7.45 and 11.33, 2xCH2), 2.15 (2H, qt, J 6.7 and 13.61), 2.12 (4H, q, J 6.82, 2xCH2), 1.68-1.52 (4H, m, 2xCH2), 1.41-1.18 (20H, m). δC (MHz, $CDCl_3$) 173.3, 172.9, 172.0, 139.1, 114.2, 114.2, 68.8, 62.5, 62.0, 34.2, 34.0, 33.8, 32.5, 32.2, 29.3, 29.2, 29.1, 29.0, 28.9, 27.6, 24.9, 24.8.

3-((4-(dimethylamino)butanoyl)oxy)propane-1,2-diyl bis(undec-10-enoate) (8)

A 2 M solution of dimethylamine in THF (**7**) (1 mL) was added to the diene bromide (**6**) (0.1022 g, 0.185 mmol) and then stirred at room temperature in a sealed round bottom flask for 4 days. The reaction mixture was concentrated in vacuo. The crude residue was purified twice by flash column chromatography on silica using 1% triethylamine to load the residue and a gradient of 5–10% methanol in DCM. The fractions were analyzed with TLC and the amine product (**8**) (0.0858 g, 86.6%) isolated and concentrated to a colourless oil. ν_{max} 3073, 2923, 2855, 1742, 1641, 1462, 1418, 1371, 1167 and 909; δH (400 MHz, $CDCl_3$) 5.89-5.75 (2H, m), 5.31-5.22 (1H, m), 5.05-4.89 (4H, m, 2xCH2), 4.35-4.05 (4H, m, 2xCH2), 2.41-2.15 (10H, m), 2.42-2.18 (4H, m), 2.15-2.01 (4H, m), 1.88-1.50 (8H, m), 1.42-1.21 (20H, m)**.** δC (100 MHz, $CDCl_3$) 139.2, 114.2, 68.9, 68.4, 65.1, 62.2, 62.1, 58.8, 45.4, 34.2, 34.1, 33.8, 31.8, 29.3, 29.2, 29.1, 28.9, 24.9, 22.8.

4-(2,3-bis(undec-10-enoyloxy)propoxy)-*N*-(3-hydroxypropyl)-*N*,*N*-dimethyl-4-oxobutan-1-aminium (10)

3-Dimethylamino-1-propanol (**9**) (0.0219 mL, 0.185 mmol, 1 eq) was added to the diene bromide (**6**) (0.1064 g, 0.185 mmol, 1 eq) and heated on sand at 90°C for 3 days. The crude residue was then purified by flash column chromatography through silica gel using 20% ethyl acetate in petroleum ether to load the crude residue and then a gradient of 10-30% methanol in DCM to give the cationic lipid (**10**) (0.0717 g, 65%). ν_{max} 3399, 3077, 2923, 2855, 1738, 1641, 1466, 1417, 1373, 1174 and 909; δH (MHz, $CDCl_3$) 5.88-5.72 (2H, m), 5.31-5.22 (1H, m), 5.15-4.89 (4H, m), 4.65 (1H, s), 4.31 (2H, dd, J 4.27 and 11.99), 4.18-4.09 (2H, m), 3.82-3.69 (4H, m), 3.62-3.51 (2H, m), 3.42-3.25 (6H, m), 2.6-4.9 (2H, m), 2.36-2.25 (4H, m), 2.2-1.98 (8H, m), 1.69-1.52 (4H, m), 1.42-1.23 (20H, m). δC (MHz, $CDCl_3$) 173.3, 173.0, 171.8, 139.1, 114.1, 68.7, 62.9, 62.6, 62.1, 58.4, 51.5, 34.2, 34.0, 33.7, 29.9, 29.3, 29.2, 29.1, 29.0, 28.8, 25.7, 24.8, 18.0.

***N*-(2,3-dihydroxypropyl)-*N*,*N*-dimethyl-4-oxo-4-(2-(undec-10-en-1-yloxy)-3-(undec-10-enoyloxy)propoxy)butan-1-aminium (12)**

3-Dimethylamino-1,2-propandiol (**11**) (0.043 g, 0.368 mmol, 1 eq) was added to the diene bromide (**6**) (0.2113 g, 0.368 mmol, 1 eq) and left on sand at 90°C for 3 days. The crude residue was then purified by flash column chromatography through silica gel using 20% ethyl

acetate in petroleum ether to load the crude residue and a gradient of 10-30% methanol in DCM. The fractions were analyzed with TLC and the product (**12**) (0.1338 g, 60%) isolated and concentrated in vacuo. ν_{max} 3322, 3080, 2923, 2858, 1738, 1639, 1464, 1417, 1377, 1173 and 907; δH (MHz, $CDCl_3$) 5.88-5.69 (2H, m), 5.29-5.1 (2H, m), 5.0-4.82 (4H, m), 4.71 (1H, s), 4.45(1H, s), 4.27 (2H, qt, J 4.0 and 12.06), 4.11 (2H, q, J 6.41 and 12.51), 4.0 (1H, s), 3.82-3.52(6H, m), 3.45-3.22 (6H, m), 2.57-2.41 (2H, m), 2.29 (4H, q, J 7.06 and 14.76), 2.19-1.91 (6H, unidentified), 1.68-1.50 (4H, m), 1.40-1.15 (20H, m). δC (MHz, $CDCl_3$) 173.3, 173.0, 171.7, 139.1, 114.1, 68.7, 66.6, 66.3, 64.7, 64.3, 62.9, 62.1, 52.5, 52.2, 34.2, 34.0, 33.7, 30.0, 29.3, 29.2, 29.1, 29.0, 28.8, 24.8, 18.1.

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Appendix

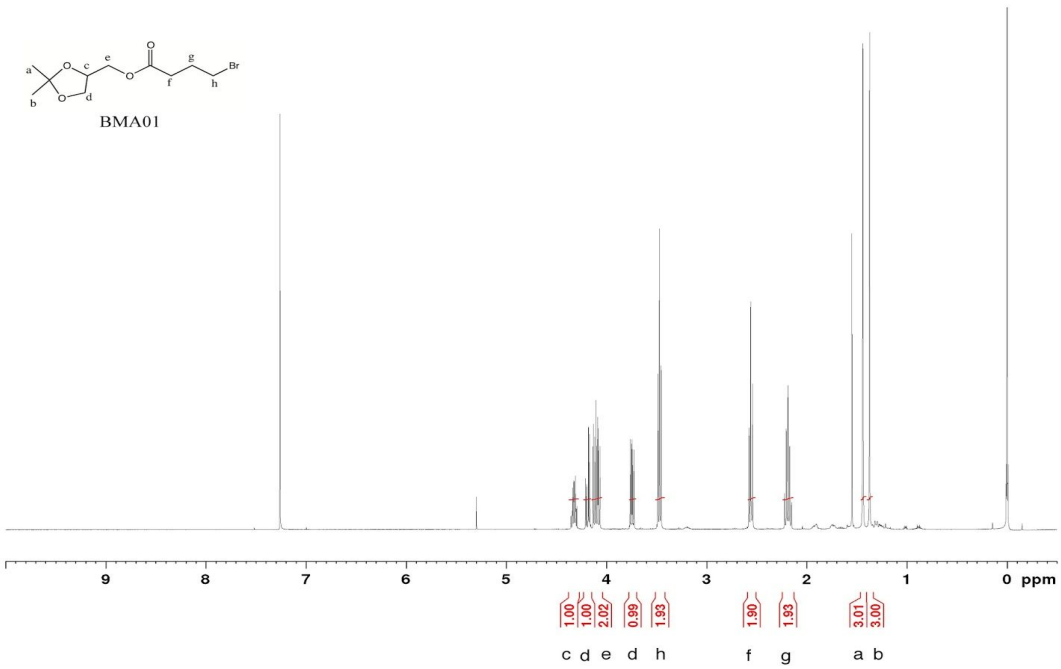

Figure 5. [1]H NMR of ester **3** with the structure obtained. The peaks are assigned by letter to their corresponding hydrogens.

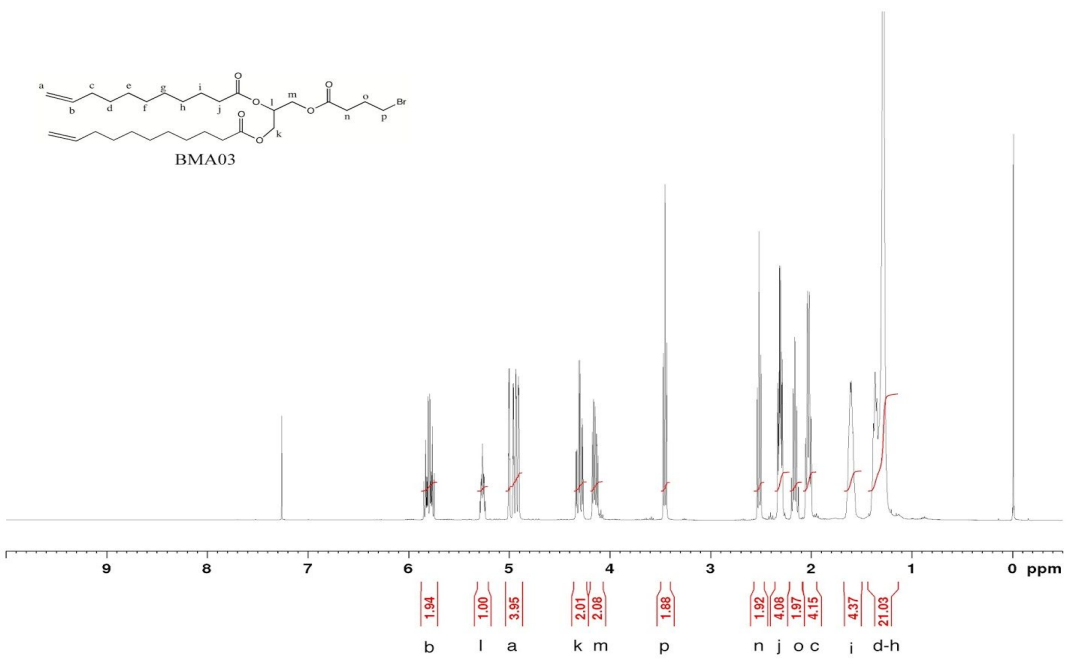

Figure 6. [1]H NMR of diene bromide **6**. The peaks are assigned by letter to their corresponding hydrogens.

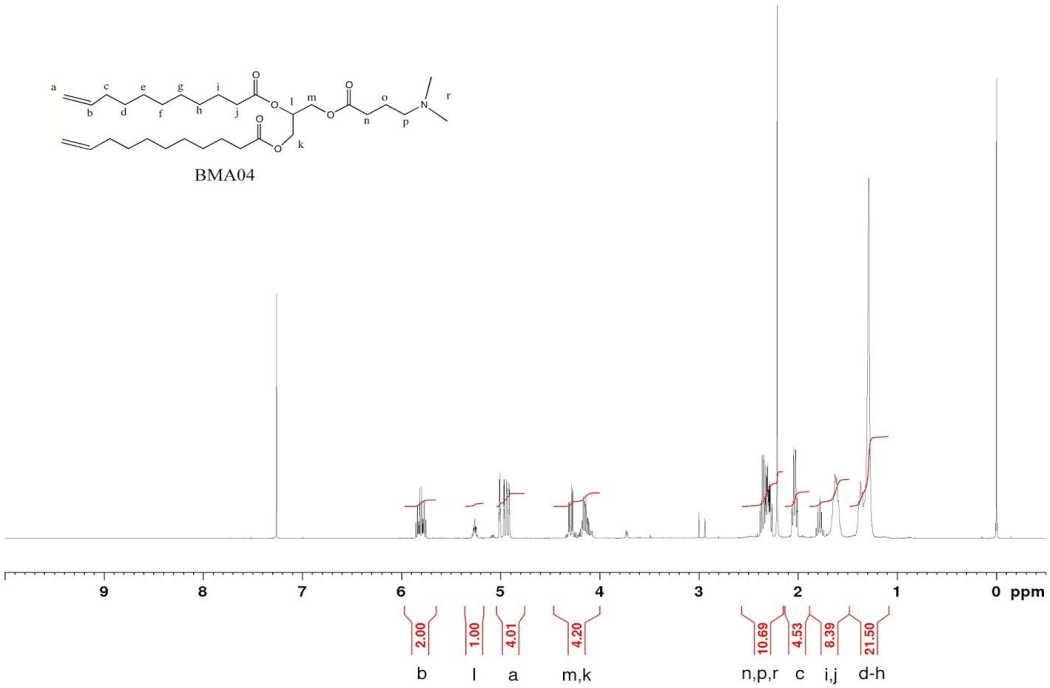

Figure 7. [1]H NMR of our first lipid **8**. The peaks are assigned by letter to their corresponding hydrogens. A clear peak is missing for two hydrogens, assigned O in the structure, that should be located in the area of. 1.2 - 2.5 ppm. In general, in the area of 1.2 - 2.5 ppm it is hard to assign the hydrogens to the integrals as they do not correspond properly.

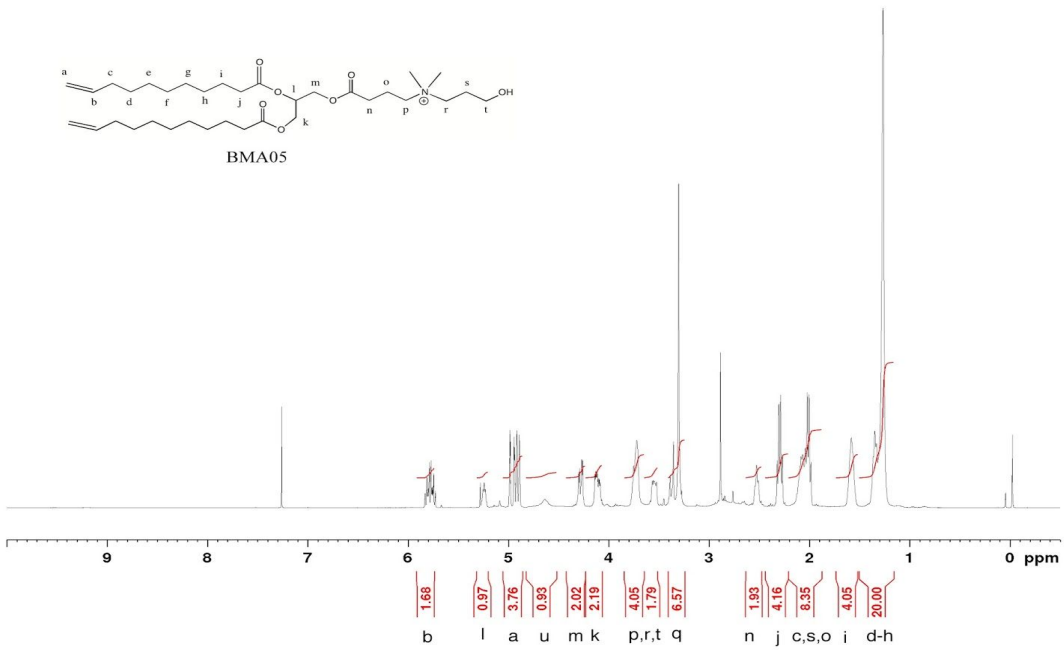

Figure 8. [1]H NMR of our second lipid **10**. The peaks are assigned by letter to their corresponding hydrogens.

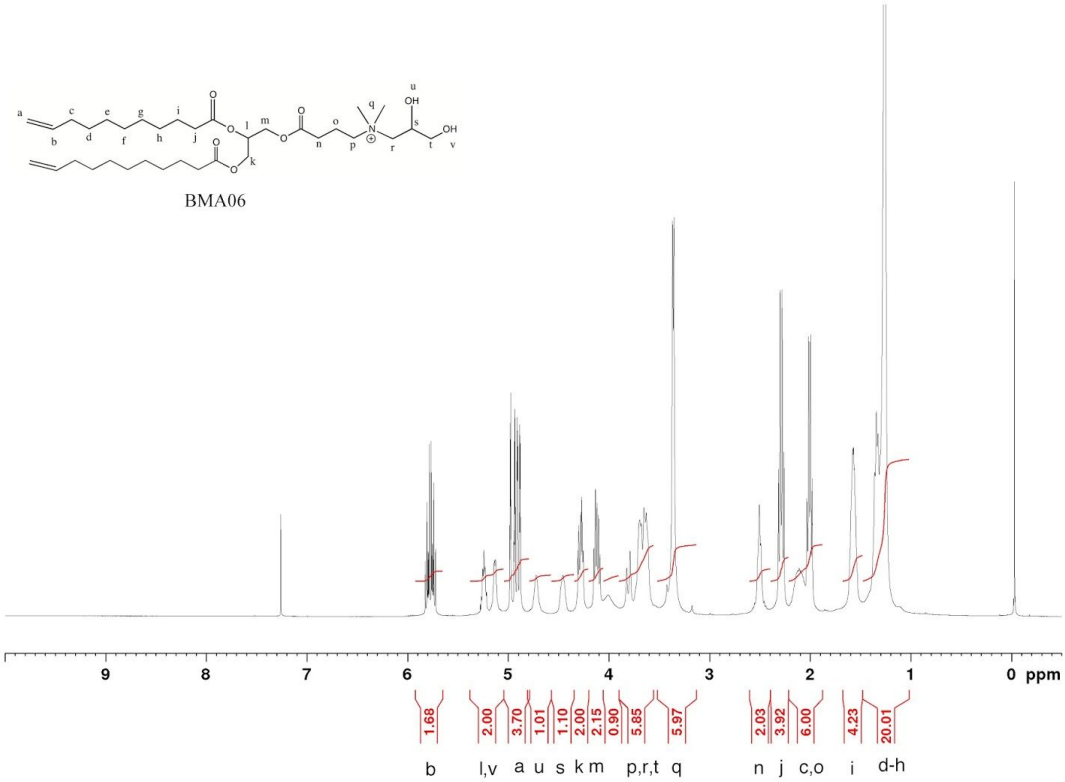

Figure 9. ^{1}H NMR of our third lipid **12**. The peaks are assigned by letter to their corresponding hydrogens. Integral corresponding to 1 hydrogen around 4.05ppm was found to be water signal.