Selection of an appropriate target gene for gene delivery research is an important aspect for the success of gene delivery systems. In order to evaluate the therapeutic potential of gene delivery systems, it is essential to determine the in vitro expression of the genes after transfection with the delivery system. This requires either direct estimation of the therapeutic gene expression in the cells or evaluation of the expression of a reporter gene that can be easily estimated through a suitable analytical tool. Estimating the expression of therapeutic gene usually requires more sophisticated tools such as western blotting, ELISA and requires use of protein specific antibodies. However, it is possible to use alternative reporter genes expression of which can easily be estimated qualitatively as well as quantitatively using easily available techniques such as fluorescence microscopy, confocal laser scanning microscopy and fluorescence activated cell sorting. Such reporter genes include several examples such as green fluorescent protein, yellow fluorescent protein, red fluorescent protein etc.

4 (A) Rationale for selection of p11 gene

p11 is a relatively new protein for the depression , but its effects has got everyone excited. p11 is a protein that controls the expression of two serotonin receptors (1). Serotonin is thought to be one of the major players in mood disorders like depression (while selective serotonin reuptake inhibitors may not work in many patients, this doesn't mean that serotonin isn't playing a role) and so the consequences of p11 became suddenly interesting. And it turned out that mice with a knockout of the p11 gene displayed what we call a depressive phenotype, where they show more immobility (which used to be called behavioral despair) in tests such as the forced swim test (FST) and the tail suspension test (TST) which are sensitive to the effects of antidepressants (2).

To start with, researchers have used a virus to insert a gene into the NAc (nucleus accumbens) which knocked down p11 in normal mice, only in the NAc. There are immobility measures that are taken into account for the tail suspension test and the forced swim test. So far, these mice look more "depressed", but they still respond to antidepressants. When they increased p11, they got a reversal of the "depressive" effects seen in p11 knockout mice (3). These mice showed more swimming in the forced swim test, more struggling in the tail suspension test, and even showed increased sucrose preference (which is thought to be a measure of anhedonia).

Advances in genomic technology have added considerable power to the search for genes associated with depression. The irony of these developments is that they paint an

increasingly complex picture. The intriguing interplay between genes and environment are at the heart of this complexity, and uncovering these dynamics promises to shed new light on the nature of cognition. In addition to the relatively large contribution of environmental factors to its etiology, the search for candidate genes for depression is further complicated by the fact that the disorder takes many different forms. The genes 5-HTT, BDNF, and TPH2 have attracted much attention in recent years but the clinical studies featuring p11 gene showed promising results tempted many researchers to treat depression using non-viral vectors containing p11 cDNA.

4 (A).1 Properties of p11 cDNA and protein

pcDNA3.1 mouse wtP11-HA

GACGGATCGGGAGATCTCCCGATCCCCTATGGTGCACTCTCAGTACAATCTGC
TCTGATGCCGCATAGTTAAGCCAGTATCTGCTCCCTGCTTGTGTGTTGGAGGTC
GCTGAGTAGTGCGCGAGCAAAATTTAAGCTACAACAAGGCAAGGCTTGACCG
ACAATTGCATGAAGAATCTGCTTAGGGTTAGGCGTTTTGCGCTGCTTCGCGAT
GTACGGGCCAGATATACGCGTTGACATTGATTATTGACTAGTTATTAATAGTA
ATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACAT
AACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTG
ACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGGGACTTTCCATTG
ACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACATCAAG
TGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCC
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CATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACAT
CAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCA
TTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAA
TGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGGTAGGCGTGTACGGTG
GGAGGTCTATATAAGCAGAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACT
GGCTTATCGAAATTAATACGACTCACTATAGGGAGACCCAAGCTGGctagagcctct
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CTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAG
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GGATGCGGTGGGCTCTATGGCTTCTGAGGCGGAAAGAACCAGCTGGGGCTCT
AGGGGGTATCCCCACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGG
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ATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGC
CTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGC
CCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAA
CGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCAC
TGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAA
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GTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTG
GCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTC
AAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCC
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CTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGT
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TGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATT
TAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCAC
CTGACGTC

Backbone

- Vector backbone: pcDNA3.1
- Backbone size w/o insert (bp): 5775
- Vector type: Mammalian Expression

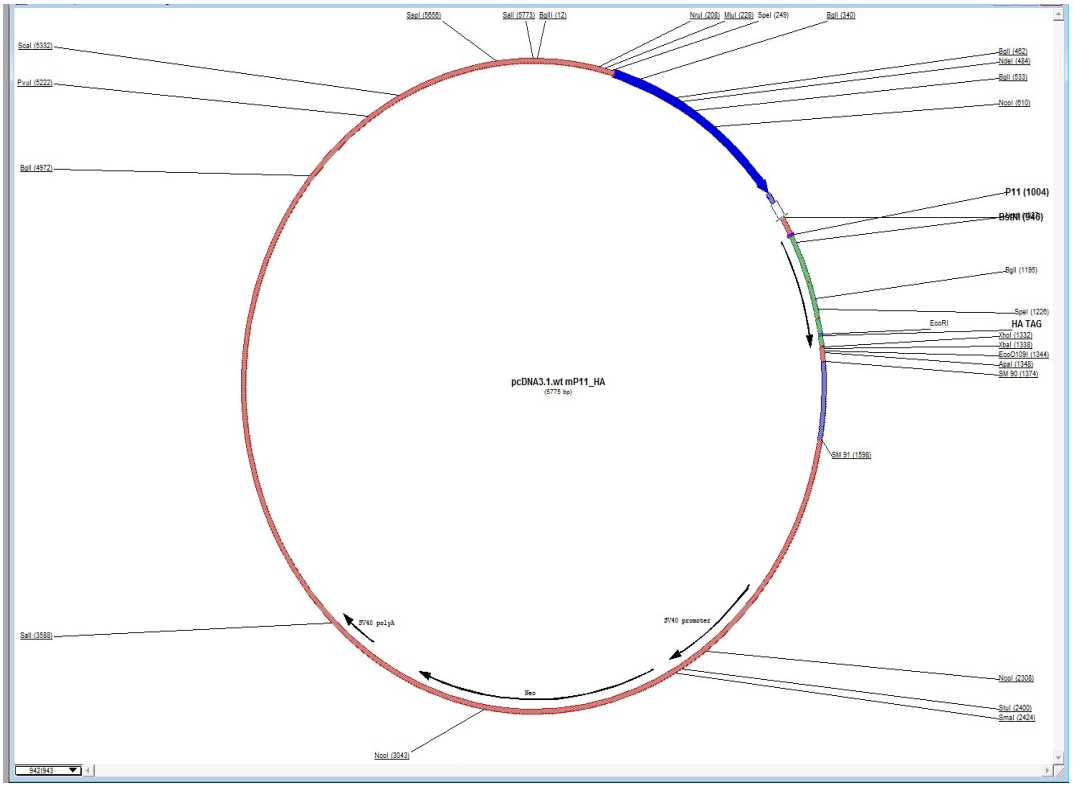

Figure 4 (A). 1 p11 plasmid vector map

Growth in bacteria

- Bacterial Resistance: neomycin
- Growth Temperature: 37°C
- Growth Strain(s): DH5alpha
- Copy number: High Copy

Gene/Insert name

- Gene/insert: p11
- Species: M. musculus (mouse)
- Promoter: CMV
- Tag / Fusion Protein: HA
- Restriction sites: Xhol (1332), Xbal (1338), Eco01091 (1344), Apal (1348), SM90 (1374)

4 (A).2 Rationale for selection of eGFP gene

Among these reporter genes, green fluorescent protein has been employed most commonly due to their wide spread established use and ease of availability (4). Hence, in context of development of gene delivery systems, green fluorescent proteins have become essential tools in order to evaluate the intracellular delivery and expression of genes through easy

fluorescence detection systems whose vector map is show in figure 4 (A). 2. Similar to the therapeutic cDNA, the cDNA for GFP can be used to develop lipoplex systems which can be used for in vitro cell line studies in order to evaluate the transfection potential of lipoplex based gene delivery system. Genes for green fluorescent protein are easily available at cost effective rates. Among the variants of Green fluorescent proteins, eGFP gene gives more robust expression of green fluorescence and as the name suggests the enhanced fluorescence activity allows sensitive detection of the gene (4)**.**

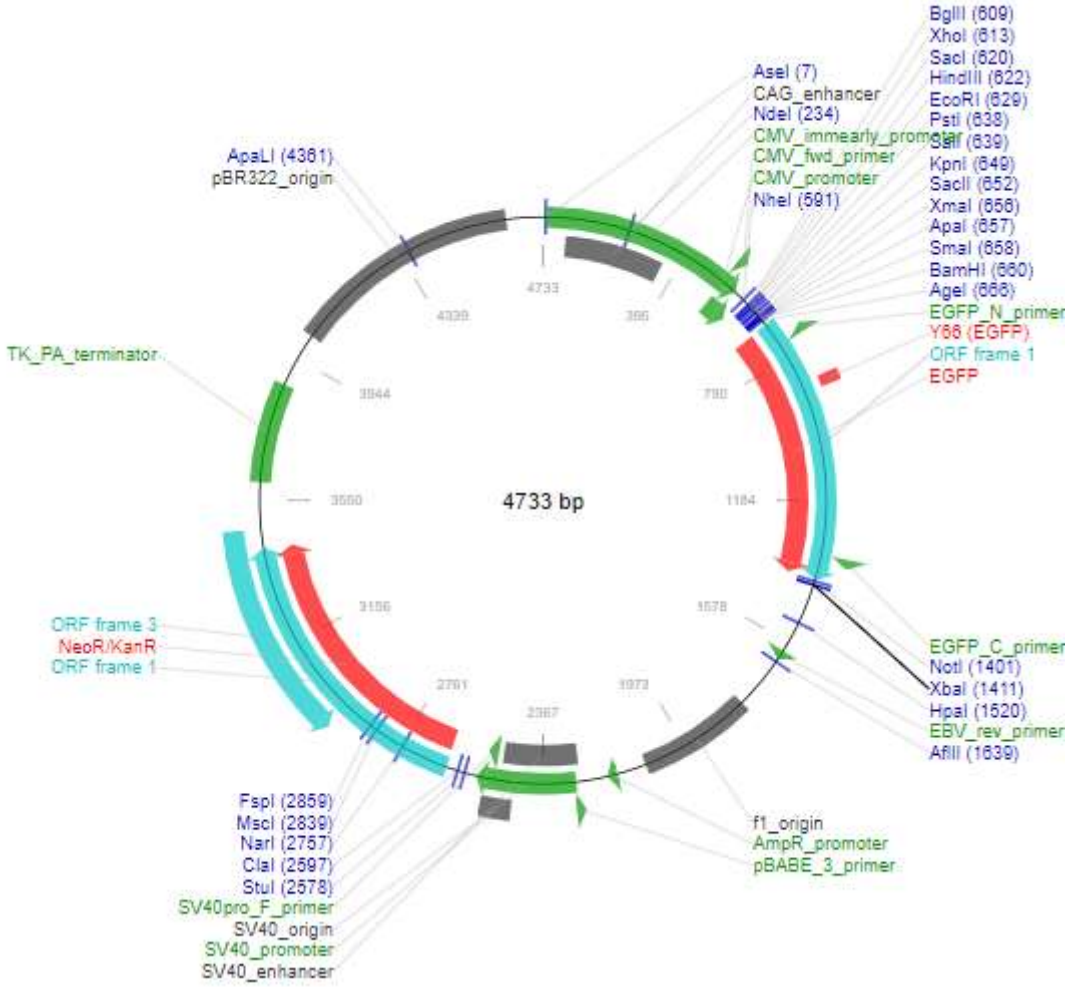

Figure 4 (A). 2 eGFP plasmid vector map

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GACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAAT
AGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACT

TGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAAT
GACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTATGGGACTT
TCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCG
GTTTTGGCAGTACATCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTC
CAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTTTTGGCACCAAAATCAA
CGGGACTTTCCAAAATGTCGTAACAACTCCGCCCCATTGACGCAAATGGGCGG
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ACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGG
CATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAG
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GCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAAC
GAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCA
CTCTCGGCATGGACGAGCTGTACAAGTAAAGCGGCCGCGACTCTAGATCATA
ATCAGCCATACCACATTTGTAGAGGTTTTACTTGCTTTAAAAAACCTCCCACA
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CAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCATAGT
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ATTCTGTGGATAACCGTATTACCGCCATGCAT

Backbone

- Vector backbone: pcDNA3
- Backbone size w/o insert (bp): 6160
- Vector type: Mammalian Expression

Growth in bacteria

- Bacterial Resistance: kanamycin
- Growth Temperature: 37°C
- Growth Strain(s): DH5alpha
- Copy number: High Copy

Gene/Insert name

- Gene/insert: eGFP
- Species: M. musculus (mouse)
- Promoter: CMV
- Tag / Fusion Protein: -
- Restriction sites: Xhol, Xbal

4 (A).3 Plasmid elements

Replication Origin (ORI): The ORI is responsible for the capability of the particular plasmid to be amplified or copied by bacteria that is the most significant distinguishable characteristic which makes it suitable for the application (5). ORI is sequence of DNA which leads the replication of plasmid by employing transcriptional devices of bacteria at work.

Antibiotic Resistance Gene: This gene delivers the advantage of endurance to the individual bacterial host containing antibiotic resistance gene. All the bacteria contains thousands of copies of particular plasmid and they would ideally get duplicated upon the process of cell division along with addition to their own DNA. Owing to this additional burden, cell division rate in bacteria get reduced. The simple logic would be the extra time needed to replicate this additional DNA results in slow cell division. This also ends up in fitness problem in bacteria, those bacteria without the additional DNA in form of plasmids has the ability to replicate faster and out-grow the bacteria with plasmids (6).

To verify the maintenance of plasmid DNA in bacteria, a gene which is resistance to antibiotics in incorporated as a part of plasmid. As an example, bacteria carrying the plasmid with ampicillin has the ability to survive and grown in presence of ampicillin. Bacteria with no plasmid DNA cannot persist with the treatment of antibiotics, under such situations, there has been an additional burden to carry the plasmid DNA, in spite of the added pressure of replication.

Insert: The insert is the gene, promoter, or other DNA fragment cloned into the MCS. The insert is typically the genetic element one wishes to study using a particular plasmid.

Promoter Region: It is the promoter region who determines the transcription through the insert. Function of promoter is the recruitment of machinery for transcription activity from a very specific organism or a set of organisms (7). If the particular plasmid in to be inserted in human, its promotor should be a human or from the mammalian sequence of promotor. Promotors also have ability for cell specific activity of expression, which can easily be attained with the use of tissue specific promoter (e.g. brain or liver or kidney specific promoters). The power or strength of the promoter is also very vital to control the expression level in particular cells.

Selectable Marker: They are used to choose particular cells who has been efficiently occupied with the plasmid for the very purpose of the expression of gene of interest. Although it is very much diverse to the bacterial cells which have taken up the plasmid for the sole resolution of replication only. This selectable marker can usually be in form of antibiotic resistance gene or some fluorescent protein which can be actively visualised under

confocal microscopy or FACS. For example HA-tag is the kind of expressive plasmids that is specifically designed to synthesis tagged proteins in cells with the human influenza hemagglutinin (HA) epitope for the detection of this tagged protein using western blot or immune blotting or immunohistochemistry or immunocytochemistry which can purify this tagged proteins with the help of affinity chromatograph (8).

Primer Binding Site: A very short, ssDNA (single stranded DNA) which can be used for the initiation for amplification in PCR reaction. Primers can also be used for the verification of the gene sequence of insert or many other regions of plasmid (9).

4 (A).4 Transformation of p11 cDNA

Plasmid or vector transformation is the process by which exogenous DNA is transferred into the host cell. Transformation usually implies uptake of DNA into bacterial, yeast or plant cells, while transfection is a term usually reserved for mammalian cells. Typically the method for transformation of a DNA construct into a host cell is chemical transformation, electroporation or particle bombardment. In chemical transformation, cell are made competent (able to take up exogenous DNA) by treatment with divalent cations such as calcium chloride, which make the bacterial cell wall more permeable to DNA (10). Heat shock is used to temporarily form pores in the cell membrane, allowing transfer of the exogenous DNA into the cell (11). In electroporation, a short electrical pulse is used to make the bacterial cell temporarily permeable. Particle bombardment, is typically used for the transformation of plant cells. Gold or tungsten particles are coated with the DNA construct and physically forced into the cell by gene gun.

4 (A).5 Transformation of competent *E. coli* using calcium chloride

As DNA is a highly hydrophilic molecule, normally it cannot pass through the cell membrane of bacteria (12). Hence, in order to make bacteria capable of internalising the genetic material, they must be made competent to take up the DNA. This can be achieved by making small holes in bacterial cells by suspending them in a solution containing a high concentration of calcium. Extra-chromosomal DNA will be forced to enter the cell by incubating the competent cells and the DNA together on ice followed by a brief heat shock that causes the bacteria to take up the DNA. Bacteria no longer become stable when they possess holes on the cell membrane and may die easily. Additionally, a poorly performed procedure may lead to not enough competence cells to take up DNA. It has been reported that a naked DNA molecule bound to the lipopolysaccharide molecule, cannot itself cross the cell membrane to enter into the cytosol. The heat shock step strongly depolarizes the cell membrane of $CaCl_2$- treated cells (13). Thus, the decrease in membrane potential lowers

the negativity of the cells inside potential which ultimately allows the movement of negatively charged DNA into the cell's interior. The subsequent cold shock again raises the membrane potential to its original value.

Protocol:

1. Grow colonies of *E. coli* (DH5-α) in 6 ml of LB medium at 37°C overnight with shaking.
2. Add the overnight culture to 300 ml of LB Medium and grow to Optical Density at 550 nanometers (OD_{550}) = 0.45 to 0.55 (about 2 hr).
3. Decant the cell suspension into six 50 ml tubes and cool on ice for 15 min with occasional swirling of the tubes.
4. Centrifuge the tubes at approximately 2,500 rpm for 15 min in a table-top centrifuge.
5. Completely remove the supernatant (use aspiration for the last few ml), and resuspend each pellet in 15 ml of cold 0.1 M $MgCl_2$ by gently vortexing.
6. Combine cells into two tubes and centrifuge as in Step #4.
7. Completely remove the supernatant, and resuspend each pellet in 20 ml of cold 0.1 M $CaCl_2$.
8. Incubate on ice for 20 min.
9. Centrifuge tubes at approximately 2,500 rpm for 10 min in a table-top centrifuge.
10. Completely remove the supernatant, and resuspend each pellet in 6 ml of 0.1 M $CaCl_2$/15% Glycerol and combine into one tube.
11. Transfer 100 µl aliquots of competent cells to microcentrifuge tubes and freeze at -80°C until used in a transformation.

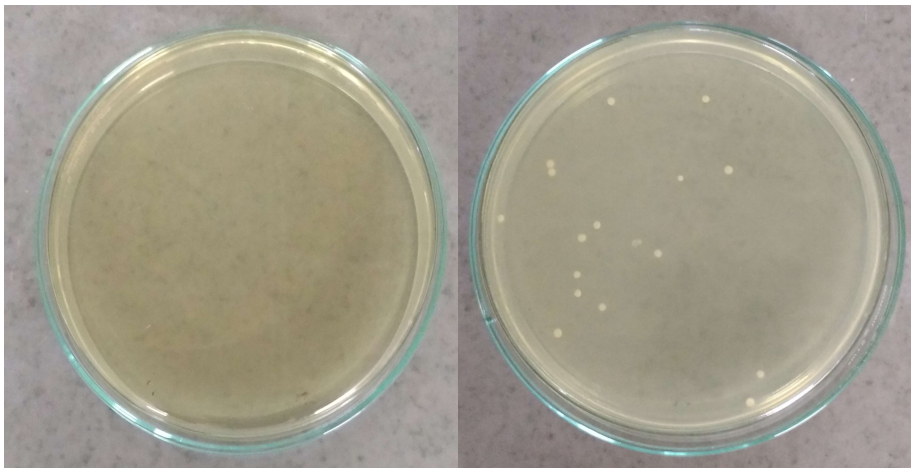


Figure 4 (A). 3 Cultures for plasmid preparation

The transformation of both the plasmids into prepared competent cells was confirmed by the growth of the competent cells on agar plates with and without antibiotic to which the plasmid DNA is resistant. In the figure 4 (A). 4, the first plate showed no growth of any colony confirming absence of antibiotic resistance gene resulting in complete suppression of competent cells by G418 (neomycin) while the second plate showed growth of colony for the antibiotic resistant plasmid. Only those cells that contain the antibiotic resistant gene for neomycin survived, grew and reproduced resulting in further isolation of pure form of plasmid.

4 (A).6 Isolation and purification of plasmid

For the isolation of plasmid DNA or RNA from the bacteria culture, alkali lysis is one of the most frequent applied method (14). It is also known as method of choice for plasmid isolation as it gives dependable, quick and results in comparatively very clean isolation. DNA obtained from this procedure can additionally be purified if necessary. This method hinges on the very exceptional property of plasmid DNA that it can easily and very quickly anneal itself following the step of denaturation (15). The following 4 important steps allow the plasmid DNA to be isolated from the chromosome of bacteria:

Cell Growth and Harvesting

This procedure as most of other methods, start usually with the step of cell culturing of bacteria harboured with plasmid containing DNA of interest. After achieving sufficient bacterial growth, they were further centrifuged to convert bacterial cells into pallet which also helps in eradiating growth medium from the cells.

Re-suspension

The pellet obtained is subsequently suspended in a solution frequently called as solution I which comprising of RNase A, EDTA, sugar (glucose) and Tris buffer. For the integrity of bacterial cell wall the presence of divalent cations like calcium or magnesium are vital. EDTA supports the process by help in decreasing the stabilization of cell wall with its ability of chelating calcium and magnesium ions present in solution, in a way that aids in prevention of damage to plasmid from DNAase (16). Glucose plays vital role in maintaining osmotic pressure such a way that cells sustain its integrity, at the same time cellular RNA released after cell lysis, is degraded by RNase (17).

Lysis

The solution II or lysis buffer comprises of NaOH (Sodium Hydroxide) and the non-ionic detergent SDS (Sodium Dodecyl Sulfate). SDS helps in solubilising the cell membrane. NaOH plays important role in disrupting the hydrogen bonds present between various DNA base pairs which converts double stranded DNA and genomic DNA to single stranded DNA along with breaking cell wall (18). This is the reason why this process is known as alkali lysis as the process of denaturation is at the core of the procedure. Proteins present in bacterial cell walls also denatured by SDS, which ultimately helps in isolation of protein from the plasmid.

Neutralisation

Solution III which is usually potassium acetate helps in increasing the pH of the mixture. At alkaline pH, re-establishment of hydrogen bonding between the single stranded DNA base pairs occur and single stranded DNA results in double stranded DNA (19). It is one of the most selective part of the process. The huge DNA material present in bacteria cannot anneal themselves but the small plasmid DNA present in cell can be easily renatured. The vigorous mixing during the mixing or the vortexing stage can affect the annealing process and genetic material other than plasmid DNA can result in shorter stretches and this can affect the purity of plasmid to be isolated. The double stranded DNA has the ability to be dissolved in solution while the single stranded DNA along with detergent SDS and denatured proteins stay together to form white precipitate with the help of hydrophobic interactions (20). The formed precipitation can be isolated using centrifugation at low RPM.

Cleaning and concentration

Even though the plasmid has been isolated from the cell, the solution contains various salts, RNase, denatured proteins, cell debris, excess of EDTA which needs a subsequent stage of cleaning up to concentrate the plasmid in solution. This cleaning step is proceed with the

use of phenol/chloroform extraction which is followed by the precipitation by ethanol and later on affinity based chromatography is performed.

Protocol

1. Inoculate 3 ml of LB containing G418 antibiotic with a single colony of transformed bacteria and grow it overnight at 37°C with shaking.
2. Pour culture into a microfuge tube. Centrifuge at 8000 rpm for 2 minutes at 4°C in a microfuge. Remove the medium and leave the bacterial pellet at bottom of the tube.
3. Resuspend the bacterial pellet in 1ml of ice-cold solution I by vortexing.
4. Centrifuge at 8000 rpm for 2 minutes and discard the supernatant.
5. Resuspend the bacterial pellet in 100 µl of ice-cold solution I by vortexing and incubate on ice for 5 minutes.
6. Add 200 µl of freshly prepared solution II to each bacterial suspension. Close the tube tightly, and mix the contents well by inverting the tube. Store the tube in ice for 5 minutes.
7. Add 150 µl of ice-cold solution III. Close the tube and disperse alkaline lysis solution III through the viscous bacterial lysate by inverting the tube several times. Store the tube in ice for 15 minutes.
8. Centrifuge the bacterial lysate for 15 minutes at 12000 rpm at 4°C in a microfuge. Collect the supernatant to a fresh tube.
9. Add equal volume of phenol: chloroform. Mix the organic and aqueous phases by vortexing and then centrifuge the emulsion at 10,000 rpm for 5 minutes at 4°C in a microfuge. Transfer the aqueous upper layer to a fresh tube.
10. Add equal volume of isopropanol and $1/10^{th}$ volume of 3M sodium acetate. Mix thoroughly by repeated gentle inversion.
11. Centrifuge at 12000 rpm for 15 minutes at 4°C.
12. Discard the supernatant leaving pellet in the tube which is DNA.
13. Add 400 µl of 80% ethanol to the pellet and centrifuge at 12000 rpm for 10 minutes at 4°C.
14. Remove all of the supernatant by aspiration. Take care with this step, as the pellet sometimes does not adhere tightly to the tube.
15. Store the open tube at room temperature until the ethanol has evaporated and no fluid is visible in the tube.
16. Dissolve the pellet in 20 µl of sterile water containing 20 µg/ml RNase A. Vortex the solution gently for a few seconds and store the DNA at -20°C.

Recipes for Buffers, Solutions and Media:

Solution I

- 50 mM glucose.
- 25 mM Tris-Cl (pH 8.0).
- 10 mM EDTA (pH 8.0).

Prepare Solution I from standard stocks in batches of approx. 100 ml, sterilize by autoclaving and store at 4°C.

Solution II

- 0.2 N NaOH (freshly diluted from a 10 N stock).
- 1% (w/v) SDS.

Prepare Solution II fresh and use at room temperature.

Solution III

- 5 M potassium acetate, 60.0 ml.
- Glacial acetic acid, 11.5 ml.
- H_2O, 28.5 ml.

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate. Store the solution at 4°C and transfer it to an ice bucket just before use.

EDTA

To prepare 0.5 M EDTA (pH 8.0): Dissolve 186.1 g of disodium EDTA•2H2O in 800 ml of Distilled water. Stir well on a magnetic stirrer. EDTA will not dissolve into solution until the pH of the solution is reached to ~ 8.0. So the pH should adjust to 8.0 with NaOH (~ 20 g of NaOH pellets) and make up the final volume to 1000ml with distilled water. Prepare the aliquots and sterilize by autoclaving.

LB Media

To prepare LB (Luria-Bertani) medium, weigh 20gm LB powder (himedia) and make up the final volume of the solution to 1 liter with deionized H2O. Then sterilize it for 20 minutes by autoclaving at 15 psi.

NaCl

To prepare 5 M NaCl : Dissolve 292 g of NaCl in 800 ml of sterile H2O and the volume is make up to to 1 liter with deionized H_2O. Prepare the aliquots and sterilize it by autoclaving.

NaOH

To 800 ml of H2O, add 400g of NaOH pellets slowly, stirring continuously. After dissolving the pellets, completely, make up the final volume to 1 liter with sterile H2O. Store the solution at room temperature.

Potassium Acetate

- 5 M potassium acetate, 60 ml.
- Glacial acetic acid, 11.5 ml.
- H_2O, 28.5 ml.

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate. Store the solution at room temperature.

SDS

Also called sodium lauryl sulfate. To prepare a 20% (w/v) solution, dissolve 200 g of SDS in 900 ml of H_2O. Heat to a temperature of 68°C and stir with a magnetic stirrer to help dissolution. Adjust the volume to 1 liter with distilled H_2O. Store at room temperature. Autoclaving not necessary.

4 (A).7 Plasmid digestion

Restriction enzymes are naturally occurring bacterial endonucleases that recognize a large range of DNA sequences (21). Given the variety of these enzymes and the unique sites they recognize, restriction digests have become the most widely used method scientists employ to selectively move a specific piece of DNA from one plasmid to another. A diagnostic restriction enzyme digest takes advantage of the fact that restriction enzymes cleave DNA at specific sequences called restrictions sites as shown in figure 4 (A). 4. Often, the size of the plasmid insert and vector backbone are known and thus this technique can be quickly used to verify plasmid. Restriction enzymes are naturally occurring bacterial endonucleases that recognize a large range of DNA sequences. Given the variety of these enzymes and the unique sites they recognize, restriction digests have become the most widely used method scientists employ to selectively move a specific piece of DNA from one plasmid to another. A diagnostic restriction enzyme digest takes advantage of the fact that restriction enzymes cleave DNA at specific sequences called restrictions sites. Often, the size of the plasmid insert and vector backbone are known and thus this technique can be quickly used to verify your plasmid.

Often, it will be enough to know that you have a 1,200 bp insert in a 5,000 bp backbone, but there are many plasmids out there that, when digested with restriction enzymes common to multiple cloning sites, will result in similar sized bands, thus making this simple digest

less informative. This is particularly true if you receive a plasmid from someone in another lab, or dig one out of the freezer and you are not 100% sure it is what you are looking for, but you have a map and know exactly what it should be. A useful restriction enzyme based technique for verifying plasmids like this is "plasmid fingerprinting", where you cut the plasmid into 3-8 pieces such that all (or most) fragments are small enough to be accurately sized on a gel and also such that they are different enough in size to be easily resolved from each other. However, by choosing an enzyme or enzymes that will cut your plasmid into multiple fragments, you can get a very unique pattern that will distinguish one 5kb backbone with a 1.2kb insert from all others.

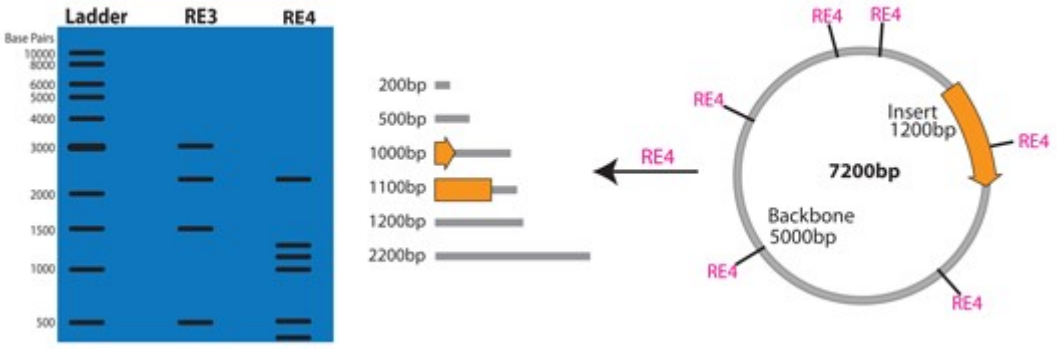


Figure 4 (A). 5 Plasmid digestion by restriction enzymes

Protocol

1. Add components to a clean tube in the order shown:
   1 µL DNA (concentration 1 µg/µL)
   2 µL 10x buffer
   1 µL restriction enzyme
   16 µL sterile water
2. Incubate the reaction at digestion temperature (usually 37°C) for 1 hour.
3. Stop the digestion by heat inactivation (65°C for 15 minutes)
4. The digested DNA is ready for further analysis.
5. the above cocktail was loaded

Along with molecular marker and supercoiled cDNA in three different wells into 1.2 % agarose gel containing ethidium bromide and the fluoroscence was detected under UV transilluminator (GelDoc Image XR+, BioRad, USA). Images were captured by ImageLab software ver. 5 (BioRad, USA). The band position of our isolated plasmid is

compared with the band of known molecular weight of marker to verify molecular weight of isolated plasmid.

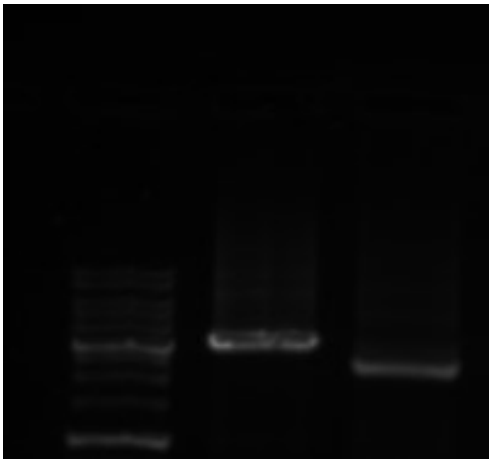


Figure 4 (A). 6 Digestion of p11 cDNA

The digestion of the isolated plasmid was carried out by restriction endonuclease enzyme for confirming the cDNA with the transformed DNA. Lane 1 shows DNA markers, lane 2 shows digested plasmid and lane 3 represents plasmid without digestion. An intact vector with or without insert is circular and under tension so it's lowest energy conformation is twisted and knotted. Lane 3 confirms the p11 plasmid, showed one band correspond to 5775 bp thereby confirming the p11 plasmid. Once the insert is cut out the tension is destroyed and only one conformation (more linear) is produced and it can be visualised as single band in agarose gel imaging. Because this digested plasmid is a different shape from the circular it runs at an apparently different size and this is smaller than the uncut plasmid (22) as depicted in figure 4 (A). 7.

4 (B) Cationic lipids for lipoplex

The use of cationic lipids for enabling the access of therapeutic cDNA and siRNA into living cells has been defined in numerous scientific literatures (23, 24). The fundamental concept is to work an arginine residue containing guanidine group and histidine, lysine containing ammonium group, as an aspirant for a cDNA delivery vehicle degraded by enzymes present in cell, and such degradation results in fragments that would be natural ubiquitous metabolites of a cell (25). The charged functional groups found on hydrophilic arm (negative/ zwitterionic in nature) are beneficial in bilayers formation at the physiological pH. Liposomal delivery is therefore more advantageous using than any other formulations. Most specifically, a compound which bears a phosphatidyl group, here it is, N-L-Arginyl-phosphatidyl-ethanolamine was used for the same disclosed in the present invention.

According to above mentioned hypothesis, we have conjugated arginine, lysine and histidine to a natural phospholipid, phosphatidylethanolamine (PE), and 3 modified lipids were synthesised. These lipids have cationic charge owing to the presence of basic groups like a guanidine group of the arginyl residue and ammonium group in lysine and histidine. These molecules would be effortlessly fragmented by peptidases present in cells into its starting components, arginine/lysine/histidine and PE, both of them are natural cellular constituents. The synthesised lipids alone cab also adept in forming liposomes in an aqueous media. As cationic liposomes has the ability for intracellular delivery of nucleic acids, the liposomes formulated using the synthesised lipids have efficacy for the gene delivery (26). This approach is used for the treatment of various disease condition including depression which can be classified under CNS disorders. Present work relates largely to a non-toxic lipid, conjugated with a cationic (alkaline) amino acids containing a guanidino in arginine or an ammonium group as in histidine and lysine. Explicitly, the naturally occurring phospholipid DOPE is conjugated with the aforementioned, naturally-occurring amino acids which can be an improvement over present compounds containing positive charge.

This increase in effectiveness with the fusogenic lipid DOPE, specifically, has been fundamentally credited to a conformational change in liposome structure from a lamellar to a more hexagonal-phase (27). This distinctive nature of DOPE coins from its structure which is an inverted cone shape, implicates its small head group comparative to its hydrophobic tails. This particular shape of the lipid is allegedly encourages lipid interaction between the membranes of endosome and the lipoplex, affecting fusion leading to endosomal escape (28). This shape averts DOPE being able to form liposomes individually

and hence it must be mixed with other lipids (most probably phosphatidylcholines) or sterols (cholesterol) with superior head groups to form liposomes (29).

4 (B).1 Use of BOC protected amino acids

Protecting group (PG) is a molecule of small size, which has the ability of protecting temporarily the host group from ongoing reaction, permitting the remaining functional groups existing in the molecule to react without disturbing the usual reactivity and separated from the molecule without disturbing the remainder functional groups (30).

One of the most prominent issue in peptide chemistry is the protection of α-amino functionality of amino acids and it is compulsory to protect amino acid polymerization after its activation. Protection of nitrogen moiety remains as a great deal of consideration in a wide range of chemical fields including activity of various amino acids. Amine group protection with tert-butyloxycarbonyl (Boc) group is an extensively used reaction because of its inertness concerning catalytic hydrogenolysis and hydrolysis resistance underneath basic conditions and nucleophilic reagents.

4 (B).2 Synthesis of amino acid modified lipid

Arginine, lysine and histidine are amino acids, alkaline in nature with positive charge, which can integrate good hydrophilic head groups to any moiety be it lipid or polymer. Due to the diverse nature of amino acids, many correspondents and their amino acid derivatives were acquaint with` gene delivery carriers. Above and beyond the cationic headgroups, the hydrophobic tail portion of cationic lipids were also an integral part in gene delivery and its transfection. Lipids-DNA complexes were stabilised by them, and also involved in intracellular lipoplexes permeation. Carbon chain/chains (saturated or unsaturated) as phospholipids, were frequently used as hydrophobic tails in positively charged liposomes, but their toxicity and stability issues do not allow them to fit the applications. For this reason, with the formation of an amide linkage between the amino group of PE and carboxy group of BOC protected amino acids, we have conjugated alkali amino acids to a natural phospholipid, phosphatidylethanolamine (PE) -DOPE. This molecule would be easily split by cellular peptidases into its original components, arginine and PE, both of which are natural cellular constituents bearing no chance for the cellular toxicity.

Procedure

Alkaline amino acids are positively charged at neutral pH; subsequently they could be good hydrophilic headgroups in cationic lipids. Three new cationic lipids were synthesised with alkaline amino acids (lysine, histidine, and arginine) headgroups and DOPE as hydrophobic

moiety. N α,N ß -di-Boc-L-lysine (L), N α ,N im -di-Boc-L-Histidine (H), and Boc-Arg(Mtr)-OH (A) were three Boc protected amino acids used in synthesis of the same.

Step I

Protected amino acids, DOPE and 4-dimethylaminopyridine (DMAP) in catalytic amount, were placed in a three necked flask (100 mL) equipped with a constant pressure dropping funnel and an inlet. The mole ratio of protected amino acid, DOPE (148.8 mg, 0.2 mmol) and DMAP was 1:2:02. Anhydrous dichloromethane (10 mL) was added in an atmosphere of nitrogen. A solution of dicyclohexylcarbodiimide (DCC, 2 equivalent of DOPE) in dichloromethane (10 mL) was added dropwise in the mixture over 1 h. After stirring for 1 h at 0º C, the mixture was stirred at room temperature for another 22 h. The white solid dicyclohexylurea (DCU) precipitate was removed by filtration. Filtrate was washed twice with 50 mL saturated sodium hydrogen sulfate, 50 mL saturated Sodium bicarbonate solution, and 50 mL saturated sodium chloride solution. DCM was dried using anhydrous potassium sulfate. Anhydrous potassium sulfate was used to dry DCM and helped it to concentrate (31).

Step II

Deprotection of protected lipids

0.2 mmol of protected lipid (for lysine and histidine modified lipids) was dissolved in 2 mL of anhydrous dichloromethane. Trifluoroacetic acid (2 mL) was added in the mixture and stirred for 2 h at 0º C in an atmosphere of nitrogen. Mixture was stirred at room temperature for another 2 h. Mixture was concentrated and recrystallized in anhydrous diethyl ether.

The μ-amino function of arginine was protected by 4-methoxy-2,3,6-trimethylbenzenesulphonyl (Mtr) group which can be easily removed by 0.20 M M methanesulphonic acid in trifluoroacetic acid–thioanisole (9:1) but is completely resistant to hydrogenolysis or treatment with neat trifluoroacetic acid hydrogen chloride. Therefore, methanesulphonic acid and thioanisole were used in addition to triflouroacetic acid in stead of only triflouroacetic acid as in case of deprotection of histidine and lysine modified lipids (31). The mechanism of DCC coupling and deprotection of BOS is represented graphically in figure 4 (B). 8, figure 4 (B). 2 and figure 4 (B). 3. Amino acid modifications of DOPE lipid is depicted in table 4 (B).1.

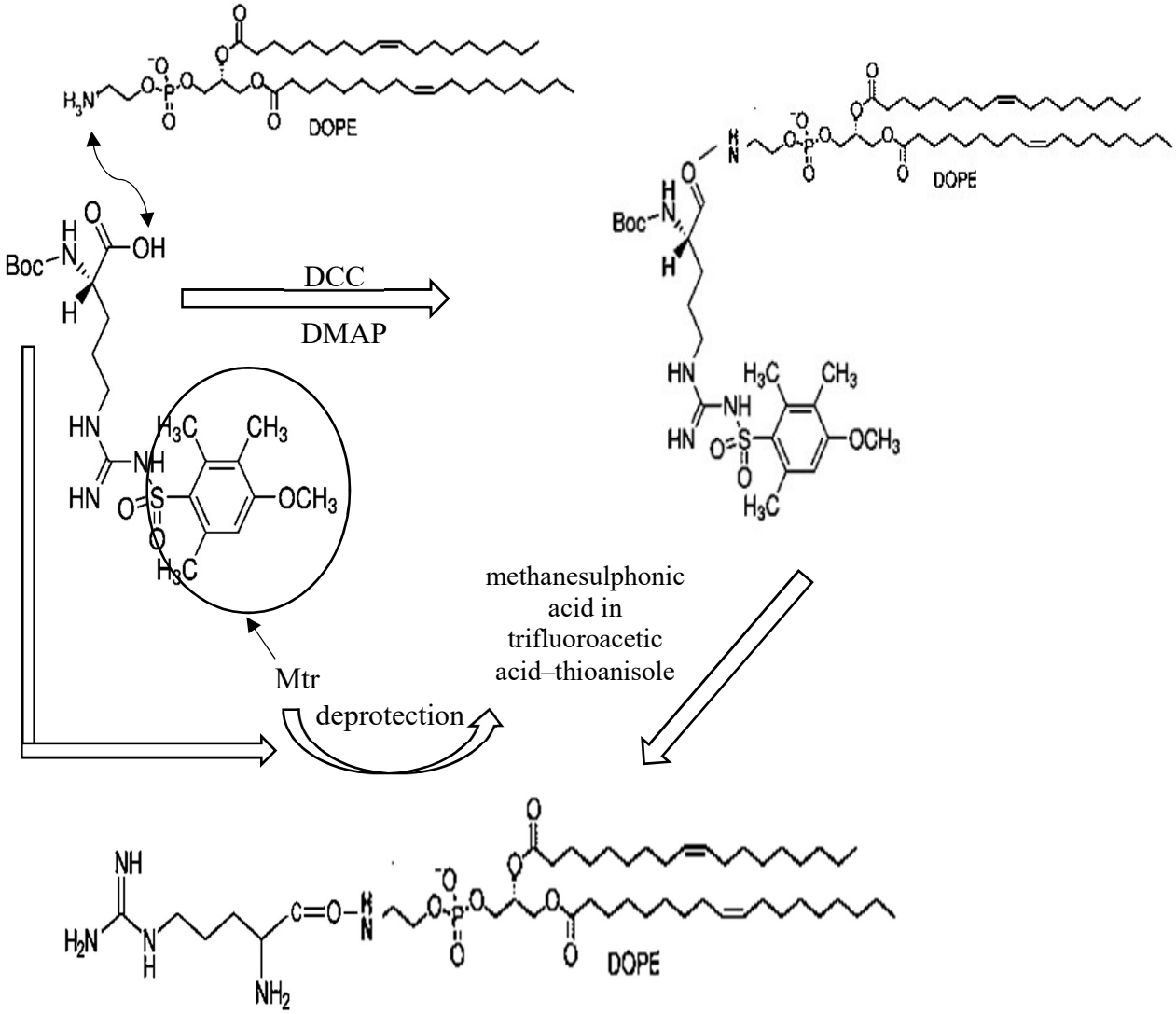


Figure 4 (B). 1 Reaction mechanism of amide formation between DOPE and N Boc- Arg (Mtr)-OH

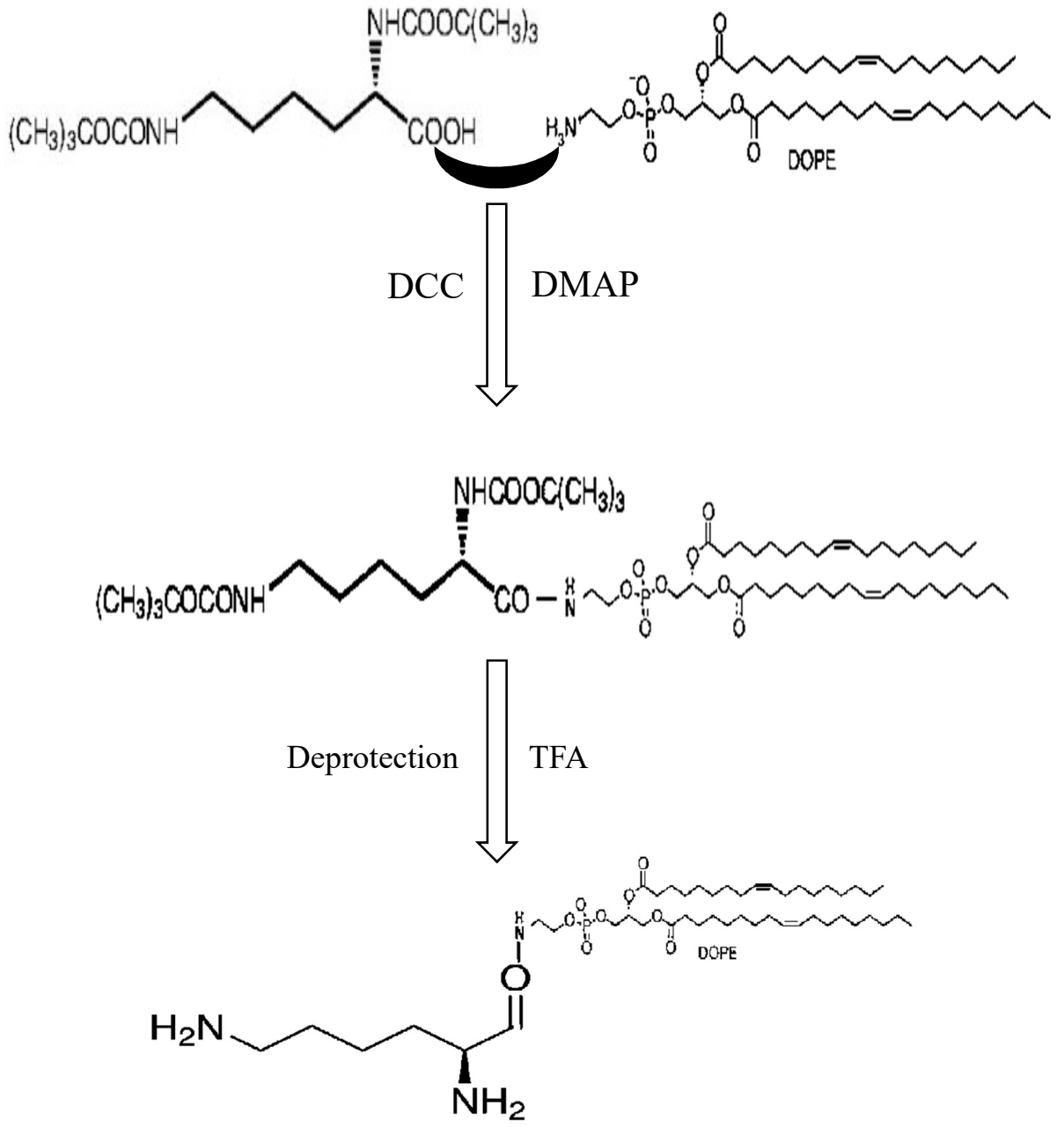


Figure 4 (B). 2 Reaction mechanism of amide formation between DOPE and N α,N ß -di-Boc-L-lysine

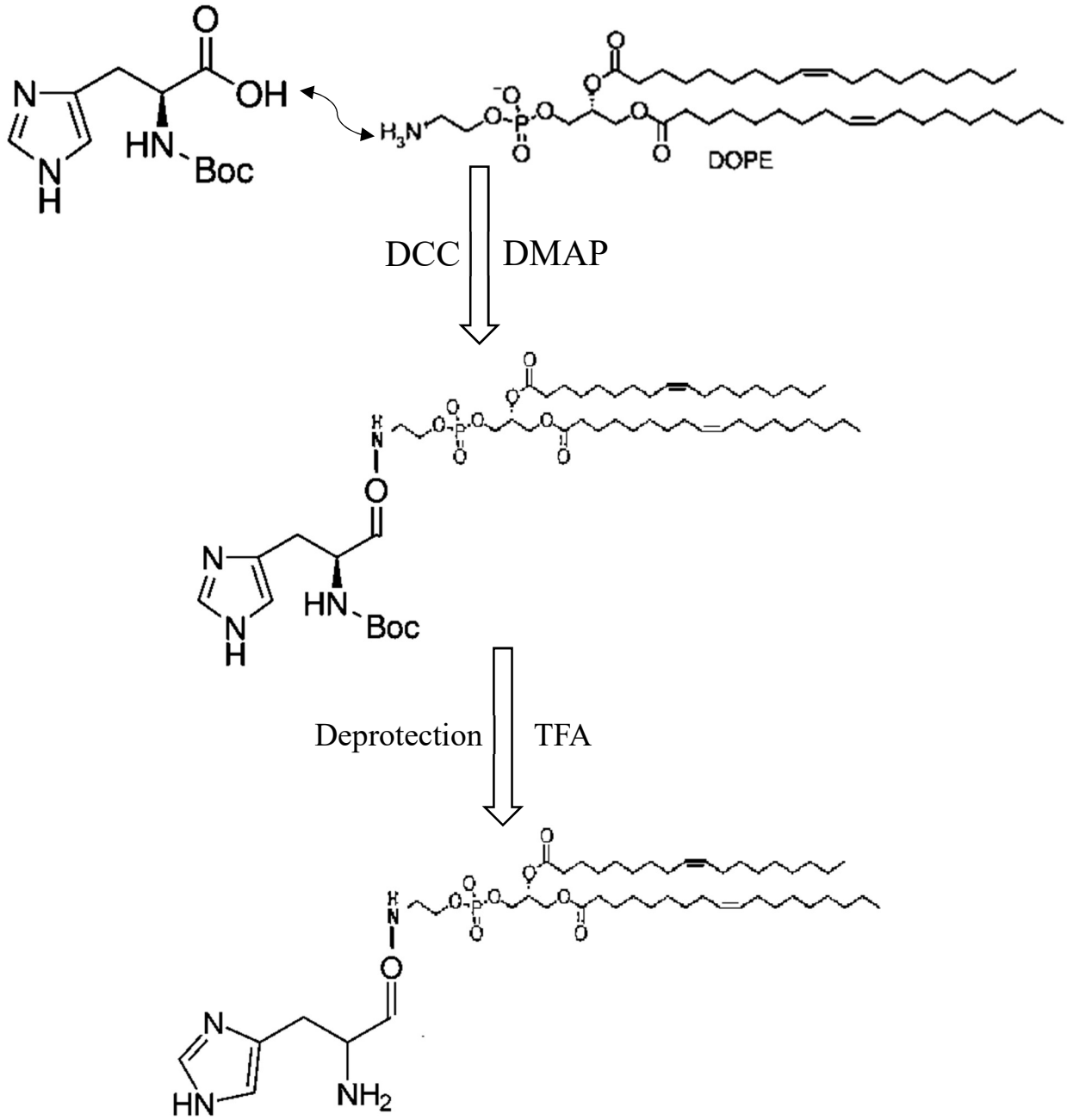


Figure 4 (B). 3 Reaction mechanism of amide formation between DOPE and N im -di-Boc-L-Histidine

Table 4(B). 1 Nomenclature of synthesised lipids for further reference

Modified lipid	Nomenclature
Arginine modified DOPE	DOPE-A
Lysine modified DOPE	DOPE-L
Histidine modified DOPE	DOPE-H

4 (B).3 TNBS assay

TNBS reacts with primary amines molecules to form an orange colour final product as shown in figure 4 (B). 4, whose absorbance at 335 to 345nm can be measured with a plate reader or spectrophotometer. TNBSA can be used for measuring amines, sulfhydryl, hydrazides, and amino groups.

TNBS reacts with primary amino group of DOPE lipid making it possible to estimate the percentage conjugation efficiency of DOPE to protected amino acids. Briefly, 500 µl of TNBS (20 mg/mL) solution was added to 1 mL of standard DOPE solution and amino acid conjugated DOPE solution in solvent system used for the development of calibration curve. After addition of 200 µl of sodium bicarbonate (0.8 M, pH 8.5), the solutions were incubated at room temperature for 60 min. 0.50 ml of 10% SDS and 0.250 ml of 1 N HCl were added to each sample after incubation and the absorbance was measured at 340 nm using UV spectrophotometer.

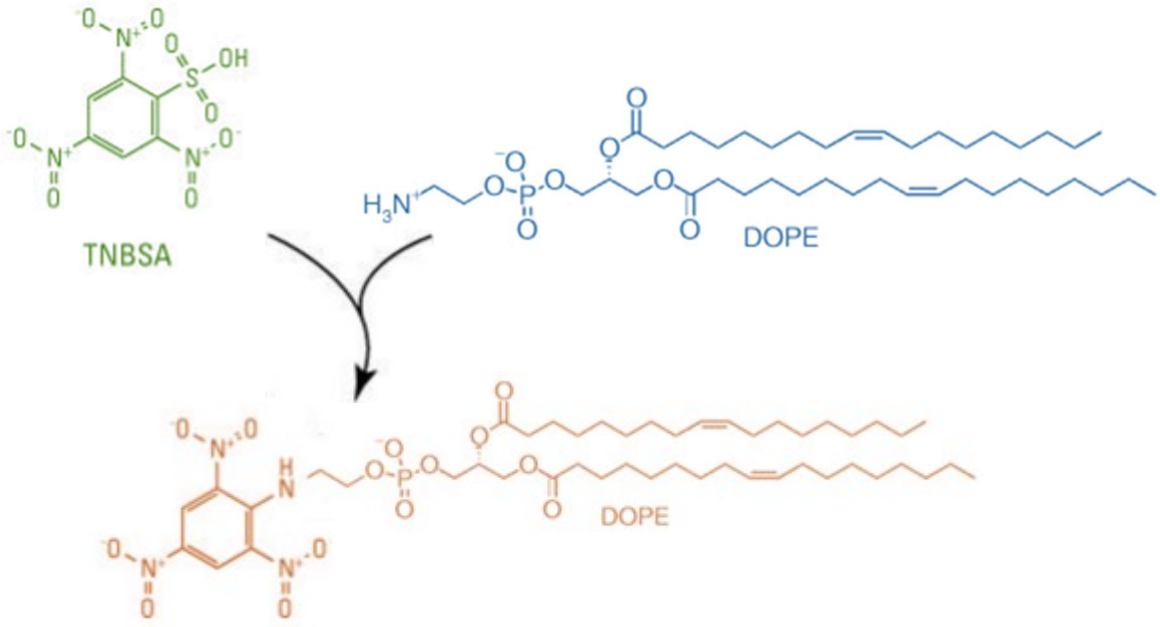


Figure 4 (B). 4 TNBS reaction scheme for the detection of primary amines present in DOPE

4 (B).4 Buffering capacity

The buffering capacity of modified lipids and DOPE from pH 10 to 3 was determined by using acid-base titration method as described in literature (32). Briefly, 10 mg of the lipid was dissolved in methanol: water in equal ratio to enable aqueous titration. The solutions were initially adjusted for pH 10 using 1 N NaOH solution and then titrated with 0.1 N HCl solution. This adjusted pH was measured using pH meter (Lab India, Mumbai). The electrodes were also calibrated with buffer solutions of pH 4.0, 7.0 and 9.2. This buffer capacity was determined as an indication of endosomal escape capacity. It was defined as the percentage of amine group protonated from pH 7.4 to 5.5 mimicking the pH change from the extracellular environment to the lower pH of the endosomes which can be calculated using following equation:

Buffer capacity (%) = $(\Delta v \times 0.1M \times 100) \div N\ mol$

Wherein V is the volume of 0.1 N HCl added to bring the pH of lipid solution from 7.4 to 5.5, and N mol is the total moles of protonable amine groups in the given portion of lipid solution. Additionally the ratio of protons consumed in intervals of 7.4 - 5.2 to 10-7.4 was also tracked as another indicator of effect of amino acid conjugation on basicity of DOPE lipid and its pKa value.

4 (B).5 Gas chromatography

Gas chromatography (GC) is used to isolate and identify small molecular weight molecules in the gas phase. The sample is applied to the injection port in vaporised form either in a gas or a liquid form. Usually it is difficult to vaporize larger compounds, the samples to be investigated are less than 1,000 Da for this method. GC is popular for industrial uses because it is very dependable and can almost run uninterruptedly. It is usually used where small, volatile molecules are identified with organic solvents. To isolate and identify larger molecules, liquid chromatography is more prevalent as the molecules need not to be vaporised. GC is preferred for nonpolar molecules while LC is usually used for the separation of polar analytes. Flame-ionization detection (FID) is a good detector for organic compounds in GC that distinguishes the amount of carbon present in a sample (33). It is a destructive detector, as the entire sample is pyrolyzed and is unaffected by noncombustible gases and water.

In our case GC is useful to detect DCM (Di Chloro Methane) i.e. persisted in the modified lipid as a residual solvent which was used as a solvent in conjugation reaction of amino acids with DOPE.

4 (B).5.1 Toxic effects of residual solvent

DiChloroMethane (DCM) is a volatile liquid which is readily absorbed into the body by the lung and the skin (34). The smell verge in humans is different, but beyond 300 ppm it is certainly noticable. It is absorbed through the skin and can produce dermatitis. It is also an irritant, and can produce burns if splashed on the skin or in the eyes and not promptly removed. In high doses methylene chloride is a mild narcotic. Its use in painting has been reported to cause headaches, faintness, lethargy, tetchiness, impassiveness, and tickling in the limbs. High revelations to DCM have been stated to cause decease in many circumstances. Elongated exposure to high DCM level has brought about kidney and liver damage. CO produced by liver metabolism of DCM binds with haemoglobin in blood to produce carboxyhaemoglobin which is toxic for the heart patients.

4 (B).5.2 Instrumentation

A Gas chromatograph (Agilent technologies 6890N) equipped with a flame ionization detector, a Headspace sampler (Agilent technologies G1888) was used to load the sample. The headspace injector and GC conditions are provided below.

4 (B).5.3 Chromate condition

1 ml of standard and sample solutions were injected into injection port of the GC. The injection port temperature was sustained at 135 °C at a split ratio of 1:8, with nitrogen gas as a carrier. The flow rate of 0.6 mL min^{-1} was kept with 14 psi pressure and the detector temperature was set at 240 °C. Temperature gradient was also upheld at 45 °C for around twenty five min and then increased at a rate of 10 °C min^{-1} up to 230 °C to a final temperature of 230 °C and maintained for 2.5 min.

4 (B).5.4 Preparation of standard and sample

DMF (N, N-Dimethyl Formamide) was selected as the standard and sample diluent because of its capability to dissolve DCM (35). It is a high boiling point solvent that does not hamper with DCM in sample to be analysed by GC. A common standard stock solution in DMF containing all known quantity of residual solvent dichloromethane was prepared in such a way that it had absolute concentration of 600 ppm.

4 (B).5.5 Specifications of Headspace injector and GC conditions

Headspace injector Gas Chromatography Oven equilibration temperature 80°C Column DB-624, Supelco, 30 m length, 0.32 mm internal diameter, and 1.8 µm film thickness, Loop temperature 100°C, Carrier gas Nitrogen Transfer line temperature 120°C, Flow rate 0.5 mL per minute (Linear velocity 26 cm/sec), GC cycle time 55 min, Injector temperature 40

°C Oven/vial, equilibration time 20 min, Detector temperature 250 °C, Pressurization time 0.5 min, Split ratio 1:5, Loop fill time 1.0 min, Oven temperature program Initial 40 °C, held for 20 minutes, Injection time 0.5 min Increase at 10 °C per minute to 240 °C, Loop equilibration time 0.5 min Held at 240 °C for 2 minutes, Vial pressure 15 psi.

4 (B).6 Result and discussion

4 (B).6.1 Synthesis of amino acid conjugated DOPE

Initial synthesis of the lipids was confirmed by TLC analysis using $CHCl_3$: MeOH: HAc mixture as mobile phase and an additional spot was detected for synthesized lipids along with spots for starting materials. The synthesised lipid was separated from starting materials with mobile phase hexane: ethyl acetate in 8: 2 ratio using column chromatography. The isolation of desired compound was checked by single spot on TLC and later confirmed by IR spectra. FTIR spectra as shown in Figure 4 (B). 5, Figure 4 (B). 6, Figure 4 (B). 7 and Figure 4 (B). 8 of synthesized lipids showed one or more of the following characteristics which confirmed the structure of the compounds:

- Primary amine peak present in starting material DOPE is converted to secondary amine peak in synthesis lipid in the region of 3200-3400 cm^{-1}
- Presence of carbonyl functional group peak around 1650-1700 cm^{-1}
- Secondary amide –NH- stretching at 1450-1550 cm^{-1}

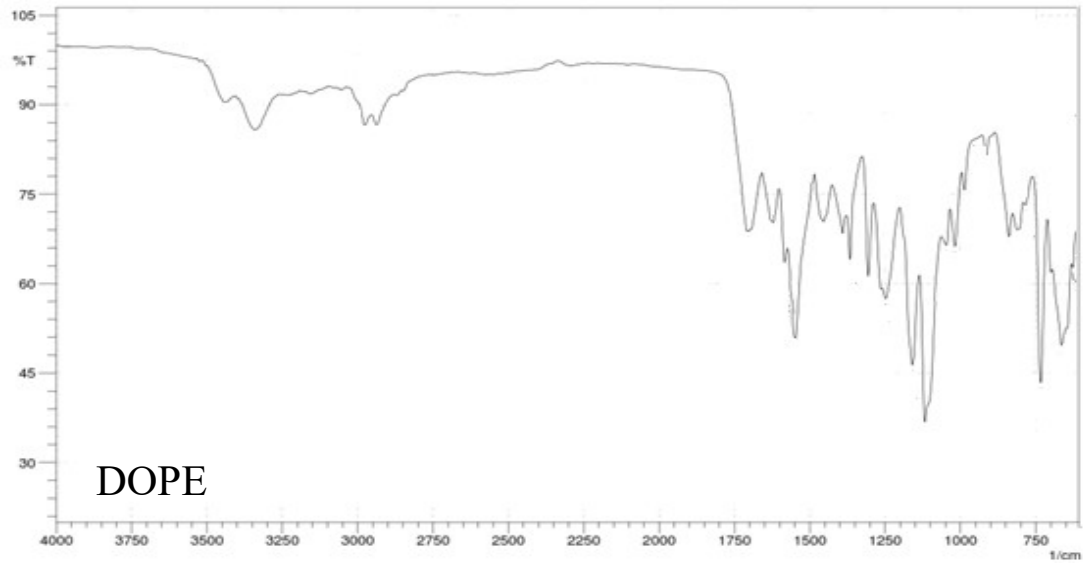


Figure 4 (B). 5 IR spectra of DOPE

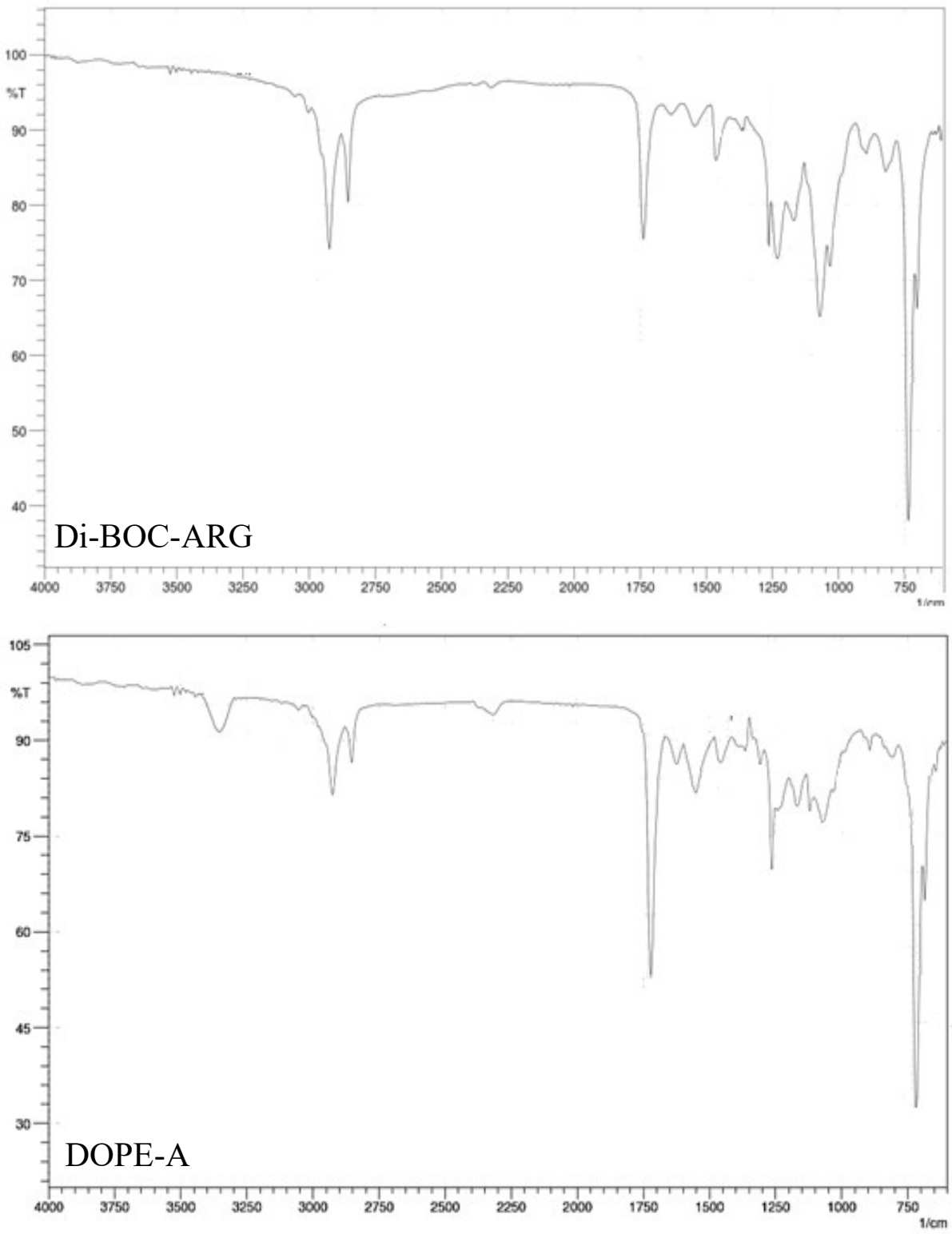


Figure 4 (B). 6 IR spectra of Di-Boc-Arginine and arginine conjugated DOPE

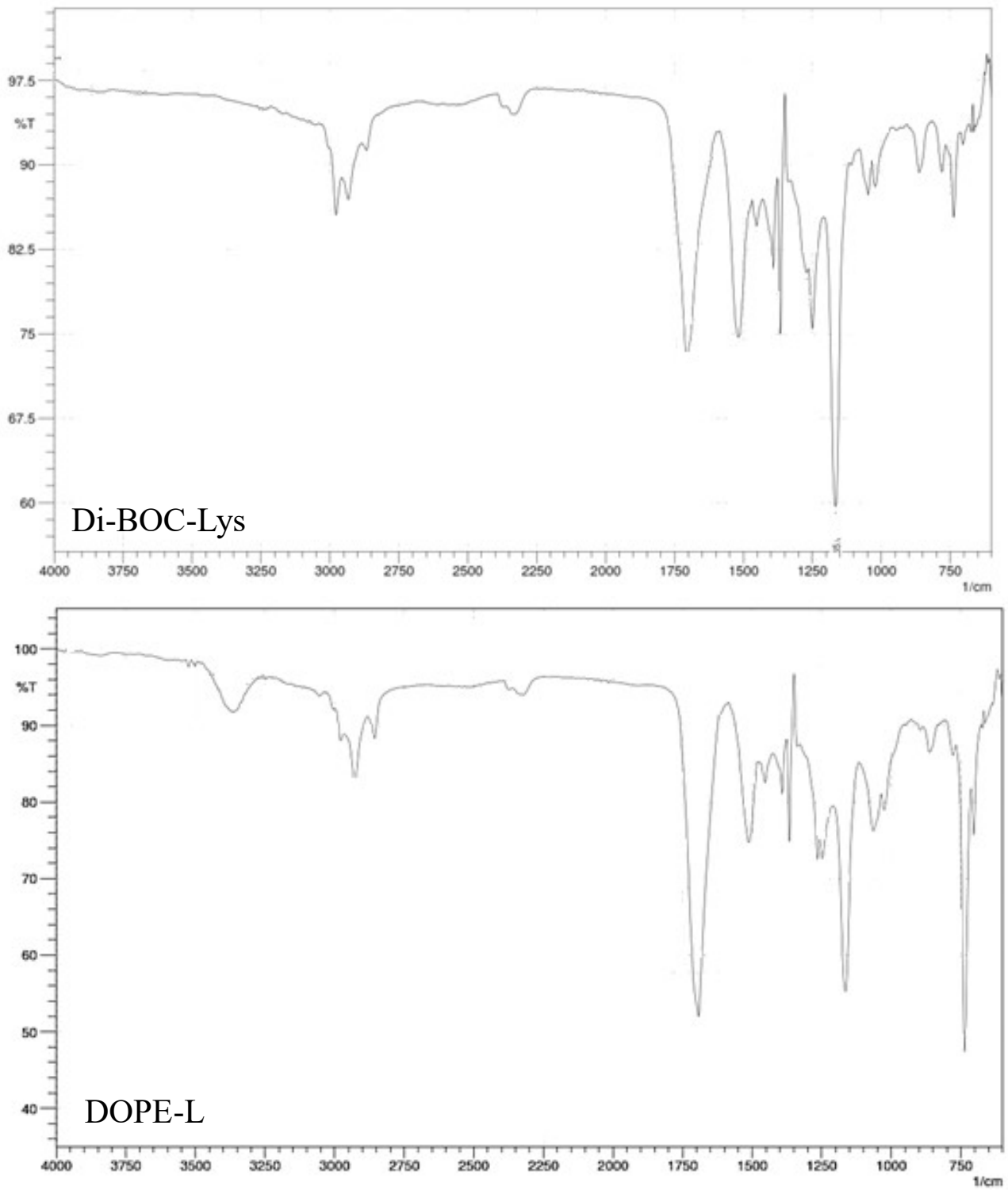


Figure 4 (B). 7 IR spectra of Di-BOC-Lysine and lysine conjugated DOPE

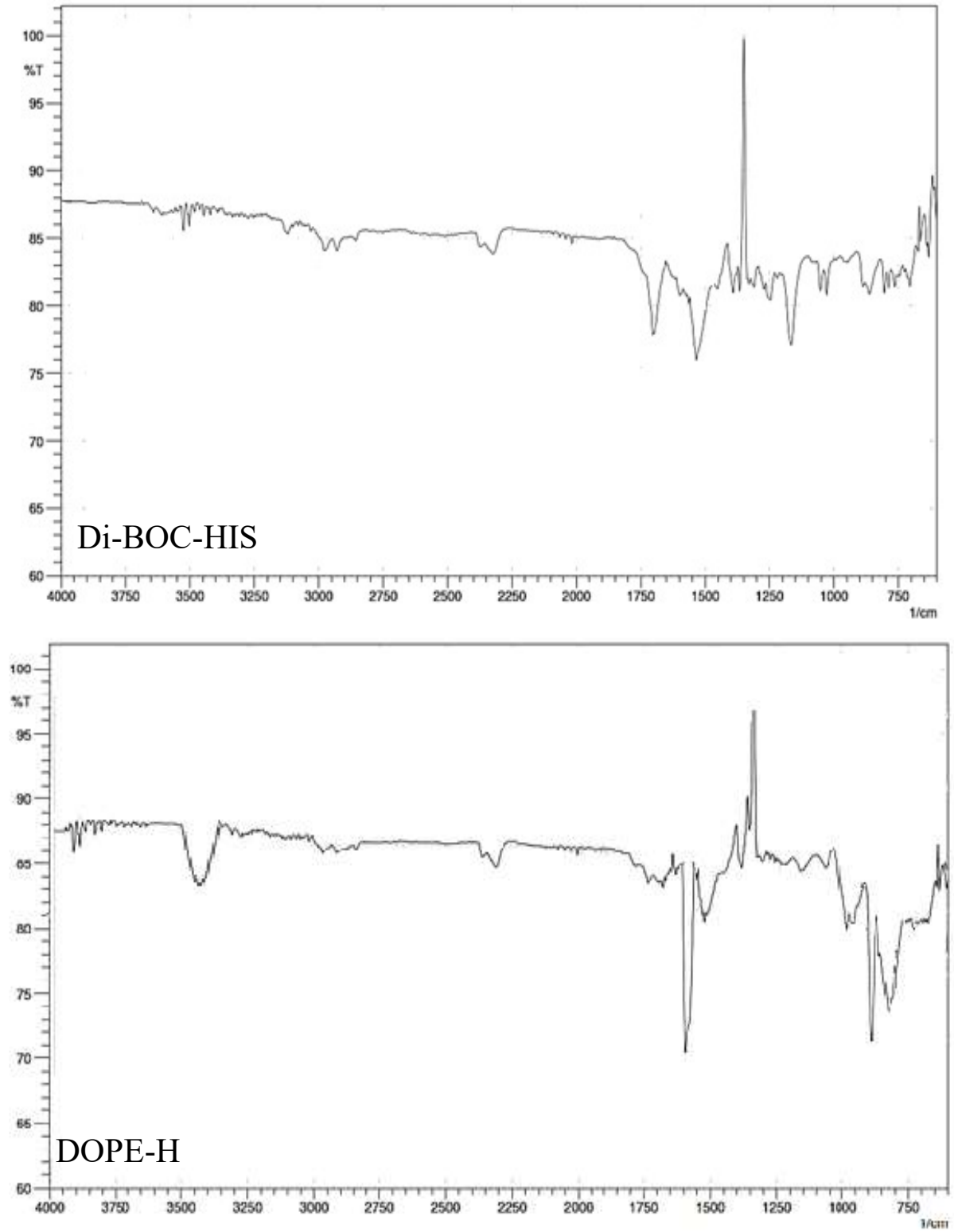


Figure 4 (B). 8 IR spectra of Di-BOC-Histidine and histidine conjugated DOPE

4 (B).6.2 Buffering capacity

Cationic liposomes on their own, without any modified lipids, fuse to the endosomes only at neutral pH, resulting in low DNA transfection due to low or lack of endosomal escape efficiency of the DNA entrapped in liposomal vesicle or DNA complexed with liposome. Buffering capacity is one of the most sought after parameter in case of gene delivery, which helps in denaturation of endosome that releases lipoplex from endosomes (36). Low buffering capacity can't induce enough endosomal disruption so transfection efficiency is low. To overcome these limitations, some modifications have been done on the lipid DOPE to improve its buffering capacity. In the present investigation, we studied the effect of conjugating amino acids to fusogenic lipid DOPE, on its buffering capacity. Histidine was one of the amino acids used for the modification of DOPE to improve buffering capacity. Use of alkaline amino acid based materials as gene carriers since the imidazole ring is a weak base with pKa of 6. Liposomes rich in amino acids attached to phospholipid, could fuse to membrane at both acidic and neutral pH (37). These studies have shown that the incorporation of amino acids into liposomal gene delivery vehicles increases the endosomal buffering capacity of the lipid, improving the efficiency of endosomal escape.

The titration curve was divided in three regions as depicted from figure 4 (B). 9 to confirm the effects of different amino acid modification on protonation behaviour of DOPE. The acid is converted to its conjugated base on addition of strong base like NaOH. At the end point, the conjugated base predominates and the total amount of base added is equivalent to the amount of acid present at the starting point.

The region 10 to 7.4 indicates the approximate amount of protonated amines at physiological pH. The figure showed the curve for arginine modified DOPE lipid was inclined in this region and therefore becomes most cationic at physiological pH. While other two modifications of DOPE i.e. lysine and histidine. Further, the comparison of ratio of protons consumed in range of pH 7.4-5.1 (endosomal pH) to the protons consumed in pH range of 10-7.4, showed an increased trend in the order of Arginine > Lysine > Histidine. The pH of DOPE conjugated with arginine, showed gradual change with respect to DOPE and DOPE conjugated histidine and lysine. This result suggested the strong tendency of DOPE-A to maintain pH on addition of 1 N HCl as a results of its strong buffer capacity.

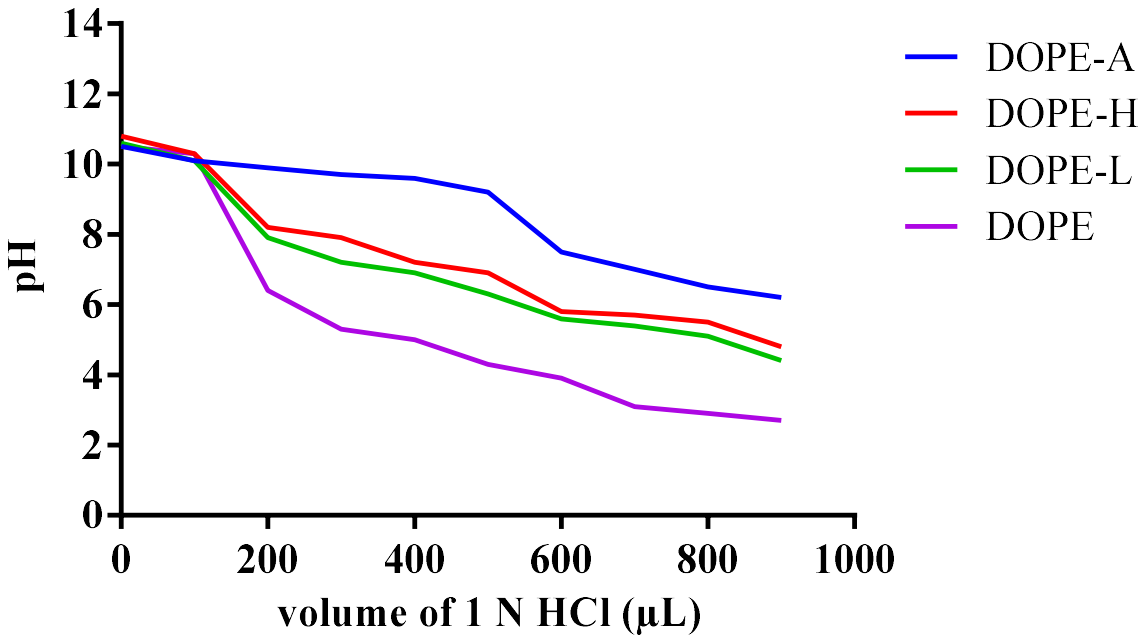


Figure 4 (B). 9 Titration of DOPE and amino acid modified DOPE with 1 N HCl

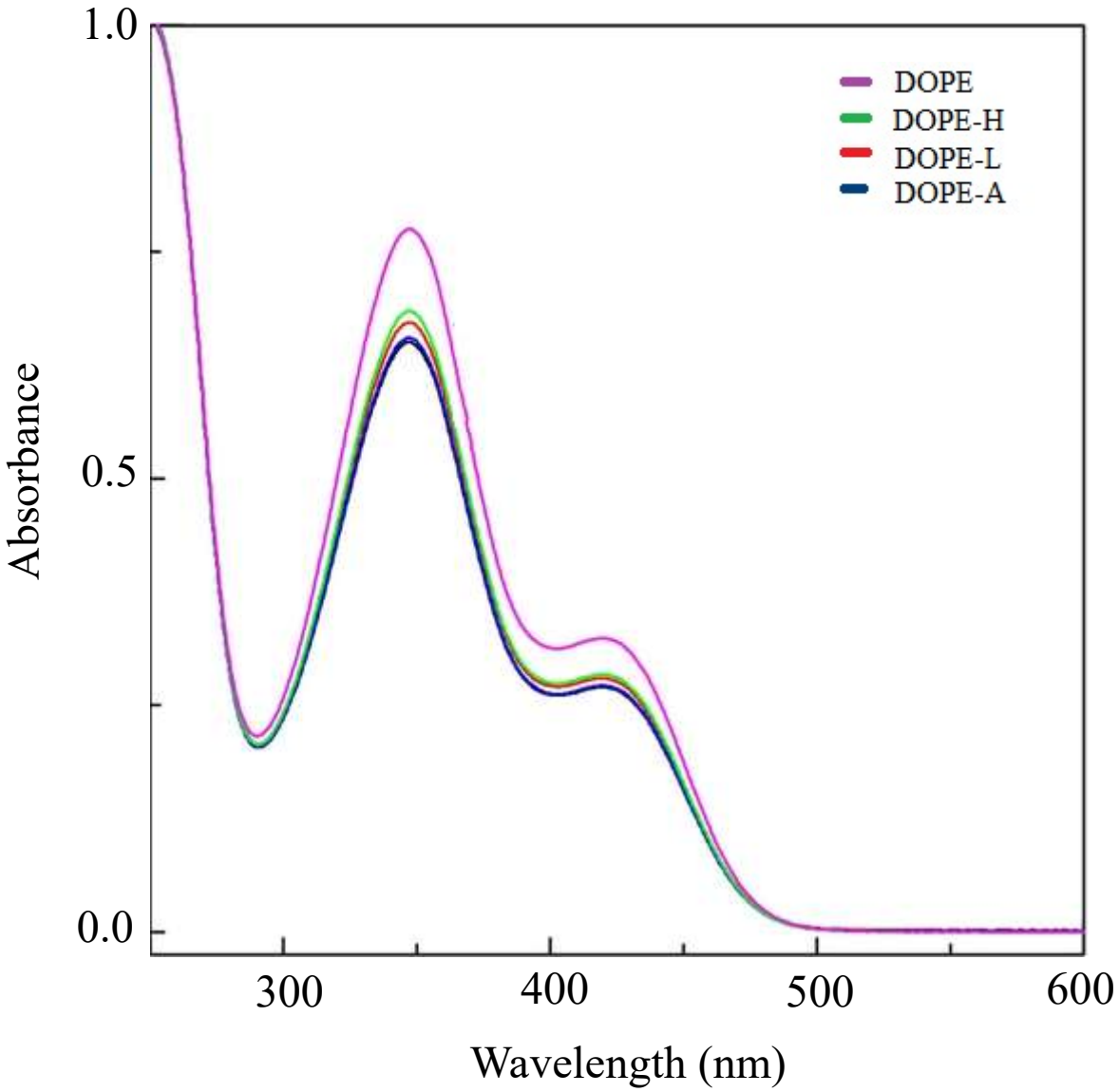
4 (B).6.3 TNBS assay

Efficiency of conjugation of the amino acid derivatives to the lipid DOPE was determined by carrying out the TNBS assay (38). After finishing the conjugation reaction, the lipid mixture was separated by column after (conjugated and unconjugated lipids) and used for analysis. For amino acids modified lipids, appropriate quantities of lipids were dissolved in reaction solvent and reaction with TNBS was carried out. Amount of free amino groups were determined and unreacted DOPE were calculated based on the calibration curve. Molar conjugation efficiency of the reaction was calculated based on the initial molar concentration of the lipid taken for reaction and molar concentration of free lipid (non-reacted) after the reaction. Blank and control experiments were performed using samples without lipid and lipid solution without TNBS respectively to negate any effect of reagent mix and lipids. In figure 4 (B).10, spectra of standard DOPE solution and unreacted DOPE after conjugation reaction with BOC protected amino acids, is evident.

Table 4(B). 2 Conjugation efficiency of protected amino acids to DOPE

Modified lipid	Conjugation efficiency (%)
DOPE-H	37.26 ± 4.95
DOPE-A	52.68 ± 5.64
DOPE-L	41.72 ± 5.09

This spectra in figure 4 (B). 11 ,shows the maximum absorption value for DOPE solution followed by unreacted DOPE after complete reaction with BOC protected amino acids in order of DOPE-H > DOPE-L > DOPE-A. From three amino acids, when DOPE was reacted



with histidine, it showed maximum absorption confirming the highest amount of DOPE

Figure 4 (B). 10 UV spectra of TNBS assay for DOPE and amino acid modified DOPE

present in reaction mixture after completion of reaction which shows low conjugation efficiency of DOPE to histidine, compared to lysine and arginine modifications as shown in table 4 (B).2.

4 (B).6.4 Gas chromatography

Rapid and highly selective HSGC method was developed and validated for the quantification of residual solvents present in synthesised lipids through an understanding of the synthetic process, nature of solvents and nature of stationary phases of columns. The residual solvent DCM (class-II solvent) was determined by this developed method. Upper limit of DCM in any sample is 600 ppm, above this concentration it is toxic to our body and may show some sign of toxicity (39). Thus it is necessary to check for the quantity of DCM in prepared amino acid conjugated DOPE lipid. DMF was used as a diluent for analysis of DCM.

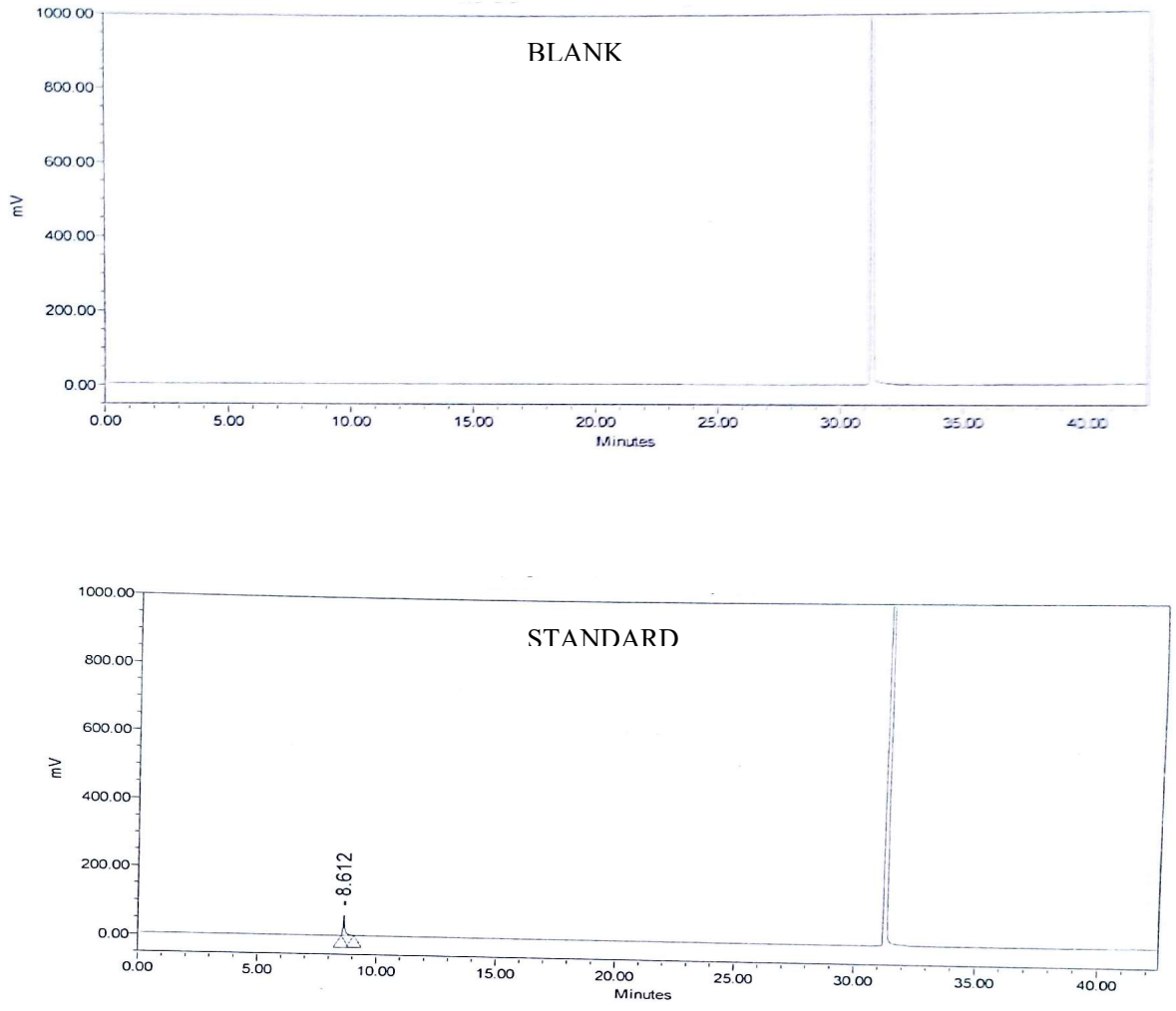


Figure 4 (B). 12 Blank and standard chromatograms generated by GC

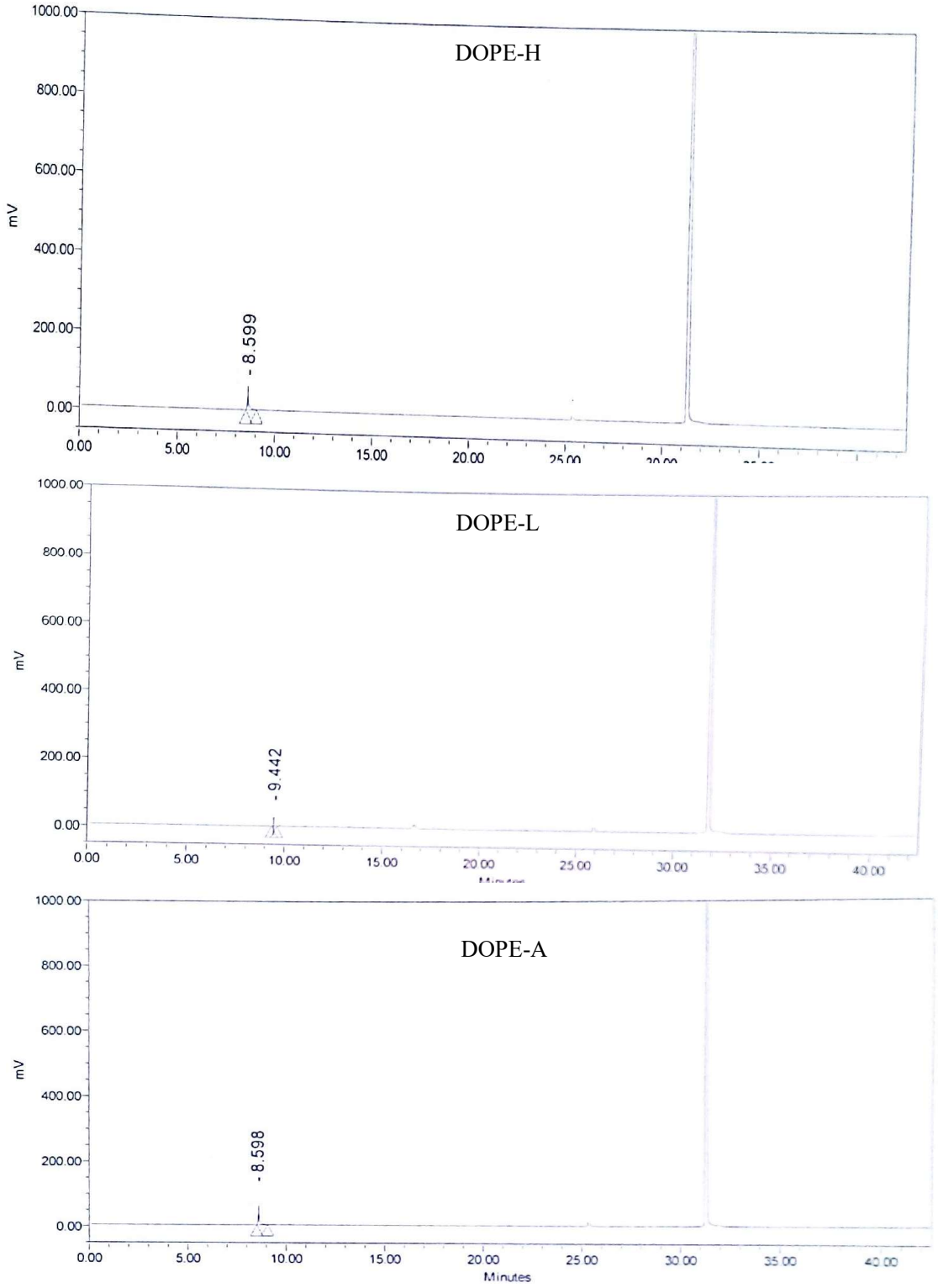


Figure 4 (B). 13 GC chromatograms of amino acid modified DOPE for detection of DCM

Standard DCM in concentration of 600 ppm was diluted in DMF and analysed using GC-FID system. DCM concentration in synthesised lipid samples was found by comparing the peak area of sample with that of standard peak area as shown in figure 4 (B). 142 and figure 4 (B). 13. The results in table 4(B). 3 showed the peak area for modified sample lipids are lesser than the peak area of standard DCM confirming that DCM was present in acceptable quantity in our synthesised sample.

Table 4(B). 3 Concentration of DCM present in samples

Name	RT	AREA	CONCENTRATION
Standard DCM	8.612	305116	600 ppm (upper limit)
DOPE-H	8.599	215827	424.1 ppm
DOPE-L	9.442	018220	35.8 ppm
DOPE-A	8.598	212722	418.3 ppm

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