Chapter 5

Synthesis of Lipids

Chapter 5

5.1 Introduction

Lipids used in gene delivery differ in their transfection efficiencies due to the cationic nature due to charge based interaction. These differences are majorly due to the differences in the physicochemical nature of the cationic lipids and different formulation strategies employed to formulate into a gene delivery vehicle. Modification of structure of the lipids can be utilized to impart certain physicochemical properties to the lipids which can be helpful in their therapeutic use. Such properties can be i.e. changed spatial structure of the lipid, change in the ionization behavior of the lipid, buffering effect. These features can help impart, into the gene delivery system, the important effects such as protection of the nucleic acid cargo from body milieu, higher transfection efficiency, low cytotoxicity etc. In the present investigation, a low molar amount of stearyl amine which has shown transfection efficiency even to the cell lines which are resistant to the transfection by some commonly used cationic lipids has been selected as cationic lipid. Modification of helper lipid, DSPE was carried out using amino acid derivatives to modify its physicochemical characteristics. The feature of these lipid is the presence of free amino group which lends us the possibility to modify it using common conjugation strategies such as EDC-NHS coupling.

5.2 Materials and Methods

5.2.1 Synthesis of modified lipids

Syntheses of Boc-histidinylated DSPE (Boc-His-DSPE) and Histidinylated DSPE (His-DSPE) were carried out by EDC/NHS coupling method (1) (**Figure 5.1**). Briefly, Boc-Histidine, Ethyldimethylaminocarbodiimide (EDC) (Merck, India) and N-hydroxysuccinimide (NHS) (Merck, India) were taken. Carboxyl group of Boc-amino acid (or Boc-carnosine for preparation of Boc-Carnosylated DSPE) was activated by carrying out reaction in aqueous media at pH ~5-5.5 (set with 10 nM MES buffer) with EDC and NHS for 30 minutes. DSPE was added to the reaction mixture and dissolved by adding sufficient quantity of chloroform:methanol mixture (4:3 by volume). The reaction was carried out for 1 day at room temperature. Synthesis was confirmed by preparative TLC analysis using $CHCl_3$:MeOH:HAc (5:4.6:0.4 volume ratio) as a mobile phase. Reaction mixture was evaporated in rotary evaporator and solid residue left was suspended in water by sonication. Aqueous suspension was centrifuged to pellet the compound and again

resuspended in water. Aqueous suspension was dialyzed against water overnight for complete removal of unconjugated Boc-Histidine, EDC and NHS. Compound was solubilized in the $CHCl_3$:MeOH:HAc (5:4.6:0.4 volume ratio) and eluted through silica gel column for purification. Isolated compound was evaporated in rotary evaporator and reconstituted in distilled water by sonication. Dispersed compound was freeze-dried until used. For synthesis of His-DSPE, Boc-His-DSPE was treated with excess of hydrochloric acid cleavage cocktail (HCl:MeOH:$CHCl_3$:Anisole 3.4:5:0.5:0.1 volume ratio) and allowed to react overnight to remove Boc protection. The reaction mixture was dried in rotary evaporator and dried residues were then washed with methanol and water 3 times to remove traces of reactants and biproducts.

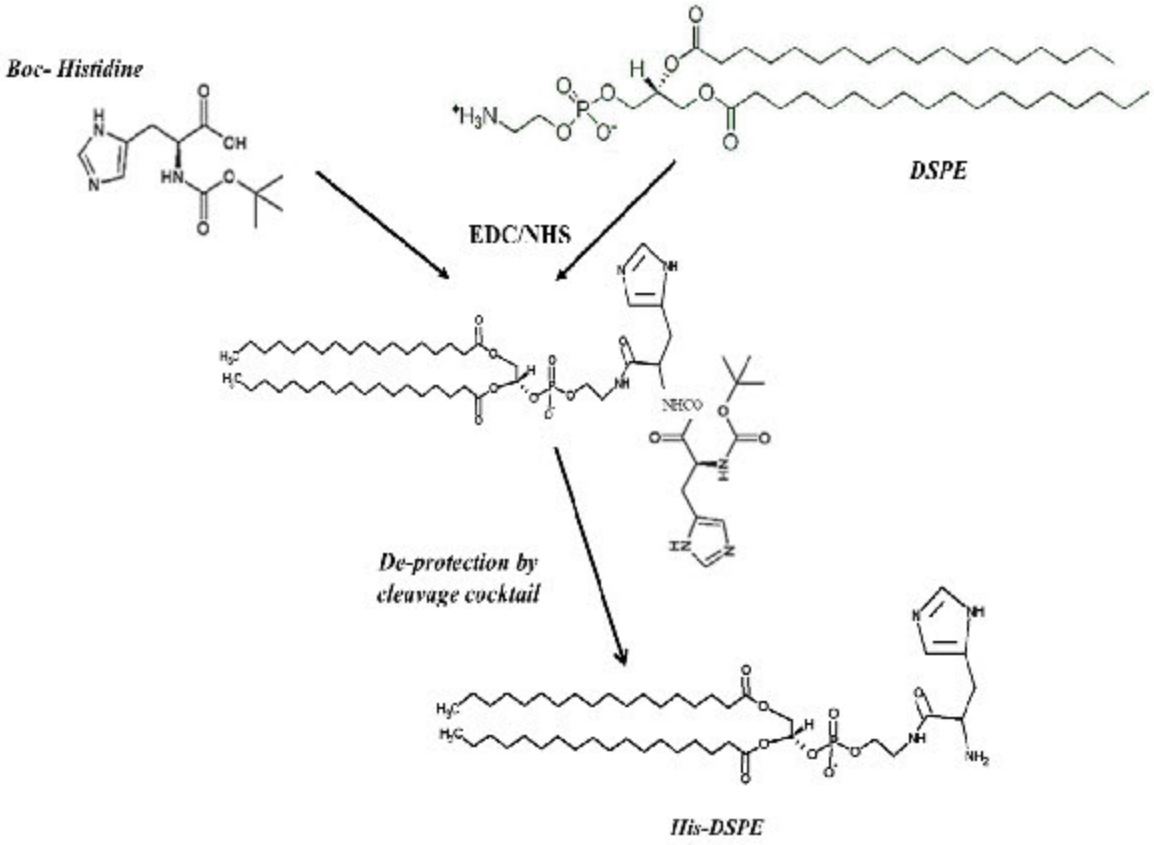
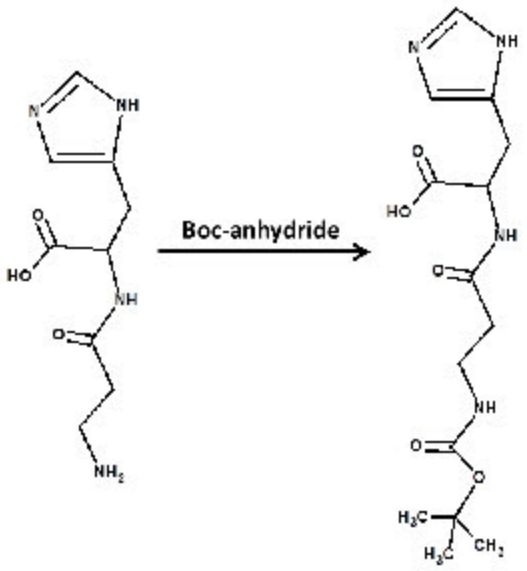


Figure 5.1 Synthesis of histidine conjugated DSPE



Carnosine *Boc-Carnosine*

Figure 5.2 Synthesis of Boc-carnosine

For synthesis of carnosine (dipeptide of alanine and histidine) modified lipids, free amino group of carnosine was protected using Boc anhydride (dibutylpyrocarbonate) **Figure 5.2**. Briefly, dibutylpyrocarbonate and carnosine (Sigma, USA) were dissolved in a mixture of ACN:MeOH:THF:H_2O (6:5:2:2 volume ratio). Reaction mixture was alkalinized by sodium bicarbonate and reaction was carried out at room temperature for 1 day. Positive reaction was confirmed through thin layer chromatography of reaction mixture using suitable mobile phase. After 1 day, reaction mixture was dried under vacuum at 60°C in rotary flask evaporator. The Boc-carnosine was isolated using methanol. Methanol extract of Boc-carnosine was centrifuged and supernatant was evaporation under vacuum in rotary flask evaporator. Three subsequent extraction and washing were performed to get pure product. The product was confirmed by FTIR and NMR spectra of the product. Synthesized Boc-carnosine was used in similar fashion as Boc-histidine to synthesize Boc-Car and Car modified lipids. Similarly, synthesis of Boc-Arg-DSPE was accomplished. Deprotection of the Boc-protected lipids was performed using same methodology to get Car-DSPE and Arg-DSPE.

Nomenclature used for different compounds and different synthesized lipids is shown in the **Table 5.1**.

Table 5.1 Nomenclature of different compounds and synthesized lipids

Lipid	Amino acid derivative	Boc Protection	Nomenclature
Distearoyl-*sn*-glycerophosphoethanolamine	-	-	DSPE
-	Histidine	-	His, H
-	Histidine	Present	Boc-His, BH
-	Carnosine	-	Car, C
-	Carnosine	Present	Boc-Car, BC
-	Arginine	-	Arg, A
-	Arginine	Present	Boc-Arg, BA
DSPE	Histidine	Present	Boc-His-DSPE, BHDSPE
DSPE	Histidine	-	His-DSPE, HDSPE
DSPE	Carnosine	Present	Boc-Car-DSPE, BCDSPE
DSPE	Carnosine	-	Car-DSPE, CDSPE
DSPE	Arginine	Present	Boc-Arg-DSPE, BADSPE
DSPE	Arginine	-	Arg-DSPE, BADSPE

Efficiency of conjugation of the amino acid derivatives to the lipids (DSPE) was determined by carrying out the TNBS assay (For Boc-His-DSPE, or Sakaguchi assay for Boc-Arg-DSPE; described in Chapter 3-Analytical Methods). Conjugation reaction was carried out and the lipid mixture (unconjugated and conjugated lipids) isolated after dialysis was lyophilized and used for analysis. For Boc-His and Boc-Car modified lipids, briefly, appropriate quantities of the lipids were dissolved in the reaction solvent and reaction with TNBS was carried out. Amount of free amino groups were determined and unreacted DSPE 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. For Boc-Arginine modified lipid, appropriate quantity of lipid was dissolved in the reaction solvent and Sakaguchi reaction was performed on the synthesized lipid. The molar concentration of the guanidine (arginine) was determined based on the standard calibration curve of the arginine. Molar conjugation efficiency was calculated based on the theoretical molar concentration of the lipid and molar concentration of lipid determined after the conjugation. Blank and control experiments were performed using samples

without any arginine and lipid without reagent mix to negate any effect of reagent mix and lipid.

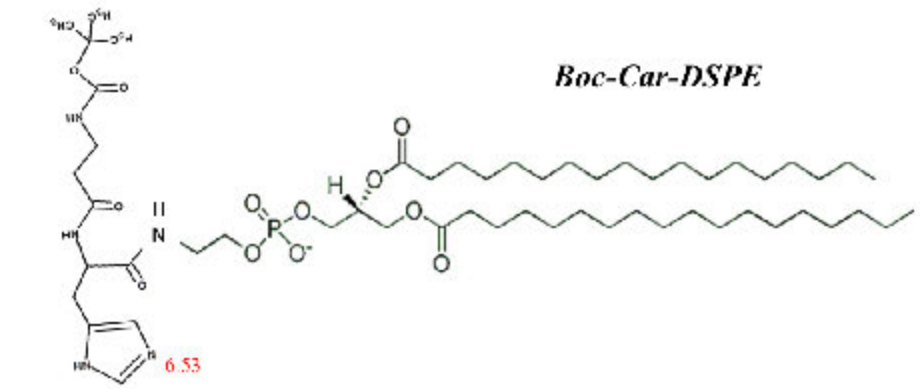
5.2.2 Physicochemical characteristics of the lipids and pH titration study:

Predicted physicochemical characteristics of the lipids were evaluated for feasibility of their use in gene delivery. pKa and log P prediction were taken according to the ChemAxon (USA). Based on the pKa characteristics, %ionization of the lipids was calculated using Henderson-Hasselbalch equation.

Synthesized lipids were evaluated for their buffering capacity and for pKa determination. Lipids were dissolved in appropriate solvent mixture comprising of MeOH:H_2O or MeOH:$CHCl_3$:H_2O to enable aqueous titration. The pH of the solvents were set to pH 10 using 0.1N NaOH solution and reaction mixture was titrated with fixed small sequential quantities of 0.1N HCl solution and pH was recorded after each addition. The pH titration curves (pH vs. volume of titrant added) were generated. Even though at some points phospholipids were showing precipitations, yet the titration was able to distinguish the pKa region of different amine functions.

5.3 Results and discussion

Structures of the synthesized DSPE base lipids are shown in the



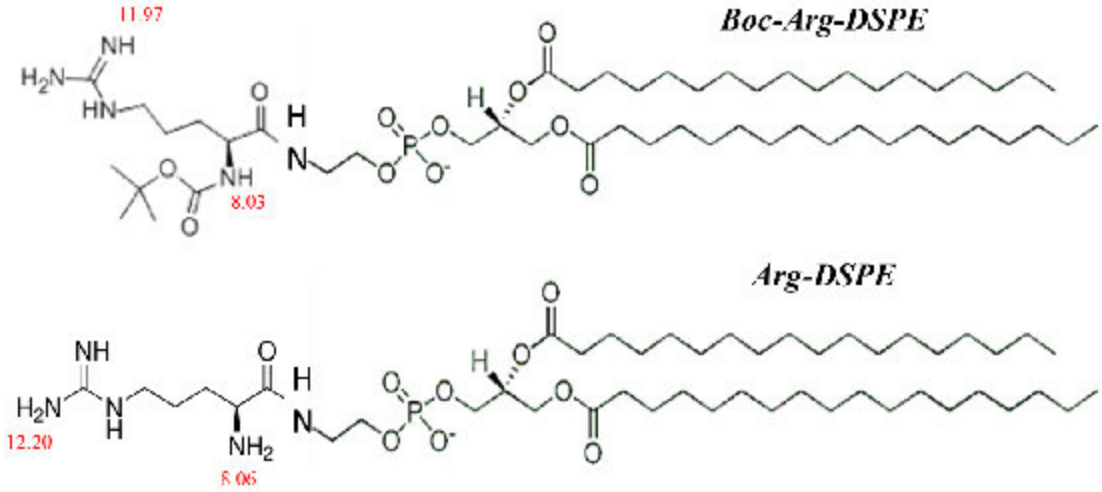
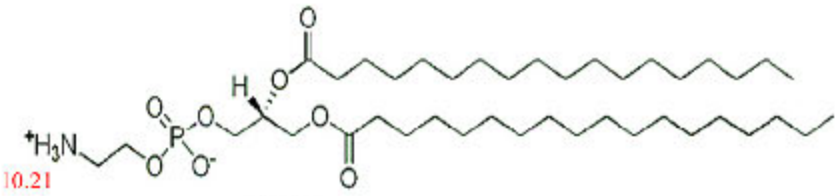
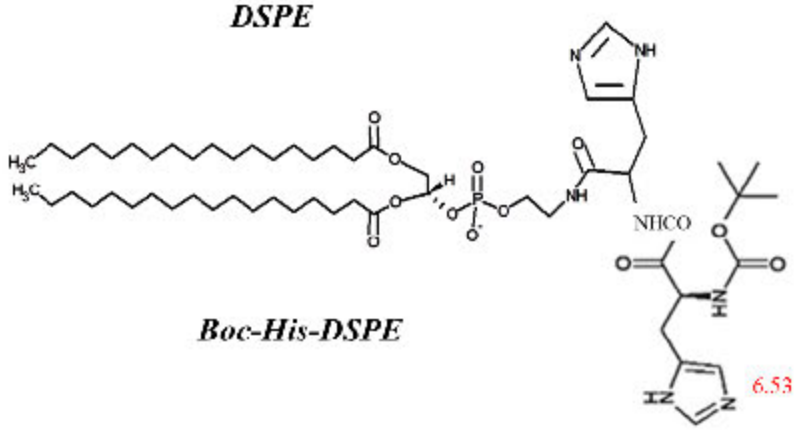


Figure 5.3. Structures of the synthesized lipids were fed to Chemicalize.org (ChemAxon) and predicted physicochemical properties of the lipids i.e. log P and pKa values of the amino functions were derived.

Initial synthesis of the lipids was confirmed by TLC analysis using $CHCl_3$:MeOH:HAc mixture as mobile phase and spots were detected for synthesized lipids. UV spectroscopy showed that the UV absorption characteristic of Boc-His was retained in the Boc-His DSPE (Figure 5.8).

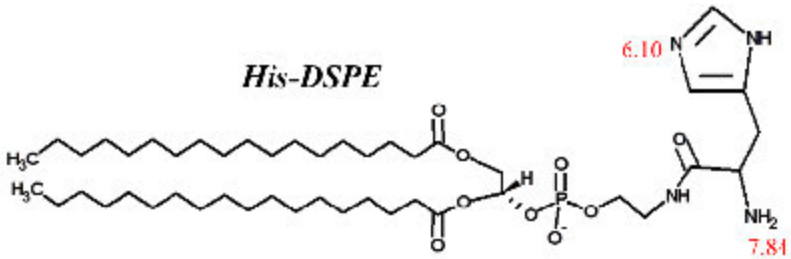


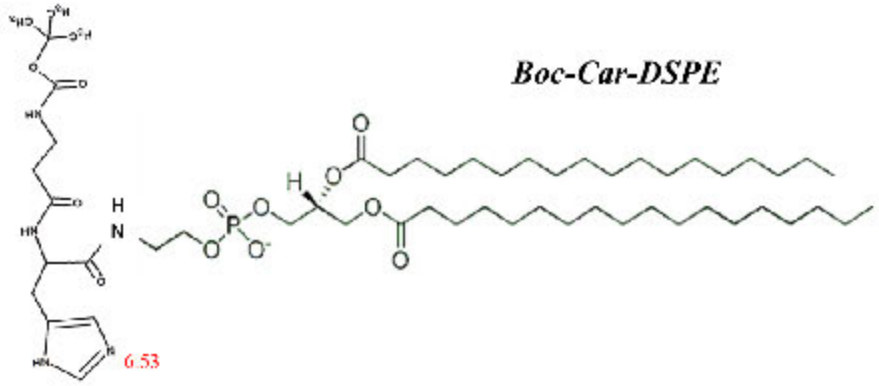
DSPE



Boc-His-DSPE

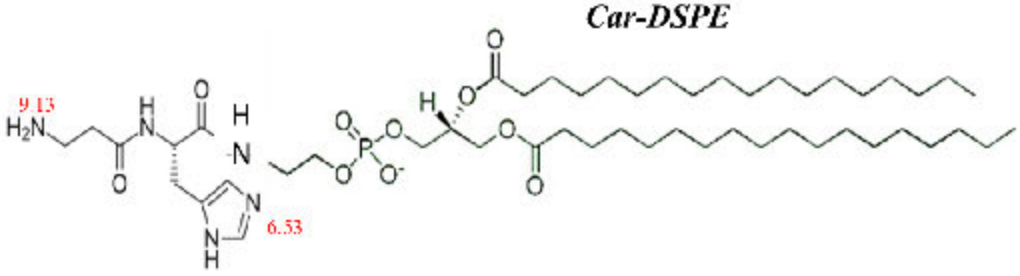
His-DSPE





Boc-Car-DSPE

Car-DSPE



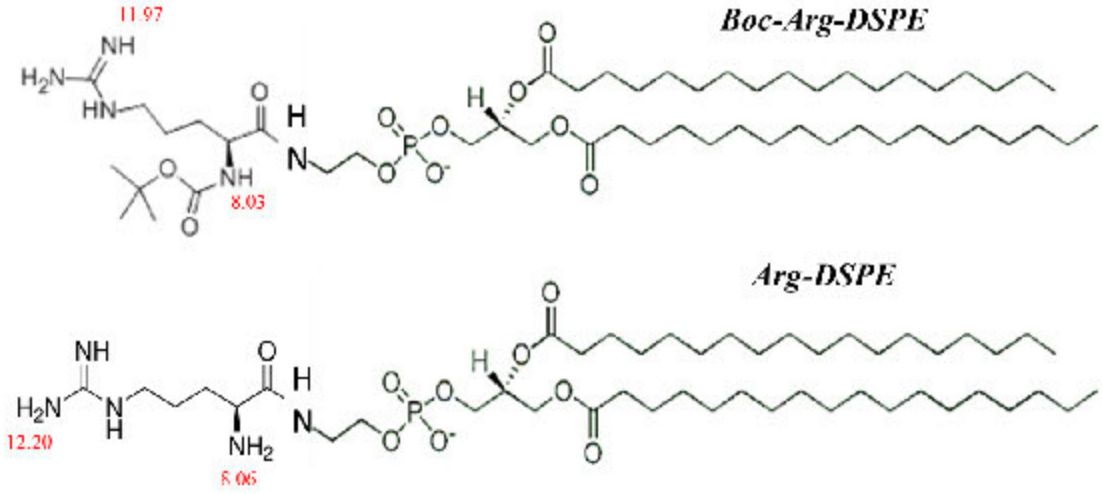


Figure 5.3 Structure of DSPE and DSPE based lipids (Value at amine groups indicates the calculated pKa of that group according to Chemaxon (Chemicalize.org)

Conjugation efficiency of the method for coupling different amino acid derivatives to the lipid after the reaction period was evaluated using either TNBS assay or Sakaguchi assay (Described in Chapter 2, Analytical Methods). Few reaction parameters for conjugation such as reaction time, ratio of lipid to amino acid derivatives were evaluated to evaluate the impact of these parameters on the conjugation efficiency. The results are summarized in the **Table 5.2**. Based on these limited screening, the reaction period of 24 hr and amino acid derivative to lipid mole ratio of 0.5:1 was used for the synthesis of lipids. Molar conjugation efficiency of the selected method for different lipids is depicted in **Table 5.3.**

Table 5.2 % molar conjugated lipids in the reaction mixture after dialysis post reaction period

Lipid	Molar ratio of amino acid derivative to lipid	Reaction period	% molar conjugated lipid
Boc-His-DSPE	1:1.5	1 day	26.6±6.2
	1:0.75	1 day	69.8±5.6
	1:0.5	1 day	80.1±3.1
	1:0.25	1 day	72.6±4.9
	1:0.5	3 days	79.6±5.2

Table 5.3 % molar conjugated lipids in the reaction mixture after dialysis post reaction period

Lipid	Molar ratio of amino acid derivative to lipid	Reaction period	% molar conjugated lipid
Boc-His-DSPE	1:0.5	1 day	80.1±3.1
Boc-Car-DSPE	1:0.5	1 day	74.8±4.7
Boc-Arg-DSPE	1:0.5	1 day	85.2±3.3

Boc-protection of carnosine was seen as rightward shift of the UV absorption spectrum of carnosine (**Figure 5.4**). NMR spectra of Boc-carnosine (Boc-Car) showed presence of BOC protons at δ 1.33 ppm (**Figure 5.5**) (2). IR spectra of Boc-carnosine show absorption at 1597 cm^{-1}, 1660 cm^{-1} and 1694 cm^{-1} indicating the presence of C=O stretch of –CONH- of peptide linkage, C=O stretch of –NHCOO- of BOC protected amine and C=O stretch of carboxylic acid respectively (**Figure 5.6**). Disappearance of doublet representing the primary amine stretch of carnosine at ~3100 cm^{-1} and 3200 cm^{-1} and strong N-H stretching absorptions at 3300 cm^{-1} and 3350 cm^{-1} representing –CONH- of peptide bond and –NHCOO- of carboxamide group of Boc protection confirm Boc protection of carnosine.

After synthesis of Boc-Car, DSPE was modified using Boc-Car. After conjugation, UV spectra of Boc-Car conjugated lipids were recorded and the presence of Boc-Car was confirmed through presence of UV absorption similar to Boc-Car in the synthesized lipids (**Figure 5.7**).

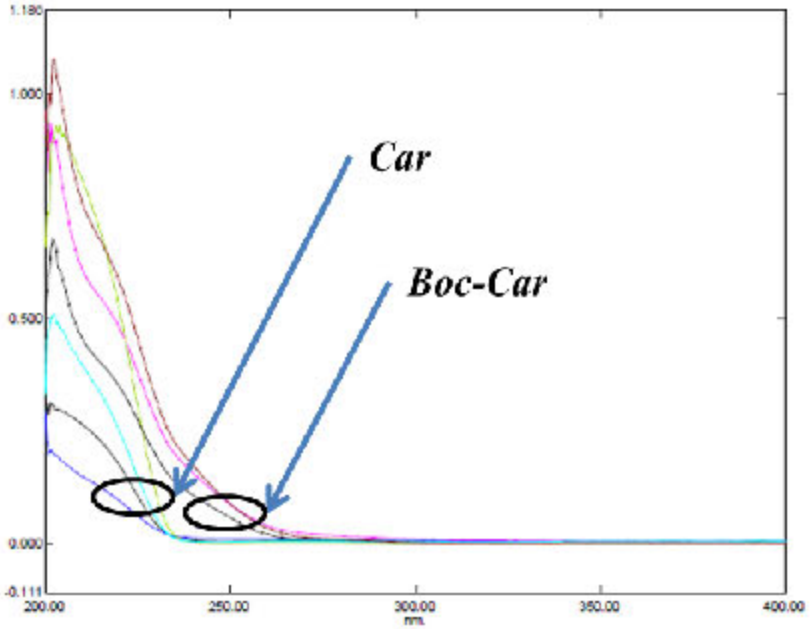


Figure 5.4 UV spectra Carnosine and carnosine isolated after Boc protection (x-axis: wavelength, y-axis: absorbance)

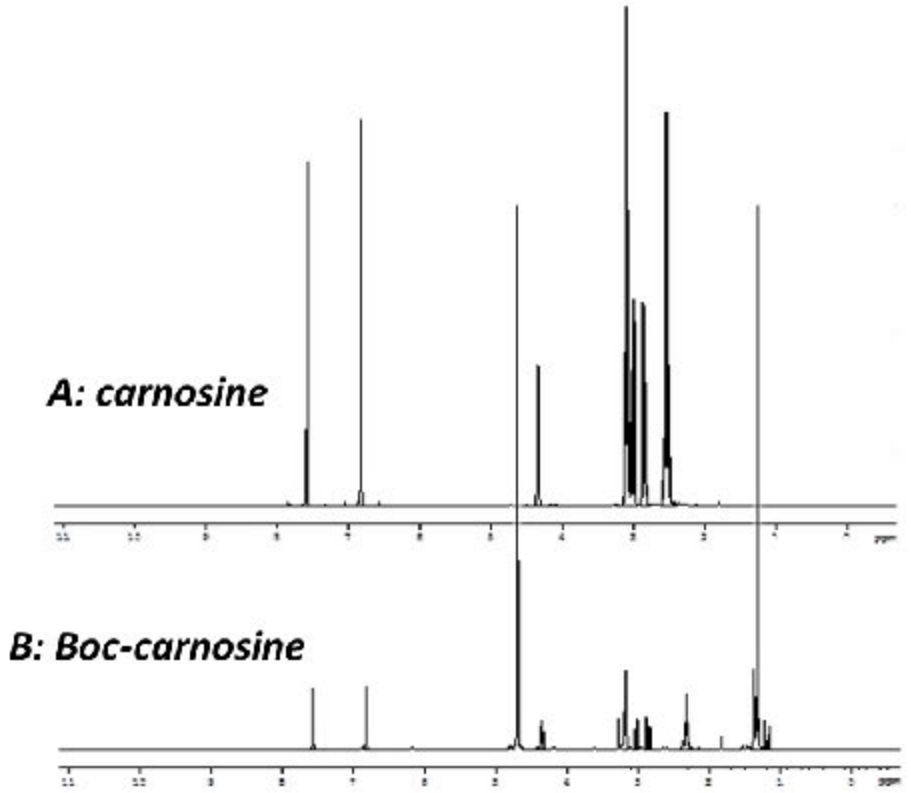


Figure 5.5 NMR spectra of Carnosine and Boc-Carnosine

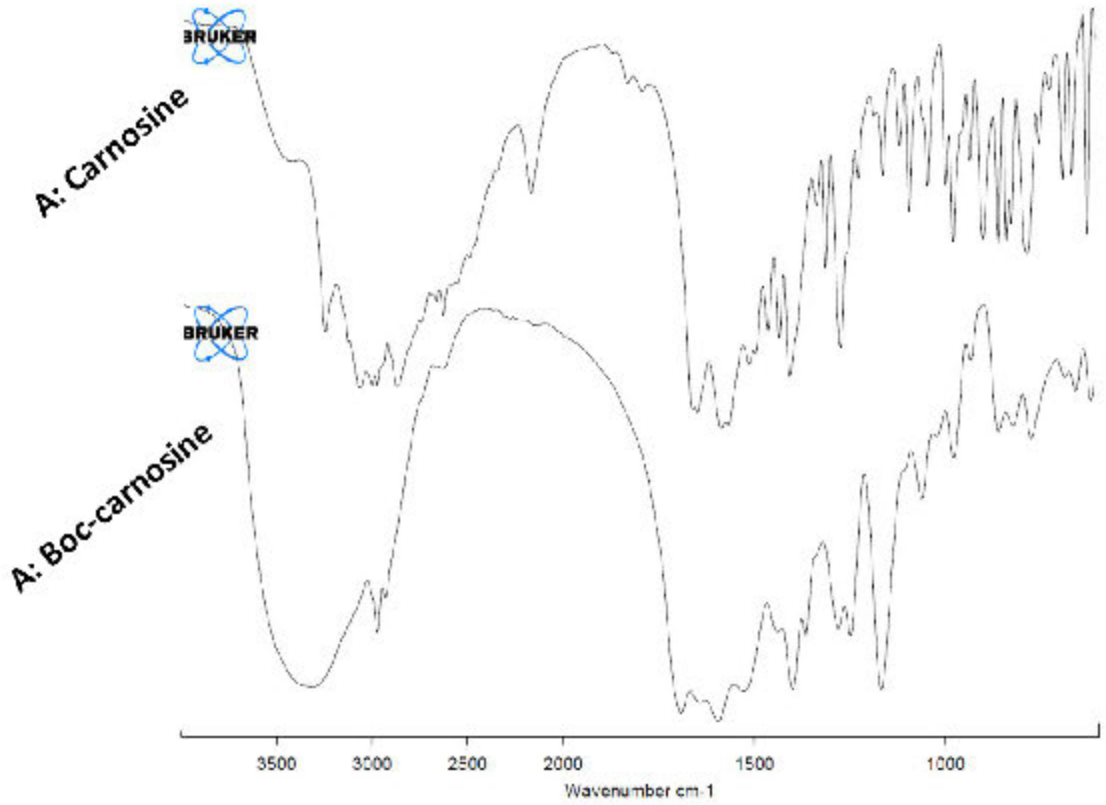


Figure 5.6 IR spectra of Carnosine and Boc-Carnosine

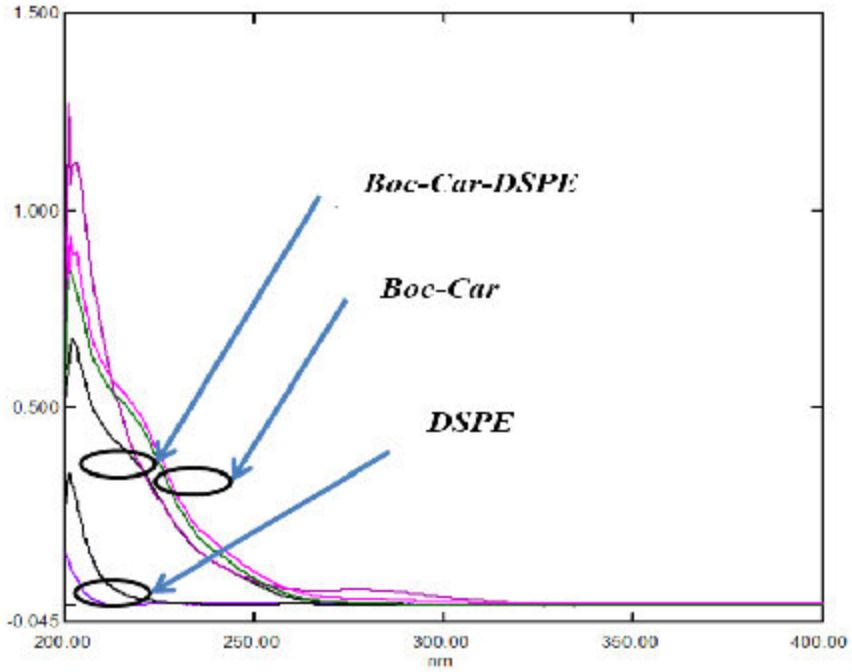


Figure 5.7 UV spectra of DSPE, Boc-Car and Boc-Car-DSPE (x-axis: wavelength, y-axis: absorbance)

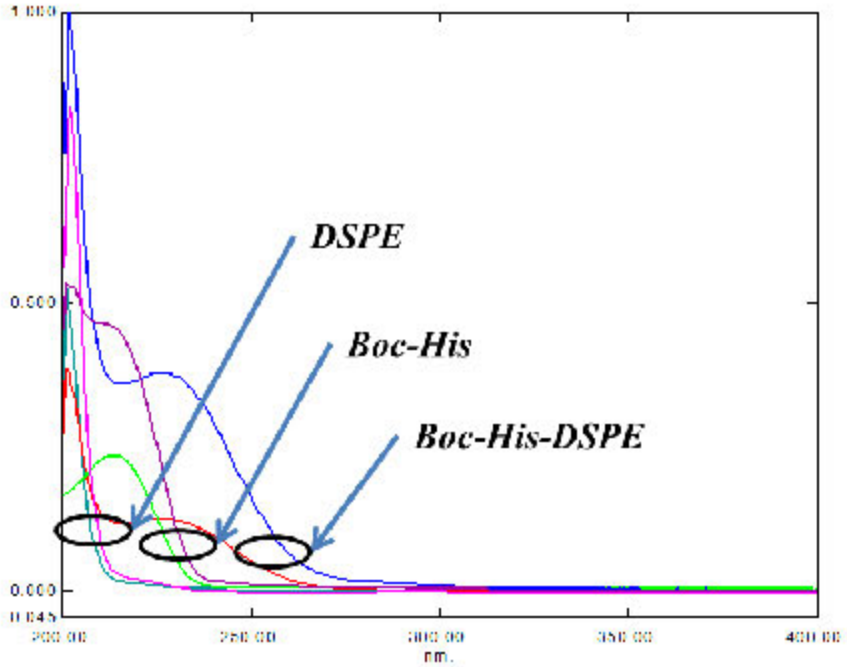


Figure 5.8 UV spectra of DSPE, Boc-His and Boc-His-DSPE (x-axis: wavelength, y-axis: absorbance)

Similarly, successful syntheses of DSPE; Boc-His-DSPE and Boc-His was confirmed by UV spectroscopy (**Figure 5.8**). Further, lipids were confirmed by FTIR spectroscopy, and mass spectrometry (See attached supplementary spectroscopy images at the end of the thesis) (3). FTIR spectra of synthesized lipids showed one or more of the following characteristics which confirmed the structure of the compounds:

- amide –NH- stretching band at ~3300-3500 cm^{-1} characteristic of the amide function of the synthesized lipids.
- amide carbonyl stretching band at ~1620 to 1680 cm^{-1}
- characteristic -C=N- and –C=C- stretching bands between 1620-1690 cm^{-1} indicating the presence of guanidine C=NH of arginine modified DSPE and -C=C- and –C=N- of aromatic imidazole ring of histidine and carnosine modified DSPE.
- secondary amide –NH- stretching at 1450-1550 cm^{-1}
- aromatic –C=C- stretching

Mass spectra of synthesized lipids were recorded to evaluate characteristic peaks of the synthesized compounds. Characteristic m/z values observed for different compounds are shown in **Table 5.4**.

Table 5.4 Characteristic mass spectra observations of synthesized compounds and some of their reactants

Name of compound	MW (g/mol)	Observed m/z values
Boc-His	255.27	256.166 (M+1), 257.103 (M+2)
Carnosine	226.10659	227.086 (M+1), 453.16 (dimer)
Boc-Car	326.35	256.5 (Boc-His fragment), 327.282 (M), 349.149 (M+Na)
Boc-Arg	274.32	289.17 (M+CH_3), 290.10 (M+CH_3+H)
DSPE	747.578	747 (M), 748.6 (M+1), 790 (M+Na)
BHDSPE	984.838	985.7 (M+1)
HDSPE	884.718	885 (M), 906 (M+Na)
BCDSPE	1051.887	1051.69 (M)
CDSPE	951.767	952 (M+), 977.5 (M+4H+Sodium), 959.56 (M+4H+Sodium-18)
BADSPE	1003.896	1004 (M), 1005.697 (M+1)
ADSPE	903.739	904 (M), 905.43 (M+1)

pH titration study was performed on the lipids to evaluate the buffering activity of the lipids. The pH titration curves for different lipids are depicted in the **Figure 5.9**, **Figure 5.10** and **Figure 5.11**.

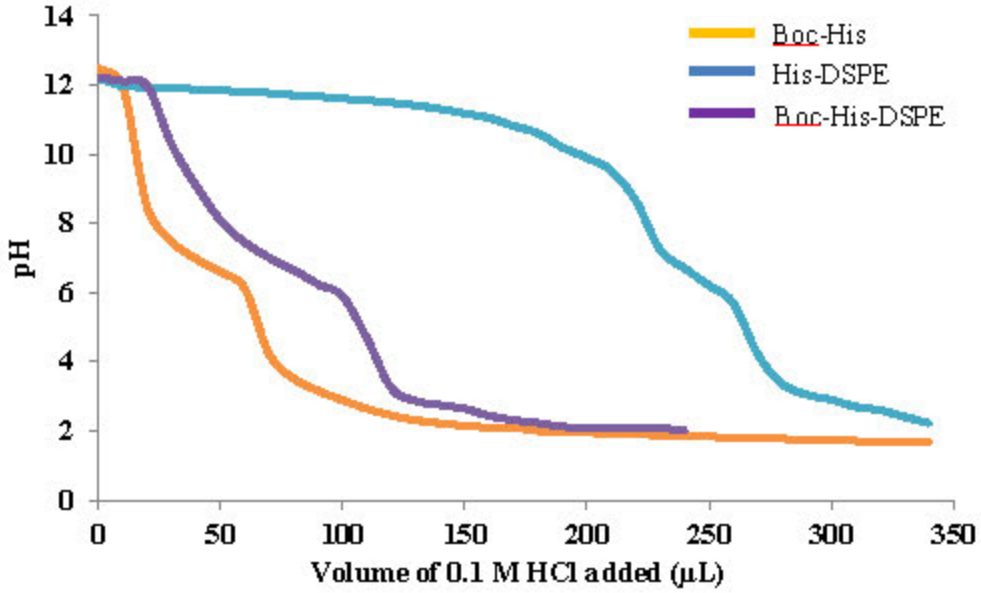


Figure 5.9 pH titration curve of Boc-Histidine, Boc-His-DSPE and His-DSPE

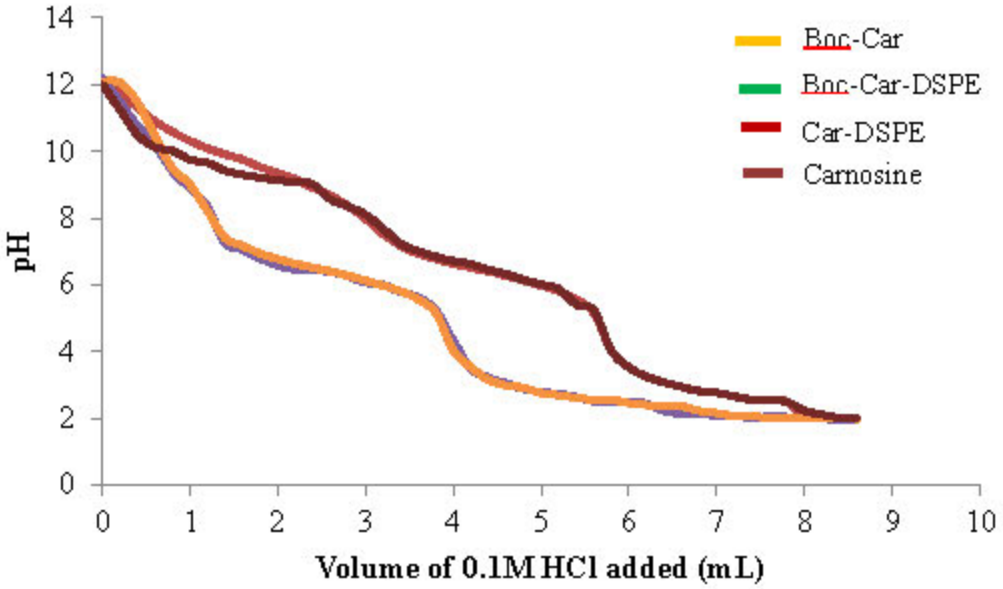


Figure 5.10 pH titration curve of Carnosine, Boc-Carnosine, Boc-Car-DSPE and Car-DSPE

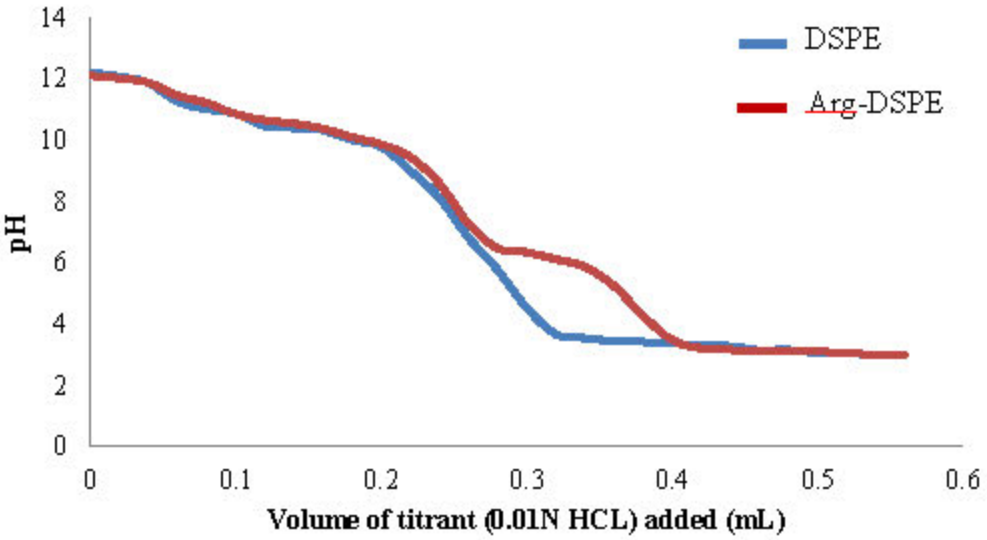


Figure 5.11 pH titration curve of DSPE and Arg-DSPE

pH titration curves of different lipids give idea of the pKa values of amino groups of the compounds which are close to the predicted values of pKa (**Table 5.5**). The results indicate that the compounds having carnosine and histidine will bear the properties of buffering due to presence of imidazole ring with nitrogen having pKa value ~6.5. Lipids with arginine derivatives will have completely ionized amino group and guanidine group at physiological pH range and hence will always be bearing two cationic charges.

Table 5.5 Physicochemical properties of different cationic lipids used in preparation of liposomes

Lipid	pKa1[@$]	pKa2[*$]	Log P[$]	%ionization of primary amine at pH 6.5-7.0[#]	% ionization of side chain amine at pH 6.5-7.0[#]
DSPE	10.00	-	12.23	>99.9	-
HDSPE	-	~6.5	7.85	-	25-51%
BHDSPE	-	6.53	7.45	-	25.3-51.7%
CDSPE	9.13	6.53	5.09	99.2-99.7%	25.3-51.7%
BCDSPE	-	6.52	6.58	-	24.8-51.1%
BADSPE	-	11.97	6.88	-	>99%
ADSPE	8.60	12.20	5.48	97.5-99.2%	>99%

[$] calculated parameters derived from Chemicalize.org (ChemAxon)
[@]pKa of primary amine group
* pKa of side chain -- imidazole ring for carnosine and histidine and guanidine ring for arginine
[#]percentage ionization calculated according to Handerson-Hasslebatch equation

5.4 References

1. Hermanson GT. Chapter 2 - The Chemistry of Reactive Groups. Bioconjugate Techniques (Second Edition). New York: Academic Press; 2008. p. 169-212.
2. Pavia D, Lampman G, Kriz G, Vyvyan J. Introduction to spectroscopy: Cengage Learning; 2008.
3. Silverstein RM, Webster FX, Kiemle DJ, Bryce DL. Spectrometric identification of organic compounds: John Wiley & Sons; 2014.