Chapter 5
Synthesis of Lipids

Sometimes things just happen, that we just can't change

-From "Terminator 3"

5.1 Introduction

Cationic lipids used in gene delivery differ in their transfection efficiencies. 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, stearyl amine which has shown transfection efficiency even to the cell lines which are resistant to the transfection by some commonly used cationic lipids have been chosen for modificatopm using amino acid derivatives. Additionally, commonly used phospholipids (DOPE and DSPE) have also been chosen. The common feature of these lipids 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 stearyl amine (Boc-His-SA) and Histidinylated stearyl amine (His-SA) 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). Carboxyl group of Boc-amino acid or Boc-carnosine 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. Stearyl amine (SA) (Merck, India) was added to the reaction mixture and dissolved by adding sufficient quantity of chloroform:methano 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-Stearyl amine, Boc-His-Stearyl amine was treated with excess of hydrochloric acid cleavage cocktail (HCl:MeOH:$CHCl_3$:Anisole 3.4:5:0.5:0.5:0.1 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.

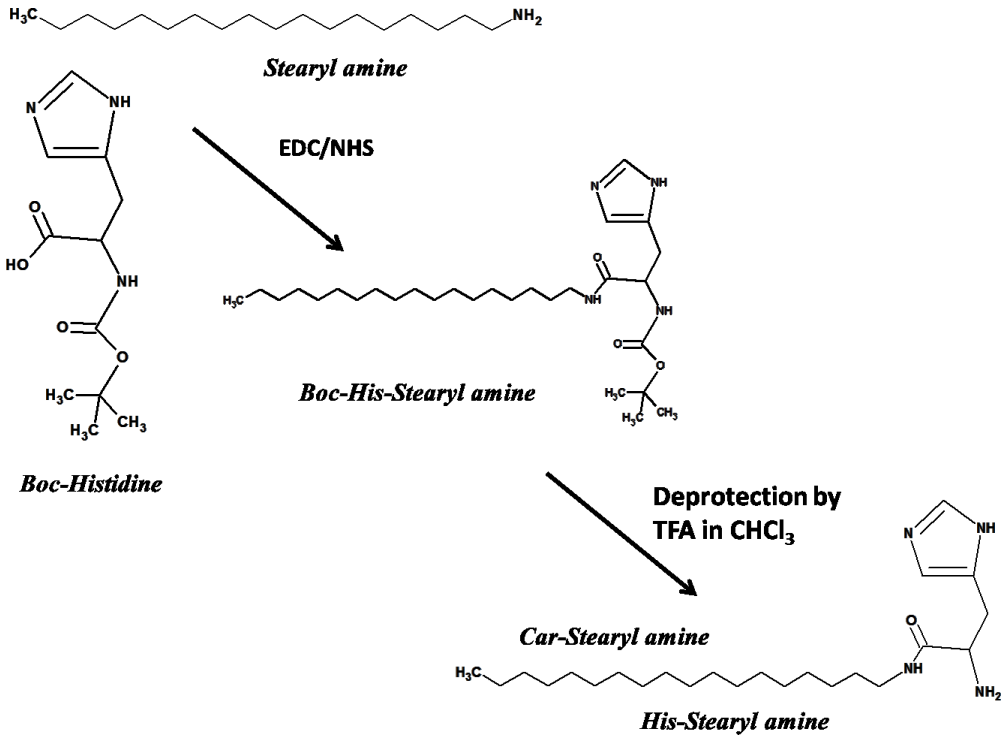


Figure 5.1 Synthesis of histidine conjugated stearyl amine

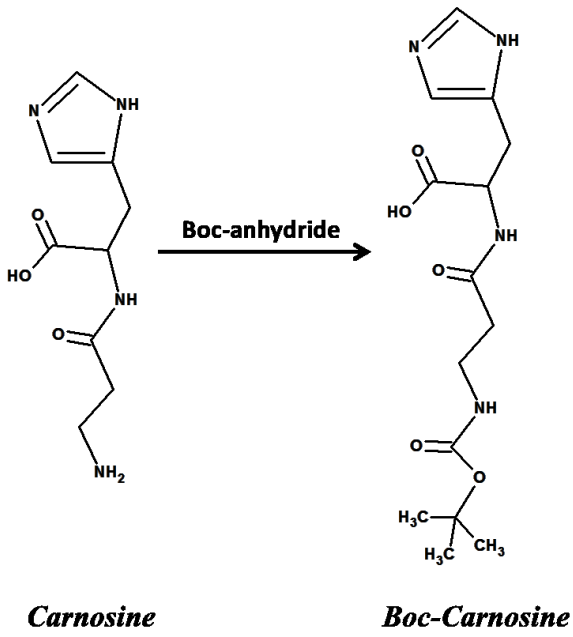


Figure 5.2 Synthesis of Boc-carnosine

For synthesis of carnosine (dipeptide of alanine and histidine) modified lipids, free amino group of carnosine was protected using Boc anhydride (dibutylpyrocarbonate). 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 5.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-SA was accomplished. Deprotection of the Boc-protected lipids was performed using same methodology to get Car-SA and Arg-SA.

Modification of dioleoyl-sn-glycerophosphoamine (DOPE) was carried out using the same strategy. Briefly, carboxyl group of Boc-amino acid or Boc-carnosine 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. After activation, DOPE was added in the

reaction mixture and dissolved by addition of mixture of chloroform, methanol and water (22:10:1 by volume). The reaction was carried out for 1 day at room temperature. Organic solvent 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 dialysed against water overnight for complete removal of unconjugated Boc-Histidine, EDC and NHS. Redispersion and centrifugation were repeated again for 2 times to ensure complete removal of unconjugated Boc-Histidine, EDC and NHS. Final pellet was again resuspended in water and freeze-dried until used. Boc-His-Stearyl amine pellet obtained was dissolved in chloroform and treated with excess of hydrochloric acid cleavage cocktail (HCl:MeOH:$CHCl_3$:Anisole in ratio of 3.4:5:0.5:0.1 by volume) and allowed to react overnight to remove Boc-protection. The reaction mixture was dried in rotary evaporator and dried residues were then washed with MeOH:H_2O (1:1 by volume) 3 times to remove traces of reactants. Carnosinylated DOPE (Car-DOPE) and Histidinylated DSPE (His-DSPE) were synthesized were also synthesized using same chemistry. For synthesis of Boc-carnosine, carnosine was Boc-protected using Boc-anhydride (Spectrochem, India).

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
Stearyl amine	-	-	SA
Dioleoyl-*sn*-glycerophosphoethanolamine	-	-	DOPE
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
SA	Histidine	Present	Boc-His-SA, BHSA
SA	Histidine	-	His-SA, HSA
SA	Carnosine	Present	Boc-Car-SA, BCSA
SA	Carnosine	-	Car-SA, CSA
SA	Arginine	Present	Boc-Arg-SA, BASA
SA	Arginine	-	Arg-SA, BASA
DOPE	Histidine	Present	Boc-His-DOPE, BHDO
DOPE	Histidine	-	His-DOPE, HDO
DOPE	Carnosine	Present	Boc-Car-DOPE, BCDO
DOPE	Carnosine	-	Car-DOPE, CDO
DSPE	Histidine	Present	Boc-His-DSPE, BHDS
DSPE	Histidine	-	His-DSPE, HDS

Efficiency of conjugation of the amino acid derivatives to the lipids (SA, DOPE and DSPE) was determined by carrying out the TNBS assay (For Boc-His-SA, or Sakaguchi assay (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 stearyl amine, DOPE and 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:

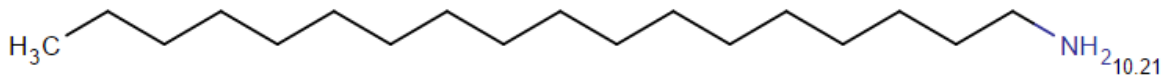
Predicted physicochemical characteristics of the lipids were evaluated for feasibility of their use in gene delivery. pKa and log P prediction were taken according to the ChemAxon (USA). Based on the pKa characteristics, %ionization of the lipids was calculated using Henderson-Hasselbalch equation.

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 titrand 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.

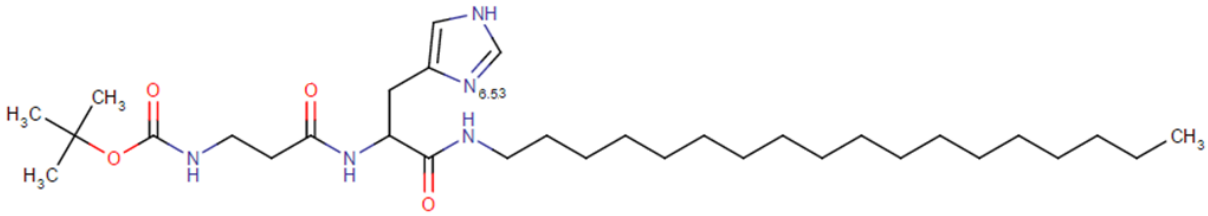
5.3 Results and discussion

Structures of the synthesized SA base lipids are shown in the **Figure 5.3** and **Figure 5.4**. 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 spectrophotoscopy showed that the UV absorption characteristic of Boc-His was retained in the Boc-His SA (**Figure 5.5**). Similar results were observed with Boc-histidine conjugated DOPE and DSPE (**Figure 5.11** and **Figure 5.13**).

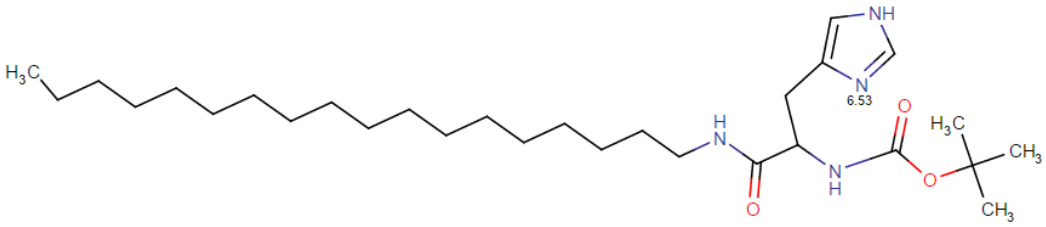


SA

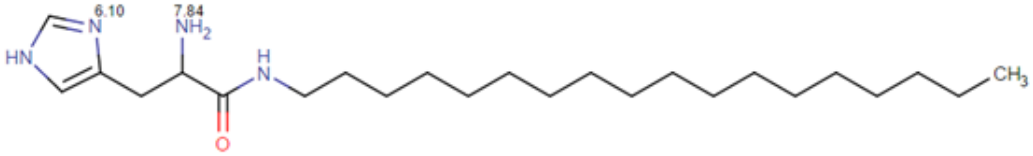


Boc-Car-SA

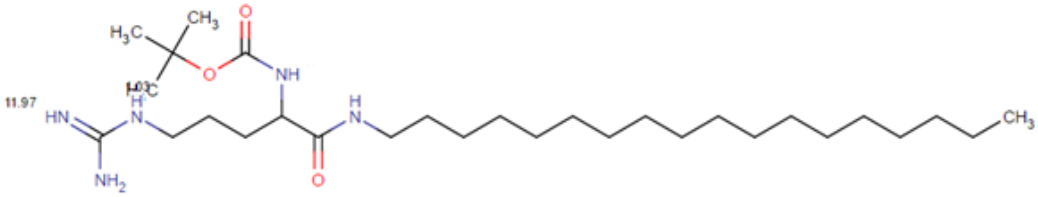
Car-SA



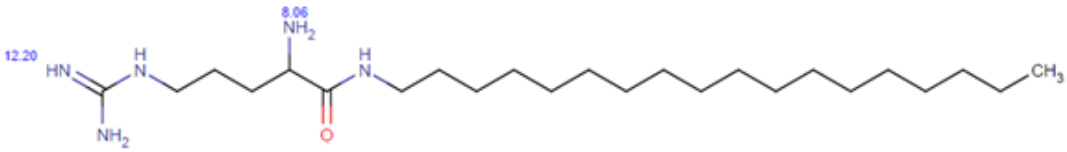
Boc-His-SA



His-SA

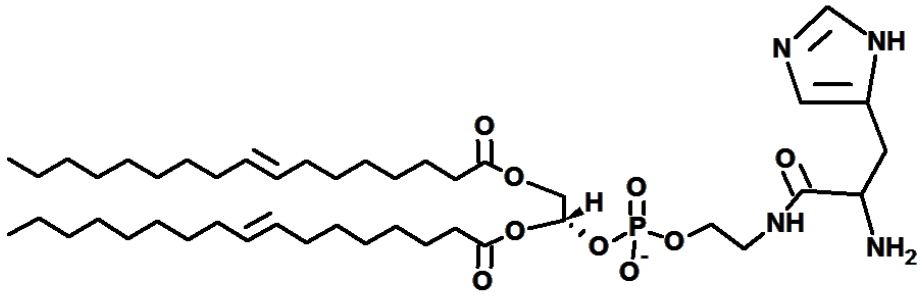


Boc-Arg-SA

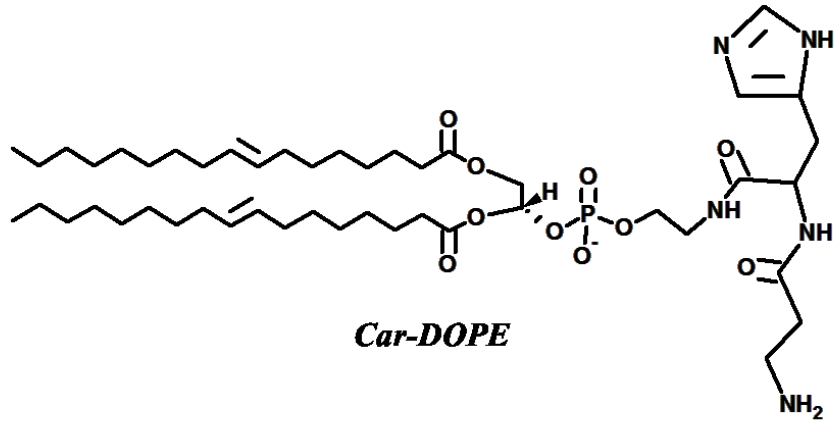


Arg-SA

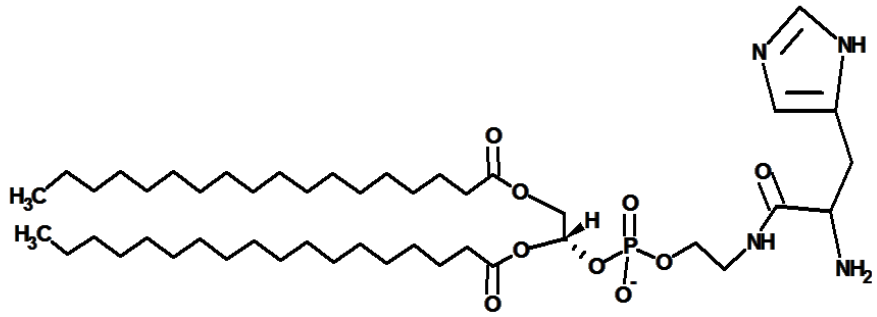
Figure 5.3 Structure of stearyl amine and stearyl amine based lipids (Value at amine groups indicates the calculated pKa of that group according to Chemaxon (Chemicalize.org)



His-DOPE



Car-DOPE



His-DSPE

Figure 5.4 Structure of Histidine and carnosine modified DOPE and DSPE

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-DOPE	1:1.5	1 day	17.64±5.5
	1:0.75	1 day	56.9±-3.1
	1:0.5	1 day	63.5±3.9
	1:0.5	3 days	66.0±4.2
Boc-His-DSPE	1:1.5	1 day	22.5±5.0
	1:0.75	1 day	57.7±4.8
	1:0.5	1 day	69.2±3.6
	1:0.5	3 days	68.5±4.2
Boc-His-SA	1:0.5	1 day	65.2±2.3
	1:0.75	1 day	55.4±3.4
	1:0.5	3 days	64.0±3.4

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-DOPE	1:0.5	1 day	63.5±3.9
Boc-Car-DOPE	1:0.5	1 day	58.9±4.2
Boc-His-DSPE	1:0.5	1 day	69.2±3.6
Boc-His-SA	1:0.5	1 day	65.2±2.3
Boc-Car-SA	1:0.5	1 day	55.9±2.4
Boc-Arg-SA	1:0.5	1 day	79.56±4.2

Boc-protection of carnosine was seen as rightward shift of the UV absorption spectrum of carnosine (**Figure 5.10**). NMR spectra of Boc-carnosine (Boc-Car) showed presence of BOC protons at δ 1.33 ppm (**Figure 5.7**) [2]. IR spectra of Boc-carnosine show absorption at 1597 cm^{-1}, 1660 cm^{-1} and 1694 cm^{-1} indicating the presence of , CO streatch of –CONH- of peptide linkage, C=O stretch of –NHCOO- of BOC protected amine and C=O stretch of carboxylic acid respectively. And 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, different lipids were 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 5.10** and **Figure 5.12**).

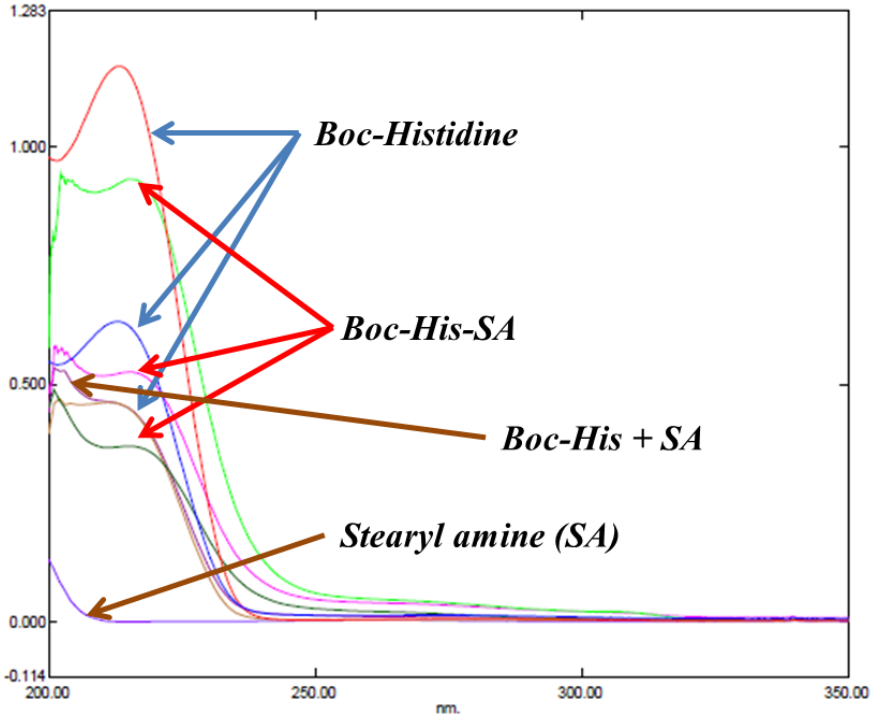


Figure 5.5 UV spectra of stearyl amine, Boc-histidine and Boc-His-SA (x-axis: wavelength, y-axis: absorbance)

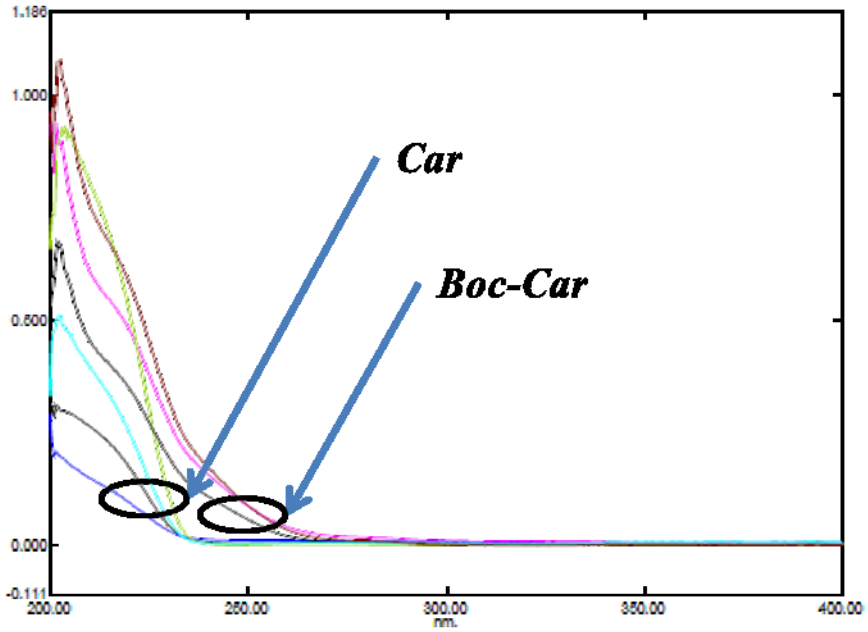


Figure 5.6 UV spectra Carnosine and carnosine isolated after Boc protection (x-axis: wavelength, y-axis: absorbance)

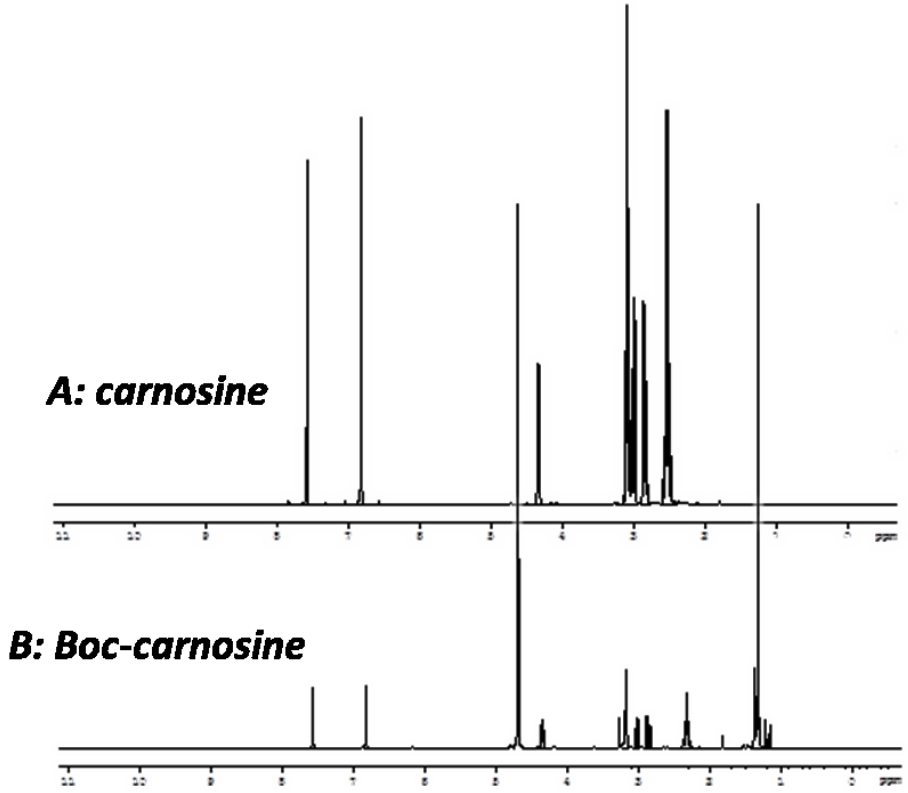


Figure 5.7 NMR spectra of Carnosine and Boc-Carnosine

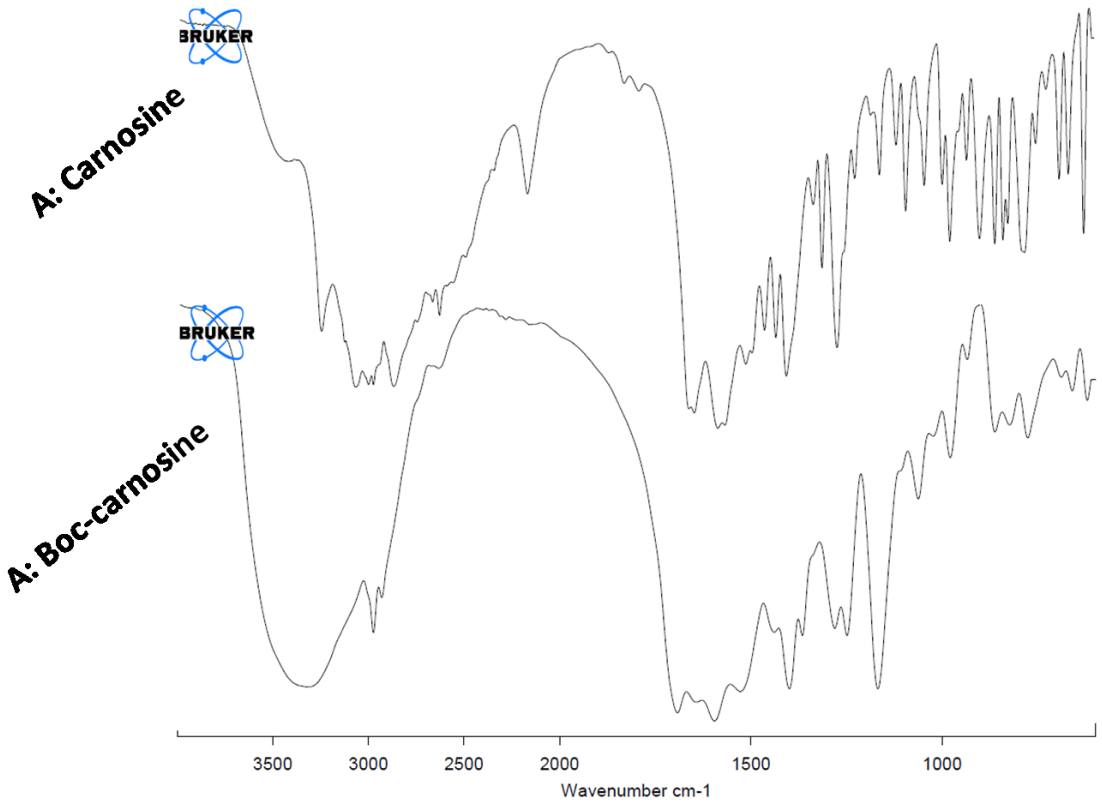


Figure 5.8 IR spectra of Carnosine and Boc-Carnosine

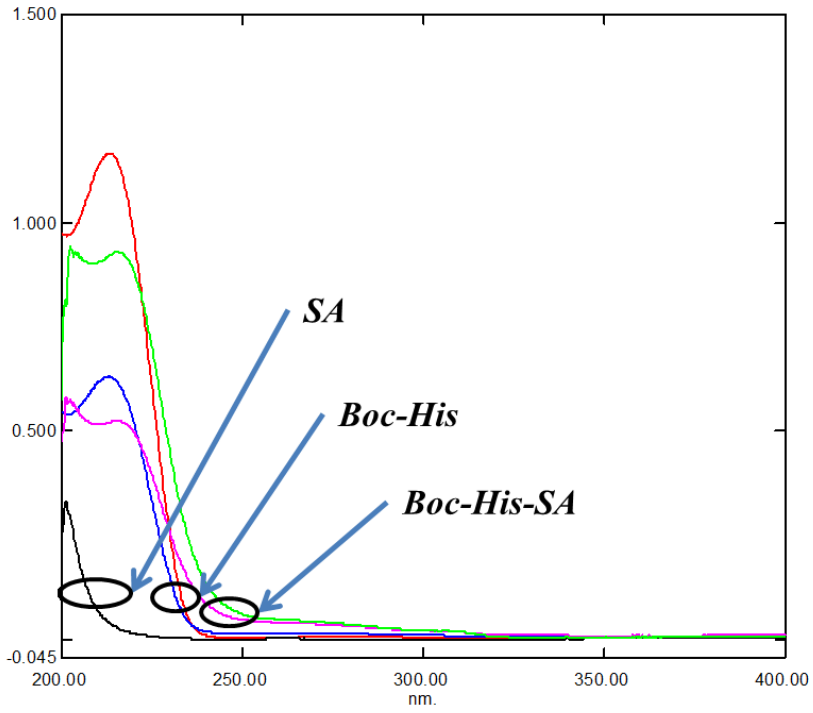


Figure 5.9 UV spectra of stearyl amine, Boc-His and Boc-His-SA (x-axis: wavelength, y-axis: absorbance)

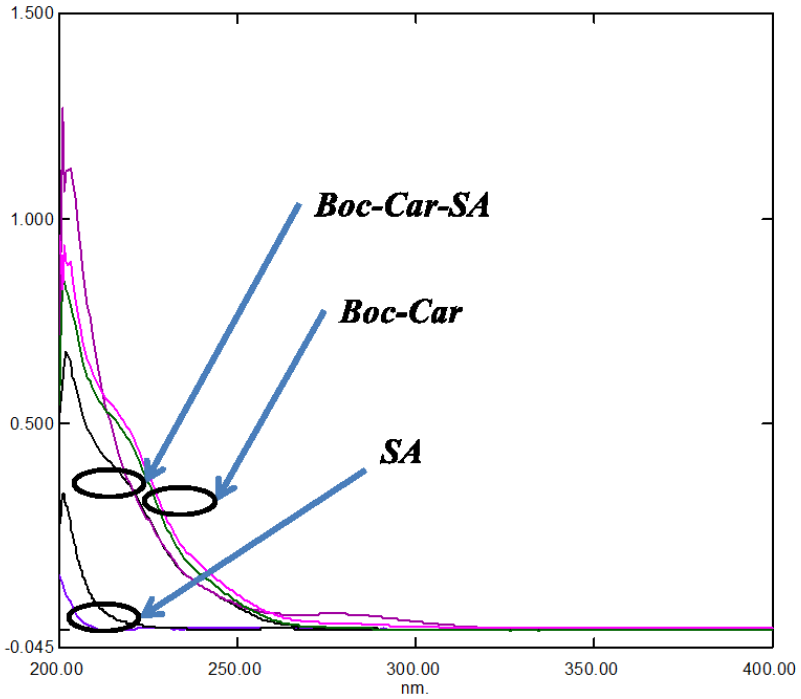


Figure 5.10 UV spectra of stearyl amine, Boc-Car and Boc-Car-SA (x-axis: wavelength, y-axis: absorbance)

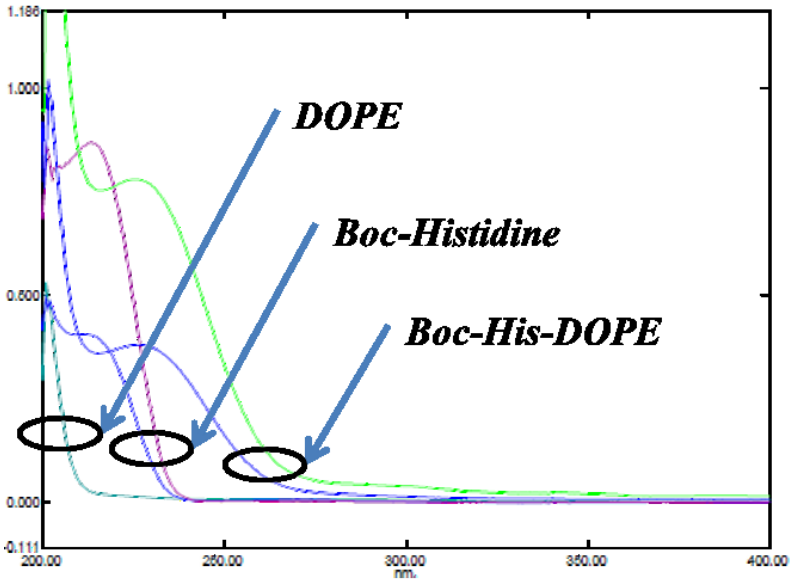


Figure 5.11 UV spectra of DOPE, Boc-histidine and Boc-His-DOPE (x-axis: wavelength, y-axis: absorbance)

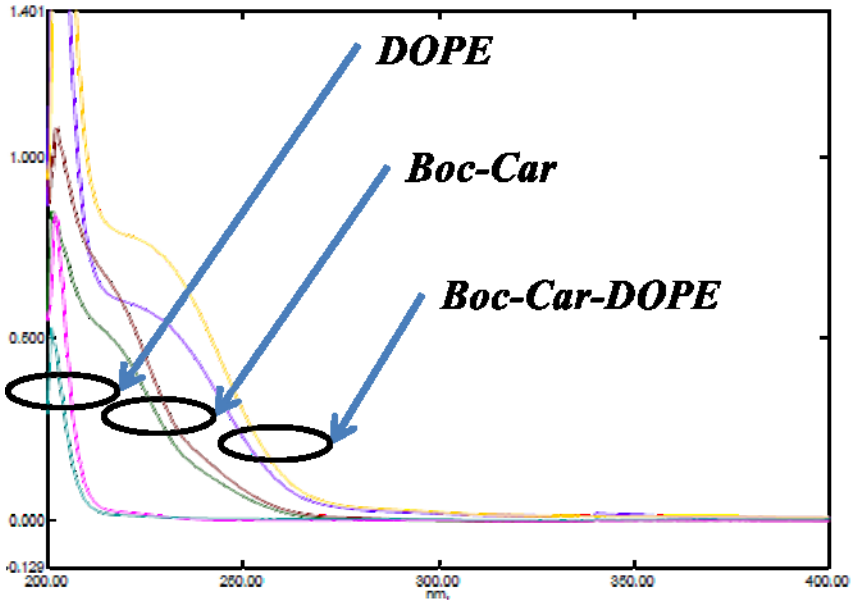


Figure 5.12 UV spectra of DOPE, Boc-Car and Boc-Car-DOPE (x-axis: wavelength, y-axis: absorbance)

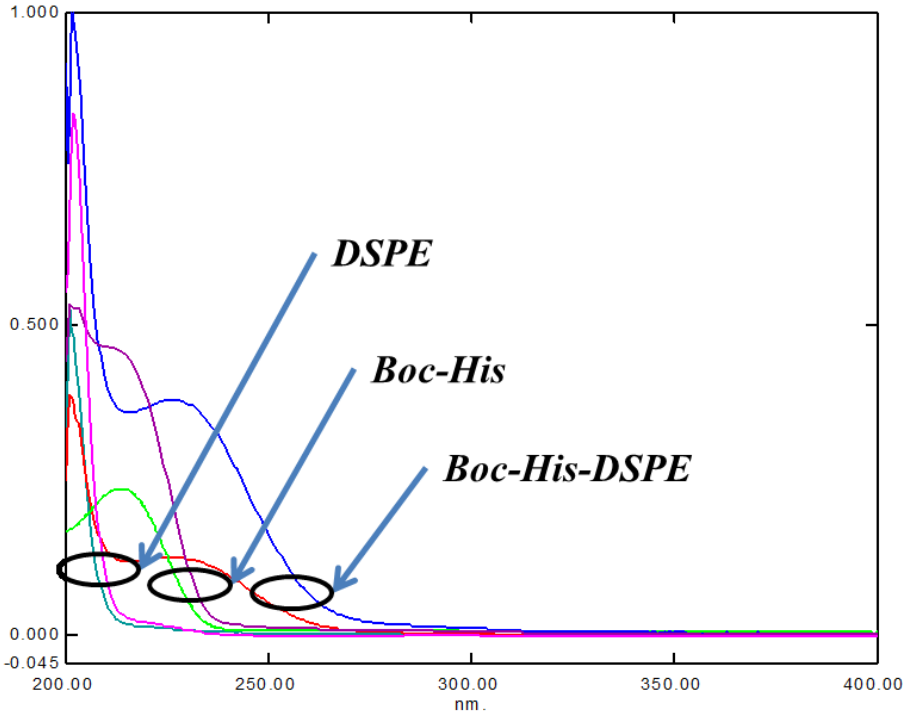


Figure 5.13 UV spectra of DSPE, Boc-His and Boc-His-DSPE (x-axis: wavelength, y-axis: absorbance)

Successful syntheses of lipids were confirmed by FTIR spectroscopy, and mass spectrometry (See attached supplementary spectroscopy images at the end of the chapter for details) [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 ~33300-3500 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 SA and -C=C- and –C=N- of aromatic imidazole ring of histidine and carnosine modified SA.
- 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 ($M+CH_3+H$)
DOPE	743.547	744.48(M), 766.7 (M+Na), 745.53 (M+1)
BHDO	980.807	981.66 (M+1 ion), 985.8 (M+4H), 1008.8 (M+4H+Na)
HDO	880.687	880.50 (M+) 881.63 (M+1), 886.6 (Hydrogenated-M+1+4H)
BCDO	1051.887	1051.69 (M)
CDO	951.767	952 (M+), 977.5 (M+4H+Sodium), 959.56 (M+4H+Sodium-18)
DSPE	747.578	747 (M), 748.6 (M+1), 790 (M+Na)
BHDS	984.838	985.7 (M+1)
HDS	884.718	885 (M), 906 (M+Na)
SA-Stearyl amine	269.51	268.33 (M), group of fragments differing by m/z of 14 characteristic of loss of sequential loss of CH2 groups
BHSA	506.77	506.61 (M+), homologous series fragments separated by m/z of 14 (loss of CH2) group
HSA	406.65	406 (M+), 441.29 (M+Cl), 425.07 (M+Cl-NH2) homologous series separated by m/z of 14
BASA	525.82	526.409 (M), 527.297 (M+1)
ASA	425.7	426.424 (M), 427.39(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 5.14**, **Figure 5.15**, **Figure 5.16** and **Figure 5.17**.

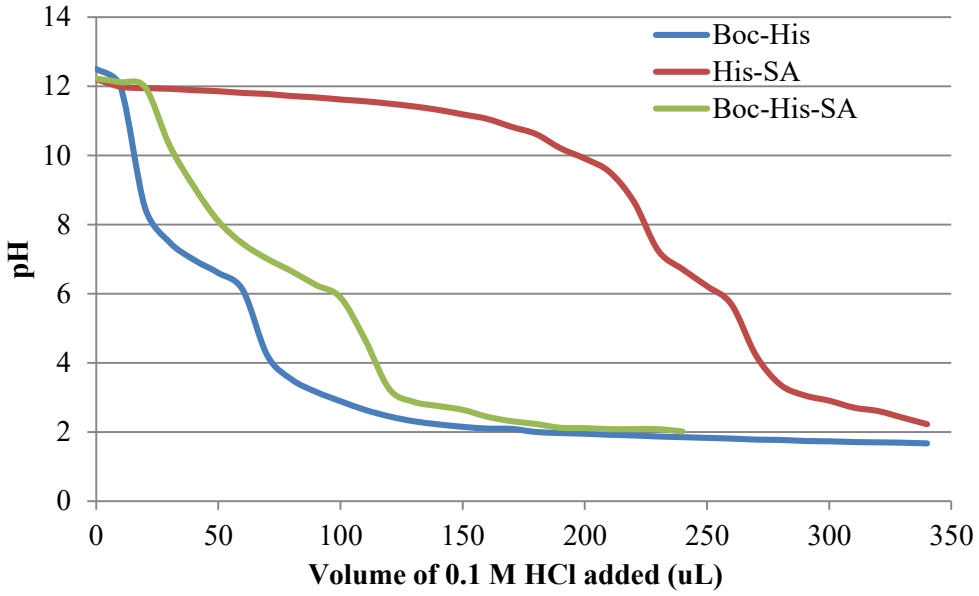


Figure 5.14 pH titration curev of Boc-Histidine, Boc-His-SA and His-SA

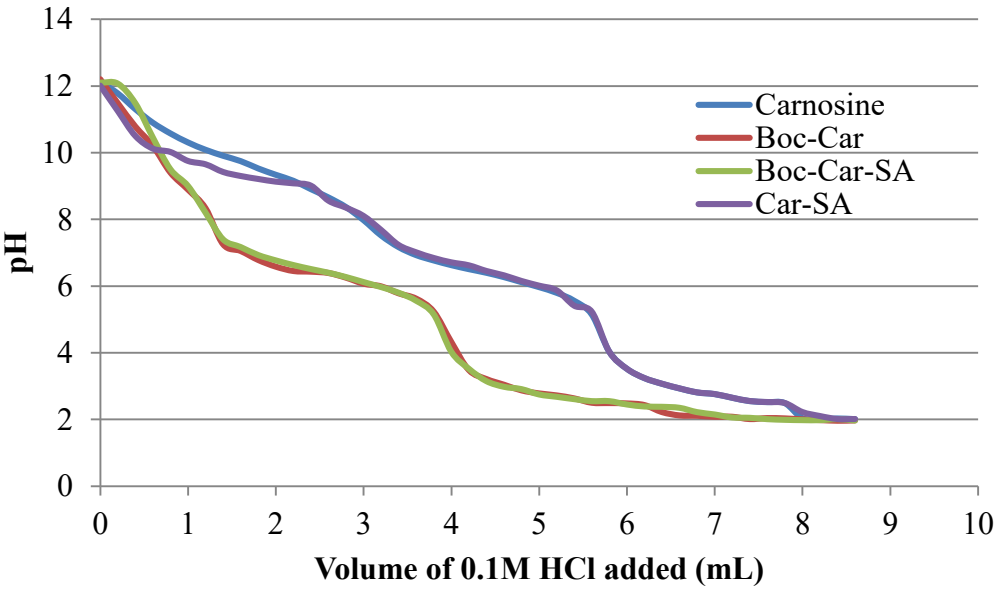


Figure 5.15 pH titration curev of Carnosine, Boc-Carnosine, Boc-Car-SA and Car-SA

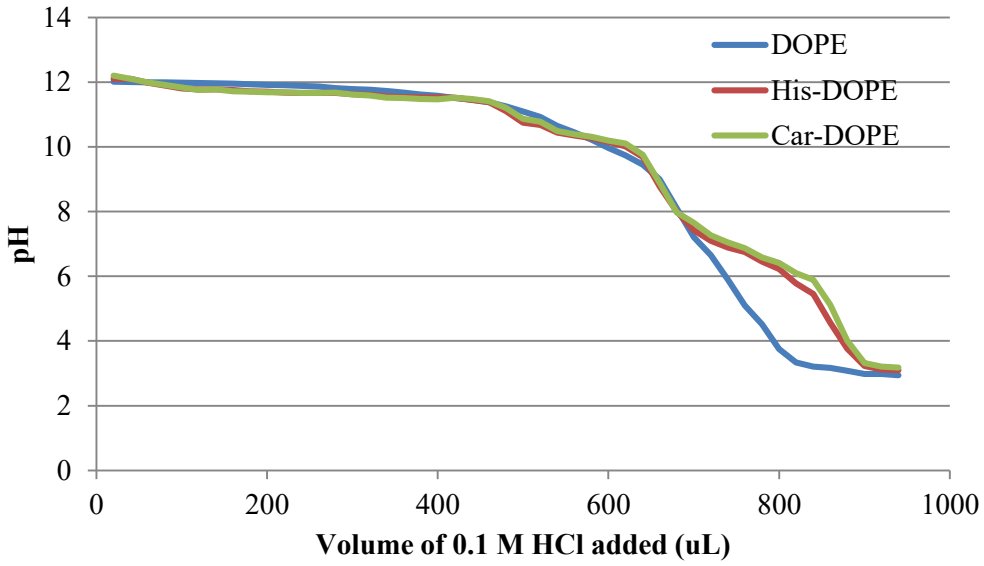


Figure 5.16 pH titration curev of DOPE, His-DOPE and Car-DOPE

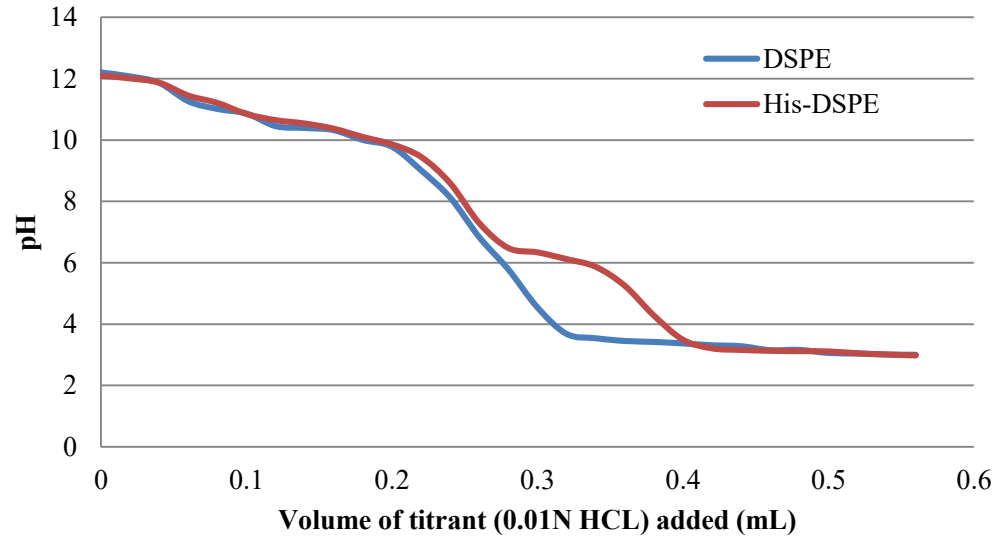


Figure 5.17 pH titration curev of DSPE and His-DSPE

pH titration curves of different lipids gives 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. Prediction was not available for His-DOPE, Car-DOPE and His-DSPE. However, the pKa

profile was similar to that observed with the imidazole (pKa ~6.5) and free amine groups (pKa ~10) of the Histidine and carnosine modified stearyl amine.

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[#]
Stearyl amine	10.21	-	6.92	>99.9%	-
Boc-His-Stearyl amine	-	6.53	7.45	-	25.3-51.7%
His-Stearyl amine	7.84	6.10	5.95	87.3-95.6%	24.4-50.5%
Boc-Car-Stearyl amine	-	6.52	6.58	-	24.8-51.1%
Car-Stearyl amine	9.13	6.53	5.09	99.2-99.7%	25.3-51.7%
Boc-Arg-Stearyl amine	-	11.97	6.88		>99%
Arg-Stearyl amine	8.60	12.20	5.48	97.5-99.2%	>99%
DOPE	10.00	-	11.51	>99.9	-
DSPE	10.00	-	12.23	>99.9	-
HDO	-	~6.5	-	-	25-51%
CDO	-	~6.5	-	-	25-51%
HDS	-	~6.5	-	-	25-51%

[$] 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

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