Chapter 7
In Vitro Physicochemical Characterization

There is no fate but what we make

-From "Terminator 3"

7.1 *In vitro* physicochemical characterization

Primary idea on performance of any gene delivery system can be obtained from the *in vitro* performance of the formulation. Such studies include the physicochemical characterization i.e. evaluation of effects of various simulated conditions which represent the in vivo environment on the physicochemical stability of the developed gene delivery system. Once the developed gene delivery systems show promising results in such studies formulation can be evaluated on the next battery of in vitro studies i.e. the cell line studies.

In the present investigation, the developed formulation will be evaluated for changes in various physicochemical parameters on exposure of electrolytes and serum which are the major encounters that the delivery systems are going to face on administration *in vivo*.

7.2 Stability of lipoplexes in presence of electrolyte (NaCl)

Lipoplexes when used in transfection studies or administered in vivo, the first challenge they encounter is the presence of serum and physiological electrolytes present in the blood or the organ [1]. Stability of lipoplexes depends on the strength of electrostatic interaction between the pDNA and cationic lipid and presence of electrolytes or serum components to which they are exposed. Competitive forces of attraction (van der Waals forces) and repulsion (either electrostatic repulsive forces or steric stabilizing barrier or both) are mainly responsible for the physical stability of a dispersed system. Additionally, various other interactions (depletion and steric interactions) also show an important role in stability of formulations [2]. The stability can be affected by either changes in the particle size [3] or may be manifested by the reduced complexation efficiencies of lipoplexes [4].

Electrostatically stabilized conventional liposomes, when exposed to electrolytes, face compression of electrostatic double layer surrounding the liposomes and subsequent aggregation followed by flocculation with increase in optical turbidity [5]. This indicates that the positive zeta potential of lipoplexes would provide the stabilization through electrostatic repulsion; however, in presence of concentrations of high salt concentrations i.e. in transfection media and in blood, the compression of double layer by electrolytes will cause the aggregation. Non-ionic polymer molecules i.e. PEGylated lipids on particulate surface prevent particles from coming close enough to allow van der Waals attractive forces between the particles to dominate and thus create steric barriers resulting

in steric stabilization [6] and the lipoplexes would remain stable even when the electrostatic double layers have been compressed. The electrolyte induced flocculation, thus, gives idea on the steric stabilization of lipoplexes through covering of surface by PEG chains [7].

Additionally, as said earlier, salts like sodium chloride can disrupt electrostatic interactions causing particle size change through aggregation and then leading to dissociation of polyplexes at certain concentration [4]. Additionally, the effects have also been noted for the differential stability in terms of their complexation efficiency of the lipoplexes prepared in presence of electrolyte as compared to that of lipoplexes prepared in absence of electrolytes followed by exposure to electrolyte [4, 8].

Dynamic light scattering can be used for estimation of influence of presence of salt on lipoplex particle size while gel electrophoresis can be used to check the effect of electrolyte on the stability as well as formation of lipoplexes.

7.2.1 Method

Lipoplexes prepared in DFW were diluted and treated with different sodium chloride solutions to get to get 1:10 v/v dilution of lipoplexes and sodium chloride concentrations in the range of 1%, 2%, 3%, 4% and 5% w/v. Samples were incubated at room temperature for 30 minutes before analysis and particle size analyses were performed in a clear sizing cuvette at 25°C temperature using 633 nm laser as a light source and 90° back-scatter photon detector using ZetaSizer Nano ZS (Malvern Instruments Ltd., UK). z-averaged diameter and PDI were determined on the Zetasize software Ver. 7.11 (Malvern Instruments Ltd., UK).

For analysis of complexation efficiency lipoplexes were prepared at different N/P ratios and were incubated at room temperature for 30 minutes at sodium chloride concentrations of 2% and 5%. After incubation samples were analyzed by gel electrophoresis method for complexation efficiency.

7.2.2 Results and Discussion

Presence of electrolytes, by disrupting the electric double layer around the lipoplexes causes aggregation and fusion leading to particle size increase. Particle sizes and PDI of the lipoplexes treated with different concentrations of sodium chloride

solutions are shown through **Figure 7.1** to **Figure 7.5**. Results show that there was a drastic increase in the particle size of the non-PEGylated SA lipoplexes as compared to the PEGylated SA lipoplexes (shown as SA lipoplexes) as well as PEGylated lipoplexes of all synthesized lipids (SA lipoplexes mentioned here represent PEGylated SA lipoplexes, unless specified). Non-PEGylated lipoplexes showed particle size increase up to micron size (1.2 μ after treatment at 5% NaCl solution), while the lipoplexes with PEGylated surfaces showed more stability against the electrolyte showing significantly less particle size increase up to 5% NaCl concentration. At all concentrations particle size remained below 170 nm for all lipoplexes except for the non-PEGylated SA lipoplexes. This can be explained by the presence of the PEG chains that even on the compromise, to some extent, of the electric double layer by electrolyte, prevent the closer approach of the lipoplexes due to steric barrier created by the PEGylation.

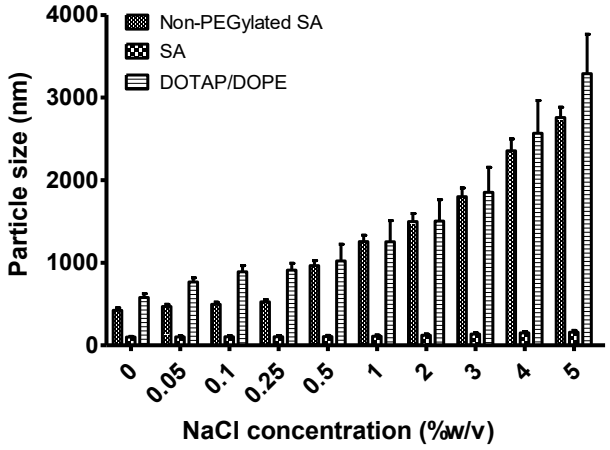

Figure 7.1 Effect of electrolyte on particle size of the SA lipoplexes and non-PEGylated SA lipoplexes and DOTAP/DOPE lipoplexes

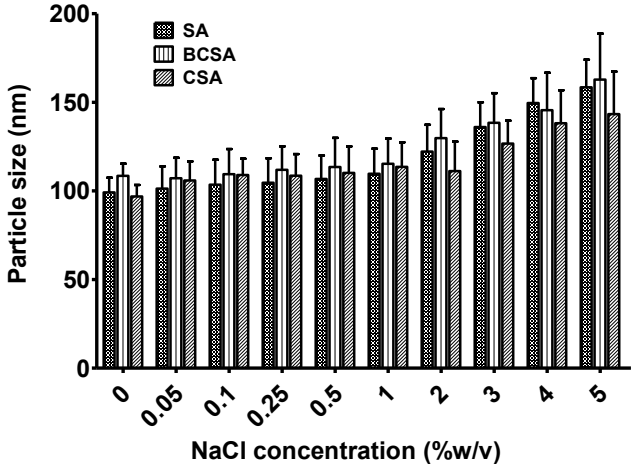

Figure 7.2 Effect of electrolyte on particle size of the SA, BCSA and CSA lipoplexes

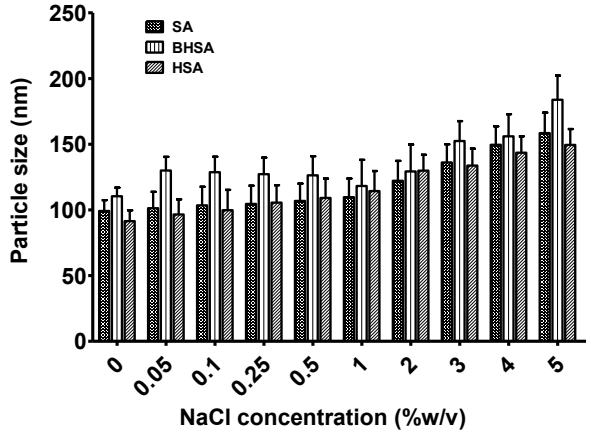

Figure 7.3 Effect of electrolyte on particle size of the SA, BHSA and HSA lipoplexes

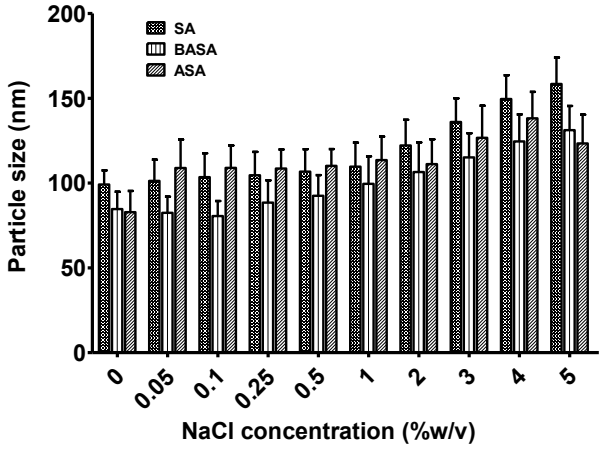

Figure 7.4 Effect of electrolyte on particle size of the SA, BASA and ASA lipoplexes

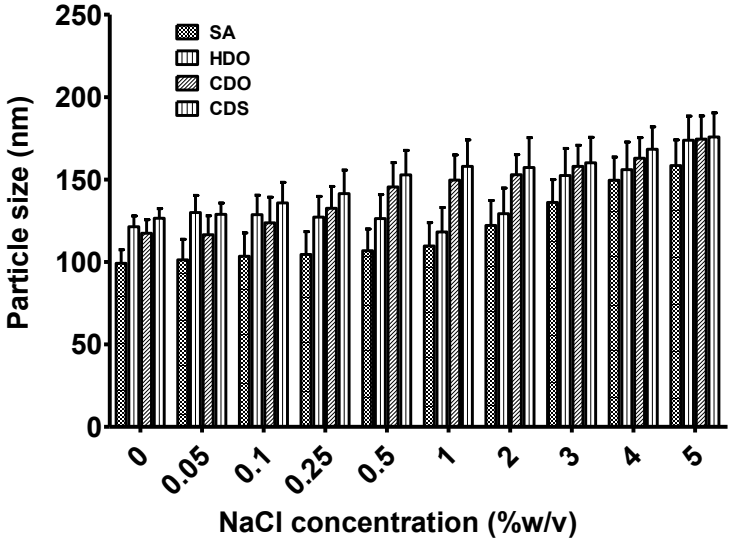

Figure 7.5 Effect of electrolyte on particle size of the SA, HDO, CDO and CDS lipoplexes

As described earlier, the disruption of electric double layer of liposomes also leads to dislodging of complexed pDNA from the surface of the lipoplexes. Hence, to evaluate the effect of incubation with electrolyte solution of lipoplexes on their complexation efficiency, gel electrophoresis was performed. Effect of presence of 2% sodium chloride was evaluated on PEGylated and non-PEGylated SA lipoplexes before complexation and after complexation i.e. lipoplexes prepared in 2% NaCl solution or lipoplexes were prepared in DFW followed by treatment with NaCl at 2% concentration. Complete complexation was not observed for both lipoplexes when complexation was performed in presence of sodium chloride. This effect can be explained by the decrease in zeta potential of liposomes on treatment with sodium chloride solution (**Figure 7.6**). On treatment with sodium chloride solutions of increasing concentrations, the zeta potential of liposomes was found to be decreasing which reached to almost neutrality after concentration of 1 or 2% NaCl and remained more or less unchanged up to5% concentration. Change in zeta potential values of non-PEGylated SA liposomes vs. that of PEGylated SA liposomes (referred to as SA liposomes) indicated that PEGylation of surface led to lower initial zeta potential which reduced rapidly over treatment with NaCl. This might be due to the compression of double layer around the PEGylated liposomes which is already destroyed to some extent by PEG chains which hinder the surface charge mediated accumulation of ions around the particle surface. Additionally, the lower zeta potential of this liposomes was easily destroyed by incubation with electrolyte with zeta potential reaching near to neutrality at 1 or 2% NaCl concentration with PEGylated liposomes while non-PEGylated liposomes still retaining the positive charge even at concentration of 5% NaCl.

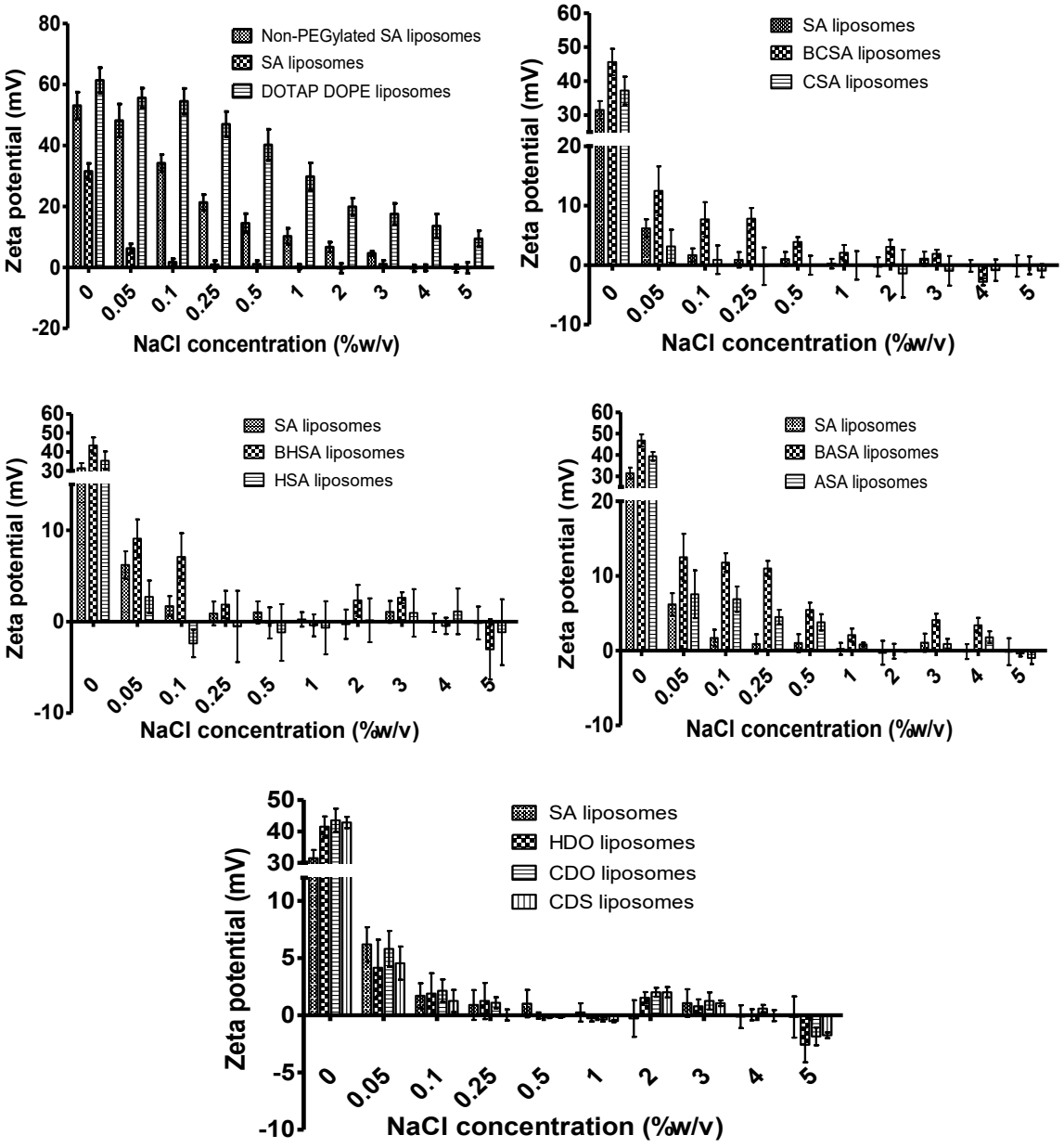

Figure 7.6 Effect of electrolyte on zeta potentials of the liposomes

Observing the trends, it was noticed that zeta potential value decrease was much more higher for initially over concentration range of 0.05 to 1% NaCl while above this concentration the zeta potential values were found to be decreasing to a lesser extent as well as a distinct observation was initial decrease of mean zeta potentials below 0 mV to concentration range of 1 to 2% NaCl which increased to positive values at 3 to 4% NaCl and again decreased below 0 mV. This observation was consistent with PEGylated SA liposomes as well as with PEGylated liposomes prepared with modified lipids. However, such decrease was not noticed with non-PEGylated liposomes which remained positively

charged over the selected concentration range suggesting some indicative role of PEG chains in such zeta potential variation.

As Boc protected and deprotected lipids would differ from each other with respect to their one and two cationic groups respectively, the difference in zeta potential was expected to be different for both these categories i.e. Boc protected and deprotected lipids with zeta potential of less magnitude for former lipids. However, the difference was not of that intensity, rather all lipids showed zeta potential between 35-45 mV. This was corroborating with the results with the zeta potentials of liposomes/lipoplexes prepared with DOTAP (head-group charge +1) and DOSPA (head-group charge 5+) which showed similar zeta potential as well as surface potential characteristics [9, 10]. This indicates that zeta potential values are irrespective of the cationic head-group charges per molecule. Giving a closer look at the effect of different lipids' behavior in electrolyte concentration in terms of zeta potential, one can notice the initial zeta potential values which are consistently higher for BOC protected lipids as compared to Boc-deprotected lipids. This seemed to be an observation opposite to the expected indicating the impact of head-group hydrophobicity/hydrophilicity balance. As two charges would be playing role in the zeta potential development of Boc-deprotected lipids as compared to Boc protected lipids, even after PEGylation zeta potential should be higher for liposomes of Boc-deprotected lipids. However, it was noticed that zeta potential of BHSA, BCSA, BASA liposomes were consistently higher than HSA, CSA and BASA liposomes. Trend remained the same over electrolyte concentrations as well i.e. decrease in zeta potential were of lesser extent for Boc-amino acid lipids as compared to deprotected lipids. Even after PEGylation, the hydrophobic portion of BHSA, BCSA and BASA which would extend over the surface of liposomes would impact the steric positioning of the PEG chains ultimately providing some hydrophobic pockets over surface that would not allow closer approach of electrolytes and hence, would protect the liposomes from electrolyte mediated loss of zeta potential. Zeta potential trend for the HDO, CDO and CDS liposomes were similar to those observed with their corresponding version of SA liposomes i.e. HSA and CSA liposomes.

Further to zeta potential, changes in the complexation efficiency of lipoplexes are also evaluated on exposure of sodium chloride. Effect of sodium chloride on complexation was higher for PEGylated lipoplexes (**Figure 7.7**). This effect can be explained by the fact

that sodium chloride does not only affect the ionic characteristics of cationic liposomes but also disrupts the electrostatic character of pDNA leading to change in its mobility [11, 12]. Hence, when sodium chloride is present before complexation, disrupted pDNA electrostatics will not allow pDNA to overcome the PEG chain barrier for complete complexation as well as the masked charge of liposomes due to presence of PEG chains, both of which will contribute to reduced complexation even at N/P ratio of 2.0. When complexes were prepared in WFD and then treated with sodium chloride, no disruption of complexes was observed even at N/P ratio of 1.0. This indicates that once the complexes are formed the complementary electrostatic interaction between the liposomes and pDNA would be so strong that complex will remain stable without any loss of pDNA from the surface. The study was carried out using 2% NaCl only and the complex may get affected by the electrolyte at higher concentrations. One study shows the complete dissociation of pDNA from lipoplexes will require 750 mM NaCl concentration which corresponds to ~4.3% NaCl concentration [4]. Additionally, here the complexes are made of cationic liposomes alone without any supporting lipids and PEGylated lipid. Thus, results obtained in the present investigation which shows complexes are stable on treatment with 2% NaCl concentration demonstrate that PEG chains of the lipoplexes which will sterically hinder the complexed pDNA from leaving surface as well as the other helper lipids which separate the charge effectively over surface of liposomes are involved in the strong complexation between pDNA and cationic liposomes. Additionally, higher complexation efficiency of non-PEGylated lipoplexes on treatment with electrolyte as opposed to that of PEGylated liposomes is corroborated by the retention of cationic charge by non-PEGylated liposomes as compared to PEGylated liposomes. In addition, some complexation efficiency shown by PEGylated liposomes which has shown almost neutral cationic charge indicate the importance of cationic surface potential and higher concentration of liposomes and pDNA that would allow sufficient collision of the particles to afford some complexation of pDNA [11].

As it can be seen from **Figure 7.8** and **Figure 7.9**, similar results were obtained with lipoplexes of BHSA, HSA, BCSA and CSA which are prepared at N/P ratio of 2.0 with electrolyte treatment before complexation or after complexation. Similarly, complexation efficiencies were not affected on electrolyte treatment of preformed BASA, ASA, HDO, CDO and CDS lipoplexes (**Figure 7.10**). Considering the zeta potential values of liposomes, it was noticed that BHSA and BCSA lipoplexes retained some mean

cationic charge of 2-4 mV magnitude. However, the effect for complexation of pDNA did not differentiate at this charge level between BHSA and HSA lipoplexes as well as BCSA and CSA lipoplexes. Treatment before complexation had higher effect on complexation efficiency of HSA lipoplexes as compared to BHSA lipoplexes and CSA lipoplexes as compared to BCSA lipoplexes for 1 hr incubation period of complexation. This can be explained, as described earlier, by the zeta potentials of BHSA and BCSA liposomes which were slightly more cationically charged than HSA and CSA liposomes respectively which might be due to presence of Boc group's hydrophobic effect. However, in all cases, preformed complexes showed no effect of electrolyte on complexation of liposomes with pDNA indicating intimate contact of pDNA with surface causing all imidazole ring nitrogens to be protonated due to acidic microenvironment created by pDNA even with the BHSA and BCSA lipoplexes.

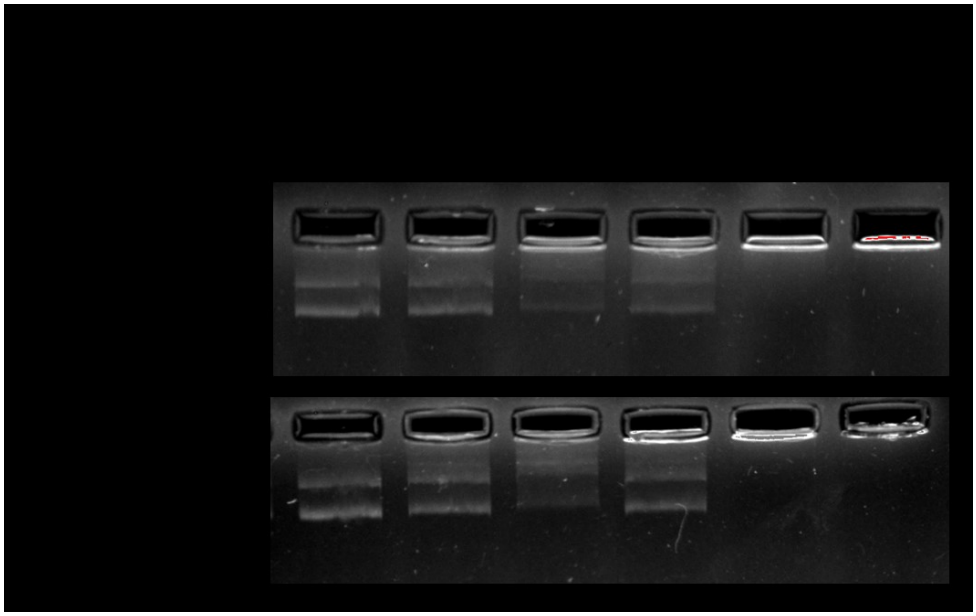

Figure 7.7 Effect of electrolyte (2% NaCl) on complexation on efficiency of non-PEGylated and PEGylated SA lipoplexes at N/P ratio of 1.0 and 2.0 (200 ng pDNA/well)

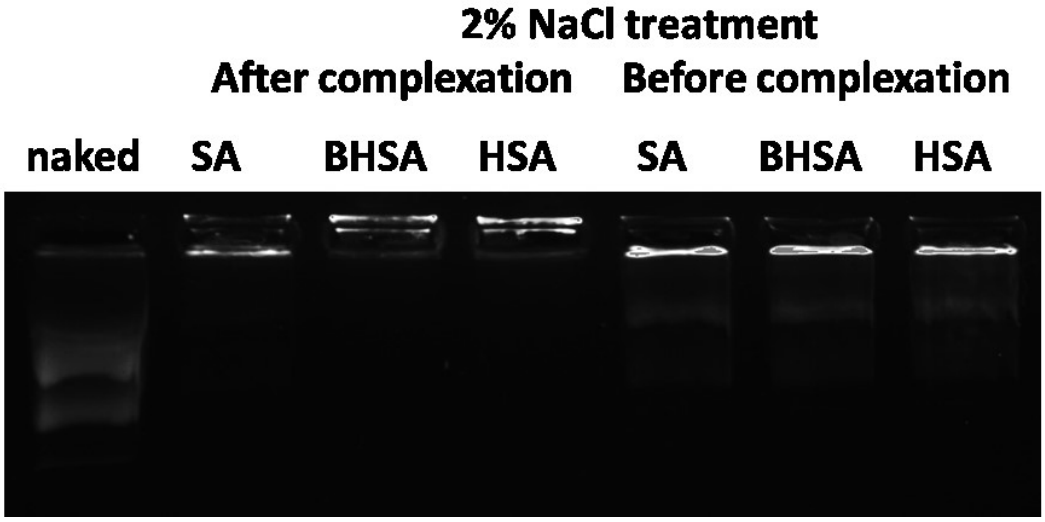

Figure 7.8 Effect of electrolyte (2% NaCl) on complexation on efficiency of SA, BHSA and HSA lipoplexes at N/P ratio of 2.0 (200 ng pDNA/well)

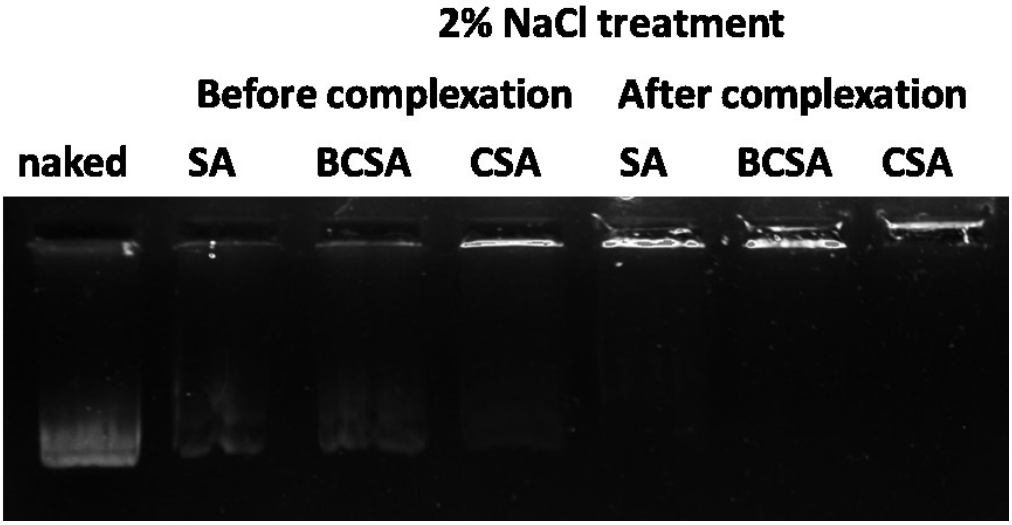

Figure 7.9 Effect of electrolyte (2% NaCl) on complexation on efficiency of SA, BCSA and CSA lipoplexes at N/P ratio of 2.0 (200 ng pDNA/well)

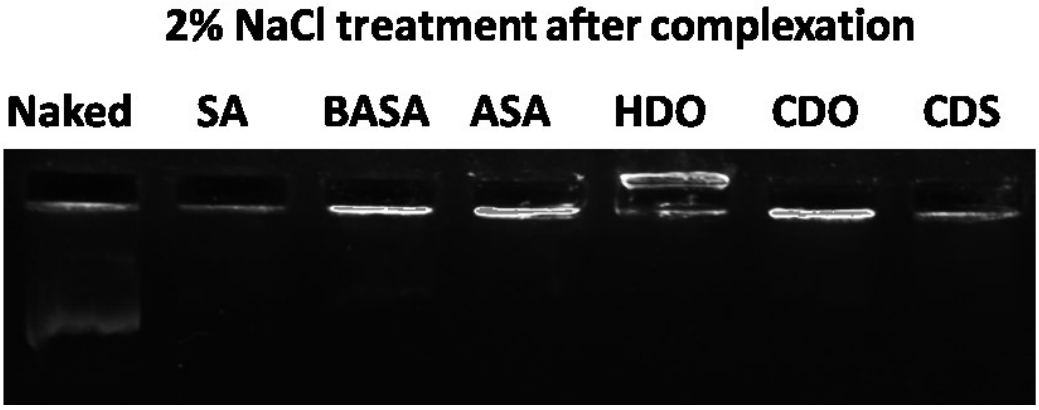

Figure 7.10 Effect of electrolyte (2% NaCl) on complexation on efficiency of SA, BASA, ASA, HDO, CDO and CDS lipoplexes at N/P ratio of 2.0 (200 ng pDNA/well)

Results indicate that the developed lipoplexes due to their PEGylated surface confer the lipoplexes stability for their in vivo survival in presence of the electrolytes present.

7.3 Serum Stability Study

For effective cellular internalization of the DNA for ensuring its functional activity, the foremost requirement is stability of the employed DNA against the nucleases present in the circulation. Even though DNA shows prolonged stability as compared to that of RNA at atmospheric conditions, the correlation of the above non-labile nature can't be extrapolated to the in vivo behaviour. The fragmentation of the DNA at inter-nucleosomal level by DNAses which are ubiquitously expressed in mammalian cell is encountered as a prominent hurdle determining the effective delivery of formulated product to the cell in intact form [13]. The circulating DNA has a short half-life of 10 – 15 min [14]. The chemical modification of the DNA by use of glycols or diols to the oligonucleotides is reported to increase their resistance to the exonucleases thereby prolong the circulation half-life of the DNA [15].

Complexation of DNA with the use of cationic lipid carriers is the most widely used approach to enhance the cellular uptake of the DNA and to target the expression of the gene to specific cells. Such complexation of DNA with cationic lipids has also demonstrated its stability against the nucleases due to prohibited access of nucleases to the complexed DNA to cause the fragmentation.

7.3.1 Methods

The DNA liposomes were studied for the integrity of complexed DNA in presence of serum for possible *in vivo* degradation because of degradation during circulation and degradation due to extracellular and intracellular nucleases. Naked DNA and lipoplexes prepared from synthesized lipids containing 10 µg of pDNA were incubated with 10 µL non-heat inactivated FBS (HiMedia Labs, India) at 37°C (Rotory shaker incubator, Scigenics-Orbitek, Tamilnadu, India) for various time periods to give a 50 %v/v serum concentration in final incubation volume. After incubation period, samples were inactivated by 10 µL of 0.5 M disodium EDTA solution to stop the degradation process. . Integrity of the pDNA at each time point was accessed using gel electrophoresis.

Incubated samples were mixed in 1:2 v/v ratio with phenol/chloroform (1:1 v/v) mixture was added to the lipoplex samples. Vigorous mixing was done to break lipoplexes and to partition the pDNA in water and lipids into phenol:chloroform layer. Samples were then phase separated by centrifugation (Remi Compufuge, Remi, India) at 14000 rpm at

4°C temperature. Separated aqueous layer is removed and treated separately with chloroform (200 µL) and vigorously vortexed and phase separated by centrifugation similarly to remove any traces of phenol in chloroform. Aqueous layer is used for determination of pDNA content using gel electrophoresis.

Aqueous layer (equivalent to 200 ng of pDNA) was directly loaded on the agarose gel along with naked pDNA samples as reference to determine intact pDNA content. Gel was run at 100 V/cm for 45 min in tank buffer using gel loading dye. Gel was visualized using UV transilluminator assembled with gel documentation system (GelDoc ImagingXR+ system, Bio-Rad, USA) and images were captured on ImageLab software as described earlier. Band densities were analyzed by ImageJ software ver. 1.50c (National Institute of Health, USA).

7.3.2 Result and discussion

Structural stability study of pDNA in serum condition was carried out at higher serum conditions. An essential property of lipoplexes designed in present investigation for further applications is the ability to protect the complexed pDNA from degradation by serum nucleases (mainly DNAses). Therefore, serum stability of lipoplex formulations were also determined by incubating them in FBS at 37°C. Naked pDNA and lipoplex formulations were incubated in 50 %v/v serum containing medium and the degradation of pDNA was analysed by gel electrophoresis. Analysis of pDNA was carried out by gel electrophoresis only, as fragments of dsDNA would interfere the analysis by QuantiFluor dsDNA detection method and fragmented dsDNA as well as ssDNA would interfere the analysis by NanoDrop. Apart from this, gel electrophoresis method of analysis will also help analyse the integrity of pDNA i.e. fragmentation of pDNA would be easy to identify as well as quantification to a certain extent.

naked pDNA was incubated with serum for 2 min, 5 min, 10 min, 20 min, 30 min and 1 hr and lipoplexes of SA liposomes (at L/P or N/P ratio of 2) were incubated with serum for 30 min, 1 hr, 2 hr, 4 hr, 8 hr, 12 hr, 24 hr. At every time point, remaining pDNA was detected (**Figure 7.11**) and results are shown in **Table 7.1**. It can be noted that degradation was started within a minute for naked pDNA which lost its complete activity within 30 min. While with the SA lipoplexes, degradation was observed to occur at a very low extent up to 4 hr with >95% of pDNA content retaining in the lipoplexes (**Table 7.2**).

However, the loss of intact pDNA was higher for non-PEGylated SA lipoplexes than for PEGylated SA lipoplexes. Results indicate that non-PEGylated SA lipoplexes are more prone to be affected by the components of serum than the PEGylated lipoplexes as indicated by the intact pDNA content of ~96% and ~82% at 8 hr and of ~81% and ~57% at 24 hr respectively. Different results observed with the lipoplexes also demonstrate the mechanism of the loss of pDNA from the lipoplexes. The higher loss of pDNA from the non-PEGylated lipoplexes is higher due to the dislodging of the surface complexed pDNA by the negatively charged components of the serum followed by the degradation by serum nucleases. Thus, PEG chains extending over the surface of the lipoplexes would be protecting the complexed pDNA from dislodging by negatively charged serum proteins and hence, would lead to lower degradation by serum nucleases.

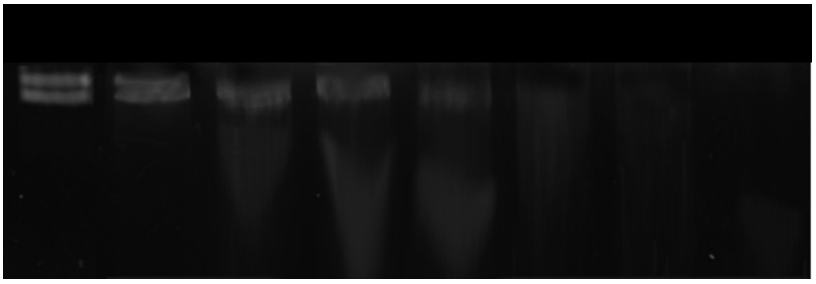

Figure 7.11 Serum stability of naked pDNA
200 ng pDNA/well, incubation time: Lane 1-naked pDNA, lane 2-0 min, lane 3-2 min, lane 4-5 min, lane 5-10 min, lane 6-20 min, lane 7-30 min, lane 8-60 min

Table 7.1 Intact pDNA content of naked pDNA incubated with serum

Time (min)	% intact pDNA remained
Naked pDNA	100
0	97.05±2.42
2	87.27±3.42
5	73.82±4.05
10	51.02±2.85
20	28.17±6.15
30	17.28±4.85
60	8.46±4.09

Table 7.2 Intact pDNA content of PEGylated and non-PEGylated SA lipoplexes after incubation with serum

Time (hr)	% intact pDNA remained	
	Non-PEGylated SA lipoplexes	PEGylated SA lipoplexes
Naked DNA	100.00	100.00
0	99.63±1.24	98.89±2.7
2	94.58±2.50	98.55±3.80
4	92.48±1.68	98.78±2.44
8	82.58±2.15	96.11±3.30
16	67.25±4.14	91.58±3.05
20	59.25±2.29	90.68±3.21
24	56.85±2.52	81.04±2.47

*Experiments were performed in triplicate.

Based on the results obtained with the PEGylated lipoplexes, lipoplexes of other liposomes prepared with synthesized lipids were incubated for 4 h and 8 h and the DNA contents were determined after incubation. The **Figure 7.12, Figure 7.13, Figure 7.14** and **Figure 7.15** shows that the pDNA was found to be stable for all lipoplexes after 8 hr period retaining >95% of pDNA in intact form as was the case with the SA lipoplexes. Results suggest that lipoplexes will protect pDNA from blood pH (7.4) and serum condition faced on intravenous administration. Enhanced stability of the lipoplexes can be attributed to both complexation with cationic lipid as well as to the PEGylated DSPE present in the lipoplexes, both of which will prevent the access of the pDNA to the nucleases present in the serum.

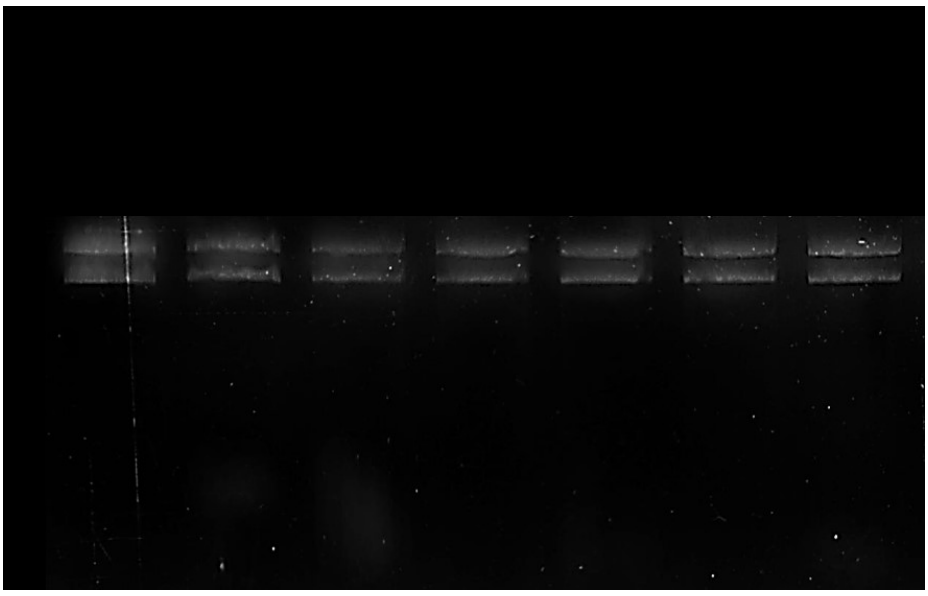

Figure 7.12 Serum stability of lipoplexes (SA, BHSA and HSA) prepared at N/P ratio of 2 after incubation with serum

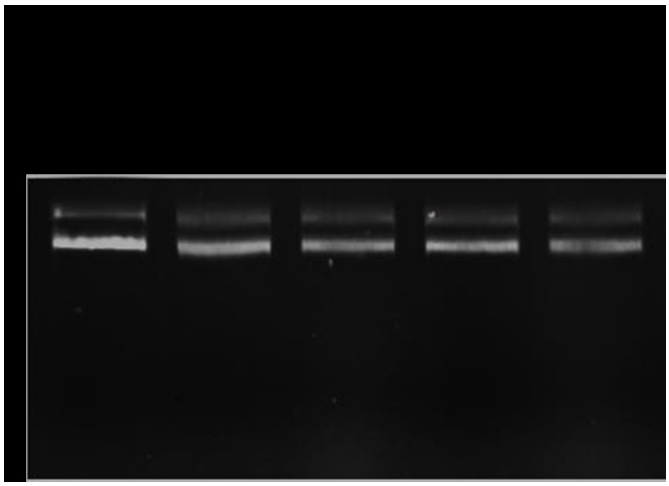

Figure 7.13 Serum stability of lipoplexes (BCSA and CSA) prepared at N/P ratio of 2 after incubation with serum

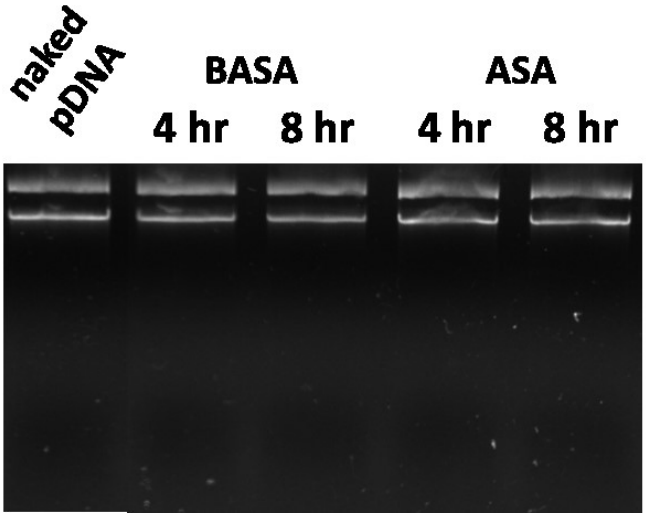

Figure 7.14 Serum stability of lipoplexes (BASA and ASA) prepared at N/P ratio of 2 after incubation with serum

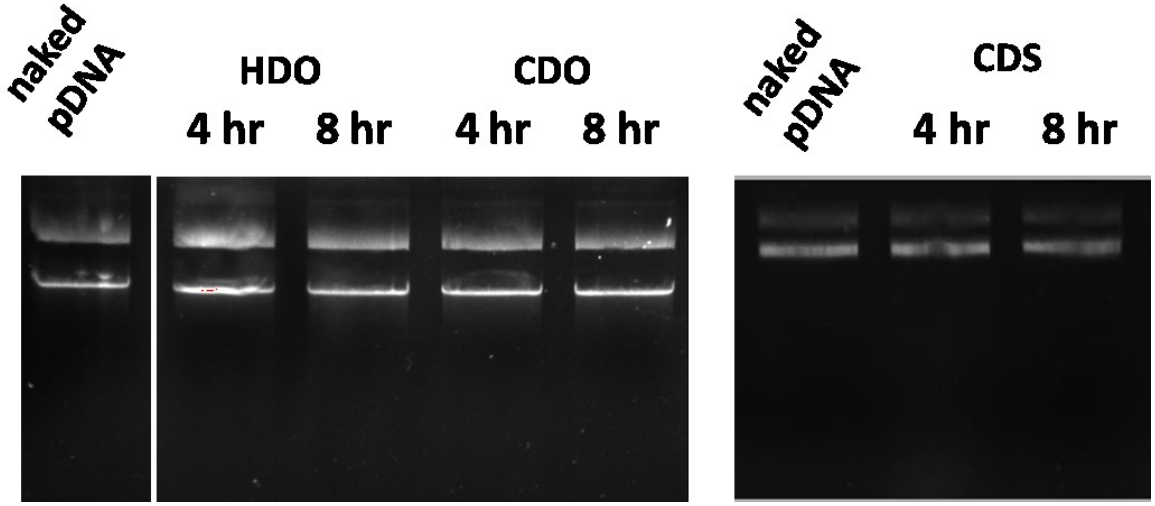

Figure 7.15 Serum stability of lipoplexes (HDO, CDO and CDS) prepared at N/P ratio of 2 after incubation with serum

Up to 8 hr, most of the pDNA was preserved in all lipoplexes; however results with SA lipoplexes show that lipoplexes failed to maintain the pDNA stability up to 24 hr retaining ~80% of pDNA. However, nanoparticulate formulations distribute after intravenous administration within few hours (<6 hr) supporting the view that after intravenous administration, developed lipoplexes will be sufficiently stable to provide for distribution in the target organ without being affected by the blood components.

Based on the effect of electrolytes and serum on the stability of pDNA in the lipoplexes, the recommended lipoplex preparation procedure should involve preparation of lipoplexes in nuclease free water without any buffer/electrolytes added. Subsequently, prepared lipoplexes can be exposed to electrolytes which will provide better complexation efficiency as well as it will provide long-lasting stability in electrolytes which are also present in the serum. Additionally, use of PEGylated lipids preserves pDNA from being released on exposure to electrolytes as well as protects it from serum nucleases, hence use of PEGylated liposomes in preparation of lipoplexes should be practiced.

7.4 References:

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