Chapter 5 Formulation development & characterization

5 Formulation development and characterization

5.1 Introduction

As mentioned previously viral vectors poses limitations like immunogenicity, mutagenicity, carcinogenicity, broad tropism, difficulty of vector production, scale-up issues and low packing efficiency [1-3] and thus research has been focused to develop non-viral vectors circumventing the aforementioned limitations. Out of several non-viral gene delivery approaches available, polymeric carriers and lipid based carriers are highly investigated. This includes polyplexes, lipoplexes and lipopolyplexes. Currently, use of lipoplexes as gene delivery carriers is not encouraged as they pose severe limitation of reduced transfection efficiency [4, 5] and thus this work mainly focuses on formulation of polyplexes and lipopolyplexes.

Polyplexes, considered as prominent candidates for non-viral gene delivery, are defined as self-assembled complexes of polymer with nucleic acid which are mainly formulated using cationic polymers [6]. These are formulated by electrostatic interaction between polycationic polymers and negatively charged nucleic acid which is widely responsible for protection of nucleic acids against degradation [7]. Polyplexes have proven efficient as a gene carrier as it can protect conjugated nucleic acids against degradation, provide efficient cellular uptake and release, translocate conjugated DNA into nucleus and assist incorporation of RNAi therapeutics into RNA induced silencing complex [8]. Polyplexes can avoid the main cellular barrier to gene transfer i.e. avoidance of degradative lysosomal enzymes which is attributed to proton sponge effect. Cationic polymers used in polyplexes have high buffering capacity and thus cause buffering inside endosomal compartment which result in influx of H^+ ions accompanied with chloride ions. The accumulation of chloride ions inside endosomes increases ionic strength which in turn causes osmotic swelling and physical rupture of endosomes. This finally leads to escape of conjugated polyplexes and protection of nucleic acids against degradative lysosomal trafficking pathway [9]. Additionally, cationic polymers used have higher capacity to condense nucleic acids and thus resulting formulation have smaller particle size and better transfection efficiency. Various cationic polymers used in formulation of polyplexes include poly-ethylenimine, poly-L-lysine, protamine, chitosan, pluronic block copolymers, poly-N-isoporpylacrylamide copolymers etc [10].

Lipopolyplexes, considered as efficient gene delivery vector, are lipid vesicles which involves encapsulation of polyplexes inside liposomes and thus has advantages of both lipoplex and polyplex [11-13]. This formulation involves core-shell like structure consisting of nucleic acids, polycation and lipids. Several advantages associated with lipopolyplexes are reduced cytotoxicity, superior stability and improved transfection due to presence of both polycation which ameliorates intracellular trafficking and lipid which can augment cellular uptake [14-16]. Aditionally, cationic polymers, as mentioned previously, have ability to form homogenous smaller conjugates which when incorporated inside lipid bilayer results in reduced blood clearance and altered distribution as it hampers phagocytic uptake [17]. Currently, due to various promising characteristics of this formulation, this is widely investigated as non-viral gene delivery carrier. Traditionally, first generation lipopolyplexes were formulated which consisted of cationic lipid, protamine based polycation and nucleic acid [18]. But these formulations showed problems related to cytotoxicity and biocompatibility which were surpassed by preparing second generation lipopolyplexes by incorporating anionic lipid instead of cationic lipid [19]. Presently, PEI is most widely investigated polycation employed in synthesis of lipopolyplexes due to its favorable characteristics and has yielded promising results. Still the cytotoxity issue of PEI can be overcome by employing biodegradable PEI synthesized using acrylates which when used in lipopolyplexes has shown reduced toxicity and higher transfection efficiency [20].

5.2 Procedure

5.2.1 Formulation of siRNA polyplexes

Polyplexes were formulated by incubating siRNA solution and copolymer/conjugate solution for specific period of time allowing to conjugate siRNA with polymer by electrostatic interaction between cationic polymeric groups and anionic phosphate groups of nucleic acid. Weighed quantity of lyophilized copolymer or conjugate was dissolved in nuclease free water (except in case of bPEI-Chi copolymer and biodegradable bPEI-Chi copolymer where copolymers were dissolved in 20% acetic acid) and diluted to achieve desired concentration. Later, siRNA stock was diluted with nuclease free water to get required siRNA concentration which was then added to copolymer/conjugate solution and volume of the mixture was made upto 10 µl. The mixture was vortexed for 2 min to allow complete mixing of the polymeric

and siRNA solution. Subsequently, the mixture was incubated for 30 min at optimal temperature away from light. siRNA was mixed with preformed copolymer and conjugates in various mole ratio to optimize the polyplexes of different copolymers and conjugates.

5.2.2 Formulation of lipopolyplexes

Liposomes were prepared by ethanol injection method as described in section 4.2.6.2. Subsequently, polyplex of biodegradable bPEI-chitosan copolymer and siRNA was prepared as described in section 5.2.1. This preformed and optimized polyplex was incubated with placebo liposomes along with ethanol to increase permeability of liposomes and volume of the mixture was made up to 10 µl. The mixture was vortexed for 2 min to get uniform mixing of liposomes and polyplexes. Later, the mixture was incubated for 30 min at optimal temperature away from light.

5.3 Characterization

5.3.1 Conjugation efficiency

The conjugation efficiency of prepared polyplexes was determined by agarose gel electrophoresis. The gel electrophoresis instrument was used according to manufacturer's specification. 1% agarose gel was prepared by dispersing the required quantity of agarose in 1x TAE (Tris-acetate-EDTA) buffer. The mixture was heated in heating mantle, with continuously shaking the flask while boiling, to dissolve the agarose. The gel tray was securely sealed at the ends by fixing it in the gel casting tray. The comb was placed over the gel tray. Later, when agarose was sufficiently cooled, ethidium bromide (0.5 µg/ml) was added, stirred and the resulting gel was poured into the gel tray to a depth of 48 mm. The gel was allowed to set at 20 °C for 30 min followed by refrigeration for further 15 min for complete solidification of the gel. The comb was removed from the solidified gel carefully. The gel was then transferred to the electrophoresis chamber and submerged into electrophoresis buffer (1x TAE buffer). Then, 10 µl polyplex formulations, along with loading dye (glycerol 30 %w/v + bromophenol blue 0.25 %w/v), were loaded in the wells and electrophoresis was performed at 100 V/cm. The gel was removed and siRNA in the agarose gel was visualized under UV light using GelDoc™ XR+ Imaging System. This technique was used to determine mole ratio of siRNA and prepared polymers necessary for complete conjugation of siRNA with copolymers/conjugates [21, 22].

Similarly, conjugation efficiency of prepared lipopolyplexes was determined using BioSpec-nano micro volume UV-Vis spectrophotometer. The conjugated siRNA from the lipopolyplexes was released by phenol-chloroform extraction. In this extraction method, lipopolyplexes and phenol: choloroform (1:1 v/v ratio) were mixed in 1:1 v/v ratio and vortexed to obtain emulsion. This emulsion was later centrifuged at 12000 x g at 4 °C for 5-10 min which resulted in separation of the mixture into 3 layers – aqueous layer, phenolic layer and chloroform layer. Aqueous layer was carefully separated avoiding contamination by phenolic layer and was used to determine conjugation efficiency of formed lipopolyplex.

5.3.2 Size distribution

Size distribution of prepared polyplexes and/or lipopolyplexes was determined using Malvern zetasizer by dynamic light scattering technique. For this, prepared polyplexes/lipopolyplexes were diluted with suitable solvent. Diluted dispersion was filled in clear disposable sample cuvettes and measurement was performed on Malvern zetasizer by helium neon lasers at 633 nm at room temperature [23, 24].

5.3.3 Electrical surface charge or potential

1 ml of prepared polyplexes/lipopolyplexes was taken in clear disposable zeta cuvette. The electrophoretic mobility of the sample was determined by laser interferometric technique called M3-PALS (Phase analysis Light Scattering) employing Malvern zetasizer. Additionally, zeta potential of pure copolymer, conjugate and placebo liposomes was determined to estimate the change in surface potential on conjugation with siRNA [23, 24].

5.3.4 Polyanion competition assay

The relative stability of formulated polyplexes or lipopolyplex was investigated by estimating the capacity of polyplexes or lipopolyplex to release siRNA in presence of competing polyanions using heparin polyanion competition assay and the siRNA released from the polyplexes was measured using gel electrophoresis. Polyplex/ lipopolyplex dispersion was prepared by incubating siRNA for 30 min at the optimized mole ratio as described in section 5.2.1 and 5.2.2. Later, the prepared polyplex or lipopolyplex were exposed to heparin sodium solution of varying concentration for 15 min. These samples, after addition of loading dye, were run on 1% agarose gel using (1x) TAE buffer, later stained and visualized as previously mentioned in section [25-27].

5.3.5 Stability of formed colloidal nanocomplexes

Nanoplexes conjugated with siRNA were formulated as mentioned previously in section. These nanoplexes were tested for stability mimicking biological condition by adding simulated nasal fluid. For this, 50 µl nanoplex dispersion was added to 0.5 ml microcentrifuge tubes and to this 50 µl of simulated nasal fluid (SNF) was added such that desired concentration was obtained in final volume. These mixtures were vortexed for uniform mixing and then incubated for 30 min at 37 °C. After incubation, the formulations were analyzed for particle size to check for aggregation. The SNF was prepared by dissolving 3 %w/v porcine mucin to buffer solution consisting of 7.5 mg/ml NaCl, 1.3 mg/ml KCl and 0.3 mg/ml $CaCl_2.2H_2O$ [28-30].

5.3.6 Ex-vivo nasal permeation study

Freshly excised sheep nasal mucosa was obtained and dipped immediately in nuclease free water to maintain hydration. Superior nasal conche was recognized and detached from the nasal mucosa and washed properly with nuclease free water to remove debris and other contaminants. This membrane was mounted on Franz diffusion cell. The tissue was stabilized using phosphate buffer (pH 7.4) in donor as well as receptor compartments for 15 min. Later, both the compartments were emptied and the receptor compartment was filled with fresh PBS (pH 7.4) such that nasal membrane touches the buffer and assembly was maintained at 37 ± 2 °C with continuous stirring. Subsequently, desired concentration of suitably diluted polyplex or lipopolyplex dispersion was added to the donor compartment. 1 ml sample was withdrawn from the receptor compartment at predefined time intervals and fresh PBS (pH 7.4) was added to receptor compartment. The withdrawn samples were suitably diluted and analyzed by fluorimetry [31].

5.4 Result and discussion

5.4.1 Conjugation efficiency

The results obtained by agarose gel electrophoresis, to estimate conjugation efficiency of polyplexes, are depicted in Figure 5. 1 to Figure 5. 4 and summarized in Table 5. 1.

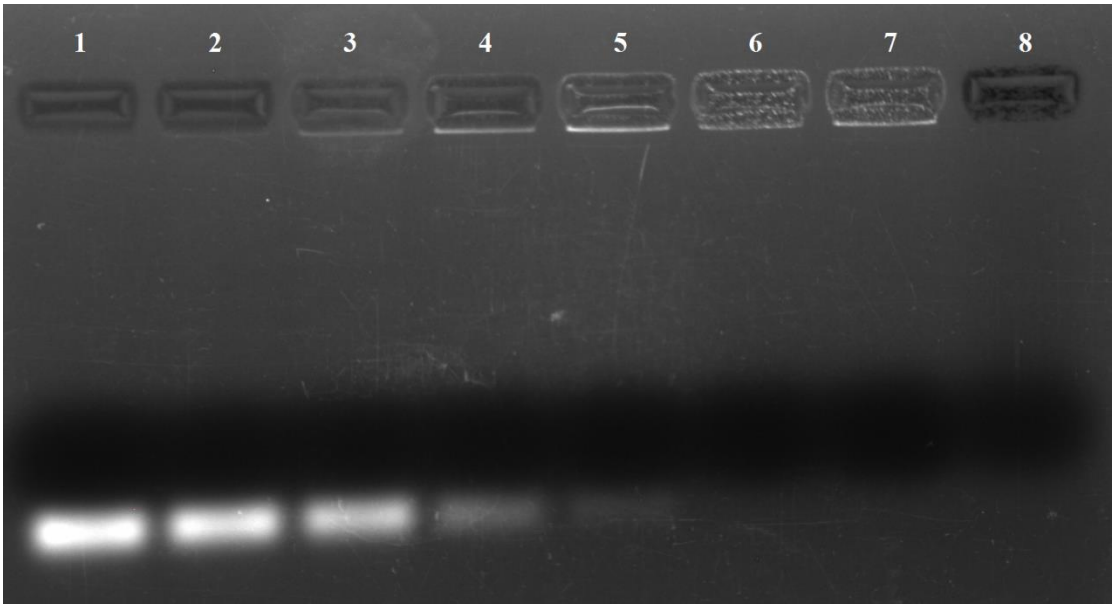

Figure 5. 1: Agarose gel electrophoresis of bPEI-HA copolymer
Polymer:siRNA mole ratio – Lane 1: Naked siRNA, Lane 2: 0.05, Lane 3: 0.1, Lane 4: 0.2, Lane 5: 0.3, Lane 6: 0.4, Lane 7: 0.5, Lane 8: 1

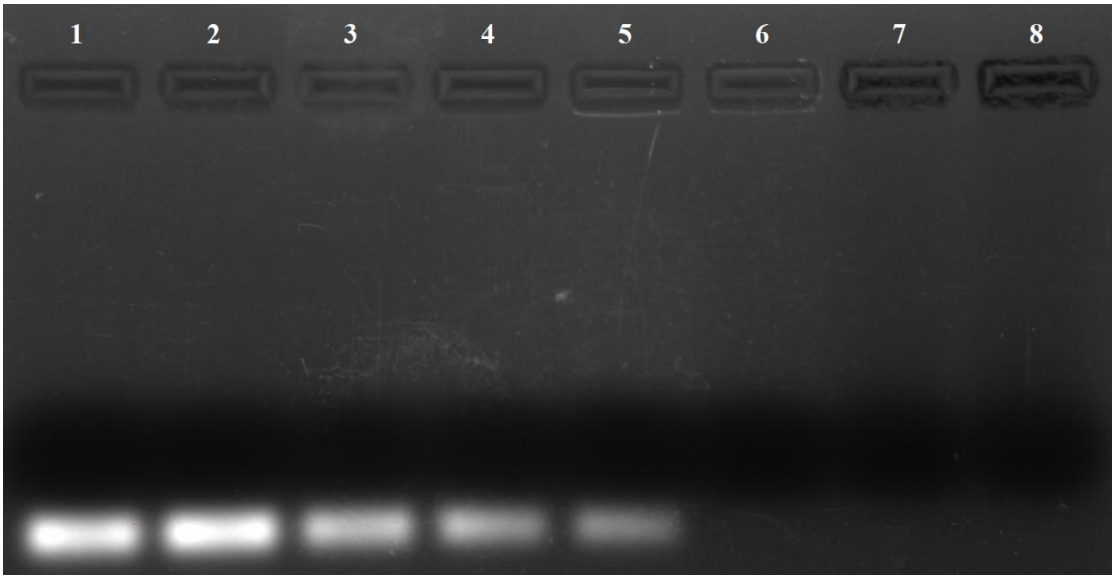

Figure 5. 2: Agarose gel electrophoresis of bPEI-Lf conjugate
Polymer:siRNA mole ratio – Lane 1: Naked siRNA, Lane 2: 0.05, Lane 3: 0.1, Lane 4: 0.2, Lane 5: 0.3, Lane 6: 0.4, Lane 7: 0.5, Lane 8: 1

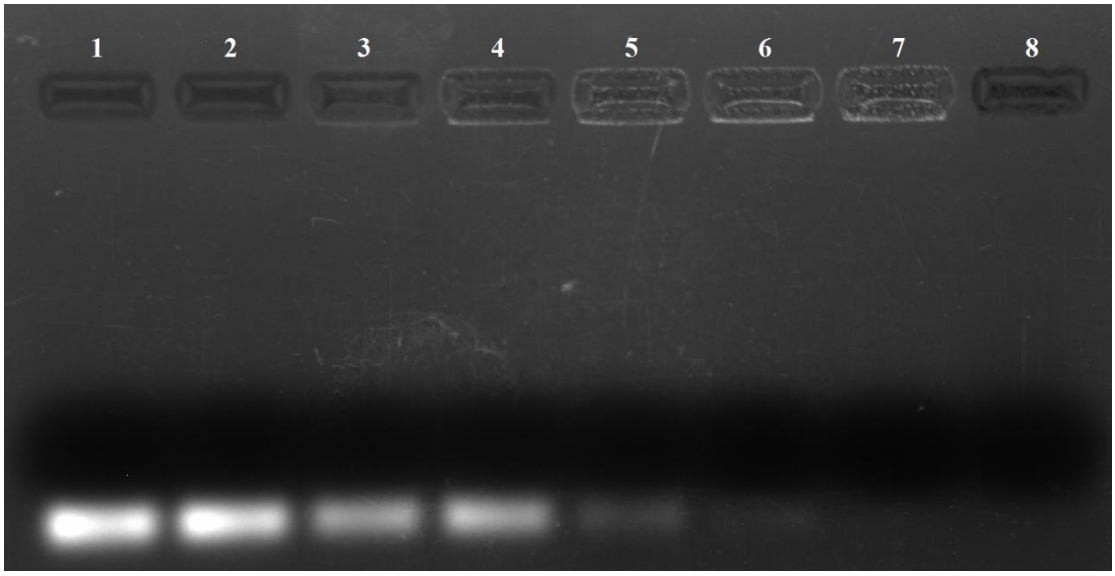

Figure 5. 3: Agarose gel electrophoresis of bPEI-Chi copolymer
Polymer:siRNA mole ratio – Lane 1: Naked siRNA, Lane 2: 0.05, Lane 3: 0.1, Lane 4: 0.2, Lane 5: 0.3, Lane 6: 0.4, Lane 7: 0.5, Lane 8: 1

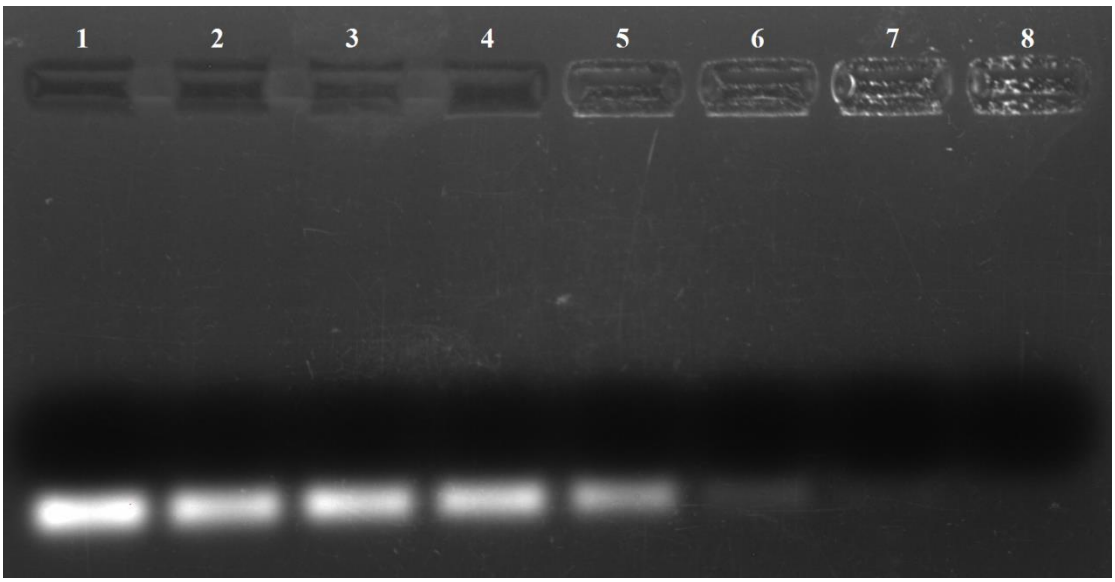

Figure 5. 4: Agarose gel electrophoresis of biodegradable bPEI-Chi copolymer
Polymer:siRNA mole ratio – Lane 1: Naked siRNA, Lane 2: 0.05, Lane 3: 0.1, Lane 4: 0.2, Lane 5: 0.3, Lane 6: 0.4, Lane 7: 0.5, Lane 8: 1

Table 5. 1: Conjugation efficiency of different vectors

Vector	Complexation ratio (mole ratio)
bPEI-HA polyplex	0.3:1
bPEI-Lf polyplex	0.4:1
bPEI-Chi polyplex	0.4:1
Biodegradable bPEI-Chi polyplex	0.5:1

The formation of polyplex was outcome of strong electrostatic interaction between cationic polymers and negatively charged siRNA [32]. This resulted in charge neutralization of siRNA, hindering its electrophoretic mobility. This effect was observed at polymer:siRNA mole ratio ranging from 0.3:1 to 0.5:1 for all the polyplexes. The surface positive charge of cationic polymers has great influence on complexation efficiency and thus biodegradable bPEI-Chi copolymer having least zeta potential (as mentioned in succeeding section) showed complexation with siRNA at higher mole ratio compared to other cationic polymers [33]. Additionally, complexation efficiency of lipopolyplex was estimated employing BioSpec-nano micro volume UV-Vis spectrophotometer which was 97.24 ± 1.07% indicating efficient complexation of siRNA with preformed vector.

5.4.2 Size distribution

The particle size of all the synthesized polyplex and lipopolyplex along with size of placebo liposomes is summarized in Table 5. 2 and depicted in Figure 5. 6 to Figure 5. 10.

Table 5. 2: Particle size of polyplexes and lipopolyplexes

Sr. No.	Formulations	Particle size (nm)
1	bPEI-HA polyplex	2583
2	bPEI-Lf polyplex	1194
3	bPEI-Chi polyplex	257.9
4	Biodegradable bPEI-Chi polyplex	126.4
5	Placebo liposomes	115.6
6	Lipopolyplex	187.4

			Size (d.nm):	% Intensity	Width (d.nm):
Z-Average (d.nm):	2583	Peak 1:	2514	100.0	380.1
PdI:	0.657	Peak 2:	0.000	0.0	0.000
Intercept:	0.799	Peak 3:	0.000	0.0	0.000

Result quality : Refer to quality report

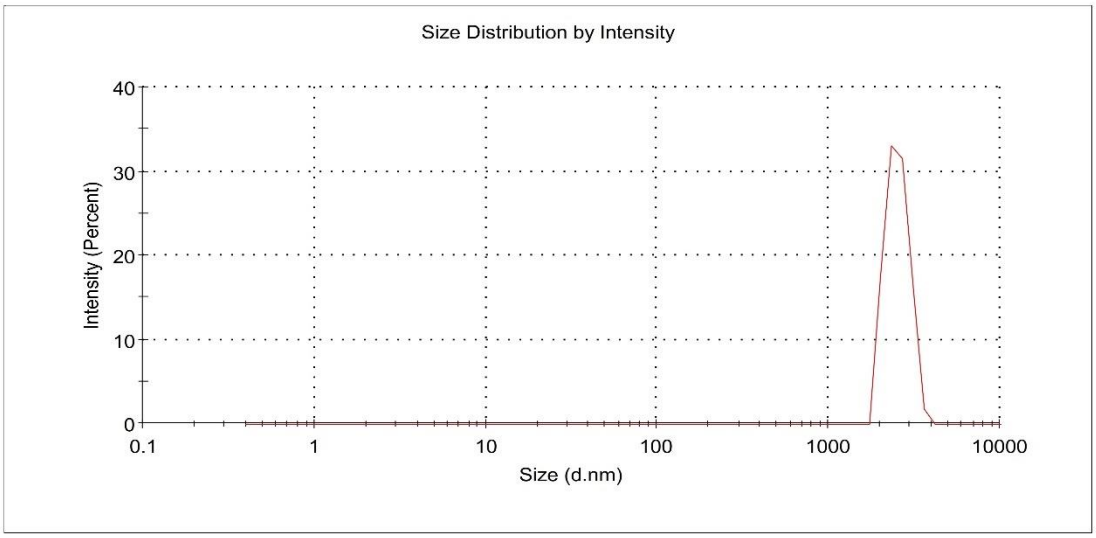

Figure 5. 6: Particle size distribution of bPEI-HA polyplexes

			Size (d.nm):	% Intensity	Width (d.nm):
Z-Average (d.nm):	1194	Peak 1:	1117	100.0	185.1
PdI:	0.717	Peak 2:	0.000	0.0	0.000
Intercept:	0.686	Peak 3:	0.000	0.0	0.000

Result quality : Refer to quality report

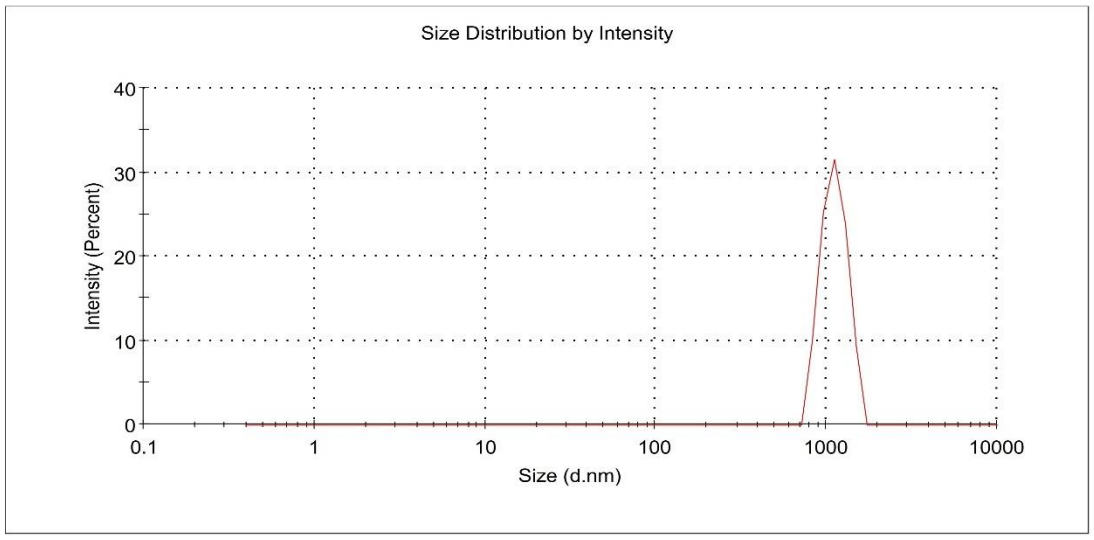

Figure 5. 5: Particle size distribution of bPEI-Lf polyplexes

		Size (d.nm):	% Intensity	Width (d.nm):
Z-Average (d.nm): 257.9	Peak 1:	256.9	100.0	74.20
PdI: 0.204	Peak 2:	0.000	0.0	0.000
Intercept: 0.943	Peak 3:	0.000	0.0	0.000
Result quality : Good				

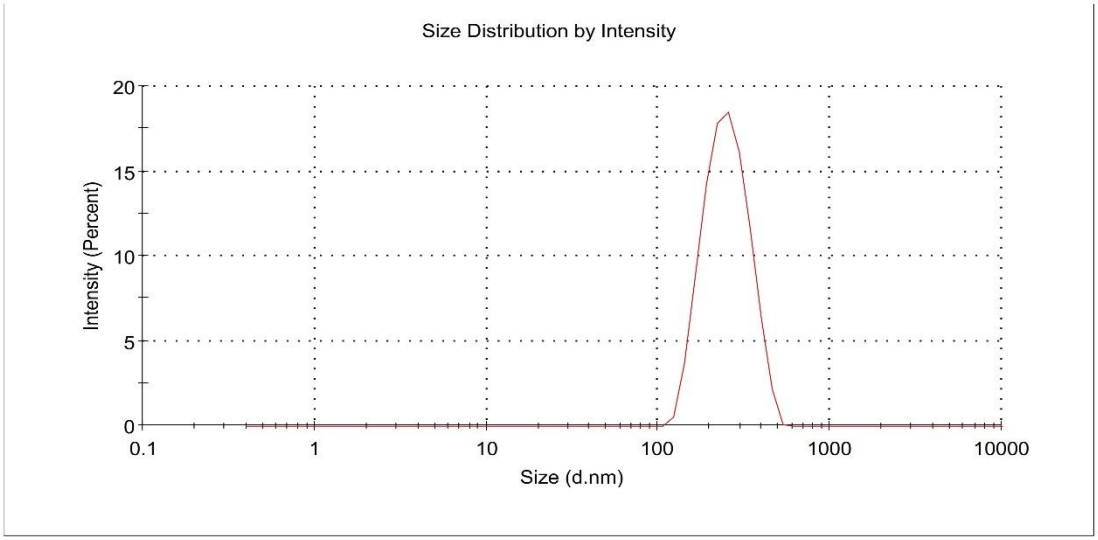

Figure 5. 7: Particle size distribution of bPEI-Chi polyplexes

		Size (d.nm):	% Intensity	Width (d.nm):
Z-Average (d.nm): 126.4	Peak 1:	147.6	97.6	61.57
PdI: 0.231	Peak 2:	4650	2.4	805.4
Intercept: 0.958	Peak 3:	0.000	0.0	0.000
Result quality : Good				

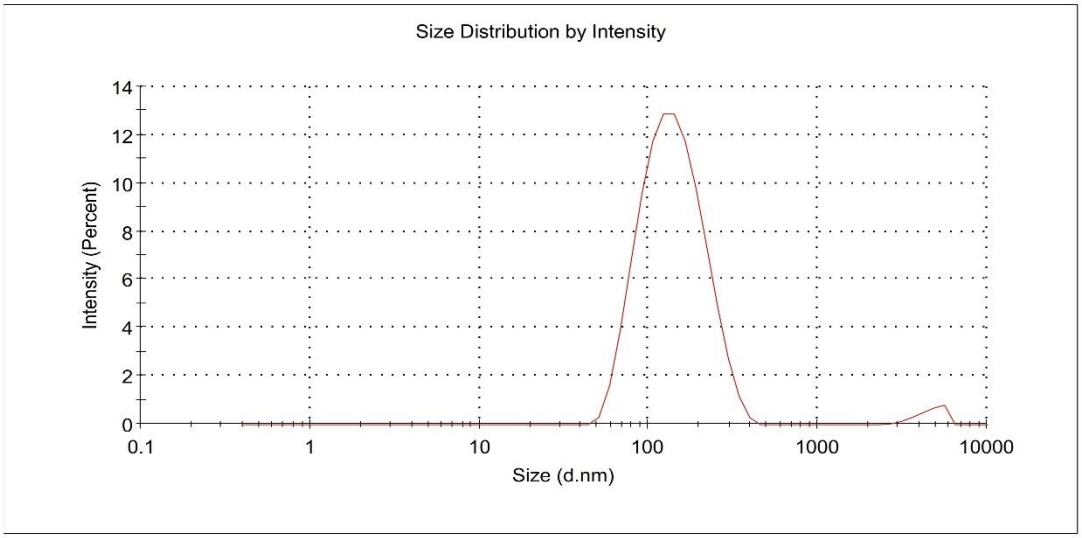

Figure 5. 8: Particle size distribution of biodegradable bPEI-Chi polyplexes

		Size (d.nm):	% Intensity	Width (d.nm):
Z-Average (d.nm): 115.6	Peak 1:	142.8	100.0	67.33
PdI: 0.170	Peak 2:	0.000	0.0	0.000
Intercept: 0.950	Peak 3:	0.000	0.0	0.000
Result quality : Good				

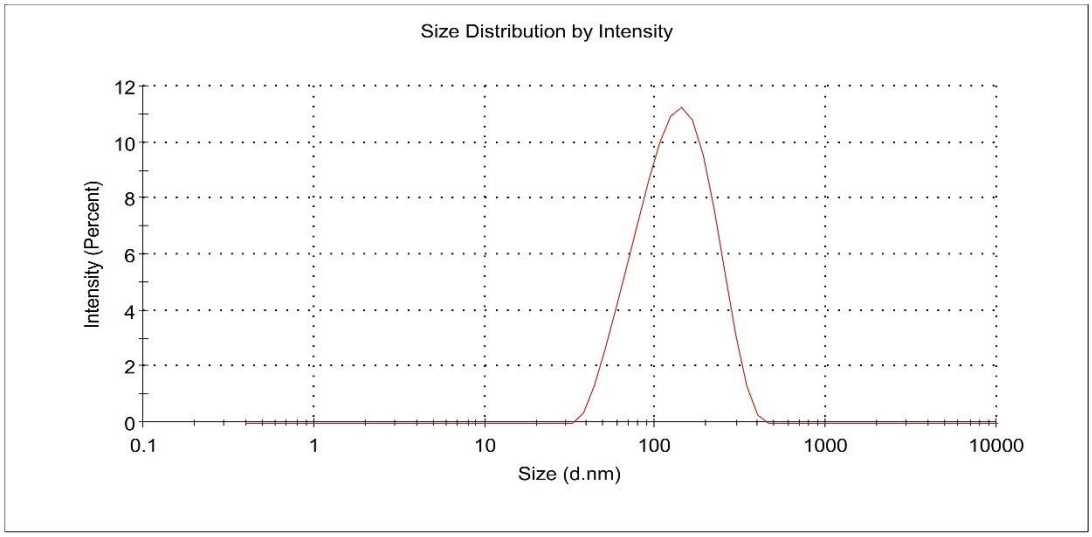

Figure 5. 9: Particle size distribution of placebo liposomes

		Size (d.nm):	% Intensity	Width (d.nm):
Z-Average (d.nm): 187.4	Peak 1:	234.4	100.0	86.16
PdI: 0.218	Peak 2:	0.000	0.0	0.000
Intercept: 0.948	Peak 3:	0.000	0.0	0.000
Result quality : Good				

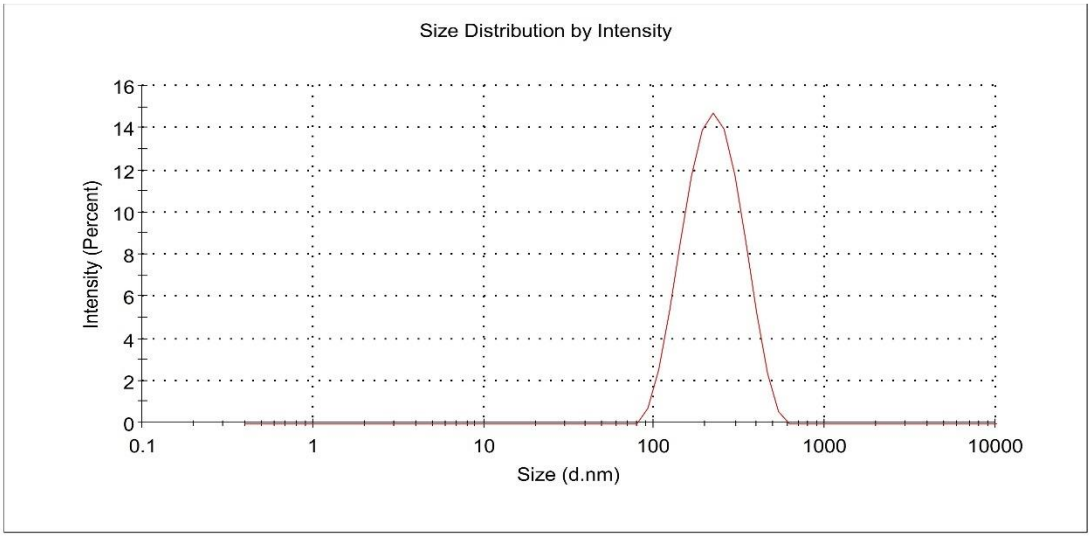

Figure 5. 10: Particle size distribution of lipopolyplexes

5.4.3 Electrical surface charge or potential

			Mean (mV)	Area (%)	Width (mV)
Zeta Potential (mV):	46.0	**Peak 1:**	46.0	100.0	4.56
Zeta Deviation (mV):	4.56	**Peak 2:**	0.00	0.0	0.00
Conductivity (mS/cm):	0.218	**Peak 3:**	0.00	0.0	0.00
Result quality	See result quality report				

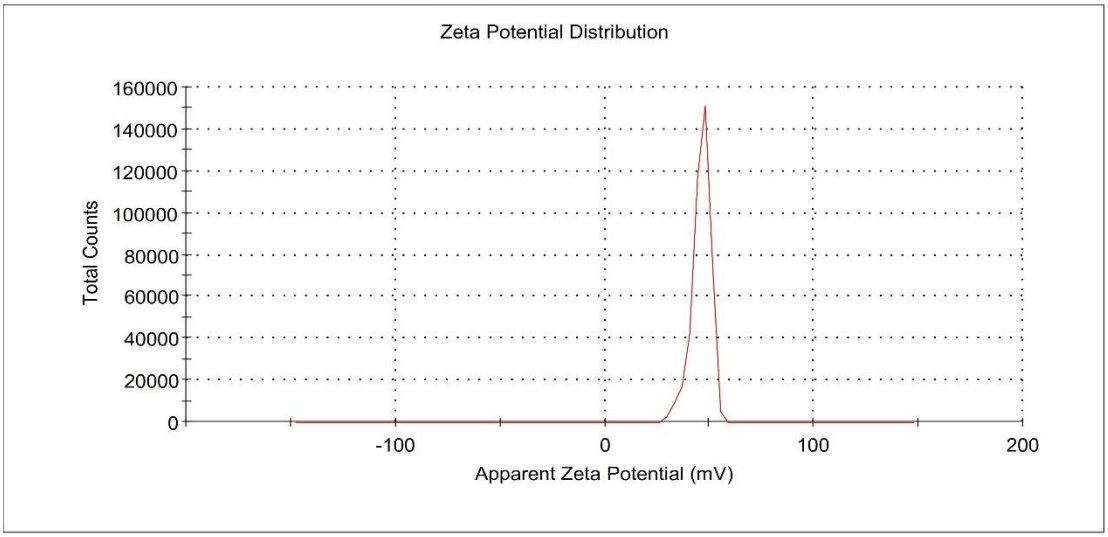

Figure 5. 11: Zeta potential of bPEI-HA copolymer

			Mean (mV)	Area (%)	Width (mV)
Zeta Potential (mV):	15.0	**Peak 1:**	28.7	49.6	5.74
Zeta Deviation (mV):	15.9	**Peak 2:**	-2.74	36.8	5.20
Conductivity (mS/cm):	0.316	**Peak 3:**	10.7	13.6	4.06
Result quality	See result quality report				

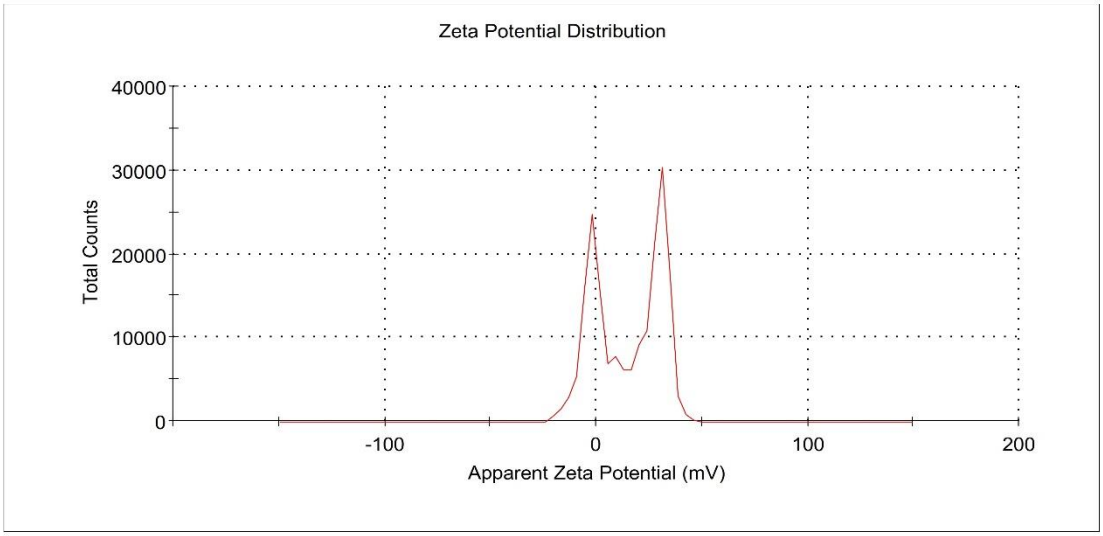

Figure 5. 12: Zeta potential of bPEI-HA polyplex

		Mean (mV)	Area (%)	Width (mV)
Zeta Potential (mV): 32.3	**Peak 1:**	32.3	100.0	4.89
Zeta Deviation (mV): 4.89	**Peak 2:**	0.00	0.0	0.00
Conductivity (mS/cm): 0.212	**Peak 3:**	0.00	0.0	0.00

Result quality See result quality report

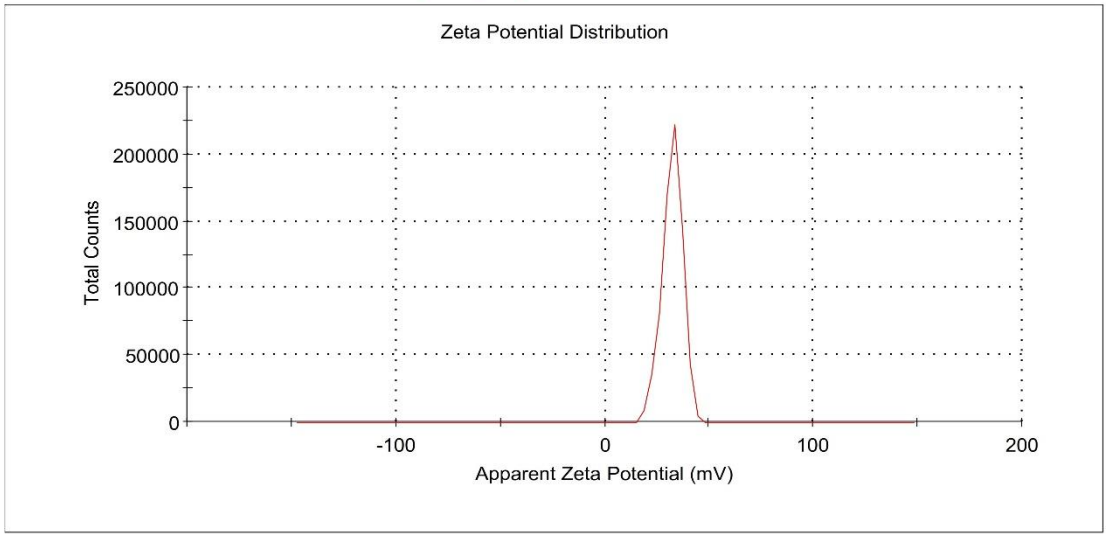

Figure 5. 13: Zeta potential of bPEI-Lf conjugate

		Mean (mV)	Area (%)	Width (mV)
Zeta Potential (mV): 23.2	**Peak 1:**	29.8	63.7	5.39
Zeta Deviation (mV): 11.7	**Peak 2:**	12.8	29.6	5.45
Conductivity (mS/cm): 0.410	**Peak 3:**	-1.46	6.0	3.13

Result quality See result quality report

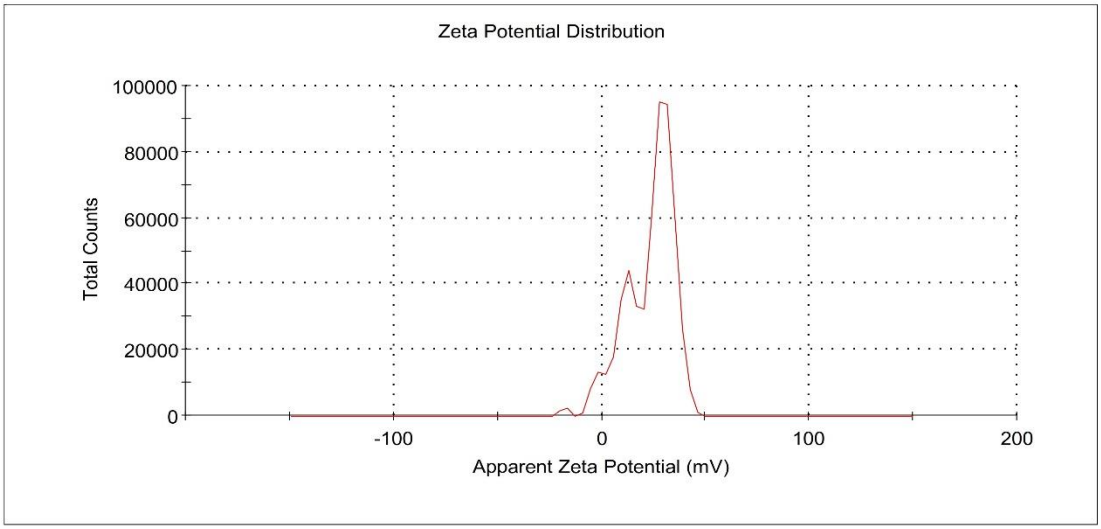

Figure 5. 14: Zeta potential of bPEI-Lf polyplex

		Mean (mV)	Area (%)	Width (mV)
Zeta Potential (mV): 36.5	**Peak 1:**	39.4	50.2	6.56
Zeta Deviation (mV): 57.7	**Peak 2:**	60.9	39.1	5.50
Conductivity (mS/cm): 1.14	**Peak 3:**	20.2	10.7	4.19

Result quality See result quality report

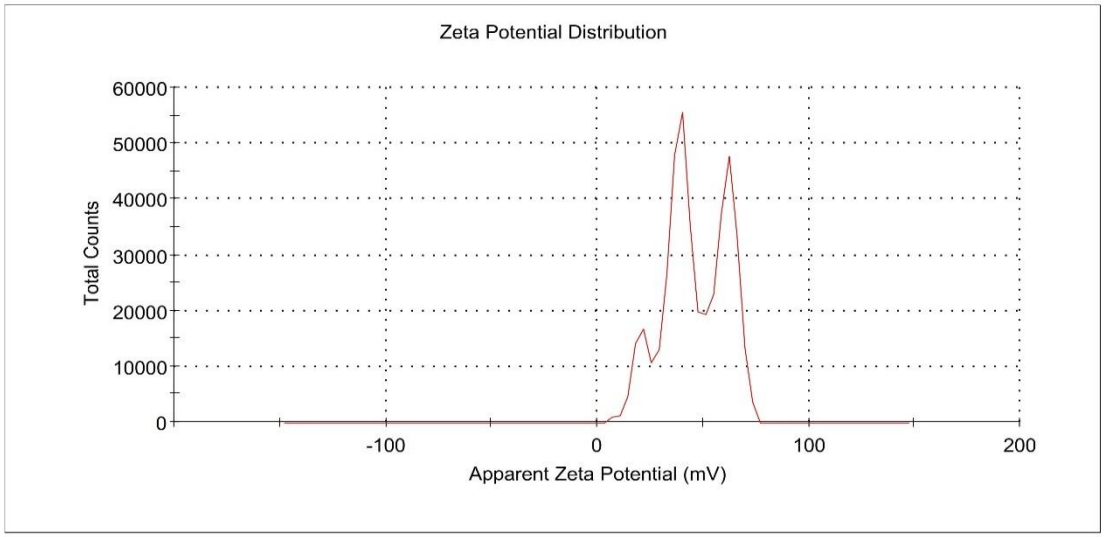

Figure 5. 15: Zeta potential of bPEI-Chi copolymer

		Mean (mV)	Area (%)	Width (mV)
Zeta Potential (mV): 21.0	**Peak 1:**	23.1	84.0	4.96
Zeta Deviation (mV): 7.48	**Peak 2:**	7.21	16.0	3.98
Conductivity (mS/cm): 0.223	**Peak 3:**	0.00	0.0	0.00

Result quality See result quality report

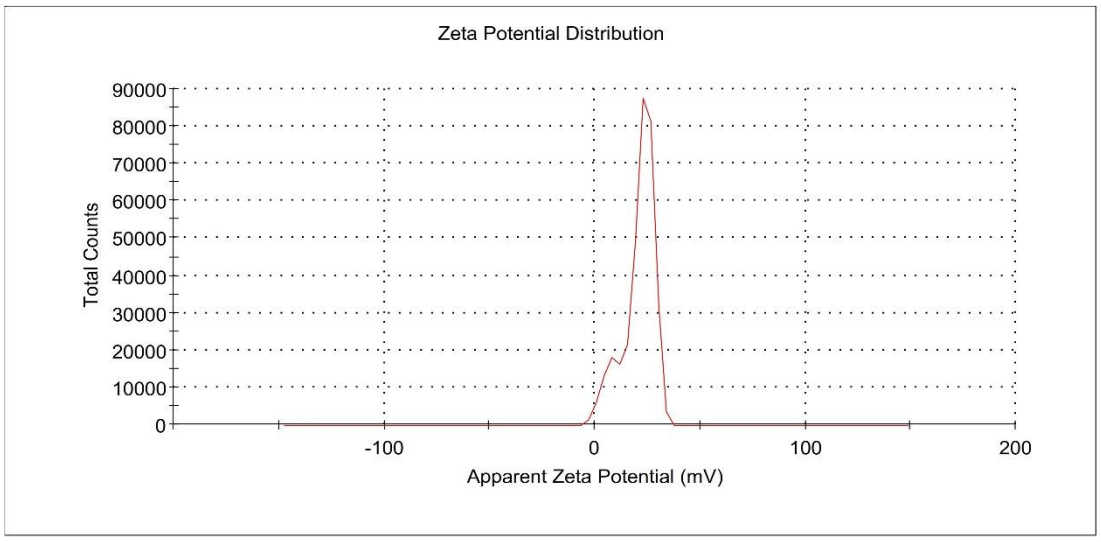

Figure 5. 16: Zeta potential of bPEI-Chi polyplex

		Mean (mV)	Area (%)	Width (mV)
Zeta Potential (mV): 21.7	**Peak 1:**	31.6	59.5	6.61
Zeta Deviation (mV): 14.1	**Peak 2:**	6.95	40.5	6.83
Conductivity (mS/cm): 0.317	**Peak 3:**	0.00	0.0	0.00

Result quality See result quality report

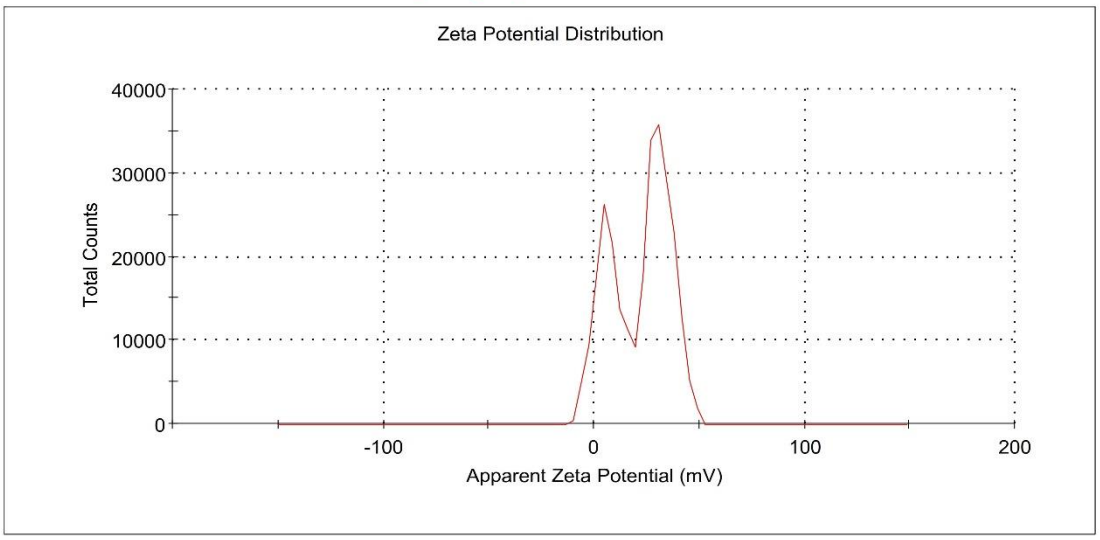

Figure 5. 17: Zeta potential of biodegradable bPEI-Chi copolymer

		Mean (mV)	Area (%)	Width (mV)
Zeta Potential (mV): 14.6	**Peak 1:**	28.8	57.0	5.23
Zeta Deviation (mV): 18.9	**Peak 2:**	0.275	33.6	8.44
Conductivity (mS/cm): 0.0549	**Peak 3:**	-23.4	9.4	3.38

Result quality See result quality report

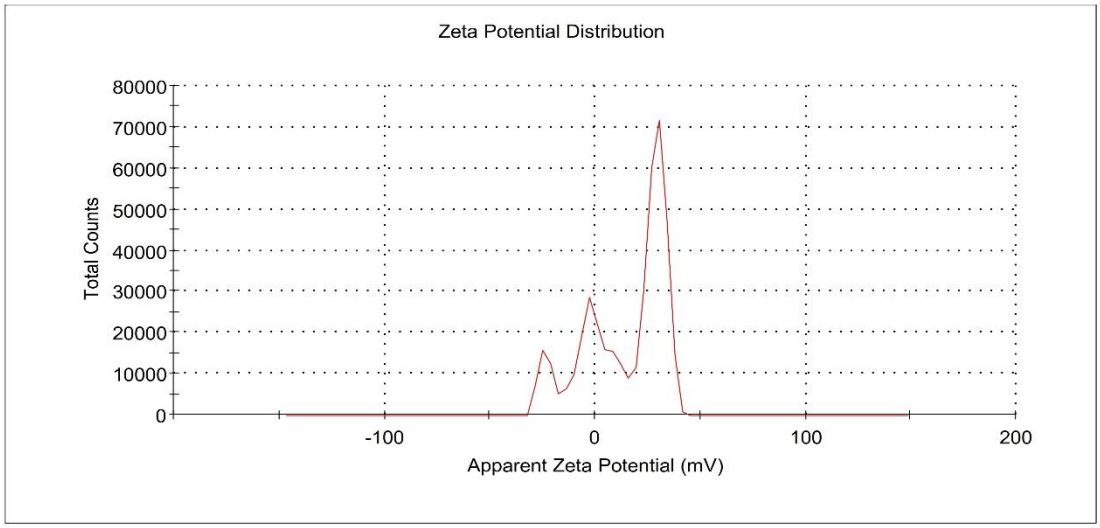

Figure 5. 18: Zeta potential of biodegradable bPEI-Chi polyplex

		Mean (mV)	Area (%)	Width (mV)
Zeta Potential (mV): -22.3	**Peak 1:**	-14.0	52.0	6.98
Zeta Deviation (mV): 11.1	**Peak 2:**	-31.5	48.0	5.28
Conductivity (mS/cm): 0.0671	**Peak 3:**	0.00	0.0	0.00

Result quality See result quality report

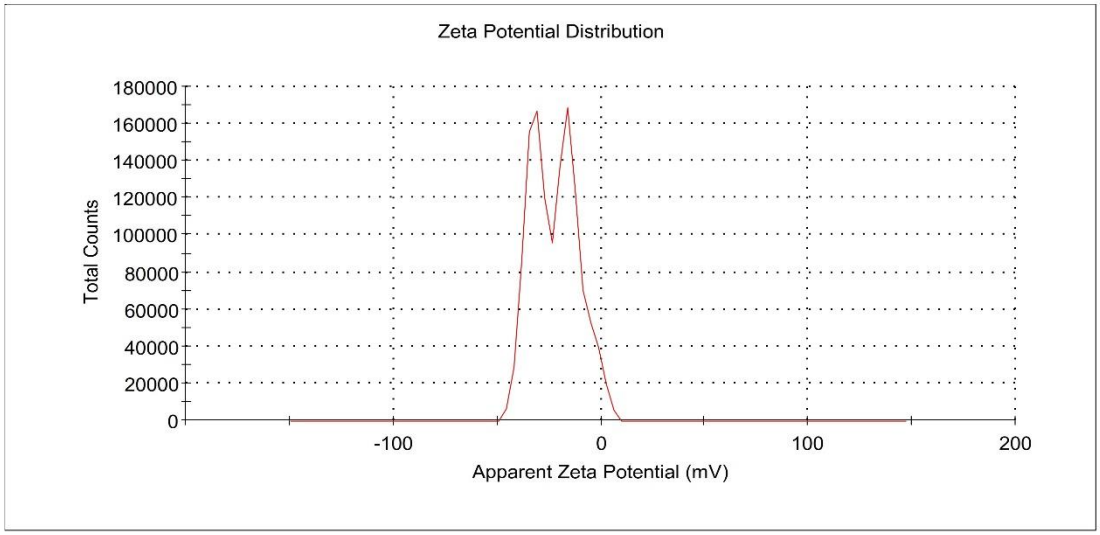

Figure 5. 19: Zeta potential of placebo liposomes

		Mean (mV)	Area (%)	Width (mV)
Zeta Potential (mV): -6.38	**Peak 1:**	-6.38	100.0	6.29
Zeta Deviation (mV): 6.29	**Peak 2:**	0.00	0.0	0.00
Conductivity (mS/cm): 0.189	**Peak 3:**	0.00	0.0	0.00

Result quality See result quality report

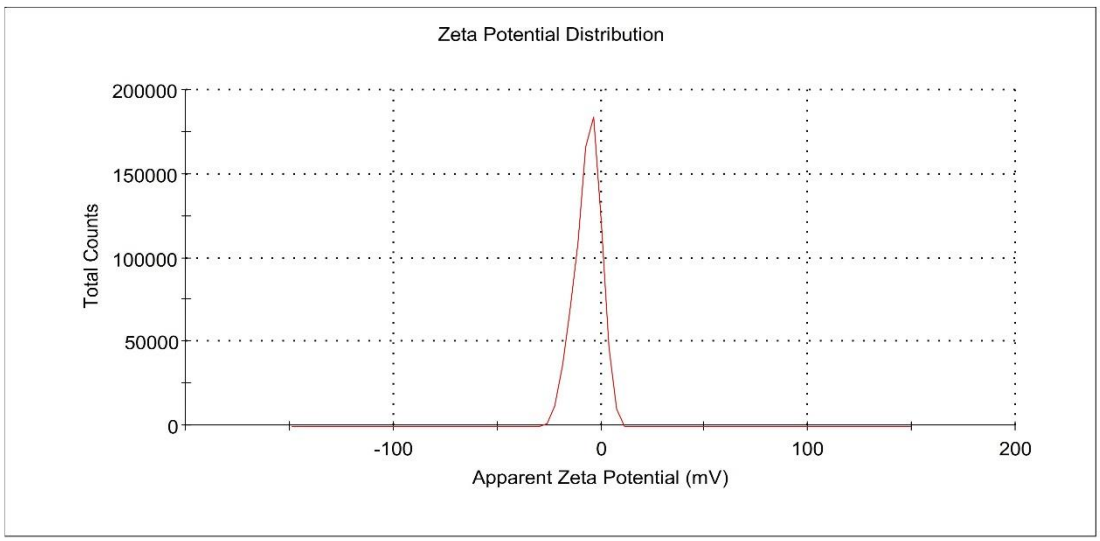

Figure 5. 20: Zeta potential of lipopolyplex

Figure 5. 11 to Figure 5. 20 depicts zeta potential values of all synthesized vectors, polyplexes and lipopolyplexes which are summarized in Table 5. 3.

Table 5. 3: Zeta potential of vectors, polyplexes and lipopolyplexes

Sr. No.	Formulations	Zeta potential (mV)
1	bPEI-HA copolymer	46.0
2	bPEI-HA polyplex	15.0
3	bPEI-Lf conjugate	32.3
4	bPEI-Lf polyplex	23.2
5	bPEI-Chi copolymer	36.5
6	bPEI-Chi polyplex	21.0
7	Biodegradable bPEI-Chi copolymer	21.7
8	Biodegradable bPEI-Chi polyplex	14.6
9	Placebo liposomes	-22.3
10	Lipopolyplex	-6.38

The results obtained proved that there was significant reduction in zeta potential of cationic vectors when complexed with negatively charged siRNA which proved conjugation of siRNA with incubated vector. In case of lipopolyplex, the placebo liposomes had -22.3 mV potential which on conjugation with biodegradable bPEI-Chi polyplex resulted in formation of lipopolyplex with resultant surface charge of -6.36 mV. This may be due to conjugation of placebo liposomes with polyplex having resultant net charge +14.6 mV.

5.4.4 Polyanion competition assay

Although, electrostatic attraction of siRNA with cationic vector is responsible for formation of polyplex or lipopolyplex, it is important to release conjugated siRNA in cytosol for its therapeutic efficiency [34]. Additionally, the formulated polyplex or lipopolyplex should remain stable during cellular uptake which if happens will result in degradation of siRNA under influence of nucleases. Furthermore, strong interaction of siRNA would hinder its release rendering it ineffective. Such electrostatic interaction of siRNA with vectors was estimated by polyanion competition assay using heparin as competing agent, the results of which are depicted in Figure 5. 21 to Figure 5. 25 and summarized in Table 5. 4.

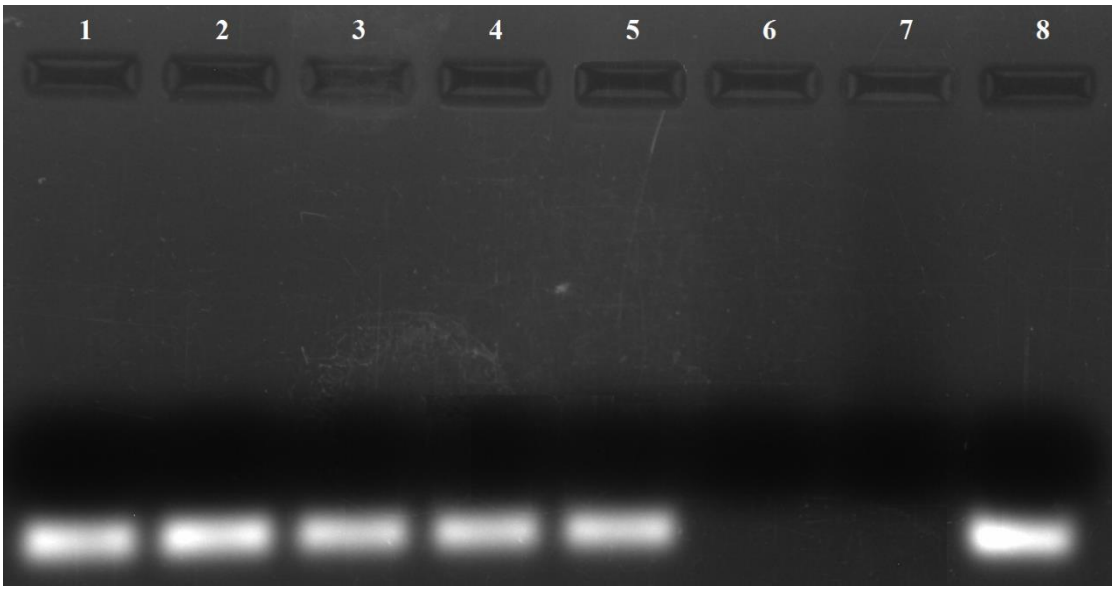

Figure 5. 21: Heparin competition assay of bPEI-HA polyplex

Heparin:siRNA w/w ratio Lane 1: 6:1, Lane 2: 5:1, Lane 3: 4:1, Lane 4: 3:1, Lane 5: 2:1, Lane 6: 1:1, Lane 7: 0.5:1, Lane 8: Naked siRNA

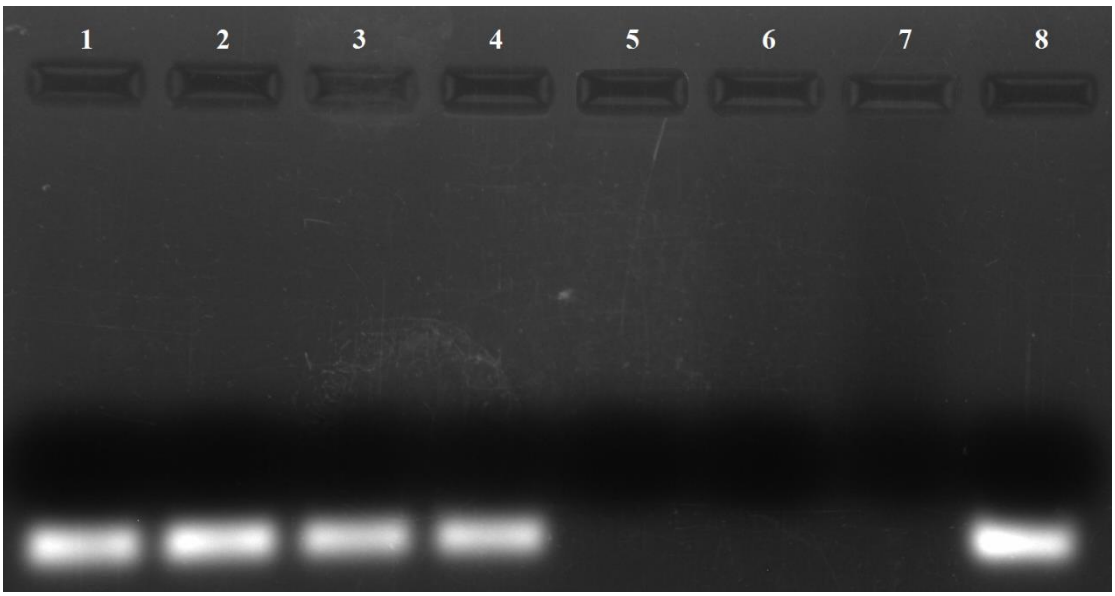

Figure 5. 22: Heparin competition assay of bPEI-Lf polyplex

Heparin:siRNA w/w ratio Lane 1: 6:1, Lane 2: 5:1, Lane 3: 4:1, Lane 4: 3:1, Lane 5: 2:1, Lane 6: 1:1, Lane 7: 0.5:1, Lane 8: Naked siRNA

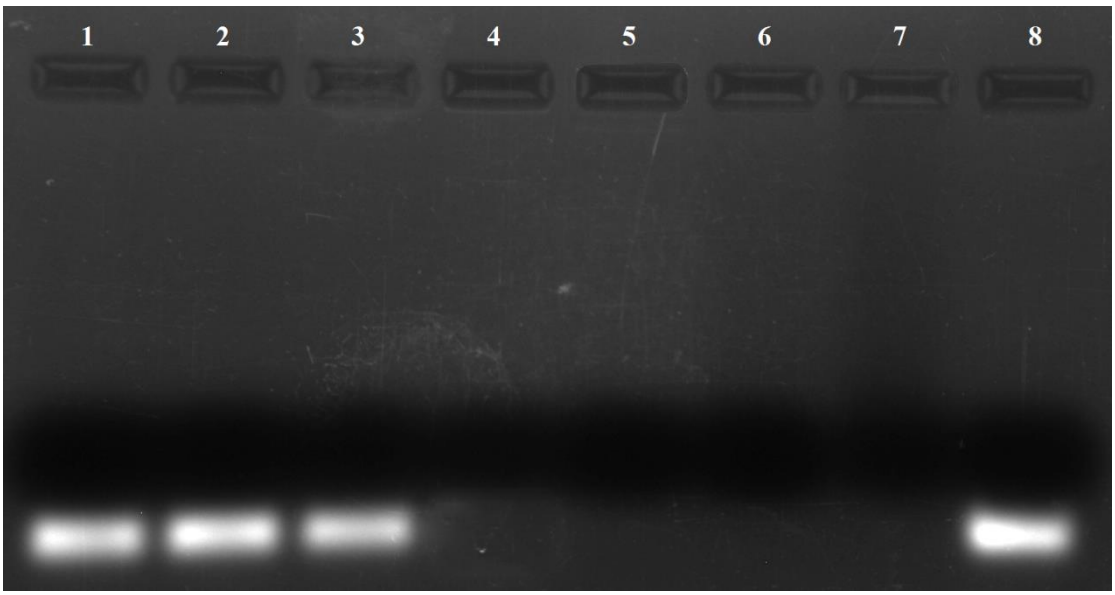

Figure 5. 23: Heparin competition assay of bPEI-Chi polyplex
Heparin:siRNA w/w ratio Lane 1: 6:1, Lane 2: 5:1, Lane 3: 4:1, Lane 4: 3:1, Lane 5: 2:1, Lane 6: 1:1, Lane 7: 0.5:1, Lane 8: Naked siRNA

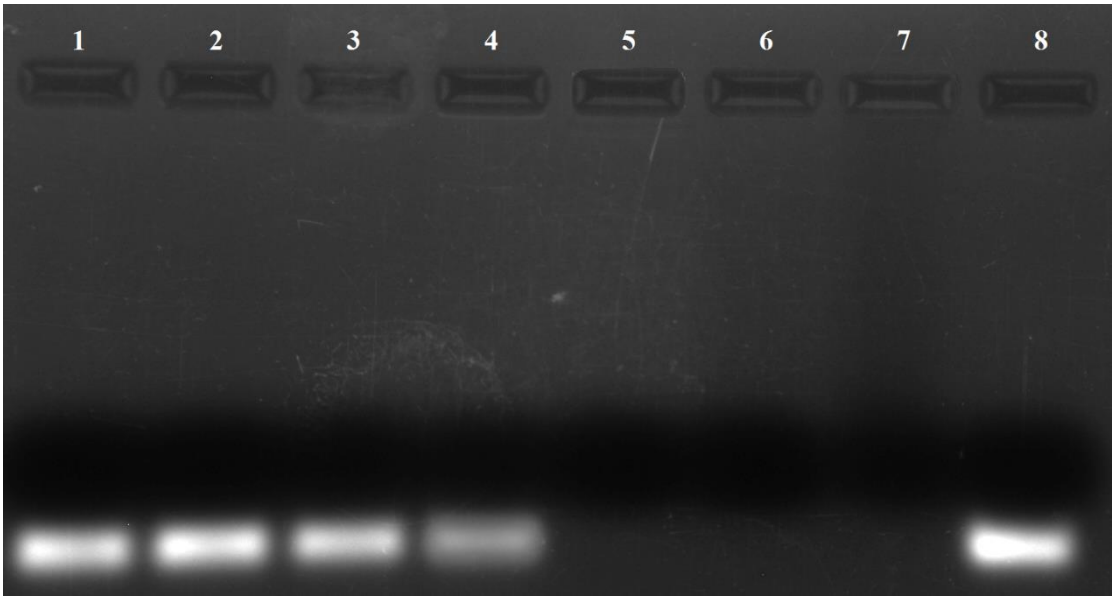

Figure 5. 24: Heparin competition assay of biodegradable bPEI-Chi polyplex
Heparin:siRNA w/w ratio Lane 1: 6:1, Lane 2: 5:1, Lane 3: 4:1, Lane 4: 3:1, Lane 5: 2:1, Lane 6: 1:1, Lane 7: 0.5:1, Lane 8: Naked siRNA

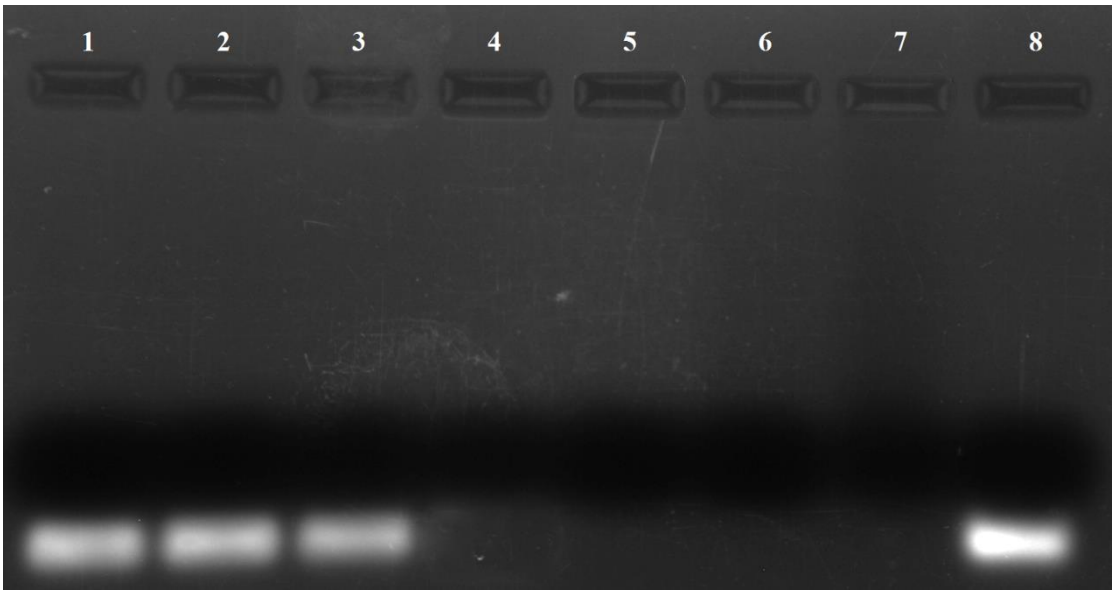

Figure 5. 25: Heparin competition assay of lipopolyplex

Heparin:siRNA w/w ratio Lane 1: 6:1, Lane 2: 5:1, Lane 3: 4:1, Lane 4: 3:1, Lane 5: 2:1, Lane 6: 1:1, Lane 7: 0.5:1, Lane 8: Naked siRNA

Table 5. 4: Results of heparin competition assay of different formulations.

Sr. No.	**Formulations**	**Heparin:siRNA (w/w)**
1	bPEI-HA polyplex	2:1
2	bPEI-Lf polyplex	3:1
3	bPEI-Chi polyplex	4:1
4	Biodegradable bPEI-Chi polyplex	3:1
5	Lipopolyplex	4:1

The results indicate that formulated nanoplexes were stable in presence of heparin up to heparin:siRNA w/w ratio of 2:1. This predicts stability of formulated nanoplexes in anionic environment in turn proving nanoplex stability in in-vivo condition in presence of serum or plasma proteins. In case of lipopolyplex, heparin causes decomplexation leading to siRNA release but this doesn't lead to complete release of siRNA. Thus, image of agarose gel electrophoresis for lipopolyplex showed bands with lower intensity compared with naked siRNA.

5.4.5 Stability of formed colloidal nanoplexes

The results of stability study performed by incubating nanoplexes with simulated nasal fluid are shown in Figure 5. 26 which when analyzed by ANOVA

proved that all the formulations resisted aggregation in simulated biological condition and thus showed non-significant change in particle size after incubation for 30 min. This may be attributed to resultant surface charge on all the formulations, as mentioned previously, which hinders aggregation by net repulsion between nanocomplexes.

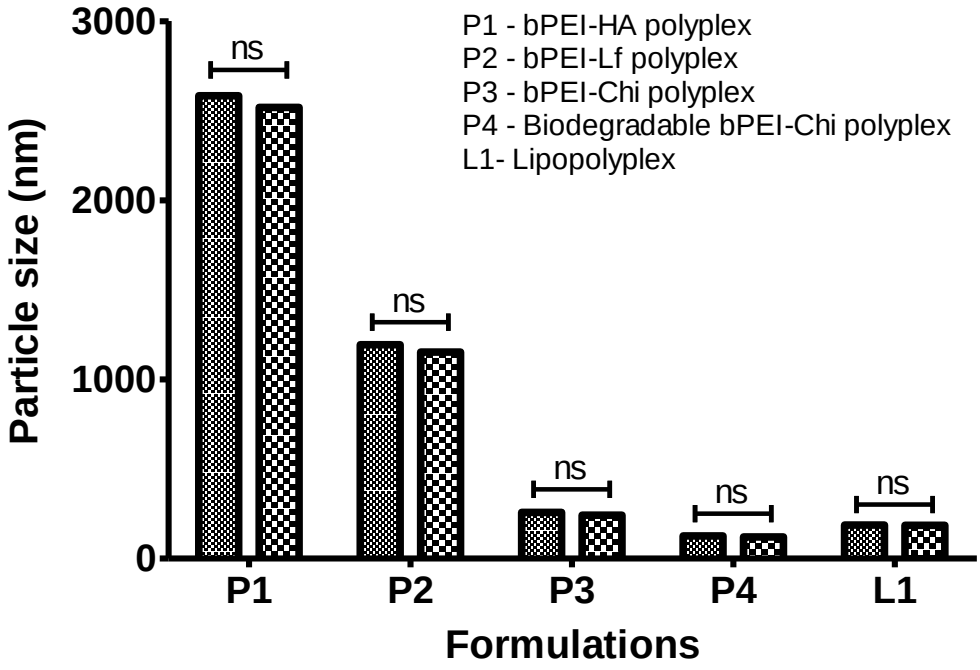

Figure 5. 26: Stability study of formed colloidal nanoplexes

5.4.6 Ex-vivo nasal permeation study

The release pattern of various polyplexes and lipopolyplexes obtained through ex-vivo nasal permeation study are depicted in Figure 5. 27 to Figure 5. 31.

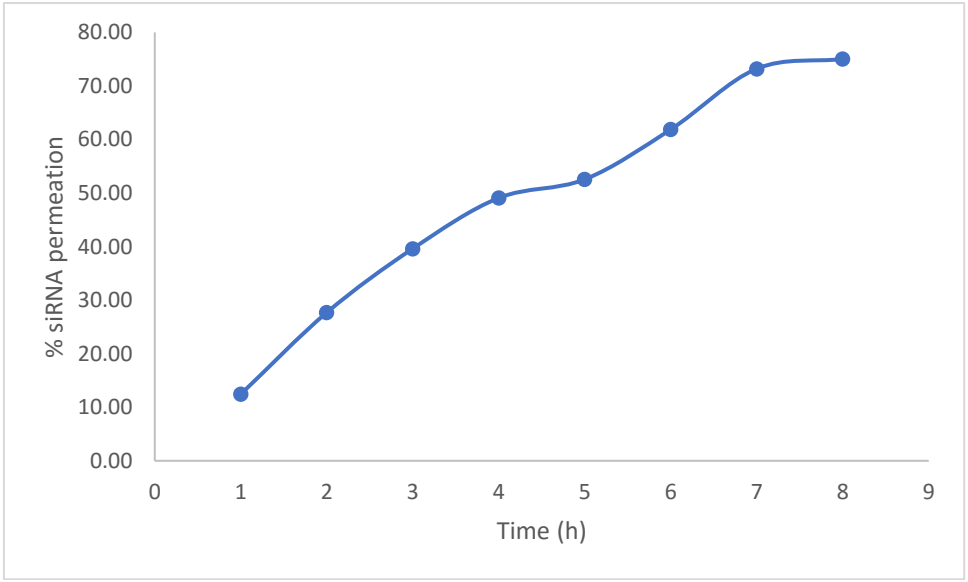

Figure 5. 27: Ex-vivo nasal permeation study of bPEI-HA polyplex

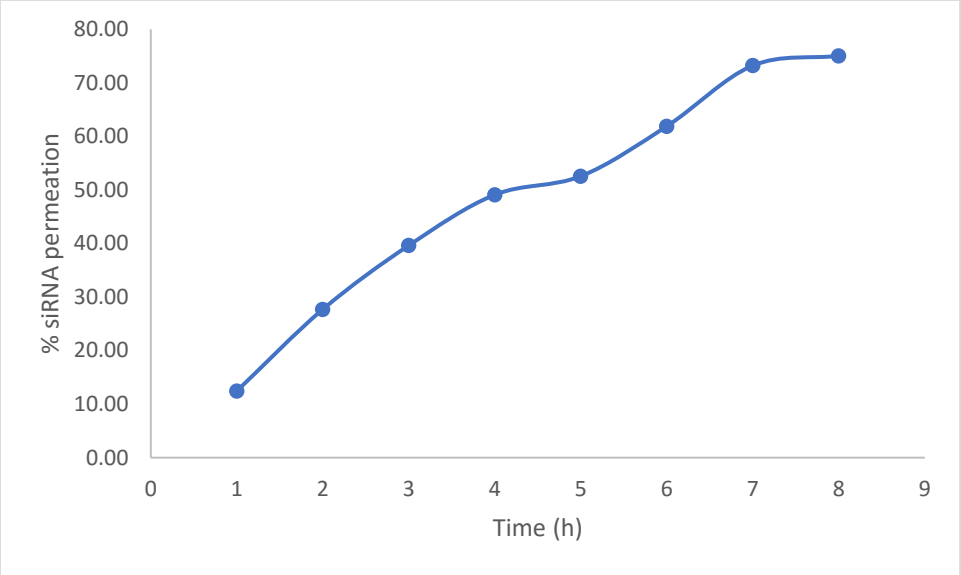

Figure 5. 28: Ex-vivo nasal permeation study of bPEI-Lf polyplex

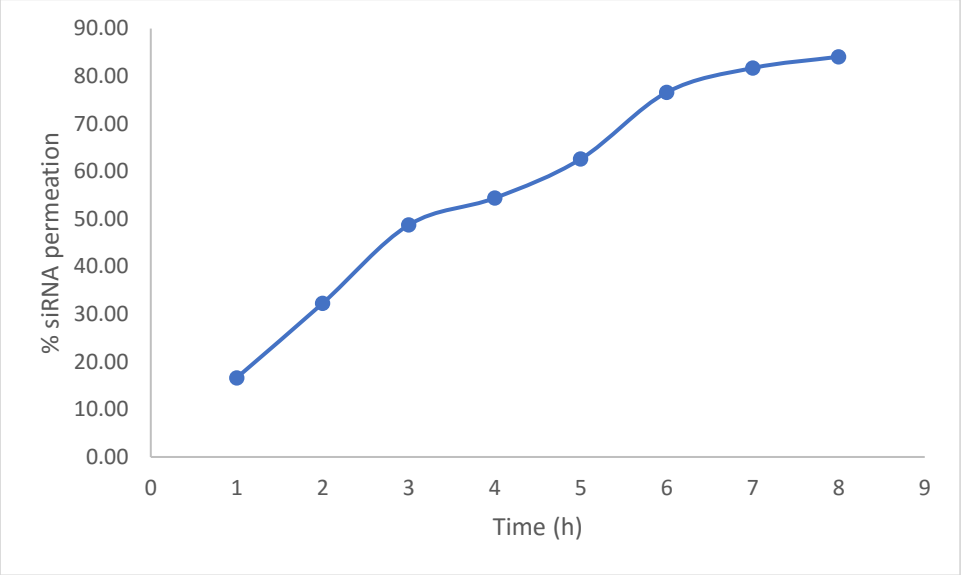

Figure 5. 29: Ex-vivo nasal permeation study of bPEI-Chi polyplex

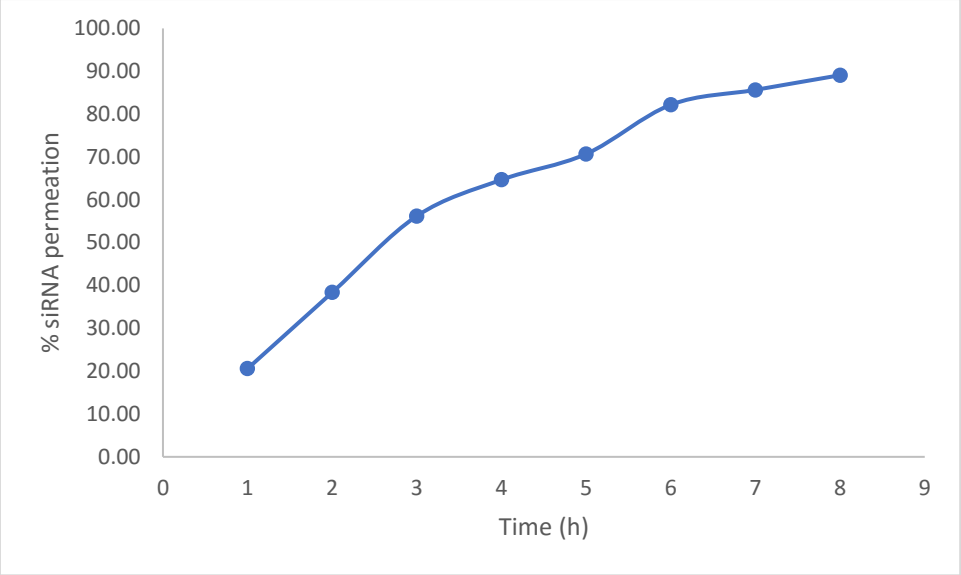

Figure 5. 30: Ex-vivo nasal permeation study of biodegradable bPEI-Chi polyplex

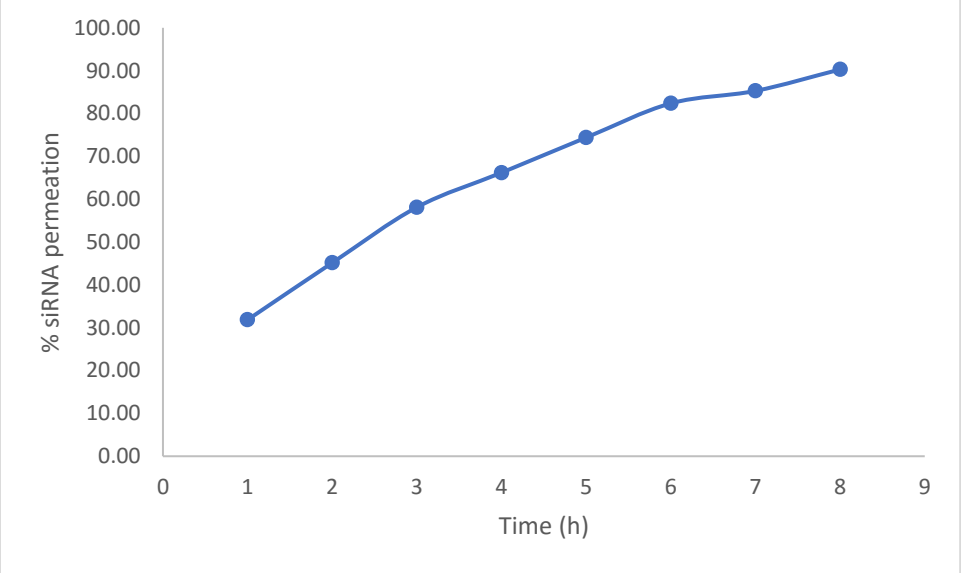

Figure 5. 31: Ex-vivo nasal permeation study of lipopolyplex

Table 5. 5: % nasal permeation of different formulations through sheep nasal mucosa

Formulations	**% permeation after 8 h**	**Mean flux ($ng/cm^2.h$)**
bPEI-HA polyplex	71.73	17.83
bPEI-Lf polyplex	75.02	18.65
bPEI-Chi polyplex	84.08	20.90
Biodegradable bPEI-Chi polyplex	89.08	22.14
Lipopolyplex	90.28	22.44

The results of ex-vivo nasal permeation study depicted permeation of significant fraction of conjugated siRNA to receptor chamber of Franz diffusion cell through excised sheep nasal mucosa after 8 h. The % siRNA permeation of different formulations through nasal mucosa is depicted in Table 5. 5.

Comparing the % permeation of different formulations, it was found that there was less permeation of bPEI-HA polyplex and bPEI-Lf polyplex which may be attributed to larger particle size hindering its free diffusion through nasal mucosa [35]. Additionally, lipopolyplex showed maximum permeation as all the polyplex showed net positive surface charge which may adhere to nasal mucosa while lipopolyplex showed charge near neutrality and thus showed better permeation [36].

5.4 References

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