6.DEVELOPMENT OFSIRNA ANCHORED CISPLATIN CAPRYLATE LOADED HNCS

6.1 FORMULATION CHALLENGES AND IMPORTANCE OF N/P RATIO

Targeted delivery of siRNA to lung tissue through IV route and pulmonary route is a challenging task due to the negative charge present on siRNAmacromolecules and stability issues of naked siRNA molecules in systemic circulation. Therefore *in vivo* actionof siRNA is still a major concern due to lack of efficiency in delivery to targeted tissues. The ideal formulation forthe delivery of siRNA to the pulmonary region should possess few characteristics, such as low off-target deposition,higher targeting at the site of action, stability, protection against enzymatic degradation of siRNA, promotingendosomal escape and low in toxicity, along with possessing the capability to deliver siRNA to the cytoplasm in asufficient concentration(1).

N/P ratio is the ratio of nmoles of nitrogen in cationic lipids/cationic polymers to nmoles of phosphate ofsiRNA. It is the parameter to optimize the complexation efficiency of nanocarriers. Incubation temperature andincubation time are the two crucial process parameters. Complete retardation of siRNA on the gel by nanocarriers is considered the optimum process output to select the optimum process parameter for maximum encapsulation and transfection efficiency. The higher N/P ratio leads to smaller size and enhancement in the ζ potentialof lipoplexes/polyplexes or nanocarriers (2, 3).

6.2 MATERIALS AND EQUIPMENTS

Table 6-1 List of Materials

Sr.no	Materials	Source
1.	Diethylpyrocarbonate (DEPC)	Sigma Aldrich, Mumbai, India
2.	Chloroform (Molecular biology grade)	Hi-media, Mumbai, India
3.	6X DNA gel loading buffer	Fermentas Life Sciences, USA
4.	Agarose	Hi-media Mumbai, India

5.	Ethidium Bromide	Hi-media Mumbai, India
6.	Phenol (Molecular biology grade)	Hi-media Mumbai, India
7.	5X TBE buffer	Hi-media, Mumbai, India

Table 6-2 List of Equipments

Sr.no	Equipment	Company
1.	Gel Doc System	BioRad Lab., USA
2.	BioSpec-nano	BioRad Lab., USA
3.	Malvern Zetasizer Nano ZS	Malvern Instruments, Malvern, UK
4.	Transmission Electron Microscopy	TECNAI G2 Spirit BioT WIN, FEI-Netherlands

6.3 METHODS

6.3.1 Development of siRNA anchored Cisplatin caprylate loaded HNCs

siRNA containing Cisplatin caprylate loaded cationic HNCs were prepared using the same method as described in section 5.5.3of chapter 5. The complexation of siRNA with Cisplatin caprylate loaded cationic HNCs was carried out based on principle of charge based interaction enabling to form complex due to cationic charge of DOTAP and anionic charge of siRNA. These HNCs were developed in two stages:

1.Preparation of Blank/cisplatin caprylate loaded cationic HNCs

2. Incubation of Cisplatin caprylate loaded cationic HNCs with siRNA

3. PEGylation using DSPE-PEG-2000 by post insertion technique

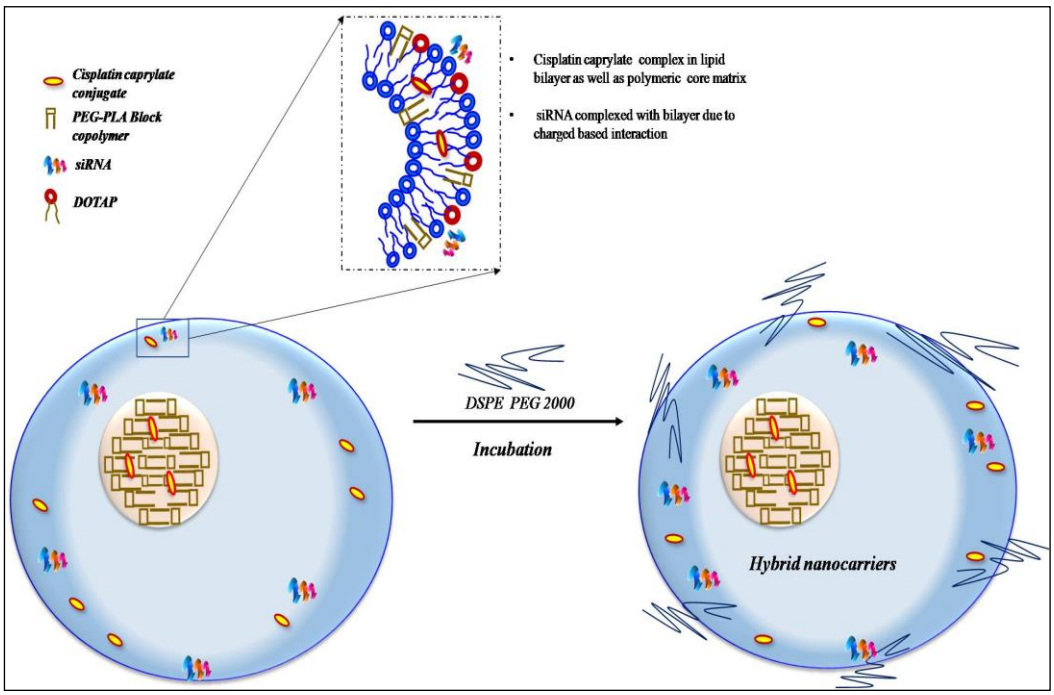

Figure 6-1 Formation of siRNA anchored cisplatin caprylate loaded HNCs

6.3.1.1 Blank/Cisplatin caprylate loaded cationic HNCs

Blank/Cisplatin caprylate loaded cationic HNCs were formulated as per procedure described in section 5.4.1 of chapter 5.Several changes have been made in reagents and apparatus due to siRNA incorporation as it is easily prone to degradation by nuclease therefore DEPC treated (nuclease free) apparatus, tips, and tubes were used and all chemicals like chloroform were of molecular biology grade. The thin lipo-polymeric film was hydrated by DEPC treated nuclease free water (NFW) and NFW was prepared.

6.3.1.2 Formulation of siRNA anchored cisplatin caprylate loaded HNCs

Cisplatin caprylate loaded cationic HNCs were formulated as per procedure described in section 5.4.1 of chapter 5. The thin lipo-polymeric film was hydrated by DEPC treated nuclease free phosphate buffer saline pH 7.4. Formulation of siRNA anchored cisplatin caprylate loaded HNCs were carried out in two different steps. First, siRNA containing cationic HNCs were prepared by incubating naked siRNA (100 nM) with preformed cisplatin caprylate loaded HNCs at different *N/P* (Nitrogen/Phosphate) charge ratio, ranging from 0 to 2.0. The mixture was gently vortexed for 2 min and incubation for different time (10, 20, 30, 60 and 120 min) and at different temperature (5° to 35°C, increment by 10°C). In second step, post

insertion technique was employed to incorporate DSPE-PEG 2000 (3 mol % of total lipid content) into preformulated siRNA anchored cisplatin caprylate loaded HNCs aqueous dispersion as per the same procedure describe in section 5.4.1 of chapter 5.

6.3.2 Size and Zeta potential measurement

The average particle size and zeta potential of siRNA loaded HNCs were determined by differential light scattering with a Malvern Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). Prior to the measurement siRNA nano-constructs were diluted with nuclease free water and measurements were carried out at 25°C±2°C. Zeta potential was calculated by Smoluchowski's equation from the electrophoretic mobility. Each sample was measured three times and the mean values were calculated.

6.3.3 Assay

Assay of the prepared formulation was carried out to confirm the amount of siRNA loaded as compared to added siRNA. Assay is important parameter to determine whether any degradation is there or not in final formulations and based on that further potency calculations can be carried out for the *in vitro* and *in vivo* studies. Below given procedure was followed for the assay of the prepared formulations:

Formulation was diluted with Diethylpyrocarbonate (DEPC; Sigma)-treated H20 to give a final volume of 100 µL (if required). The samples were then vortexed with 200 µL of phenol/chloroform (1:1 v/v) and were subsequently spun at 14,000 rpm at 4°C for 10 min. From this centrifuged samples, aqueous layer was separated out and quantified using below given two methods:

1. 5 µL of the sample was diluted with DEPC treated water up to 25 µL and mixed with 5 µL loading buffer and was loaded onto 2% agarose gel. Afterwards, siRNA was visualized by UV transillumination and gel photography using a Gel Doc System (BioRad Lab., USA).

2. Aqueous layer was mixed further with DEPC treated water up to 1 mL and absorbance was taken at 260 nm using nanodrop.

6.3.4 Entrapment efficiency of siRNA in HNCs

6.3.4.1 Gel Retardation Assay

Gel retardation assay was carried out to determine siRNA incorporation amount in HNCs. Following incubation period, siRNA nano-constructs were

subjected to gel electrophoresis to assess encapsulation of siRNA within HNCs. siRNA nano-constructs were mixed with 2 µL of 6X DNA gel loading buffer (Fermentas Life Sciences, USA) and loaded onto a 2% agarose gel containing 0.5 µg/mL ethidium bromide, and separated by electrophoresis for 20 min at 100 V in TBE buffer. Afterwards, siRNA was visualized by UV trans-illumination and gel photography using a Gel Doc System (Bio-Rad Lab., USA). Amount of free siRNA was visualized onto the gel as diffused white band.

6.3.4.2 Ultracentrifugation

HNCs were added to the centrifuge tube and centrifuged at 70000 x g for 4 hr at 4°C. Pellet was vortexed with 200 µl of phenol/chloroform (1: 1 v/v) and was subsequently spun at 14,000 rpm at 4°C for 10 min. From these centrifuged samples, aqueous layer was separated out and quantified using methods described in the Assay section **(Section 6.3.3)** above (4, 5).

6.3.5 Cryo TEM and Freeze fracture TEM studies

Morphology and size of HNCs were evaluated using Transmission Electron Microscopy (TECNAI G2 Spirit BioT WIN, FEI-Netherlands) operating at 200 kV with 0.27 nm resolution. The hydrophobic grid was converted into hydrophilic by glow discharge. The samples were spread on grid and cryo-freezed in liquid ethane at -180°C. The grid was inserted into microscope using a cryo holder and images were taken at 70,000X magnification. Cryo-TEM and Frezze fractured TEM experiments were carried out at CSMCRI, Bhavnagar and department of pharmaceutics, Institute of Pharmacy, Friedrich schiller university of Jena, Germany respectively (4, 6).

6.3.6 Serum stability study

Assay of the prepared formulation was carried out to confirm the amount of siRNA loaded as compared to added siRNA. Assay is important parameter to determine whether anydegradation is there or not in final formulations and based on that further potency calculations can be carried out for the *in vitro* and *in vivo* studies. Below given procedure was followed for the assay of the prepared formulations.

Formulation was diluted with Diethylpyrocarbonate (DEPC; Sigma)-treated H20 to give a final volume of 100 µL (if required). The samples were then vortexed with 200 µL of phenol/chloroform (1:1 v/v) and were subsequently spun at 14,000 rpm at 4°C for 10 min. From this centrifuged samples, aqueous layer was separated

out and quantified using below given two methods: 1. 5 µL of the sample was diluted with DEPC treated water up to 25 µL and mixed with 5 µL loading buffer and was loaded onto 2% agarose gel. Afterwards, siRNA was visualized by UV transillumination and gel photography using a Gel Doc System (BioRad Lab., USA) 2. Aqueous layer was mixed further with DEPC treated water up to 1 mL and absorbance was taken at 260 nm using nanodrop.

Cisplatin loaded siRNA anchored HNCs were checked for integrity of complexed siRNA in presence of serum for degradation during circulation due to RNAse. Naked siRNA and HNCs containing 26.6 µg of siRNA with PEGylation and without PEGylation (batch V3) were incubated with 50 µl non-heat inactivated FBS at 37 C for different time periods to give a 50 % serum concentration in final incubation volume having pH of 7.4. Incubated samples were processed and quantified in gel documentation system as per procedure mentioned above (7).

6.3.7 Atomic force microscopy (AFM) analysis

AFM was done on AFM-NT-MDT (Model No. NT-MDT NTEGRA Prima) with silver nitride cantilever at IIT, Gandhinagar. AFM images were recorded for LPHNPs using NT-MDT NTEGRA Prima Scanning Probe Microscope (SPM) in tapping mode using 100µ X 100µ micron scanner with tapping mode and NSG tip with size at the edge around 10 nm at 1.01 Hz frequency. Typical substrate used for the study was Silicon wafer, Si (100) with average root mean square roughness (RMS) of 0.065 for bare surface. A drop of dilute solution of NPs was deposited on Si (100) substrate, which was dried under ambient conditions for 24 hrs. The coated area of substrate was subjected to 10µ x 10µ and 5µ X 5µ scan under SPM. The topography image was developed with Nova software supplied with instrument. Average roughness analysis and particle size distribution in the scanned area were studied. The atomic force microscope (AFM) system has evolved into a useful tool for direct measurements of micro-structural parameters and unravelling the intermolecular forces at Nanoscale level with atomic-resolution characterization.

6.4 RESULTS AND DISCUSSIONS

6.4.1 Development of siRNA anchored Cisplatin caprylate loaded HNCs

6.4.1.1 Process optimization

Process parameter optimization such as incubation time and temperature were optimized for desired results. The effect of one variable was studied at a time keeping other variables constant. The results are recorded in Table 6.3 from which the following conclusions were drawn:

Incubation Time: Incubation time for the complexation between siRNA and pre-formed HNCs is very vital. Many reports suggest that incubation should be between 20-30 min at ambient temperature. Three different levels were studied as variables i.e. 15 min, 30 min and 45 min. At 15 min less than 65 % of siRNA were complexed with HNCs, confirmed by gel electrophoresis. Incubation at 30 and 45 min generated same results and showed more than 85 % of complexation.

Incubation temperature: less than 70 % of siRNA was complexed at 20°C and more than 85 % siRNA was complexed at 25°C and 37°C therefore 25°C was selected as incubation temperature.

Table 6-3Selection of process parameters for preformed Cisplatin caprylate loaded siRNA-HNCs complexes

Incubation time	
Time (min)	% siRNA entrapped* #
15	<65
30	>85
45	>85
Incubation temperature	
Temp (c)	% siRNA entrapped* #
20	<70
25	>85
37	>85
* N/P - 2 for all formulation and % entrapment was measured by Gel retardation assay # - Experiments were performed in triplicates.	

6.4.1.2 Formulation Optimization

The complexation of siRNA with cisplatin caprylate loaded cationic HNCs was carried out based on principle of chargebased interaction enabling to form complex due to cationic charge of DOTAP in HNCs and anionic charge of siRNA. Incubation time for the complexation between siRNA and pre-formed liposomes is

very vital. Maximum siRNA complexation was obtained at incubation time of 30 mins and incubation temperature of 25 C. The spontaneous process of incorporation of PEGylated lipids into preformed HNCs is governed by the hydrophobic interactions of lipids and hydrophobic end of lipopolymers. The concentrations of PEGylated lipids were maintained below their critical micelle concentration (CMC) to prevent the self-assembly of PEGylated lipids. A positively charged lipid DOTAP was a key lipid for encapsulation of siRNA. The gel retardation pattern of siRNA was affected mainly by *N/P* ratios. N is thequaternary nitrogen of the cationic lipid, DOTAP andPis the phosphate group of nucleic acid base of siRNA.

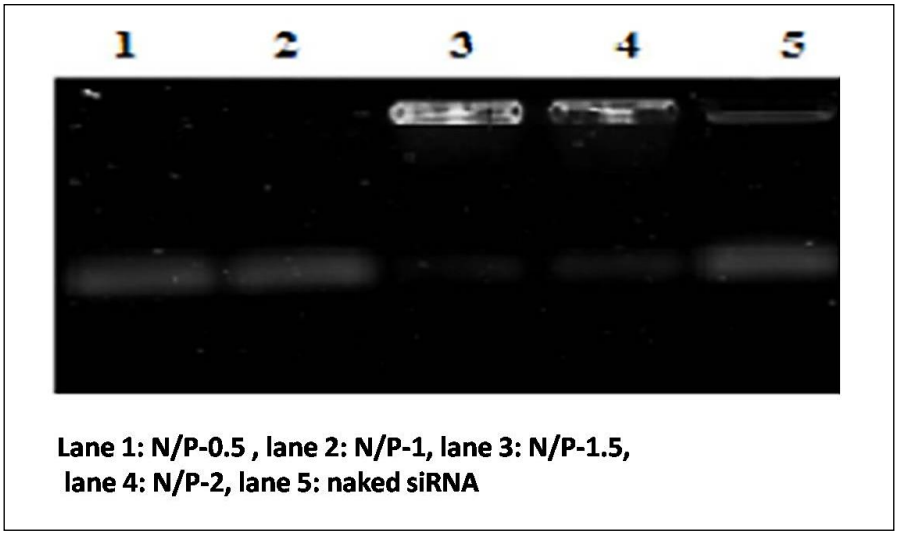

Figure 6-2 Gel retardation assay siRNA-HNCs at different N/P ratio

Below *N/P* ratio of 1.0, a considerable amount of the siRNA migrated as free siRNA on agarose gels towards positive electrode. Below*N/P* of 1, siRNA complexation with HNCs occurred (table 6.4) but obtained complex was very loose. This loose nanoconstructcomplex may release siRNA before entering the cell and result into inactivation. However, complete complexation was found to occur at 1.5 and 2 and there was no visible band (Figure 6.2) detected after gel electrophoresis run thus free siRNA was not present at *N/P* of 1.5 and 2.0. These results suggest the ability of HNCs for complete complexation of siRNA at N/P ratio of 1.5.

6.4.2 Nanocarrier Size and Zeta Potential

Size of the cationic HNCs after siRNA complexation and PEGylation was mainly dependent on the rigidity of pre-formed HNCs obtained in step-1. Post insertion technique of PEGylation slightly increased the nanocarrier size due to

increase in the hydration volume on the surface (table 6.4). Increment in nanocarrier size is due to increment in thickness of outer PEG layer. Zeta potential was found to be decreased after siRNA complexation (Table 6-4) and further confirms the surface interaction between positively HNCs and negatively charged siRNA.

Table 6-4 Cisplatin loaded siRNA anchored HNCs characterization

Formulation	**N/P ratio**	**Effect of PEGylation Nanocarrier size (nm)**		**Effect on siRNA complexation on Zeta Potential (mV)**		**siRNA % complexation efficiency**
		Before	After	Before	After	
R1	-	162.2±3.45	-	+43.18±1.83	-	-
S1	0.5	175.6±1.48	186.5±1.59	41.79±3.78	25.93± 1.69	34.78± 2.69
S2	1	158.2±0.96	169.9±0.39	48.37±1.79	27.29± 2.95	43.95± 1.58
R2	1.5	161.7±2.18	171.2±2.74	+41.86±1.83	25.39± 1.68	95.83± 2.68
S3	2	158.4±1.94	169.8±1.95	+39.06±1.83	22.61± 0.17	92.69± 1.57
S4	2	153.4±1.18	NA	34.95±2.70	-	90.36± 2.78
All batches S1 to S4 have similar formulation composition as batch R1 except S4 which was formulated without addition of DSPE PEG 2000& Experiments were performed in triplicate.						

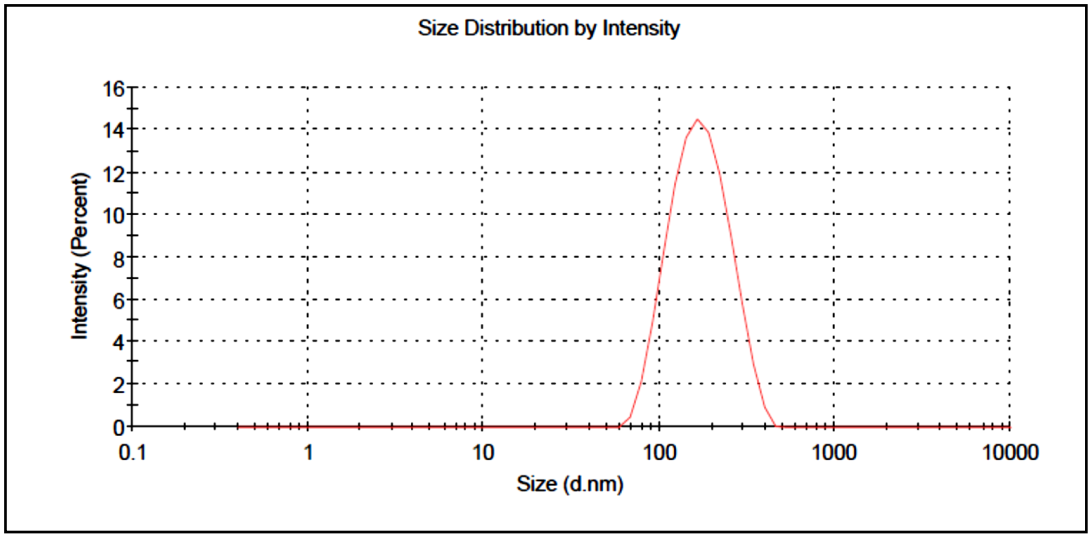

Figure 6-3 Nanocarrier Size of developed HNCs after siRNA complexation

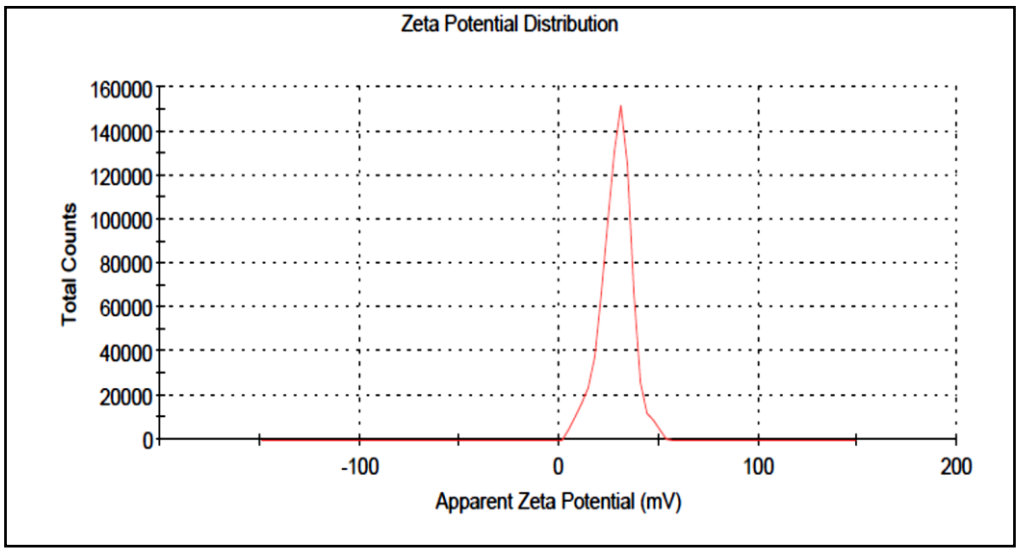

Figure 6-4Zeta potential of developed HNCs after siRNA complexation

6.4.3 Cryo-Transmission Electron microscopy

Further confirmation of HNCs structure was done by Cryo-TEM (Figure 6-5 and Figure 6-6) which revealed unilamellarity in structure and having particle size below 200 nm. This size range assists in EPR effect for tumor internalization of nano materials and also avoiding RES uptake. Formations of distinct PEG-PLA particles along with matrix formation inside cavity of HNCs were also confirmed by Cryo-TEM. Further insight into the HNCs cavity was captured by freeze fractured Cryo-TEM (Figure 6-7) and it revealed solid polymeric matrix core inside HNCs.

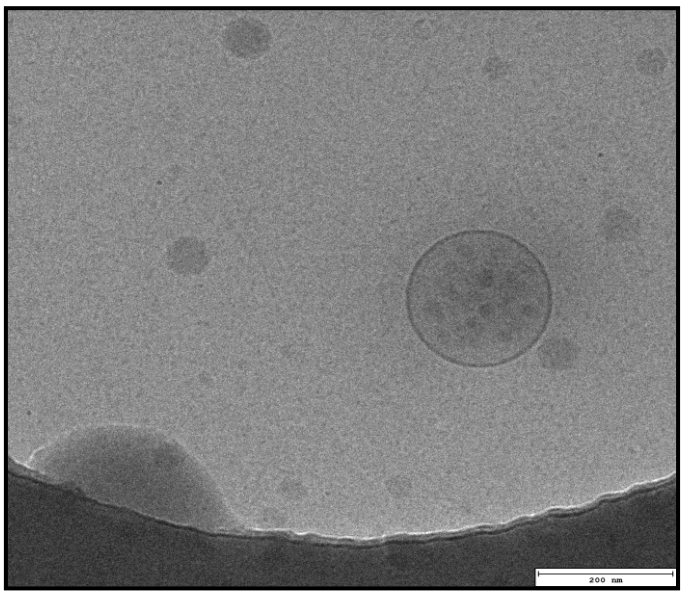

Figure 6-5HNCs Cryo TEM

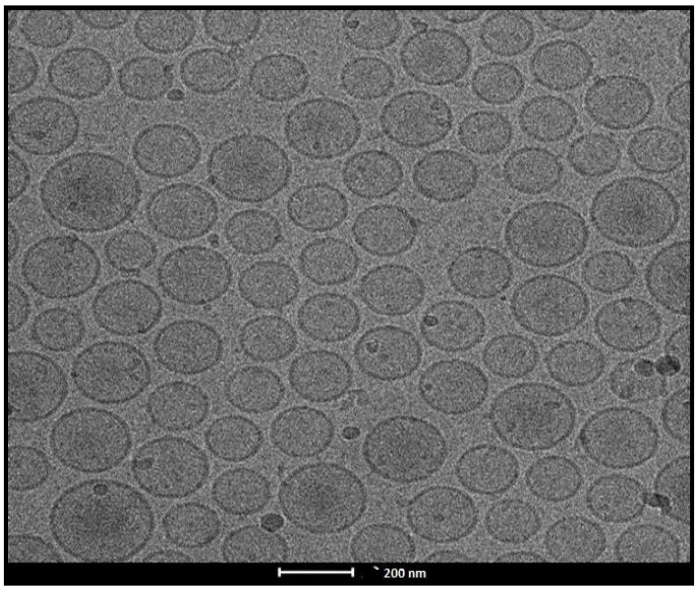

Figure 6-6HNCs cryo TEM

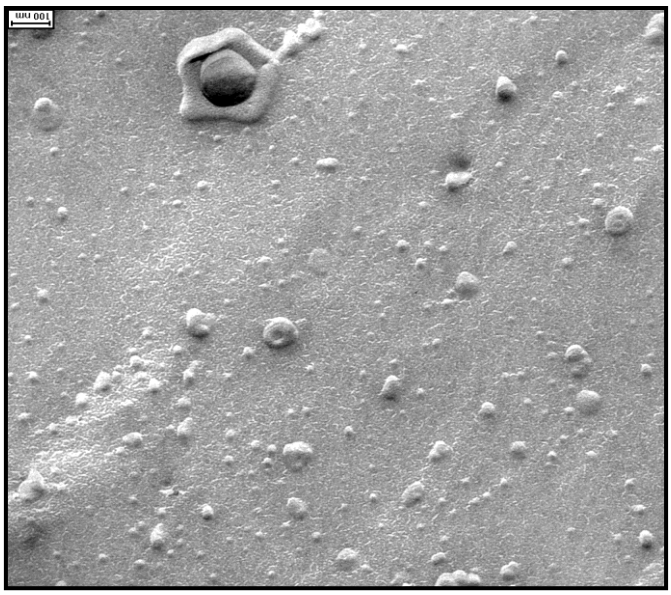

Figure 6-7freeze fracture TEM

6.4.4 Serum stability study

From the results of band density of gel electrophoresis (fig. 6.2), it can be concluded that HNCs retained integrity of siRNA thereby protecting it from nucleases of external environment. Additional protection might be provided by post insertion technique of PEGlyation employed after attachment of siRNA with HNCs. Post insertion technique of PEGylation provide coating having good encapsulation and protection in blood however it should also retain an efficient delivery of siRNA

and drug to cancer cells. Moreover, it leads to very less premature siRNA release due to its protection inside PEGylation layer.

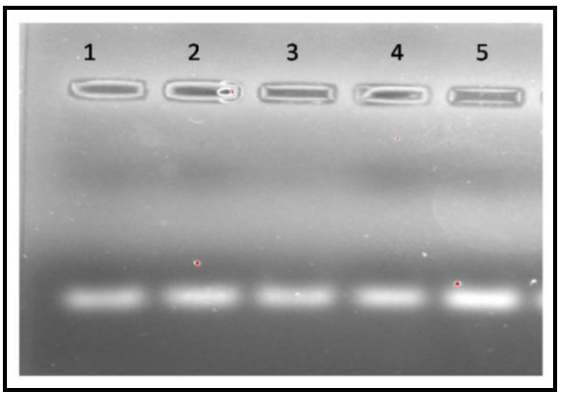

Figure 6-8 HNCs serum stability in absence of PEGylation layer

Lane: 1= 0 hr, 2=4 hr, 3= 8 hr, 4= 12 hr, 5= 24 hr

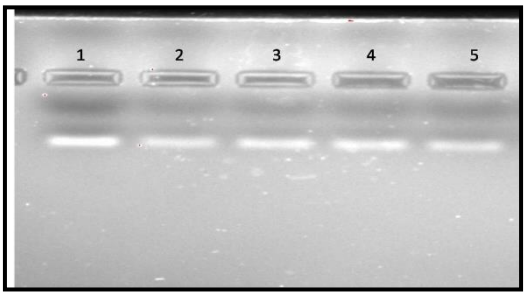

Figure 6-9 HNCs serum stability (with PEGylation layer)

Lane: 1= 0 hr, 2=4 hr, 3= 8 hr, 4= 12 hr, 5= 24 hr

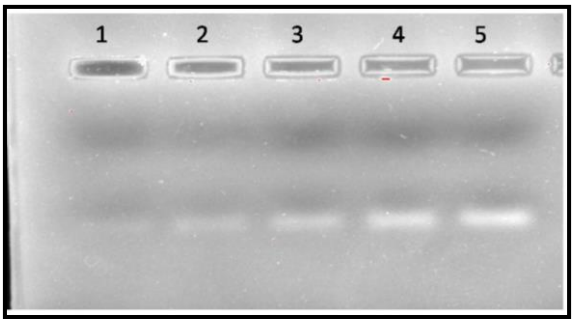

Figure 6-10 Naked siRNA serum stability

Lane: 1= 24 hr, 2=12 hr, 3= 8 hr, 4= 4 hr, 5= 0 hr

Stability study of siRNA in serum condition was carried out and the degradation of siRNA was analyzed by gel electrophoresis (Figure 6-8). Gel retardation assay showed that degradation started within 30 mins after incubation of

siRNA with FBS and only 20 % of siRNA remained intact as compared to initial loading. HNCs encapsulated siRNA was stable even after 24 hr and more than 80 % of siRNA was detected on agarose gel (Figure 6-9 and Figure 6-10). It was confirmed that prepared HNCs successfully protected the encapsulated siRNA from enzymatic degradation. There was significant increase in stability of PEGylated HNCs compared to bare HNCs (Figure 6-11). It may be due to outer PEGylated layer providing steric shielding effect thereby keeping away serum nucleases.

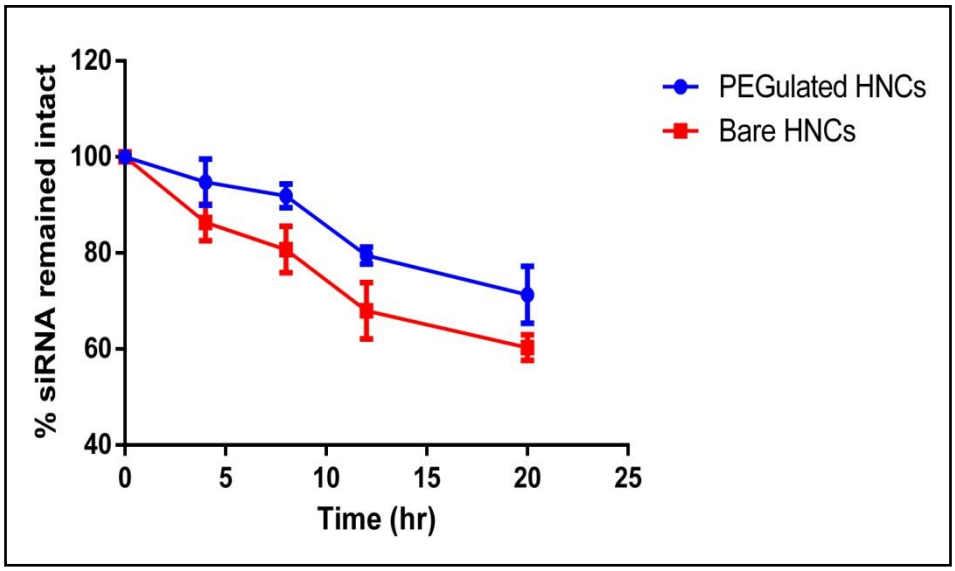

Figure 6-11 in vitro release of siRNA from HNCs

6.4.5 Atomic force microscopy (AFM) analysis

The morphological studies performed by AFM showed uniform and spherical shaped discrete particles (Figure 6-13) without aggregation with approx. Size of 150 nm. The height histogram (Figure 6-12) shows fairly narrow distribution of peak heights from 5 to 20 nm corresponding to lipid bilayer thickness and PEGylation layer. The average roughness (Ra) was observed to be 10.2 nm. These values indicated a slightly rougher surface. The increase in roughness could be attributed to the presence of PEGylation layer due to DSPE-PEG-2000 in HNCs which is necessary for stability and long circulation time in-vivo.

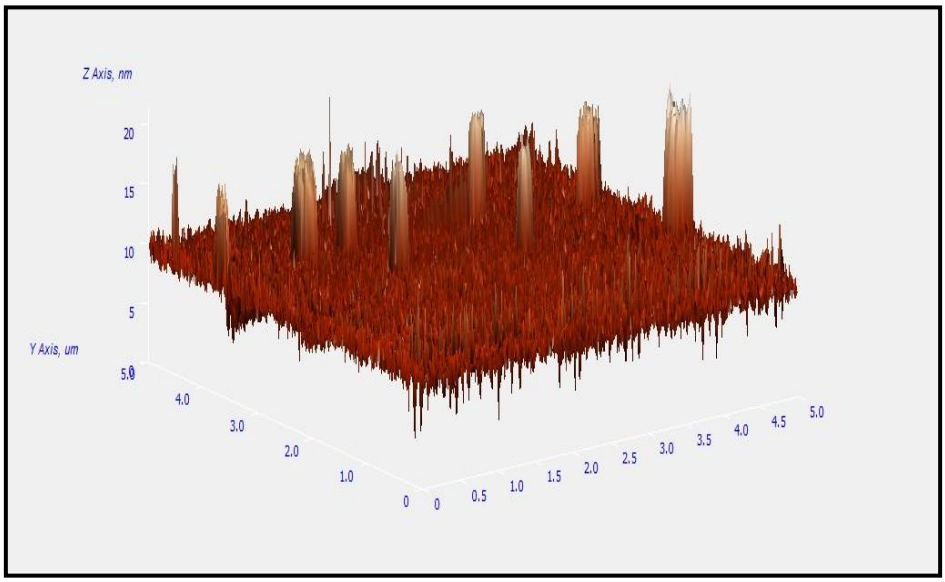

Figure 6-12 AFM HNCs-1 PEGylation layer thickness

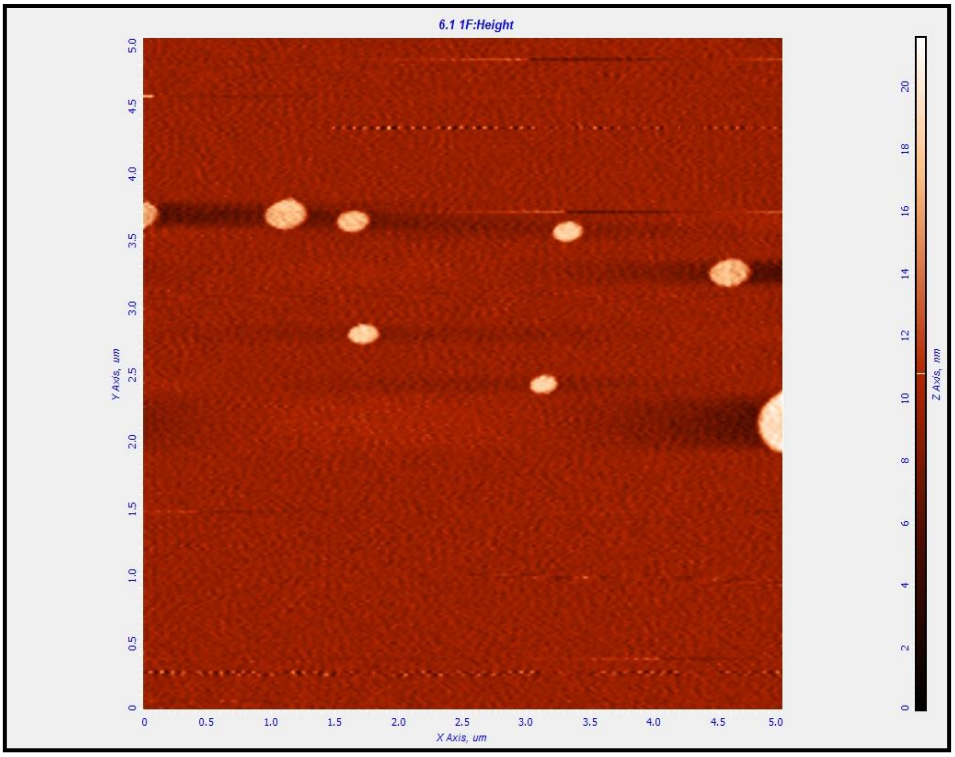

Figure 6-13 AFM HNCs-2

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