6 DEVELOPMENT OF LYOPHILIZED DRY POWDER FORMULATION

6.1 INTRODUCTION

The application of chemotherapy directly to the lungs for the treatment of lung tumors has been proposed for many decades. Inhalation was achieved by nebulizing liquid drug formulations, i.e. intravenous solution-like formulations (in the first trials) and liposomal dispersions, as nebulization is the safest way to administer a drug to the lungs (1). Compared to other non-oral routes, inhalation path is very useful for management, and allows a large delivery window in the airway. It also accounts for lower structural adverse effects in comparison to its local actions (2).

Lyophilization is the most popular method of transforming thermo-labile bioactive materials including proteins and gene vector complexes into powders. The effect of lyophilization on the bioactivity of siRNA-lipoplexes was studied in presence of non-ionic cryo-protectant and results proved that it could be lyophilized and reconstituted without loss of transfection efficacy (3). It has no effect on intrinsic biological activity and functionality is preserved by lyophilized siRNA. The dry powder aerosols of polyethylenimine (PEI)-based gene vectors prepared in the presence of cryo-protectant by lyophilization and powderization were tested and found to be well tolerated by Brus et al (4). The choice of cryoprotectant along with lyophilization steps affects the stability of the formulation but it should not affect the transfection efficacy of shRNA pDNA.

In preserving integrity and avoiding freeze fracturing during the period of lyophilization, the cryoprotectant plays an essential role. If we use it in sufficient concentration, it also stops the accumulation at the freezing stage of lyophilization (5). The theory of particle isolation based on the separation of unfrozen particles into the unfrozen matrix has been suggested as a possible stabilization mechanism and there is no role for polymer-induced vitrification (6).

The powder-based DPI formulations may give several benefits, such as improved stabilization, low drug leakage during administration, improved processing and accessibility, and improved delivery of drugs to the intended pulmonary area (7, 8). Therefore, it would be of great advantage to produce safe dry powder aerosols for pulmonary gene therapy. The inhaled formulation particles could not exceed 3 μm in size, as particle of this size will enter

the alveoli, are picked up by epithelial cells, and are brought through and discharged between the epithelial cells through the circulating blood stream and the extracellular space (9).

6.2 MATERIALS AND EQUIPMENTS

6.2.1 Materials

Table 6-1 List of Materials

Sr no.	Name	Supplier of Material
1	Trehalose, Maltose, Sucrose, Dextrose, Lactose	Procured from Hi-Media, Mumbai
2	Glycine, Albumin	Merck life science, USA.
3	Respitose SV003, Respitose ML006, Lactohale 200, Lactohale LH 300, Lactohale LH 230, Lactohale ML 001	kind gift from DFE Pharma, USA
4	Inhalac 200	Procured from Meggle Pharma, Germany
5.	Acetonitrile	SD fine chemicals. India
6.	DEPC treated water	Laboratory prepared
7.	Double distilled water	Laboratory prepared

Table 6-2 List of Equipments

Sr no.	Name of equipment	Company
1.	Virtis Advantage Plus Lyophilizer	Virtis, India.
2.	HPLC	Agilent Technologies 1260 infinity II, India.
3.	Andersen cascade impactor (ACI)	Copley Scientific, USA.
4.	SEM instrument- JSM-6380LV	JEOL, India.
5.	Gel Doc System	BioRad Lab., USA
6.	TECNAI G2 Spirit BioT WIN,	FEI, Netherlands
7.	Philips EM 301	BAL-TEC Inc, Balzers, Liechtenstein
8	Malvern Mastersizer	Malvern Panalytical Ltd, UK

6.3 PREPARATION AND CHARACTERIZATION OF LYOPHILIZED PLHNCS

6.3.1 Lyophilization procedure

Lyophilization of the developed hybrid nanocarriers were carried out using Virtis advance lyophilizer to achieve high percentage drug retention (PDR). The overall lyophilization method involves freezing of PLHNCs at a hold temperature of -40 °C for a duration of 5h. Primary drying and secondary drying was carried out at various temperatures and time segments, as shown in Table 6-3. For the planning ordeal, the chamber pressure and cold trap temperature were held at 10 Pa and 50°C. The freeze-dried PLHNCs had been resuspended in 2 mL of Type-1 water and particle size was measured to determine stability after lyophilization. Different cryoprotectants i.e., lactose, maltose, sucrose, dextrose and trehalose along with polypeptides were used to prevent freezing damage. For the lyophilization, the achieved pellet of the hybrid nanocarriers were suspended in water and mixed with different cryoprotectants. Different grade of lactose were mixed in different mass ratio with sieved lyophilized hybrid nanocarriers and resultant powder was filled in size "2" capsule. Percentage drug retention studies were carried out on developed dry powder of PLHNCs.

Details of the various steps involved in lyophilization such as primary and secondary drying, freezing time ramp and hold temperature are mentioned in the Table 6-3.

Table 6-3 Thermal cycle for freeze drying

Stage	Temp. (°C)	Ramp (Min)	Hold (Min)	Pressure (Pascal)
Freezing	5	10	10	NA
	-40	120	240	NA
Primary drying	-15	150	320	10 Pascal
	0	180	220	10 Pascal
	10	90	220	10 Pascal
Secondary drying	20	90	220	10 Pascal
	28	90	540	10 Pascal

6.3.2 Sf/Si ratio

Ratio of PLHNCs size after lyophilization with size before lyophilization is termed as Sf/Si ratio. Sf/Si ratio 1 is considered ideal theoretically. Though it is almost impossible to achieve Sf/Si ratio 1 for any pharmaceutical vesicle containing product, Sf/Si ratio of 1.3 is considered safe to prove efficient lyophilization without damaging vesicles (10).

6.3.3 Percentage drug retention

The PLHNCs formulation were withdrawn from lyophilized vial after lyophilization and rehydrated with double distilled water for assessment of amount of Docetaxel retained in PLHNCs using HPLC (Method as mentioned in chapter 3). The samples were also analyzed for the evidence of hard-caking and discoloration.

6.3.4 Moisture content analysis

For the estimation of moisture in any material, Karl-Fischer titration is commonly employed and it is also widely known for the lyophilized formulation (11). Using known amounts of water, commercially available reagents (pyridine free) were standardized (0.25 kg). 40 ml of anhydrous methanol was titrated with a Karl-Fisher reagent for the correction of residual water found in methanol. After this, sample was introduced and the water content was measured with titrimetric analysis.

6.3.5 Cryogenic and Freeze fracture transmission electron microscopy (cryo-TEM & FF-TEM)

Cryo-TEM was used to evaluate the size, lamellarity, shape, and morphology of the optimized lyophilized PLHNCs. This technique was performed at 0.27 nm of resolution with 200 kV on TECNAI G2 Spirit BioT WIN, FEI-Netherlands. PLHNCs reconstituted suspension was spread on the grid after the cryo-freeze at -180 ˚C in liquid nitrogen and subjected to imaging at 70,000× magnification. The image was then processed and analysed using TECNAI software.

The suspension of lyophilized PLHNCs were sandwiched between copper plates for freeze fracture TEM and the gold grid was used as a spacer. Then with the help of liquid ethane (-180 °C), the lyophized suspension was frozen and then fractured at -150 °C at a pressure of $2x10^{-7}$ mbar in BAL-TEC Inc, Balzers, Liechtenstein. Samples were coated with a Pt/C grid at different angles and examined with a (Philips EM 301) electron/TEM microscope.

6.4 PREPARATION AND CHARACTERIZATION OF DRY POWDER FORMULATION FOR INHALATION

6.4.1 Development of dry powder formulation

The lyophilized cake was passed through 120# and 240# sieve, which turned the cake into a fine powder. With a differential laser scanning system called Malvern Mastersizer 2000, Malvern UK, the particle size of the fine powder was measured. The powder collected was appropriately combined with the few inhalational carriers with distinctive flow properties at a

different weight ratio. On the basis of improved dispersibility of the DPI during inhalation, ratios of different grade of fine and course lactose were optimized. In capsule size 2, the dry powder formulation was filled. As mentioned above, the lyophilized bulk was refined and filled with hard gelatin capsule size 2 to study aerodynamic behavior.

The in-vitro deposition resulting from the aerodynamic characteristics of the dry powder formulations was assessed by using Andersen Cascade Impactor (ACI). The respirable fraction, Fine Particle Fraction (FPF), was defined as particle mass below 5 µm. The larger particle fraction or carriers particles would settle in the oropharynx while very small particles are exhaled before undergoing lung deposition. The amount of dose emitted from the capsule through the inhalation device in to the apparatus is another key parameter which is used for optimization. Ciplahaler was selected as a sampling device for performing In-vitro lung deposition studies on Anderson cascade impactors.

6.4.2 In-vitro deposition studies

The aerodynamic features of the dry powder inhaler were calculated using the Anderson cascade impactor (ACI) developed by Copley Scientific. Powder for inhalation was filled in capsules size 3 for the dispersion of the powder through a cascade impactor and the capsule was mounted in actuators (ciplahaler). The flow rate was regulated for 4 seconds to 60 L/min so that the amount of 4 L was drawn considering the inhlaler, which causes a 4 kPa pressure drop. The purpose of the whole method was to imitate the action of human respiration. To avoid undue dilution, the volume of Docetaxel PLHNCs dry powder deposited on each plate and sieve was carefully collected with a minimum amount of solvent. By the analytical process (HPLC), the set of all the sieves and plates were further examined.

6.4.3 Compendial and non-compendial tests of developed PLHNCs dry powder.

Physical properties of the developed dry powder formulation were evaluated with density (Bulk and tapped), Carr's index, Hausner ratio and mean particle size, mass median aerodynamic diameter (MMAD) and Geometric Standard Deviation (GSD). Even compendial tests like Fine Particle Fraction (FPF), Emitted dose (ED), and device fraction were also considered while optimizing the dry powder formulation.

- **Carr Index:** The Carr Index of a material is calculated with the following formula :

$$\mathbf{Carr\_Index = (\rho_{tapped} - \rho_{bulk})/\rho_{tapped} * 100}$$

With ρtapped: the tapped bulk density of the material (kg/m3) and ρbulk : the loose bulk density of the material (kg/m3)

- **Hausner Ratio:** The Hausner Ratio of a material is calculated with the following formula:

$$\textbf{Hausner ratio} = \rho_{tapped}/\rho_{bulk}$$

- **Emitted dose (ED):** The sum of the dosage released into the device from the capsule by the inhalation tool.
- **Fine powder fraction (FPF):** Fraction of dose found below cut-off diameter < 5 μm (12).
- **Recovered dose (RD):** As a proportion of the overall assay, the total amount of the dosage recovered from the inhalers, capsule shell and the system.
- **Mass Median Aerodynamic Diameter (MMAD):** The diameter of 50 percent of the particle mass is greater and 50 percent smaller. On the likelihood scale to log of aerodynamic dimension, it was graphically calculated from the plot of total percent mass less than the size specified. At 50 percent cumulative percent, the MMAD is the benefit of intersecting (13).
- **Geometric Standard Deviation (GSD):** The GSD was obtained as slope of line or using the formula,

$$\mathrm{GSD} = \frac{\sqrt{\mathrm{d84}}}{\mathrm{d16}}$$

Where, d84 and d16 are diameters corresponding to 84% and 16% undersize mass, respectively (14).

- **Effective index (EI):** The effective index can be calculated by

$$Effective\ index = \sqrt{(100 - DF)\ x\ FPF}$$

Where, DF is the device fraction and FPF is the fine particle fraction.

6.4.4 Scanning electron microscopy

SEM was performed to achieve a better insight into the surface properties and characteristics of the developed dry powder. PLHNCs dry powder (1-2 mg) was subjected on double-sided adhesive tape and attached with an aluminum stub. It was then exposed for SEM with 20 kV of accelerating voltage. The SEM images of the sample was performed by the

Electrical Research and Development Association (ERDA), Vadodara with instrument-JSM-6380LV, JEOL.

6.4.5 Powder X-ray diffraction (PXRD)

To study the crystallinity, PXRD studies were performed to understand the crystalline nature of dry powder, as the nature of crystallinity along with amorphousness may affect the behavior of dry powder i.e., interactions between adhesiveness and cohesiveness, fluidization of dry powder in the lung, hygroscopicity, etc. The powder X-ray diffraction pattern was collected from 2-Theta values ranging from 4 to 40.

6.4.6 DSC

The physical state of Docetaxel loaded PLHNCs was determined by comparing the DSC thermogram of Docetaxel and Lyophilized D-PLHNCs .The DSC study was done using DSC 60 under Nitrogen gas at flow rate 40-100 ml/min .Then, 5 mg of sample was heated from 0 to 300 °C at temperature rising speed 10°C/min.

6.4.7 FT-IR

The Lyophilized Docetaxel PLHNCs and Docetaxel were studied through Infrared spectrum. This study was carried out to check the compatibility or any significant changes in drug properties during formulation.

6.5 INTEGRITY OF shRNA pDNA AFTER DRY POWDER FORMULATION

The integrity of shRNA pDNA after conversion to powder form for inhalation was determined using gel electrophoresis method as described in chapter 3 section 3.8.7.

6.6 RESULTS AND DISCUSSION

6.6.1 Optimization of lyophilization

The Sf/Si ratio of 1.1 (as seen in Table 6-4) in both formulations (PLHNC1 and PLHNCs 2) supports the trehalose as a cryoprotectant. But only **68.9 ± 1.2 and 72.1 ± 1.3** of the drug has been retained in both PLHNC1 and PLHNC2 respectively if only sugar is added to the formulation. Hence there is a need for another cryoprotecting agent i.e. proteins and polypeptides which can together provide better drug retention, less aggregation upon reconstitution, and practically no fusion. The cryoprotecting nature of polypeptides and proteins can be explained by their ability to coat the surface of nanocarriers (15). Polypeptides and proteins prevent fusing and aggregation of PLHNCs, as they resist the close juxtaposition of the outer lipid surface of PLHNCs, which occurs during the primary drying and freezing

steps. Albumin with trehalose revealed the most favorable results in terms of the stability (Sf/Si ratio less than 1.3) and percentage drug retention as seen in Table 6-4.

From the results, it was concluded that trehalose with albumin delivered the maximum drug retained in PLHNCs and acceptable Sf/Si ratio. The optimum phospholipid to trehalose ratio of 1:6 along with phospholipid to albumin ratio of 1:1 was found to be optimized lyophilized formulation with PDR of 89.8 ± 1.8 and 94.6 ± 1.8 for PLHNC1 and PLHNC2, respectively. Integrity of the shRNA pDNA possess notewothy importance after the lyophilization process. Hence, D-sh-PLHNCs were developed once the lyophilization process and formulation parameters were optimized with lipid to trehalose ratio 1:6 and lipid to albumin ratio 1:1. The developed D-sh-PLHNCs shows shRNA pDNA integrity of 98.1 ± 1.2 along with % complexation of 95.2 ± 1.6. The combination of minimum one sugar along with one protein/polypeptide provide the eminent results compared to sugar alone due to bioprotective nature of protein and polypeptides. Both formulations were physiochemically stable with no aggregation and/or fusion, which could be confirmed by the Sf/Si ratio of less than 1.3 as shown in Table 6-4.

Table 6-4 Optimization of cryoprotectant

Variable used	**Percentage drug retained of PLHNC Optimized batch 1**	**Sf/Si ratio**	**Percentage drug retained of PLHNC Optimized batch 2**	**Sf/Si ratio**
Choice of cryoprotectant (Mass ratio 1:2)				
Lactose	48.2 ± 1.8	1.3 ± 0.2	50.4 ± 1.5	1.4 ± 0.1
Maltose	46.5± 1.2	2.4 ± 0.4	49.7± 1.4	2.5 ± 0.2
Sucrose	58.7 ± 2.3	1.4 ± 0.1	59.6 ± 2.3	1.3 ± 0.6
Dextrose	52.4 ± 1.0	1.8 ± 0.4	56.5 ± 2.4	1.7 ± 0.1
Trehalose	**68.9 ± 1.2**	**1.1 ± 0.2**	**72.1 ± 1.3**	**1.1 ± 0.1**
Choice of protein/polypeptide (Mass ratio 1:1)				
Gelatin	69.2 ± 1.3	2.5 ± 0.5	72.9 ± 2.1	2.4 ± 0.3
Glycine	71.5 ± 2.6	1.4 ± 0.3	73.1 ± 1.6	1.4 ± 0.2
Albumin	**76.9 ± 1.8**	**1.2 ± 0.1**	**78.6 ± 2.3**	**1.1 ± 0.2**
Casein	Precipitation in formulation			
Lipid to sugar mass ratio (protein ratio 1:1)				
1:2	80.6 ± 1.5	1.1 ± 0.2	81.6 ± 1.2	1.1 ± 0.1
1:4	83.5 ± 1.7	1.1 ± 0.1	83.7 ± 1.3	1.1 ± 0.1
1:6	**89.8 ± 1.2**	**1.1 ± 0.1**	**94.6 ± 1.8**	**1.2 ± 0.1**
1:8	90.1 ± 1.1	1.2 ± 0.2	94.8 ± 2.1	1.2 ± 0.4
1:10	91.6 ± 1.3	2.4 ± 0.4	95.4 ± 2.0	1.3 ± 0.3
Lipid to protein ratio (Sugar ratio 1:6)				
1:0.5	76.2 ± 1.4	1.1 ± 0.1	82.6 ± 1.9	1.1 ± 0.2

1:1	**89.5 ± 1.1**	**1.1 ± 0.1**	**94.6 ± 2.0**	**1.2 ± 0.1**
1:1.5	89.1 ± 1.2	2.1 ± 0.5	94.8 ± 2.3	2.3 ± 0.5
1:2	91.8 ± 1.9	2.2 ± 0.6	95.6 ± 2.6	2.6 ± 0.6

6.6.2 Water content analysis

The moisture content values for both optimized batch of PLHNCs were 2.0 and 1.90 % which was within acceptable limit (not more than 3%). Hence both formulations were selected for further optimization of dry powder for inhalation.

Table 6-5 Determination of moisture content of lyophilized samples

Sr no.	Sample Name	% Water content
1	PLHNC optimized batch 1	2.00
2	PLHNC optimized batch 2	1.90

6.6.3 Cryo TEM and freeze-fracture TEM studies

The PLHNCs morphology was confirmed by cryo-TEM. All the nanocarriers were found to be spherical in shape, monodisperse, and possessed a smooth surface. Very interestingly, the cryo-TEM signature of a flat lipid domain over a thick polymer core of PEG-PCL was visualized in all PLHNCs. Images in Figure 6-1 revealed that the particles were possessing unilamellarity on the outer surface along with a matrix-type system at the core of the particle, which was examined by zooming the image. The size of the particles ranged between 100-200 nm, which supports the enhanced permeation and retention effect to assist the PLHNCs into a tumor vasculature. The diffuse ring over the dark shadow obtained by the diffraction of the electron pattern justified the lipid layer over the polymer matrix. Another image in the box has shown the matrix structure of the polymer in the lipid coat. A sharp single flow type pattern was visible in freeze fracture TEM studies proved the unilamellarity of the outer lipid layer. A sharp single flow pattern avoids the probability of multilamellar outer lipid coat and confirms the presence of unilamellar structure over the polymeric core.

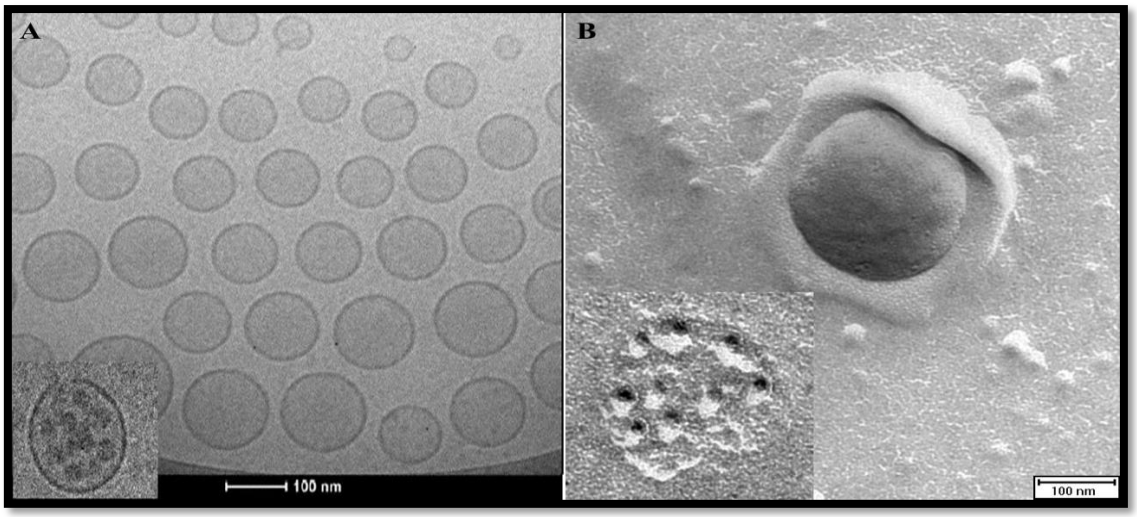

Figure 6-1 A) Cryo-TEM of lyophilized PLHNCs (B) freeze fracture TEM of lyophilized PLHNCs.

6.6.4 Optimization of Dry powder formulation

Various grades of coarse and fine lactose have been used to achieve high respiratory fraction from the developed dry powder formulation as shown in Table 6-6. Coarse lactose (Respitose SV003) in a ratio of 1:6 has shown a fine particle fraction of only ~48 %. Hence, it was necessary to add fine lactose with it. The addition of 15 % fine lactose (Lactohale LH 300) with Respitose SV003 increased the fine particle fraction up to ~68%, which was maximum. The FPF ratios of the developed PLHNCs DPI was 2.25 and 2.40 and the ED ratios were 1.84 and 1.88 for PLHNCs1 and PLHNCs2 DPI respectively. The fine lactose particles were tightly bound with the strongest bounding site of the coarse lactose and left loosely binding site for the PLHNCs molecules (16). As a result, during the aerosolization, PLHNCs particles were easily detached from the loosely binding sites and generated a higher portion of respirable aerosol which accounted for the higher FPF.

Table 6-6 Optimization of Dry powder formulation. (Control sample: Ashthalin by Cipla Ltd., India. delivery device: Ciplahaler by Cipla Ltd., India FPF=28.4±1.6; ED=52.1±1.8.)

Variable studied	Grade of lactose	Emitted dose for PLHNC1 (%)	FPF for PLHNC1 (%)	Emitted dose for PLHNC2 (%)	FPF for PLHNC2 (%)
Lyophilized dry powder: Coarse	Lactohale 200	41.8 ± 1.8	25.6 ± 2.1	40.6 ± 2.1	25.8 ± 3.4
	Lactohale ML 001	48.2 ± 2.1	28.5 ± 3.6	50.0 ± 4.3	27.4 ± 2.9
	Inhhalac 200	42.6 ± 3.5	21.4 ± 4.1	43.1 ± 3.9	21.1 ± 2.1
	Respitose SV003	58.2 ± 19	35.6 ± 2.2	62.5 ± 3.1	35.5 ± 3.2

lactose (w:w) (1:2)					
Fine lactose (5 % w/w of coarse lactose)	Lactohale LH 230	69.5 ± 2.1	36.5 ± 3.9	68.1 ± 2.9	38.1 ± 2.7
	Lactohale LH 300	72.6 ± 1.9	41.2 ± 2.1	76.2 ± 2.8	45.2 ± 1.6
	Respitose ML 006	73.5 ± 3.2	29.5 ± 4.6	73.2 ± 4.5	29.1 ± 1.8
Effect of PLHNCs to lactose ratio (Lactohale LH 5 %)					
Respitose SV 003	1:2	72.6 ± 1.2	41.2 ± 2.6	71.2 ± 4.8	42.5 ± 2.9
	1:4	81.9 ± 3.6	42.6 ± 3.1	83.2 ± 1.6	42.6 ± 3.7
	1:6	95.6 ± 2.1	48.5 ± 2.9	96.1 ± 3.8	47.5 ± 2.8
	1:8	96.1 ± 1.9	49.2 ± 3.9	96.9 ± 4.6	49.5 ± 3.1
	1:10	96.8 ± 1.1	49.5 ± 2.1	96.9 ± 3.9	50.6 ± 4.5
Effect of % of Fine lactose mixed with Respitose SV003.,					
Lactohale LH 300	5 %	95.6 ± 2.1	48.2 ± 4.1	96.1 ± 2.1	50.3 ± 4.9
	10 %	95.4 ± 2.8	55.6 ± 3.7	95.6 ± 3.9	51.6 ± 2.5
	15 %	96.3 ± 1.7	63.9 ± 2.1	98.3 ± 3.1	68.3 ± 2.5
	20 %	97.1 ± 2.7	65.2 ± 4.2	98.1 ± 3.5	67.9 ± 5.1

D-sh-PLHNCs were prepared using the respitose SV003 (1:6) and lactohale LH 300 to identifiy the integrity and % complexation of shRNA pDNA. The developed D-sh-PLHNCs shows shRNA pDNA integrity of 97.9 ± 1.5 along with % complexation of 95.3 ± 1.2 after the formuation of dry powder inhaler.

6.6.5 Evaluation of dry powder for inhalation

As compared to PLHNC1, the PLHNC2 dry powder has shown good flowability and foldability as described in point score evaluation by Carr in1964 (17) for the angle of repose, Carr's index, and Hausner ratio which is shown in Table 6-7. The moisture content (by Karl Fischer titration) of both the formulations has been found below 1.5%. FPF of the PLHNC2 dry powder was higher, when compared to PLHNC1 dry powder, suggesting a higher amount of drug has been residing in lower stage and plate (stage/plate 3 to 7) of cascade impactor (As seen in Figure 6-2). The effective index of PLHNC2 (78.66 ± 1.8) dry powder formulation has been found better compared to PLHNC1 (74.90 ± 2.1) dry powder formulation, which implies effective deposition of the Docetaxel PLHNC2 into lungs. Turboelectrification, as well as charge generation during dispersion (by Ciplahaler) is responsible for the higher lung deposition and lesser device fraction. The lower effective index of the PLHNC1 has suggested that a higher proportion of the dispersed PLHNCs dry powder was deposited in the upper respiratory tract.

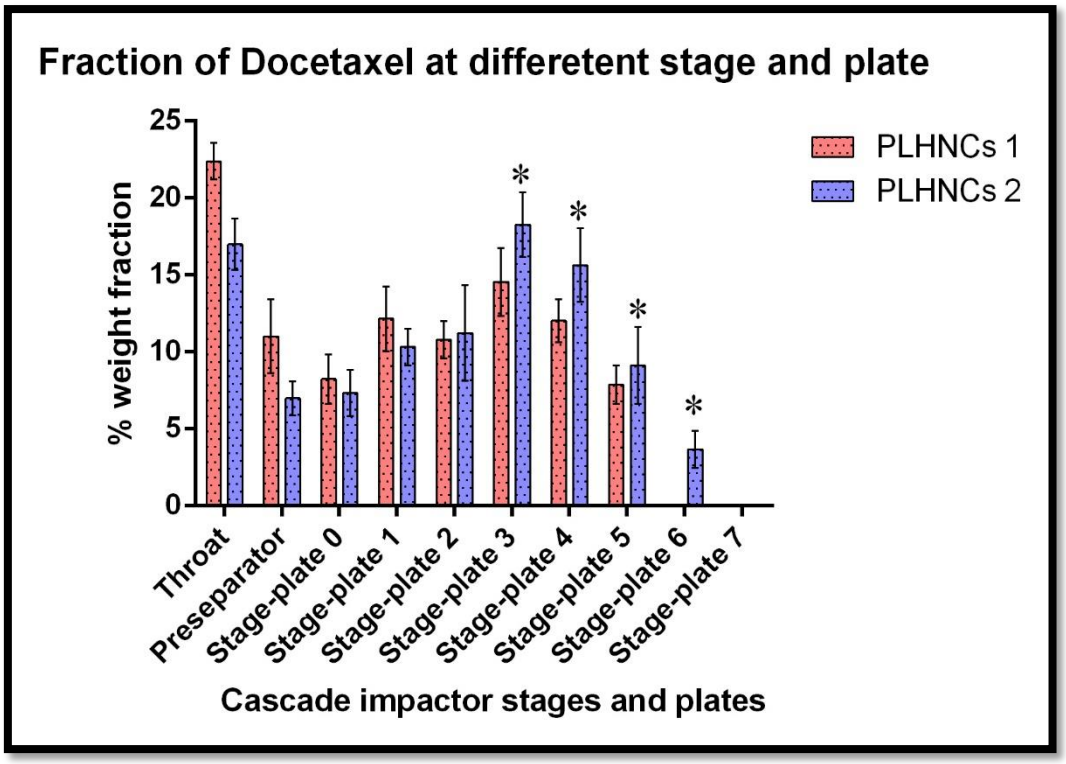

Figure 6-2 Weight fraction of Docetaxel at different stages and plates in Anderson cascade impactor.

*** shows a significant increase in Docetaxel fraction at the respirable range of particle size**.

Table 6-7 Evaluation of PLHNC dry powder for inhalation (N=3)

Variable studied	PLHNC 1 dry powder	PLHNC 2 dry powder
Mean particle size (d50)	5.95 ± 2.1	3.78 ± 2.5
Angle of repose (θ)	25.8 ± 2.8	28.7 ± 1.6
carr's Index,	16.28 ± 1.6	15.28 ± 1.7
Hausner ratio	1.62 ± 0.12	1.18 ± 0.10
Moisture content (%)	1.4 ± 0.2	1.2 ± 0.1
Mass median aerodynamic diameter (MMAD),	4.95 ± 0.36	3.56 ± 0.21
Geometric Standard Deviation (GSD).	3.16 ± 1.18	2.22 ± 0.26
device fraction (%)	12.2 ± 2.5	9.4 ± 1.9
Fine particle fraction (%)	**63.9 ± 2.1**	**68.3 ± 2.5**
Effective index	74.90 ± 2.1	78.66 ± 1.8

6.6.6 Scanning electron microscopy

The aerosolization performance of the dry powder inhalers is largely dependent on the size and shape of the particles. In Figure 6-3, maximum ferret diameter (length) is almost twice the minimum ferret diameter (width). Hence, it can be concluded that the elongation ratio is more than 2 for the developed DPI of PLHNCs. A higher elongation ratio is directly responsible for the higher delivery of DPI to the respiratory region, which is associated with higher fine particle fraction (18). Tomahawk-shaped particles have a higher elongation ratio resulting in the higher drug deposition in small airways which is required for the anti-cancer drug. However, size and morphology of the PLHNCs have opposite effects i.e., increase in the size of the nanocarriers have little effect on the emitted dose, while it has a significant effect on the fine particle fraction. On contrary, the sparse surface of dry powder has little effect on emitted dose, but it can improve the fine particle fraction.

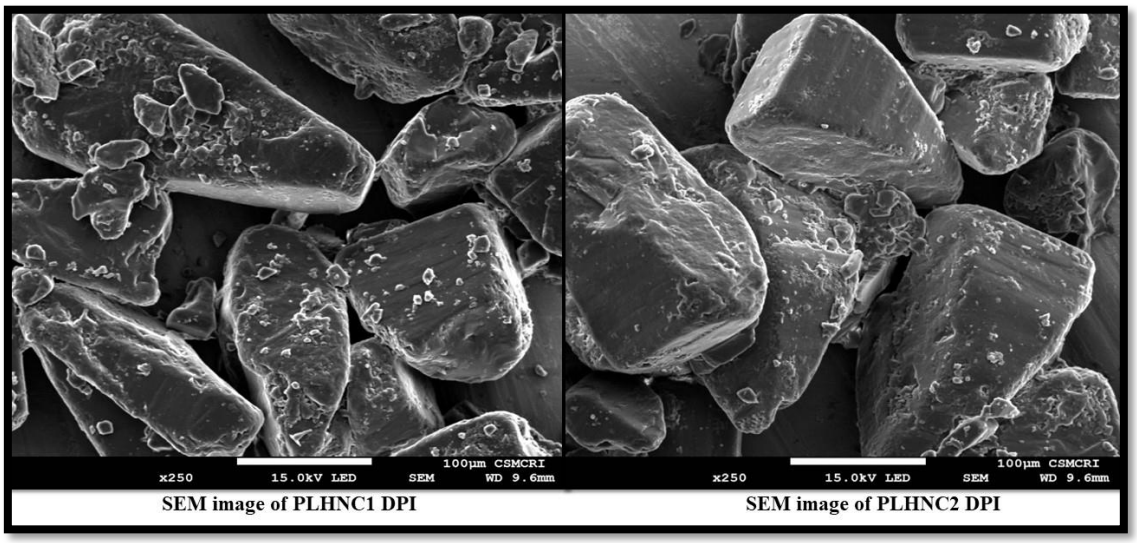

Figure 6-3 SEM images of developed PLHNCs

6.6.7 Powder X-ray diffraction (pXRD)

The pXRD findings indicate that the composition of dry powder was crystalline in nature, which may be attributed to trehalose during lyophilization and respitose powder processing. The presence of crystallinity is suitable for agglomeration and adsorption onto the surface of carrier materials (As shown in Figure 6-4). Crystallinity is also helpful during shelf storage for improved flow dynamics and better chemical stability. When intensity of 100% relative peak was compared between Drug and DPI formulation PLHNCs, it showed that DPI formulation has the highest intensity. The 100% peak of the DPI is almost 10 times higher than drug's highest intensity which shows higher crystallinity of the DPI compared to Docetaxel and proves the importance of powder processing in pulmonary delivery. The presence of crystallinity is

suitable for agglomeration and adsorption of PLHNCs onto the surface of carrier materials. Amorphous regions have higher energy for surface adhesion than crystalline regions, contributing to weak de-aggregation after air stream fluidization. In comparison, during patient inspiration, crystalline regions interact weakly with and are readily overcome by turbulent shear. The presence of the coarse and fine grade of lactose also accounts for the crystallinity which accelerates the adsorption of the PLHNCs on the carrier surface and loose agglomeration

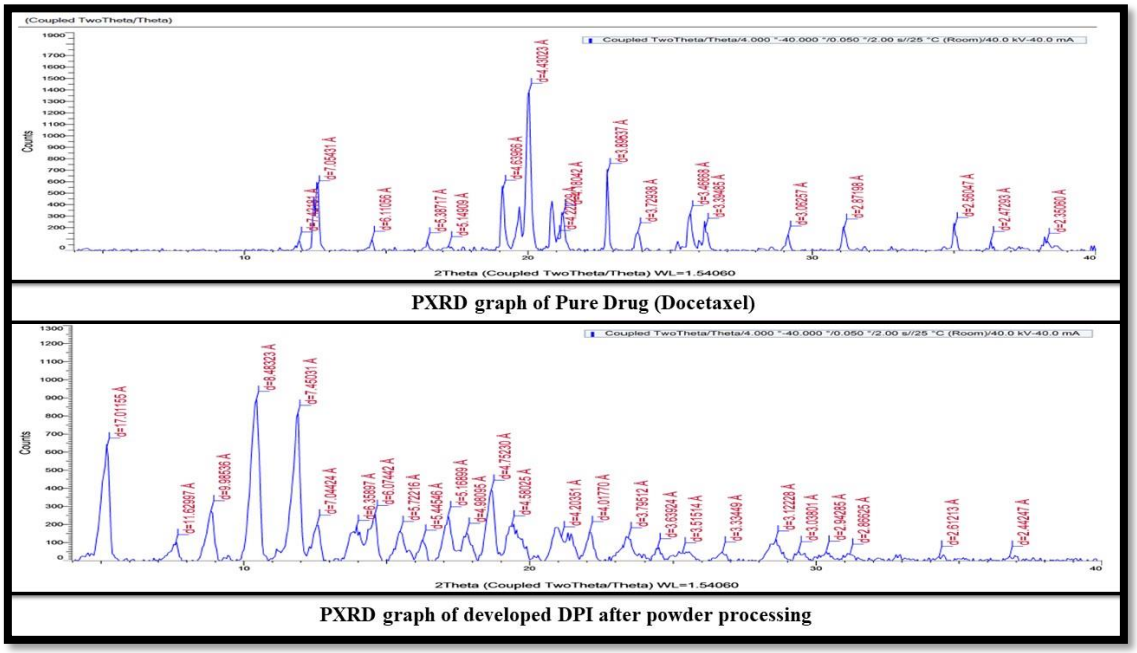

Figure 6-4 Comparison of PXRD graph of Docetaxel and PLHNC DPI formulation

6.6.8 DSC

The DSC thermograms of Docetaxel and D-PLHNCs DPI are shown in Figure 6-5. The DSC thermogram of Docetaxel showed sharp endothermic peak at 179°C indicating its melting point. The DSC thermogram of D-PLHNCs DPI did not show any sharp peaks at 179°C indicating no free crystalline drug was present over the surface of the dry powder. The endothermic peak present between range of 110 to 120 °C in DPI formulation was due to melting of lactose monohydrate present in the Lactohale LH 230 and Respitose SV003.

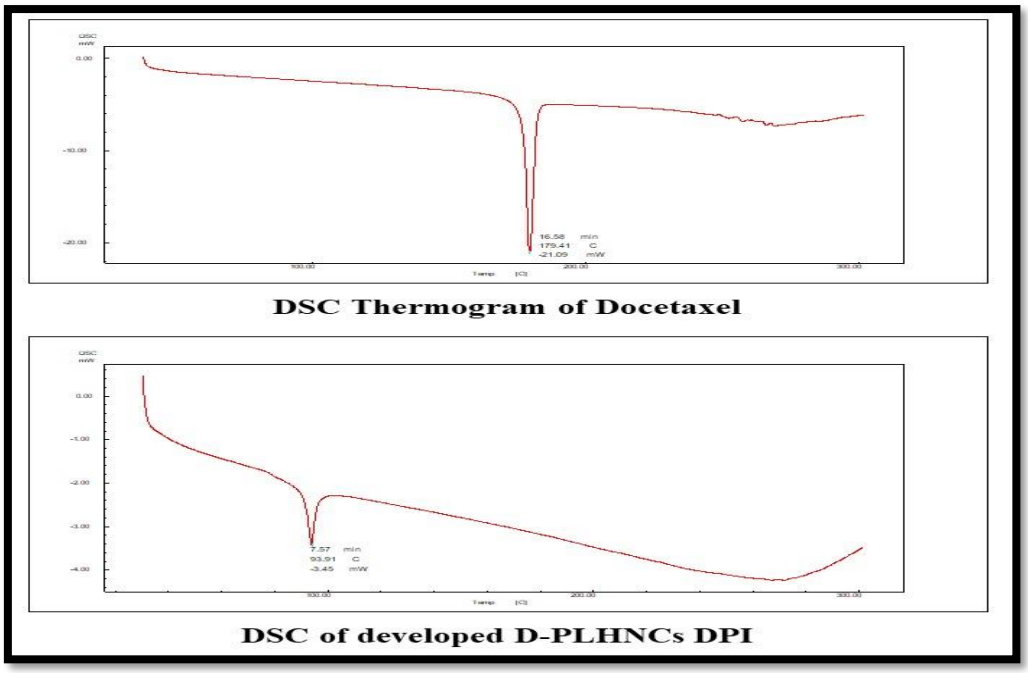

Figure 6-5 DSC graph comparison of Docetaxel and D-PLHNCs DPI

6.6.9 FT-IR

Figure 6-6 exhibited characteristic peaks at 3377.50 cm^{-1} for OH stretching, 3487.00 cm^{-1} for NH stretching, 1452.57 cm^{-1} for C=C stretching, 1711.85 cm^{-1} for C=O stretching, and 2982.00 cm^{-1} for CH Stretching Vibrations. All these peaks are considered characteristic to Docetaxel and are prominently observed. Whereas, in case of DPI, all characteristic peaks pertaining to docetaxel were absent, which proves encapsulation of Docetaxel in the PLHNCs.

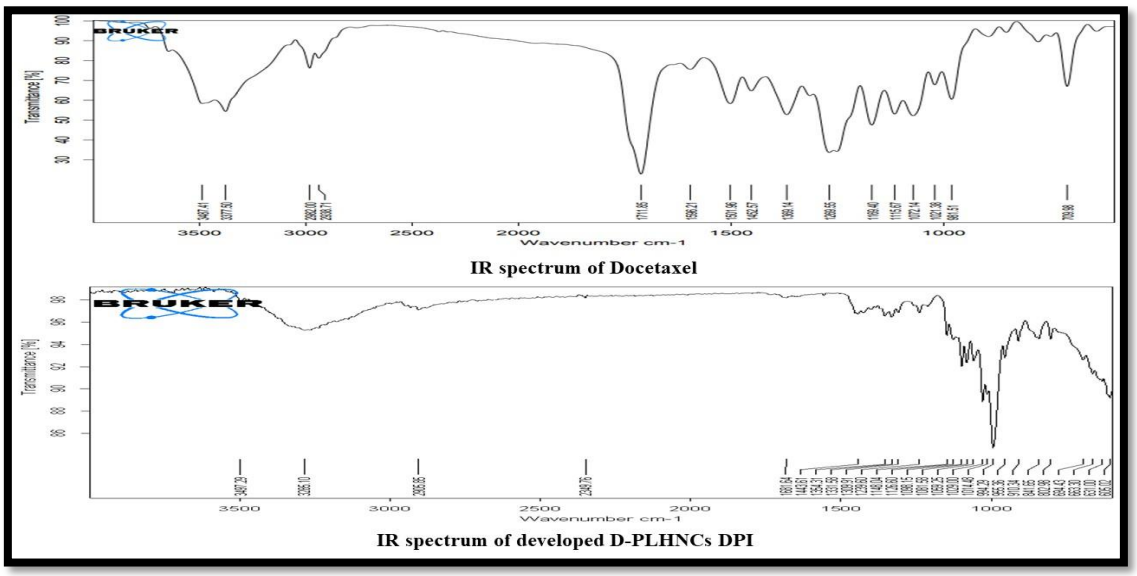

Figure 6-6 IR graph comparison of Docetaxel and developed D-PLHNCs DPI

6.6.10 Integrity of shRNA pDNA after development of DPI

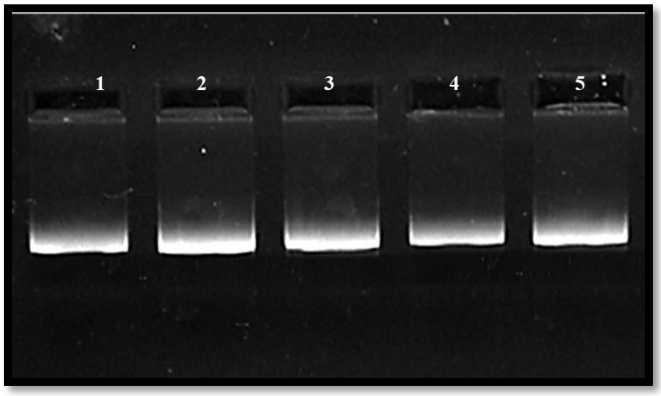

Figure 6-7 Integrity of shRNA pDNA

Lane 1: naked shRNA pDNA, Lane 2: shRNA pDNA band after lyophilization of PLHNC1, Lane 3: shRNA pDNA band after powder processing (DPI) of PLHNC1, Lane 4: shRNA pDNA band after lyophilization of PLHNC2, Lane 5: shRNA pDNA band after powder processing (DPI) of PLHNC2

From results (As shown in Figure 6-7) of band density obtained by gel electrophoresis, it can be concluded that the developed DPI formulations of PLHNCs retained the integrity of the shRNA pDNA after the lyophilization and powder processing as band density of both formulations were remained similar after the lyophilization as well as dry powder processing. Even results of Table 6-8 confirms that more than 95 % ABCB1 shRNA pDNA remained intact after the process of lyophilization and powder processing.

Table 6-8 Relative band density of shRNA pDNA with PLHNCs after Lyophilization and powder processing

Formulation	Relative band density		
	Before lyophilization	**After Lyophilization**	**After powder processing (DPI)**
PLHNC1	**0.984 ± 0.092**	**0.978 ± 0.081**	**0.972 ± 0.074**
PLHNC2	**1.054 ± 0.039**	**1.035 ± 0.046**	**1.032 ± 0.042**

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