Chapter 7:

Development of Dry Powder for Inhalation

7.1 Introduction

Therapeutic siRNA has been tried in clinical trials in various other diseases including cancers which proves there clinical application. However; in case of lung diseases like CF no clinical trials with siRNA have been started yet. The potential reason restricting the clinical application is the unavailability of the suitable local administration technique, preferably inhalational route [1]. The inhalational route is highly convenient and patient compliant and it allows one to widely distribute the therapeutics agent along the airways. Further, it gives localized action and reduces the systemic side-effects, as opposed to the case of amiloride therapy [2]. These combined attributes indicate that inhaled siRNA therapeutic targeting Na^+ channel, has the potential to show a better therapeutic index and significantly improved duration of action than small molecule inhibitors and appears to be most relevant from future clinical application in treatment of CF. The powder based DPI formulations offer many advantages such as improved stability, low drug loss during administration, improved handling and portability and efficient drug delivery to the pulmonary targeted region [3]. Therefore, the development of stable dry powder aerosols for pulmonary gene therapy would be of great benefit.

Lyophilization has been the most common method for converting thermo labile bioactive materials such as proteins and gene vector complexes into powders. Various studies have demonstrated freeze drying of gene delivery vectors such as polyethyoleneimine [4]. Seville et al. have performed comparison of Dry powder prepared by lyophilization and spray drying [5]. They reported the only drawback of the lyophilization as poor respirability of such powder when not sufficiently processed, while spray drying produces particles with good aerodynamic properties. Yadava et al. have studied the effect of lyophilization on the bioactivity of siRNA-lipoplexes and they reported that the lyophilization in presence of non-ionic cry-protectant could be lyophilized and reconstituted without loss of transfection efficacy. It has no effect on intrinsic biological activity and lyophilized siRNA retains functionality. Corinna et al. studied the dry powder aerosols of polyethylenimine (PEI)-based gene vectors prepared by lyophilization and powderization in the presence of lyoprotectants and found that they were well tolerated. The choice of lyoprotectant and subsequent lyophilization and/or powderization did not affect the in vitro transfection efficiency. Even *in vivo* screening of

powderized samples in the form of insufflation showed highest gene expression in case of lactose [6].

The freezing step of lyophilization is operated through cooling of shelves through the glass container in contact. The conduction, convection and radiation are the normal heat transfer modes during lyophilization. The sample has to be cooled from ambient conditions to sub-freezing temperature. During cooling first ice-nucleation occurs several degrees below equilibrium point of sample which is known as "supercooling". Subsequent to nucleation ice starts to grow and leads to freeze concentration of the sample consisting of two phase i.e. ice and freeze concentrated solution [7]. The composition of such sample can be obtained by equilibrium freezing-curve of water in the presence of solute. In case of crystalline solutes, the solute crystallization occurs when temperature falls below the eutectic point.

The cryoprotectant play an important part in maintaining the characteristics of the lyophilized polyplex or lipoplex. The aggregation of non-viral vectors is proposed to occur during the freezing step of lyophilization process, which can be avoided by using suitable concentration of cryoprotectant [8]. The particle isolation hypothesis based on separation of unfrozen particles in the unfrozen matrix has been proposed as probable mechanism of stabilization and there is no role of vitrification induced by polymers. After lyophilization the solid cake needs to be subjected to powderization process involving shear forces to convert them into freely flowable nature. Studies attributed the stabilization effect to pDNA condensation by cationic vectors to minimize the damage caused by shearing forces during powderization.

The powder processing of the lyophilized cakes needs to be performed to convert the poorly flowing mass of solid into a freely flowing, easy to fluidize powder bed which can be dispersed into air stream of respiratory tract in response to the actuation force by the patient during inhalation. Further, literature reports that pDNA condensation induced by cationic agents may minimize damage to pDNA by shear-related forces during powderization [8]. Successful gene delivery to the lungs was reported in several studies using polyplex based gene delivery vectors. Among these cationic agents, bPEI has been most commonly used in gene delivery to the lung, however a systematic developmental

approach involving through in vitro characterization is essential to convert them into practical applications [6].

7.2 Materials

The previously optimized polyplex formulations were used. Sucrose, Trehalose, Mannitol were purchased from Himedia, Mumbai. Respitose SV001 was a kind gift from DFE Pharma, USA. Inhalac 230 was procured from Meggle Pharma, Germany. Rhodamine B was purchased from Himedia, Mumbai.

7.3 Preparation and characterization of DPI

7.3.1 Optimization of lyophilization

The siRNA polyplex were prepared and dispersed in nuclease free water to which different cryo-protectant (mannitol, trehalose, sucrose) were added and filled in Type-1 borosilicate glass. The vials were partially closed with double slotted grey bromo butyl rubber stopper and lyophilized using VerTis advantage plus lyophilizer (SP Scientific, New York, USA). The cycle parameters were selected based on subject knowledge of the matter consisting of optimum freezing rate and subsequent drying temperature below the glass transition of the cryo-protectant, so as to obtain elegant, fluffy cake and retain desired product attributes of particle size, zeta potential, moisture content etc. Due to analytical considerations for siRNA, the aerosol behaviour of developed dry powder was based on the rationale given by Pfeifer et al. [6]. For assessment of aerodynamic behaviour of powder, Rhodamine B at a concentration of 1 nM was added to the solution before lyophilization.

The freezing stage was begun by ramping the shelf temperature to 5 ^{o}C and then holding it for 20 min for equilibrium to minimize supercooling effects in subsequent freezing stage. Then temperature was ramped to -40^{o}C and maintained for sufficient time to ensure complete freezing. During primary drying the temperatures were maintained below the collapse temperature of the bulking agents. Finally, secondary drying was carried out to remove the bound water and achieve desired moisture content by maintaining the cake at 25 ^{o}C. The lyophilized vials were kept at 2-8 ^{o}C until use. The cryo-protectant out of mannitol, trehalose and sucrose was selected by studying effect on particle size, zeta potential and integrity of siRNA as per the procedures in chapter 4.

Thermal Cycle for Freeze Drying

Current Recipe: C:\Virtis\Sushil siRNA.rcw
Recipe Name :

Thermal Treatment Steps

	Temp	Time	Ramp/Hold
Step # 1	5	20	H
Step # 2	-40	120	R
Step # 3	-40	180	H
Step # 4	0	0	R
Step # 5	0	0	H
Step # 6	0	0	H
Step # 7	0	0	H
Step # 8	0	0	H
Step # 9	0	0	H
Step # 10	0	0	H
Step # 11	0	0	H
Step # 12	0	0	H

Freeze Temp	-40 °C
Additional Freeze	0 min
Condenser Setpoint	-60 °C
Vacuum Setpoint	100 mTorr

Primary Drying Steps

	Temp	Time	Vac	Ramp/Hold
Step # 1	-30	120	100	H
Step # 2	-15	120	100	R
Step # 3	-15	240	100	H
Step # 4	-5	120	100	R
Step # 5	-5	240	100	H
Step # 6	10	60	100	R
Step # 7	10	300	100	H
Step # 8	20	60	100	R
Step # 9	20	360	100	H
Step # 10	0	0	0	R
Step # 11	0	0	0	H
Step # 12	0	0	0	R
Step # 13	0	0	0	H
Step # 14	0	0	0	R
Step # 15	0	0	0	H
Step # 16	0	0	0	R
Post Heat	25	900	100	

7.3.2 Moisture content

The moisture content in Lyophilized bulk and DPI formulations was determined using Karl-Fischer titration. The pyridine free reagent available commercially was standardized by titrating with known quantity of water (250 mg). 40 mL of anhydrous methanol was titrated with reagent to correct for residual water in methanol. After this sample was added and water content was determined by titrimetric procedure.

7.3.3 Powder processing and preparation of DPI

The lyophilized bulk powder was passed through 120# and 240# to convert it to fine size. The particle size was evaluated over Malvern Mastersizer 2000, Malvern UK. The obtained fine powder was blended with different inhalational carriers such as inhalac 230 and respitose SV001 at different weight ratios of lyophilized bulk/carrier, ranging from 1/1 to 1/6 to improve the dispersibility of the powder during inhalation process. The dry powder formulation was filled in capsule size 3. The lyophilized bulk was processed as described above and filled in hard gelatin capsule size 3 to study aerodynamic behaviour.

7.3.4 In-vitro deposition studies

The *in-vitro* deposition resulting from the aerodynamic characteristics of the dry powder formulations was assessed by using Andersen cascade impactor (ACI) of Copley Scientific. The size 3 capsule filled with processed powder containing rhodamine B were actuated using Aerolizer as a dispersing device. The flow rate was kept to achieve pressure drop of 4 kPa, so as to mimic normal inspiratory force of patient. The apparatus was operated at a flow rate of 60 L/min for 4 sec so that volume of 4 L was drawn thought the inhlaler. The content of rhodamine was determined using developed analytical method.

7.3.5 Spectrofluorometric estimation of rhodamine B

A calibration curve was prepared for rhodamine B in saline solution at a concentration of 20 ng/mL to 200 ng/mL using spectrofluorometer. The relative fluorescence units were measured at each concentration with excitation wavelength of 553 nm and emission was measured at wavelength of 576 nm. The method was validated for accuracy, specificity, freedom from any interference from powder excipients.

7.3.6 Aerodynamic particle size

ACI has been the traditional choice for classification of particles based on the aerodynamic size. The ACI, typically consists of classification stages consisting of nozzle and an impaction plate. The aerosol stream passes through each stage via nozzle and impacts on the plate. The large inertial particles impact on the plate while smaller particles stay entrained in air stream and pass onto the next stage. The ACI designs each successive stages to impart higher velocity in nozzle leading to collection of smaller diameter in successive stages. The every stage is characterized by cut-off size for unit density sphere at the given flow rate, i.e. change in flow rate shifts the cut-off size to larger or smaller diameters [9]. The sharpness of the collection efficiency curve for given cut-off size in different impactor designed is function of Reynolds number which is generally in the range of 500-3000 to get sharp values.

The respirable fraction, Fine Particle Fraction (FPF), was defined as particle mass below 5 um. The larger particle fraction or carriers particles would settle in the oropharynx while very small particles are exhaled before undergoing lung deposition. The Aerolizer was primed by connecting with the induction port. Flow meter was installed downstream to impactor and negative pressure was applied to achieve desired flow rate. The ACI was operated at flow rate of 60 L/min and stability was ensured throughout operation. The capsule containing DPI was loaded into the Aerolizer and connected to the induction port and a total of 5 capsules, meeting the requirements of analytical sensitivity, were fired at selected flow rate. At the end the assembly was dismantled and samples were collected by rinsing the walls and collection plate with saline. The fractions collected were analysed by spectrofluorometry to calculate:

1. Emitted dose (ED): the amount of dose emitted from the capsule through the inhlation device in to the apparatus.
2. Fine powder fraction (FPF): as the fraction of dose found below cut-off diameter $< 4.7\ \mu$.
3. Recovered dose (RD): The total amount of dose recovered from the inhalers, capsule shell and the apparatus as % of average assay.

4. Mass Median Aerodynamic Diameter (MMAD): The diameter at which 50% mass of particles are larger and 50% smaller. It was determined graphically from the plot of cumulative % mass less than given size on probability scale to log of aerodynamic size. The MMAD is the value of intersect at 50% cumulative percent.
5. Geometric Standard Deviation (GSD): The GSD was obtained as slope of line or using the formula,

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

Where, d_{84} and d_{16} are diameters corresponding to 84% and 16% undersize mass, respectively.

7.3.7 Scanning electron microscopy

In order to get insight into the surface properties and powder characteristics of the prepared blend the particle were subjected to electron microscopy. For this purpose, samples (1-2 mg) were put on double sided adhesive tape attached to the aluminium stub. Then it was exposed to the SEM under 15 KV accelerating voltage. The photographs were taken using Jeol JSM-5610LV (Japan) scanning electron microscope.

7.3.8 X-ray diffraction (XRD)

The XRD studies were performed to study the crystalline behaviour of the powder, since the degree of crystallinity and amorphous nature may influence the powder behaviour such as adhesive cohesive interactions, flow and fluidization properties, hygroscopicity etc. The powder X-ray diffraction pattern was collected from 2-Theta values ranging from 5 to 55.

7.3.9 Integrity of siRNA

The Integrity of siRNA after conversion to powder form for inhalation was determined by procedure described in chapter 4.

7.3.10 Animal studies

All experiments and protocol described in the present study were approved by the Institutional Animal Ethical Committee (IAEC) of Pharmacy Department, The M. S.

University of Baroda and with permission from committee for the purpose of control and supervision of experiments on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

7.3.10.1 Animals:

Female, Sprague–Dawley rats weighing between 200-350 g were used. The female sex was chosen because of their more sensitivity to such studies [10]. The animals were housed two per cage in air conditioned room maintained at 20-25 ^{o}C and illuminated with artificial light cycling at 12 h light and 12 h dark. They were supplied with water and commercial rodent diet. The animals were chosen for the experiment randomly. They were acclimatized to the laboratory conditions for 5-7 days before the dosing or start of the experiment.

7.3.10.2 Administration of doses:

The animals were fasted 12 h before dosing to avoid the effect of diet. Prior to dosing the animals were weighed and doses were calculated based on body weight. Further, rats were anaesthetized by intraperitoneal injection of xylazine (5 mg/kg) and ketamine (50 mg/kg). Trachea was surgically exposed on the ventral side of the neck of the rat and tracheal puncture was performed by a needle just below the larynx [11]. Animals were dosed with formulation of 1) saline, 2) LPS, 3) LPEI 4) HELPEI-35, 5) LMWC-29-PS-12. Lipopolysaccharide (LPS, 0.1 µg/ml) was used as positive control. Rats were placed in dorsal recumbency during recovery form anaesthesia to ensure penetration into lower airways and lungs. After complete dosing the animals were withheld from food for 1-2 h. The dosing of each carrier was chosen based on the amount required for delivery of therapeutic concentration of siRNA i.e. 3 nmoles/kg.

7.3.10.3 Clinical observations

After instillation animals were closely monitored for any changes in skin or fur, behaviour patterns, tremors, convulsions, diarrhoea, sleep, salivation, coma etc. Respiratory distress signs like dyspnea, bradypnea, apnea or hyperpnoea, etc. were checked if present.

7.3.10.4 Bronchoalveolar fluid examination

After 24 h of administration, animals were euthanized by an overdose of intraperitoneal injection of pentobartital (75 mg/mL). Lungs were surgically removed, cleaned, and weighed to check edema formation. The trachea was exposed and cannulated with a 20-gauge catheter. The lungs were then lavaged with 5 mL normal saline instilled through the trachea and collected after 30 sec. The obtained BAL fluid was centrifuged at 1500 rpm for 10 min and then the supernatant was stored at -20°C. The enzymatic activities of lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) in BAL fluid was determined by using commercial kits for assay of LDH and ALP (Abcam, Mumbai, India) and the enzyme levels were reported as fold increase compared with saline treated groups. The lung weights were reported as g/100 g body weight.

7.3.10.5 Histopathological examination of lung

After recovery of BAL fluid, the lungs were inflated with 2 ml PBS. Lung tissue was excised from the whole lung, fixed with 10% formalin, and embedded in paraffin. Various paraffin sections (1-3 µm thick) were cut using microtome (MICROM) and mounted on slide and stained with hematoxylin and eosin. Then sections were observed under phase contrast microscope (Nikon Corporation, Japan) and photographs were taken using NIS-Elements software. Sections were evaluated for the presence of inflammatory reactions, musculinization of arteries etc.

7.3.11 Stability studies

Dry powder formulations of polyplexes were evaluated for stability as per ICH guidelines. Briefly, 20 mg of formulations were filled in hard gelatin capsules kept in tightly closed HDPE container. At different time points, formulations were reconstituted with nuclease free water and evaluated for complexation efficiency, particle size, and water content.

7.3.12 Statistical analysis

Experiments were performed in triplicate. Unless stated, data are expressed as the mean ± standard deviation (SD). The statistical significance of the results was determined using a Student's t-test where $p<0.05$ denotes significant difference.

7.4 Result and Discussion

7.4.1 Optimization of lyophilization

The siRNA polylex were converted into powder formulation using lyophilization. The process consists of freezing the sample and then removing the water by subliming or desorption through vacuum. The stages are categorized into freezing, primary drying and secondary drying. However, first step of freezing the sample into a solid form is most determinant of the integrity of the final product as it impacts the type and size of formed ice crystals, the way of mass transfer during primary and secondary drying [12]. It is also said to be the most aggressive step of lyophilisation responsible for size growth, aggregation, product concentration etc. Therefore, for successful lyophilisation the design of lyo-cycle is important aspect for maintaining product features. There are two types of freezing behaviours. The liquid phase suddenly solidifies at eutectic point, depending on the type of solids in the solution or liquid phase forms a glassy state (does not solidifies), and becomes more and more viscous to form a stiff mass [13].

The Fig. 7.1, shows the effect of freezing rate on super-cooling and nucleation. The larger area of exothermic curve indicates formation of larger ice crystals. Therefore, cooling rate was kept optimum to avoid formation of either very large ice crystals when frozen slowly or formation of very small crystals due to rapid cooling. During freezing, sensible heat is first removed to lower the temperature to the freezing point. The gradual decrease in temperature with time will continue until reaching the eutectic temperatures for major product components.

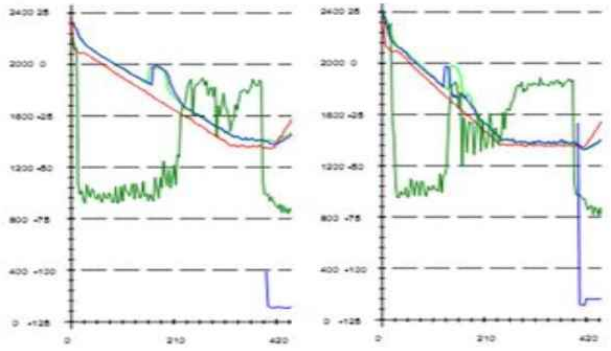

Fig. 7.1: Effect of freezing rate on super cooling

The formulations characteristic should be retained after stressful processing of lyophilisation. Therefore, after lyophilzation the formulations were tested for particle size, zeta potential and physical appearance on hydration of cake. The Table 7.1 and Table 7.2 shows the effect of lyophilization on the physicochemical properties. It was observed that, lyo-cycle selected resulted in aesthetic appearance of cake with good redispersibility. At the end of the cycle the water content was < 2% w/w in all the cases. All the selected cryoprotectants: sucrose, mannitol and trehalose were able to retain particle size of siRNA formulations. The zeta potential values where unaffected after reconstitution. In both the cases, i.e. HELPEI-35 and LMWC-29-PS-12 (i.e. stabilized LMWC-29-PS-12) there was no significant effect on size and zeta potential of the formulations.

Table 7.1: Optimization of lyophilization for HELPEI-35

Type of Cryoprotectant	Moisture Content (%w/w)	Before Lyophilization		After Lyophilization	
		Particle Size (nm)	Zeta Potential (mV)	Particle Size (nm)	Zeta Potential (mV)
Sucrose	1.97	152.3 ±4.2	20.6±3.6	162.2±3.7	21.35±2.9
Trehalose	1.57			158.6±2.2	20.66±2.6
Mannitol	1.88			160.5±2.8	21.31±1.7

*Values represented as mean±SD (n= 3)

Table 7.2: Optimization of lyophilization for LMWC-29-PS-12

Type of Cryoprotectant	Moisture Content (%w/w)	Before Lyophilization		After Lyophilization	
		Particle Size (nm)	Zeta Potential (mV)	Particle Size (nm)	Zeta Potential (mV)
Sucrose	1.7	147.2 ±2.8	13.85±2.3	154.7±4.1	13.75±1.7
Trehalose	1.9			153.2±3.3	14.38±2.3
Mannitol	2.11			153.4±3.8	14.12±2.2

*Values represented as mean±SD (n= 3)

Fig. 7.2: Optimization of lyophilization for HELPEI-35 polyplexes: 1) mannitol, 2) trehalose, 3) sucrose; LMWC-29-PS-12 polyplexes: 4) mannitol, 5) trehalose, 6) sucrose

Fig. 7.2 shows the physical appearance of lyophilized cakes. Although all the cryoprotectants were successfully lyophilized to yield an elegant dry cake, there were differences in physical nature of cake. The cake from sucrose showed shrinkage. On the other hand trehalose and mannitol behaved equally in terms of cake formation. The cake formed by mannitol was more homogenous and porous in nature. Therefore, it was expected that mannitol would show better product characteristics. Further, mannitol is known as osmogen and could help in reducing viscosity of mucous secretions through transfer of water to airways.

7.4.2 Powder precessing and preparation of DPI

Therefore, lyophilized formulation containing mannitol was subjected to powder processing using our previously developed techniques [15-17]. The lyophilized cake was size reduced to fines by passing through 120# and 240# (Hitco sieves, Hind Trading Company, Bar-oda, India). Fig. 7.3 shows that particle size after sieving. The D50 for the obtained fines was 31.58 µm. Thus obtained fine powder was mixed separately with respitose SV001 and inhalac 230 at different weight ratios, as shown in Table 7.5 and Table 7.6. The blending was performed in geometric mass ratio. The obtained powder

mass were then filled in hard gelatin capsules (size 3) and stored in HDPE bottles containing silica bags as desiccant. Meanwhile, the powder prepared was subjected to analysis of powder chracteristics for selection of optimum carrier mass ratio.

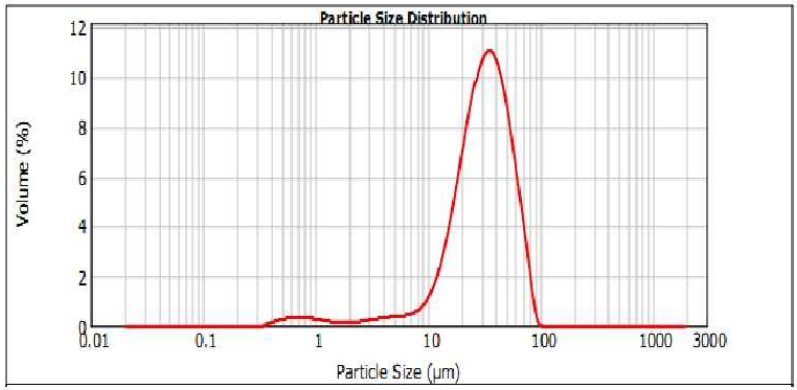

Fig. 7.3: Result analysis report of Malvern Mastersizer after powder after size reduction

7.4.3 Spectrofluorometric estimation of rhodamine B

In order to detect aerodynamic properties of the prepared DPI, rhodamine B was used to study relative distribution in cascade impactor. Therefore, lyophilized formulation containing rhodamine B were prepared and subjected to powder processing as described above. Prior to this, a calibration curve for estimation of rhodamine B was prepared in saline. The Relative fluorescent unit (RFU) were recorded using spectrofluorometer (Shimadzu, Kyoto, Japan). Fig. 7.4 shows the obtained overlay of spectra of calibration standards of rhodamine B. Further, the calibration plot (Fig. 7.5) of concentration vs RFU gave a correlation coefficient (R^2) of 0.999 and calibration equation of y = 2.2052x + 16.65. Thus method was linear and had acceptable range.

The methods was validated for analytical parameters. Table 7.3 Results of accuracy measurements. The accuracy of the method was determined in terms of % recovery and % RSD. The % recovery was 101.5 to 103.0, which was within acceptable limits. The precision was determined in terms of % RSD for inter and intra-day precision as shown in Table 7.4. The % RSD was in the range of 1.34 to 2.62. Further, no interference of mannitol, sucrose and trehalose was observed with rhodamine B and hence, the spectrofluorometric method was concluded to be reliable and proposed for analysis of aerosolization properties of developed DPI.

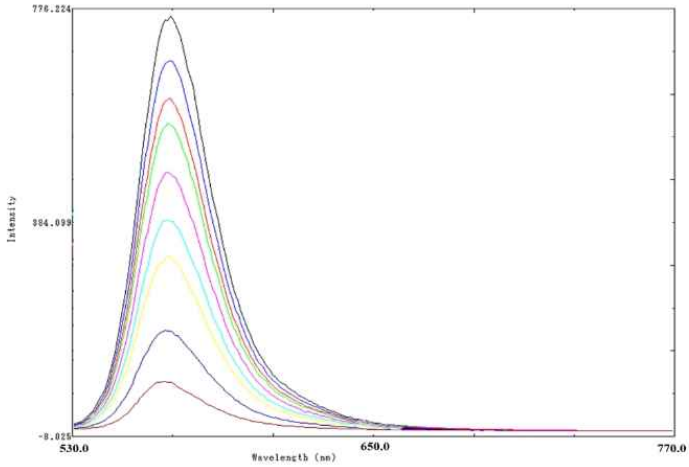

Fig. 7.4: Spectrofluorometric spectra of Rhodamine B.

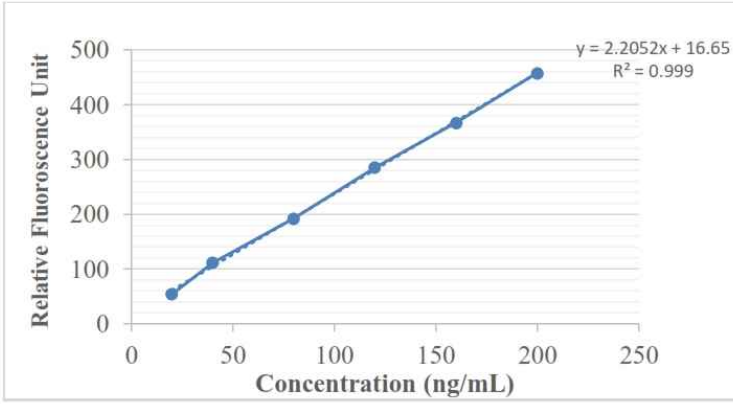

Fig. 7.5: Calibration curve for Rhodamine B

Table 7.3 Results of accuracy measurements

Prepared Concentration (ng/mL)	Observed Concentration (ng/mL)	Standard Deviation (SD)	%Recovery
40	41.20	1.15	103.0
80	82.15	2.45	102.68
120	121.8	2.85	101.50

*All the measurements were performed in triplicates i.e. n= 3

Table 7.4 Inter and intra-day precision of the method

	Observed Concentration ± SD		%Relative Standard Deviation	
Prepared Concentration (ng/ml)	**Intraday precision**	**Interday Precision**	**Intraday precision**	**Interday Precision**
40	41.81	43.48	1.94	2.62
80	83.21	81.56	1.85	1.99
120	119.13	123.80	1.34	1.28

*All the measurements were performed in triplicates i.e. n= 3

7.4.4 Aerosolization performance of dry powder for inhalation

ACI was used to assess the aerodynamic properties of the processed lyophilized bulk containing rhodamine B. The powder processing leads to enhancement of powder characteristics such as cohesive-adhesive interactions, flow properties, fluidization etc. of the lyophilized bulk. During powder processing the lyophilized bulk was size reduced to fines through #240, while the subsequent mixing with coarse powder results in adsorption of these fine particle on the surface, making them carriers for fine particle fraction. Now the bulk properties of the powder are derived from bulk properties of respitose SV001/ inhalac 230.

When the data of aerodynamic behaviour was compared it revealed the critical role played by coarse carriers in development of DPI. As shown in Table 7.5 and Table 7.6, at lower carrier mass ratios, in case of both respitose SV001 and inhalc 230, there was low emitted dose i.e. 56.35±5.27% and 54.62±5.1% for inhalac and respitose, respectively. This indicates that bulk properties were dominated by lyophilized bulk with sticky/cohesive nature and was poorly fluidized. The incorporation of coarse carrier at higher mass ratio, led to improvement in emitted dose up to 84% and 91% for inhalac and respitose, respectively due to improved fluidity. However, the FPF and consequently the MMAD observation revealed differences in inter-particulate forces when using two different carrier. Inhalac 230 showed higher FPF than the respitose i.e. 37.48 ±3.82% and 28.10 ± 1.12 respectively at carrier mass ratio of 1:6. This clearly indicated the obvious choice of inhalc 230 as carrier for present formulation. Further, in case of inhalc 230

there was no significant difference in FPF at carrier mass ratio of 1:5 and 1:6, therefore, 1:5 carrier mass ratio was considered optimal for the developed formulation.

Respitose, which has D50 in the range of 200 – 250 µm, has good flow properties. However, the large size leads to the major deposition in the upper respiratory region of cascade impactor. In addition, the presence of large number of active sites would have resulted in stronger adhesive interaction between respitose and lyophilized powder. As the particles could not separate, they travel the trajectory of carrier without getting detached under the influence of de-aggregation forces such as turbulent shear stresses and inertial separations. Further, the large particles have large surface discontinuities which are also said to shelter the fine adherents from the de-aggregation forces. On the other hand, the D50 of inhalc was between 70-110 µm, which imparts deeper penetrability in sequential stages of cascade impactor, even for the particles which are not stripped off the surface of the carrier. In addition, the adhesive interactions might be weak to allow the dispersion into primary respiratory particles under the influence of mentioned de-aggregation forces.

Table 7.5 Characterization of aerodynamic behavior using inhalac 230 as carrier

Sr. no	Carrier mass ratio (w/w)	Recovered Dose (%)	Emitted Dose (%)	MMAD (µm)	FPF (%)	GSD
1.	1:0	90.3±4.3	56.35±5.27	9.6±1.8	8.56 ± 1.76	4.44±1.2
2.	1:1	91.25±3.9	62.4±6.34	6.8±1.2	10.45 ± 2.03	3.2±0.9
3.	1:3	93.54±3.5	73.6±4.65	4.8±1.2	19.7 ± 1.48	4.4±1.2
4.	1:5	95.6±4.4	91.23±4.3	2.9±0.4	36.15 ± 3.15	2.1±0.8
5.	1:6	96.3±5.1	91.6±4.9	2.8±0.5	37.48 ±3.82	1.9±0.5

*Values represented as mean±SD (n= 3)

Table 7.6 Characterization of aerodynamic behaviour using respitose SV001 as carrier

Sr. no	Carrier mass ratio (w/w)	Recovered Dose (%)	Emitted Dose (%)	MMAD (µm)	FPF (%)	GSD
1.	1:0	92.3±5.2	54.62±5.1	11.76±1.1	9.62 ± 0.81	5.34±1.3
2.	1:1	94.3±4.3	58.42±5.5	6.54±1.4	12.59 ± 1.45	4.56±1.1
3.	1:3	94.6±4.5	64.4±5.8	4.9±0.9	16.21 ± 1.07	2.9±0.7
4.	1:5	94.8±4.1	76.5±4.8	3.8±0.5	21.88 ± 1.45	2.1±0.5
5.	1:6	95.2±4.6	84.3 ± 2.9	3.3±0.4	28.10 ± 1.12	1.88± 0.2

*Values represented as mean±SD (n= 3)

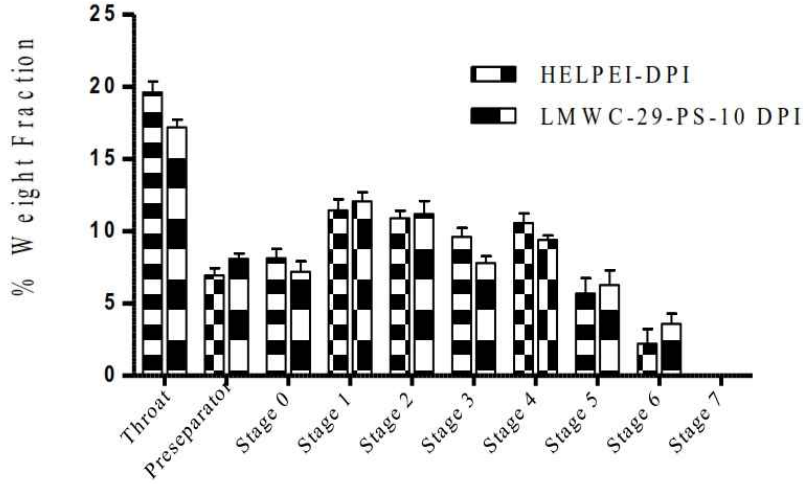

Fig. 7.6: In vitro pulmonary deposition pattern of dry powder formulations

The developed DPI formulations of polyplex retained the integrity of the siRNA after the lyophilization and powder processing.

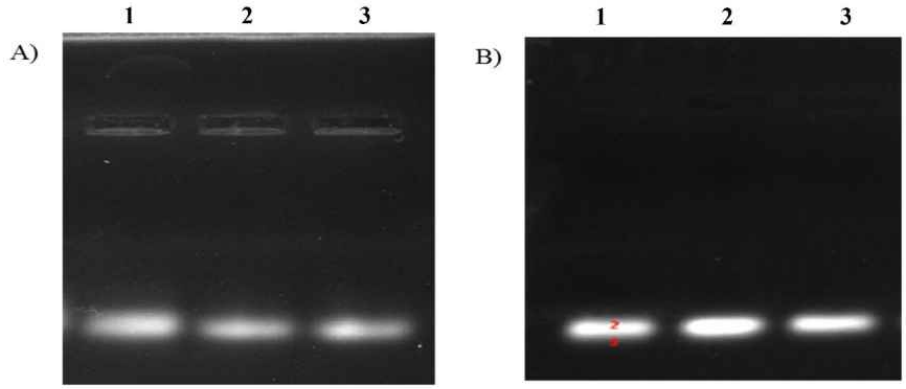

Fig. 7.7: A) Integrity of siRNA from HELPEI-35 DPI formulation (Lane 1: Naked siRNA, Lane 2: After lyophilization, Lane 3: After powder processing); **B)** Integrity of siRNA from CSPS conjugate DPI formulation (Lane 1: Naked siRNA, Lane 2: After lyophilization, Lane 3: After powder processing)

7.4.5 Scanning electron microscopy

The SEM were performed to study the microscopic features of the dry powder. It can be observed in Fig. 7.8 that fines were adsorbed on the surface of carrier particles. The small particles adhere on the energy rich active sites present on the surface of carrier. The carrier particles are easily entrained in the inspiratory air flow from which the adhered particles are then stripped off due to turbulent shear stress and inertial separation mechanisms.

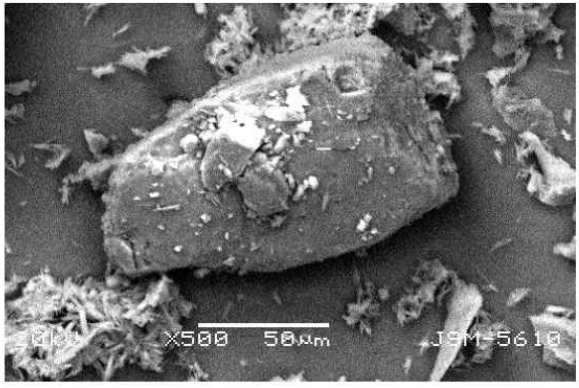

Fig. 7.8: SEM image of the optimized blend for DPI formulation

7.4.6 XRD

The XRD results show that the dry powder formulation was crystalline in nature, which could be due to mannitol during lyophilization and powder processing with Inhalc 230 (Fig. 7.9). The presence of crystallinity is suitable for agglomeration and adsorption onto the surface of carrier materials. Amorphous regions have higher surface adhesion energy than crystalline regions which leads to poor de-aggregation after fluidization in air stream. In contrast, crystalline regions interact weakly with and are easily overcome by turbulent shear during inspiration by patient. The crystallinity is also helpful for better flow properties and better chemical stability during shelf storage.

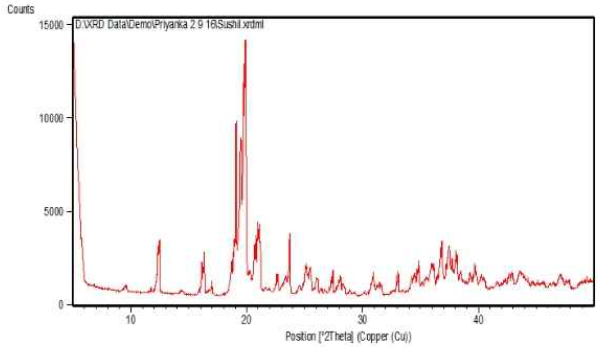

Fig. 7.9: XRD image of developed DPI after powder processing

7.4.7 Animal studies

All animals were found healthy and no mortality was observed neither any sign of clinical toxicity was observed. The weight of wet lungs was normalized to 100 g body weight (L/B) for comparison purpose. For saline treated animals, L/B was 0.49 while that for LPS treated animals was 0.81 (Fig. 7.10). The increased L/B for LPS treated animals indicates edema due to extracellular fluid accumulation in respiratory mucosae. The L/B ratio for LPEI formulation was 0.65, indicating lung injury or edema formation, while for HELPEI-35 formulation it was 0.53, with no significant difference from saline control ($p>0.05$). Similarly, L/B for stabilized LMWC-29-PS-12 formulation was 0.51 with no significant difference from control ($p>0.05$).

The enzymatic activities of LDH and ALP in BAL collected from the treated animals were in accordance with L/B data. As expected, the levels of ALP and LDH in

LPS-treated animals were ~2.6 fold higher than that of saline control. For LPEI also, the activity of LDH and ALP in BALF was significantly different from that of the control ($p<0.05$), while for HELPEI-35 and stabilized LMWC-29-PS-12 there was no significant difference ($p>0.05$). In general, data indicate a favorable safety profile for the hydroxyethyl substituted LPEIs and LMWC-29-PS-12 formulations.

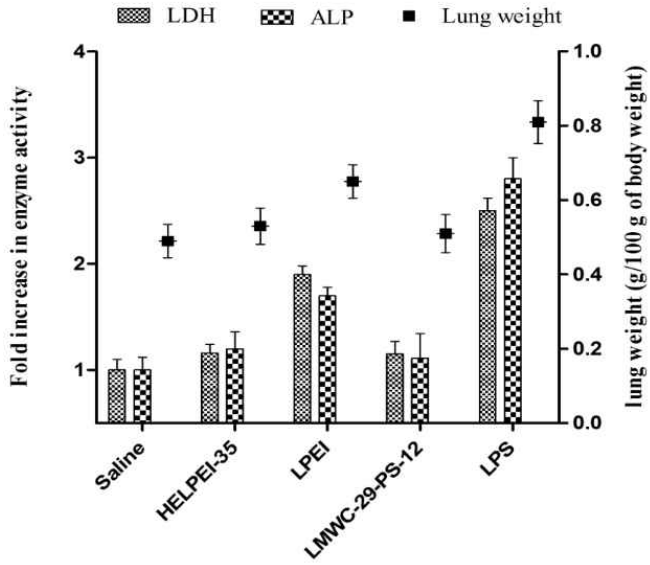

Fig. 7.10: Results of lung weight analysis and BALF examination

Histopathological analyses performed to evaluate the toxicity of particles yielded results that were in good agreement with those obtained by BAL analysis (Fig. 7.11). A high level of inflammation was observed with LPS as expected, it shows bronchial epithelial with infiltrated leucocyte migration, epithelium shows degeneration with necrosis and exudation. On the other hand saline treated samples showed no inflammatory infiltration. The LPEI formulation showed comparatively more inflammatory cell migration while HELPEI-35 and LMWC-29-PS-12 formulations were similar to control.

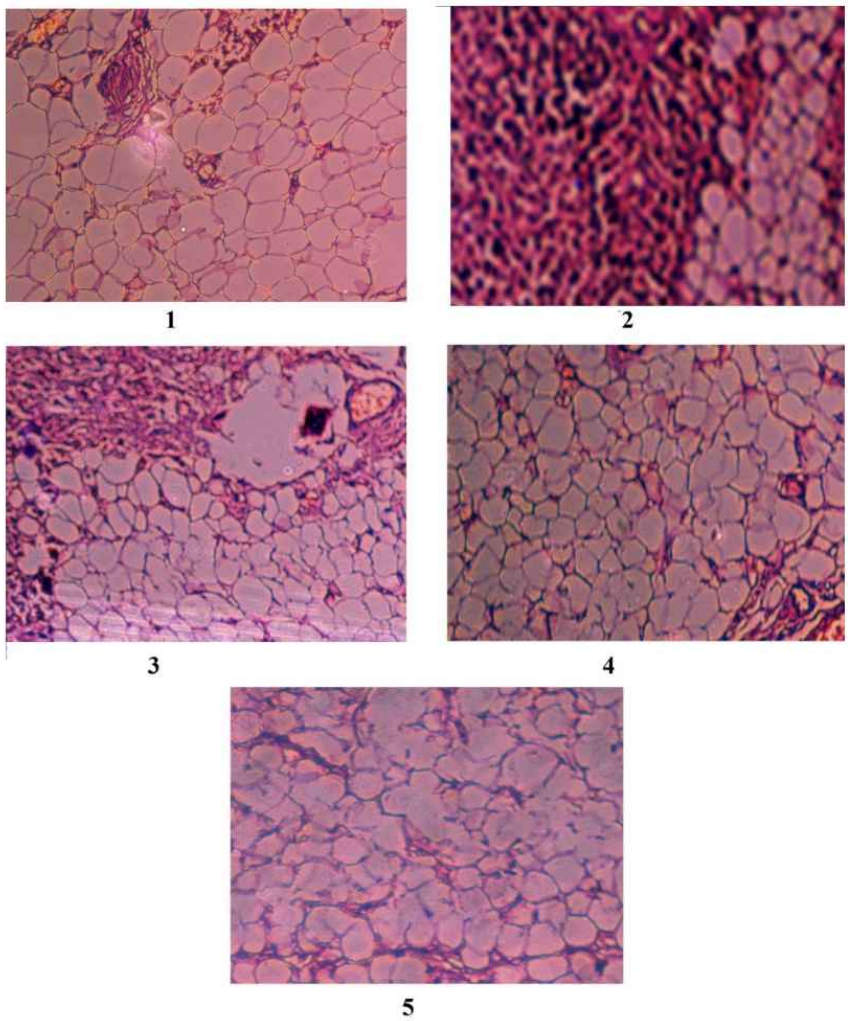

Fig. 7.11: Histological analyses of lung in hematoxylin and eosin stained sections after treatment with (1) Saline, (2) LPS, (3) LPEI formulation, (4) HELLPEI-35 formulation, (5) LMWC-29-PS-12 formulation.

7.4.8 Stability studies

The stability studies were carried out, for optimized formulations, in accordance with the ICH guidelines to evaluate the suitability of the storage condition for the developed formulations. The critical parameters of the formulations such as particles size, water contents have to be preserved during stability studies. The particle size and complexation

efficiency was determined after reconstitution at each time point. All formulations were easily dispersed and retained particle size (Table 7.7). There was no significant difference in complexation efficiency of formulations at different storage time points. It can be recommended to store such formulations at refrigerated conditions under dry state. The water content was attributed to water of crystallization of carrier. However, such water is not available for reactions as it is below the water activity. Increase in water content may influence complexation efficiency as siRNA are prone to hydrolytic degradation. Therefore, it is recommended that water content of such formulations be strictly controlled during stability.

Table 7.7: Results of stability study (mean± SD)

Storage conditions	Time (Months)	siRNA Complexation Efficiency (%)		Particle size (nm) ± SD		Water content (%w/w)	
		A	B	A	B	A	B
5 ± 3°C	Initial	97.16±2.37	97.44±1.81	162.2±2.7	151.7±2.3	3.52	3.56
	1M	96.57±2.12	96.88±1.77	165.1±3.5	157.8±4.1	3.56	3.85
	3M	95.38±2.88	96.46±2.25	171.6±4.7	159.5±3.3	3.90	4.09
25 ± 2°C /60 ± 5% RH	1M	95.19±2.75	97.06±1.68	168.3±3.6	163.2±2.8	3.57	3.52
	3M	94.53±2.51	95.35±2.12	179.9±3.6	165.4±4.5	4.26	4.41

A= HELPEI-35 polyplex formulation; B = LMWC-29-PS-12 polyplex formulation

7.5 References

1. Clark KL, Hughes SA, Bulsara P, Coates J, Moores K, Parry J, et al. Pharmacological Characterization of a Novel ENaCalpha siRNA (GSK2225745) With Potential for the Treatment of Cystic Fibrosis. Mol Ther Nucleic Acids 2013; 2: e65.
2. Guillaume C, Delepine P, Droal C, Montier T, Tymen G, Claude F. Aerosolization of cationic lipid-DNA complexes: lipoplex characterization and optimization of aerosol delivery conditions. Biochem Biophys Res Commun 2001; 286(3): 464-71.
3. Labiris NR, Dolovich MB. Pulmonary drug delivery. Part II: the role of inhalant delivery devices and drug formulations in therapeutic effectiveness of aerosolized medications. British journal of clinical pharmacology 2003; 56(6): 600-12.
4. Brus C. Physicochemical and Biological Properties of Polyethylenimine Based Delivery Systems for Oligonucleotides and Ribozymes: Cuvillier Verlag; 2004.
5. Seville P, Kellaway I, Birchall J. Preparation of dry powder dispersions for non-viral gene delivery by freeze-drying and spray-drying. J Gene Med 2002; 4(4): 428-37.
6. Pfeifer C, Hasenpusch G, Uezguen S, Aneja MK, Reinhardt D, Kirch J, et al. Dry powder aerosols of polyethylenimine (PEI)-based gene vectors mediate efficient gene delivery to the lung. J Control Release 2011; 154(1): 69-76.
7. Kasper JC. Lyophilization of nucleic acid nanoparticles: lmu; 2012.
8. Allison SD, dC Molina M, Anchordoquy TJ. Stabilization of lipid/DNA complexes during the freezing step of the lyophilization process: the particle isolation hypothesis. Biochimica et Biophysica Acta (BBA)-Biomembranes 2000; 1468(1): 127-38.
9. Marple VA, Willeke K. Impactor design. Atmospheric Environment (1967) 1976; 10(10): 891-96.
10. Lipnick RL, Cotruvo JA, Hill RN, Bruce RD, Stitzel KA, Walker AP, et al. Comparison of the up-and-down, conventional LD50, and fixed-dose acute toxicity procedures. Food Chem Toxicol 1995; 33(3): 223-31.

11. Thrall RS, Peterson LB, Linehan JH, Abramoff P, Moore VL. The effect of immunization on the uptake of intratracheally administered antigen. Clinical Immunology and Immunopathology 1978; 10(2): 136-47.
12. Wei W, Mo C, Guohua C. Issues in freeze drying of aqueous solutions. Chinese Journal of Chemical Engineering 2012; 20(3): 551-59.
13. Mujumdar AS. Handbook of industrial drying: CRC Press; 2014.
14. Pyne A, Surana R, Suryanarayanan R. Crystallization of mannitol below Tg′ during freeze-drying in binary and ternary aqueous systems. Pharmaceutical research 2002; 19(6): 901-08.
15. Mahesh Kumar T, Misra A. Formulation and evaluation of insulin dry powder for inhalation. Drug Dev Ind Pharm 2006; 32(6): 677-86.
16. Shah SP, Misra A. Development of liposomal amphotericin B dry powder inhaler formulation. Drug Deliv 2004; 11(4): 247-53.
17. Joshi M, Misra A. Dry powder inhalation of liposomal Ketotifen fumarate: formulation and characterization. Int J Pharm 2001; 223(1-2): 15-27.