Chapter 6
Development of Dry powder formulations and characterization

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6.1 Introduction

Respiratory tract is a promising route of administration for therapeutics for many inherited and acquired pulmonary diseases. Pulmonary delivery of therapeutics have been designed to treat both acute and chronic diseases such as cystic fibrosis (CF), alpha 1-antitrypsin deficiency, surfactant protein deficiencies, acute respiratory distress syndrome (ARDS), cancer and asthma (1). Low systemic absorption, reduced side effects, prevention of therapeutics from hepatic metabolism and reduced dose of therapeutics are the important advantages of inhalation route of therapeutic delivery (2). Moreover, the comparatively easy, non-invasive accessibility also makes the lungs attractive for such innovative therapeutic interventions. Despite the advantages mentioned above, the pulmonary delivery of therapeutics present several challenges which include airway geometry, pulmonary defense mechanisms, including the mucociliary escalator, as well as the macrophagic and enzymatic activities (3, 4), also the presence of lung disease might also affect therapeutic outcomes. However, direct delivery via inhalation is of great advantage since local application minimizes serious adverse effects caused by systemic immune response. Thus the suitable therapeutic carriers are required to overcome these barriers.

In recent years, polymeric NPs have been shown to be effective in delivery of therapeutics to the lung. However to facilitate delivery of NPs through respiratory tract, it is necessary to incorporate NPs into micron size carriers like sugars with aerodynamic diameter around 5 μm. Delivery of these particles in dry powder inhaler form confer many advantages as increased stability, reduced drug loss during administration, improved portability and efficient delivery to the pulmonary targeted region (5). Though various techniques has been utilized for development of dry powders Freeze-drying is currently the most common method for preparing dry formulations of thermosensitive drugs, proteins and gene vector complexes (6). However to preserve of particle size and therapeutics efficiency, stabilizers was added during freeze drying process (7).

A variety of excipients have been used routinely for freeze-drying to achieve suitable stability for biopharmaceuticals. These excipients are serving as stabilizer and bulking agent. Sugars such as lactose, sucrose, trehalose, and mannitol are most commonly used for these purpose as cryoprotective agents. They are also attractive as excipients due to their influence on the glass transition temperature (Tg) (8). Tg value is of particular importance for the development of a

robust and effective lyophilization process, obtaining stable amorphous solids and consequently to achieve the desired properties, such as a high redispersion speed, an acceptable storage stability, and also an appropriate residual moisture content (9).

These sugars are known to vitrify at a specific temperature denoted Tg′. The immobilization of nanoparticles within a glassy matrix of cryoprotectant can prevent their aggregation and protect them against the mechanical stress of ice crystals. Generally, freezing must be carried out below Tg′ of a frozen amorphous sample or below Te (eutectic crystallization temperature) which is the crystallization temperature of soluble component as a mixture with ice, if it is in a crystalline state in order to ensure the total solidification of the sample (10). This process generates various stresses during freezing and drying steps. So, protectants are usually required to protect the nanoparticles from freezing and desiccation stresses. There are literature about nanoliposomal dry powder formulations with different aerodynamic properties prepared using different proportions of carriers, cryoprotectants, and antiadherents (11, 12). Also freeze-dried dry powder formulations of therapeutic molecules such as budesonide, ketotifen, amphotericin B, leuprolide acetate, and levonorgestral have been formulated (11-13).

However, these smaller particles shows stronger cohesive forces, resulting in increased adhesion between the particles and subsequently poor flow properties (14). So, one way to improve the flow properties of a drug is through the addition of excipients. Excipients were used to enhance the physical or chemical stability of the active pharmaceutical ingredient, its mechanical properties, and/or its pharmaceutical properties, such as dissolution and permeation. In DPI formulations, excipients function first and foremost as carrier particles. Usually, no more than a few milligrams of drug need to be delivered (e.g. corticosteroids for asthma therapy), and excipients provide bulk, which improves handling, dispensing, and metering of the drug. Various carrier molecules used for this purpose are lactose, glucose, mannitol and trehalose (15-18). Lactose is the most commonly used carrier in DPIs. Lactose has become an ideal carrier for DPIs because of its well-established safety profiles, ready availability, and low cost. Furthermore, crystalline lactose has smooth surfaces, a regular shape, and good flow properties (17, 19). These therapeutic molecules to the carrier particles and on inspiration they get separated from carrier particles and deposited in lungs. The larger carrier particles deposit on the oropharynx, while the fine drug particles partly reach the deep lung. This chapter discuss the preparation of dry powder formulation of nanoparticles, their characterization and in vivo evaluation.

6.2 Materials

Optimized nanoparticles formulation as developed previously were used. Lactose monohydrate, sucrose and trehalose were purchased from Himedia. Lactohale 201 was obtained as gift sample from DFE Pharma, USA. Moisture test kit was obtained from Sigma Aldrich, Mumbai, India. Assay kits for LDH and ALP was obtained from Abcam, Mumbai, India. All other chemicals were purchased of analytical grade and were used as discussed earlier.

6.3 Preparation and characterization of dry powder inhaler formulation

Freeze drying technique was used for preparation of the dry powder inhalation formulation by using suitable cryoprotectant. The optimized nanoparticles were mixed with different cryoprotectants like lactose monohydrates, sucrose, trehalose at different ratio to preserve the size and shape. The nanoparticulate dispersion containing cryoprotectant was frozen at - 80 °C for at least 24 hour and subsequently freeze dried for 2 days using Heto Drywinner. The freeze dried NPs were stored at 4 °C. Influence of cryoprotectant on size and percent drug retained after re-suspension in water was evaluated. Based on particles size evaluation, suitable cryoprotectant was selected for preparation of dry powder inhalation. After freeze-drying, samples were divided in two fractions, one fraction is sized through 120# sieve while other fraction is sized through no. 230 sieve. Capsules (size 3) were filled with obtained dry powder and stored in bottles containing silica bags as dehumactant. The bottles were stored in a desiccator until further use. Further effect of the carrier on aerodynamic parameters of dry powder was also evaluated. The sieved lyophilized NPs powders were mixed with lactose carrier (Lactohale 201) in varying mass ratios from 1:0.5 to 1:3. The *in-vitro* deposition studies of these formulations were determined by using Anderson cascade impactor Apparatus 3, as specified in the United States Pharmacopoeia (USP) after aerosolization of 5 capsules at 28.3 L/min for 10 sec using Aerolizer as the delivery device. The flow rate was adjusted to a pressure drop of 4 kPa, as is typical for inspiration by a patient. Apparatus were operated at airflow rates 28.3 L/min for 10 sec so that a volume of 4 L of air was drawn through the inhaler as recommended by the pharmacopoeias (20).

Powder deposited in the device (mouthpiece adapter, inhaler and capsule), the induction port simulating the throat and the each stage of the impactor were collected and evaluated for drug/pDNA content by using UV spectrophotometry method, while Rhodamine B or 6-Coumarin content was analyzed by spectrofluorimetry. Analysis of the amount of drug deposited on each stage was done to determine recovered dose (RD), emitted dose (ED), fine powder fraction

(FPF), dispersibility, Mass Median Aerodynamic Diameter (MMAD) and Geometric Standard Deviation (GSD). Recovered dose (RD) was determined as the total amount of NS/pDNA/Rhodamine B/6-coumarin recovered from the inhaler, capsule shell and the apparatus and was expressed as the percentage of the average assay amount. The emitted dose (ED) was calculated by accurately weighing the capsule before and after Aerolizer actuation. Fine particle dose (FPD) was considered as the amount of NS found below effective cut-off diameter < 4.7μ. Dispersibility, was calculated from the percentage amount of drug particles with effective cut-off diameter < 4.7μ in the emitted dose, Fine particle fraction (FPF) was the ratio of FPD to RD, expressed as percentage. Mass Median Aerodynamic Diameter (MMAD) is defined as the diameter at which 50% of the particles by mass are larger and 50% are smaller. The mass median aerodynamic diameter (MMAD) and the geometric standard deviation (GSD) were calculated according to USP (20) by deriving a plot of cumulative mass of powder detained at each stage (expressed as percent of total mass recovered in the impactor) versus cut-off diameter of the respective stage. Geometric Standard Deviation (GSD) is a measure of the spread of an aerodynamic particle size distribution. Typically calculated as: $GSD = (d84/d16)^{1/2}$

Where, d84 and d16 represent the diameters at which 84% and 16% of the aerosol mass are contained, respectively.

6.3.1 Residual water content

Residual water content in the dry powder formulations was analytically quantified by using Karl Fischer titration method using an Automatic titrator instrument (model AT 38 C with KF attachment, Spectra lab, India). The measured moisture content was expressed in percentage for the known weight of the sample.

6.3.2 Scanning electron microscopy (SEM)

Morphology of formulations was studied by scanning electron microscopy (SEM). The samples for SEM were prepared by sprinkling a small amount of powdered formulation (1-2 mg) onto a double sided adhesive tape attached to an aluminum stub. The accelerator voltage for scanning was 15.0 kV. The formulations were then examined by SEM and photograph were taken by using Jeol JSM-5610LV (Japan) scanning electron microscope.

6.3.3 Antibacterial activity

To evaluate effect of lyphilization and powderization on antibacterial activity of the NS loaded NPS the minimum inhibitory concentration (MIC) test was carried out as described in earlier

chapter. Briefly formulations were reconstituted in PBS then diluted further with Trypticase soy broth (TSB) to make a final NS concentration ranging from 50 µg/mL to 10 µg/mL (calculated from % drug loading) in a final volume of 100 µL. Then, 100 µl of diluted *P. aeruginosa* inoculum were added to each well and the samples were incubated at 37 °C for 12 h. The MIC endpoints were determined by reading the OD of the plate wells at 600 nm and confirmed by visual inspection. The lowest concentration that yielded OD ≤ 0.1 was determined as the MIC.

Minimum bactericidal concentration (MBC) is the drug concentration where no visible growth appears on agar plates. NS and NS-loaded NPs treated bacterial cultures showing growth or no growth in the MIC tests were used for this test. Bacterial cultures that were used for the MIC test were inoculated onto the agar and incubated at 37°C for 12 h. Microbial growth was assessed by counting colonies.

6.3.4 Luciferase assay

Further effect of lyphilization and powderization on luciferase expression activity of the pDNA loaded NPs were also evaluated as described in earlier chapter. The NPs were reconstituted in Tris EDTA buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8). The CFBE 41o- cells were seeded in 96 well plate (Falcon, Cell Growth Area: 0.16 cm²) at a density of 20000 cells / well in 100 µl of MEM with Earle's salts and L-glutamine, and containing 10 % (v/v) FBS, 1 % L-glutamine 20mM and 1% Penicillin–Streptomycin. After 24 h, the culture medium was replaced with fresh MEM medium (serum free) containing NPs containing the quantity of pDNA (LUC WT) constant (500 ng). After 24 h of incubation of the NPs were removed and the cells were washed with PBS pH 7.4 and were replaced with complete media. In all the experiments, naked pDNA transfected cells were used as negative control and the Lipofectamine plus (Invitrogen) transfected cells were used as a positive control. After 48, 96, 120 h of the post transfection luciferase detection was performed on the transfected cells using a chemiluminescent assay (Promega, USA). Tests were carried out as described by the manufacturer and Luciferase activity in the supernatant was quantified in relative light units (RLU) using TD-20/20 Luminometer, version 2, Turners designs, CA. Results were normalized to total cell protein using Pierce® BCA protein assay kit (Thermo Scientific BioRad Protein Assay) with bovine serum albumin as protein standard. (The amount of protein in the supernatant was determined, after a suitable dilution).

6.3.5 Animals

Male, pathogen-free, Sprague–Dawley rat weighing 200-350 g were obtained from Torrent Research Centre (TRC), Gandhinagar, India. All experiments described in present study was approved by the Institutionl Animal Ethical Committee (IAEC) of Pharmacy Department, The M. S. University of Baroda, India and from Committee for purpose of Control and Supervision of Experiment on Animals (CPCSEA), Ministry of Social Justice and Empowerment, Government of India with number MSU/PHARM/IAEC/2011/16. Animals were housed three per cage in a room maintained at 25 ± 2°C with an alternating 12 h dark/12 h light cycles. Animals will be allowed for free access to water and commercial rodent diet under standard laboratory conditions. Animals will be acclimatized to the laboratory environment 5 to 7 days prior to the study.

6.3.6 Particles uptake by rat alveolar macrophage

Particles uptake by macrophages was evaluated by incubating the particles with rat alveolar macrophages collected from the lungs of anesthetized (Ketamine (100 mg/Kg)) male Sprague–Dawley rats (200–250 g). Briefly, 1 mL phosphate buffered saline containing 0.5 mM disodium EDTA was perfused through the trachea using 24-guage catheter. The resulting BAL fluid was centrifuged at 1500 rpm for 5 min to obtain pellets of macrophages, which was then suspended in Hanks Balanced Salt Solution (HBSS). The cells at a density of 4×10^5 cells/mL were then put onto cover slips placed over 12-well plates and incubated in a humidified chamber at 37° C for an hour. Following incubation, cells were washed with PBS and then an aliquot of fluorescent PLGA NPs (reconstituted with HBBS) suspended in HBSS was added into the wells (1 mg/mL) and incubated for an hour at 37 °C. The cells were then washed with PBS and fixed with formalin 10 %. Then cells were again washed with PBS and the nuclei were finally labeled with a Hoechst dye for 10 min at room temperature. Upon washing with PBS, glass coverslips were placed on a glass slide covered with a drop of anti-fade solution and sealed. Uptake of the fluorescent particles was observed under confocal microscope (Carl Zeiss, Confocol LSM 510 META, ×63, NA 1.4, oil immersion).

6.3.7 Particle deposition and clearance in rat lungs

For studying particle deposition and clearance, fluorescent NPs were used. Prior to study, Sprague–Dawley rat were fasted for 12 h but had free access to water. The rats were divided into four groups containing 13 rats in each group. Rats were anesthetized by intraperitoneal

administration of Ketamine (100 mg/Kg) and about 10 mg of the Fluorescent NPs particles were administered intra-tracheally by syringe as described by Okamoto et al., 2000 with some modification (21). Four groups of rat received following treatment: i) Rho-PLGA NPs (Formulation G); ii) PEG Rho PLGA NPs (Formulation H); iii) 6 C- PEI PLGA NPs (Formulation O); iv) PEG 6 C- PEI PLGA NPs (Formulation P). To study particle deposition, 1 rat from each group was sacrificed 24 h post administration and lungs were isolated. Lungs were sectioned and visualized under confocal microscope (Carl Zeiss, Confocol LSM 510 META, ×63, NA 1.4, oil immersion). To study the clearance of NPs, rats were sacrificed and lungs were isolated on day 0, 1, 3 and 6 after treatment. The lungs were homogenized with 2 mL of saline and marker molecules (Rhodamine B & 6-coumarin) in the NPs deposited in lung were extracted using 5 mL of a mixed solution of chloroform and methanol at a ratio of 1:1 (v/v) with a continuous shaking for 1 h. The resulted dispersion was centrifuged to separate the organ precipitate and then the concentration of 6-coumarin was measured at excitation and emission wavelengths of 458 and 505 nm, respectively; while the concentration of Rhodamine B at excitation and emission wavelengths of 560 and 595 nm, respectively using Spectrofluorometer (RF-5301PC, Shimadzu). The elimination of nanocomposite particle from the lung was evaluated by measuring the amount of Rhodamine B or 6-coumarin remaining in the respiratory organs at 0, 1, 3 and 6 day after the intratracheal administration.

6.3.8 Safety studies

6.3.8.1 Cytotoxicity study

The cytotoxicity of formulations were studied by an MTT (3-(4, 5-Di methylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay upon incubating the particles with CFBE41o- cells, a bronchial epithelial cell line. Briefly, CFBE41o- cells were seeded into 96 well plates (Falcon 3072) at density 4×10^4 cells/well in EMEM containing 10 % FBS and 1% antibiotic and incubated at 37°C, 5% CO_2 for 24 h. After 24 h, the cells were exposed to different concentration of formulations (0.5mg/mL to 5 mg/mL) in EMEM for 24 h. Cells treated with EMEM and 0.1% sodium dodecyl sulfate (SDS) were used as negative and positive controls, respectively. After 24h treatment, cells were rinsed thoroughly with phosphate buffer saline (PBS), treated with 100 µL MTT solution, and incubated for 4 h. After 4 h of incubation, the medium with MTT was replaced with a solubilization solution (100 µL DMSO) to dissolve the formazan crystals formed after internalization of MTT by live cells. The resulting colored solution was analyzed using a

microplate reader (Synergy Mix, Biotek) at 570 nm. Cell viability was expressed as the percentage of absorbance of test samples relative to that of cells treated with EMEM (n=3).

6.3.8.2 TEER analysis

Transepithelial electrical resistance (TEER) analysis was performed on CFBE41o- cells to determine the integrity of the cell monolayer upon exposure to NPs suspension. The cells were seeded onto Transwell® clear permeable filter inserts as described above. 500µL of medium was added to the apical side of the cell monolayer and 1,500 µL were added to the basolateral side. TEER across the insert was measured using an ohm meter using chopstick electrodes. TEER was measured from till day 2 to ensure that the monolayer was confluent, and this was confirmed by light microscopy. The resistance of an insert lacking cells was subtracted from all measurements to correct for the resistance of the Transwell. On day 2, the apical medium was removed to expose the layer to air and air-interfaced culture (AIC) was introduced. On day 3 prior to dry powder impingement, TEER was measured. For appropriate measurement, the volume of AIC condition was adjusted with prewarmed medium when measuring the resistance, then AIC was returned to its original condition. Dry powder impingement consisted of spraying 1 mg of powder onto the cells via a syringe (1 mL syringe, 18 G needle). Light microscopy was used to verify dispersion of the particles across the cell monolayer. TEER was measured immediately following formulation impingement and again after 1, 2, 3, 4 and 5 days.

6.3.8.3 Bronchoalveolar lavage (BAL) studies

BAL studies were also performed to evaluate the safety of the formulations (22, 23). Sprague–Dawley rats were anesthetized by intraperitoneal administration of Ketamine (100 mg/Kg) and divided into six groups (n = 3) to receive following treatments intratracheally (i) Saline, (ii) Formulation D, (iii) Formulation F, (iv) Formulation L, (v) Formulation N and (vi) lipopolysaccharide (LPS, 0.1 µg/ml, positive control). 24 h after administration, lungs were surgically removed, cleaned and weighed to investigate the possibility of edema formation. The lungs were then lavaged with 5 mL normal saline instilled through the trachea and collecting 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.

6.3.9 Lung histology

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) were cut and stained with hematoxylin and eosin (H&E). Then sections were observed under Fluorescence microscope (Nikon Corporation, Japan) and photographs were captured.

6.3.10 Stability study

As per the ICH guidelines for stability studies, the physical stability of the formulations was assessed for 6 months (24, 25). The formulations (Capsules) were stored in HDPE bottle at 5 ± 3°C and 25± 2°C and 60±5% relative humidity for a period of 6 months. At predetermined time points, samples were evaluated for the particle size, residual moisture content and % EE.

6.3.11 Statistical analysis

The experiments were performed in triplicate, unless otherwise stated. All data were expressed as mean ± standard deviation. The statistical significance of the results was determined using a Student's t-test where $P<0.05$ as minimum level of significance.

6.4 Results and discussion

6.4.1 Lyophilization of NPs

The nanoparticulate formulations were lyophilized with sugars such as Lactose, Sucrose and Trehalose at a weight ratio of 1:0.5, 1:1, 1:1.5 and 1:2 of polymer: sugar and were checked for particle size after resuspension in water. The results of particle size observed after lyophillization and resuspension are reported in Table 6.1 & 6.2. After lyophillization at different Polymer: cryoprotectant ratio, particle size of the NPs in the cake was determined after resuspension in 2 ml of water followed by vortexing to break the lumps. The NPs showed comparatively lower particle size with Trehalose than Lactose as well as sucrose and at ratio 1:1. Further increase in ratio of Trehalose results in non-significant improvement in particle size, hence NPs: Trehalose ratio of 1:1 was optimized and used for all formulations and these formulations were further used to develop DPI formulation.

6.4.2 Characterization of DPI

The Polymer: Trehalose ratio of 1:1 was used for further evaluations. Formulations were freeze dried at 1:1 ratio of Polymer: Trehalose. After freeze-drying, samples were divided in two fractions, one fraction is sized through 120 sieve while other fraction is sized through no. 230

sieve. The obtained two fractions were mixed and then filled in capsules (size 2) and stored in bottles containing silica bags as desiccant. Further effect of carrier on aerodynamic properties of the formulations were evaluated by mixing of the dry powder with lactose carrier (Lactohale 201) in varying mass ratios from 1:0.5 to 1:3. *In-vitro* aerosolization properties of the dry powder formulations were evaluated using Andersen cascade impactor (ACI) Apparatus 3, as specified in the USP (20) after aerosolization of 5 capsules at 28.3 L/min for 10 sec using Aerolizer as the delivery device. The flow rate was adjusted to a pressure drop of 4 kPa, as is typical for inspiration by a patient. Apparatus were operated at airflow rates 28.3 L/min for 10.0 sec so that a volume of 4 L of air was drawn through the inhaler as recommended by the USP (20).

Table 6.1: Particle size of NS loaded NPs before and after lyophillization (n=3) (Mean± SD)

NPs + Cryo-protectants used	**Polymer: cryo-protectant ratio**	**Initial particle size (nm)**	**Particle size after lyophilization (nm)**
NS-DS PLGA NPs	--	140.83 ± 2.4	746.70± 26.45
NS-DS PLGA NPs + Lactose	1.0 to 0.5		558.03± 25.16
	1.0 to 1.0		512.66±13.57
	1.0 to 1.5		484.63± 19.65
	1.0 to 2.0		459.96± 15.69
NS-DS PLGA NPs + Sucrose	1.0 to 0.5		481.90± 08.50
	1.0 to 1.0		418.66± 08.08
	1.0 to 1.5		365.20± 17.32
	1.0 to 2.0		262.60± 08.54
NS-DS PLGA NPs + Trehalose	1.0 to 0.5		294.43± 09.01
	1.0 to1.0		**253.13± 06.50**
	1.0 to 1.5		246.60± 08.54
	1.0 to 2.0		243.33± 06.11
PEG-NSDS PLGA NPs + Trehalose	1.0 to 1.0	150.13±3.20	241.66±07.50
Rho-PLGA NPs + Trehalose	1.0 to 1.0	143.18±3.3	256.66±09.07
PEG Rho-PLGA NPs + Trehalose	1.0 to 1.0	149.51±4.2	244.33±06.02

Different aerosolization parameters like recovered dose (RD), emitted dose (ED), Fine particle fractions (FPF), MMAD, GSD were determined and were expressed as percent of the average assay amount of NS/pDNA/Rhodamine B/6-Coumarin discharged from the inhaler. The results of the study is depicted in Table 6.3 & 6.4. In case of NS loaded NPs, Dry powder

formulation without lactose carrier particles, only 75.96 ± 3.03 % of the particles are successfully emitted off.

Table 6.2: Particle size of pDNA loaded NPs before and after lyophillization (n=3) (Mean± SD)

NPs + Cryo-protectants used	Polymer: cryo-protectant ratio	Initial particle size (nm)	Particle size after lyophilization (nm)
PEI PLGA NPs	--	178.9 ± 4.33	663.13± 14.97
PEI PLGA NPs + Lactose	1.0 to 0.5		504.72± 19.96
	1.0 to 1.0		476.66± 10.01
	1.0 to 1.5		462.13± 07.75
	1.0 to 2.0		423.30± 10.14
PEI PLGA NPs + Sucrose	1.0 to 0.5		515.23± 23.58
	1.0 to 1.0		438.66± 13.61
	1.0 to 1.5		365.20± 17.32
	1.0 to 2.0		269.26± 05.13
PEI PLGA NPs + Trehalose	1.0 to 0.5		268.43± 07.50
	1.0 to 1.0		**217.46± 06.50**
	1.0 to 1.5		212.26± 06.50
	1.0 to 2.0		207.33± 05.03
PEG-PEI PLGA NPs + Trehalose	1.0 to 1.0	189.5 ± 4.22	201.23± 06.20
6-C PLGA NPs + Trehalose	1.0 to 1.0	181.6 ± 4.05	210.23± 07.65
PEG 6-C PLGA NPs + Trehalose	1.0 to 1.0	193.8 ± 5.20	206.20± 07.30

The low ED is caused due to the agglomeration of the particles and may be due to their small diameter, making them difficult to be aerosolized off the percentage of initial dose. Even for the drug particles that are successfully aerosolized, only a small fraction of them (Dispersibility: 17.36 ± 0.69 %) possess below effective cut-off diameter < 4.7μm indicating that a majority of the aerosolized drug particles remain agglomerated entering the ACI resulting in MMAD 6.53±0.29 μm. The combination of the low ED and low dispersibility also leads to a low FPF (13.19±0.86 %), which is much lower than the typical FPF of ≈ 20 –30% for commercial DPI products. The higher MMAD also results in phagocytic uptake and are not desirable for prolonged drug delivery to the lung when macro-phages are not the intended target (26). Also particles with MMAD > 5 μm would not reach the lower airway regions as they tend to deposit in the upper airways, whereas particles with MMAD bellow 1 μm would be exhaled without depositing. Thus, it important to have MMAD between 1-5 μm for effective distribution of particles in lung.

Thus the carrier particles are required to improve the aerosolization of the drug nanoparticle aggregates. Hence, lyophilized NPs were mixed with different ratio of the inhalable lactose (Lactohale 201). The lyophilized powder of the PLGA NPs were adsorbed onto the surface of the inhalable lactose carriers during the mixing process. The obtained nanocomposite particles were composed of the PLGA NPs and the inhalable lactose carriers. During inhalation, the PLGA NPs could be easily separated from the surface of the inhalable lactose carriers under the airstream generated by patients and deposited in the deep lung. Subsequently the lyophilized PLGA NPs could be reconstituted in the lung fluid, and release the therapeutic moiety (27).

It was observed that, addition of the inhalable lactose significantly improve ED, dispersibility and thus % FPF, while lower the MMAD bellow 5 μm (Table 6.3). Carrier to mass ratio of 1:1.5 show high %FPF (34.64±0.42 %) and MMAD (3.49±0.1 μm); however further increased in carrier to mass ratio doesn't significantly improved %FPF, thus carrier to mass ratio 1:1.5 was used for further preparation of the dry powder formulations. Dry powder formulations of PEGylated NPs and Rhodamine loaded NPs also showed similar aerosolization properties (Table 6.3). Similar results were observed with pDNA loaded NPs (Table 6.4). Dry powder formulation without lactose carrier showed low ED (71.72±2.54 %), as well as dispersibility (17.41±3.23 %). The combination of the low ED and low dispersibility also leads to a low FPF (12.43±1.94 %) and high MMAD (6.64±0.46 μm). While further addition of the inhalable lactose significantly improved % FPF and lower the MMAD. Carrier to mass ratio of 1:1.5 show high %FPF (34.25±0.50 %) and MMAD (3.82±0.04 μm) however further increased in carrier to mass ratio doesn't significantly improved %FPF, thus carrier to mass ratio 1:1.5 was used for preparation of the other dry powder formulation. Dry powder formulations of PEGylated NPs and 6-Coumarin loaded NPs were also showed similar aerosolization properties (Table 6.4).

Figure 6.1 show SEM image of lyophilized NPs and nanocomposite particles. It was observed that the lyophilized NPs shows aggregations. However, after mixing with inhalable lactose carriers, the lyophilized NPs powders were adsorbed on the surface of inhalable lactose carriers and form nanocomposite particles. The lyophilized NPs appear to be gently in contact with the surface of inhalable lactose carriers and will be separated easily from the carriers in an air-stream. The residual water content of the formulations was measured by using Karl Fischer titration. The residual water content plays crucial role in long-term stability of formulation, both physically and chemically. The water content of formulations may change significantly during

storage due to a variety of factors, like stopper moisture release and leakage, crystallization of an amorphous excipient, or moisture release from an excipient hydrate (28). Increased water content also facilitates the crystallization of formulation, may accelerate formulations' instability (29). High moisture content also facilitates the crystallization of formulation of excipients such as various sugars. The moisture content observed was 1.24 ± 0.31, 1.07 ± 0.12, 1.26 ± 0.20, 1.20 ± 0.04 for Formulation D, Formulation F, Formulation N, Formulation L respectively. It was observed that water content in all formulations was less than 1.5 % which was apt for long term stability

6.4.3 Antibacterial activity

To evaluate effect of lyophilization and powderization on antibacterial activity of the NS loaded NPs the minimum inhibitory concentration (MIC) test was carried out. Briefly formulations were reconstituted in PBS then diluted further with Trypticase soy broth (TSB) to make a final NS concentration ranging from 50 µg/mL to 2 µg/mL (calculated from % drug loading) in a final volume of 100 µL. Bacteria were exposed to different concentrations of initial and reconstituted NPs for 24 h and optical density was measured at 600 nm (OD 600). In addition, microbial growth was ascertained by growing bacteria on agar plates after suitable dilution. The antibacterial activity of NS-loaded NPs was determined as MIC on *P. aeruginosa*. A MIC value for NSDS PLGA NPs (Initial) was found approximately equal to 18 µg/mL. In the same experimental conditions, the MIC of Formulation D (Reconstituted) ~17 µg/mL (Figure 6.2), while the complete eradication of the bacteria was observed at 44 µg/mL and 42 µg/mL for NSDS PLGA NPs (Initial) and PEG NSDS PLGA NPs + Carrier (1:1.5) (Reconstituted), respectively. While, MIC value for PEG NSDS PLGA NPs (Initial) was found approximately equal to 14 µg/mL. In the same experimental conditions, the MIC of Formulation F (Reconstituted) ~15µg/mL (Figure 6.2), while the complete eradication of the bacteria was observed at 36 µg/mL and 38 µg/mL for NSDS PLGA NPs (Initial) and PEG NSDS NPs + Carrier (1:1.5) (Reconstituted), respectively. We have observed that there is no significant difference between MIC of initial and reconstituted NPs ($p > 0.05$; $p=0.174$ for PEG NSDS PLGA NPs (Initial) & Formulation F (Reconstituted); $p=0.109$; $p> 0.05$ for NSDS PLGA NPs (Initial) & Formulation D (Reconstituted)), which indicates that the lyophilization and powderization doesn't affect the antibacterial activity of the NS loaded NPs.

Table 6.3: Aerodynamic properties of dry powders containing NS loaded and Rhodamine loaded PLGA NPs (mean ± SD, n=3).

Lyophilized Formulations	Carrier mass ratio	Formulation code	Recovered dose (% ± SD)	Emitted dose (%± SD)	Dispersibility (% ± SD)	FPF (% ± SD)	MMAD (µm ± SD)	GSD
NSDS PLGA NPs	1:0	A	99.49 ± 0.52	75.96 ± 3.03	17.36 ± 0.69	13.19 ± 0.86	6.53 ± 0.29	3.54 ± 0.26
	1:0.5	B	99.33 ± 0.43	82.91 ± 3.18	25.02 ± 0.79	20.74 ± 0.81	5.46 ± 0.06	3.57 ± 0.32
	1:1	C	99.66 ± 0.44	88.54 ± 1.00	30.00 ± 1.99	26.55 ± 1.47	4.37 ± 0.36	2.97 ± 0.05
	1:1.5	D	99.53±0.40	90.90 ± 0.65	38.11 ± 0.73	34.64 ± 0.42	3.49 ± 0.10	2.62 ± 0.01
	1:2	E	99.09 ± 0.45	92.25 ± 1.15	39.48 ± 1.54	36.41 ± 0.96	3.56 ± 0.25	2.87 ± 0.08
PEG-NSDS PLGA NPs	1:1.5	F	99.13 ± 0.16	89.34 ± 0.26	40.12 ± 0.93	35.84 ± 0.74	3.60 ± 0.17	2.97 ± 0.16
Rho-PLGA NPs	1:1.5	G	99.02 ± 0.25	89.35 ± 0.38	40.09 ± 0.23	35.83 ± 0.05	3.45 ± 0.04	2.90 ± 0.01
PEG Rho-PLGA NPs	1:1.5	H	98.68 ± 0.30	89.91 ± 0.20	41.00 ± 0.91	36.86 ± 0.80	3.39 ± 0.03	2.88 ± 0.06

Table 6.4: Aerodynamic properties of dry powders containing pDNA loaded and 6-coumarin loaded PLGA NPs (mean ± SD, n=3).

Lyophilized Formulations	Carrier mass ratio	Formulation code	Recovered dose (%± SD)	Emitted dose (%± SD)	Dispersibility (% ± SD)	FPF (% ± SD)	MMAD (µm ± SD)	GSD
PEI PLGA NPs	1:0	I	99.13 ± 0.44	71.72±2.54	17.41±3.23	12.43±1.94	6.64±0.46	2.88±0.02
	1:0.5	J	99.22 ± 0.18	77.48±0.86	25.02±3.84	19.37±2.82	5.54±0.30	2.84±0.08
	1:1	K	99.51 ± 0.43	82.59±0.58	31.96±1.08	26.40±0.80	4.65±0.04	2.71±0.19
	1:1.5	L	99.57 ± 0.49	86.26±1.13	39.72±0.99	34.25±0.50	3.82±0.04	2.55±0.06
	1:2	M	99.24 ± 0.49	88.18±0.85	42.23±2.56	36.23±1.89	3.77±0.18	2.58±0.03
PEG-PEI PLGA NPs	1:1.5	N	99.39 ± 0.26	88.23±1.38	39.17±1.77	34.54±1.09	3.83±0.10	2.43±0.08
6-C-PLGA NPs	1:1.5	O	99.38 ± 0.41	88.08±0.49	38.14±1.57	35.10±1.55	3.84±0.01	2.37±0.03
PEG 6-C-PLGA NPs	1:1.5	P	99.31 ± 0.26	87.05±1.11	40.14±1.86	34.93±1.22	3.77±0.08	2.42±0.05

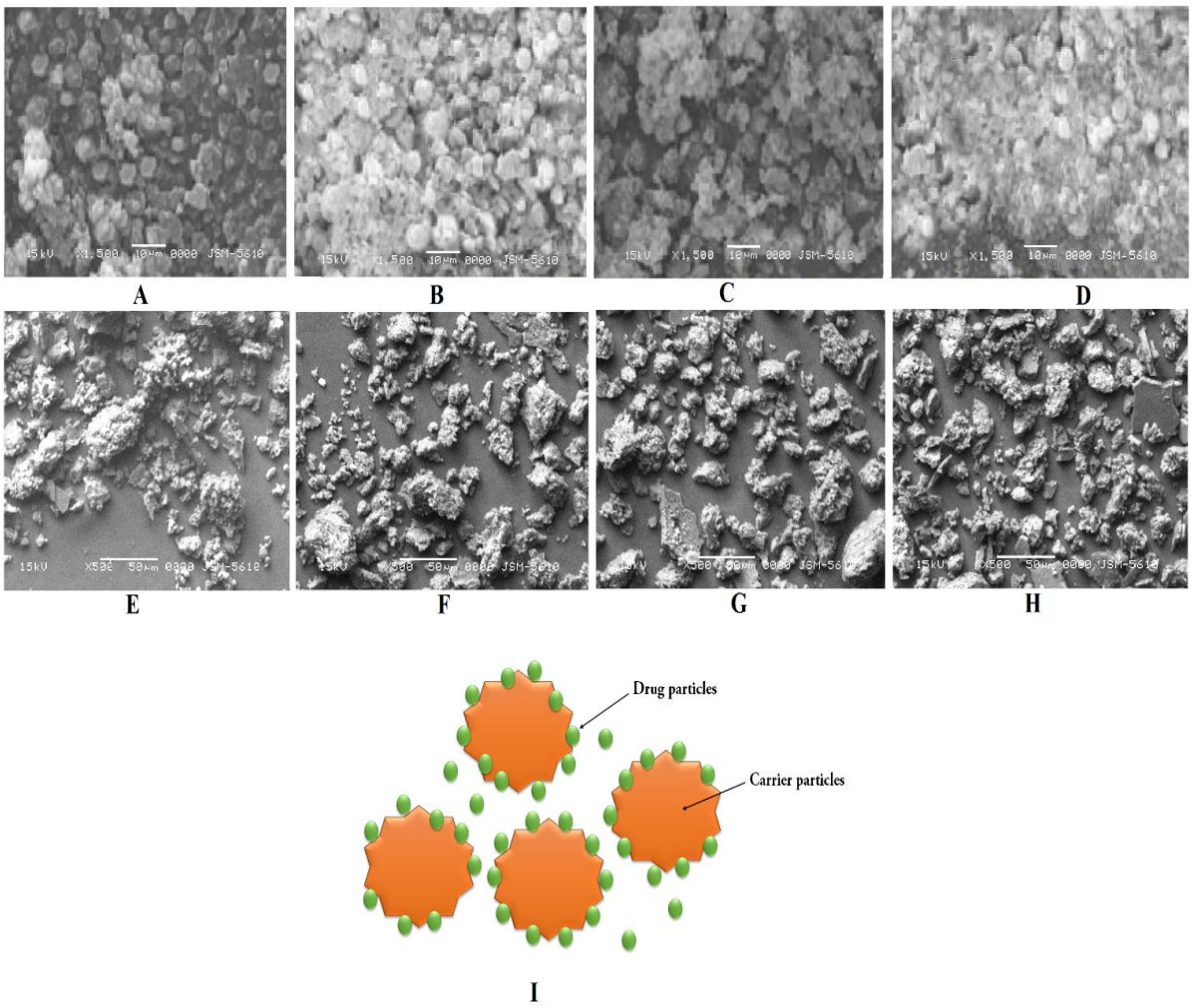

Figure 6.1: SEM image of A) Lyophilizeed NSDS PLGA NPs; B) Lyophilizeed PEG NSDS PLGA NPs; C) Lyophilizeed PEI PLGA NPs; D) Lyophilizeed PEG PEI PLGA NPs; E) Lyophilizeed NSDS PLGA NPs + Lactohale 201 (1:1.5 ratio); F) Lyophilizeed PEG NSDS PLGA NPs + Lactohale 201 (1:1.5 ratio); G) Lyophilizeed PEI PLGA NPs + Lactohale 201 (1:1.5 ratio); H) Lyophilizeed PEG PEI PLGA NPs + Lactohale 201 (1:1.5 ratio); I) Schematic representation of drug NPs loaded in the crevices of the carrier particle

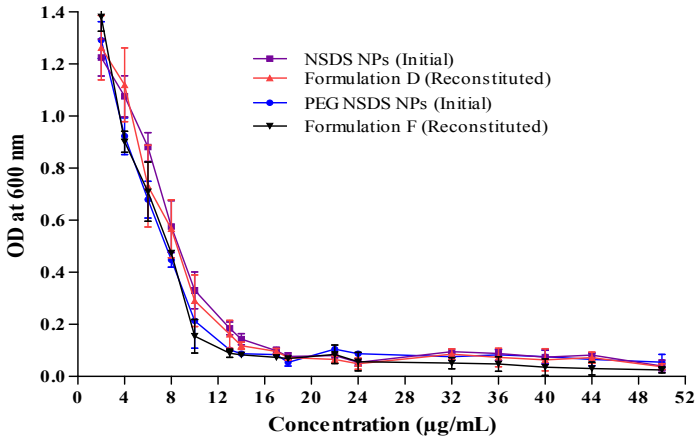

Figure 6.2: In vitro antibacterial activity of NSDS PLGA NPs (Initial), PEG NSDS PLGA NPs (Initial), Formulation D (Reconstituted) and Formulation F (Reconstituted) against *P. aeruginosa*. (OD = absorbance at 600 nm of UV-spectrophotometer measurement). We observed that there is no significant difference in MIC values for initial and reconstituted formulation. ($p > 0.05$; $p=0.174$ for PEG NSDS PLGA NPs (Initial) & Formulation D (Reconstituted); $p=0.109$; $p> 0.05$ for NSDS PLGA NPs (Initial) & Formulation F (Reconstituted))

6.4.4 Luciferase assay

Further effect of lyphilization and powderization on luciferase expression activity of the pDNA loaded NPs were also evaluated as described in earlier chapter. The NPs were reconstituted in Tris EDTA buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8). Reconstituted formulations were evaluated for their ability to transfect cells in culture. CFBE41o − cells were chosen as a relevant target cell line for therapy. Transfection efficacy for all particle preparations was determined by quantifying the expression of luciferase encoded on the plasmid on 2, 4 and 6 days after transfection and compared with initial formulations. Naked pDNA transfected cells were used as negative control and the Lipofectamine plus (Invitrogen) transfected cells were used as a positive control. Both dry powder formulations and initial formulations showed similar transfection efficiencies in the cell lines used. In general, dry powder formulations seemed to increase gene expression slightly, but this increase was not significant (Figure 6.3). This indicates that the lyophilization and powderization doesn't affect the bioactivity of the pDNA loaded NPs.

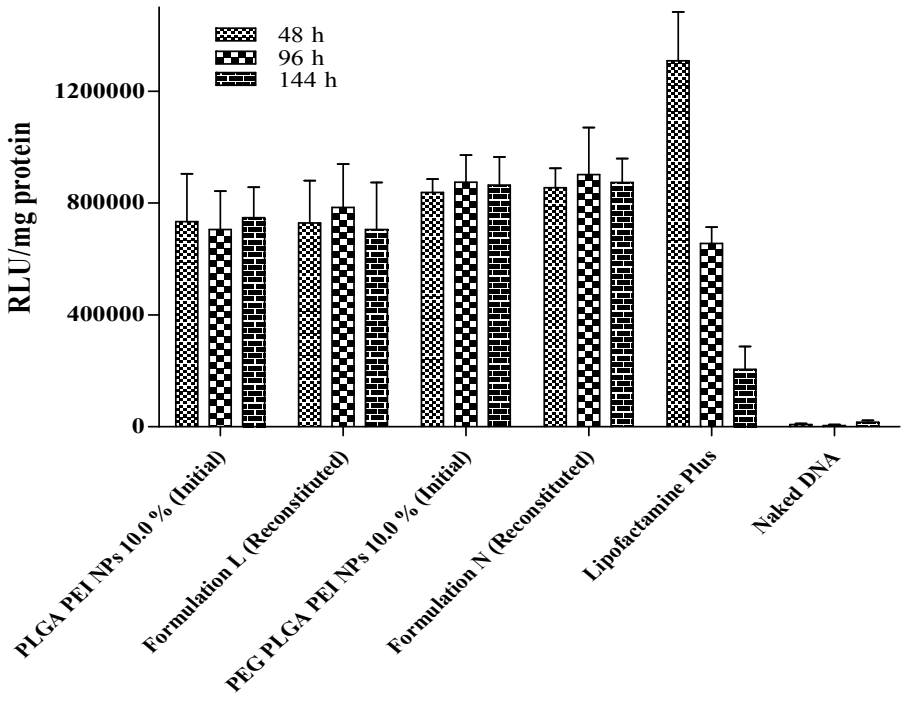

Figure 6.3: Transfection of CFBE cells treated with NPs encapsulating pCDNA-3 LUC-WT plasmid along with large T antigen and normalized to total protein content. Particle formulations contained PEI-PLGA NPs10 % (Initial), Formulation L (Reconstituted), PEG-PEI-PLGA NPs 10 % and Formulation N (Reconstituted). Transfection efficiencies were compared to Lipofectamine plus (prepared according to the manufacturer's instructions) as a positive control and pDNA as a negative control.

6.4.5 Particles uptake by rat alveolar macrophage

Macrophages are known to effectively ingest large particles (≥ 500 μm) by phagocytosis (30). MMAD between 1–5 μm is ideal for the effective distribution of the particles in the lung. However this optimal particle size range for inhalation is also ideal for phagocytosis and in-vivo inhaled dry-powder particles in this size range may be taken up by the resident macrophages as early as 1 hour post-delivery (31, 32). Thus here, we investigated the influence of the PEGylation of the polymeric particles with MMAD between 1–5 μm on the uptake by alveolar macrophages that reside on lung epithelial surfaces and are responsible for engulfing and removal of the inhaled particles. Here we used two types of the fluorescent particles one is PEGylated and other is non-PEGylated. Fluorescent markers used are Rhodamine b and 6-Coumarin. Rho-DS PLGA

NPs (Formulation G), PEG Rho-DSPLGA NPs (Formulation H), 6-C-PEI PLGA NPs (Formulation O), PEG 6-C-PEI PLGA NPs (Formulation P) were reconstituted in HBSS and used for evaluation of the macrophage uptake. We have observed that PEGylation of the particles reduces the macrophage uptake and thus their removal (Figure 6.4). It was reported that particles within the size range of 20–200 nm are favorably taken up by dendritic cell, whereas those within the range of 0.2–5 μm are preferentially engulfed by macrophages (33).

Also being a hydrophilic molecule, PEG reduces NPs electrostatic interaction with mucin, promote formation of hydrogen bonding with mucin thus promote NPs diffusion through mucus and mucus penetration of the particles and deliver active molecule to the epithelial cells. Thus PEGylation promote the mucus penetration of the inhaled particles and also prevent the removal by the phagocytic uptake.

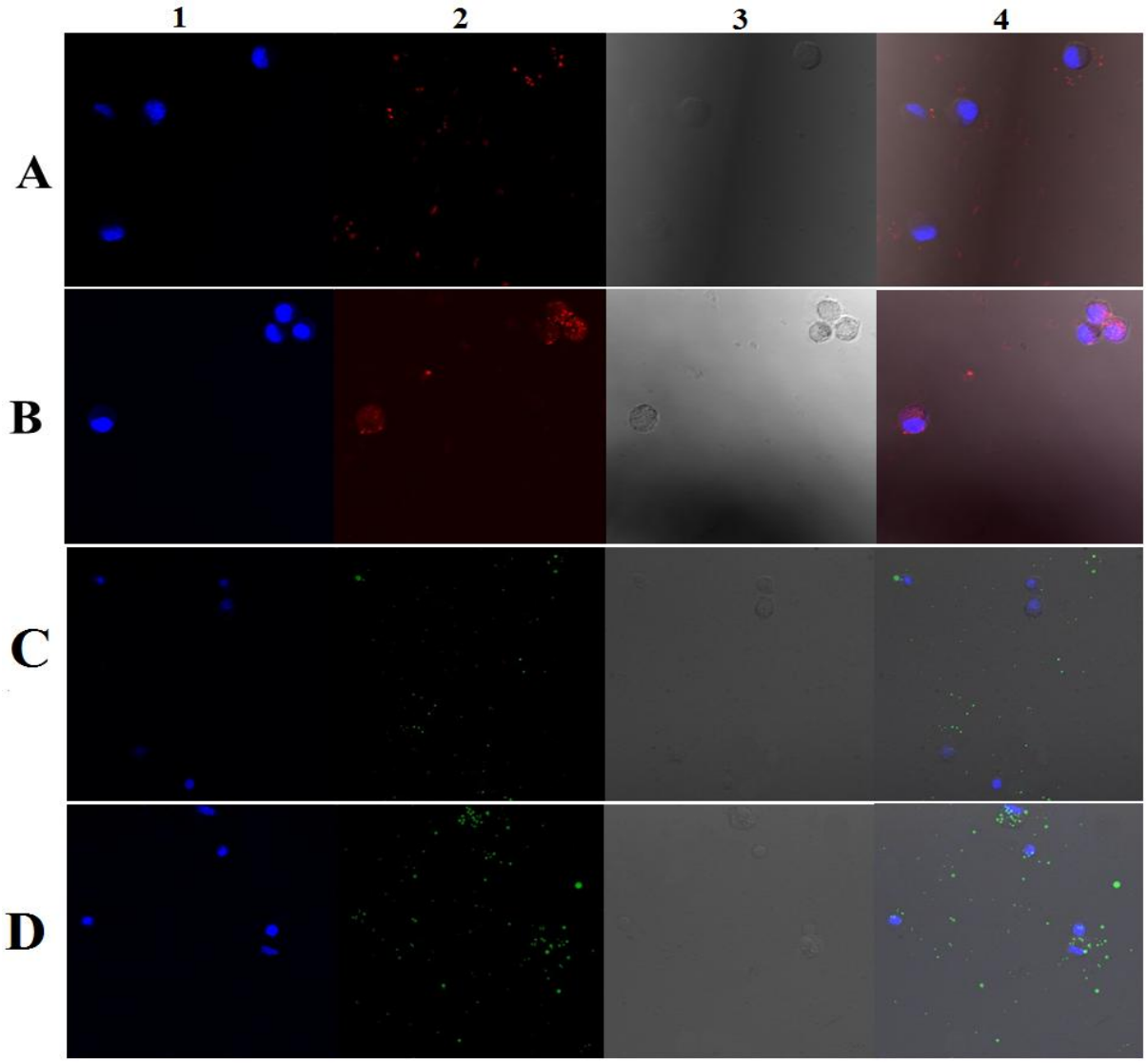

Figure 6.4: Uptake of particles by rat alveolar macrophages 1 h after incubation with (A) PEG Rho-DSPLGA NPs (Formulation H); (B) Rho-DS PLGA NPs (Formulation G); (C) PEG 6-C-PEI PLGA NPs (Formulation P); (D) 6-C-PEI PLGA NPs (Formulation O). Panel 1 shows macrophages nucleus stained with Hoechst dye, panel 2 shows fluorescent particles, panel 3 indicate the bright filed, and panel 4 indicate the merge image.

6.4.6 Particle deposition and clearance in rat lungs

The deposition pattern of the nanocomposite particles was assessed in vivo after administration of the selected formulations in rats by a syringe as described by Okamoto et al., 2000 with some modifications. It was observed that PEGylated formulations were distributed more extensively in

the lung tissues as compared with non-PEGylated particles (Figure 6.5). In addition to this qualitative studies we have also quantitated fluorescent intensity in the lungs on day 1, 3 and 6 post administrations of fluorescent particles. In case of Rhodamine B loaded particles, ~18% of PEGylated particles (Formulation H) were eliminated from the lungs, while ~35 % of non-PEGylated particles (Formulation G) were cleared off one day post administration of particles. While in case of 6-Coumarin loaded particles, ~14% of PEGylated particles (Formulation P) were eliminated from the lungs, while ~37 % of non-PEGylated particles (Formulation O) were cleared off one day post administration of particles. This difference in the clearance of the particles is consistent with particles uptake data presented in Figure 6.4 and distribution of particles observed in qualitative data presented in Figure 6.5. However, on 6^{th} day, about ~ 86 % of nonPEGylated particles were out of the lungs while about ~70 % of PEGylated particles were cleared out. The previous studies showed that ~ 90% of inhaled particles cleared of within 72 h (34). It is well known that inhaled particles are cleared from lung mainly by macrophage uptake and mucociliary clearance results in low therapeutic efficiency. These defense mechanism are main hurdles in effective lung delivery. Based on the data presented it is clear that PEGylated particles present desirable lung retention pattern, can produce sustained therapeutic effects with reduced accumulation of particles exhausted of drug.

6.4.7 Safety study

Cytotoxicity of the formulations was assessed by MTT (3-(4, 5-Di methylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay in CFBE 41o- cells, a human bronchial epithelial cell line. Results of the MTT assay are reported in Figure 6.6 (A & B). The percentage of cell viability was compared to control cells (100%) obtained at the same time under the same experimental conditions For both type of formulations (drug & pDNA loaded), positive control SDS treatments showed cell viability of only 10.74±1.92% (Figure 6.6 A) and 7.35±1.80% (Figure 6.6 B) after 24h of incubation when compared with control cells. All Formulations showed cell toxicity in dose-dependent fashion (Figure 6.6 A & B). These doses used were too high than the required dose. In case of drug formulations, though Figure 6.6 A shows improvement in the cell viability for PEGylated formulation (Formulation F) but it is not statistically significant. While in case of pDNA loaded formulations, it was observed that PEGylation significantly improved cell viability of formulation N (as discussed earlier). This effect could be due to reduced surface

charge of the cationic particles by DSPE-PEG addition (Figure 6.6 B). PEG shield the particle surface, reduces surface charge thus improve the cell tolerance.

Further, toxicity of the formulations was confirmed from TEER analysis on cells under air-interfaced culture (AIC) conditions (Figure 6.6 C). The presence of an intact cell monolayer was confirmed by steady TEER values (450−500 Ωcm^2) after 3 days of culturing and the monolayer was visible by light microscopy. After exposure to the formulations, TEER was measured at a defined time interval for 5 days. TEER depends on tight junctions between cells, which can be disrupted by noxious stimuli (35) and thus provides a convenient measure of barrier function (36). In case of drug loaded formulations, incubation of monolayers with Formulation D at a concentration of 1mg/mL, showed decrease in TEER but it was not significant, while Formulation F showed slight enhance in TEER indicating the safety of the formulation. While in case of the pDNA loaded formulations, formulation L showed decrease in TEER which is significantly different from the controlled while there was no significant difference between control and formulation N (for Formulation L and control, $p<0.05$; $p=0.005$; for formulation N and control, $p>0.05$; $p=0.09$). Taken together, these results suggest that the PEGylated formulations do not interfere with the integrity of the airway epithelial cells in vitro and may have little acute toxicity when used in vivo to treat lung disorders.

To evaluate in vivo safety, Bronchoalveolar lavage (BAL) studies was performed. The weight of wet lungs, expressed as ratio of lung weight per 100 g of body weight (L/B). For saline treated animals, lung weight was 0.47 while that for LPS treated animals was 0.89 (Figure 6.6). The increased weight for LPS treated animals suggested formation of edema due to accumulation of extracellular fluid into the epithelial cells of the respiratory wall. For formulations D and L, L/ B ratio were 0.61 and 0.63 respectively, indicating considerable lung injury or edema formation, while for formulation F and N L/B ratio were 0.50 and 0.49 respectively indicating no substantial lung injury. The enzymatic activities of lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) (injury markers) in BAL collected from the treated animals supports the obtained data of L/ B ratio. For Formulation D and L, activity of LDH and ALP in BAL was observed significantly different from that of control treatment, while that for Formulation F and N, no significant difference was observed between control and test formulation (LDH activity: For Saline and Formulation D, $p<0.05$; $p=0.01$; For Saline and Formulation F, $p>0.05$; $p=0.18$; For Saline and Formulation L, $p<0.05$; $p=0.03$; For Saline and Formulation L, $p>0.05$; $p=0.12$) (ALP

activity: For Saline and Formulation D, $p<0.05$; $p=0.01$; For Saline and Formulation F, $p>0.05$; $p=0.40$; For Saline and Formulation L, $p<0.05$; $p=0.03$; For Saline and Formulation L, $p>0.05$; $p=0.07$). However, level of ALP and LDHs in LPS treated animals were ~ 2.75 fold higher than those observed after administration of saline. Overall, data observed indicate the safety profile of PEGylated formulation.

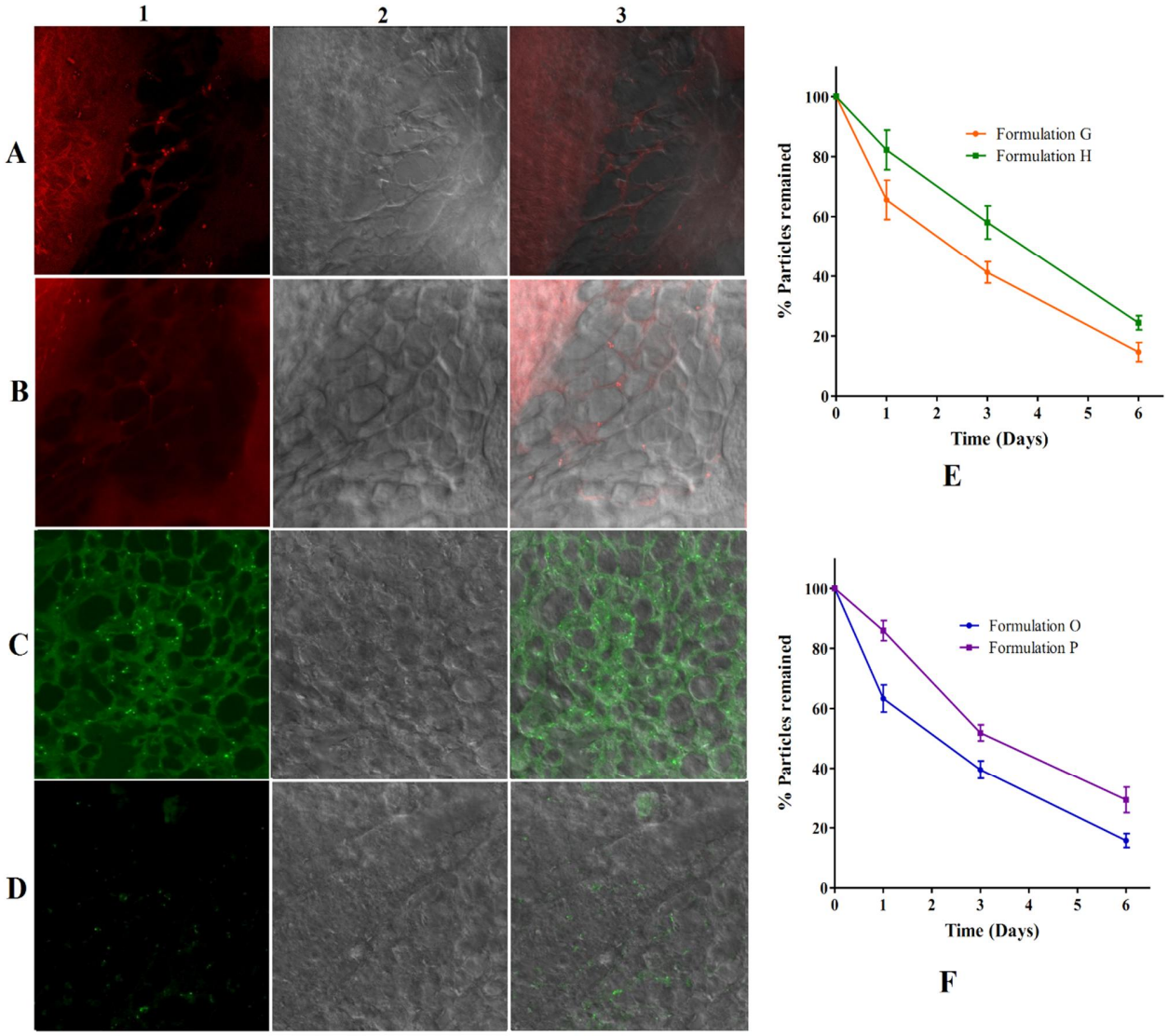

Figure 6.5: (A-D) Lung tissue sections imaging 24 h after administration of fluorescent particles: A) Formulation H; B) Formulation G; C) Formulation O; D) Formulation P; (E & F) Clearance of the particles from rat lungs after intratracheal administration

Histopathological analyses were also performed to evaluate the toxicity of the particles. After recovery of BAL fluid, Lung tissue was excised from the whole lung, fixed with 10% formalin, sectioned and stained with hematoxylin and eosin (H&E). Histopathological results showed good correlations with BAL analysis. An increase in the infiltration of inflammatory cells was observed with Formulation D and L as compared to the Formulations F and N and saline (Figure 6.6). While high level of inflammation was observed with LPS.

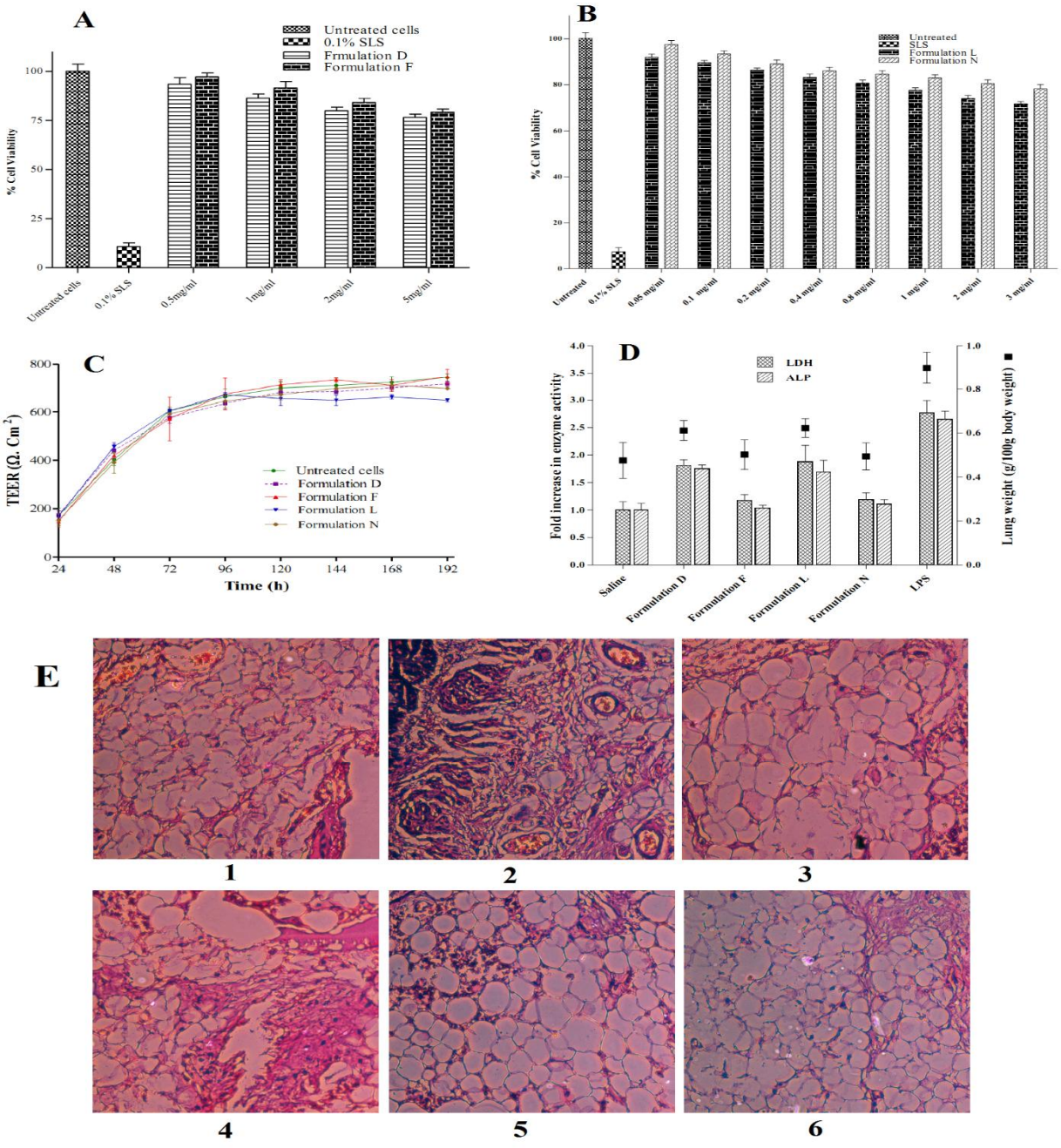

Figure 6.6: A) & B) Effects of formulations on viability of CFBE 41 o- cells after 24 h incubation. Data represent mean ± SD, (n=6); **C)** TEER analysis of CFBE 41 o- cells layers exposed to formulations; **D)** BAL fluid analysis post administration of formulations for lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) activities. ■ indicates wet lung weight per 100 g of rat weight. Data represents mean ± SE (n = 3) **E)**: Histological analyses of lung in hematoxylin and eosin stained sections. (1) Saline, (2) LPS, (3) Formulation D, (4) Formulation F, (5) Formulation L, (6) Formulation N.

6.4.8 Stability study

The stability studies were carried out, for optimized formulation F and N, in accordance with the ICH guidelines to evaluate the suitability of the storage condition for the developed formulations. It is very important to preserve the critical parameters i.e. mean particles size, water contents, % EE to ensure its stability during storage. After each time points, formulations were reconstituted and evaluated for particle. All formulations were easily dispersed and showed no significant change in particle size (Table 6.5). There was no significant difference in % EE of formulations at different storage time points (Table 6.5). There was no significant difference observed in moisture content of formulations on storage (Table 6.5). The lower moisture content in formulation on storage indicates the long term stability of formulations.

Table 6.5: Results of stability study (mean ± SD)

Storage Condition	Time	Particle size (nm) ± SD		% Water content ± SD		% EE ± SD	
		Formulation F	Formulation N	Formulation F	Formulation N	Formulation F	Formulation N
5 ± 3°C	Initial	241.66±7.50	201.86±6.20	1.07±0.12	1.20±0.04	96.01±1.02	99.90±0.17
	3 M	239.63±4.89	200.20±4.01	1.14±0.12	1.29±0.11	95.35±2.10	99.83±0.28
	6 M	241.43±5.27	205.23±4.13	1.19±0.17	1.32±0.15	96.05±1.68	99.75±0.42
25 ± 2°C /60 ± 5% RH	1 M	243.00±4.35	202.20±3.53	1.16±0.14	1.25±0.14	95.11±2.35	99.72±0.47
	2 M	241.80±4.01	204.20±4.57	1.23±0.07	1.27±0.13	95.81±1.33	99.69±0.53
	3 M	244.66±6.11	202.86±3.55	1.29±0.16	1.30±0.16	95.58±1.71	99.79±0.36
	6 M	242.83±4.25	203.53±4.22	1.32±0.10	1.31±0.14	95.31±2.15	99.82±0.30

6.5 Conclusion

Particulate drug delivery system is a good option to overcome the limitations of the lung delivery. Here we prepared dry powder formulations by lyophilizing nanoparticles and subsequently sieved to obtain dry powder formulations. However poor aerosolization properties of these formulations depicted the need of the carriers to improve the aerosolization properties of the lyophilized powder. It was observed that addition of the inhalable carrier significantly improved the aerosolization properties of the formulations. SEM image clearly showed that the nanoparticles were adhered on the surface of the carrier and formed nanocomposite particles. It was observed that, there was no impact of lyophilization and powderization on the luciferase activity of the pDNA loaded particles, indicate the efficiency of the process. Further, it was observed that PEGylated particles show lower macrophage uptake as compare to non-PEGylated particles. PEGylated particles present desirable lung retention pattern, showed better safety profile, can produce better therapeutic effects.The results of the study depict that dry powder formulations of PEGylated nanoparticles have better therapeutic efficacy and can be used for effective treatment of the cystic fibrosis.

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