"Development of siRNA therapeutics for the treatment of obstructive airway disorders"

Synopsis of the PhD thesis to be submitted to

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1. Introduction

Asthma and chronic obstructive pulmonary disease (COPD) represent the leading obstructive airway disorders in the world, owing to their high prevalence and high burden on the health system. Asthma is a chronic disorder of the airways generally characterised by airway inflammation, airflow obstruction and airway hyper responsiveness. Allergen sensitization is the most important risk factor for the development of the asthma. As per WHO report, it is estimated that currently 235 million people suffers from asthma and about 250,000 annual deaths worldwide.Chronic obstructive pulmonary disease (COPD) is characterized by a progressive airflow limitation that is partially reversible, a chronic lung inflammation and systemic effect.

Asthma is characterized as recurrent reversible airway obstruction, with wheezing, breath shortness and frequently night-time cough and its main feature involves the airway inflammation, which causes the airway hyper-responsiveness and results into in reversible airway obstruction. Pathogenesis of the asthma involves, following the exposure of the allergens or stimuli activates the T cells with a Th2 profile of cytokine production, in mucosa, which attract inflammatory granulocytes following release of several other inflammatory mediators which cause damage to epithelium and one of the cause of airway hyper responsiveness.Key mediators such as leukotriene B_4 and cysteinyl leukotrienes (C_4 and D_4); interleukins IL-4, IL-5, IL-13; and tissue-damaging eosinophil proteins are released and mediates an strong, instantaneous inflammatory reactions.

Neurotrophins, growth factors family includes Nerve growth factor, Brain Derived Neurotrophic Factor, NT-3 and NT-4/5 at first found in the nervous system, mediate inflammatory response through immune and structural cells. It has been alsofound that neurotrophins express outside the nervous system as well, particularly in immune cells. Nevertheless, majorly studies focused on inflammatory and allergic conditions and so their outcome reflects rather the underlying disease pathophysiology than the normal physiology. Neurotrophins trigger such neuroimmune interactions as well.

1.1 RNA Interference by silencing RNA (siRNA)

siRNAs, which are small molecules of RNA with exogenous origin, have demonstrated gene silencing of the over expressed gene very effectively and specifically. siRNAs having greater gene-silencing ability demonstrated stability in biological fluids. Silencing of the gene can be

provoked by siRNAs by specific cleavage of complementary mRNA. Delivery of siRNA to the airways can occur without the use of any vector or transfection agent (naked siRNA), or siRNA can be complexed with different vectors to increase stability and improve cellular uptake (viral vectors, cationic lipids, polymers, nanoparticles, and chemical modifications).

Neurotropins,the key mediators in the neuronal plasticity and neurogenic inflammation, have been found to be increased in bronchoalveolar lavage fluid of patients with allergic asthma and COPD. In an animal model, Neurotropins production was shown to be upregulated in cells infiltrating asthmatic airways, including macrophages and T cells. So, neurotropins may be potential targets in the airway inflammation and hyperreactivity conditions to treat asthma and chronic airway obstruction. So, gene silencing by the RNA interference mechanism by siRNA is promising approach in the airway inflammation and hyperresponsiveness by targeting the neurotropic factor (BDNF, which is highly upregulated in the inflammation).

Proposed delivery of the selected siRNA therapeutics via pulmonary administration by formulating siRNA into the suitable nanocarriers systems to treat airway inflammation and airway obstruction in the asthma and COPD. Due to the negative charge of the siRNA, it cannot cross the cell membrane. So by utilising different non-viral vectors or carriers like cationic polymers or polycations making complex with siRNA, it can be deliver to specific cellular target. These vectors or carriers assist to siRNA to escape from endosomes after cellular internalisation and thereby preventing lysosomal degradation and releasing the siRNA therapeutics payload into the cytoplasm, where down regulation and gene silencing of the target mRNA occurs by RNA interference mechanism.

Objective of the research work

The objective of the proposed research work is development of safe non viral vectors and characterization of therapeutic siRNA delivery system for treatment of obstructive airway disorders via pulmonary route.

Hypothesis

It is hypothesized that development of safe non-viral vectors or carriers to deliver siRNA therapeutics by pulmonary route will improve the therapeutic efficacy of the treatment of obstructive airway disorders with minimum toxicity.

Research Plan:

Development of non viral vectors or carriers based siRNA therapeutic delivery in asthma and COPD requires the higher transfection efficiency with minimum toxicity and preserving stability of formulation during aerosolization.

So, objectives of the research work are,

1. Development of the suitable non-viral vectors or carriers focusing the safety and complexing ability of the carriers.
2. Characterization of the developed carriers in terms of buffering capacity and proton sponge effect, condensation ability to siRNA by gel retardation assay and physicochemical characteristics.
3. Development and evaluation of the formulations containing siRNA in terms of *In vitro* cytotoxicity by MTT assay using suitable cell line and cellular uptake.
4. Development of nebulised form of siRNA formulation and evaluation in terms of siRNA integrity and formulation stability and performance during nebulisation.
5. Evalution of the formulation in suitable animal model.

2. siRNA Profiling and Analytical Method Development

2.1 .Selection of siRNA

Selection of the siRNA (brain derived neurotropic factor (BDNF) a member of neurotropin family) which is highly up regulated in the airway inflammatory conditions of bronchial asthma and in chronic obstructive airway conditions and also believed to be involved in the neurogenic inflammation and also involves in the smooth muscle contractility of the airways results in airway hyper reactivity and airway obstruction. Neurotropins also involves in crosstalk between nervous systems and immune system. So by knocking down the expression of BDNF mRNA by siRNA can be promising therapeutic approach to treat obstructive airway disorders.

2.2 siRNA Profile

There are several thermodynamic and biophysical necessities such as GC content, Tm, MW, purity, which all confirms the siRNA functionality. The guanosine/cytosine (G+C) content of each duplex in most highly functional siRNAs ranges between 36% and 52%.

The relative stability and propensity to form internal hairpins can be estimated by the predicted melting temperatures (T_m). Sequences with high T_m values favor internal hairpin structures reducing their activity. Duplexes exhibited T_m< 60°C or predicted hairpin structures are better silencers.

The characteristics of selected siRNA:

- ✓ MW [g/mol]: 13300
- ✓ T_m [°C]: 57.5
- ✓ Purification: HPLC
- ✓ GC-Content [%]: 42.8
- ✓ Length: 21-mer

The siRNA was purchased from Sigma Aldrich. The obtained siRNA as a dry pellet form was desalted,analyzed by the HPLC and diluted by the siRNA dilution buffer to make required concentration of stock solution for the experimental work. The molecular weight of siRNA was 13300 g/mole which will be used for calculation of N/P ratios of the complex.

2.3. Analytical Method Development

2.3.1. UV Spectrophotometric Analysis of siRNA

(i)Preparation of nuclease free water:

1 ml of diethyl polycarbonate was mixed with the 1 litre of double distilled water and kept for stirring overnight on stirrer. Further, prepared water was autoclaved at 121°C for the 15 minutes at 15 psi.

Apparatus were washed by DEPC treated water to neutralize the RNases and DNases. Purity of the siRNA was checked using the Nanodrop UV spectrophotometer.

To estimate the known concentration of siRNA, UV spectrophotometric analysis was performed. Different serial dilutions of siRNA were made in nuclease free water (NFW)from stock solution prepared in NFW.Absorbances of these solutions as well as stock solution were recorded at 260 nm on a NanoDrop UV spectrophotometer.

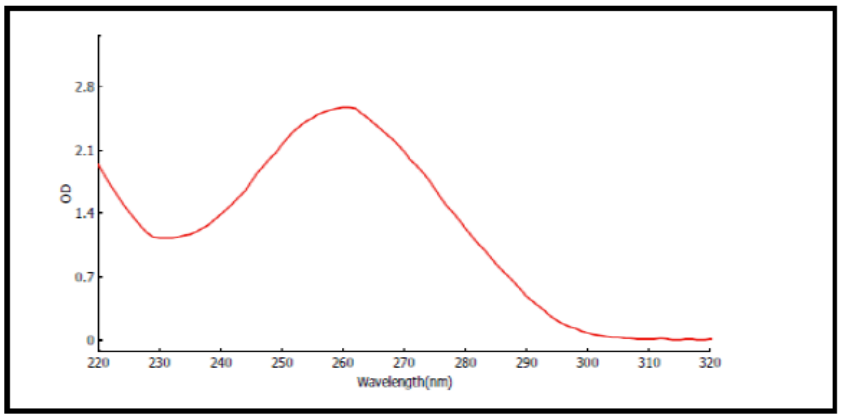


Fig.1: Characteristic peak of siRNA at 260 nm

2.3.2. siRNA Gel electrophoresis: Gel retardation assay.

Owing to the negative charge of siRNA, it migrates on the gel towards positive charge under the applied electric current. Based on this principle, agarose gel electrophoresis was used for the relative quantification of free siRNA migrated on the gel based on the size. Cationic non-viral vectors for siRNA should be able to complex with negatively charged siRNA.

To check the ability of the developed vectors for its complexation ability with siRNA, agarose gel electrophoresis assay was performed by using ethidium bromide as a RNA binding dye.

Depending upon the size of the molecule,DNA and RNA migrate through an agarose gel matrix. The smaller the molecules are, the farther they will migrate on the gel. Ethidium bromide, fluorescent dye is generally used to visualize DNA or RNA on the gel, which intercalates into the minor groves of double-stranded nucleic acid strands. Complex of the siRNA and developed cationic vectors retard the migration of siRNA. And the quantity of the migrated free siRNA would give a direct idea of the quantity of complexed siRNA.

2.3.3. Method of Analysis

Agarose gel was prepared by the dissolving agarose in100 ml of 1x TBE (Tris-Borate-EDTA) buffer with the aid of heating on a heating mantle until clear solution is obtained with intermittent shaking. Melted agarose was allowed to cool to a pourable consistency.

Till then, the gel tray was tightly secured at both the ends with tape to fluid-tight seal and comb was placed in the gel casting tray. Ethidium bromide was added (0.5µg/mL) to the

cooled agarose (to about 60°C) and mixed properly. Ethidium bromide loaded gel was poured in the gel casting tray up to 4-8 mm height. Gel was allowed to set at 20°C for 30 min followed by 15 min refrigeration for complete solidification of gel. Comb was removed from solidified gel and tapes were taken off the edges of gel tray. Gel was submerged in electrophoresis chamber with 1x TBE buffer.

Fig 2: Agarose gel

2.3.4. Determinationof quantifiable amount of siRNA

Different concentrations (10-200 pmole) of the siRNA solutions were prepared and evaluated by agarose gel electrophoresis for the determination of quantification amount of the siRNA. siRNA solutions were mixed with the gel loading buffer containing bromophenol blue 0.25% w/v and sucrose 50% w/v in 0.5 ml Eppendorf tubes for loading into agarose gel. 15 µLof each siRNA solution was loaded in to the wells and electrophoresed at 100 V/cm voltage for 20 mins. Post-run, gel was removed and migrated siRNA was visualized under UV light using GelDoc™ XR^+ Imaging System. Quantifiable amount of siRNA was found to be 30 pmole concentration.

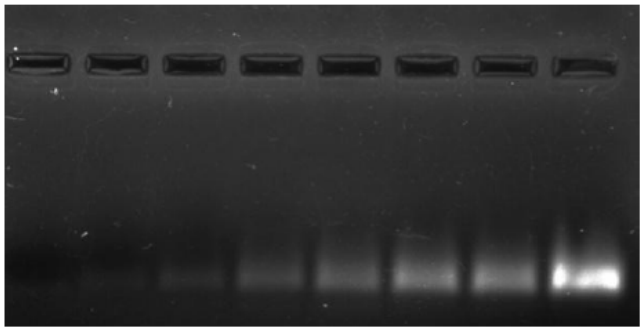


Fig 3: Determination of Quantifiable Range of siRNA

[Lane (L→R) 10 pmole, 20 pmole, 30 pmole,40 pmole, 50 pmole, 75 pmole,100 pmole,200 pmole.]

2.3.5.Relative Quantification

siRNA solutions of different concentrations (30-50 pmoles) were prepared and mixed with gel loading buffer and performing the gel electrophoresis as described above. Relative band densities of other siRNA concentrations were determined by taking the band density at 50 pmole concentration as 1 and evaluating other band densities relative to 50 pmole conc.

Concentration of siRNA (pmole)	Relative Band Density	%RSD
30	0.58±0.011	2.41
35	0.67±0.012	1.82
40	0.78±0.01	1.52
45	0.89±0.010	1.08
50	1.000±0.000	0.00

Table 1: Relative band densities at different siRNA concentrations

3. Formulation and development

3.1. Selection of carriers or vectors

[A] PEI based vectors

For Delivery of the negatively charged siRNA to the lung via pulmonary route requires the suitable nontoxic cationic charged delivery vectors or carriers which having adequate complexation ability. Several types of non-viral polymeric vectors have been extensively studied for the gene delivery for the treatment of the various diseases including lung diseases. Polycations such as Polyethelenimine(PEI), chitosan, poly-l-lysine etc. are the most explored carriers for the siRNA or DNA delivery. However, polyethylenimine was extensively studied and one of the best transfection agent for gene delivery as its buffer capacity and proton sponge effect, its cytotoxicity limits their applications. And to overcome the problem of cytotoxicity and to improve transfection efficiency, several modification and conjugation strategies for the PEI and have been sought.

3.2.Modification of PEI

PEI was dissolved in 5 mL of MES buffer (0.1 M, pH 6.0) and Urocanic acid was dissolved in 5 mL of DMSO. The dissolved Urocanic acid was added to the PEI solution at 4°C. NHS/EDC was mixed in 5 mL of 0.1 M MES buffer. The prepared NHS/EDC was

added to the PEI and Urocanic acid mixture solution. The reaction was performed overnight. The resulting solution was adjusted to pH 7 with 1M HCl. The product was dialyzed against water for three days and consequently lyophilised to solid form.

3.3. TNBS Assay

Free primary amine concentrations of the modified PEI was estimated by trinitrobenzene sulfonic acid (TNBS) assay. TNBS reacts with primary amino groups of polymers making it possible to estimate the percentage of substituted primary amines in modified PEI as compared to unmodified PEI. Briefly, 250 µl of freshly prepared aqueous TNBS solution (0.01%w/v) was added to 500 µL of modified PEI dissolved in distilled water. Mixed well and incubated it for 2 hrs at 37°C. After that 250 µl of 10% SDS solution and 125 µl of 1 N HCL were added to the sample.Histidine was used for the development of standard calibration curve of concentration ranging from 5-30 ppm in 0.1 M sodium bicarbonate buffer pH 8.5.The absorbance was read at 410nm using a UV spectrophotometer. Different percentage of conjugation was achieved of 50-60%on the PEI.

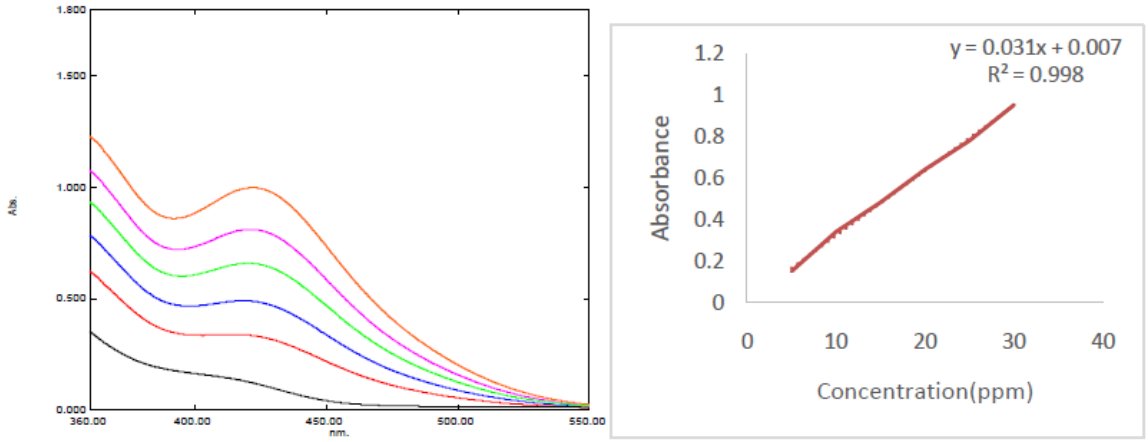


Fig 4: Calibration curve for TNBS assay

3.4. FTIR Analysis

Modified PEI

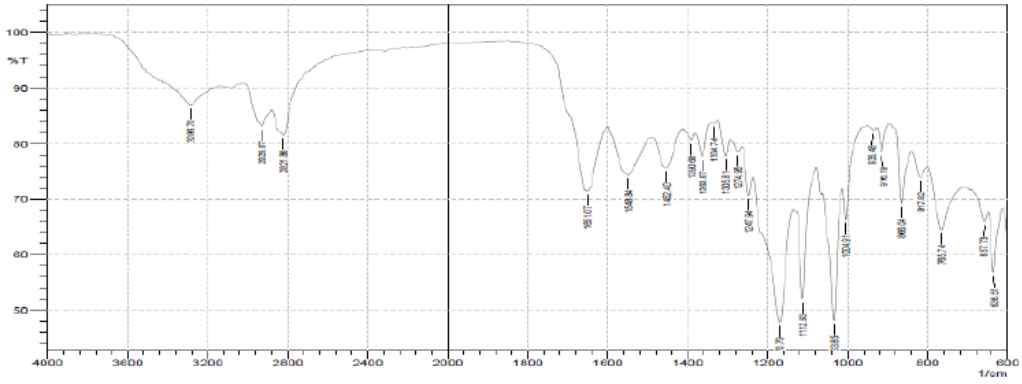


Fig 5: FTIR of Modified PEI

3.5. siRNA complexation ability by Gel retardation assay

Minimum amount of vectors required to make complex with siRNA was estimated by gel retardation assay and gel was visualised under UV light using GelDoc™ XR$^+$ Imaging System.

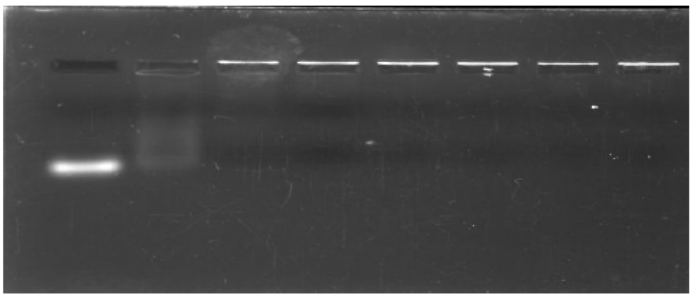


Fig 6: Gel retardation assay

3.6. Proton sponge effect

After internalisation of siRNA-carrier complex into the cell, endosomal escape is important step to release the siRNA payloads into the cytoplasm. Therefore it is essential for carriers to have efficient buffer capacity in the endosomal pH range. Proton sponge effect study was performed for developed carriers by dissolving the carriers into the 150 mM Nacl solution and titrated against the 0.1 N HCL/NaOH. Consumed volume of titrant was calculated.

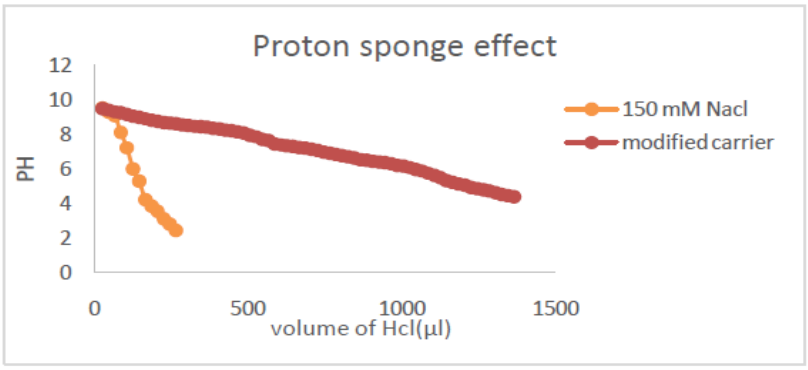


Fig 7: Proton sponge effect

3.7. Haemolytic potential : Developed vectors are characterised for the biocompatibility and toxicity profile and so for that vectors are subjected to haemolysis study.Study was carried out by collecting the blood samples from the rat in heparinized tube and blood was washed with saline solution and subjected to centrifuge until supernatant was clear. Pellet of RBCs was collected and suspended in saline solution. Prepared vector solution was incubated with RBC suspension at 37°C for 30 min and after that sample was centrifuged and supernatant was analyzed by UV spectrophotometer at 540 nm for hemolysis.

Modified vectors did not exhibit the any significant hemolytic potential while unmodified form of polymer shows the hemolytic activity at some extent which is due to cationic primary amines.

3.8. Erythrocyte aggregation: Aggregation behaviour of erythrocytes collected from the rat blood was studied under microscopy. Developed vectors did not show any aggregation of erythrocytes.

3.9 Preparation of Polyplex

Polyplex formulation was prepared by the incubating the polymer solution with the siRNA for specific period of time. Briefly, in 0.5 ml micro centrifuge tube, DEPC treated nuclease free water was taken and required concentration of polymer solution was added after suitable dilution. The mixture was vortexed for 10 mins and then, siRNA was added and incubated for 30 min at 37°C. Various ratios of polymer and siRNA were used to optimize the polyplexes with different N/P ratios.

Fig 8: siRNA polyplexes at different N/P ratios from 1 to 20

3.10 Particle size and zeta potential

The Particle size and zeta potential of polyplex was determined by dynamic light scattering with a Malvern Zetasizer. Appropriate dilution of polyplex formulation was made with nuclease free waterprior to the measurement and then measurement was carried out at 25°C. Zeta potential calculation was performed by ZetaSizer software using Smoluchowski's equation from the electrophoretic mobility. Particle size was found to be in range to 100-300 nm with different ratios and zeta potential was found to be positive.

[B] Chitosan based vectors

Chitosan is biodegradable and biocompatible polymer with polycationic in nature and due to its positive charge, it can be bound with the negatively charged siRNA or DNA and thereby it can protect the nucleic acids from the degradation by nucleases. Nevertheless,chitosan has its limitations due to his low water solubility at physiological pH, which may be challenging for the *in vivo* delivery of the siRNA. Chemical modifications have been sought for chitosan to improve the solubility and also transfection. Quarternization (trimethylation) of the amino groups of chitosan is one of the approach to improve the solubility at wide pH range.

3.11 Synthesis of the Trimethylated Chitosan

Quaternization was based on a reductive methylation procedure using methyl iodide in an alkaline environment. 0.5 g of chitosan was dissolved in 20 ml N-methylpyrrolidinone (NMP) in a waterbath at 60°C. Quantity of 7ml of a 5% (w/v)NaOH solution was added which provides the required alkaline environment. The methylation started by adding 6ml of CH_3I to the solution. Reaction mixture was stirred for 60-90 min. The product was precipitated using a mixture of ethanol and ether (1:1), isolated by centrifugation at 10,000

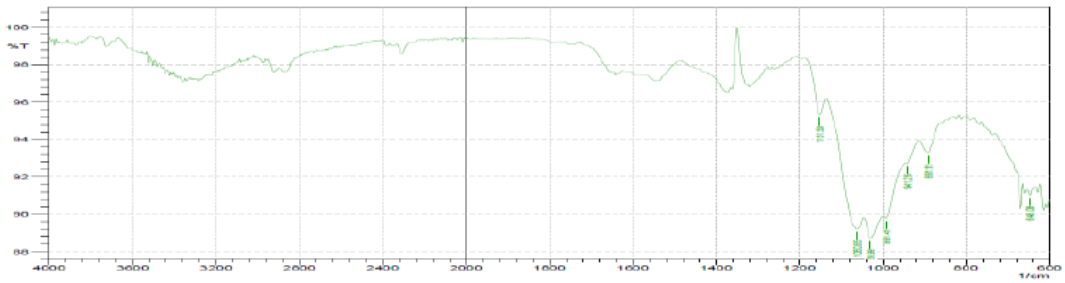
RPM by Remi centrifuge and thoroughly washed with ethanol and ether twice. The product was dried at 40°C and further characterized. Reaction parameters were optimized to get desire degree of quarternization of the 20-40%.

3.12 Conjugation of modified chitosan

Modified chitosan was coupled with urocanic acid in different mole ratio via an active ester intermediate using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) chemistry. Briefly, Modified chitosan was dissolved in 50 ml of MES buffer (25 mM, pH 6.0). Moreover, the carboxyl group of urocanic acid was activated by the N-hydroxysuccinimide (NHS)/EDC dissolved in 10 ml of MES buffer. The activated UA solution was added to the chitosan solution and allowed to react with stirring for 24 h at room temperature. The resulting polymer solution was dialyzed against distilled water. After dialysis, the polymer solution was centrifuged at 10,000 rpm for 10 min, and the precipitant was obtained and washed by repeated centrifugation and water addition. The resulting solution was lyophilized. In addition, the free amino groups of conjugated chitosan in solution were determined by TNBS assay.

3.12 FTIR Analysis

Chitosan



Modified chitosan

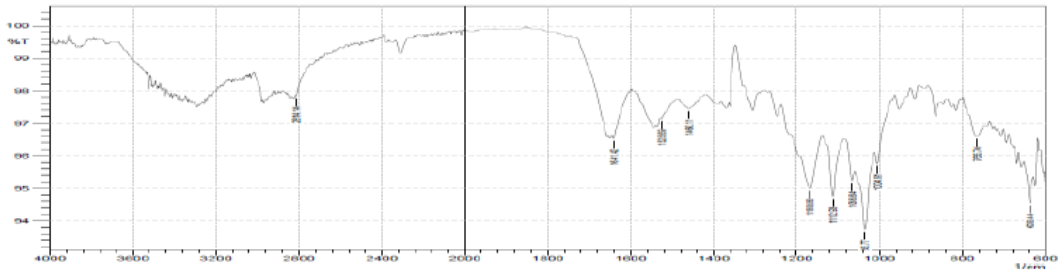


Fig 9 : FTIR of Chitosan and Modified chitosan

3.13 Solubility improvement

Solubility improvement of modified chitosan was studied by turbidimetric method. Briefly, chitosan was dissolved in 0.3% acetic acid solution (1 mg/ml), the pH of the solution was adjusted with1N NaOH and the transmittance of the resulting solution was measured at 600 nm as a function of pH value. The results showed that the modified chitosan produce higher transmittance.

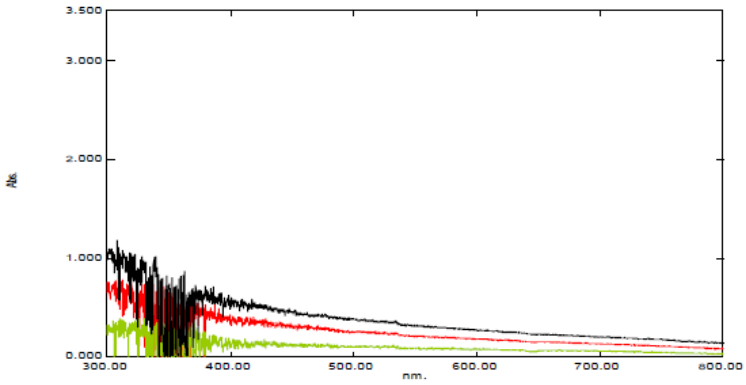


Fig 10: Solubility analysis by turbidimetry
(**1-** Chitosan at pH 7.0, **2-** chitosan at pH 6.5, **3-** Modified chitosan at pH-6.5)

3.14 Proton sponge effect

Proton sponge effect study was performed for developed carriers by titrating against the 0.1 N HCL/NaOH. Consumed volume of titrant was calculated. It is very important to have good buffering capacity and proton sponge effect for the escape of the siRNA complex for the release of its therapeutic payload into the cytoplasm. Modified carriers demonstrated good proton sponge effect.

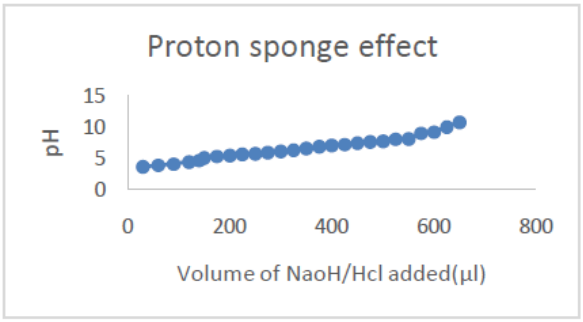


Fig 11: Proton Sponge Effect

3.15 siRNA binding ability of the modified chitosan

siRNA binding ability of the developed vectors were assessed by the gel retardation assay by with different ratios of the vectors with siRNA. Results demonstrates that vectors efficient bind with the negatively charged siRNA.

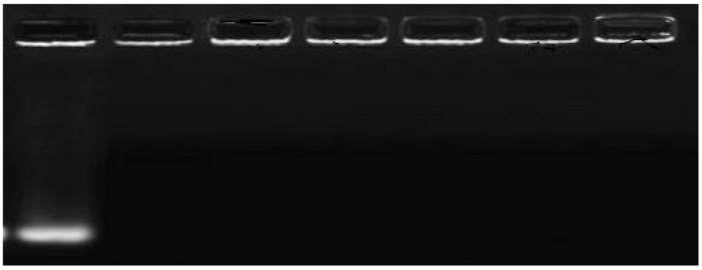


Fig 12: Gel retardation assay

3.16 MTT assay

MTT (3-(4, 5-dimethylthiazole-2-yl)-2,5-di-phenyl tetrazolium bromide assay was used for determination of cytotoxicity of various polyplex formulations. Alveolar epithelial cells were seeded onto a 96-well plate at a cell density of 5×10^3 cells per well and allowed to grow in DMEM medium containing antibiotics and 10% FBS in humidified air at 5% CO_2 concentration for 24 hrs.

After 24 hrs of incubation, cells were separately treated in triplicate with developed formulations. Then, the transfecting medium was replaced with complete medium. The cells were incubated for 48 hrs and then treated with 20µL of 5 mg/mL solution of MTT. After 4hr of incubation with MTT solution, the culture medium was removed and 200 µL of DMSO was added and solubilized formazan dye dissolved in DMSO was measured by colorimetry at 570 nm using an ELISA plate reader and cell viability was calculated.

Results demonstrates that the chitosan and chitosan modified vectors exhibited the overall greater than 90% of the cell viability due to the nontoxic and biocompatible nature of the vectors. On the other hand, modified PEI based vectors revealed high cell viability than unmodified one,which is due to the cationic charge of the primary amines cause cytotoxicity.

Cellular uptake

In order to perform cell uptake studies FAM labelled negative control siRNA (FAM-NC-siRNA) was used. The lyophilized stock supplied was reconstituted with NFW in amber

coloured tubes and used for experiments. For quantitative measurements flow-cytometry was used while qualitative evaluation was conducted using confocal microscopy.

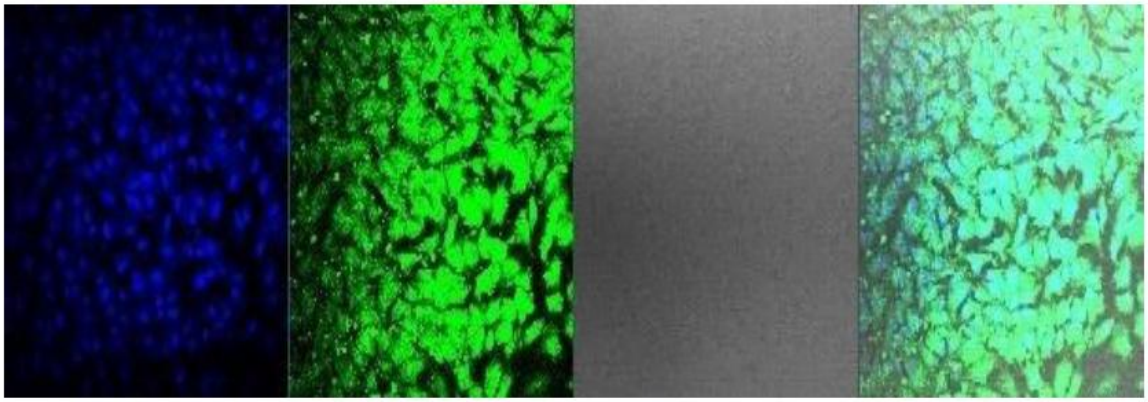
Confocal microscopy

The laser scanning confocal microscope (LSCM) is an important element of modern day biomedical research. In a conventional microscopy, the entire specimen or samples illuminated from mercury of xenon source. Nevertheless, in confocal microscopy the illumination is achieved through scanning one or more laser beams across the specimen to create an optical section of specimen in a non-invasive way. Further, It utilizes confocal pinholes that allow light coming only from the plane of focus to reach the photomultiplier tube detector and excludes the 'out of focus' light coming to the detector. This enables imaging of the living specimens and generation of 3-dimensional (3D) data in the form of Z-stacks.

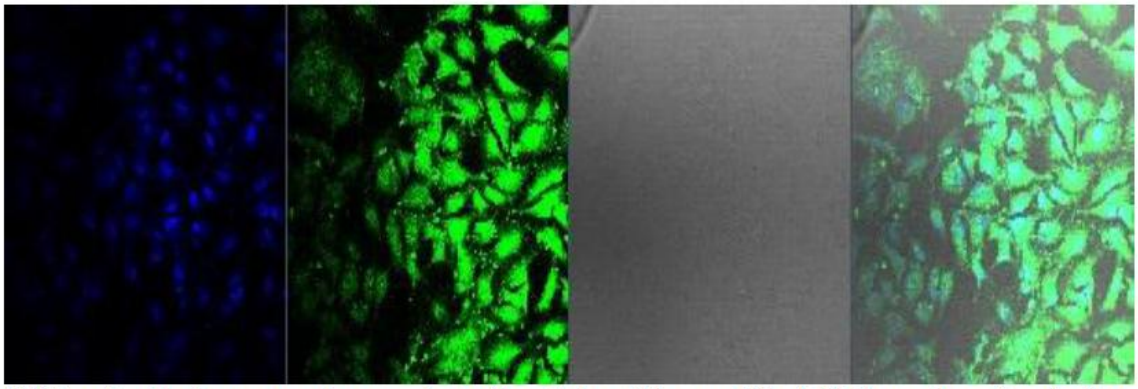
The optical path is based on conventional reflected light wide-filed epifluorescence microscope with a point light source and a pinhole in front of detector which are confocal with each other. The specimens are labeled with one or more fluorescent probes. The confocal microscopy also offers the advantage greater resolution due to use of highly sensitive photomultiplier tube detectors. In cellular biology, Confocal microscopy has been used for visualizing intracellular organelles, cellular uptake, intracellular localization of drugs and drug delivery systems using fluorescent probes. Cellular uptake studies were performed as per following section on A549 alveolar epithelium cell line.

Protocol: Alveolar epithelial Cells-A549 were seeded at a density of 10^4cells/well in a 24 well plate containing 0.17 mm thick flame sterilized cover glass and were allowed to grow for 24 hr DMEM at 37 °C temperature and 5% CO_2. After 24 h cells the media was removed and cells were washed with sterile PBS. Then the cells were exposed to formulations containing FAM labeled negative control siRNA (FAM-NC-siRNA) at siRNA concentration of 100 nM. After 6 h of exposure cells were washed with PBS for twice to ensure removal of residual formulation. Then the cells were fixed with 4% paraformaldehyde solution (1 mL/well) and incubated at room temperature for 3-5 min. The paraformaldehyde was immediately removed after exposure time and cells were washed with PBS three times accompanied by intermittent shaking for each wash to remove the traces of paraformaldehyde. Then the nuclei of the cells was stained with 4',6-diamidino-2-

phenylindole (DAPI) at 1 ug/mL concentration with enough volume to cover the cells and kept for 15 min at room temperature for dye permeation under protection by aluminum foil. Then cells were washed once with PBS. The coverslips were mounted on glass slide using PBS:glycerin solution (50:50 and confocal microscopy was performed using confocal laser scanning microscope.The negative control for the experiment was Naked FAM-NC-siRNA and positive control was Lipofectamine 2000 (L2K) complexed siRNA. The results of confocal microscopy showed the cell uptake of different polyplex prepared from modified PEI.



(A) **Confocal microscopy images for cell uptake of the PEI based polyplex**



(B)**Confocal microscopy images for cell uptake of the modified Chitosan based polyplex**

FACS Analysis (Flow Cytometry Analysis)

Flow-cytometry is one of the useful techniques for characterizing cells in clinical diagnosis and biomedical research for quantifying aspects about their size, internal complexity and surface markers. In a flow cytometer the cell suspension is hydrodynamically

focused in a single cell wide stream of fluid containing a fast-moving sheath fluid around the slow moving cell suspension emerging through a 70 um nozzle.

Protocol: cells were seeded with cell density of 5 X 105 cells/well in 24 well plate. The cells were permitted to grow for 24 h in DMEM culture media. After 24 h the cells were treated with polyplex formulations with FAM-NC-siRNA at a 100 nM concentration and kept for 6 h in incubator maintained with 5% CO2 at 37°C in humidified conditions. During this time, the cells were supposed to internalize the formulations depending on the transfection efficiency. After incubation, the cells were washed with 7.4 pH cold PBS thrice to eliminate the residual polyplex formulations and collected using trypsin to obtain a cell suspension in 7.4 pH PBS. Before analysis the cell suspension was passed through 70 µm cell strainer to disperse any cell aggregates and analyzed for % cell uptake using fluorescence activated cell sorter (FACS BD, USA). The naked FAM-NC-siRNA and Lipofectamine 2000 (L2K) complexed siRNA were used as negative and positive control respectively for the experiment.

Results: After evaluation for cell uptake the formulations were subjected to flow cytometry analysis using FAM-NC-siRNA at 100 nM concentration for all the formulations to get quantitative details of cell uptake.

It was apparent from the results that, cells treated with naked FAM-NC-siRNA and showed very low level of uptake, while PEI based positively charge polyplexes showed higher uptake inside cells.

- **Development of nebulised formulation**

Nebulisers are used extensively for the administration of medication by inhalation, typically generating aerosol particles less than 5 mm in diameter that can reach the lower respiratory tract. Thus nebulisation is an attractive option to deliver genes directly to the affected epithelial cells in the lungs of patients with Cystic fibrosis, Asthma, COPD etc. A crucial step towards this goal is to find an appropriate formulation and a compatible nebuliser. Vector suspensions should maintain their stability and biophysical characteristics, protecting the siRNA from shearing forces during nebulisation so that its biological activity is preserved. In addition, the yield from the nebuliser must be maximized.

The aerodynamic particle sizes of siRNA aerosols were determined by sample deposition in a twin stage impinger. The stage at which the aerosol is deposited in the two stage impinger, reveals the terminal settling velocity of the nebulised suspension dependent upon the aerodynamic diameter of the nebulised globules.

Conclusion

The present research work demonstrates an attempt to develop siRNA therapeutics in treatment of obstructive airway disorders such as asthma and chronic obstructive pulmonary disease(COPD). At present, there are no ideal vector available which is both safe and effective in delivering highly unstable siRNA molecules. Here, we have tried to impart these features to two cationic polymers i.e.PEI and Chitosan. Both characterization studies revealed that both the vectors can be used after proposed modifications as a vector for siRNA delivery.

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List of Publications

Research/Review Articles:

1. Jitendra Amrutiya, Rohan Lalani, Priyanka Bhatt, Krupa Siddhapura, AmbikanandanMisra, Nucleic acid carriers for pulmonary gene delivery, Journal of Pharmaceutical Sciences and Technology Management, July 2015,volume (1), issue (2), Pages 37-69.

2. SushilkumarPatil, Imran Vhora, Jitendra Amrutiya, Rohan Lalani and AmbikanandanMisra, Role of Nanotechnology in Delivery of Protein and Peptide Drugs,Current Pharmaceutical Design,2015, 21(29),Pages 4155-4173.**Impact factor:3.452.**

3. Imran Vhora, SushilkumarPatil, JitendraAmrutiya and AmbikanandanMisra,Liposomes and Lipid Envelope-Type Systems for Systemic siRNA Delivery,Current Pharmaceutical Design,2015,21(31),Pages 4541-55.**Impact factor:3.452**.

4. Priyanka Bhatt, Imran Vhora, SushilkumarPatil, JitendraAmrutiya, Chandrali Bhattacharya, AmbikanandanMisra, RajashreeMashru, Role of antibodies in diagnosis and treatment of ovarian cancer: Basic approach and clinical status, Journal of Controlled Release,Volume 226, 28 March 2016, Pages 148–167.**Impact factor:7.705.**

5. Rohan Lalani,AmbikanandanMisra, JitendraAmrutiya, Hinal Patel, Priyanka Bhatt and SushilkumarPatil,Approaches and Recent trends in gene delivery for treatment of Atherosclerosis. Recent Patents on Drug Delivery& Formulation,Volume 10,2016.

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9. Priyanka bhatt, , Rohan Lalani, Imran Vhora, Sushilkumar Patil, Jitendra Amrutiya, Ambikanandan Misra, Rajashree Mashru, "Liposomes encapsulating native and cyclodextrin enclosed Paclitaxel: Enhanced loading efficiency and its pharmacokinetic evaluation."International Journal of Pharmaceutics,2017, (Manuscript Submitted).

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11. Imran Vhora, SushilkumarPatil, Hinal Patel, JitendraAmrutiya, Rohan Lalani, and AmbikanandanMisra.Overview of Polyester Nanosystems for Nasal Administration, Pan Stanford Publishing, Singapore (2015).