Summary Report

Schizophrenia is chronic incapacitating mental disorder represented by number of positive and negative symptoms including delusions, hallucinations, disorganized speech or behavior and impaired cognitive ability. It also results in disintegration of thinking process and emotional responsiveness. Although, intense research has been carried out in this area, the precise cause for schizophrenia is unknown so it is accepted that it arises mainly due to interaction among genetic and environmental factors. Various studies, carried out to estimate the etiology of schizophrenia, strongly suggest genetics as predisposing factor for schizophrenia. The statistics also states about 10% risk for a first-degree relative while 3% risk for a second-degree relative supporting hereditary cause for schizophrenia. Additionally, for monozygotic twins, the risk of schizophrenia is 48% while it reduces to 12% to 14% in dizygotic twins. Furthermore, if both the parents have schizophrenia then the risk of schizophrenia increases by 40% in the child.

Currently, schizophrenia is included in the top 10 causes of disability worldwide and has prevalence of 0.6% to 1.9% worldwide; diagnosed in about 5.1 per 1000 lives annually and lifetime morbidity of 7.2 per 1000. Although, medical science has evolved greatly, mortality rate in schizophrenic patients has increased which may be attributed to lifestyle (i.e., lack of exercise, unhealthy diet, and excessive smoking and alcohol intake), adverse effects, suboptimal treatments of the related adverse effects and suicide. Additionally, pathophysiology of this disease still remains unclear which is considered as the main cause for failure of currently available therapies. Even though, improvements on existing drugs and drugs acting at novel neurotransmitters will yield improved therapy, this approach to therapy is not as intellectually satisfying as efforts to find causes. Thus, researchers have diverted to target the root cause of the disease and have focused on developing gene therapy. With these efforts various susceptibility genes for schizophrenia were discovered some of which may be listed as RGS4, DISC1, NRG1, DAOA, CHRNA7, COMT, PRODH, AKT1, DRD3, DTNBP1, G30/G72, HTR2A, SLC6A4, ZDHHC8 etc.

Overexpression of NRG1 gene was considered to play important role in pathogenesis of schizophrenia. It was hypothesized to regulate NMDA receptor expression and thus was considered as a vital therapeutic target for management of schizophrenia. Thus, this research focuses to target the aforementioned gene using RNA

interference (RNAi) via. siRNA delivery to reduce its overexpression and thus offer anticipated curative approach for schizophrenia. The foremost cause of concern in siRNA delivery is its degradation by nucleases which would render the therapy ineffective which can be overcome by conjugating siRNA with suitable vector and then delivering that conjugated siRNA to the target site. This work mainly targets development of non-viral gene delivery vector for siRNA delivery.

siRNA targeting NRG-1 was procured from Sigma Aldrich having sense strand sequence - 5'-CUCAUAAAGUGUGCGGAGA[dT][dT]-3'. The purity of procured siRNA was determined by measuring ratio of A260/A280 which was 2.03 ± 0.11 indicating highly pure sample. Quantification of siRNA was performed by UV spectroscopy method and the linearity of siRNA calibration plot in concentration range of 5-50 pmole was expressed by equation y = 1.3212x + 0.683 with regression coefficient value of 0.9991. The UV method was validated by determining accuracy and precision and the results obtained depicted % recovery within 98-102% while %RSD less than 2% which met the acceptance criteria of ICH guidelines. Furthermore, agarose gel electrophoresis was performed to determine conjugation efficiency of the formed complexes, for which calibration curve was linear expressed by equation y = 155.61x – 2258.1 with regression coefficient value of 0.9982. Accuracy and precision of the technique were estimated by determining % recovery and % RSD which were found to be 99.96 ± 0.53 and 0.533, respectively.

In case of non-viral vectors, PEI is broadly explored for nucleotide-based therapies like DNA, siRNA and oligonucleotides. It has been also used for administering siRNA by local routes including intrathecal, subcutaneous, intraperitoneal etc. Although its widespread use there is huge concern regarding its toxicity and thus researchers have diverted their focus to modify PEI resulting in polymer having desired transfection with less toxicity. This work basically deals with 2 basic modifications of 25 kDa bPEI to form copolymers with mucoadhesive properties or to synthesize conjugates with brain targeting ligand. For mucoadhesive property bPEI was conjugated with hyaluronic acid and chitosan while targeted vector was synthesized by conjugating bPEI with lactoferrin. All the synthesized copolymers and conjugates were analyzed by NMR and FT-IR. Additionally, molecular weight of synthesized copolymers and conjugates was determined using Malvern Zetasizer by static light scattering technique.

Furthermore, using hexane diol diacrylate biodegradable bPEI was synthesized which was further used to synthesize mucoadhesive copolymer by conjugating with chitosan. This was analyzed by NMR, FT-IR and Malvern zetasizer for molecular weight determination. The NMR, FT-IR and molecular weight results supported successful conjugation of bPEI with respective moiety.

Moreover, mucoadhesion study of copolymers confirmed mucoadhesive property of them. The % mucin binding efficiency of bPEI-HA, bPEI-Chi and biodegradable bPEI-Chi copolymers were 28, 16.5 and 14.5 % respectively which showed mucoadhesive properties thereby improving nasal residence time in-turn augmenting therapeutic effect.

For siRNA delivery, the synthesized vectors were conjugated with siRNA to form polyplexes. Additionally, siRNA conjugated mucoadhesive polyplex was further complexed with anionic liposomes to form lipopolyplexes. All the formulations were evaluated for conjugation efficiency using agarose gel electrophoresis which concluded complexation of siRNA with formed copolymers and conjugates at 0.3:1 to 0.5:1 (polymer:siRNA mole ratio). Conjugation efficiency of lipopolyplex was determined by nanodrop UV which was 97.24 ± 1.07% for the optimized formulation.

The synthesized polyplexes and lipopolyplexes were subjected to particle size analysis and the biodegradable PEI-Chi polyplex having particle size of 126.4 and lipopolyplex having size of 187.4 were screened for further analysis. Zeta potential of all the polyplexes was in positive range which proved excess of positively charged copolymer or conjugates in formed complexes. The zeta potential of lipopolyplex was -6.38. Furthermore, conjugation of siRNA with synthesized cationic vector resulted in reduction of copolymer or conjugate surface charge depicting conjugation by electrostatic interaction.

Stability of formed polyplex in anionic environment was estimated by polyanion competition assay the results of which depicted stability of formed polyplex till 2:1 (heparin:siRNA mole ratio). This in turn proved nanoplex stability in-vivo in presence of serum or plasma proteins. For lipopolyplex, heparin didn’t have any effect on the entrapped siRNA but can release surface conjugated siRNA. Thus, image of agarose gel electrophoresis for lipopolyplex showed bands with lower intensity compared with control. Additionally, stability of polyplexes in simulated nasal fluid and was checked by estimating particle size which showed negligible aggregation of the formulations under biological conditions. Ex-vivo nasal permeation study was

performed using fluorescent labelled siRNA polyplex, depicting permeation of significant fraction of conjugated siRNA to receptor chamber of Franz diffusion cell through excised sheep nasal mucosa. When the results of different formulations were compared, it was found that there was less permeation of bPEI-HA polyplex and bPEI-Lf polyplex which may be attributed to larger particle size hindering its free diffusion through nasal mucosa. Additionally, lipopolyplex showed maximum permeation as all the polyplex showed net positive surface charge which may adhere to nasal mucosa while lipopolyplex showed charge near neutrality and thus showed better permeation.

The formulations were further screened by in-vitro tests using SK-N-MC cells. In-vitro cell cytotoxicity study using MTT was performed which proved that that % cell viability after incubation of SK-N-MC cells with maximum concentration of bPEI-HA copolymer, bPEI-Lf conjugate, bPEI-Chi copolymer and biodegradable bPEI-Chi copolymer (i.e. 50 nmole) for 24 h was 95.11 ± 1.16, 95.27 ± 1.36, 96.97 ± 4.00, 95.16 ± 1.54, respectively. The results concluded that all the synthesized polymers exhibited negligible toxicity and thus could provide better and safe alternative for delivery of gene therapeutics to such neuronal cells.

The results of in-vitro permeation study using transwell showed that there was significant improvement in permeability when conjugated with polyplex and lipopolyplex, resulting in improved therapeutic efficacy. However, there was no enhancement in permeability of bPEI-HA and bPEI-Lf polyplex which might be attributed to higher particle size and molecular weight interfering with its transfer across RPMI 2650 cells. Maximum permeability enhancement, as evident from enhancement ratio, was 3.06 for lipopolyplex which might be due to lowest particle size and enhancement of transfection efficiency.

Qualitative cell uptake was determined using confocal microscopy which showed improved cell uptake of polyplex and lipopolyplex as compared to naked siRNA. This may also prove prevention of siRNA degradation due to nucleases by providing shielding effect to the conjugated siRNA. Additionally, cellular uptake amongst polyplexes was dependent on particle size of them and thus bPEI-HA polyplex having particle size of 2583 nm showed lowest cellular uptake as evident from fluorescence intensity while biodegradable bPEI-Chi polyplex having particle size of 126.4 nm showed maximum uptake amongst polyplexes. Furthermore, cellular uptake of lipoplexes conjugated with biodegradable bPEI-Chi polyplexes was found maximum amongst all siRNA conjugated vectors. This was supported by the fact that

lipopolyplexes caused enhancement of clathrin mediated cellular uptake as compared to polyplexes. The results of quantitative cell uptake using flow cytometry supported the data of cellular uptake study obtained using confocal microscope and showed that polyplex and lipopolyplex transfected 71.3% and 69.9% of cellular population with mean fluorescence intensity of 55.4 and 117, respectively.

The results of gene expression study conclude that there was 38.72% and 39.95% relative gene expression when the cells were treated with NRG1 siRNA complexed polyplexes and lipopolyplexes, respectively. The resultant knockdown efficiency of the treated cells was 61.28% and 60.05%, respectively. The results depicted significant change in relative gene expression and thus significant knockdown efficiency when cells were exposed to treatment. This may be due to increased transfection of complexed therapeutic siRNA into the resulting cultured cells which further binds to target mRNA and results in its degradation.

The formulated polyplex and lipopolyplex were administered intranasally by formulating nasal spray. The pH of optimized formulation for nasal spray was 5.12 ± 0.01 with osmolality of 535 ± 5 mOsmol/kg both of which complied FDA specified limit for nasal spray. The viscosity of nasal spray was 40.5 ± 1.5 cP at 10 RPM. Additionally, viscosity decreased proportionally as shear increased thus proving shear thinning properties of formulated nasal spray.

Nasal spray pump of CPS Technology Platform (Aptar Pharma, Illinois, USA) was used to deliver formulated nasal spray. The average pump delivery volume of nasal spray from the selected nasal spray pump was 73.524 ± 0.995 µl. Additionally, in order to achieve uniformity in pump delivery, this nasal spray pump should be primed with 5 actuations and delivers accurately up to 110 sprays. The average % pump delivery of 110 actuations was 99.58 ± 1.25% which was well within 10% limit recommended by FDA for average pump delivery. The data of repriming concluded that % pump delivery of nasal pumps was within FDA's recommended limit i.e. 85-115% but after 24 h the % pump delivery falls beyond the allowed range. Thus, such pump can be used without priming if kept unused for not more than 24 h. Additionally, it was found that 3 actuations were required for repriming of the nasal spray pump.

The results of spray pattern showed that the shape of the spray pattern was ellipsoidal in shape with no axis longer than 4.7 cm and 7.2 cm at distance of 3 and 6 cm, respectively. Further, ovality ratio of developed nasal spray was 1.054 ± 0.015 and 1.067 ± 0.039 at 3 and 6 cm, respectively which depicts ideal and uniform spray pattern.

Additionally, measurement distance from nozzle does not have significant effect on spray pattern as evident from ovality ratio.

The results of plume geometry showed that plume angle and plume width were 35.4 ± 2.5° and 19.2 ± 1.4 mm, respectively at 3 cm. Similarly, plume angle and plume width at 6 cm were 28.1 ± 0.4° and 30.0 ± 0.5 mm, respectively. This optimized nasal spray may improve nose to brain delivery of therapeutics was supported by various in-vitro and in-vivo studies, which were carried out to determine influence of plume angle on nasal spray deposition characteristics.

The droplet size distribution of formulated nasal spray was determined and effect of various parameters including spraying angle, actuation force, actuation distance and hold time on droplet size distribution was studied. The optimized actuation parameters selected were 30° spraying angle, 45 N actuation force, 3 cm actuation distance and hold time of 2 s. The individual parameters i.e. D_{10}, D_{50}, D_{90}, span value and % droplets having size less than 10 µm at optimized actuation parameters were 19.28 µm, 52.95 µm, 123.54 µm, 1.97 and 2.27%, respectively.

The results of weight loss concluded that there was no significant change in weight of nasal spray filled pump even after 3 months at various orientations i.e. inverted, upright and horizontal. Thus, it was concluded that there was no dripping or leakage from the nasal spray pump and thus qualifies sealing characteristics of nasal spray pumps.

The performance of nasal spray formulation at near completion stage of the nasal spray in the pump was determined by estimating tail-off characteristic parameters including droplet size distribution, spray pattern and shot weight. The results confirmed augmentation in droplet size distribution with altered spray pattern along with reduction in shot weight influencing nasal spray characteristics.

The results of microbial limit test demonstrated approximately 31 CFU on TSA plates when 1 ml formulation was spread on plates while CFU on SDA plates were not found indicating absence of yeast and mold. Both TAMC and TYMC count complied USP defined limits along with absence of *S. aureus* and *P. aeruginosa* fulfilling criteria for passing microbial limit test for nasal spray.

Finally, the data of preservative efficacy test concluded that the log reduction profiles of both bacteria and fungi including yeast and molds were complying the limit specified by USP at all the time points i.e. 7 days, 14 days and 28 days. Thus, the nasal

spray formulation was found to have desired preservative action fulfilling the criteria for passing preservative efficacy test.

In-vivo pharmacological study comprised of acute toxicity study, efficacy study using suitable disease model, distribution study and nasal ciliotoxicity study were performed to establish safety and efficacy of developed vectors and formulations. The results of acute toxicity concluded that the synthesized vectors and formulations were safe, up to 10 times the therapeutic dose, in the animals tested. Nasal ciliotoxicity study was performed to prove biocompatibility of developed formulation along with its safety. The results depicted insignificant inflammation of nasal mucosa treated with optimized formulations signifying biocompatibility of developed formulations.

The results of brain distribution study concluded that naked siRNA when delivered through nasal route showed insignificant delivery to the target site which might be attributed to their hydrophilicity with net negative surface charge and higher molecular weight, both of which impede its brain distribution. Additionally, naked siRNA has very low half-life, around 10 min, due to its degradation by nucleases. Even after its endocytosis, it is uptaken by lysosomes where it is cleaved resulting in loss of its therapeutic action. Conversely, there was drastic improvement in brain uptake of siRNA when administered in form of polyplex or lipopolyplex which was around 2-fold than naked siRNA. Such improved uptake might be due to augmented endosomal escape preventing its hydrolytic degradation from lysosomal enzymes. Furthermore, such formulation enhances cellular uptake of siRNA and its release and thereby facilitate its accumulation in cytosol. All these factors together contribute in improved brain distribution of conjugated siRNA as compared to naked siRNA.

Moreover, efficacy study was performed by developing animal model overexpressing NRG-1 and later performing western blot and RT-PCR. The results of western blot proved that there was considerable reduction of protein expression from 89.29 ± 1.52% to 71.33 ± 0.99% (Polyplex) and 89.29 ± 1.52% to 51.49 ± 1.14% (Lipopolyplex) when normal protein level in control group was about 73.96 ± 1.36% which depicted considerable reduction in protein expression on siRNA administration. Furthermore, gene expression levels in disease model and treatment groups, performed by RT-PCR, showed approximately 60% gene knockdown in treated groups as compared to disease control group. This improved efficacy in polyplex and lipopolyplex formulation may be attributed to increased uptake and release of siRNA from the conjugated vector and thereby results in higher accumulation of siRNA in

cytosol. This along with reduced degradation of siRNA causes higher therapeutic efficiency at same concentration as compared to naked siRNA. Thus, vector conjugated siRNA can effectively result in higher gene knockdown, in turn downregulate overexpressed NRG1 gene during schizophrenic condition and thereby maintaining optimum gene expression.

Finally, stability study of polyplex and lipopolyplex was performed by analyzing siRNA integrity and particle size distribution. The data concluded that there was no significant change in both % recovery and particle size of both the formulation proving siRNA integrity in the conjugated form and physical stability of polyplex and lipopolyplex in terms of particle size. Stability of nasal spray was determined by estimating various parameters including pH, osmolality, viscosity, pump delivery volume, plume geometry and droplet size distribution. The data of physicochemical characterization indicated stability of formulated nasal spray in terms of pH and other physicochemical parameters. The data of plume geometry after 3 months showed that plume angle and plume width were 32.6 ± 3.8° and 20.3 ± 1.1 mm, respectively at 3 cm. Similarly, plume angle and plume width at 6 cm were 27.6 ± 0.7° and 29.5 ± 0.3 mm, respectively. The data represented that there was no significant change in plume geometry of nasal spray even after 3 months indicating its stable spray characteristics in turn providing reproducible nasal spray deposition and therapeutic effect. Furthermore, data of droplet size distribution showed that there was non-significant change in all the parameters including D_{10}, D_{50} and D_{90} thus supporting uniform viscosity of nasal spray throughout stability period. Additionally, it also verified absence of aggregation and other instability indicating processes proving that the formulated nasal spray would have uniform characteristics throughout stability period.

Conclusively, the work focused on developing novel approach for brain delivery of gene therapeutics by intranasal administration. To achieve this, various vectors having either mucoadhesive or targeting property were prepared by synthesizing copolymers or conjugates which included bPEI-HA copolymer, bPEI-Lf conjugate, bPEI-Chi copolymer, biodegradable bPEI-Chi copolymer and anionic liposomes. The prepared vectors were conjugated with siRNA by electrostatic interaction and the resultant polyplex and lipopolyplex were screened. The preliminary investigation ruled out possibility of use of bPEI-HA, bPEI-Lf and bPEI-Chi polyplex and thus biodegradable bPEI-Chi polyplex and lipopolyplex were selected for further characterization. These selected formulations were further delivered by nasal spray which was optimized and

characterized to yield desired nasal deposition and delivery. In-vitro cell line study proved safety, better cellular uptake and better transfection yielding desired gene expression for the screened formulations. Further, in-vivo study of the selected formulations verified safety, biocompatibility, brain distribution and efficacy of the developed formulations. Finally, the formulations were exposed to stability study for 3 months to get crude idea about stability of the formulations. All the results supported the intended goal of the study and thus this study can provide new therapeutic approach for treatment of schizophrenia. Additionally, when properly optimized this model vectors as well as formulation can serve as platform technology for brain delivery of gene therapeutics in future.