7. Summary and conclusion

Major depressive disorders (MDD), normally known as depression is a serious mental disorder of mammoth social and clinical relevance. Depressive disorders are commonly occurring psychiatric conditions that tend to run in families. They are heterogeneous and complex, predicted to involve the interplay between multiple genes and non-genetic risk factors, such as stressful life events. Their phenomenology and biological substrates are not clearly distinguished from those of normal mood states on the one hand and frequently comorbid conditions such as the anxiety disorders on the other, further complicating research into their aetiology. Preliminary molecular genetic studies performed over the past several decades have implicated only a modest number of specific candidate genes in major depression, thus far offering little insight into its pathophysiological basis. This leaves open the way for a new wave of large-scale, genome-wide association studies that are providing vastly increased amounts of preliminary data for understanding these important conditions. Medications for depression can be classified as MonoAmine Oxidase inhibitors (MAOIs), tricyclic antidepressants, Selective serotonin reuptake inhibitors, atypical anti-depressants. These marketed treatmets have many limitations including low emission rates, high rate of reccurance, non-adherance to therapy, many side effects as well as interactions between drugs. None of the traditional treatments of depression depicted above are particularly effective. Last few years have seen astonishing developments in the field of genetics and neuronal science, along with new advancements in the molecular biology and nano science. The results from such studies allowed the researches to improve their knowledge about brain and its functioning. In the past decade, with the improved knowledge of brain functioning, various gene delivery approaches have been studied for the treatment of depression. Such gene delivery approaches include increase in the serotonin neurotransmission, enhancing serotonin sensitivity, increasing glucocorticoid susceptibility and increasing BDNF level in the brain. Genes that are responsible for depression include but not limited to SLC6A4, S100A10, FKBP5, NR3C1, CRHR1, BDNF and NTRK2. Among all these genes, S100A10 commonly known as p11 gene is most sought after gene. Greengard and colleagues found that 5-HT1B interacts with p11, and according to Greengard, p11 plays a role in the recruitment of receptors to the cell surface where they are more functional. By using a virus to deliver an extra dose of the gene p11 to the adult mouse brain, the protein expressed by the gene is thought to bind to serotonin receptor molecules and ferry them to the cell surface, positioning them to receive serotonin's signals from neighboring cells. While the gene therapeutics for depression have not been studies in humans or they are not in clinical studies

level, the primary studies in mice involving p11 gene showed its efficiency. Recent researches have evidenced that a particular protein involved in progression of depression in many patient is known as p11 or S100A10 which involved in changing the modulation and effectiveness of serotonin in brain. Therefore in this project we have attempted to formulate the liposomal delivery system with the alkali amino acid modified DOPE lipid, carrying p11 cDNA which has the capability to modulate level of p11 protein in brain cells which was further confirmed using interferon induced animal model of depression.

For the successful development of any carrier system, a suitable analytical method should be developed which should be validated. For this purpose, gel electrophoresis and UV spectrometric method using Nanodrop were developed for the quantification of cDNA complexed to established nano carrier as well as for the purity of plasmid isolated from bacteria. The calibration curve of plasmid DNA was generated using Nanodrop for the linearity range of 10-50 ng/µL and accuracy, precision of method was established. The purity of plasmid was assessed by the ratio of OD260/OD280 and OD260/OD230. The detectability of p11 cDNA in gel electrophoresis method was found to be 5 ng and calibration curve was developed in linearity range of 10 to 60 ng by measuring the peak area of bands. Analytical method was also developed for the assessment of lipid which was used in the detection of unconjugated lipid.

p11 plasmid and eGFP (enhanced green fluorescent plasmid) which is used for the detection of intracellular detection and transfection efficiency of newly developed carrier, were isolated and purified using alkali lysis method in DH5 alpha strain of E.Coli bacteria. The isolated plasmid was digested using restriction enzyme which recognised the DNA sequence present in plasmid. The digestion of plasmid released the tension present in circular plasmid and showed the single band present at 5775 bp, confirming the identity of p11 cDNA.

For the development of novel carrier, DOPE was modified using alkali amino acids histidine, lysine and arginine. To serve the purpose of conjugating amino acids to DOPE, BOC protected amino acids were used to avoid side reactions. After carefully choosing protected amino acids, the coupling reaction was carried out using DCC as a coupling agent in dry DCM as solvent. The side product of this reaction, DCU, is insoluble in DCM making it easier to separate it from main product. Completion of reaction was confirmed using TLC in mobile phase containing CHCl3: MeOH: HAc which showed spot of newly conjugated lipid. The conjugation was further confirmed using IR spectroscopy and presence of residual solvent DCM in final product was checked by gas chromatography which was found to be

well below toxic level. The conjugation efficiency of DOPE to individual amino acids were evaluated using TNBS assay and the conjugation efficiency for DOPE-A, DOPE-H and DOPE-L was found to be 52.68 ± 5.64, 37.26 ± 4.95 and 41.72 ± 5.09 respectively. The synthesised lipids were checked for their buffering capacity and results showed the maximum buffering capacity for arginine based liposomal formulation.

The synthesised lipids were used in the formulation of positively charged liposomes as they carry positive charge due to presence of alkali amino acids. Thin film hydration method was used for the preparation of liposomes with HSPC, DPPE, cholesterol and synthesised lipids as a part of liposome formulation. The formulation was optimised using D-optimal design with mole % of HSPC, cholesterol and DPPE as independent variables while size and PDI of liposomes were chosen as the dependent variables based on the preliminary screenings. Based on the design, the optimal criteria for final formulation was applied and predicted as well as experimental values for size and PDI were evaluated using statistical analysis and results showed values of $p< 0.05$, indicated no significant difference existed between practical value and standard value, concluding the suitability of the selected model for optimization. The optimised formulation of liposome was then incubated with p11 cDNA and assessment was carried out for complexation using gel electrophoresis and Nanodrop. The different liposomal formulations prepared from different synthesised lipids showed difference in their complexation efficiencies with p11 cDNA with minimum L/P ratio of 4 was required for the arginine based liposomes and L/P ratios of 6 and 8 were required for lysine and histidine based liposomal formulations respectively. Effects of temperature and pH was also assessed on the conjugation efficiency and due to difference in charged density of their headgroups, temperature and pH had different effects on individual liposomal formulations. The lipoplexes were then challenged for stability studies and biocompatibility studies. For the stability challenges, lipoplexes were incubated with up to 5 % concentration of sodium sulphate, as much as up to 4 times the heparin weight ratio to cDNA and 5 % v/v of serum. All the formulations, were stable even when they were incubated in such exaggerated conditions of sodium sulphate, heparin and serum as mentioned above. During biocompatibility studies, when lipoplex formulations were incubated with erythrocytes, they showed no sign of aggregation and devoid of haemolytic potential.

Lipoplex formulated from amino acid modified lipids were screened using *in vitro* cell line studies. As a part of this cell line studies, the cytotoxicity of the alkali amino acid based cationic liposomes was investigated in SHSY5Y cells. At N/P ratios required for maximum complexation efficiency, lipoplex formulated from DOPE-A showed minimum cytotoxic

effects compared to other two formulations. Confocal microscopy was carried out to check for the cellular uptake efficiency of SHSY5Y cells to internalise liposomal formulations complexed with eGFP. This study proved the better cellular uptake of DOPE-A lipoplex in comparison to other formulations and naked DNA. Permeation studies were carried out to replicate the hurdles presented by blood brain barrier and permeation studies also backed the results obtained from cellular uptake studies that arginine modified liposomes have better penetration abilities than other formulations. Effects of all the formulations on protein level was measured using western blot analysis. The level of p11 protein was diminished using interferon in SHSY5Y cell line which was elevated using p11 containing lipoplex formulations. Among all the formulations, arginine containing lipoplex showed superior performance in elevating p11 protein level. Gene expression level was also checked for all the formulations using RT-PCR and the same liposomal formulation containing p11 gene showed maximum % of gene expression in interferon treated cell line.

Among all the liposomal formulations formulated from different amino acids modified DOPE lipid, liposome composed of arginine modified DOPE lipid showed most promising results as confirmed by *in vitro* cell line studies. As a result, liposomal formulation formed from DOPE-A was only selected for further studies.

For p11 gene to efficiently treat the depression, it is important for the lipoplex containing p11 gene to cross the BBB and achieve maximum therapeutic concentration in BBB. For this purpose, DOPE-A lipoplex containing p11 gene was delivered through nasal mucosa using nasal spray. Nasal spray was formulated using monobasic and dibasic sodium phosphate as buffer in quantity (0.5525 % and 0.0975 %), 0.03 % Di Sodium EDTA, 0.5 % glycerine, 0.8 % Sodium Chloride and 0.1 % Benzalkonium Chloride. Nasal spray was then characterised for pH, viscosity, osmolality, droplet size distribution, plume geometry, spray pattern and shot weight as per the criteria of US FDA. DOPE-A containing lipoplex was further evaluated for its nasal permeation efficacy and nasal toxicity. The penetration of naked cDNA was found to be significant lower as compared to lipoplex formulation after 8 h. After 8 h, DOPE-A lipoplex showed 89.37 % of cDNA permeating nasal mucosa while only 19.82 % of naked DNA permeate the nasal mucosa in same time period. Moreover, DOPE-A lipoplex formulation showed no sign of toxicity on nasal mucosa and it is safe to administer this formulation though nasal cavity.

Other than nasal route, systemic route was also explored for the p11 gene delivery to the brain. For systemic delivery, DOPE-A containing p11 gene was conjugated to monoclonal antibody IGF-II using DSPE-mPEG$_{2000}$-meleimide as a linker lipid. For the conjugation of

mAb to lipoplex, first important step is the functionalization of lipoplex using DSPE-$mPEG_{2000}$-meleimide. This functionalisation was achieved using pre-insertion and post-insertion methods. The efficiency of pre-insertion and post-insertion method was analysed by quantification of sulfhydryl group and the results showed almost double amount of functionalisation was achieved using post-insertion method. After functionalisation of liposomes, antibody was thiolated using Traut's reagent. Then the attachment of mAb to lipoplex formulation was confirmed using Bradford's method and SDS-PAGE analysis. Using bradford's method, the amount of mAb on pre-insertion and post-insertion functionalised liposome was found to be around 16.9 µg and 11.4 µg. Thus, % mAb conjugation over liposomal surface was found to be 84.5 % for post-insertion method and 57.0 % for pre-insertion method. Coomassie staining method was used for detection of antibody present on liposomes after SDS-PAGE analysis which showed more intense blue colour due to presence of more antibody attached to lipoplex for post insertion method. After surface modification, lipoplex formulation showed maximum complexation efficiency at N/P ratio of 6 which was originally 4. The physicochemical properties of the lipoplexes were evaluated for the mAb conjugated lipoplexes. It was observed that the particle size of the lipoplexes changed after conjugation; however, zeta potential of the lipoplexes was not changed drastically for the lipoplexes. During the cell cytotoxicity study it was observed that there was no significant difference in the cytotoxicity of the lipoplexes before and after conjugation to the targeting mAb. Qualitative cellular uptake of targeted formulation was carried out using live imaging of confocal microscopy to see the effects of antibody conjugation on the cellular internalization. From the results, it was observed that the cellular uptake of targeted formulation changed drastically after mAb conjugation to the same formulation.

After optimizing DOPE-A formulation for nasal and targeted systemic delivery, they were further assessed in animals for their toxicity and efficacy. Sighting study was performed before performing actual toxicity study at dose of 100 µg/kg, 250 µg/kg and 500 µg/kg of plasmid DNA. All doses were safe in sighting study so toxicity study was performed at 250 µg/kg and 500 µg/kg dose including one animal from sighting study. At these 250 µg/kg and 500 µg/kg there was no morbidity recorded in any animal. Food habits and weight of animals were also taken in consideration and no change in food habits as well as in weights of animals were found after treatment with formulation. CBC study was also taken into account after systemic treatment to evaluate targeted formulation for its allergic potential and no change in any blood cells were found. Transportation across nasal mucosa for

intranasal route was performed using cryosectioned nasal mucosa and then observed under confocal microscope which proved the efficient permeation or transportation of cDNA complexed with liposome formulation in comparison to naked cDNA.

During the systemic administration of formulations, more intense fluorescence for IGF-II targeted lipoplex formulation confirmed the role of IGF-II receptors present in BBB for transportation of targeted formulation through BBB which was proved using the cryosectioned brain tissue visualised under confocal microscope. For intranasal route, there is no role for receptor mediated transcytosis to play for the formulation to accumulate in brain. So, targeted delivery from systemic route is proved to be more effective than the local delivery of liposomes through nasal route.

During the induction of depression in mice, studies proved that the dose of 1200 IU/day was selected as minimum dose required for the induction of depression like behaviour. At this particular dose, there was drastic change in the immobility time, around 105 seconds for FST and 123 seconds for TST; representing change in the response of animals, indicating towards their depressive behaviour. This dose of interferon reduce the level of p11 protein by 70 % to its original level. Treatment with non-targeted liposomal formulations with both systemic and intranasal route did not significantly improve the p11 level while treatment with targeted liposomes significantly improve the protein level with noticeable improved behaviour pattern and enhanced p11 protein level. Analysis of gene expression in animals after interferon treatment for 15 days through RT-PCR showed around 34 % of gene expression to its original gene expression level which was improved to 70 % with systemic delivery of targeted formulation while nasal delivery of lipoplex formulation showed only around 43 % of gene expression.

The results show that all the modification applied to DOPE is excelled in their nucleic acid carrying property, buffering capacity, toxicity profile, better resistant against electrolytes, serum and heparin in comparison to DOPE itself. Further studies including *in vitro* cell line studies proved the better efficiency of lipoplex formulated form arginine modified DOPE which was later delivered to brain using two different approaches comprising of intranasal route and systemic route. The optimised formulation dispersed in nasal spray for the intranasal delivery and same formulation was attached to IGF-II mAb through maleimide linker for systemic delivery. *In vivo* studies further concluded the superior ability of IGF-II conjugated lipoplex system to enhance p11 protein in selective brain area when compared to liposomal formulation delivered through intranasal route which was evident by increased level of p11 protein level in interferon induced depression model. This bodes for the

potential efficiency of systemic route enabled with monoclonal antibody conjugated liposomes for the treatment of depressive disorders.