Chapter 12
Summary and Conclusion

I gave up on giving up.
-From "Land of the Lost"

12.1 Summary and Conclusion

Osteoporosis a disease of bones is a silent disease due to its character of occurring without symptomatic changes in the body. It is regarded as a global healthcare and accounts for over 8.9 million fractures annually. Worldwide, 1 in 3 women and 1 in5 men above 50 years' age are affected by osteoporotic fracture. Imbalance between bone resorption and bone formation is the underlying mechanism in all cases of osteoporosis. Various hormonal level defects contribute to the disease condition leading to cascades of processes in normal aetiology of bone formation and cause increased bone resorption by osteoclasts and/or decreased bone generation by osteoblasts.

Osteoporosis treatment includes lifestyle changes and medication and the therapy is to be continued lifelong. Medication includes supplemental calcium, vitamin D, calcitonin, bisphosphonates, bone morphogenetic proteins and several others. However, analyses show that calcium supplementation alone and vitamin D supplementation alone are not effective in preventing fractures in osteoporotic patients as the combination thereof. Glucocorticoid-induced osteoporosis and osteoporosis related to aging are mainly outcome of reduced bone formation due to reduced number of osteoblasts. A concurrent approach involving medications for prevention of bone loss and promotion of bone formation may provide a model therapeutic strategy for such case. There is therefore an important need to develop therapeutic strategies capable of promoting bone formation in osteoporotic subjects.

In the past decade various gene delivery approaches have been studied for the treatment of osteoporosis. Such gene delivery approaches particularly act either by inducing or one or other growth factors, cytokines, transcription factors, other mediators or their receptors that are implicated in osteoporosis. Bone morphogenetic factors (BMPs), mainly BMP-2, BMP-4, BMP-6, BMP-7 and BMP-9, are other osteogenic proteins that have been studied for bone regeneration in fractured bone healing, osteoporosis and osteopenia. BMP-9 has been shown to provide most robust and effective osteogenic activity in animal studies and was thus selected for delivery by use of lipidic vectors to bone cells. Plasmid containing BMP-9 gene was procured from Sinobiologicals in lyophilized form in tubes.

For delivery of gene therapeutics in osteoporosis, few instances have been reported where non-viral gene delivery have been used in vivo preclinically. These vectors include calcium phosphate nanoconstructs and lipoplexes. This opens a possibility to develop and use liposomal vectors among which lipoplexes offer inherited low toxicity characteristics of biocompatible bilayer structure. Further, lipoplexes can be modified in order to provide advantages such as ability to target various organs by modifying liposome surface by attaching appropriate ligands, reduced immunogenic response, differential release characteristics and protection of the complexed gene. However, delivery of lipoplexes containing therapeutic gene to the cell faces various challenges and thus focus of the research carried was also on overcoming toxicity, issues of loss of nucleic acid in systemic circulation, targeting to specific cell for preventing non-specific distribution and enhancing transfection efficiency for cellular uptake along with endosomal escape.

Analytical methods required in successful development of lipoplex formulations were developed and partially validated for suitability of analysis. Three methods were used for estimation of purity of the isolated plasmid and for quantification of pDNA namely, UV spectrophotometric method, Spectrofluorometric method and Gel electrophoresis. From UV spectroscopic analysis, the purity of the DNA isolated was confirmed to be high along with determination of linearity in concentration range of 2 – 400 ng/µL and further quantitation of the band was performed from 10 – 200 ng pDNA concentration relative to 200 ng band. Electrophoresis study of agarose gel showed that ≥ 10 ng concentrations of pDNA are detectable. Analytical methods used in the synthesis of lipids were TNBS assay (which was used to detect free unconjugated lipids) and Sakaguchi assay (which was used to determine quantitate guanidine groups) in order to determine the conjugation efficiency of the synthesis method employed for conjugation of lipid with Boc-amino acid derivatives. Accuracy and precision was evaluation for both the methods.

In context of development of gene delivery systems, green fluorescent proteins (GFP) have become essential tools in order to evaluate the intracellular delivery and expression of genes through easy fluorescence detection systems and the pDNA for GFP was used to develop lipoplex systems and was used for in vitro cell line studies in order to evaluate the transfection potential of lipoplex based gene delivery system. The plasmid DNA was isolated from the working culture of the *E. Coli* transformed strains using the

alkaline lysis method. The digestion of the isolated plasmid was carried out by restriction endonuclease enzyme for confirming the pDNA with the transformed DNA. The isolated pDNA after linearizing with restriction digestion showed a single strong band on the agarose gel while undigested intact pDNA showed typical two band characteristic. The BMP-9 plasmid showed a linear band corresponding to molecular weight of 5657 bp and eGFP plasmid showed the linear band corresponding to its molecular weight of 5446 bp there by confirming the plasmid DNA.

For preparation of lipoplexes, syntheses of Boc-histidinylated stearyl amine (Boc-His-SA) and Histidinylated stearyl amine (His-SA) were carried out by EDC/NHS coupling method. For synthesis of carnosine (dipeptide of alanine and histidine) modified lipids, free amino group of carnosine was protected using Boc anhydride (dibutylpyrocarbonate). Modification of dioleoyl-sn-glycerophosphoamine (DOPE) was carried out using the same strategy. Efficiency of conjugation of the amino acid derivatives to the lipids (SA, DOPE and DSPE) was determined by carrying out the TNBS assay (For Boc-His-SA), or Sakaguchi. Further, Synthesized lipids were evaluated for their buffering capacity and for pKa determination and identification by Mass, FTIR, NMR and UV spectroscopy. pH titration study was performed on the lipids to evaluate the buffering activity of the lipids.

Liposomes were developed using cationic lipids i.e. DOTAP, stearyl amine and modified cationic stearyl amine, DOPE and DSPE and optimization of the processing condition for formation of liposomes was carried out for parameters like solvent evaporation time, vacuum applied, rotation speed of flask for film formation, hydration time and temperature etc. Stearyl amine liposomes were initially developed using different other lipids i.e. HSPC, DSPC, DMPC, DPPC, Egg PC, DOPE, DSPE, and Cholesterol. Different molar ratios of lipids were tried to develop stearyl amine liposomes. All liposomes were developed using 3 mole% of mPEG2000-DSPE to develop PEGylated liposomes. Thin film hydration method was used for liposome preparation. The prepared liposomes were characterized for particle size and size distribution wherein the target was minimum particle size with lowest PDI. The prepared liposomes batches S15, S18 and S20 exhibiting monomodal distribution with low PDI and a positive zeta potential were selected. Similarly, liposomes containing DOPE and DSPE were prepared and optimized lipid composition (batch 20 and 24) was selected. For further optimization of DSPE –

DOPE liposomes, DOE was employed using D-optimal design by taking HSPC, EPC and cholesterol as variables and Particle size and PDI as response. The optimized batch parameters based on desirability plot was obtained as HSPC:EPC:Chol::35:30:12 and exhibited particle size of 109.7 nm and PDI of 0.186 with 0.832 desirability. The predicted response was confirmed experimentally. Optimized batch obtained through DoE was further used for preparation of the liposomes of cationic DOPE and DSPE wherein equimolar amounts of DOPE was replaced with His-DOPE or Car-DOPE to prepare cationic DOPE liposomes and equimolar amounts of the DSPE was replaced with DSPE to prepared cationic DSPE liposomes.

The pDNA was complexed with the liposomes to form lipoplexes and characterization was carried out for complexed DNA by gel electrophoresis, UV spectroscopy and spectrofluorimetry. Further, developed lipoplexes were characterized for particle size, zeta potential, morphology by Cryo-TEM, assay and residual water content. Optimization parameter for effective complexation involved selection of optimum incubation time (30 - 60 min) and incubation temperature (25°C, 37°C, 45°C) for non-PEGylated and PEGylated lipoplexes and was confirmed by gel electrophoresis. Optimization of the formulation parameters was done by determining the complexation efficiency in terms of ratio of moles of modified lipid to moles of phosphate of pDNA (L/P ratio). Complexation efficiencies of liposomes prepared with different lipids synthesized from SA were determined at different L/P ratios. In case of DOTAP/DOPE lipoplexes, complete retardation required L/P ratio of 1.50 and higher. L/P ratio of 2 for BHSA/BCSA lipoplexes and L/P ratio of 1 for HAS/CSA lipoplexes was used. Also, complete complexation of pDNA at L/P ratio of 1 for BASA lipoplexes and L/P ratio of 0.5 for ASA lipoplexes was observed. All the prepared lipoplexes showed pDNA content ranging from 99.5 to 101.7%. Complexation efficiencies of lyophilized lipoplexes were determined by UV spectrophotometry and spectrofluorometry after ultracentrifugation of the samples. Complexation efficiencies of lipoplexes of SA based liposomes prepared at L/P ratio of 2.00 and showed no significant change in the complexation efficiencies of lipoplexes after lyophilization indicating that the cryoprotectant-bulking agent (sucrose), the freezing process and the drying cycles did not affect the electrostatic interaction between the liposomes and pDNA. Similarly, lipoplexes of DOPE and DSPE based liposomes were prepared and optimized as done for liposomes containing stearylamine. Lipoplex formulations prepared were evaluated further for physicochemical properties.

L/P rations were chosen to attain the N/P ratio of 2.00 which helped in making comparisons of the physicochemical properties of the lipoplexes as well as in vitro and in vivo outcomes of the lipoplexes.

Formulation was evaluated in vitro for physichochemical characteristics in presence of simulated conditions i.e. presence of electrolytes and serum. The formulation was exposed to different percentage of electrolytes and it was demonstrated that the developed lipoplexes maintained the particle size well below 150 nm for the lipoplexes prepared with synthesized lipids. Additionally, it was observed that the effect of electrolytes on complexation was predominantly dependent on the presence of electrolyte before complexation process or after complexation process and on the PEGylation characteristics of the lipoplexes. Results demonstrated that there was no effect of 2% sodium chloride on the complexation efficiency of lipoplexes when exposure was made after the lipoplexes are formed. However, presence of electrolytes before complexation with pDNA significantly affected the pDNA complexation efficiency of lipids.

On the next stage the lipoplexes were evaluated for the cell line studies in which cytotoxicity of the formulations were evaluated through hemolysis study by exposing the formulation with RBCs in vitro and through exposing the adherent C2C12 mouse muscle cell line. Thorough cytotoxicity studies evaluated that the developed lipids were less toxic as compared to the widely used cationic lipids. Also, the toxicity of the developed lipoplexes were less than the lipoplexes prepared with the DOTAP and stearyl amine indicating the potential use of amino acid derivatives to conjugate on the stearyl amine and phospholipids of choice. Fluorescent microscopy and FACS analyses confirmed qualitatively and quantitatively the cellular uptake and subsequent expressed on the complexed pDNA. It was observed that developed series of formulations significantly enhanced the cellular expression of the gene even more than the commercially available standard lipid formulation Lipofectamine 2000. This is attributed to the structural features of lipids and on the formulation compositions. Further to this, lipoplexes were evaluated for in vitro calcium deposition studies which validated in vitro the therapeutic potential of lipoplexes. All the lipoplexes were successful in inducing the transdifferentiation of the C2C12 cells to ostoblastic cells which were capable of matrix deposition of calcium. All developed lipoplex formulations demonstrated better in vitro performance over Lipofectamine 2000 as well as common cationic agents such as DOTAP and stearyl amine. As a next step to cell line studies, formulations were developed using the same

composition to target the bone marrow using anionic peptide with $(Asp\text{-}Ser\text{-}Ser)_6$ sequences to effectively deliver the therapeutic gene in the site of action.

Acute toxicity studies in mice demonstrated safety attribute of the selected cationic lipid based lipoplex formulations which enabled high dosage of cationic lipids. *In vivo* studies in osteoporotic rats were performed to determine the *in vivo* performance i.e. bone induction in osteoporotic rats. Results indicate that selected formulations were able to induce bone formation in osteoporotic rats leading to increase in cortical thickness, cancellous bone formation. However, targeted lipoplexes were more effective in the recovery of the bone than the non-targeted lipoplexes. The results conclude the effectiveness of the developed formulation for osteoporosis treatment through intravenous administration. It was demonstrated that the targeted formulations, even though with their little compromised uptake and expression *in vitro*, were more effective for in vivo therapeutic purpose.

Among developed targeted formulations, lipoplexes that were peptide conjugated and which represented each group of synthesized lipids (amino acid derivative modified stearyl amine and amino acid derivative modified phospholipid) were evaluated for stability studies i.e. HSA-$(DSS)_6$ lipoplexes and HDS-$(DSS)_6$ lipoplexes. Due to similar compositions of the lipoplexes except for the modified lipid, the stability data can be extended to have an idea on the stability of the other lipoplexes as well. Both formulations in lyophilized form exhibited sable characteristics over the tested period of 3 months.

Conclusively, it can be stated that synthesized lipids can be used as cationic gene delivery vectors for treatment of osteoporosis due to their advantages of buffering effect, effective cellular expression, over current lipid systems. Thorough further animal studies need to be performed for evaluation of the synthesized lipids for the other toxicological parameters, developed lipids can be established in the treatment of genetic diseases including osteoporosis. Established performance and stability profiles also demonstrate that the cationic lipids developed in these processes can also be used in gene delivery approaches for other diseases also.