6.1 SUMMARY

The cholic acid (ChA) conjugates of polyethylenimine 2 kDa (PEI2), polyethylenimine 25 kDa (PEI25) and polyallylamine 15 kDa (PAA15) were prepared using carbodiimide chemistry. In each polymer conjugate series, by controlling lipid:polymer feed ratio (mol/mol), four different substitution levels of lipid:polymer were achieved. The synthesized polymer conjugates were characterized and confirmed by 1H-NMR spectra (Bruker 300 MHz). Specific peaks were confirmed by unmodified samples of PEI, PAA and ChA prior to conjugate analysis. The unique and characteristics peak of polymers (PEI2, PEI25 and PAA15) and cholic acid (ChA) were used for calculating substitution level of cholic acid per polymer unit. The degree of substitution was determined by characteristic peak integrations corresponding to PEI (-NH-$\underline{C}H_2$-$\underline{C}H_2$-NH-; δ~2.5-3.0 ppm) and PAA (NH_2-$C\underline{H}_2$-CH; ~δ 1.3-1.7 ppm) with respect to terminal methyl group of the ChA (-$\underline{C}H_3$; δ~0.95 ppm). It was found that the number of lipids (cholic acid) substituted per PEI increased with increased molecular weight of the polymer (PEI2 to PEI25). For a polymer (PEI2):lipid feed ratio of 0.2 to 4, number of lipids per PEI was increased from 0.53 to 1.90, whereas for PEI25 4.9 to 14.3 lipid per PEI25 were obtained when feed ratio (polymer:lipid) 0.2 to 16 was used. The lipid substitution for PAA15 was also increased from 0.89 to 6.7 for lipid:PAA15 feed ratio of 1.25 to 10. All the polymer conjugates were found to be soluble in water.

The ability of polymer conjugates and unmodified polymers (PEI2, PEI25 and PAA15) to bind the pDNA was calculated by SYBR Green I assay and BC50 values (i.e., polymer:pDNA weight ratio required to bind 50% of pDNA) were calculated using SigmaPlot 11.0 software. The pDNA binding capacity of polymer conjugates for PEI25 and PAA15 remained relatively unaltered while the binding capacity of PEI2 conjugates were found to be significantly decreased with increasing cholic acid substitution. The results of electrophoretic mobility shift assay (EMSA) also confirmed the same finding as observed in SYBR Green I assay. The ability pDNA complexes with polymer conjugates exposed to cleavage by DNase I was carried out to test the ability of these nanoplexes in protecting DNA from digestion by DNase present in serum. It was observed that all polymer conjugates at polymer:pDNA weight ratio of <5 (the ratio used for transfection studies), protected at least ~50% of pDNA up to 1 h treatment with DNase I.

Nanoplexes are expected to demonstrate stability in the presence of anionic serum proteins encountered in vivo. Therefore, a qualitative evaluation of dissociation of complexes by anion (heparin) challenge at various concentrations (0.01, 0.05, 0.1, 0.5 mg/ml) was monitored. It was found that unmodified PEI2, PEI25 and PAA15 were so tightly bound to pDNA that dissociation of complexes required 1 mg/ml heparin solution. Polymer conjugates of PEI25 and PAA15 were slightly less tightly bound to plasmid DNA (0.5 mg/ml heparin required for complete dissociation), whereas PEI2-ChA was less tightly bound to pDNA requiring 0.1 mg/ml heparin concentration. Based on DNase I protection and dissociation study, it can be concluded that the nanoplexes prepared with PEI2-ChA would protect pDNA extracellularly and would release it intracellularly after their uptake, thereby improving transfection efficiency.

The cytotoxicity of the chosen polymers and their ChA conjugates in 293T cells by MTT assay show that conjugation of ChA with PEI2 did not indicate an increase its cytotoxicity. On the other hand, the cytotoxicity of PAA15-ChA and PEI25-ChA is significantly higher which discourages their *in vivo* use. The transferrin containing nanoplexes of polymer conjugates with pDNA were prepared to serves two purposes, firstly, it is used as a brain targeting ligand and secondly it can be used as a shielding agent against nonspecific interaction with anionic serum proteins.

The dynamic light scattering analysis showed that the hydrodynamic diameter of various nanoplexes (with three different polymer conjugate series) measured was within the range of 100-230 nm. The particle size distribution pattern indicated that all particles are of nanometer size and devoid of any aggregates. The incorporation of transferrin to the nanoplexes of polymer or polymer conjugate with pDNA showed an increase in particle size ~80 nm. The transmission electron microscopy (TEM) images were revealed that particles in nanometer dimension (nanoplexes) were formed by unmodified polymer or polymer conjugates with pDNA. Also it was found that the particles were compact, solid and spherical in shape. The zeta potential (ζ) pattern indicated that nanoplexes prepared with all polymer conjugates were having positive zeta potential and the zeta potential of transferrin containing nanoplexes was also positive.

Transfection efficiency of ChA conjugates in 293T cells using the gWIZ-GFP showed that PAA15-ChA conjugates did not show significant transfection efficiency and hence they

were not used for further studies. But a general trend of increased transfection efficiency with increase in lipid substitution was observed for both types of polymer conjugates viz PEI2 and PEI25. The higher lipid substituted (1.9 lipids/PEI2) polymer conjugate of PEI2 showed highest transfection efficiency (~68%) in terms of GFP positive cells at higher weight ratio. The nanoplexes also demonstrated GFP expression in CB-MSC cells in terms of GFP fluorescence. It means that PEI2-ChA conjugates work better in terms of transfection efficiency than PEI25. The fluorescent microscopic images also confirmed the similar trend. The GFP expression by transferrin containing nanoplexes of PEI2-ChA conjugates at higher polymer:plasmid ratio (weight ratio 12) produced 79.41% GFP positive cells. An increased transfection efficiency of the nanoplexes can be attributed to the overexpression of TfR on U87MG cells.

It was observed that the transferrin containing nanoplexes of PEI2-ChA showed transfection efficiency of ~25% and ~30 % in NT8e cells and ~17% and ~19% in SKOV3 cells. The fluorescent microscopic images revealed a similar trend of transfection as observed by flow cytometric analysis using GFP as reporter gene. The transferrin containing nanoplexes of PEI2-ChA with plasmid p53 showed increased apoptosis in NT8e cells and the increased apoptosis clearly suggested restored p53 function. The western blot analysis revealed that p53 was expressed significantly more in NT8e cells in comparison with the p53 expression in SKOV-3 cells when treated with transferrin containing nanoplexes of PEI2-ChA with pDNA. The naked plasmid p53 could not show any p53 expression in both the cell lines.

The animals injected intravenously with transferrin containing nanoplexes of PE(PEI2-ChA with gWIZ-GFP showed localized yellow colored signal corresponding to highest GFP intensity in brain region. In addition to in vivo imaging, ex vivo imaging for organs isolated from animals treated clearly showed a strong signal for GFP in brain. At 48 days post-tumor induction, animals treated with transferrin containing nanoplexes of PEI2-ChA with plasmid p53 showed significantly smaller (~5 times) tumors as compared with tumors of control animals.

6.2 CONCLUSIONS

The cholic acid modified PEI2 conjugates were synthesized successfully and were established as an efficient transfecting agent by evaluating in several different cell lines (from 293T, a screening cell line to NT8e, hard to transfect cell line). The transferrin containing nanoplexes of synthesized novel PEI2-ChA conjugates with pDNA formed compact particles in nanometer dimension, showed excellent pDNA binding capacity and demonstrated their ability to protect pDNA against DNase I. The PEI2-ChA conjugates were found to be apparently nontoxic in 293T cells at the concentrations tested and yet their transfection efficiency was comparable with PEI25 in all the cell lines tested. The transferrin containing nanoplexes of PEI2-ChA conjugate with plasmid p53 demonstrated *in vitro* p53 protein expression and showed significant apoptosis indicating their ability to the restore normal functional p53 function in NT8e cells. The transferrin containing nanoplexes further demonstrated the brain targeting efficiency *in vivo* whereby the gene expression was found to be localized in the brain region. The nanoplexes of PEI2-ChA also showed significant tumor regression in xenograft model of nude mice with NT8e cells.

Thus, based on the results obtained in present research work, it can be concluded that the transferrin containing nanoplexes of PEI2-ChA conjugates with plasmid p53 warrants clinical trials in humans after exhaustive animal studies for use as a novel gene delivery system.