Abstract

The research work described in this thesis encompasses the syntheses, characterization and bioactivity determination of various novel systems crafted for achieving high efficacy and targeted gene therapeutic effect in cancer cells *in vitro* and *in vivo*. Cancer being one of the prominent causes of mortality over the world, has gained tremendous attention of scientists over the past few decades. The latest approach has been to target the disease at the genetic level itself, a field of research known as 'Gene therapy'. Several favorable results have been obtained in cancer gene therapy, but each accompanied by a set of limitations, the most common being the lack of gene transfection efficiency *in vivo*. A chemist's approach to solving this problem is by designing an efficient gene delivery vector that can bind the nucleic acid cargo in a reversible manner to ensure the subsequent release at the target site. It should provide protection from nucleases and should also meet the very important criteria of biocompatibility. In the studies described herein, a range of naturally occurring biomolecules *i.e*. a pH-sensitive tripeptide, a biodegradable polysaccharide and an essential vitamin (alone and in conjugation with a set of receptor targeted antibodies) have been explored for their ability to deliver therapeutically relevant agents inside cancer cells. These have been derivatized using simple and cost-effective synthetic methods for fine-tuning of their physicochemical properties. The aggregation behavior of these derivatives, in solution, was studied along with their nucleic acid binding abilities. After complete characterization of the systems by various biophysical techniques, their delivery efficacy was gauged by performing various biological assays involving cancer cells in culture. The promising systems were taken for studies in animal model (nude mice) for assessment of *in vivo* anti-cancer therapeutic potential. Target-specific gene transfection has been attempted using immunoliposomes guided by monoclonal antibodies decorated on the surface. Taken together the thesis presents a potential cisplatin prodrug, a safe and efficient gene transfecting polysaccharide for co-delivery of gene and drug, besides an amphiphilic vitamin for efficacious delivery of short therapeutic oligonucleotides and for cell-specific DNA delivery allowing effective gene transfer in the hard to transfect suspension cells.

Chapter 1: A Concise Overview of Cancer Gene Therapy, the Futuristic Medicine Gene therapy has been proposed to be the future of medicine in case of most genetic disorders like cancer. The scope of this technique has widened over the years and the easiest approach devised has been to alter the regulation patterns of certain oncogenes, tumour suppressor genes or the genes responsible for modulating drug sensitivity. After giving a throwback into the birth and growth of the concept of gene therapy, the first chapter of this thesis covers the different facets of this promising mode of therapy while briefly summarizing the advances made in the field thus far. First, a comprehensive description of the mechanism of action of cisplatin, which is the first line of treatment for cancer, is provided followed by the advantages of a Pt(IV) prodrug over a Pt(II) drug. This section then surveys various cell-cycle dependent drugs based on a Platinum core,

some of them even being clinically approved. The readers are also introduced to the Nobel Prize winning technique of RNA interference (RNAi) along with its implications in cancer gene therapy. The utilization of small interfering RNA (siRNA) for downregulation of oncogenes, and for identification of putative gene targets for effectively countering malignancy, is described therein. Putting the siRNA-based strategies to effect requires an efficient vector system for intracellular transfer of the exogenous short oligonucleotides. The designing of such vectors needs a comprehensive understanding of the process of nucleic acid delivery and the various impedances encountered in its pathway to bring about the desired therapeutic effect. The most important ones among these are: nucleic acid complexation, cellular binding, cellular uptake, endosomal escape, intracellular trafficking, nuclear entry and release of the complexed nucleic acid. These have also been highlighted along with the steps that have been taken to overcome the limitations of non-viral gene delivery and development of newer systems for enhancement of delivery efficacy. Some of the most promising non-viral transfect ants belong to the category of cationic polymers that condense genes into compact polyelectrolyte complexes, by virtue of formation of which, they offer protection against nuclease induced damage. A plethora of natural and synthetic polymers that have demonstrated gene transfection abilities have been described along with an important class of amphiphilic gene vectors *i.e.* cationic lipids. The way their self-assembly drives the formation of nano-sized vesicles in aqueous medium and their strong nucleic acid binding and release properties make them attractive candidates for highly effective gene transfection. Since vectors of synthetic origin exert immunostimulatory effects, the focus of this thesis is to utilize naturally occurring unique systems for attaining safe, effective and target specific gene transfection.

Chapter 2: New Cisplatin Prodrugs Originating from the pH-Sensitive Self-Assembling Natural Tripeptide In this chapter, a pH-sensitive self-assembling tripeptide, KFG (Lysine-Phenylalanine-Glycine) is used for preparation of a Pt(II) core based cisplatin analogue and a Pt (IV) core based cisplatin prodrug, both of which were completely characterized. They demonstrated higher potency than the native drug in cell lines that are resistant to clinically relevant concentrations of cisplatin. Additionally, when used in combination with Doxorubicin encapsulated tripeptide vesicles, Pep- DOX, they were found to lower the effective dosage of the anti-cancer drug *in vitro* exhibiting synergistic enhancement in cytotoxicity. The more active Pep-Pt(IV) complex was taken for *in vivo* studies where the combinatorial therapy with Pep-DOX was able to cause complete flattening of xenograft tumours within a week of treatment in nude mice model (**Figure I**). Although found quite potent, the cisplatin analogues have been reported to exhibit several side effects at the medicinal stage, which may be attributed to the heavy load of renal clearance and accumulation of metal-ion based by-products. **Figure I.** Molecular structure of the Pep-Pt (II) and Pep-Pt(IV) complexes used for preparation of dual drug delivery systems that exhibit synergistically enhanced drug internalization *in vitro* and tumour regression *in vivo*.

Chapter 3: Exploration of Derivatives of Chitosan, a Naturally Occurring Polymer, as a Biocompatible, Potential Vector for Sustained Gene Delivery Chapter 3A: Chitosan Imine Derivative for Sustained Gene Transfection The common concern of biocompatibility of a gene delivery vector was met by designing a water-soluble derivative of a naturally abundant carbohydrate polymer for safe and efficient gene delivery. Chitosan, a partially deacetylated derivative of the second most abundant natural polymer, chitin that is found in the shells of crustaceans was chosen due its biodegradability and ability to condense DNA by virtue of proton able amine functionalities (pKa = 6.5) running along the length of the polymer backbone. It was solubilized in water by *O*-carboxymethylation and then used for imine formation with pyridine-4-carboxaldehyde. The pyridine imine derivative, (py)CS(CH2COOH) was observed to form nano-sized spherical polyplexes with pDNA having positive surface charge in aqueous medium. The gene transfection ability of this derivative was found to be comparable to that of commercial standard, Lipofectamine 2000. However, it displayed sustained transfection over longer durations with minimal loss of cell viability as opposed to the commercial standards which became toxic with increased dosage or time of incubation (**Figure II**). This chapter presents an in-depth study of the biocompatible, non-toxic, chitosan derivative for efficient and sustained gene transfection owing to the formation of nuclease tolerant and serum stable polyplexes with pDNA. **Figure II.** Molecular structure of chitosan imine derivative, (py)CS(CH2COOH) and its application as a safe and efficient gene delivery carrier.

Chapter 3B: Chitosan Oxime Ether with Doxorubicin for Gene and Drug Co-Delivery Having succeeded in developing a safe, effective and biocompatible polymeric gene delivery carrier, we embarked upon using it in combination with a drug bearing polymer for co-delivery of gene and drug. A novel oxyamine derivative of chitosan, CS(ONH2) was first synthesized which was then used for preparation of oxime ether with pendant drug entities anchored on to a DNA complexing polymer backbone. The oxime ether linkage, being stable under biologically relevant conditions and hydrolysable at the endosomal pH, conferred pH sensitive drug release properties to the Doxorubicin tethered polymer, CS(Dox). When mixed with pDNA carrying (py)CS(CH2COOH) polyplexes, enhanced internalization of both, the gene and drug. The extent of cell death caused by the polymer-drug conjugate was improved when used in combination with (py)CS(CH2COOH) polyplexes carrying the tumour suppressor gene, p53. The improvement in potency relative to the free drug pointed to the advantageous role of the oxime ether linkage. Fluorescence based cytometry and microscopy studies have shown that pH-sensitive linkage indeed elevated the cellular internalization of the anti-cancer drug. The extent of co-delivery is demonstrated to increase as compared to the use of free drug with the polyplexes (**Figure III**). **Figure III**. Molecular structures of CS(ONH2) and CS(Dox) along with a schematic representation of novel nanocomposites for gene and drug co-delivery.

This chapter, thus, evaluates a versatile delivery vector capable of efficiently delivering both, a drug and a gene to bring about an enhanced anti-cancer therapeutic response. Polymeric vectors

are known to deliver polynucleotides by virtue of formation of polyelectrolyte complexes. A vector for adept delivery of short oligonucleotides requires a vector of non-polymeric origin. **Chapter 4: Assessment of siRNA Delivery using Tocopherol Gemini Lipids Terminated with Hydroxyethyl Head Groups Chapter 4A: Efficacy of siRNA Transfection by Hydroxyethyl Terminated Tocopherol Gemini Lipids *in vitro* and its use for Induction of Apoptosis in Cancer Cells** Owing to high positive surface charge, cationic lipids are known to efficiently condense small oligonucleotides into compact nano-aggregates called lipoplexes. Since synthetic lipid vectors are usually associated with immunogenic responses, a vector molecule that is essential to the target cells and does not possess a biosynthetic pathway inside the cell, was chosen for the study. Vitamin E or α-Tocopherol was taken for construction of a hydroxyethyl terminated Gemini lipid, TH8S that has proven its ability to deliver pDNA effectively in combination with a helper lipid, DOPE, at a molar ratio of 2:1. Chapter 4A discusses the use of this efficient co-liposomal formulation for delivery of short interfering RNA (siRNA) of therapeutic importance. Selection of an appropriate target gene is crucial for achieving a therapeutically relevant gene knockdown. Since surviving is one such gene that is essential for the survival of cancer cells and inhibition of their apoptotic pathway, the TH8S-DOPE co-liposomes were used for transfection of anti-surviving siRNA in HepG2 cells. Effect of hydroxyl functionality, at the head group, on the efficiency of siRNA delivery was explored using MTT assay and apoptosis detection assay in HepG2 cells. Validation of the same was obtained by RT-PCR and Western blot studies where a mechanistic insight into the route of apoptosis induction by surviving downregulation was obtained (**Figure IV**). Downregulation of surviving by the interfering nucleotide was also found to sensitize the transfected cells towards the chemotherapeutic agent, Doxorubicin. The details of the *in vitro* studies have been described in this chapter.

Figure IV. Molecular structures of TH8S Gemini and DOPE helper lipids used for the formation of co-liposomes that demonstrate GFP knockdown and induction of apoptosis by surviving downregulation *in vitro* along with tumour regression by intratumoral delivery of anti-surviving siRNA leading to chemo sensitization. **Chapter 4B: Transfection of siRNA Mediated by Hydroxyethyl Terminated Tocopherol Gemini Lipids for Tumour Regression *in vivo*** This chapter describes the study assessing the ability of afore mentioned tocopherol Gemini lipids with hydroxyethyl terminated head groups, to regress xenograft tumours in nude mice model system. For any anti-cancer gene therapeutic to be clinically relevant, it must be exhibit high efficiency of gene transfection *in vivo*. Tumorigenic cells, HepG2 explant cells were used for generating xenografts in athymic nude mice. After validating the TH8S/DOPE co-liposome mediated internalization of anti-surviving siRNA in the cells of the tumour tissue by RT-PCR, the ability of lipoplexes to lower the rate of tumour growth was determined. The effect of inhibition of tumour growth, by way of post-transcriptional gene silencing of surviving in the tumour cells, by the interfering oligonucleotide was further supplemented by a sub-optimal concentration of Doxorubicin (**Figure IV**). The improvement of responsiveness of the tumour cells towards anti-

apoptotic agents by downregulation of surviving was, thus, used to achieve complete inhibition of tumour progression in the animal model studied.

Chapter 5: Targeted Gene Delivery Mediated by Receptor-Guided Immunoliposomes

Chapter 5A: Targeting Hepatocellular Carcinoma using Anti-LDLr mAb Tagged Gemini Tocopherol Liposomes

A promising strategy to enhance the therapeutic potential of liposome mediated gene delivery is by directing the liposomes to the target cells. This strategy was employed making use of a very strong and specific interaction, the antigen-antibody interaction. For targeting hepatocellular carcinoma, liposomes prepared from the highly efficient Tocopherol Gemini lipids with hydroxyethyl headgroups were decorated with anti-LDLr antibody which recognizes and binds to the Low-Density Lipoprotein receptor (LDLr) that is particularly abundant on the surface of

Figure V. Molecular structures of TH8S and TME used for construction of immunoliposome by conjugation with anti-LDLr mAb achieving p53 delivery specifically in liver cancer cells.

liver cancer cells. Its involvement in cholesterol homeostasis and metabolism of hepatic tumour tissue allows it to possess a special mechanism for intracellular transport of LDL, called 'receptor-mediated endocytosis'. Making use of the thiol-maleimide addition chemistry, anti- LDLr monoclonal antibody (mAb) was conjugated to monomeric tocopherol lipid (TME) that was incorporated in TH8S liposomes (**Figure V**). Detailed in this chapter are the methods of purification of the mAb, its conjugation to TME and construction of immunoliposomes. The immunoliposomes, upon complexation with the pCEP4-p53, have been demonstrated to effect p53 inhibition of cancer cell proliferation and induction of apoptosis by reinstatement of the tumour suppressor gene, p53. While being able to selectively transfect liver cancer cells, these tocopherol based immunoliposomes offer an additional advantage due to the presence of the liver cytosolic protein, α-TTP (α-Tocopherol transfer protein) that possesses a specific binding affinity for tocopherol, facilitating vector bioavailability.

Chapter 5B: Efficient and Targeted Gene Transfection in Suspension Cells using Anti- CD4 mAb Directed Immunoliposomes As opposed to adherent mammalian cells in culture, suspension cells are known for being hard to transfect using the traditional non-viral agents like cationic polymers or lipids. Since the difficulty in transfection arises out of the inability of the transfection complex to attach to the surface of the cells floating in the culture medium, the strategy of mAb guided targeting was utilized to facilitate this binding interaction (**Figure VI**).

Figure VI. CD4 targeted gene transfection in suspension cells using OKT4 tagged TH8S immunoliposomes. A well-established suspension cell culture system was used as a model, with the T-cell receptor, CD4 being the guidance cue for cellular binding and subsequent internalization of p53 gene carrying TH8S immunolipoplexes tagged with OKT4 (anti-CD4 mAb). The chapter goes through a series of assays for understanding the T-cell specific gene delivery by the constructed immunoliposomes, while throwing light on their capacity as being among the few in the league of efficacious suspension cell transfect ants.