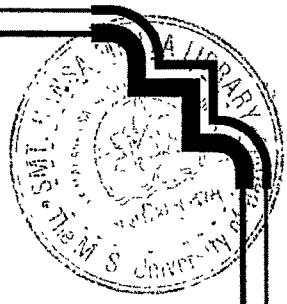

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

Recently, with the advance of biotechnology, an increasing number of proteins and peptides have been developed as therapeutic drugs. Biotechnology has enabled several new categories of molecules to be used as therapeutic agents. For example, peptide based drugs and DNA based therapeutic agents (gene therapy, DNA vaccines and antisense oligonucleotides) are now being routinely investigated. Due to the low bioavailability after oral delivery, these drugs are usually administered by the parenteral route. However, parenteral administration has some limitations. First, the short biological half-life of peptide drugs and DNA result in an inconveniently high dosing frequency. Second, the high blood concentrations of some peptide drugs after intravenous administration or after chronic dosing could cause significant toxicity. To prolong the biological half life of the drug, to prevent from enzymatic degradation and to reduce the injection frequency and toxicity of intravenously administered drugs, it would be necessary to develop safe and sustained injectable delivery systems containing peptide drugs and DNA.

Particulate systems, such as liposomes, micro or nanoparticles, have attracted a great deal of attention for peptide drugs and DNA as safe and sustainable drug delivery systems. Liposomes have considerable potential as drug carriers for peptide drugs and DNA to prevent from enzymatic degradation. Liposomes are biodegradable and essentially non-toxic, can encapsulate hydrophilic, hydrophobic and amphipathic drugs. Delivery of agents to the reticuloendothelial system (RES) is easily achieved since most conventional liposomes are trapped by RES. As one approach to avoid the phagocytosis of liposomes by RES, coating the liposomes with chains of polyethylene glycol (PEG) has been used to prolong the circulation time of the liposomes, based on a sterical hindrance or an increase of liposomal surface hydrophilicity. For the purpose of delivery of agents to target organs other than RES, long circulating liposomes have been developed by modifying the liposomal surface and its surface charge.

Various types of systems (liposomes, emulsions, micelles and nanoparticles) were developed over the last decade in order to achieve controlled release

and targeting of therapeutic agents (peptide drugs and DNA) to specific tissues. Liposomes are microscopic lipid vesicles consisting of one or more concentric lipid (usually phospholipids) bilayers enclosing discrete aqueous spaces. They can be constructed so that they can entrap the drug both within their aqueous compartment or intercalated into the lipid phase. These liposome-encapsulated drugs often have biodistribution and toxicities, which differ greatly from the free drug. The value of liposomes as model membrane systems derives from the fact that liposomes can be constructed of natural constituents such that the liposome membrane forms a bilayer structure which is in principal identical to the lipid portion of natural cell membranes- the 'sea of phospholipids' in the Singer and Nicholson model. The similarity between liposome and natural membranes can be increased by extensive chemical modification of the liposome membrane, and may be exploited in areas such as drug targeting or immune modulation, both *in vitro* and *in vivo,* where the ability to mimic the behaviour of the natural membranes, and also degraded by the same pathways, makes them a very safe and efficacious vehicle for medical applications. The main objectives and advantages of using liposomes as drug delivery systems are:

1. Reduce the distribution to sensitive tissues leading to toxicity
2. Significant dose reduction
3. Favorably alter the pharmacokinetics
4. Permit targeting to the desired tissue
5. Reduce metabolism or degradation
6. Improve solubility
7. Better therapeutic index and enhanced cellular uptake
8. Biodegradability
9. Low toxicity and low immunogenicity
10. Ability to trap drugs without the necessity of covalent bonding
11. Agents can be delivered to tissues in a sustained, continuous and predictable fashion
12. Repeated drug administration is not necessary
13. Ease of preparation.

For some applications liposomes have been limited by a number of factors, which include:

- Their relatively fast clearance, which show a pronounced dependent on size.
- Their marked tendency to phagocytosise and localize in the tissues of the mononuclear phagocytic system (MPS), particularly in the liver and spleen.
- Stability.

Thus conventional liposomes along with their associated drugs often eliminated from the circulation by cells of the MPS before effective delivery of drugs to extravascular and some intravascular target sites can be achieved. Uptake by the MPS cells generally leads to irreversible sequestering of the unencapsulated drugs thereby eliminating any beneficial effects as well as posing potential risk of toxicity to these cells. Interestingly, the composition of the liposomes can be altered by the inclusion of cholesterol, rigid lipids and/or charged components in the liposomes which generally have effects on the clearance and the biodistribution of liposomes. Altering the surface charge of the neutral liposomes has been shown to markedly influence their rate of distribution out of circulation. Some negatively charged carbohydrate containing lipids like Gangliomonoside 1, Sulfogalactosylceramide and Phosphatidylinositol and recently, N-glutaryl/adipyl derivatives of Dioleyl phosphatidyl ethanolamine (DOPE) prolong liposome circulation times. However, liposomes containing other negatively charged phospholipids such as phosphatidylserine (PS), Phosphatidyl glycerol (PG), Phosphatidic acid (PA) or cardiolipin are rapidly distributed to liver and spleen compared to their neutral and positively charged counterparts. This was followed by findings that a hydrophilic polymer-lipid conjugate, polyethyleneglycol-phosphatidyl ethanolamine (PEG-PE), can provide better protection from uptake and prolonged blood circulation. The hydrophilicity was considered as a main requirement, but it turned out not to be sufficient one. Indeed liposomes were coated with series of hydrophilic polymers, among which maltopentaose, estimated to be more hydrophilic than PEG5000, but they were still removed from blood circulation very rapidly in mice. Dextran-

coated liposomes circulate shorter than PEG-coated ones, inspite of more hydrophilic nature of dextran compared to PEG. It has been proposed that besides hydrophilicity, chain flexibility is another major feature necessary for the coating of polymers to provide prolonged circulation. The protective layer of PEG is considered as a "cloud" of possible chain conformations, with a density high enough to prevent the interactions of opsonins with the surface of the particles. Only if a polymer chain possesses both hydrophilicity and flexibility properties it can serve as an effective protector/coat for particles against opsonization. For drug delivery, the PEG-PE containing liposomes have several important advantages but the major three are:

1. Inhibits RES mediated clearance directly and indirectly through prevention of opsonization.
2. Hydrophilicity and flexibility of PEG based coatings significantly increase the liposome blood circulation time.
3. It is easy to prepare and use without modification of the many well-known liposome methodologies.

The aim of the present study was to develop a sustained and a safe injectable drug delivery system for peptide drugs (cyclosporine and leuprolide acetate) and DNA by exploring the potential of charged and sterically stabilized liposomes.

CYCLOSPORINE

Cyclosporine (also known as Cyclosporine A), a poorly water soluble cyclic peptide comprising 11 amino acids, has been utilized clinically as a potent immunosuppressive agent, such as the prevention of rejection following transplantation of kidney, liver, bone marrow and pancreas. The use of CsA has been limited due to a broad toxicity profile. Serious side effects, including both acute and chronic nephrotoxicity are associated with its use; however, particularly in the initial post-surgical phase where it must be administered intravenously at relatively high dose. Liposomes are microscopic lipid spheres that accumulate preferentially at sites of infection and inflammation and also at the tumor sites after intravenous administration. Therefore by incorporating CsA into a liposomal carrier, it

should be possible to achieve elevated drug levels at sites of tissue rejection, as a result of involvement of macrophages and other components of the immune response, while lowering drug exposure to sensitive organs such as kidney and prolonged systemic circulation.

LEUPROLIDE ACETATE

Leuprolide acetate, a highly water soluble nonapeptide and a potent GnRH analogue utilized clinically for the treatment of prostate carcinoma in men, ovarian and breast carcinoma in women. Leuprolide acetate has been used extensively in clinical oncology because these agents exert anti tumor effects against a variety of tumor cells both *in vitro* and *in vivo*. These effects may be mediated by several mechanisms including direct antiproliferative activity toward malignant cells. GnRH agonists induce cell cycle arrest in the Go/G1 phase but the detailed molecular mechanism is unknown. One of the proposed mechanisms of the anti-tumor effect of GnRH agonists is the down regulation of GnRH receptor (GnRH-R) activity in the pituitary gland that results in inhibition of sex steroid secretion in patients with premenopausal breast cancer or prostate cancer. The other probable mechanism of action is the direct action of GnRH agonists via the GnRH-R, which is expressed in malignant tumors that differs from the pathway mediated via the pituitary. This direct action promotes the antiproliferative effect of these agents on the tumors. GnRH-based system is expressed in prostate cancer, which might participate in the local regulation of tumor growth. This hypothesis has been later confirmed by showing that the activation of locally expressed GnRH-R by means of potent GnRH super agonists significantly reduces the proliferation of prostate cancer cells, both *in vitro* and *in vivo*. Reports revealed that the nucleotide sequence of GnRH receptors in human breast and ovarian tumors is identical with that found in pituitary. However, the direct antiproliferative activity of GnRH analogue requires the continuous presence of this molecule at the site of tumor, therefore a delivery system that prolongs the biological half-life of the drug, prolongs the exposure of target cells (such as malignant cells) to GnRH analogue into malignant cells would be advantageous, such goals can be achieved by liposomal delivery system.

DNA

Calf thymus DNA was encapsulated in the liposomes as a model DNA. Upon systemic administration, naked DNA was rapidly cleared from the circulation. Within the short period, DNA will be available as a pharmaceutical for gene delivery, vaccination and other applications in molecular medicine. The generally poor efficiency of delivery remains one of the limitations to the development of gene therapy and vaccination. Much attention is therefore being paid to the design of new formulations of DNA using liposomes as an alternative attractive carrier for the controlled delivery of therapeutic agents (including DNA). Cationic liposomes have been widely used for gene delivery systems and various cationic liposomes are commercially available. A major limitation in development and *in vivo* applications of the cationic liposomes is the rapid uptake of particulate liposomes, following intravenous administration, by the RES. Therefore a delivery system that prolongs the circulation in blood and diminished uptake by the liver and spleen cells would be advantageous, such goals can be achieved by sterically stabilized liposomal delivery system.

1.2 PROPOSED PLAN OF WORK

The proposed plan of work is as follows:

CYCLOSPORINE: (CsA)

1. Preparation of neutral and charged liposomes of CsA and optimization of the process and formulation variables.
2. Characterization of the conventional liposomes of CsA by methods useful for ascertaining the well documented liposomal parameters (size, shape, lamellarity, entrapment, etc.).
3. *In vitro* drug release studies, using an appropriate method, for evaluation of the sustained release of CsA from conventional liposomes.
4. *In vitro* immunosuppressive studies of the conventional liposomes containing cyclosporine using mouse splenocytes (splenic lymphocytes) by Flow cytometry.
5. Optimization of radiolabeling of the free drug (CsA) and their prepared liposomal formulations using Technetium (^{99m}Tc).

6. *In vivo* biodistribution studies of the prepared liposomes for the evaluation of sustained and targeted delivery using suitable animal models.
7. Comparison of Nephrotoxicity of CsA and their prepared liposome formulations.

LEUPROLIDE ACETATE

8. Preparation of conventional liposomes of Leuprolide acetate and optimization of the process and formulation variables.
9. Synthesis and characterization of methoxy polyethylene glycol derivatives using cyanuric chloride as coupling agent.
10. Preparation of sterically stabilized liposomes by incorporating the prepared polymers (mPEG5000-CC-PE and mPEG2000-CC-PE).
11. Preparation of sterically stabilized liposomes of Leuprolide acetate and optimization of the process and formulation variables.
12. Characterization of the conventional and sterically stabilized liposomes of leuprolide acetate by methods useful for ascertaining the well documented liposomal parameters (size, shape, lamellarity, entrapment, entrapped volume etc.) and the extent of steric stabilization.
13. *In vitro* drug release studies, using an appropriate method, for evaluation of the sustained release of leuprolide acetate from conventional and sterically stabilized liposomes.
14. *In vitro* cytotoxicity studies of the conventional and sterically stabilized liposomes of leuprolide acetate using cancer cell lines by MTT assay.
15. Optimization of radiolabeling of the free drug (leuprolide acetate) and their prepared liposomal formulations using Technetium (^{99m}Tc).
16. *In vivo* biodistribution studies of the prepared liposomes for the evaluation of sustained and targeted delivery using suitable animal models.

DNA

17. Preparation of conventional liposomes of DNA and optimization of the process and formulation variables.

18. Characterization of the conventional and sterically stabilized liposomes of DNA by methods useful for ascertaining the well documented liposomal parameters (size, shape, lamellarity, entrapment, entrapped volume etc.) and the extent of steric stabilization.
19. *In vitro* drug release studies, using an appropriate method, for evaluation of the sustained release of DNA from conventional and sterically stabilized liposomes.
20. Optimization of radiolabeling of the free drug (DNA) and their prepared liposomal formulations using Technetium (^{99m}Tc).
21. *In vivo* biodistribution studies of the prepared liposomes for the evaluation of sustained delivery using suitable animal models.