Chapter 2
Literature Review

2.1 CANCER: A BRIEF OUTLINE

2.1.1 INTRODUCTION

Cancer is an English term derived from the Greek word for crab, Karkinos, which was believed to be first used by Hippocrates, who attributed the affliction to an excess of black bile. Cancer was known in antiquity, being described in the early writings of Greek *and* Romans.

Cancer is not a disease, but a group of diseases affecting different organs and systems of the body. Cancer can be defined very broadly as a disease in which there is uncontrollable multiplication and spread within the body of abnormal forms of the body's own cells (Montogomery, 1979).

Cancer cells differ from normal cells in behaviour in that they manifest three characteristics not seen in normal cells, which are uncontrolled proliferation, invasiveness and capacity to metastasize.

2.1.2 THE MAGNITUDE OF THE PROBLEM

There is no worldwide cancer registry; therefore, the incidence of cancer is estimated on the basis of the National Cancer Institute's Surveillance, Epidemiology, and End Results (SEER) database, which tabulate cancer incidence and death figures from the U.S. population. In 2000, 1.22 million new cases of invasive cancer (619,700 men, 600,400 women) were diagnosed and 552,200 people (284,100 men, 268,100 women) died of cancer. The percent distribution of new cancer cases and cancer deaths by site for men and women are shown in Table 2.1.

Tumors can be benign or malignant.

Benign tumors are not cancer. They can often be removed and, in most cases, they do not come back. Cells from benign tumors do not spread to other parts of the body. Most important, benign tumors are rarely a threat to life.

Malignant tumors are cancer. Cells in these tumors are abnormal and divide without control or order. They can invade and damage nearby tissues and organs. Also, cancer cells can break away from a malignant tumor and enter the bloodstream or the lymphatic system spreads from the original cancer site to form new tumors in other organs. The spread of cancer is called metastasis. Leukemia and lymphoma are cancers that arise in blood-forming cells. The abnormal cells circulate in the bloodstream and lymphatic system. They may also invade (infiltrate) body organs and form tumors.

2.1.3 TYPES OF CANCERS

Most cancers are named from the organ or type of cell in which they begin. The classification of cancer given below are based on its frequency of occurrence and based on the occurrence in the organs (Sausville and Longo, 2001).

2.1.3.1 Most Common Cancers

- Bladder Cancer
- Breast Cancer
- Colorectal Cancer
- Endometrial Cancer
- Head and Neck Cancer
- Leukemia
- Lung Cancer
- Lymphoma
- Melanoma
- Non-Small-Cell Lung Cancer
- Ovarian Cancer
- Prostate Cancer

2.1.3.2 Less common Cancers

- Acute Lymphocytic Leukemia
- Adult Acute Myeloid Leukemia
- Adult non-Hodgkin's
- Brain Tumour
- Cervical Cancer
- Childhood Cancers
- Childhood Sarcoma
- Chronic Lymphocytic
- Chronic Myeloid Leukemia
- Esophageal Cancer
- Hairy Cell Leukemia
- Kidney Cancer
- Liver Cancer
- Multiple Myeloma
- Neuroblastoma
- Oral Cancer
- Pancreatic Cancer
- Skin Cancer
- Small-Cell Lung Cancer

2.1.3.3 Body location/ System

- Bone
- Brain
- Breast
- Childhood Cancers
- Digestive/Gastrointestinal
- Endocrine
- Eye
- Germ Cell
- Gynaecologic
- Head and Neck
- Leukemia
- Lung
- Lymphoma
- Neurologic
- Respiratory / Thoracic
- Sarcoma / Musculoskeletal

TABLE. 2.1 Distribution of cancer incidence and deaths for 2000[a]

MALE			FEMALE		
Sites	**%**	**Number**	**Sites**	**%**	**Number**
CANCER INCIDENCE					
Prostate	29	180,400	Breast	30	182,800
Lung and bronchus	14	89,500	Lung and bronchus	12	74,600
Colon and rectum	10	63,600	Colon and rectum	11	66,600
Bladder	6	38,300	Endometrium	6	36,100
Lymphoma	5	31,700	Ovary	4	23,100
Melanoma	4	27,300	Lymphoma	4	23,100
Oral Cavity	3	20,200	Melanoma	3	20,400
Kidney	3	18,800	Bladder	2	14,900
Leukemia	3	16,900	Pancreas	2	14,600
Pancreas	2	13,700	Thyroid	2	13,700
All other	19	119,300	All other	22	130,500
CANCER DEATHS					
Lung and bronchus	31	89,300	Lung and bronchus	25	67,600
Prostate	11	31,900	Breast	15	40,800
Colon and rectum	10	27,800	Colon and rectum	11	28,500
Pancreas	5	13,700	Pancreas	5	14,500
Lymphoma	5	13,700	Ovary	5	14,000
Leukemia	4	12,100	Lymphoma	5	12,400
Esophagus	3	9,200	Leukemia	4	9,600
Liver and bile duct	3	8,500	Endometrium	2	6,500
			Brain	2	5,900
Bladder	3	8,100	Stomach	2	5,400
Stomach	3	7,600	Myeloma	2	5,400
All other	22	62,200	All other	21	57,500

[a] Data exclude basal and squamous cell skin cancers and carcinoma in situ except the bladder (Greenlee, 2000).

2.1.4 CANCER TREATMENT

The goal of cancer treatment is first to eradicate the cancer. If this primary goal cannot be accomplished, the goal of cancer treatment shifts to palliation, the amelioration of symptoms, and preservation of quality of life while striving to extend life. The dictum *primum non nocere* is not the guiding principle of cancer therapy. Every cancer treatment has the potential to cause harm, and treatment may be given that produces toxicity with no benefit. The therapeutic index of many interventions is quite narrow, and most treatments are given to the point of toxicity. The guiding principle of cancer treatment is *primum succerrere*, first hasten to help. Radical surgical procedures, large-field hyperfractionated radiation therapy, high-dose chemotherapy, and maximum tolerable doses of cytokines such as interleukin (IL) 2 are all used in certain settings where 100% of the patients will experience toxicity and side effects from the intervention, and only a fraction of the patients will experience benefit. One of the challenges of cancer treatment is to use the various treatment modalities alone and together in a fashion that maximizes the chances for patient benefit.

2.1.5 METHODS OF TREATMENT

Treatment for cancer can be either local or systemic. Local treatments affect cancer cells in the tumor and the area near it. Systemic treatments travel through the bloodstream, reaching cancer cells all over the body. Surgery and radiation therapy are types of local treatment. Chemotherapy, hormone therapy, and biological therapy are examples of systemic treatment.

It is hard to protect healthy cells from the harmful effects of cancer treatment. Because treatment does damage healthy cells and tissues, it often causes side effects. The side effects of cancer treatment depend mainly on the type and extent of the treatment. Also, the effects may not be the same for each person, and they may change for a person from one treatment to the next.

2.1.6 CHEMOTHERAPY

2.1.6.1 Historic Background

The treatment of patients with cancer using chemicals in the hope of causing regressions of established tumours or to slow the rate of tumor growth arose by analogy to the proposition of Ehrlich that bacteria could be killed selectively by compounds acting as "magic bullets." Candidate compounds that might have selectivity for cancer cells were suggested by the marrow-toxic effects of sulphur and nitrogen mustards and led, in the 1940s, to the first notable regressions of haematopoietic tumours following use of these compounds by Gilman and colleagues. As these compounds caused covalent modification of DNA, the structure of DNA was thereby identified as a potential target for drug design efforts. Biochemical studies demonstrating the requirement of growing tumor cells for precursors of nucleic acids led to nearly contemporaneous studies by Farber of folate analogues. The cure of patients with advanced choriocarcinoma by methotrexate in the 1950s provided further impetus to define the value of chemotherapeutic agents in many different tumor types. This resulted in efforts to understand unique metabolic requirements for biosynthesis of nucleic acids and led to the design, rationale for the time, of compounds that might selectively interdict DNA synthesis in proliferating cancer cells. The capacity of hormonal manipulations including oophorectomy and orchiectomy to cause regressions of breast and prostate cancers, respectively, provided a rationale for efforts to interdict various aspects of hormone function in hormone-dependent tumours. The serendipitous finding that certain poisons derived from bacteria or plants could affect normal DNA or mitotic spindle function allowed completion of the classic armamentarium of "cancer chemotherapy agents" with proven safety and efficacy in the treatment of certain cancers.

Chemotherapy is the use of drugs to kill cancer cells. Neoadjuvant chemotherapy refers to drugs given before surgery to shrink a tumor; adjuvant chemotherapy refers to drugs given after surgery to help prevent the cancer from recurring. Chemotherapy also may be used (alone or along with other forms of treatment) to relieve symptoms of the disease. Chemotherapy is usually given in cycles: a treatment period (one or more days when treatment is given) followed by a recovery period (several days or weeks), then another treatment period, and so on. Most anticancer drugs are administered by injection into a vein (IV); some are injected into a muscle or under the skin; and some are administered by oral route.

Generally, anticancer drugs affect cells that divide rapidly. In addition to cancer cells, these include blood cells, which fight infection, help the blood to clot, and carry oxygen to all parts of the body. When blood cells are affected, patients are more likely to get infections, may bruise or bleed easily, and may feel unusually weak and very tired. Rapidly dividing cells in hair roots and cells that line the digestive tract may also be affected. As a result, side effects may include loss of hair, poor appetite, nausea and vomiting, diarrhoea, or mouth and lip sores.

2.1.6.2 Classification of Cytotoxic Agents

They may be grouped into six categories based on chemical and mechanistic properties (Calabresi and Chabner, 1996).

A. Alkylating agent	- e.g. Cyclophosphamide, Mephalan, Busulfan, etc.
B. Antimetabolites	- e.g. Methotrexate, Cytarabine, 5-Fluorouracil, etc.
C. Natural products	- e.g. Vincristine, Etoposide, Doxorubicin, etc.
D. Hormones and antagonists	- e.g. Tamoxifen, Flutamide, Leuprolide, etc.
E. Radioactive isotopes	- e.g. Iodine-131, Strontium-89 Samarium-153, etc.
F. Miscellaneous agents	- e.g. Cisplatin, Mitoxantrone, Procarbazine, etc.

2.1.7 REFERENCES

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2.2 LIPOSOMES

2.2.1 INTRODUCTION

A liposome is defined as a structure consisting of one or more concentric spheres of lipid bilayers separated by water or aqueous buffer compartments (Lasic *et. al.,* 1998). Liposomes form spontaneously when lipids are dispersed in aqueous media, giving rise to a population of vesicles, which may range in size from tens of nanometers to tens of microns in diameters (New, 1990). They were brought to the attention of the scientific world by A.D.Bangham in 1960's. (Bangham *et. al.,* 1965). In the 1960's and 1970's various liposome preparation methods were developed to study biological processes of membranes and membrane bound proteins. By 1970, liposomes were proposed as drug carriers to modify the therapeutic index of a drug by reducing toxicity or increasing efficacy (or both) of the parent drug. The potential use of liposomes as biodegradable, or biocompatible drug carriers to enhance the potency and reduce the toxicity of therapeutics was recognised only in the late 80's and early 90's. Only then there were the first series of liposome-based therapeutics approved for human use by the U.S. Food and Drug Administration (FDA).

Thus we find that the history of liposomes can be divided into three periods: genesis, middle ages and modern era (Swarbrick and Boylan, 1994)

The period of genesis (1965-1975) dealt mainly with large multilamellar vesicles (MLV) obtained by the thin film hydration method (Amseelam et.al., 1993; Bangham, 1974; Lichtenberg and Barenholz, 1988; Nassender *et. al.,* 1990). Most efforts in this period were dedicated to the physicochemical characeterization of liposomes in their relevancy to biological membranes.

The middle ages (1975-1985) included the period in which many alternative methods of liposome preparation were developed. The first attempts were to develop quality control assays, relevant for liposomes as pharmaceutical dosage forms. A vast amount of information on liposomal physical properties, interactions with cells and performance as drug carriers was collected (Gregoriadis, 1988; Lichtenberg and Barenholz, 1988; Szoka, 1991).

The modern era is characterized by scale-up of liposomal production and a better selection of lipid raw materials for liposome preparation. Many sophisticated lipids, allowing control over the physicochemical and biological fate of liposomes became available. This includes lipids that change the integrity of liposomes as a response to changes in temperature and pH, polymerizable lipids, lipids that reduce or prevent the uptake of liposomes by the mononuclear phagocyte system (MPS). Finally chemical trails with liposome-based formulations were initiated.

Now the period has been reached where scientific, technological and pharmaceutical achievements afford a complete evaluation of the potentials and limitations of liposomes as pharmaceutical devices.

2.2.2 CLASSIFICATION OF LIPOSOMES

Liposomes can be classified either on the basis of their structural properties or on the basis of the preparation method (Crommelin and Schreir, 1994). These two classifications are, in principle, independent of each other. Some of the important members of each class are presented in Table 2.2.

2.2.3 COMPONENTS OF LIPOSOMES

Liposomes are generally composed of the following classes of materials:

2.2.3.1 Phospholipids (Barenholz and Crommelin, 1994; Crommelin and Schreir, 1994):

Phospholipids are one of the major components of liposomes. Five groups of phospholipids that can be used for liposome preparation have been described. These include phospholipids from natural sources (e.g. egg phosphatidyl choline, egg phosphatidyl ethanolamine), modified natural phospholipids (e.g. partially or fully hydrogenated phosphatidyl cholines), semi synthetic phospholipids (e.g. phospholipids obtained from the natural sources whose acyl chains are removed and chemically replaced by defined acyl chains), fully synthetic phospholipids (e.g. phospholipids made by complete pathways) and phospholipids with non-natural head groups (e.g. polyethylene glycol-phosphatidyl ethanolamine).

Phosphatidyl choline

The most common phospholipid is phosphatidyl choline molecule (PC). They are amphipathic molecules in which a glycerol bridge links a pair of hydrophobic acyl hydrocarbon chain with a hydrophilic polar head group, phosphocholine. Molecules of PC are not soluble in water in the accepted sense, and in aqueous media they align themselves closely in a planar bilayer sheets in order to minimize the unfavourable interactions between the bulk of the aqueous phase and the long hydrocarbon fatty acid chains. Such interactions are completely eliminated when the sheets fold on themselves to form closed sealed vesicles.

Table 2.2 Classification of liposomes

a) Based on structural parameters (size and number of bilayers)

Type	Abbreviation	Size range
Small unilamellar vesicles	SUV	20-100 nm
Large unilamellar vesicles	LUV	> 100 nm
Giant unilamellar vesicles	GUV	> 1 μm
Oligo lamellar vesicles	OLV	0.1 –1 μm
Multi lamellar vesicles	MLV	0.1 –1 μm

b) Based on method of liposome preparation

Abbreviation	Type
REV	Single or oligo lamellar vesicles made by the reverse phase evaporation method
MLV-REV	Multi lamellar vesicles made by the reverse phase evaporation method
SPLV	Stable plurilamellar vesicles
FATMLV	Frozen and thawed MLV
VET	Vesicles prepared by extrusion methods
FUV	Vesicles prepared by fusion
DRV	Dehydration-rehydration vesicles
BSV	Bubblesomes

Phosphatidyl cholines, also called as lecithin can be derived from both natural and synthetic sources. They are readily extracted from egg yolk and soya bean but less readily from bovine heart and spinal cord. Lecithin, from natural sources is a mixture of phosphatidyl cholines of high level of poly unsaturation in the fatty acyl chains while that from mammalian sources contains a higher proportion of fully saturated chains (New, 1990a).

Phosphatidyl ethanolamine (PE) is another neutral phospholipid possessing an unsubstituted quaternary ammonium group. Negatively charged phospholipids like dipalmitoyl phosphatidyl glycerol (DPPG), phosphatidyl serine or dipalmitoyl phosphatidyl acid (DPPA) can also be used. Here all three possible forces regulating head group interactions of bilayer membrane come into play--steric hindrance, hydrogen bonding and electrostatic charge. The inclusion of these lipids will tend to increase the interlamellar repeat distances between successive bilayers in the MLVs, swelling the structure with the greater proportion of the aqueous phase. The presence of charge lipids also reduces the likelihood of aggregation followed by formation of MLVs.

2.2.3.2 Sphingolipids

Important members of this group include spingomyelin and gangiosides (Lasic *et. al.,* 1998).

2.2.3.3 Sterols

Cholesterol and its derivatives are quite often included as components of liposomal membranes (Lasic *et. al.,* 1998).

Cholesterol

Cholesterol is a major component of natural membranes and its incorporation into liposome bilayers causes major changes in the properties of vesicles. Cholesterol does not, by itself, form bilayer structures but it can be incorporated into phospholipid membranes in high concentrations. Being an amphiphatic molecule cholesterol gets inserted into the membrane with its hydroxyl group oriented towards the aqueous surface and with the aliphatic chain aligned parallel to the acyl chains in the centre of the bilayer. The rigid steroid nuclei alongside the first 10 or so carbons of the phospholipid chain has the effect of reducing the freedom of motion of these carbons, while at the same time creating space for a wide range of movement for the remaining carbons towards the terminal end of the chain. Thus it enhances the rigidity of membrane. They tend to increase vesicle stability on the shelf and in the body (Gabizon et.al., 1989; Gabizon and Barenholz, 1988; Gabizon and Papahadjopoulos, 1988; Hwang, 1987; Senior, 1987). They increase the fluidity or micro-viscosity of the bilayer, decrease the permeability of the membrane to water soluble molecules and stabilize the membrane in the presence of biological fluids.

2.2.3.4 Other substances

A variety of other lipids and surfactants can be used to form liposomes such as single chain surfactants in combination with cholesterol; non ionic lipids such as a variety of polyglycerol and polyethoxylated mono- and di- alkyl amphiphiles for topical pharmaceutical preparations; some recently prepared single and double chain lipids having fluorocarbon chains which can form very stable liposomes; stearyl amine and dicetyl phosphate which impart either a positive or a negative surface charge to liposomes and a number of compounds having a single long-chain hydrocarbon and an ionic head group such as quaternary ammonium salts and dialkyl phosphates which have been found to be capable of forming vesicles (Lasic *et. al.*, 1998). A large variety of polymerizable lipids including lipids containing conjugated diene, methacrylate and thiol groups as polymerizable surfactants have also been synthesized. Cationic lipids. Such as DODAB/C (dioctadecyl dimethyl ammonium bromide / chloride), DOTAP (dioleoyl trimethyl ammonium propane), DOTMA (dioleoyl propyl trimethylammonium chloride), analogues of DOTMA *and* cationic derivatives of cholesterol have been synthesized for preparing cationic liposomes (Lasic *et. al.*, 1998). α-tocopherol and other such antioxidants are often included as components of liposomal membranes to minimize lipid degradation by oxidation (New, 1990a). A chelating agent is also included to sequester heavy metals which act as catalysts for the oxidation reaction (New, 1990a).

2.2.4 MECHANISM OF LIPOSOME FORMATION

The exact mechanisms involved in liposome formation are still not fully understood. It has been suggested that the large free energy change between water and a hydrophobic environment is responsible for the preference of typical lipids to assemble in bilayer structures excluding water as much as possible from the hydrophobic core in order to achieve the lowest free-energy level and hence the highest stability for the aggregate structure (thermodynamic basis of bilayer assembly or the hydrophobic effect) (Lasic *et. al.*, 1998). A high degree of surface activity of a given molecule does not guarantee its ability to form bilayer structures in the presence of water. An attempt was made to identify potential bilayer compounds on the basis of molecular shape by Israelachvili and co-workers (Israelachvili *et. al.*, 1980) but it was a simplification. Yet, this approach to look at packing constrains still helps to predict the behaviour of a new lipid molecule in water. More recent statistical thermodynamic approaches to predict the state of aggregation of (phopho) lipids have been discussed (Seddon, 1990). While such models can give a useful explanation of various liposome morphologies, they obviously had to be expanded. The reasoning of an open bilayered fragment as a transition structure in the vesiculation process was used to explain liposome formation and preparation techniques of liposomes. It is likely that all high-energy treatments of large multilamellar vesicles cause bilayer fragmentation and fragments, after fusion, self-close into unilamellar vesicles (Lasic *et. al.,* 1998).

An alternative 'budding off" model used to explain the formation of large multilamellar liposomes from the swelling of dried lipid bilayer. The difference between the area of polar heads on the outer and the inner side of bilayers induce curvature, causes area mismatches and results in budding off when excess of the outer monolayer surface area over the inner one exceeds some critical value. The difference in surface areas can be induced by various gradients such as pH, other ions and the asymmetric presence of molecules that can adsorb or insert into the membrane (Lasic *et. al.,* 1998).

The consensus today is that liposomes are produced wither by self-closure of small bilayered fragments or by fission due to a surface difference between the two opposing monolayers. Some energy input is normally required to form liposomes, which can be described as a kinetic trap that is advantageous as compared to systems at thermodynamic equilibrium.

2.2.5 BASIC PROPERTIES OF LIPOSOMES

Variations in liposome size, charge, surface hydration, membrane fluidity and clearance of lipid-associated drug explain the variations in cellular and Physiological mechanisms and liposomal stability in storage.

2.2.5.1 Surface charge

Based on the head group composition of the lipid and pH, liposomes bear a negative, neutral or positive charge on the surface. The nature and density of charge on the surface of the liposomes influences stability, kinetics and extent of biodistribution, as well as interaction with and uptake of liposomes by target cells.

Liposomes with a neutral surface charge have a lower tendency to be cleared by cells of the reticuloendothelial system (RES) after systemic administration and the highest tendency to aggregate. Although negatively charged liposomes reduce aggregation and have increased stability in suspension, their non-specific cellular uptake is increased *in vivo*. Negatively charged liposomes containing phosphatidylserine (PS) or phosphatidyl glycerol (PG) were observed to be endocytosed at a faster rate and to a greater extent than neutral liposomes (Allen et.al., 1991; Lee and Low, 1995). Negative surface charge is recognised by receptors found on a variety of cells, including macrophages (Allen et.al., 1991; Lee and Low, 1994). Inclusion of some glycolipids, such as the ganglioside GM1 or phosphatidyl inositol (PI), inhibits uptake by macrophages and RES cells and results in longer circulation times. It has been suggested that a small amount of negatively charged lipids stabilize neutral liposomes against an aggregation-dependent uptake mechanisms (Drummond et.al., 1999). High doses of positively charged liposomes have been shown to produce varying degrees of tissue inflammation (Scheule et.al., 1997).

2.2.5.2 Surface hydration or steric effect

The surface of the liposome membrane can be modified to reduce aggregation and avoid recognition by RES using hydrophilic polymers. This strategy is often referred to as surface hydration or steric modification. Surface modification is often done by incorporating gangliosides, such as GM1 or lipids that are chemically conjugated to hygroscopic or hydrophilic polymers, usually poly (ethylene glycol) (PEG). This technology is similar to protein PEGylation. Instead of conjugating PEG to therapeutic proteins such as adenosine deaminase to reduce immune recognition and rapid clearance, PEG is conjugated to the terminal amine of Phosphatidylethanolamine (Beauchamp et.al., 1984). This added presence of hydrophilic polymers on the liposome membrane surface provides an additional surface hydration layer (Torchilin, 1994). The resulting liposomes cannot be recognised by macrophages and RES as foreign particles, and spared phagocytic clearance.

2.2.5.3 Fluidity of lipid bilayer

Lipid bilayers and liposome membranes exhibit a well-ordered or gel phase below the lipid phase transition temperature (T_c) and a disordered or fluid phase above the T_c. the lipid phase transition is measured and expressed as

T_c, the temperature at which equal proportions of the two phases coexist. At temperature corresponding to T_c, a maximum leakiness is observed in liposome (Risbo *et. al.,* 1997). The phase behaviour of a liposome membrane determines permeability, aggregation, protein binding and to a lesser degree, fusion of liposomes. Because the T_c varies depending on the length and nature (saturated or unsaturated) of the fatty acid chains, the fluidity of the bilayer can be controlled by selection and combination of lipids. Incorporation of cholesterol at a low concentration into the bilayer leads to an increase in the transmembrane permeability, whereas incorporation of higher amounts (> 30 mol%) of cholesterol can eliminate phase transition and decrease the membrane permeability at a temperature above T_c (Corvera *et. al.,* 1992).

Various phase transitions of lipid bilayers have been designed to induce liposome fusion and drug release. Encapsulated drugs can be released into the target tissue by modulating local tissue temperature by external heating using various sources of energy, such as infra red, microwave, or laser light. However, drugs bound to lipid membranes or protein bound lipid membranes may shift the transition temperature or abrogate the phase transition behaviour altogether (Jorgensen *et. al.,* 1993; Krill *et. al.,* 1998). Binding of serum proteins also influences the phase transition behaviour and release of the aqueous contents of liposomes (Sullivan and Huang, 1986). In addition, to fluidity, in particular liposomes that exhibit phase transition behaviour at or near physiologic temperatures (~37°C), may enhance phospholipase activity at the cell surface, generating lysophospholipids.

2.2.5.4 Liposome size

Early research has demonstrated that liposome size affects vesicle distribution and clearance after systemic administration. The rate of liposome uptake by RES increases with the size of the vesicles (Hwang, 1987). Whereas RES uptake *in vivo* can be saturated at high doses of liposomes or by pre-dosing with large quantities of control liposomes, this strategy may not be practical for human use because of the adverse effects related to the impairment of RES physiological functions. The general trend for liposomes of similar composition is that increasing size results in rapid uptake by RES (Senior, *et. al.,* 1985). Most recent investigations have used unilamellar vesicles, 50-100 nm in size, for systemic drug delivery applications.

Serum protein binding is an important factor that affects liposome size and increases the rate of clearance *in vivo.* Complement activation by liposomes and opsonization depend on the size of the liposomes (Devine *et. al.,* 1994; Liu *et. al.,* 1995). Even with the inclusion of PEG in the liposome compositions to reduce serum protein binding to liposomes, the upper size limit of long circulation PEG-PE liposomes is ~ 150-200 nm. Due to biological constraints, development of long circulating large (>500 nm) liposomes using steric stabilization methods has not been successful. Hence, considerations of liposome size and its control in manufacturing at an early stage of development provide a means to optimise efficiency of liposome drug delivery systems.

2.2.6 PREPARATION OF LIPOSOMES

Literature abounds in methodologies used to prepare liposomes. In the different preparation procedures, a general pattern can be discerned:

1. The lipid must be hydrated.
2. The liposomes have to be sized.
3. Non-encapsulated drug has to be removed.

Crommelin and Schreir (1994) have given an excellent brief outline of the major methods along with the type of liposome product formed, which is reproduced below for the first two steps.

2.2.6.1 Hydration stage

A. Mechanical Methods

1. Vortexing or hand shaking of phospholipid dispersions (MLV)
2. "Microfluidizer" technique (mainly SUV)
3. Bubbling inert gas through aqueous phospholipid dispersions (MLV, LUV)
4. High shear homogenisation (mainly SUV)

B. Methods based on replacement of organic solvent(s) by aqueous media

1. Removal of organic solvent(s) before hydration (MLV, OLV, SUV)
2. Reverse phase evaporation (LUV, OLV, MLV)
3. Use of water immiscible solvents ether and petroleum ether infusion (solvent vaporization) (MLV, OLV, LUV).
4. Use of water miscible solvents such as ethanol injection (MLV, OLV, SUV)

C. Methods based on detergent removal by

1. Gel exclusion chromatography (SUV)
2. "Slow" dialysis (LUV, OLV, MLV)
3. Fast dilution (LUV, OLV)
4. Miscellaneous related techniques (MLV, OLV, LUV, SUV)

D. Methods based on size transformation and fusion

1. Spontaneous fusion of SUV in the gel phase (LUV)
2. Freeze-thawing (MLV)
3. Freeze-drying (MLV)
4. Dehydration of SUV followed by rehydration with or without sizing.
5. Ca^{2+} ion induced fusion (LUV, OLV, MLV)
6. Detergent-induced growth (LUV, OLV)

E. Methods based on pH adjustment (SUV and possibly LUV)

2.2.6.2 Sizing Stage

A. Low pressure extrusion

B. High pressure extrusion (SUV)

C. Ultrasonic treatment (SUV 20 nm in diameter)

2.2.7 COMMONLY USED METHODS OF PREPARATION OF LIPOSOMES

2.2.7.1 Thin film hydration

In the laboratory, a mixture of lipids in volatile organic solvent is deposited on the surfaces of a round bottom flask as the solvent is removed by rotary evaporation under reduced pressure. MLV's ranging in tens of micrometers to several tenths of a micrometer form spontaneously when an excess volume of aqueous buffer is added to the dry lipid and the flask is agitated.

2.2.7.2 Reverse phase evaporation

LUV's can be prepared by forming water in oil emulsions of phospholipids and buffer in excess organic phase followed by removal of the organic phase under reduced pressure. Removal of the organic phase under vacuum causes the phospholipid-coated droplets of water to coalesce and eventually form a viscous gel. Removal of the final traces of solvent under high vacuum or mechanical disruption (vortexing) results in collapse of gel into a smooth suspension of LUV's.

2.2.7.3 pH induced vesiculation

SUV's can be prepared from mixed depressions of PC and PA provided that the molar proportion of PC is 70 % or less. These liposomes are formed when the phospholipid mixtures are dispersed wither directly in sodium hydroxide at pH 10 or in water the pH of which is rapidly increased. However, the technique is limited to charged phospholipids and mixtures of these with neutral phospholipids.

2.2.7.4 Injection of water miscible solvents

Water miscible solvents like ethanol, glycerine and polyglycols have been employed in preparation of liposomes. The solvent containing the lipid is diluted by an excess amount of the aqueous phase rather than being vaporized. As the solvent concentration is reduced by diafiltration or ultrafiltration, liposomes form.

2.2.7.5 Injection of water immiscible solvents

The lipid mixture was injected into an aqueous solution of the material to be encapsulated at 55-65°C or reduced pressure. Vaporization of the solvents leads to the formation of single layer vesicles (Deamer and Bangham, 1976).

2.2.7.6 Detergent dialysis method

Removal of detergent molecules from aqueous dispersions of phospholipid/ detergent mixed micelles is another approach to produce liposomes. As the detergent is removed the micelles become progressively richer in phospholipids and finally coalesce to form closed, single-bilayer vesicles.

2.2.8 SEPARATION OF UNENTRAPPED DRUG FROM LIPOSOMAL SUSPENSION

Many lipophilic drugs exhibit a high affinity to the bilayer and are completely liposome associated. However, for other compounds the encapsulation efficiency is less than 100 %. The unentrapped material can be removed using the following techniques.

2.2.8.1 Dialysis and Ultrafiltration

Conventional dialysis membranes can be used with molecular weight cut off characteristics dependent on the molecular weight of the free'compound to be removed from the liposome dispersion. During ultrafiltration, the dispersion is stirred or circulated by a pump. This convection process must not induce leakage of encapsulated material (Crommelin and Schreir, 1994).

2.2.8.2 Ultracentrifugation

Ultracentrifugation can be used for removal of dissolved nonliposomal associated material and for separation of heterogeneous colloidal material, be it liposomes with different sizes and / or subfractions with different densities (New, 1990a; Crommelin and Schreir, 1994). It can be used for concentrating dilute liposome dispersions (Barenholz *et. al.*, 1977).

2.2.8.3 Gel Permeation Chromatography

Sephadex (e.g. G-50 or G-100), Sepharose or Bio-Gel columns are regularly used to separate liposome-associated material from non-encapsulated material. The liposomes are not retained by the gel bed and elute in the void volume, the free material elutes in the later fractions (New, 1990a; Crommelin and Schreir, 1994).

2.2.8.4 Ion-exchange resins

The potential of ion exchange resins to remove nonencapsulated material from liposomal dispersions has been studied (Storm *et. al.*, 1985). Ion exchange resins can be utilised for efficient and fast removal of nonliposome-associated material without dilution of the dispersion if they have a high affinity for the nonliposome-associated compound.

2.2.8.5 Protamine aggregation

This method is useful in developmental studies and is effective for neutral and negatively charged liposomes. The inclusion of protamine cause aggregation of liposomes and thereby precipitates upon centrifugation and the free drug will be present in the supernatant, which can be analysed after separation. However, the free drug should not be precipitated by protamine (New, 1990b).

2.2.9 CHARACTERIZATION OF LIPOSOMES

Both physical and chemical characteristics of liposomes influence their behaviour *in vivo* and *in vitro.* Several examples demonstrating the importance of proper selection of liposome structures to obtain optimum and reproducible therapeutic effects have been published (Goren *et. al.,* 1990; Senior, 1987; Storm *et. al.,* 1989). Therefore, it is essential to characterize liposomes properly. The nature of characterization of liposomes can be divided into two broad categories.

2.2.9.1 Physical Characterization

Physical characterization of liposomes includes evaluation of the following parameters:

A. Size

The size distribution of liposomes is often difficult to estimate because often no one technique can monitor all sizes present. Electron microscopy techniques used for this purpose include negative staining electron microscopy (New, 1990b), bare grid electron microscopy (Crommelin and Schreir, 1994) and freeze fracture electron microscopy (Betagiri *et. al.,* 1993). Cryofixation methods for liposomes have been described (Crommelin and Schreir, 1994). The potential of scanning tunnelling microscopy for liposome characterization is yet to be determined (Fowler *et. al.,* 1992). Dynamic light scattering, which has become quite popular, is usually used for 'sized' liposomes with diameters between a few nanometers and a few micrometers (New, 1990b). For heterogeneous liposome dispersions in the supra-micrometer range Coulter counter techniques can be used. Other techniques that may have advantages for analysing heterogeneous dispersions are based on size exclusion chromatography, high performance gel exclusion chromatography, ultracentrifugation and sedimentation field flow fractionation (Betagiri *et. al.*, 1993; Crommelin and Schreir, 1994).

B. Lamellarity

The average number of bilayers in liposomes can be found using a number of techniques such as "bare grid" electron microscopy (Crommelin and Schreir, 1994), freeze fracture electron microscopy (Betagiri *et. al.*, 1993a) and negative staining electron microscopy (New, 1990b), ^{31}P-NMR (Jousma *et. al.*, 1987), trinitrobenzene sulphonic acid method (Barenholz *et. al.*, 1977; New, 1990b) and small angle X-ray scattering (SAXS) (Jousma *et. al.*, 1987). Combination of these methods has also been used (Talsma *et. al.*, 1987).

C. Trapped Volume

Trapped volumes are usually determined experimentally by dispersing lipid in an aqueous medium containing nonpermeable radioactive solute such as ^{22}Na or ^{14}C inulin. The proportion of solute trapped is determined by removing external radioactivity by centrifugation, dialysis or gel filtration and determination of residual radioactivity per lipid (Hope *et. al.*, 1985). The Karl Fischer method can also be used for measuring the trapped volume when used in conjunction with methods like centrifugation to separate external aqueous phase (Betagiri *et. al.*, 1993b). NMR techniques utilising deuterium oxide have also been used (New, 1990b). Electron spin resonance (ESR) stopped-flow technique is yet another procedure utilised for estimating trapped volume (Anzai *et. al.*, 1990).

D. Bilayer fluidity

The bilayer fluidity' depends on the bilayer composition, the temperature and the aqueous environment. In particular, in the fluid-crystalline state, molecular motion occurs in bilayer. Information on molecular motion in a bilayer can be collected by fluorescence polarization techniques. Probes such as diphenyhexatriene (DPH), trimethyl amino-DPH (TMA) or transparinaric acid (NPA) are used. Manipulation of the physico-chemical nature of the probe offers the opportunity to gain information on particular parts of the bilayer structure (Ben-Yashar and Barenholz, 1989; Jones and Cossins, 1990; Shinitzky and Barenholz, 1978). Electron spin resonance (ESR) spectroscopy has been used to determine bilayer fluidity (Crommelin and Schreir, 1994)

E. Charge

The charge density on liposomes can be estimated from mobility measurements of liposomes in an electrical field (microelectrophoresis). The mobility data can be converted to zeta potentials by using the Helmholtz-Smoluchowski equation or the Henry equation. The zeta potentials can be used to calculate the charge density at the hydrodynamic plane of shear using the Gouy-Chapman equation (Cerv, 1990; Crommelin and Schreir, 1994). Methods that determine that mobility of the individual liposomes in an electrical field visually are now replaced by methods where liposome mobility in an electrical field in a capillary is followed by dynamic light scattering (Grit and Crommelin, 1993).

2.2.9.2 Chemical Characterization

A. Analysis of phospholipids and their degradation products

The quantitative determination of phospholipids can be done by methods like the Barlett assay (New, 1990b), the Stewart assay (Stewart, 1980), the rhodamine complexation and thin layer chromatography (New, 1990b). Lysolecithin, a major product of the hydrolysis of lecithin, has been estimated using densiometry. The hydrolysis products of other phospholipids can be estimated in the same way. Estimation of phospholipid oxidation can be obtained using UV absorbance method, thiobarbituric acid method, iodimetric method and gas-liquid chromatography (New, 1990b).

B. Analysis of cholesterol, α-tocopherol and other bilayer components

Cholesterol purity can be estimated using gas-liquid chromatography (New, 1990b). Quantitation of cholesterol is done using ferric perchlorate method (New, 1990b) or Zlatkis, Zak *and* Boyle's method (Zlatkis *et. al.,* 1953). High performance liquid chromatography is used to quantify α-tocopherol (New, 1990b). Different types of chromatography can be used to separate bilayer components such as thin layer chromatography, gas-liquid chromatography, high performance thin layer chromatography and high performance liquid chromatography (Crommelin and Schreir, 1994).

C. Drug entrapment

The amount and location of a drug within a liposome depends upon a number of factors such as partition coefficient of the drug between aqueous compartments and lipid bilayers, solubility of the drug in each phase, total amount of lipid used (for nonpolar drugs), internal volume of liposome (for polar drugs) and the method of preparation (Lasic *et. al.,* 1998). The distribution of drug within the liposome may also contribute to level of entrapment and correction of this distribution can increase the entrapment efficacy of the corresponding liposomes (Betagiri *et. al.,* 1993a).

2.2.10 STABILITY OF LIPOSOMES

The stability of liposomes can be discussed under the following headings:

2.2.10.1 Physical stability

Changes in the size of the liposomes can take place over a period of time. These changes can be a result of aggregation (formation of larger units of liposomal material which is reversible) and sedimentation or fusion (irreversible formation of new colloidal structures). The methods used to characterize the size of liposomes can also be used to follow these changes in size. However, to differentiate between aggregation and fusion, fluorescent markers such as terbium citrate-sodium dipicolinate, calcein-cobalt complex in association with EDTA etc. are used. Liposome membrane fusion may also be detected through fluorescence resonance energy transfer between two lipids originally present in different sets of liposomes (Jones and Cossins, 1990).

Drug molecules can leak from the liposomes. The leakage rate strongly depends on the bilayer composition and the physicochemical nature of the drug. Changes in the bilayer permeability can occur as a result of chemical degradation processes as described below (Crommelin and Schreir, 1994).

2.2.10.2 Chemical Stability

As phospholipids usually form the backbone of the bilayer, their chemical stability is important. Two types of chemical degradation reactions can affect the performance of phospholipid bilayers:

1. Hydrolysis of the ester bonds
2. Peroxidation of unsaturated acyl chains (if present)

The stability of phospholipids can be ascertained using the methods discussed earlier in the section on characterization of liposomes (Section 2.2.9). The factors which affect the stability of phospholipids (such as pH, ionic strength, temperature and other such factors) will therefore also affect the stability of the liposomes formed from them.

Oxidation of cholesterol can be monitored through gas liquid chromatography using a silica capillary column to separate cholesterol from its oxidate products (New, 1990b). The stability of the drug must also be considered since the stability profile of the free drug may be quite different from its profile in the encapsulated state. There is little information available on the effect of entrapped drug on the stability of phospholipids.

2.2.10.3 Approaches to improve liposomal stability

To be commercially feasible, liposomes should have a satisfactory shelf life. In general, a shelf life of at least one year is a minimum prerequisite in the pharmaceutical industry. Approaches to improve the physical and chemical stability of liposomes are:

A. Selection of bilayer components

Stability of liposomes can be improved by minimum use of unsaturated phospholipids. If unsaturated lipids are used, addition of antioxidants such as α- tocopherol or butylated hydroxytoluene and use of argon or nitrogen to minimise exposure to oxygen are recommended. Oxidation of unsaturated phospholipids, catalysed by heavy metals may be inhibited by addition of metal chelators such as EDTA and use of light resistant containers. Processing and storage of liposomes at low temperatures can add to liposome stability. With aqueous liposome dispersions, the selection of a proper pH range (pH 6-7) will reduce hydrolysis and lipid peroxidation mediated damage. Addition of substantial fractions of cholesterol decreases the rate of leakage during storage by rendering the bilayer structure more rigid. Use of saturated phospholipids is a useful technique to improve the stability. To reduce the probability for liposome aggregation or fusion, a charge-inducing agent is often included into the bilayer. Phosphatidyl glycerol (PG), Phosphatidic acid (PA) and charged cholesterol esters such as cholesterol hemisuccinate are negatively charged lipids at neutral pH that have proven to be good candidates for inducing a negative charge on the liposome bilayers. Conversion of small vesicles to larger structures as a result of fusion processes can be markedly reduced by the presence of the trace amounts of the phospholipid isomer 1,3-diacyl-2-phosphatidylcholine. Various methods have been employed which may increase the stability of liposomes by cross linking membrane components covalently using methods such as glutaraldehyde fixation, osmification or polymerisation of alkyne-containing phospholipids. The incorporation of long aliphatic-branched chain polymers (e.g. polyvinyl alcohols esterified with palmitic or stearic acid), up to about 10 % by weight into PC membranes, can substitute for cholesterol in reducing the leakage of medium-sized solutes. Another type of cross linking, involving hydrogen bonding, is probably responsible for the increased stability of liposomal membranes containing sphingomyelin or 2-carbomoyl PC derivatives.

B. Freeze-drying of liposomes

Freeze-drying of liposomes is an excellent method to increase the shelf life of liposomes. Cryoprotectants such as sucrose, maltose, trehalose, lactose, proteins, amino acids and polyalcohols have been shown to be effective. Other variables affecting freeze-drying of liposome dispersions include nature of the bilayer and of the liposome-associated compound, liposome size and technological parameters such as freezing rate, freezing temperature and freezing time.

C. Proliposome approach

The lipids from an organic solvent are deposited on finely powdered sodium chloride or sorbitol. Upon hydration, liposome dispersion is formed. This approach can only be used for compounds that are fully liposome associated upon hydration and if a rather wide particle size distribution is acceptable in the clinical situation.

2.2.11 APPLICATIONS OF LIPOSOMES

The literature concerning the applications of liposomes is so vast that a detailed review is not possible. An idea of the varied applications of liposomes can be obtained from Table 2.3, which shows the liposomal products developed or under development (Kirby and Gregoriadis, 1999).

Table 2.3 Liposome-based products developed or under development

Product	Drug	Target disease
Liposomal nystatin	Nystatin	Systemic fungal infections
Liposomal tretinoin	All-trans retinoic acid	Leukemia
Liposomal annamycin	Annamycin	Kaposi's sarcoma Retractory breast cancer
Newcastle disease vaccine	Newcastle disease virus (killed)	Newcastle disease (chicken)
Avian Rheovirus vaccine	Avian Rheovirus (killed)	For vaccination of breeder chickens; for passive protection of chicks against rheovirus infection
AmBisome	Amphotericin B	Systemic fungal infections; Visceral leishmaniasis
Amphotec	Amphotericin B	Systemic fungal infections
Abelcet	Amphotericin B	Systemic fungal infections
DaunoXome	Daunorubicin	First line treatment for advanced Kaposi's sarcoma, Breast cancer and other solid tumours
Doxil	Doxorubicin	Kaposi's sarcoma, Refractory ovarian, recurrent breast, prostate and primary liver cancers
D99	Doxorubicin	Metastatic breast cancer
MiKasome	Amikacin	Serious bacterial infections
VincaXome	Vincristine	Solid tumors
E. coli 1257:H7 vaccine	E. coli 0157:H7 (killed)	E. coli 0157 infection
Shigella flexneri 2A vaccine (oral)	S. flexneri 2A (killed)	S. flexneri 2A infection
Depocyt	Cytarabine	Lymphomatous meningitis

Product	Drug	Target disease
C53	Prostaglandin E_1	Systemic inflammatory disease
Epaxal-Berna vaccine (IRIV* liposomes	Inactivated hepatitis A virions (HAV) (antigen; RG-SB strain	Hepatitis A
Trivalent influenza vaccine	Hemagglutinin and neuraminidase from H_1N_1, H_3N_2 and B strains according to recommendations by WHO	Influenza
HAV/HBS_s-IRIV* Combined vaccine	HAV, genetically engineered hepatitis B antigens (HBs)	Hepatitis A and B
Diphtheria/ tetanus/ hepatitis A combined vaccine	Diphtheria and α and β tetanus toxoids; inactivated HAV virions	Diphtheria, tetanus, hepatitis A
Hepatitis A and B/ diphtheria/ tetanus/ influenza supercombined vaccine (intramuscular)	Inactivated HAV virions, HB_s, diphtheria, α and β tetanus toxoids, hemagglutinin and neuraminidase from influenza starins as in trivalent influenza vaccine	Hepatitis A and B, diphtheria, tetanus and influenza

*IRIV- Immunopotentiating reconstituted influenza virosome

2.2.12 REFERENCES

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2.3 STERICALLY STABILIZED LIPOSOMES

2.3.1 INTRODUCTION

The pharmaceutical and pharmacological justifications of the use of liposomes as drug carriers are as follows (Barenholz and Crommelin, 1994):

1. Liposomes supply both a lipophilic environment and aqueous ' milieu interne' in one system and therefore suitable for the delivery of hydrophobic, amphipathic and hydrophilic drugs and agents.
2. Liposomes are chemically and physically well-characterized entities.
3. The biological fate of liposomes after their administration is related to their composition and physical properties.
4. Liposomes are biocompatible due to their biodegradability, low toxicity and lack of immunogenicity.
5. Liposomes can serve as a device for controlled release of drugs in the bio environment (microreservoir concept) including intracellular spaces (after endocytic uptake).
6. Liposomes help to reduce exposure of sensitive tissues to toxic drugs.
7. Liposomes can be administered through most routes of administration including ocular, pulmonary, nasal, oral, intramuscular, subcutaneous, topical and intravenous.
8. Pharmacokinetics and *in vivo* biodistribution of liposomes can be controlled by their port of entry combined with their lipid composition and size.

Until recently, one of the main drawbacks to the use of liposomes as a parenteral drug delivery system was their rapid uptake from the circulation by cells of the mononuclear phagocytic system (MPS) as a result of liposome opsonization in the blood.

Due to this, the therapeutic use of liposomes had been limited mainly to delivery of drugs to the MPS. e.g. manipulation of macrophage function and treatment of diseases involving macrophages like leishmaniasis. However, this scenario changed with the advent of what are known as sterically stabilized liposomes as a means to impede opsonization and MPS uptake.

2.3.2 THE IMMUNE SYSTEM

To understand the behaviour of sterically stabilized liposomes, a brief overview of the human immune system is necessary.

The human body has the ability to resist almost all types of organisms or toxins that tend to damage the tissues and organs, which is called immunity. While a portion of the immunity results from general processes and is known as innate immunity, the major portion of the immunity results from a special immune system, which produces antibodies and activated lymphocytes that attack and destroy specific organisms or toxins. This latter type of immunity is known as acquired immunity.

2.3.2.1 Innate immunity

Innate immunity includes the following-

A. Phagocytosis

Phagocytosis of bacteria and other invaders by white blood cells and cells of the tissue macrophage system. The white blood cells or leukocytes are the mobile units of the body's protective system. The various types of leukocytes include polymorphonuclear neutrophils, polymorphonuclear eosinophils, polymorphonuclear basophils (collectively these three types are known as granulocytes), monocytes, lymphocytes and plasma cells. In addition, there are a large number of platelets, which are fragments of a seventh type of white cell found in bone marrow, the megakaryocyte.

It is mainly the neutrophiles and monocytes that attack and destroy injurious agents. The neutrophils are mature cells that can attack and destroy such agents in the circulating blood. However, the blood monocytes are immature cells that have very little ability to fight with injurious agents. However, once they enter tissues, they swell to much larger sizes to become macrophages. While mainly these cells are mobile, a large portion of these macrophages become attached to the tissues and remain attached for months or perhaps even years unless they are called upon to perform specific protective functions. When appropriately stimulated, these fixed macrophages can break away from their attachments and become mobile macrophages. The combination of mobile macrophages and fixed tissue macrophages is collectively called the reticuloendothelial system or the RES.

The tissue macrophages in various tissues differ in appearances and they are known by different names: Kupffer cells in the liver; tissue macrophages in the lymph nodes, spleen and bone marrow; alveolar macrophages in the alveoli of the lungs; tissue histocytes in the subcutaneous tissues and microglia in the brain. The term mononuclear phagocytic system (MPS) has been proposed to replace RES.

The various types of macrophages and neutrophils have the properties of diapedesis, amoeboid motion and chemotaxis. The most important function of neutrophils and macrophages is phagocytosis. Phagocytosis involves three selective procedures:

1. If the surface of a particle is rough, the likelihood of phagocytosis is increased.

2. Most natural substances of the body have protective coats that repel phagocytes. Dead tissues and foreign particles frequently have no protective coats and many are also strongly electrically charged, which also makes them susceptible to phagocytosis.

3. The immune system develops antibodies against infectious agents. These antibodies then adhere to their membranes and make them especially susceptible to phagocytosis. This results from the antibody molecule binding with another factor called complement, which is an additional part of the immune system. Some of the products of the complement then 'cover' the bacteria and make them very susceptible to phagocytosis, the process known as opsonization. Complement can also attach to some bacteria even in the absence of antibodies and this too can lead to opsonization.

B. Destruction by the acid secretions of the stomach and by the digestive enzymes of organisms swallowed into the stomach.

C. Resistance of the skin to invasion by organisms.

D. Presence of certain chemical compounds in the blood that attach to foreign organisms or toxins and cause their destructions. Some of these are

1. Lysozyme, a mucolytic polysaccharide that attacks bacteria and causes them to dissolute.

2. Basic polypeptides, which react with and inactivate certain types of gram-positive bacteria.
3. The complement complex, a system of about 20 proteins that can be activated in various ways to destroy injurious agents.

2.3.2.2 Acquired immunity

The human body also has the ability to develop extremely powerful specific immunity against individual agents such as lethal bacteria, viruses, toxins and even foreign tissues from other animals. This is called acquired immunity. Two basic, but closely allied, types of acquired immunity occurs in the body:

A. Humoral immunity: Here, the body develops circulating antibodies, which are globulin molecules that are capable of attacking the invading agent.

B. Cell-mediated immunity: Here, large numbers of activated lymphocytes are formed that is specifically designed to destroy the foreign agents.

Both the antibodies and the activated lymphocytes are formed in the lymphoid tissue of the body, which is located most expensively in the lymph nodes, spleen, submucosal areas of the gastrointestinal tract and in the bone marrow. The lymphoid tissue is distributed very advantageously in the body to intercept the invading organisms or toxins before they can spread too widely. The lymphocytes if normal lymphoid tissues are divided into two major populations:

A. T-lymphocytes: These forms activated lymphocytes and thus are responsible for cell-mediated immunity.

B. B-Lymphocytes: These forms antibodies and thus are responsible for humoral immunity.

A. T-cell mediated immunity

Upon exposure to the proper antigen, the T-lymphocytes of the lymphoid tissue proliferate and release large numbers of activated T-cells into the lymph. These then pass into the circulation and are distributed throughout the body passing through the capillary walls into the tissue spaces, back into the lymph and blood, thus circulating throughout the body. Some of these newly formed lymphocytes are preserved in the lymphoid tissue as T-lymphocyte memory cells. Therefore, on subsequent exposure of the same antigen, the release of activated T-cells occur far more rapidly and much more powerfully than in the first response. The antigens bind with receptor molecules on the surfaces of T-cells.

T-cells are of different types such as:

1. Cytotoxic T cells

These are direct attack cells capable of killing micro organisms and at times even the body's own cells. For this reason, they are frequently called killer cells.

2. The Helper T cells

These cells are the most numerous of all the T cells. They help is functions of the immune system in various ways.

a. By increasing the activation of B cells, cytotoxic T cells and suppressor T cells by antigens. This is done by secretion of lymphokines by the helper T cells once they are activated.

b. Stimulation of activity of other T cells by secretion of a substance called interleukin-2, one of the lymphokines, that increases the activity of other T cells, including cytotoxic T cells, some suppressor T cells and probably even some of the other helper T cells.

c. Activation of the macrophage system by secretion of another lymphokine, called macrophage migration inhibition factor that slow or stops the migration of macrophages that have been chemotactically attracted into the infected tissue area thus causing a great accumulation of macrophages. This factor also activates the macrophages to cause far more efficient phagocytosis allowing them to attack and destroy greatly increased numbers of invading organisms.

3. Suppressor T cells

These cells regulate the activities of the other cells keeping them from causing excessive immune reactions that might be severely damaging to the body. They are therefore also called regulatory T cells. These cells also play a role in immune tolerance.

B. B-cell mediated immunity

Upon entry of a foreign antigen, the lymphoid tissue macrophages phagocytize the antigen and then present it to the adjacent B cells. In addition, the antigen may also be presented to T cells at the same time, the consequences of which have been discussed earlier. The B-lymphocytes specific for the antigen develop to give plasma cells, which produce antibodies. These antibodies are gamma globulins (also known as immunoglobulins). Some of the progeny of the B-lymphocytes do now form plasma cells but form moderate numbers of new B-lymphocytes similar to the original cell (memory cells). Due to the presence of these cells, subsequent exposure to the same antigen will cause a much more rapid and much more potent antibody response.

There are five general classes of antibodies namely IgM, IgG, IgA, IgD and IgE. Antibodies act mainly, in two different ways to protect the body against invading agents:

1. By direct attack on the invader: Antibodies can inactivate the invader by agglutination, precipitation, neutralization or lysis.

2. By activation of the complement system that then destroys the invader: Complement is a collective term to describe a system of about 20 different proteins, many of which are enzyme precursors. All these are present normally among the plasma proteins that leak out of the capillaries into tissue spaces. The enzyme precursors are normally inactive but they can be activated through two separate ways:

a. The Classical Pathway

This pathway is activated by an antigen-antibody reaction. i.e. when an antibody binds with an antigen, a specific reactive site on the antibody is uncovered or activated and this, in turn, binds with a molecule of the complement system setting in motion, a "cascade" of sequential reactions which, starting from a small amount, lead to the production of large amounts of a number of end products. These cause effects such as opsonization and phagocytosis, lysis, agglutination, neutralization of viruses, chemotaxis, activation of mast cells and basophils and inflammatory effects, all of which help to prevent damage by the invading organism or toxin.

b. The Alternate Pathway

The complement system can sometimes be activated without the intermediation of an antigen-antibody reaction. All the same final products of the system are formed as in the classical pathway and these cause the same effects as those just described above in protecting the body against the invader. Since the alternate pathway does not involve an antigen-antibody reaction, it is one of the first lines of defence against invading agents.

2.3.3 OPSONIZATION AND PHAGOCYTOSIS

Opsonization is a process whereby materials adsorb to a foreign surface in such a way as to prepare the surface for recognition as foreign and thus for phagocytosis by the MPS. The word 'opsonin' is of Greek origin and means a relish, a seasoning or a sauce. It has been quoted as being "the preparation of meal" (Buckton, 1995). The materials involved in opsonization are known as opsonins. Opsonins are poorly defined plasma factors, which bind to foreign particles and promote phagocytosis. The opsonins facilitate attachment of the phagocyte to the antigen by forming a bridge between the phagocyte and antigen. These opsonins can be specialized or non-specific. Specialized opsonins contain at least one receptor-recognizable site and often more than one binding site (so that they can cross link). They also play important roles in immune response regulation and may alter vascular permeability. Specialized opsonins, bound to an antigen, expose receptor-recognizable sites and, thus mediate further immunocomplex interaction with cells. The latter process depends on factors such as the number and type(s) of incorporated opsonins molecules. Those immunocomplexes, which contain, a small number of opsonins molecules are known to circulate for hours and even days, while, others are immediately arrested by phagocytes. (e.g. in liver, spleen, bone marrow or lungs). Thus phagocytosis is triggered faster by multiple rather than single opsonins - receptor interactions (Papisov, 1995).

Major specialized opsonins of normal blood include:

1. **Immunoglobulins** –which have already been discussed before.

2. **Complement** – As discussed earlier, complement comprises a set of proteins that work to eliminate microorganisms and other antigens from blood. This task is achieved by complement cooperation with cells that express complement have historically assigned numbers which do not completely correspond to the sequence of their actions. The third complement protein, C3, has a central role in complement function. One of the most important functions of complement is to mark antigens with fragments of C3, thus making them recognizable to phagocytes bearing C3 receptors (Papisov, 1995). To be bound to an antigen, C3 should be first activated by C3 convertases (which are antigen bound) that produce C3b, a C3 fragment containing an active cyclothioester group; the latter is chemically active and may covalently bind nucleophilic functional groups, preferentially OH groups (Law *et. al.,* 1980). C3 convertases are protein complexes formed either with participation of activated C1 complex, C2 and C4 (C4b2a, classical activation pathway) or by C3 itself and factors B and D (C3bBb, alternative pathway). Subsequent C3 activation produces more antigen-bound C3b and more C3 convertase complex (Papisov, 1995). Other complements such as C4 also seem to be involved in the phagocytic process (Gref *et. al.,* 1995). C4, which is less abundant, has a thioester group, similar to that in C3, which binds, however, preferentially to amino groups (Law *et. al.,* 1984). The antigen ability to activate complement presumably depends on the presence of nucleophilic functional groups that may bind C3 or C4 and/or domains that may bind C1. The C1 complex consists of three proteins C1q, C1r and C1s where C1q substances including IgG and IgM –containing immune complexes, lipopolysaccharides and porins from gram-negative bacteria, ligand-bound C-reactive protein, nucleic acids and other highly charged polymers (Papisov, 1995).

3. Fibronectins

Fibronectins are large proteins abundant in plasma and extra cellular matrix. These are indications that the fibronectin molecule in the solution is a disk having a diameter of 30 nm and thickness of 2 nm (Benecky *et. al.,* 1990). However, the fibronectin molecule consists of several domains connected by relatively flexible joints and believed to be capable of unfolding, in particular, as a result of substrate binding (Khan *et. al.,* 1990; Wolff and Lai, 1989). Fibronectin is believed to participate in hepatic uptake of particulates (Papisov, 1995).

Minor or non-specific opsonins are proteins present in blood in small concentrations. These are recognition proteins bearing binding sites, receptor –recognizable domains and sometimes, sites for binding another recognition molecule. Some minor opsonins are acute-phase proteins (i.e. their concentration in plasma increases as a result of inflammation or trauma). Many minor opsonins trigger complex responses that often include histamine release (Papisov, 1995). Most minor opsonins are lectins. e.g. conglutinin is a 330 kDa lectin with four binding sites and high specificity to terminal (α 1-2) oligomannose (Young and Leon, 1987). Mannose – binding protein (MBP), a protein that was recently found to be identical to a component of Ra-reactive factor (RaRf), binds terminal non-reducing mannose, N-acetylglucosamine and glucose residues. MBP was reported to enhance phagocytosis mediated by C1q receptor; in addition, MBP was found to trigger complement activation via activating $C1r_2$-$C1s_2$ complex. MBP was suggested to be an acute phase protein (Papisov, 1995). Lipopolysaccharide binding protein (LBP) was recently shown to directly recognize lipopolysaccharides and to trigger remarkable biological responses (Tobias *et. al.,* 1995). Vitronectin, a component of extracelluar matrix, participates in complement reactions (often named protein S) and probably may also act as an opsonins (Savill et.al., 1990). Serum amyloid P component (SAP) is a normal human serum protein with the non-fibrillar amyloid P component found in amyloid deposits. SAP is composed of 10 non-covalently associated globular subunits, each of 25 kD arranged as two face-to-face cyclic pentameric discs (Pinteric *et. al.,* 1976). SAP binds to a number of apparently unrelated substances including phospholipids (Saxena *et. al.,* 1987). Another pentraxin, C-reactive protein (CRP) is an acute phase protein. Human CRP interacts with fibronectin, fibrinogen and phosphorycholine; rat CRP binds phosphorylethanolamine as well (Kottgen *et. al.,* 1992). Another minor opsonins are tuftsin, which has been shown to enhance recognition of various particulates by different macrophages (Stolnik *et. al.*, 1995).

Recently, the presence of 'organ-specific' opsonins has been proposed (Moghimi and Patel, 1988) where, for instance, the liver specific opsonins can enhance the uptake of particulates by Kupffer cells whereas spleen specific opsonins could mediate the uptake of particulates by spleen macrophages.

Another hypothesis suggests that some opsonins are recognised by all macrophages whilst others are recognised only by specific subpopulations of macrophages. It follows that the adsorption of different (or different combinations of) blood proteins would lead to the particle having a different fate in the body (Buckton, 1995).

It has been suggested that phagocytosis is regulated by the presence and balance between two groups of blood components: opsonins, which promote phagocytosis and dysopsonins that, suppress the process. Immunoglobulin IgA and Secretory IgA are the best-known dysopsonins. The mode of their action is not known although a high hydrophilicity of the molecule has been suggested as a possible explanation (Stolnik *et. al.*, 1995).

From the above discussion, it can be seen that the opsonization process is a consequence of multiple adsorption and displacement processes, probably involving conformational changes, which ultimately lead to the final opsonization process. The net effect of opsonization is to make the particle surface more hydrophobic and it is reasonable to assume that this may be the probable reason for phagocytosis.

2.3.4 STRATEGIES FOR MPS AVOIDANCE BY LIPOSOMES

The *in vivo* biodistribution of liposomes has been exclusively studied in the last 2 decades. Studies have been focussed on the mechanism underlying the efficient uptake of liposomes by the RES (Harashima *et. al.*, 1993; Moghimi, 1998). It is now generally understood that the mononuclear phagocytes of the RES, principally the Kupffer cells of the liver and secondarily the splenic macrophages, are responsible for the clearance of liposomes from the circulation. In addition, the parenchymal cells of the liver are also involved in liposome uptake. Furthermore, serum factors (opsonins) coating the liposome surface are believed to promote the specific uptake of liposomes by the liver and the spleen even though liposomes have a comparatively hydrophilic surface. These interactions depend on the physical and chemical characteristics of liposomes (Mori *et. al.,* 1992). Efforts are therefore being directed towards identification of these characteristics and their subsequent manipulation for avoidance of the MPS uptake.

The surface charge has been recognised as an important determinant of particulate clearance from the circulation. Biological particles are usually negatively charged whilst administered carrier systems can be positively or negatively charged and that charge can be changed as a consequence of changes in the fluid. Charged particles are surrounded by associated charged species of the opposite charge creating the electrical double layer. When a charged carrier and a biomolecule are in close proximity, their associated charged layers will overlap. The DLVO theory can be used to describe the net interaction between two particles of the same charge as they approach each other. It is a general view that negative surface charge increases the clearance of particulates from the circulation, relative to neutral or positively charged one (Stolnik *et. al.,* 1995). Particles with neutral surfaces seem to be the most appropriate with regard to blood persistence (Petrak̆, 1993).

Liposome size plays an important role in determining their biodistribution. There is an inverse relationship between liposome size and the rate at which they are distributed to MPS cells such as kupffer cells. Larger liposomes have dramatically more rapid clearance into MPS cells than smaller liposomes. Two parallel pathways have been proposed to account for the difference in hepatic uptake rates between large and very small liposomes. Large liposomes are deposited in a high affinity, low capacity saturatable system, predominantly involving kupffer cells. It was suggested that the smaller liposomes avoid, to some extent, the first pathway but are removed by a low affinity, high capacity system involving the parenchymal cells (Allen *et. al.,* 1995).

The hydrophobicity / hydrophilicity of the surface affects the attractive forces experienced by the particle, thereby influencing the opsonization process and interaction forces that govern adhesion of the particle to the cell (Stolnik *et. al.,* 1995). Particles with hydrophilic surfaces, upon which water molecules can readily absorb, have longer blood half lives (Blume and Cevc, 1990; Klibanov *et. al.,* 1990; Troster and Kreuter, 1992; Allen, 1994).

Several studies have also shown the importance of bilayer rigidity in prolonging the circulation time of liposomes. Decreasing the fluidity of the liposomal bilayer in the presence or absence of stealth components can further increase the circulation half-lives of the liposomes (Allen and Chonn, 1987; Gabizon and Papahadjopoulos, 1988; Allen, 1988; Allen *et. al.,* 1989). The mechanism is probably due to the inability of plasma opsonins to effectively insert into more tightly packed (rigid) bilayers (Allen, 1988; Allen *et. al.,* 1989).

Appropriate combinations of these two or more strategies will ensure that liposomes will not be sequestered by the cells of the MPS, as any one of these strategies alone may not be adequate for preventing uptake of liposomes by the cells of the MPS.

2.3.5 AGENTS FOR STERIC STABILIZATION

Poly ethylene glycol (PEG) is one of the most popular polymeric materials to alter and control the biodistribution of liposomes. PEG possess an ideal array of properties for use in liposomes (Zalipsky, 1995):

1. Very low toxicity.
2. Excellent solubility in aqueous solutions.
3. Extremely low immunogenicity and antigenicity
4. Non-biodegradable yet readily excretable after administration.
5. Excellent pharmacokinetic and biodistribution behaviour: when injected into animals, it shows high persistence in blood compartment and low accumulation in RES organs. PEG excludes proteins, other macromolecules and particulates from its surroundings.
6. Readily available in a variety of molecular weights.
7. Interness of polyether backbone in biological environments as well as in most chemical reaction conditions under which the end groups of PEG can be subjected to chemical modification and/or conjugation reactions.

For drug delivery, PEG coated liposomes have several advantages (Woodle, 1995; Woodle, 1997):

a. Prolonged circulation independent of the bilayer physicochemical properties thereby permitting separate control of drug loading / leakage and blood circulation.

b. Plasma pharmacokinetics independent of lipid dose thereby simplifying dose escalation.

c. Compatibility with ligands or other chemical functionalities at the outer surface of the PEG coating.

d. Ease of preparation and use without modification of the many well-known liposomes methodologies.

e. Amenable to pharmaceutical product regulatory requirements.

f. Economy as compared to the use of monosialogangliosides.

Most of the PEG-lipids used for preparation of long circulating liposomes are prepared by derivatization of phosphatidylethanolamines (PEs), which act as an anchor to graft the PEG chain to the liposome surface. Monomethyl ether of PEG (mPEG) is often used for conjugation. It is particularly useful when multiple chains of the polymer have to be linked to the intended substrate. Due to its structural simplicity and possession of only one derivatizable end group, the use of mPEG minimizes crosslinking possibilities and leads to improved homogeneity of conjugates. Figure 2.1 shows various reagents used for derivatization of PE and the end products so obtained (Zalipsky, 1995).

The chlortraizine approach (A, Figure 2.1) to the preparation of PEG-PE (Blume and Cevc, 1990; Tilcock *et. al.,* 1993) is not attractive due to the toxicity of the reagent and its degradation products. The presence of slightly reactive chloride in the PE conjugate with (A) is another drawback of this method.

Klibanov and coworkers used succinimidylsuccinate-PEG (SS-PEG) (B, Figure 2.1) derived from mPEG-5000 for preparation of mPEG-succinamide-dioleoyl-PE (Klibanov, *et. al.,* 1990). Succinate ester linkage present in this conjugate is known to undergo hydrolysis under physiological conditions

(Zalipsky, 1995). Parr and coworkers (Parr, *et. al.,* 1994) demonstrated that this type of conjugate readily releases free mPEG-OH after a few hours of incubation at 37°C in mouse serum.

Urethane linked mPEG-PE conjugates were obtained by reacting mPEG-oxycarbonylimidazole (C, Figure 2.1) or succinimidyl carbonate (SC-PEG) (D, Figure 2.1) with distearoyl-PE (Allen *et. al.,* 1991; Woodle *et. al.,* 1992a). The reagent (D, Figure 2.1), being more reactive, allows preparation of mPEG-DSPE within 10-15 minutes at 45°C.

Figure 2.1 Methods for the preparation of mPEG- phosphatidyl ethanolamine conjugates

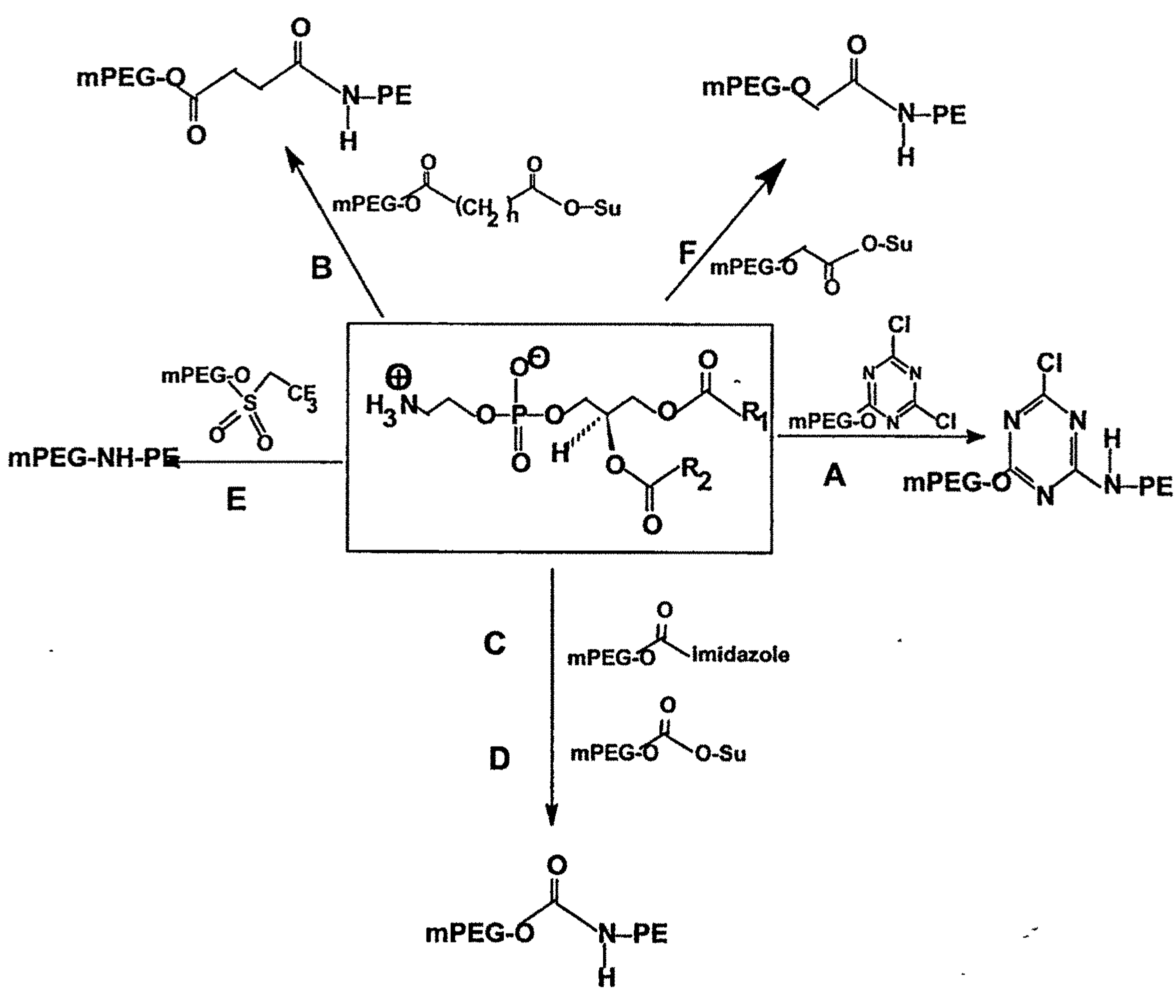
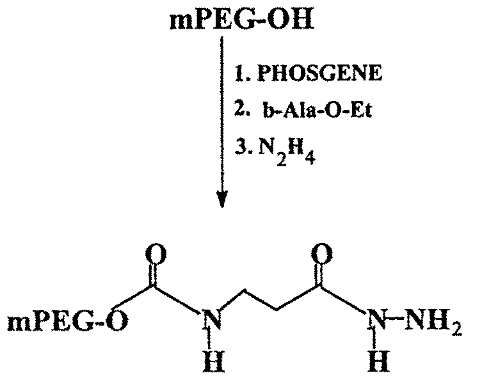


Figure 2.2 Synthesis of hydrazone-linked mPEG-lipid



Urethane linkages are quite stable and thus are more attractive than other linkages (Zalipsky, 1995).

DSPE (distearoylphosphatidylethanolamine)-PEG conjugates derived from reagent (E) (mPEG-tresylate) of molecular weight 5000 were prepared for formulation of long circulating liposomes (Tilcock *et. al.,* 1993; Blume and Cevc, 1990). Instead of using a PEG-lipid conjugate to form liposomes, Senior and coworkers (Senior *et. al.,* 1991) modified surface amino groups of DSPC (distearoylphosphatidylcholine) -DPPE (dipalmitoyl-PE)-cholesterol vesicles with this reagent. The attractive feature of this approach is in its selective grafting of the polymer on the exterior of the vesicles. It avoids the presence of mPEG residues inside the liposomes.

Amide linked mPEG_DSPE was prepared by coupling succinimidylester of carboxymethylated PEG (F, Figure 2.1) to the amino group of PE (Parr *et. al.,* 1994).

Oxidation of the diol residue of distearoylphosphatidylglycerol (DSPG) with periodate followed by reaction with mPEG-hydrazide (Figure 2.2) led to a hydrazone-linked mPEG-lipid. For some applications, it might be useful to have an acid-labile hydrazone linkage connecting the conjugate components (Zalipsky, 1995).

PEG-PE can form micelles giving clear solutions in water even upto concentrations as high as several mg/ml. Thus, PEG-PE has the potential to dissociate from the liposome and form micelles in equilibrium with bilayers. This 'problem' has been taken advantage of to achieve a slow loss of PEG-coating by selection of the lipid anchor. A reversal of this process, insertion of PEG-PE from micelles into preformed liposomes, also has been used as a means to prepare liposomes with a PEG coating (Woodle, 1997).

Introduction of PEG-PE replacing the distal methoxy with chemical functionalities useful for conjugation is an important advancement. The need for such functionalised PEG lipids, shown in Figure 2.3, was created by their usefulness in attachment of various biological relevant ligands to the exterior of the PEG-grafted liposomes. Some of the end group functionalised PEG-lipids have already been successfully used in various conjugation protocols resulting in attachment of immunoglobulins, peptides and other ligands to the distal ends of liposome-grafted PEG chains (Zalipsky, 1995).

Carboxy-PEG-DSPE was prepared by coupling bis-carboxyl-PEG to the amino group of PE followed by isolation of the desired product (I, Figure 2.3) by silica gel chromatography. It has been used for conjugation of plasmogen to liposomes for targeting to fibrin. Longevity in plasma circulation was well preserved (Blume and Cevc, 1993; Blume *et. al.,* 1993). This was the principle methodology for preparing some of the other conjugates (II-IV) (Figure 2.3).

Amino-PEG-DSPE (II, Figure 2.3) has proved useful as starting material for synthesis of other functionlilzed PEG-lipids. E.g. 2-pyridylthiopropionamide (IV, Figure 2.3) and bromoacetamide (V, Figure 2.3) derivatives. Amino-PEG-DSPE was cleanly derivatized with heterobifunctional reagents N-succinimidyl pyridyl dithiopropionate) (SPDP) and p-nitrophenyl bromoacetate respectively. Placing an amino acid residue as part of Linker A (Figure 2.3) is helpful for the final chemical characterization of the conjugates by amino acid analysis. This was utilized in case of hydrazide-PEG-DSPE (III, Figure 2.3). Amino-PEG-DSPE, apart from being used for preparing other end group fuctionalized PEG-DSPE, also forms long circulating liposomes, behaving as positively charged particles (Zalipsky *et. al.,* 1994).

Hydrazide-PEG-DSPE (III, Figure 2.3) form hydrazone linkages with aldehydes. It is used for conjugation of antibodies, oxidized on their carbohydrate residues, to distal ends of PEG chains on surfaces of liposomes. It is also used for conjugation of periodate treated N-terminal Serine or Theorine peptides (Zalipsky *et. al.,* 1996).

2-pyridyldithiopropionamide-PEG-DSPE (IV, Figure 2.3) has been used as a prescursor for HS-PEG-DSPE. It is very efficient for attachment of maleimide-containing antibodies to end groups of PEG on liposomal surfaces. It is used for binding HS-containing ligands through disulfide linkage (Allen *et. al.,* 1995a).

Bromoacetamide-PEG-DSPE (V, Figure 2.3) is an efficient moiety for thioether forming reactions with HS-ligands. It is utilized for Fab-SH attachment to end groups of PEG chains on liposomes (Zalipsky, 1995).

Linker B in most of the above cases (Figure 2.3) was a stable urethane linker formed by reacting PE with an appropriate succinimidyl carbonate-PEG-derivative (SC-PEG-X) where X represents a functional residue or a protected form thereof. This might allow further control of the *in vivo* properties of the relevant conjugated liposomes (Zalipsky 1995).

Certain new polymers have also been suggested for the steric protection of liposomes. The list is presented as Figure 2.4 (Torchilin and Trubetskoy, 1995).

Figure 2.3 End group functionalized PEG lipids

X –Linker A –PEG –Linker B –DSPE

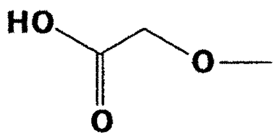
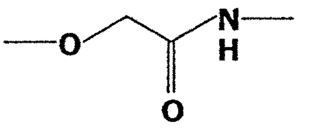
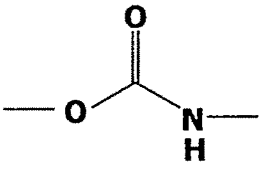
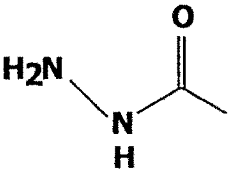
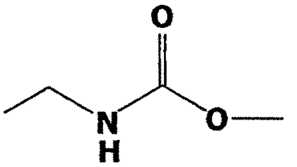
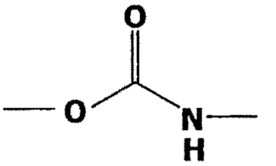
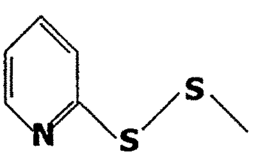
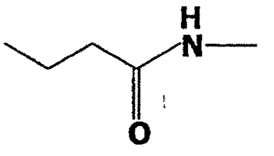
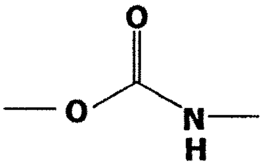
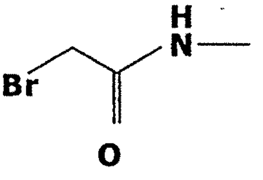
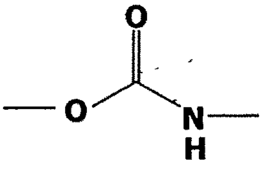
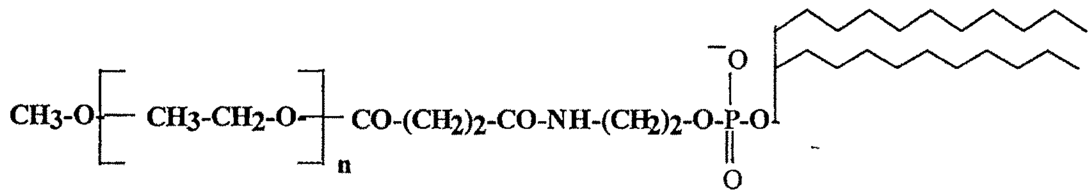
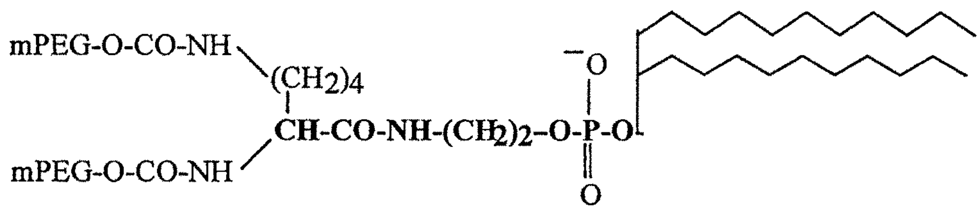
No.	**X-**	**–Linker A –**	**–Linker B –**
I		None	
II	H_2N —	None	
III			
IV			
V		None	

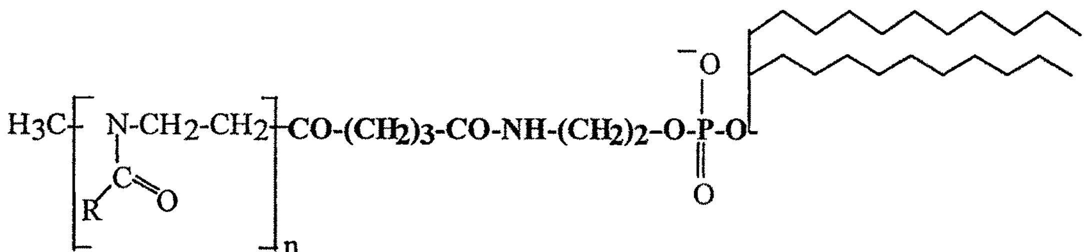
Figure 2.4 Amphiphilic synthetic polymers used for steric protection of liposomes



PEG-PE

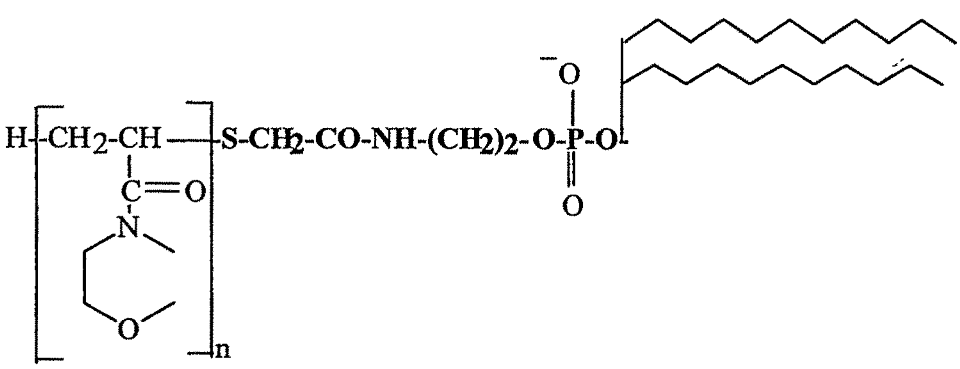


Branched PEG-PE

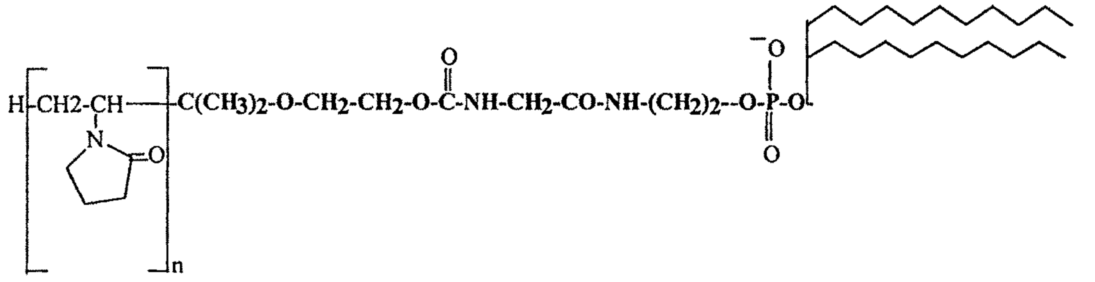


R = CH_3, C_2H_5

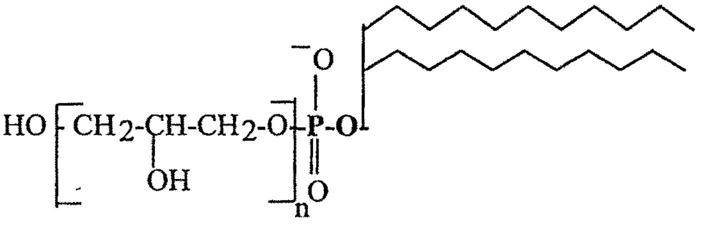
Poly(2-alkyl-2-oxazoline-)-PE



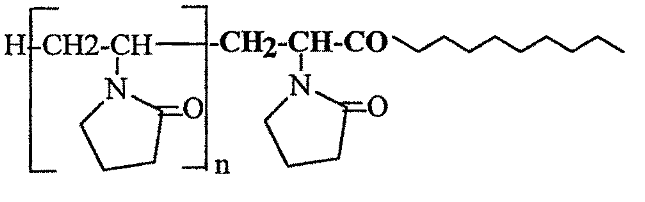
Poly(acryloyl morphine)-PE



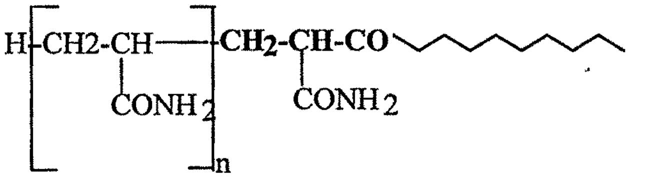
Poly (vinyl pyrrolidone)-PE



Poly (glycerol)-phosphatidyl glycerol



Poly (vinyl pyrrolidone)-palmitate



Poly (acryl amide)-palmitate

2.3.6 MECHANISM OF PROLONGED CIRCULATION OF LIPOSOMES

Since the advent of long circulating liposomes, intensive studies have been conducted to determine the mechanism of prolonged circulation. The prolonged systemic circulation effect of GM_1 was suggested to be related to the reduction in opsonization brought about through increasing surface hydrophilicity, suppression of complement dependent phagocytosis and a barrier effect of the carbohydrate chains (Stolnik *et. al.,* 1995). The stabilizing effect of gangliosides depends on the number of sialic acid residues: the higher order the gangliosides, the greater the activity in enhancing the stability of liposomes. However, among various gangliosides, GM_1 is unique in terms of having a strong activity in prolonging the circulation time of liposomes; other gangliosides such as GD_{1a} and GT_{1b} exhibits little or no activity (Mori *et. al.,* 1992). A liposome agglutination assay was used to assess the degree of steric barrier produced on the liposome surface by GM_1 (Mori *et. al.,* 1991). The study indicated that the steric barrier activity of GM_1 is weaker than that of PEG 2000-PE, however, its activity in prolonging the circulation time of liposomes is greater than that of PEG 2000-PE. The above two findings, in conjunction, indicate that the stabilizing effect of GM_1 via the steric barrier provided by the oligosaccharide residues is probably not a major mechanism in prolonging the circulation time of liposomes. The mechanism of high melting phospholipids in improving the circulation time of GM_1 containing liposomes has been attributed to the increasing bilayer rigidity, as a result of diminished rotational and translational diffusions of the lipids at the physiological temperature and thus the enhanced stability of liposomes. However membrane rigidity is not a strong requirement in liposome construction for prolonged circulation (Mori *et. al.,* 1992).

The mechanism of PEG protective action is under continuous investigation (Gabizon and Papahadjopoulos, 1992; Needham *et. al.,* 1992; Senior *et. al.,* 1991; Woodle *et. al.,* 1992). The general opinion is that, on the biological level, coating liposomes with PEG sterically hinders interactions of blood components with the liposome surface. This slows down inhibition of liposome destruction by lipoproteins and prevents liposome interaction with opsonins resulting in fast capture of liposomes by the reticuloendothelial system (RES). To confirm this, the reduced binding of plasma proteins with PEG liposomes was demonstrated (Senior *et. al.,* 1991; Chonn *et. al.,* 1992; Allen, 1994). The role of the supposed inhibition of opsonization of PEG liposomes in the prolongation of their circulation time also seems evident (Senior, 1987). From the properties of colloids, it is known that repulsive interactions between colloidal particles can be enhanced by coating these particles with soluble, well-hydrated and chemically inert polymers. Such modifications might decrease surface hydrophobicity and interaction of particles with RES. Thus, hydrophilicity and surface charge was considered as main requirement for long circulation (Gabizon and Papahadjopoulos, 1992). However, surface hydrophilicity and repulsive interactions alone cannot explain all the phenomena observed.

Thus, for example, liposomes coated with well soluble and highly hydrophilic dextran did not demonstrate prolonged circulation time or ability to protect the liposome surface from interacting with proteins from the solution (Blume and Cevc, 1993; Torchilin *et. al.,* 1994). Liposomes coated with maltopentose, estimated to be more hydrophilic than PEG 5000 were, still, rapidly removed from blood circulation in mice (Gref *et. al.,* 1995). It has been proposed that, besides hydrophilicity, the important feature of protective polymers is their flexibility (free rotation of individual polymer units around inter- unit linkages). The molecular mechanism of polymer protective action is determined by the properties of a flexible polymer in solution and includes the formation of the polymeric layer over the liposome surface which is impermeable for other solutes even at relatively low polymer concentrations (Torchilin *et. al.,* 1994; Torchilin and Papisov, 1994).

It has been proposed that the transient, flexible and rapidly changing structure of PEG makes the modelling of an antibody around it by the immune system difficult (Woodle and Lasic, 1992). The confirmation of PEG on the liposome surface has been predicted using the model of interactions between polymer-coated surfaces developed by De Gennes (Bedu-Addo and Huang, 1995). This model distinguishes between a model involving low grafting densities, known as the mushroom model, and another involving high grafting densities known as the brush model, both describeing the configuration of polymer on the surface. The ether oxygen of the (CH_3-CH_2-$O)_n$ group of PEG hydrogen bonds with water in aqueous solution. This preference of PEG for water supposedly causes the PEG to extend into a brush or mushroom conformation on the liposome surface even at low polymer grafting densities (Needham *et. al.,* 1992).

The liposomes are therefore sterically stabilized by a uniform coating of polymer layer which increases repulsive forces while reducing attractive forces between the bilayers (Woodle and Lasic, 1992). The model successfully predicts the steric repulsion and hence the stability of polymer coated colloidal particles (Woodle and Lasic, 1992). Lasic and coworkers (Lasic *et. al.,* 1991) proposed that the PEG chains also mask the electrostatic charge that would otherwise be associated with the liposome surface thus contributing to the steric stabilization.

2.3.7 PHARMACOKINETICS OF STERICALLY STABILIZED LIPOSOMES

An understanding of the influence of liposome composition, size, charge, dose and route of administration on the absorption, distribution and elimination of sterically stabilized liposomes and their associated drugs is critical to the development of rational therapeutic applications for such carriers.

The markers used for examining the pharmacokinetics of liposomes fall into two major classes: those associated with the liposome bilayer (e.g. radiolabeled lipids) and those associated with the liposome interior aqueous compartment (e.g. ^{3}H or ^{14}C-inulin, ^{67}Ga-desferoximine).

2.3.7.1 Absorption

Absorption or the fraction of drug and /or that reaches the systemic circulation depends on the liposome composition, the characteristics of the drug, the route of administration and the physiology of the absorption site. The absorption of sterically stabilized liposomes is quick and complete after intraperitoneal injection but is incomplete and takes some time when they administered subcutaneously, the mechanism being via the lymphatic drainage pathway. The rate and extent of absorption of these liposomes from a subcutaneous site depends on the actual site (Allen *et. al.,* 1991). In contrast, conventional liposomes administered subcutaneously are not released into the blood to a significant degree and there is some evidence that they are retained in the lymph node probably as a result of ingestion by macrophages.

2.3.7.2 Distribution

Due to their large size, the distribution of liposomes and other drug carriers is highly restricted as compared to the distribution of their associated drugs. The distribution of liposome-associated drugs is dependent on both the rate of release of drug from carrier and the restricted accessibility of the carrier to the tissues and interstitial fluids and the release of the drug therein.

The distribution kinetics of conventional and sterically stabilized liposomes has been extensively studied by many investigators (Allen and Chonn, 1987; Allen *et. al.,* 1989; Allen *et. al.,* 1991; Blume and Cevc, 1992; Klibanov *et. al.,* 1990; Liu *et. al.,* 1991; Mori *et. al.,* 1991; Parr *et. al.,* 1994; Senior *et. al.,* 1991). The rate and extent of distribution of liposomes from the circulation into tissues depends on liposome composition, surface charge, bilayer fluidity, liposome size, liposome dose and dosing frequency. The principle tissue of distribution for liposomes is the MPS and there is only a limited ability of liposomes to extravasate and diffuse into the interstitial fluids bathing the cells of other tissues. There is an inverse relationship between the liposome size and the rate at which they are distributed to MPS cells. Increasing the rigidity of conventional by incorporating high phase transition temperature lipids (e.g. DSPC) and more rigid phospholipids like sphingomyelins decreases their rate of distribution into the MPS (Allen *et. al.,* 1989; Woodle *et. al.,* 1992a). Similarly, modifying the bilayer composition with the addition of cholesterol has been shown to decreases uptake into the MPS tissues (Senior, 1987). Altering the surface charge of neutral liposomes has been shown to markedly influence their rate of distribution out of the circulation.

Sterically stabilized liposomes, stabilized using PEG and other such moieties, reduces uptake by MPS and prolongs liposome circulation times. Decreasing the molecular weight (or chain length) of PEG to less than 750Da increases clearance from circulation (Allen *et. al.,* 1991; Maruyama et. a. 1991; Woodle *et. al.,* 1992a). However, increasing the molecular weight of PEG above 1000 Da did not further increase the survival times of liposomes in circulation. Incorporation of PEG_{1000} or PEG_{2000} into liposomes resulted in higher blood levels compared to PEG_{5000}. Sterically stabilized liposomes also distribute into MPS tissues but at a slower rate and to a lesser extent than plain liposomes. There is also increased distribution of these liposomes into tumors (Allen *et. al.,* 1995; Gabizon, 1995) and into areas of infection (Bakker-Woudenberg *et. al.,* 1992; Allen *et. al.,* 1995).

2.3.7.3 Elimination

The major route of elimination of liposomes is by the cells of the MPS (Woodle and Lasic, 1992). The clearance of sterically stabilized liposomes follows first order elimination (log-linear) kinetics while clearance of conventional liposomes follows saturation-type, Michaelis- Menton pharmacokinetics (Allen and Hansen, 1991). It was proposed that there are two distinct elimination systems. A high affinity, low capacity saturable system likely corresponds to the recognition and binding of liposomes by cells of the MPS. A low affinity, high capacity system also appears to exist. This system is undoubtedly multifaceted and likely contains components of lipid metabolism, new MPS cell recruitment, binding site turnover and recycling and uptake into other tissues. The elimination of sterically stabilized liposomes by this system appears to be slower than the capacity of this system to 'turn over'. The low affinity, high capacity system may also be saturable but only at very high lipid doses (Allen and Hansen, 1991). Sterically stabilized liposomes ultimately localize in the liver and spleen, but at a reduced rate and with decreased total uptake (Allen *et. al.,* 1995).

2.3.8 APPLICATIONS OF STERICALLY STABILIZED LIPOSOMES

Sterically stabilized liposomes provide an entirely new and unique drug delivery vehicle because of their ability to evade quick clearance by the immune system. Using such liposomes, the goal of specific cell targeting becomes theoretically possible. The accessible sites are in the vascular compartment, at sites of increased vascular permeability, which occurs at sites of increased inflammation, infections, tumors and other traumatic conditions on systemic administration and in the peritoneal cavity and in the CNS on localized administration.

Most of the applications of the sterically stabilized liposomes are related to the delivery of chemotherapeutic agents and biomolecules.

2.3.9 STERICALLY STABILIZED LIPOSOMES IN CANCER THERAPY

2.3.9.1 Tumour Target Delivery

The rationale for the use of water soluble polymers for anticancer drug delivery include: the potential to overcome some forms of multidrug resistance, preferential accumulation in solid tumors due to enhanced permeability and retention (EPR) effect, biorecognizability, and targetability. The accumulation and retention of polymeric drugs are greatly enhanced in tumor tissue compared with those in normal tissue due to a phenomenon known as the EPR (enhanced permeability and retention) effect of macromolecules and lipidic particles in solid tumor (Maeda *et. al.,* 2000; Matsumara *et. al.,* 1986). This phenomenon, the EPR effect, is now recognized as a general characteristic of viable and rapidly growing solid tumor. Another general characteristic is the architectural defectiveness of tumor blood vessels, which also causes enhanced leakiness.

The EPR effect is also observed around the periphery of the tumor, i.e., in normal tissues surrounding the tumor, because of the variety of vascular mediators found there. Polymeric drugs may be cleared more rapidly from normal tissue than from tumor tissue via lymphatic drainage.

Factors affecting the EPR effect of macromolecular drugs in solid tumor are

1. Active angiogenesis and high vascular density in tumor.
2. Extensive production of vascular mediators that facilitate extravasation includes:
   (a) bradykinin
   (b) nitric oxide
   (c) VPF/VEGF
   (d) prostaglandins
   (e) collagenase (MMP)

(f) peroxynitrite

3. Defective vascular architecture, for example, lack of smooth muscle cells, lack of or reduced numbers of receptors for angiotensin II, large gap in endothelial cell-cell junctions, anomalous vascular conformation (e .g., branching or stretching)
4. Impaired lymphatic clearance of macromolecules and lipids from interstitial tissue (resulting in their retention).

Long-circulating (high-molecular weight) polymer conjugates have the potential to accumulate efficiently in tumor tissue due to the leaky tumor vasculature and the enhanced permeability and retention (EPR) effect. All of the previous studies indicate that tumor vasculature can be an ideal target for tumor-selective delivery of macromolecular anticancer agents (Noguchi et.al 1998, Sawa *et. al.,* 2000).

Many attempts have been made to achieve good selectivity to targeted tumor cells by preparing specialized carrier agents that are therapeutically profitable for anticancer therapy. Among these, liposomes are the most studied colloidal particles thus far applied in medicine and in particular in antitumor therapy. Although they were first described in the 1960s, only at the beginning of 1990s, the first therapeutic liposomes appear on the market. The first-generation liposomes (conventional liposomes) comprised a liposome-containing amphotericin B, Ambisome (Nexstar, Boulder, CO, USA), used as an antifungal drug, and Myocet (Elan Pharma Int, Princeton, NJ, USA), a doxorubicin-containing liposome, used in clinical trials to treat metastatic breast cancer. The second-generation liposomes ("pure lipid approach") were long-circulating liposomes, such as Daunoxome, a daunorubicin-containing liposome approved in the US and Europe to treat AIDS-related Kaposi's sarcoma. The third-generation liposomes were surface-modified liposomes with gangliosides or sialic acid, which can evade the immune system responsible for removing liposomes from circulation. The fourth-generation liposomes, pegylated liposomal doxorubicin, were called "stealth liposomes" because of their ability to evade interception by the immune system, in the same way, as the stealth bomber was able to evade radar. Actually, the only stealth liposome on the market is

Caelyx/Doxil (Schering-Plough, Madison NJ, USA), used to cure AIDS-related Kaposi's sarcoma, resistant ovarian cancer and metastatic breast cancer. Pegylated liposomal doxorubicin is characterized by a very long-circulation half-life, favorable pharmacokinetic behavior and specific accumulation in tumor tissues. These features account for the much lower toxicity shown by Caelyx in comparison to free doxorubicin, in terms of cardiotoxicity, vesicant effects, nausea, vomiting and alopecia. Pegylated liposomal doxorubicin also appeared to be less myelotoxic than doxorubicin (Cattel *et. al.,* 2003).

The influence of diameter on the pharmacokinetic and biodistribution of stealth liposomes into the tumor (4T1 murine mammary carcinoma) and cutaneous tissues (skin and paws) of mice was studied to ascertain the time course of liposome accumulation and to determine if a preferential accumulation of liposomes into tumor over skin or paws could be achieved by altering liposome size. All formulations delayed tumor growth, with liposomes of 100 or 157 nm being equally efficacious and superior to liposomes of 255 nm (Charrois and Allen, 2003).

A Phase I study of stealth liposomal cisplatin (SPI-077) concurrent with RT for head and neck squamous cell carcinoma (HNSCC) was conducted to test the potential advantages over the free drug when combined with radiation therapy selective tumor localization, improving the therapeutic ratio, and prolonged half-life, allowing more radiosensitization (Rosenthal *et. al.,* 2002).

A multicenter clinical study was designed to compare the efficacy of adjuvant stealh liposome-encapsulated cisplatin (SPI-77) to "standard-of-care" carboplatin therapy in dogs with osteosarcoma (OSA) in the context of a randomized study design. Stealth liposome encapsulation of cisplatin allowed the safe administration of five times the maximally tolerated dose of free cisplatin to dogs without concurrent hydration protocols (Vail, *et. al.,* 2002).

Hamilton and coworkers (2002) had performed a phase I study of polyethylene glycol (pegylated, Stealth) liposomal doxorubicin (Caelyx, Doxil) using a prolonged (6-week) dose interval to reduce the incidence of skin toxicity that was dose-limiting at more conventional dose intervals, and which appeared to be schedule dependant. Pegylated liposomal doxorubicin (Doxil, Caelyx) is a formulation of doxorubicin in poly(ethylene glycol)-coated (stealth) liposomes with a prolonged circulation time and unique toxicity profile. Doxil toxicity profile is drastically different from that of doxorubicin, and is characterized by dominant and dose-limiting mucocutaneous toxicities, mild myelosuppression, minimal alopecia, and no apparent cardiac toxicity. Although the single maximum tolerated dose (MTD) of Doxil is actually lower than that of conventionally administered doxorubicin, the cumulative MTD dose of Doxil may be substantially greater than that of free doxorubicin (Gabizon, 2001).

Phase I study was conducted to test the safety aspect of stealth liposomes encapsulated cisplatin, which proved that cisplatin encapsulated in Stealth liposomes (SPI-77) retains anti-tumour activity, but has a much reduced toxicity, compared to native cisplatin (Veal *et. al.,* 2001).

Studies were conducted to investigated the relative accumulation of ^{99m}Tc-DTPA radiolabelled stealth liposomal doxorubicin (Caelyx) in patients with metastatic brain tumours and with brain glioblastoma undergoing radiotherapy showed that stealth liposomal drugs selectively overcome the blood-brain barrier in the tumoral areas (Koukourakis *et. al.,* 2002).

The combined chemo-radiotherapy with stealth liposomal doxorubicin for locally advanced sarcomas is feasible and promising, the benefit expected from the unique ability of the stealth liposomes to accumulate selectively in the tumoral tissue (Koukourakis *et. al.,* 2000).

The relative cardiotoxicity of pegylated (stealth) liposomal doxorubicin (PL-DOX; Doxil) was compared to nonliposomal doxorubicin (Adriamycin) in rabbits and dogs confirmed that PL-DOX is also less cardiotoxic than the same dose of unencapsulated doxorubicin in humans (Working *et. al.,* 1999).

The pharmacokinetics, biodistribution and therapeutic efficacy of cisplatin encapsulated in long-circulating pegylated (Stealth) liposomes (SPI-077) were compared with those of nonliposomal cisplatin in two murine (C26 colon carcinoma and Lewis lung) tumor models. The result summarized that encapsulation of cisplatin in long-circulating pegylated liposomes (SPI-077) has a prolonged circulation time and increased tumor Pt disposition, and its antitumor effect was significantly improved compared to cisplatin in murine colon and lung cancer models (Newman *et. al.,* 1999).

Encapsulation in polyethylene glycol-coated (pegylated; Stealth) liposomes alters the pharmacokinetic characteristics, and hence the safety and tolerability profile, of doxorubicin. Pegylated liposomal doxorubicin administered as a single agent is generally well tolerated (Alberts and Garcia, 1997).

Polyethylene glycol (PEG)-coated (pegylated; Stealth) liposomes are stable, long-circulating drug carriers useful for delivering doxorubicin to the sites of solid tumours. Compared with conventional liposomes, pegylated liposomes are less extensively taken up by cells of the reticuloendothelial system (RES) and have a reduced tendency to leak the drug while in circulation. The pharmacokinetics of PEG-liposome encapsulated doxorubicin is characterized by an extremely long circulating half-life, slow plasma clearance and a reduced volume of distribution compared with conventional liposomal doxorubicin or free doxorubicin. The long circulation and ability of pegylated liposomes to extravasate through 'leaky' tumour vasculature results in localization of doxorubicin in tumour tissue (Gabizon and Martin, 1997).

Studies showed that stealth liposome encapsulated vincristine (S-Vinc) prolonged the drug's distribution phase plasma half-life in rats from 0.22 to 10.5 hours, while there was no significant difference in LD_{50} between encapsulated and free drug (Allen *et. al.,* 1995b).

The use of long-circulating liposomes as cytotoxic drug (doxorubicin) carriers in brain tumor results in enhanced drug exposure and improved therapeutic activity, with equal effectiveness against early small- and large-sized brain tumors (Siegel *et. al.,* 1995).

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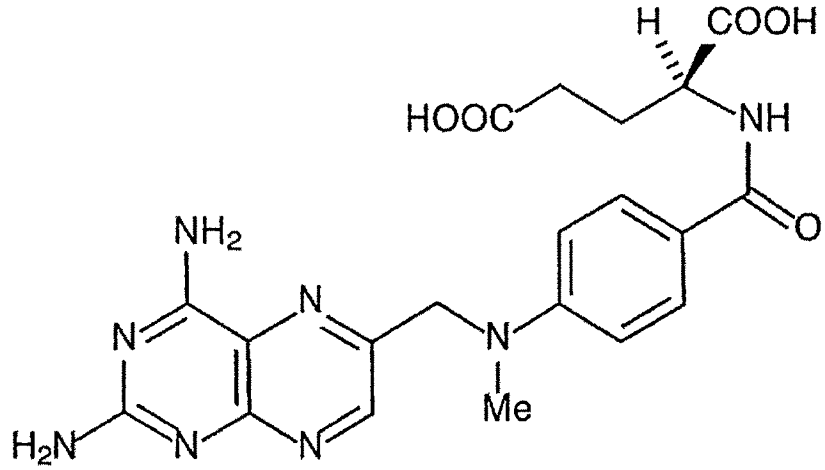
2.4 PROFILE OF METHOTREXATE

Methotrexate is an antimetabolite of dihydrofolic acid and it thus prevents conversion of deoxyuridylate to thymidylate and blocks the synthesis of new DNA needed for cellular replication.

2.4.1 OTHER NAMES: MTX, Amethopterin, 4-amino-10-methyl folic acid

2.4.2 MOLECULAR FORMULA: $C_{20}H_{22}N_8O_5$

2.4.3 STRUCTURAL FORMULA AND CHEMICAL NAME:



(*S*)-2-[4-[[(2,4-diaminopteridin-6-yl)methyl]methylamino]benzoylamino] pentanedioic acid

2.4.4 MOLECULAR WEIGHT: 454.4

2.4.5 APPEARANCE AND COLOUR: A yellow or orange, crystalline, hygroscopic powder.

2.4.6 SOLUBILITY: Methotrexate is practically insoluble in water, in alcohol and in methylene chloride. It dissolves in dilute solutions of mineral acids and in dilute solutions of alkali hydroxides and carbonates and slightly soluble in methanol.

2.4.7 PHARMACOPOEIAL STATUS: Official in British Pharmacopoeia (2001), United States Pharmacopoeia (2002), Indian Pharmacopoeia (1996).

2.4.8 IDENTIFICATION:

A. Dissolve 0.250 g in a 14g/l solution of sodium carbonate R and dilute to 25.0 ml with the same solution. The specific optical rotation is +19° to +24° calculated with reference to the anhydrous substance.

B. Dissolve 10 mg in a 4g/l solution of sodium hydroxide R and dilute to 100 ml with the same solvent. Dilute 10 ml of this solution to 100 ml with a 4g/l solution of sodium hydroxide R. Examined between 230 nm and 380 nm, the solution shows three absorption maxima, at 258 nm, 302 nm and 371 nm, respectively. The ratio of the absorbance measured at the maximum at 302 nm to that measured at the maximum at 371 nm is 2.8 to 3.3.

C. Examine by infrared absorption spectrophotometry, comparing with the spectrum obtained with methotrexate CRS.

2.4.9 CATEGORY: Antineoplastic

2.4.10 MECHANISM OF ACTION: (Dorr and Fritz, 1980;Haskell, 1990)

Methotrexate is an antimetabolite of the folic acid analog type. Methotrexate is cell cycle-specific for the S phase of cell division. Activity is due to inhibition of DNA synthesis, repair, and cellular replication; inhibition occurs as a result of relatively irreversible binding of methotrexate with dihydrofolate reductase, which prevents reduction of dihydrofolate to the active tetrahydrofolate.

Growth of rapidly proliferating cells (malignant cells, bone marrow, fetal cells, buccal and intestinal mucosa, cells of the urinary bladder, spermatogonia) is affected more severely than growth of most normal tissues and skin.

2.4.11 PHARMACOKINETICS: (Krogh, 1993; Wang *and* Fujimoto, 1984; Bennett, 1988; Nelson *and* Frank, 1981; Ballis *et. al.,* 1989)

2.4.11.1 Absorption

Methotrexate is generally completely absorbed from parenteral routes of injection. After intramuscular injection, peak serum concentrations occur in 30 to 60 minutes.

2.4.11.2 Distribution

After intravenous administration, the initial volume of distribution is approximately 0.18 L/kg (18% of body weight) and steady-state volume of distribution is approximately 0.4 to 0.8 L/kg (40 to 80% of body weight). Methotrexate competes with reduced folates for active transport across cell membranes by means of a single carrier mediated active transport process. At serum concentrations greater than 100 mM, passive diffusion becomes a major pathway by which effective intracellular concentrations can be achieved. Methotrexate in serum is approximately 50% protein bound. Laboratory studies demonstrate that it may be displaced from plasma albumin by various compounds including sulfonamides, salicylates, tetracyclines, chloramphenicol, and phenytoin. Methotrexate does not penetrate the blood-cerebrospinal fluid barrier in therapeutic amounts when given orally or parenterally. High CSF concentrations of the drug may be attained by intrathecal administration.

2.4.11.3 Metabolism

After absorption, methotrexate undergoes hepatic and intracellular metabolism to polyglutamated forms, which can be converted back to methotrexate by hydrolase enzymes. These polyglutamates act as inhibitors of dihydrofolate reductase and thymidylate synthetase. Small amounts of methotrexate polyglutamates may remain in tissues for extended periods. The retention and prolonged drug action of these active metabolites vary among different cells, tissues and tumors. A small amount of metabolism to 7-hydroxymethotrexate may occur at doses commonly prescribed. Accumulation of this metabolite may become significant at the high doses used in osteogenic sarcoma. The aqueous solubility of 7-hydroxymethotrexate is 3 to 5 fold lower than the parent compound. Methotrexate is partially metabolized by intestinal flora after oral administration.

2.4.11.4 Half-Life

The terminal half-life reported for methotrexate is approximately 3 to 10 hours for patients receiving treatment for psoriasis or low-dose antineoplastic therapy (less than 30 mg/m^2). For patients receiving high doses of methotrexate, the terminal half-life is 8 to 15 hours.

2.4.11.5 Excretion

Renal excretion is the primary route of elimination and is dependant upon dosage and route of administration. With IV administration, 80 to 90% of the administered dose is excreted unchanged in the urine within 24 hours.

There is limited biliary excretion amounting to 10% or less of the administered dose. Enterohepatic recirculation of methotrexate has also been proposed. Renal excretion occurs by glomerular filtration and active tubular secretion. Nonlinear elimination due to saturation of renal tubular reabsorption has been observed in psoriatic patients at doses between 7.5 and 30 mg. Impaired renal function, as well as concurrent use of drugs such as weak organic acids that also undergo tubular secretion, can markedly increase methotrexate serum levels. Excellent correlation has been reported between methotrexate clearance and endogenous clearance. Methotrexate clearance rates vary widely and are generally decreased at higher doses. Delayed drug clearance has been identified as one of the major factors responsible for methotrexate toxicity. It has been postulated that the toxicity of methotrexate for normal tissues is more dependent upon the duration of exposure to the drug rather than the peak level achieved.

2.4.12 INDICATIONS AND USAGE: (Schwenn *et. al.,* 1991)

2.4.12.1 Neoplastic Diseases

Methotrexate is indicated in the treatment of gestational choriocarcinoma, chorioadenoma destruens and hydatidiform mole. In acute lymphocytic leukemia, methotrexate is indicated in the prophylaxis of meningeal leukemia and is used in maintenance therapy in combination with other chemotherapeutic agents. Methotrexate is also indicated in the treatment of meningeal leukemia. Methotrexate is used alone or in combination with other anticancer agents in the treatment of breast cancer, epidermoid cancers of the head and neck, advanced mycosis fungoides, and lung cancer, particularly squamous cell and small cell types. Methotrexate is also used in combination with other chemotherapeutic agents in the treatment of advanced stage non-Hodgkin's lymphomas.

Methotrexate in high doses followed by leucovorin rescue in combination with other chemotherapeutic agents is effective in prolonging relapse-free survival in patients with non-metastatic osteosarcoma who have undergone surgical resection or amputation for the primary tumor.

2.4.12.2 Psoriasis

Methotrexate is indicated in the symptomatic control of severe, recalcitrant, disabling psoriasis which is not adequately responsive to other forms of therapy, but only when the diagnosis has been established, as by a biopsy and/or after dermatologic consultation. It is important to ensure that a psoriasis 'flare" is not due to an undiagnosed concomitant disease affecting immune responses.

2.4.13 DOSE:

(Bennett, 1980; Perry, 1992; Simon *and* Rivera, 1984;Magrath, 1989).

Adults:

Oral:	daily: 0.625-2.5 mg/kg	
	q1-2w: 15-30 mg x 5 days (or IM)	
	biw (twice a week): 10-15 mg/m^2/dose (or IM)	
	q2w: 2.5 mg/kg	
	daily: 3.3 mg/m^2 x 4-6 weeks	
Intravenous:	q1w: 25-40 mg/m^2	
	q1-3w: 100 mg-30 g/m^2 followed by leucovorin rescue within 2-24 hours	
Intrathecal:	q4-7d: 12 mg in 6 ml preservative-free NS	

Children:

Oral:	q1w: 15-20 mg/m^2	
Intravenous:	q4w: 300-3000 mg/m^2	
	q3-4w: 8-12 g/m^2 IV (top dose 20 g)	
Intrathecal:	<1 year - 6 mg	2-3 year - 10 mg
	1-2 year - 8 mg	>3 year - 12 mg

2.4.14 SIDE EFFECTS AND TOXICITY

ONSET	SIDE EFFECT AND TOXICITY
IMMEDIATE	anaphylaxis (rare),
(hours to days)	nausea and vomiting (dose related, 40% with high dose),

	allergic (fever and chills, rare), radiation recall reaction (rare), chemical meningitis (intrathecal use) *and* lung problems (pulmonary edema, pleuritic chest pain).
EARLY (days to weeks)	low WBC, RBC, platelets (myelosuppression, nadir 7-14 days, recovery 14-21 days), mouth sores (dose related, stomatitis), gastrointestinal problems (diarrhoea, bleeding, perforation), liver problems (elevated liver function tests), skin problems (pigmentation, photosensitivity, rash), loss of appetite (anorexia), hair loss (alopecia, usually mild), lung problems (interstitial pneumonitis), eye problems (conjunctivitis), CNS problems (acute encephalopathy), blood problems (megaloblastosis) *and* kidney problems (toxic neuropathy, with high doses).
DELAYED/LATE (weeks to years)	liver problems (fibrosis, cirrhosis) central nervous system problems (leukoencephalopathy)

2.4.15 CONTRAINDICATIONS:

Methotrexate can cause foetal death or teratogenic effects when administered to a pregnant woman. Methotrexate is contraindicated in pregnant women with psoriasis and should be used in the treatment of neoplastic diseases only when the potential benefit outweighs the risk to the foetus. Women of childbearing potential should not be started on methotrexate, until pregnancy is excluded and should be fully counselled on the serious risk to the foetus. Pregnancy should be avoided if either partner is receiving methotrexate; during and for a minimum of 3 months after therapy for male patients, and during and for at least one ovulatory cycle after therapy for female patients. Because of the potential for serious adverse reactions from methotrexate in breast fed infants, it is contraindicated in nursing mothers. Patients with psoriasis with alcoholism, alcoholic liver disease or other chronic liver disease should not receive methotrexate. Patients with psoriasis who have overt or laboratory evidence of immunodeficiency syndromes should not receive methotrexate.

Patients with psoriasis who have pre existing blood dyscrasias, such as bone marrow hypoplasia, leukopenia, thrombocytopenia, or significant anemia, should not receive methotrexate. Patients with known hypersensitivity to methotrexate should not receive the drug.

2.4.16 DRUG INTERACTIONS: (Adamson et.al., 1991; Balis et.al., 1987; Dorr and Von Hoff, 1994; Frenia and Long, 1992; Jarosinski *et. al.,* 1988; McEvoy, 1994; Tatro, 1992)

AGENT	EFFECT	MECHANISM
Alcohol	Enhanced hepatotoxicity	Additive
Asparaginase	Enhanced hepatotoxicity	Additive
Carboxypeptidase-G	Decreased toxicity of MTX	Cleaves the MTX molecule to inactive fragments
Co-trimoxazole	Increased risk of hematological toxicity from MTX	Possibly displacement of MTX from plasma protein binding sites or inhibition of renal tubular secretion

Etretinate	Increased serum methotrexate levels and/or enhanced hepatotoxicity	Unknown
Leucovorin	Decreased toxicity of MTX	Leucovorin "rescues" normal cells from toxic effects of methotrexate
Non-steroidal anti-inflammatory drugs (NSAIDs) [eg, ASA, ibuprofen, indomethacin, ketoralac, ketoprofen, naproxen]	Severe, occasionally fatal toxicity (GI and hematologic) may follow the concomitant administration of NSAIDs and MTX, especially with high-dose MTX	NSAIDs may inhibit renal elimination of MTX, possibly by inhibition of renal prostaglandin synthesis (resulting in decreased renal perfusion) or by competition for renal tubular secretion
Neomycin, polymyxin B, nystatin, vancomycin	MTX serum concentrations may be decreased	Reduced absorption of oral MTX
Phenytoin	Decreased effect of phenytoin	Possible decreased absorption of phenytoin
Probenecid	Increased effect of MTX	Inhibition of renal excretion of MTX
Thiazide diuretics	Prolonged leukopenia	Unknown
Thiopurines (azathioprine, mercaptopurine)	Increased effect of thiopurines	Inhibition of first-pass metabolism of thiopurines
Warfarin	Increased pharmacological effect of warfarin	Possibly altered hepatic metabolism of warfarin

2.4.17 ANALYTICAL TECHNIQUES:

Several analytical techniques have been reported for estimation of methotrexate. A brief review of methods has been given below.

2.4.17.1 Chromatographic Techniques

Various HPLC methods are available for the estimation of methotrexate, which is tabulated below (Table 2.4). HPLC based methods are useful for estimation in biological fluids and in dosage form using UV detector ranging from 254 –313 nm.

Table 2.4 Chromatographic techniques for determination of methotrexate

Column used	λmax (UV)	Medium	Reference
Phenyl reverse phase	254 nm	Dosage form	Arthur *et. al.*, 1976
Nucleosil 100 SB	310 nm	Biological fluids	Mader *et. al.*, 1993
LiChrosorb RP-18	254 nm	Biological fluids	Emara *et. al.*, 1998
Supelcosil LC-18	310 nm	Hospital surfaces	Floridia et.al., 1999
HP Lichrosphere C_{18}	313 nm	Biological fluids	Ge and Lu, 2001

Paper chromatography

Paper chromatographic method is official in Indian Pharmacopoeia. The mobile phase used is 15.6% w/v solution of $Na_2H_2PO_4$ (pH 5.8) and separated components were detected at 254 nm (I.P., 1996).

Ion exchange chromatography

Ion exchange based separation of folic acid analogs including methotrexate was reported by Copenhaver and Bring (1964). The cation exchange resin was AG 50W-X4 and the developing solvent was 15% $Na_2HPO_4.12H_2O$ (pH 8.5) buffer containing 0.1M mercaptoethanol.

2.4.17.2 Fluorimetric methods

Salamoun and Frantisek (1986) determined MTX and its metabolites 7-hydroxymethotrexate and 2,4-diamino-N10-methylpteroic acid in biological fluids by liquid chromatography with fluorimetric detection at 417 nm (excitation at 282 or 360 nm).

Kuroda *et. al.,* (2000) have reported a fluorimetric method for determination of methotrexate in plasma, bile or urine in rats and monkeys. Fluorescence was measured at 435 nm with an excitation maximum at 350nm.

2.4.17.3 Spectrophotometric methods

Sastry and Lingeswara Rao (1996) have reported three spectrophotometric methods for the determination of methotrexate. Method (*i*) Reduction of Fe(III) to Fe(II) by the drug to form a coloured complex with 1,10-phenanthroline (λmax at 510 nm). Method (*ii*) Reduction of Folin-Ciocalteu reagent by the drug to form a blue complex (λmax at 770 nm). Method (*iii*) Coupling reaction through an aromatic secondary amino group in *p-N*-methylaminobenzoylglutamic acid released on reduction of the drug with Zn and HCl with 1,2-naphthaquinone-4-sulfonic acid sodium salt to form a coloured complex (λmax = 500 nm).

Ellaithy *et. al.,* (1983) reported determination of MTX in 0.1M HCl at 306 nm and 0.1M NaOH at 258 nm.

2.4.17.4 Polarographic methods

Ebel *et. al.,* (1985) reported polarographic method for estimation of MTX using saturated calomel electrode.

Dias and Dhadke (1994) reported a polarographic method for determination of methotrexate using Clark's and Cubb's buffer (pH 10.8) as supporting electrolyte.

2.4.17.5 Radioimmunoassay

Tracey *et. al.,* (1983) described radioimmunoassay of MTX using hydroxy-ethyl methacrylate gel impregnated with MTX.

Howell *et. al.,* (1980) also described the use of radioimmunoassay in determination of MTX in human plasma.

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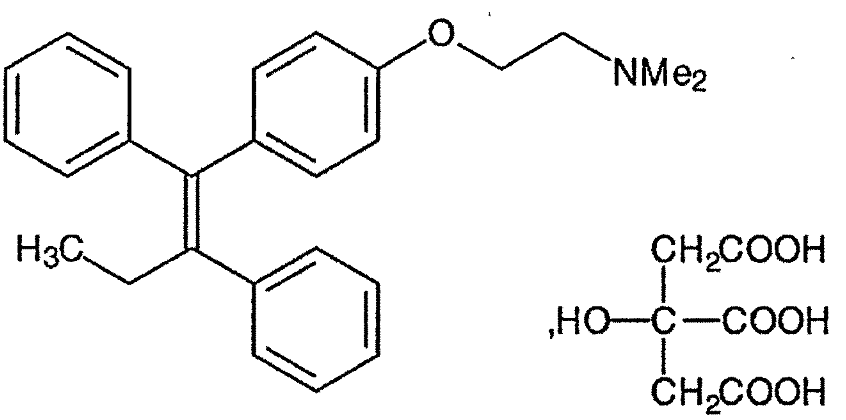
2.5 PROFILE OF TAMOXIFEN CITRATE

Tamoxifen citrate is an effective, palliative treatment for patients with advanced disease whose tumour is estrogen receptor (ER) positive. It can also be used as an adjuvant therapy in postmenopausal women to prevent disease recurrent and currently under study as a chemo preventive in women at risk for developing breast cancer.

2.5.1 MOLECULAR FORMULA: $C_{26}H_{29}NO$, $C_6H_8O_7$

2.5.2 STRUCTURAL FORMULA AND CHEMICAL NAME:



Z)-2-[4-(1,2-Diphenylbut-1-enyl) phenoxy]-N,N-dimethylethylamine citrate

2.5.3 MOLECULAR WEIGHT: 563.6

2.5.4 APPEARANCE AND COLOUR: A white or almost white, crystalline powder.

2.5.5 SOLUBILITY: Tamoxifen citrate is slightly soluble in water, soluble in methanol and slightly soluble in acetone.

2.5.6 PHARMACOPOEIAL STATUS: Official in British Pharmacopoeia (2001), United States Pharmacopoeia (2002), Indian Pharmacopoeia (1996).

2.5.7 IDENTIFICATION:

A. Dissolve 20 mg in methanol R and dilute to 50.0 ml with the same solvent. Dilute 5.0 ml of the solution to 100.0 ml with methanol R. Examined between 220 nm and 350 nm, the solution shows two absorption maxima, at 237 nm and 275 nm. The ratio of the absorbance measured at the maximum at 237 nm to that measured at the maximum at 275 nm is 1.45 to 1.65.

B. Examine by infrared absorption spectrophotometry, comparing with the spectrum obtained with tamoxifen citrate CRS. Examine the substances prepared as discs. If the spectra obtained in the solid-state show differences, dissolve the substance to be examined and the reference substance separately in acetone R, evaporate to dryness and record new spectra using the residues.

C. Examine by thin-layer chromatography, using as the coating substance a suitable silica gel with a fluorescent indicator having an optimal intensity at 254 nm.

2.5.8 CATEGORY: Anti-estrogen, Antineoplastic.

2.5.9 MECHANISM OF ACTION:(Dorr and Fritz, 1980; Krogh, 1993)

Tamoxifen is a synthetic nonsteroidal anti-estrogenic analog of clomiphene. It is a competitive inhibitor of estradiol binding to the ER. When bound to the ER, tamoxifen induces a change in the three dimensional shape of the receptor, inhibiting its binding to the estrogen-responsive element (ERE) on DNA. Under normal physiological conditions, estrogen stimulation increases tumor cell production of transforming growth factor β (TGF-β), an autocrine inhibitor of tumor cell growth. By blocking these pathways, the net effect of tamoxifen treatment is to decrease the autocrine stimulation of breast cancer growth, capturing the cell in G_1. In addition, tamoxifen decreases the local production of insulin-like growth factor 1 (IGF-1) by surrounding tissues; IGF-1 is a paracrine growth factor for the breast cancer cell.

Other biochemical effects of tamoxifen include interaction with protein kinase C and stimulation of human NK cells. Approximately 25% of malignant melanomas have demonstrable estrogen receptors. Although the clinical significance of this is not known, occasional objective responses to tamoxifen have been reported. Similarly, the presence of estrogen receptors and estrogen binding proteins has been reported in normal and malignant pancreas.

2.5.10 PHARMACOKINETICS: (Martindale, 1989; Buckley *and* Goa, 1989; Lien *et. al.,* 1989)

2.5.10.1 Absorption

Tamoxifen is orally well absorbed. Peak concentrations occur 4-7 h after oral dosing. Peak concentrations after single oral doses of 20 mg are about 40 μ/l. There is no information on absolute bioavailability.

2.5.10.2 Distribution

Tamoxifen is more than 99% protein-bound in serum, predominantly to albumin. In patients with breast cancer, concentrations of tamoxifen and its metabolites in pleural, pericardial and peritoneal effusion fluid are between 20 and 100% of those in serum, but only trace amounts enter the cerebrospinal fluid. Concentrations in breast cancer tissue exceed those in serum. The volume of distribution is 50-60 l/kg. High concentrations in uterus, endometrium, breast, prostate and ovary is found.

2.5.10.3 Metabolism

Tamoxifen citrate undergoes extensive hepatic metabolism hepatic microsomal enzyme oxidation system, causes autoinhibition of metabolism to 1-(4-ethanolyloxyphenyl)-1,2-diphenylbut-1-ene (the primary alcohol), N-desmethyl tamoxifen, 4-hydroxy tamoxifen, 4-hydroxy-N-desmethyl tamoxifen and N-desdimethyl tamoxifen.

2.5.10.4 Half-life

The elimination is biphasic, with an initial half-life of around 7 h and a terminal half-life of 7-11 days.

2.5.10.5 Excretion

The major excretory route is via the bile as metabolites and enterohepatic recirculation occurs and slowly excreted in feces. Less than 1% is excreted in the urine.

2.5.11 INDICATIONS AND USAGE: (Fisher *et. al.,* 1998; Jaiyesimi *et. al.,* 1995; Jordon, 1995)

Tamoxifen citrate is the endocrine treatment of choice for postmenopausal women with metastatic breast cancer or at high risk of recurrence from the disease. It is also used in premenopausal women with ER-positive tumors. Tamoxifen has been used alone as an adjuvant therapy for women at risk for recurrence following initial diagnosis and treatment of primary breast cancer. Tamoxifen is under study as a possible chemotherapeutic agent in women who are at risk of developing breast cancer.

2.5.12 DOSE: (Buzdar *et. al.,* 1994; McEvoy, 1993; Vogel, 1987)

Adults:	20 mg po daily
	20 mg/m^2po daily
	10 mg po bid
	40 mg po qid x 7 days then 20 mg po bid (for malignant melanoma)

2.5.13 SIDE EFFECTS AND TOXICITY: (Andersson *et. al.,* 1991; Fisher *et. al.,* 1994; Drugs., 1993; Kreisman and Wolkove, 1992; Love *et. al.,* 1991)

ONSET	**SIDE EFFECT *AND* TOXICITY**
IMMEDIATE	nausea and vomiting (25%),
(hours to days)	distaste for food.
EARLY	
(days to weeks)	hot flushes (25%),
	headache (rare),
	flare response (transient increase in bone or tumor pain and/or hypercalcemia),
	menstrual irregularities,
	swelling of extremities (edema),
	vaginal discharge or bleeding (rare),
	vulvar itching (rare, pruritis vulvae),
	dizziness (rare),
	low WBCs, platelets (rare, transient, myelosuppression),
	skin problems (rare, rash, dermatomyositis),

	liver problems (elevated liver function tests, rare, transient; liver damage, rare).
DELAYED/LATE (weeks to years)	blood clots (infrequent, pulmonary emboli or thrombosis),
	mental depression (rare),
	eye problems (more common with high doses, retinopathy, corneal opacities),
	endometrial cancer (rare).

2.5.14 CONTRAINDICATIONS: (Dorr and Van Hoff, 1994)

Tamoxifen is contraindicated in patients with a history of significant thromboembolic disease, in pregnant and breast-feeding mothers. In premenopausal patients it may be prudent to suggest barrier forms of contraception because, initially, tamoxifen therapy can effectively induce ovulation. Oral contraceptives (birth control pills) are relatively contraindicated in breast cancer. Breast-feeding is not recommended due to their potential secretion into breast milk.

2.5.15 DRUG INTERACTIONS: (Hansten and Horn, 1992; McEvoy, 1993)

AGENT	EFFECT	MECHANISM
Aminoglutethimide	Decreased tamoxifen concentrations	Increased metabolism of tamoxifen
Oral anticoagulants (eg, warfarin)	Significant increase in anticoagulant effect	Unknown
Thyroid function test	Elevate T4 (thyroxine)	Increased thyroxine-binding globulin

2.5.16 ANALYTICAL TECHNIQUES:

Several analytical techniques have been reported for estimation of tamoxifen citrate. A brief review of methods has been given below.

2.5.16.1 Chromatographic Techniques

Various HPLC methods are available for the estimation of tamoxifen citrate, which is tabulated below (Table 2.5). HPLC based methods are useful for estimation in biological fluids and in dosage form using UV detector ranging from 238 – 310 nm.

Table 2.5 Chromatographic techniques for determination of tamoxifen citrate

Column used	λmax (UV)	Medium	Reference
Ultrasep ES Pharm RP8	270 nm	Dosage form	Gliesing *et. al.,* 1997
SPE Bond-elut C_{18}	238 nm	Biological fluids	Merle *et. al.,* 1998
Bondapak C_{18}	265 nm	Biological fluids	MacCallum *et. al.,* 1997
Phenomenex Kromasil C_{18}	310 nm	Biological fluids	Koskinen *et. al.,* 1997
BDS-Hypersil	280 nm	Biological fluids	Lim *et. al.,* 1993

Gas Chromatography

Guetens and coworkers (2002) had reported capillary gas chromatography (cGC)-mass spectrometry (MS) method for the determination of tamoxifen citrate in biological samples.

A method for identification of tamoxifen and metabolites in urine, using the gas chromatography and mass spectrometry system (GC-MS) was described by Mihailescu *et. al.,* (2000).

2.5.16.2 Spectrophotometric methods

Sastry *et. al.,* (1995) have reported simple, rapid and sensitive spectrophotometric methods for the determination of tamoxifen citrate, based on the formation of chloroform soluble ion-association complex between Tamoxifen and the dye (napthalene Blue 12 BR or Alizarin Red-S) in acidic medium. The coloured species exhibit maximum absorbance at 620 and 440 nm respectively.

Hewala (1993) have demonstrated the determination of tamoxifen citrate in powder and tablet form. The first method is based on using the first-derivative spectra of UV absorption spectra. The second method, was a colorimetric method (acid dye) involving the formation of coloured ion-pair compounds between the drugs and methyl orange (C. I. Acid Orange 45) in $CHCl_3$; absorbance measured at 420 nm.

2.5.16.3 Potentiometric method

Stephen *et. al.,* (1997) and Wang *et. al.,* (1997) had demonstrated the potentiometric determination of tamoxifen from biological fluids.

2.5.16.4 Nuclear magnetic resonance method

The use of NMR for quantitative analysis of pharmaceuticals was described by Lindgren and Martin (1993). The method was illustrated by the relative determinations of 'cis' and 'trans' isomers of clomiphene and tamoxifen.

2.5.16.5 Polarographic method

The electrochemical behaviour of tamoxifen citrate was studied by polarography in the fast-scan and differential pulse modes, cyclic voltammetry and micro-coulometry (Fijalek et. al, 1989).

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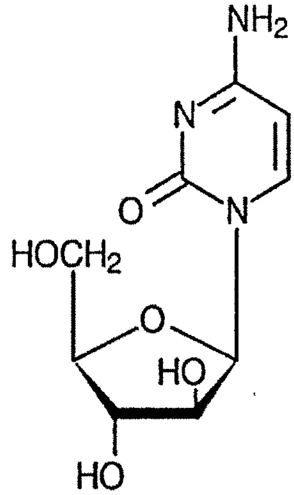
2.6 PROFILE OF CYTARABINE

Cytarabine is the most important antimetabolite used in the therapy of acute myeloid leukemia. It is single most effective agent for induction of remission in the disease.

2.6.1 OTHER NAMES: ara-C and cytosine arabinoside

2.6.2 MOLECULAR FORMULA: $C_9H_{13}N_3O_5$

2.6.3 STRUCTURAL FORMULA AND CHEMICAL NAME:



1-β-D-arabinofuranosylcytosine

2.6.4 MOLECULAR WEIGHT: 243.2

2.6.5 APPEARANCE AND COLOUR: A white or almost white, crystalline powder

2.6.6 SOLUBILITY: Cytarabine is freely soluble in water, very slightly soluble in alcohol, chloroform and in methylene chloride.

2.6.7 PHARMACOPOEIAL STATUS: Official in British Pharmacopoeia (2000), United States Pharmacopoeia (2002), Indian Pharmacopoeia (1996) and European Pharmacopoeia (1997).

2.6.8 IDENTIFICATION:

A. Dissolve 20.0 mg in 0.1M hydrochloric acid and dilute to 100.0 ml with the same acid. Dilute 5.0 ml of the solution to 100.0 ml with 0.1M hydrochloric acid. Examined between 230 nm and 350 nm, the solution shows absorption maximum at 281 nm. The specific absorbance at the maximum is 540 to 570.

B. Examine by infrared absorption spectrophotometry, comparing with the spectrum obtained with cytarabine CRS. Examine the substances prepared as discs.

C. Examine the chromatograms obtained in the test for related substances in ultraviolet light at 254 nm. The principal spot in the chromatogram obtained with test solution (b) is similar in position and size to the principal spot in the chromatogram obtained with reference solution (a).

2.6.9 CATEGORY: Antineoplastic.

2.6.10 MECHANISM OF ACTION: (Chabner and Myers, 1989; Haskell, 1990)

Cytarabine is metabolized intracellularly into its active triphosphate form (cytosine arabinoside triphosphate). This metabolite then damages DNA by multiple mechanisms, including the inhibition of alpha-DNA polymerase, inhibition of DNA repair through an effect on beta-DNA polymerase, and incorporation into DNA. The latter mechanism is probably the most important. Cytotoxicity is highly specific for the S phase of the cell cycle.

2.6.11 PHARMACOKINETICS: (Balis and Poplack, 1989; Ho and Frei, 1971; Knoben and Anderson, 1988; Knoester *et. al.,* 1993; Reynolds, 1982).

2.6.11.1 Absorption

Cytarabine is ineffective orally; less than 20% of a dose is absorbed from the gastrointestinal tract. Constant i.v. infusions of cytarabine produce relatively constant plasma concentrations of the drug in 8 to 24 hours. Following s.c. administration of cytarabine, peak plasma levels of the drug are attained about 20 to 60 minutes after injection and fall below steady-state infusion levels after about 100 minutes.

2.6.11.2 Distribution

Cytarabine is rapidly and widely distributed into tissues. After rapid i.v. injection of cytarabine, approximately 13% of the drug is bound to plasma proteins. Cytarabine crosses the blood-brain barrier to a limited extent. During a continuous i.v. or s.c. infusion, cytarabine concentrations in the cerebrospinal fluid (CSF) are higher than those attained after rapid i.v.

injection and are about 40 to 60% of plasma concentrations. Most of an intrathecal dose of cytarabine diffuses into the systemic circulation but is rapidly metabolized and usually only low plasma concentrations of unchanged drug occur. The drug apparently crosses the placenta. The protein binding of cytarabine is low (15%).

2.6.11.3 Metabolism

Cytarabine disappears rapidly from the plasma following i.v. injection. I.V doses of cytarabine exhibit biphasic elimination with an initial half-life of approximately 10 minutes in which a major fraction of a dose is hepatically metabolized by cytidine deaminase to inactive 1-beta-D-arabinofuranosyluracil (uracil arabinoside).

2.6.11.4 Half-life

Varies among individuals; may relate to cytotoxicity.

Alpha phase -10 minutes.

Beta phase- 1 to 3 hours (about 2 hours after intrathecal administration).

2.6.11.5 Excretion

Cytarabine and its metabolite, uracil arabinoside, are excreted in urine. After rapid i.v., s.c. or intrathecal injection or continuous i.v. infusion, about 70 to 80% of the dose is excreted in urine within 24 hours. Approximately 90% of the urinary drug excretion occurs as the metabolite and about 10% as unchanged cytarabine.

2.6.12 INDICATIONS AND USAGE: (Crogh, 1992; Robert *et. al.,* 1985; Winter *et. al.,* 1985)

Primarily for induction and maintenance of remission in acute leukemia in both adults and children. It has found use in the treatment of acute myelocytic leukemia (AML), chronic myelocytic leukemia (blast phase), acute lymphocytic leukemia (ALL) and erythroleukemia. It may be used alone or in combination with other antineoplastic agents, superior results being obtained with combination therapy. Cytarabine is often used with daunorubicin, doxorubicin, thioguanine or vincristine. The drug is also used in combination therapy for acute Myelogenous leukemia, Meningeal leukemia, non-Hodkin's lymphomas in adults and in children and for treatment of relapses of acute lymphocytic leukemia in both age groups.

2.6.13 DOSE: (Cappizzi *et. al.,* 1994; Perry, 1992; Riddell *et. al.,* 1988)

Adults:

Induction: q2w: 200 mg/m2/day x 5 days - continuous infusion

q2-4w: 100 mg/m2/day x 7-10 days - continuous infusion

100 mg/m2 q12h x 7 days - rapid injection

2-6 mg/kg/day x 5-10 days - continuous infusion or in 2-3 divided doses - rapid injection

daily: 100-200 mg/m2 until remission

Maintenance: q1-4w: 1-1.5 mg/kg IM/SC

q4w: 70-200 mg/m2/day x 2-5 days - continuous infusion or rapid injection

High dose: q2-3w: 2-3 g/m2 over 1-3 hours q12h x 4-12 doses

Intrathecal: daily: 5-75 mg/m2 or 30-100 mg x 4-5 days

biw: 50 mg in 6 mL NS alternating with methotrexate twice weekly x 6 doses (ie, 3 doses of each over 3 weeks)

Children:

Intravenous: q4w: 75-150 mg/m2/day or 5 mg/kg/day x 5 days

biw: 300 mg/m2/day (biw = twice weekly) x 4 weeks

q4w: 150 mg/m2/day x 5 days by continuous IV or SC infusion

1-3 g/m2/dose q12h x 4-12 doses (<3 years: 100 mg/kg/dose)

Intrathecal: <1 year: 20 mg

1-2 years: 30 mg

2-3 years: 50 mg

>3 years: 70 mg

2.6.14 SIDE EFFECTS AND TOXICITY: (Baker *et. al.,* 1991; Grossman *et. al.,* 1983; Herzig *et. al.,* 1987; Higa *et. al.,* 1991)

ONSET	SIDE EFFECT AND TOXICITY
IMMEDIATE (hours to days)	anaphylaxis (rare), increased uric acid levels (hyperuricemia), CNS problems (cerebellar toxicity, cerebral dysfunction, seizures), CNS toxicity (chemical arachnoiditis, seizures, paraplegia, IT route, rare), nausea and vomiting, flu-like syndrome and injection site problems (thrombophlebitis, pain, inflammation, rare).
EARLY (days to weeks)	low WBCs, RBCs, platelets (myelosuppression, first nadir 7-9 days, second nadir 15-24 days, recovery 25-34 days), lung problems (pulmonary edema), eye problems (conjunctivitis), mouth sores (stomatitis), hair loss (alopecia, more common with higher doses), gastrointestinal problems (hematemesis, diarrhoea, abdominal pain), liver problems (elevated liver function tests, hepatotoxicity), nerve damage (peripheral neuropathy, rare), skin problems (rash, freckling) and blood problems (megaloblstosis).
DELAYED/LATE (weeks to years)	infertility.

2.6.15 CONTRAINDICATIONS:

Cytarabine is contraindicated in patients with a history of known hypersensitivity to cytarabine. It is also contraindicated in pregnancy and breast-feeding.

2.6.16 DRUG INTERACTIONS: (McEvoy, 1993)

AGENT	EFFECT	MECHANISM
Flucytosine	Decreased flucytosine effect	Possible competitive inhibition of the uptake of flucytosine by fungi
Aminoglycosides	Decreased aminoglycoside effect	Unknown
Fludarabine	Decreased metabolism of fludarabine; cytarabine given first appears to inhibit antineoplastic effect of fludarabine. Fludarabine given first appears to stimulate rather than inhibit metabolic activation of cytarabine	Fludarabine competes for deoxycytidine kinase, needed to convert both drugs to their active form clinical importance is yet unknown

2.6.17 ANALYTICAL TECHNIQUES:

Several analytical techniques have been reported for estimation of cytarabine. A brief review of them has been given below.

2.6.17.1 Chromatographic Techniques

Various HPLC methods are available for the estimation of cytarabine, which are tabulated below (Table 2.6). HPLC based methods are useful for estimation in biological fluids and in dosage form using UV detector ranging from 238 –310 nm.

Table 2.6 Chromatographic techniques for determination of Cytarabine

Column used	λmax (UV)	Medium	Reference
Supelcosil LC-18	272 nm	Work place	Floridia *et. al.,* 1999
Nucleosil C_{18}	275 nm	Biological fluids	Rentsch *et. al.,* 1995
Octadecyl Si 100 Polyol	265 nm	Biological fluids	Galushko *et. al.,* 1990
Nucleosil C18	280 nm	Biological fluids	Schleyer *et. al.,* 1989
Ultrasphere ODS	280 nm	Biological fluids	Riccardi *et. al.,* 1989

Thin layer chromatography

Cytarabine was analyzed by thin layer chromatography in plasma sample using bakerbond C18 column was reported by Paw and Misztal, 1997.

2.6.17.2 Spectrophotometric methods

The determination of cytarabine by second-derivative spectrometry was investigated by Tuncel *et. al.,* (1991) which is proved to be rapid, accurate and practical.

Differential, first- and second-derivative differential UV spectrophotometric methods were described for the determination of cytarabine in formulations, such as injections and creams by Mahrous and coworkers (1992).

2.6.17.3 Radioimmunoassay

Sato *et. al.,* (1984) described the use of radioimmunoassay in determination of cytarabine in human plasma.

2.6.17.4 Polarographic method

Dogrukol-ak and Tuncel (1994) had described about the analysis of cytarabine in ampoules by polarographic technique.

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3.1 INTRODUCTION

The analytical methods used in studies of drug-containing liposomes should, in addition to possessing the desired characteristics of accuracy, precision, reproducibility, ruggedness, robustness etc., should also possess the ability to be used in conjunction with techniques common to liposomal adjuvants. Techniques, which ensure minimum interference from the other components of the formulation, include methods like Bligh-Dyer two-phase extraction, dialysis, Sep-Pak minicolumn extraction or ultrasonic disruption (New, 1990). The methods used should be stability indicating which would, when used, draw attention to any potential incompatibility between the various components of the liposomes.

3.2 EXPERIMENTAL

3.2.1 Drugs

Methotrexate was gifted by Sun Pharma Advanced Research Centre, Vadodara and Tamoxifen citrate and Cytarabine were gifted by Dabur Research Foundation, Ghaziabad.

3.2.2 Reagents

Egg Phosphatidyl choline was purchased from Sigma Chemical Co., St.Louis, M.O.; Cholesterol, ferric chloride hexahydrate, ammonium thiocyanate, sodium nitrate, picric acid, sodium chloride, anhydrous sodium sulphate, glacial acetic acid (aldehyde free), chloroform, methanol, concentrated hydrochloric acid and sodium hydroxide (analytical reagent grade) were purchased from S.D.fine chemicals, Boisar, Thane. Potassium dihydrogen phosphate and disodium hydrogen phosphate (ExcelR grade) were purchased from Qualigens fine chemicals, Mumbai.

3.2.3 Apparatus

Cyclomixer and tabletop centrifuge (Remi Equipments, Mumbai), electrically heated thermostatically controlled water bath (Superfit Equipments, Mumbai), Vibronics Ultrasonic cleaner 120W (Vibronics Co. Pvt. Ltd., Mumbai), Shimadzu UV-1601 UV-Visible spectrophotometer (Shimadzu Corporation, Japan).