CHAPTER 1.
INTRODUCTION AND LITERATURE REVIEW

Contents

List of Figure

List of Table

INTRODUCTION

In recent years lipid-based carriers, such as liposomes, have successfully encapsulated chemotherapeutic agents ameliorating some toxicity issues, while enhancing the overall therapeutic activity in cancer patients. The goal of this thesis was to design, characterize and optimize liposomal formulations for anticancer agent such as gemcitabine hydrochloride.

1.1. Cancer

Worldwide, cancer is one of the leading causes of mortality and cancer rates are set to increase at alarming rate globally. According to the World Cancer Report, cancer rates could further increase by 50% to 15 million new cases in the year 2020. The most prevalent types of cancer are prostate cancer and breast cancer in men and women, respectively. In addition, Lung cancer is the most frequent cause of death for both sexes. As evidenced by the high incidence and mortality rates, novel treatments strategies for this formidable disease are warranted. Canceris a disease characterized by uncontrollable, irreversible, independent, autonomous, uncoordinated and relatively unlimited and abnormal over growth of tissues **(Figure 1.1)**. Cancer spreads by invasion to the surrounding tissues and by metastasis to distant sites.

Tumors can be of two major types:

- **Benign tumors are not cancer:** generally slow growing expansive masses often with a “Pushing margin” and enclosed within a fibrous capsule
- **Malignant tumors are cancer:** usually rapidly growing, invading local tissue and spreading to distant sites.

In all the types of cancer one of most leading cancer is Lung cancer which is a commonest cancer caused due to tobacco smoke, affects 1.5 million people worldwide annually, with 80% mortality within a year of diagnosis. Lung cancer is currently treated with intravenous administration of chemotherapeutic agents but is non-selective as it cannot differentiate between host cells and cancer cells leading to normal cell toxicity. Further, the diagnostic tools available currently can inadequately detect the tumors and hence render the condition dejected. This provides impetus to pursue the research for effectively treating the lung cancer. Lung cancer is the most common cancer in developed and developing nations like India. India faces about 10% of the world lung cancer incidents. The most common etiological factor for the cause of lung cancer is smoking, which is on the rise in India. Against this backdrop, the proposed project will significantly impact effective treatment of lung cancer.

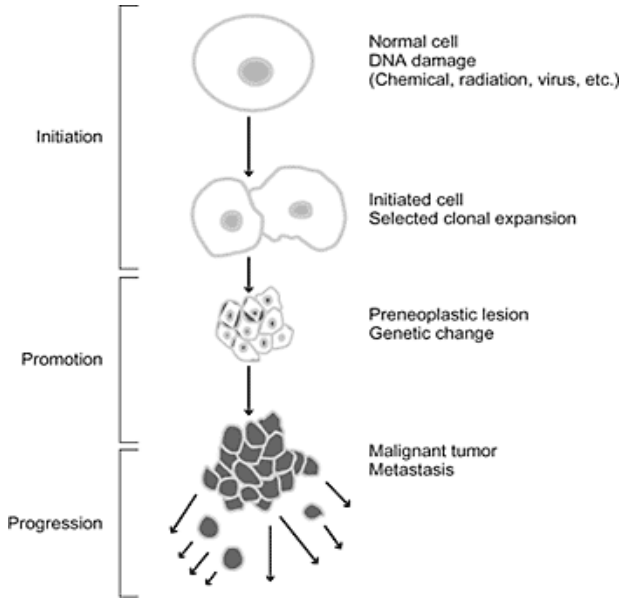


FIGURE 1 1 Development of cancer

Types of Lung Cancer

Lung cancers are classified according to histological type. This classification has important implications for clinical management and prognosis of the disease. The vast majority of lung cancers are carcinomas—malignancies that arise from epithelial cells. The two most prevalent histological types of lung carcinoma, categorized by the size and appearance of the malignant cells seen by a histopathologist under a microscope: non-small cell and small-cell lung carcinoma. The non-small cell lung carcinomas are grouped together because their prognosis and management are similar. There are three main sub-types: squamous cell lung carcinoma, adenocarcinoma, and large cell lung carcinoma. Accounting for 25% of lung cancers, squamous cell lung carcinoma usually starts near a central bronchus. A hollow cavity and associated necrosis are commonly found at the center of the tumor. Well-differentiated squamous cell lung cancers often grow more slowly than other cancer types. Adenocarcinoma accounts for 40% of non-small cell lung cancers. It usually originates in peripheral lung tissue. Most cases of adenocarcinoma are associated with smoking; however, among people who have never smoked ("never-smokers"), adenocarcinoma is the most common form of lung cancer. A subtype of adenocarcinoma, the bronchioloalveolar carcinoma, is more common in female never-smokers, and may have different responses to treatment.

Small cell lung carcinoma is less common. It was formerly referred to as "oat cell" carcinoma. Most cases arise in the larger airways (primary and secondary bronchi) and grow rapidly, becoming quite large. The small cells contain dense neurosecretory granules (vesicles containing neuroendocrine hormones), which give this tumor an

endocrine/paraneoplasticsyndrome association. While initially more sensitive to chemotherapy and radiation, it is often metastatic at presentation, and ultimately carries a worse prognosis. Small cell lung cancers have long been dichotomously staged into limited and extensive stage disease. This type of lung cancer is strongly associated with smoking.

1.1.1. Therapeutic Interventions

Most often cancer is treated with a myriad of therapeutic interventions including surgery, ionizing radiation and chemotherapy, the combination of which depends on the type and stage of disease. The goal of treatment is to reduce local tumor burden and eliminate all malignant cells. Surgery and radiation therapy are effective for local or contained disease, and is often curative at early stages of disease, but not all types of cancer can be treated by these methods.

Treatment of NSCLC generally requires partial surgery along with radiotherapy and chemotherapy. Both, radio and chemotherapy cause painful toxicity to the patient thereby requiring premature end of the therapy leaving the treatment halfway even though the tumor cells are successfully killed. The reason for this is that all cytotoxic drugs kill normal cells as well as cancerous cells resulting in severe side effects. Furthermore, because of the blood circulation in the body, only a small fraction of the drug actually reaches the target tumor tissue and most of the drug acts on normal tissues or is rapidly eliminated. Therefore, to obtain a therapeutic effect, a relatively high dose of drug must be administered and usual drug formulations are used in a balance between killing the tumor (efficacy) and killing the patient by causing lower toxicity to normal organs. The use of combination chemotherapy has been used in current treatment of NSCLC and is associated with a response rate of over 50% and a median survival of 8-12 months [1]. This chemotherapy comprises only symptomatic management and partial cure. The major problems associated with chemotherapeutic agents are inadequate tumor specificity, narrow therapeutic indices and emergence of resistant cancer cells. Extensive side effects due chemotherapeutic anticancer drugs on normal dividing cells as hair follicles, germ cells and hematopoietic cells are well known resulting in dose limiting toxicity and incomplete therapy.

The continual progress in survival outcomes and advancement in treatments have strongly paralleled the acquired scientific knowledge in tumor biology; and this is highlighted by the development of combination chemotherapy regimens that take into consideration mechanisms of drug action and developmental resistance [4]. Common classes of chemotherapeutic agents are listed in **Table 1.1.** In addition, recent advances in research using liposomal and nanoparticulate systems in treatment of cancer have led to development of many products for more efficacious means of treating the lung cancer. Development of

liposomal doxorubicin (DOXIL), daunorubicin, nanoparticulate paclitaxel (Abraxane), monoclonal antibody based Herceptin formulation have been the most successful and effective formulations or treatment of solid tumor and metastatic cancer with higher selectivity at site of cancer and lower systemic side effects. However, direct targeting of these drugs to the lung tissue without systemic side-effects has still been a mystery without any suitable treatment solution.

TABLE 1 1 Some chemotherapeutic agents used in the treatment of cancer

Drug Class	**Chemical Name**	**Mechanism(s) of Action**
Antitumor Antibiotics	Anthracyclines	Stabilize topoisomerase II-DNA cleavable complexes, DNA intercalation
	Actinomycin D	Inhibits DNA-directed RNA synthesis
Plant Alkaloids	Taxanes	Interfere with microtubules
	Vinca alkaloids	Bind tubulin, disrupt mitotic spindle formation
Nucleoside Analogues	Gemcitabine/Cytarabine/ Fludarabine	Inhibits DNA synthesis
Antimetabolites	5-Fluorouracil	Inhibits thymidylate synthase
	Methotrexate	Competitive inhibitor of DHFR, Inhibits DNA synthesis
Alkylating Agents	Cyclophosphamide	Intra-strand DNA crosslinker
	Temozolomide	Methylates guanine residues in DNA
Camptothecin Derivatives	Chlorambucil/Melphalan/Topo tecan	Intrastrand DNA crosslinker Stabilizes topoisomerase I-DNA complex
Epipodophyllot-oxins	Etoposide	Stabilizes topoisomerase II-DNA cleavable complex
Platinum-Based Compounds	Cisplatin / Carboplatin	Intra-strand DNA crosslinker

1.2. Drug Delivery

Antineoplastic agents used in the treatment of lung cancer have often associated with number of severe toxicities such as bone marrow depression results in granulocytopenia, agranulocytosis, throbocytopenia, and aplastic anaemia, lymphocytopenia and inhibition of lymphocyte function results in suppression of host immunity and etc.

- Currently Campothecin, Paclitaxil, Carboplatin, Cisplatin, Docetaxel, Topotecan, Etoposide, Gemcitabine are the most widely used anticancer agents in treatment of lung cancer with their known reported toxicities.
- The medications are available as injections for systemic use and result in hazardous side effects due to their non-specificity on the dividing cells in the body.
- Intracellular transport of different biologically active molecules is one of the key problems in drug delivery in general. Currently the anticancer agents have poor intracellular concentration in the cancer cells.

In view of the light of above factsassociated with many of the available chemotherapeutic agents, drug delivery systems have been used as one of the promising strategy to improve pharmacological effects of these drugs. Amongst the many delivery systems designed for intravenous use such as micelles, lipid emulsions, liposomes, polymer-drug conjugates, polymer microspheres, nanoparticles, niosomes, and osmotic pumps, liposome technology has been successful with several products currently available for human use. These liposomal products encapsulate various drugs including the antifungal agent amphotericin B, and the anti-cancer agents like daunorubicin, doxorubicin, and cytarabine and have been summarized in **Table 1.2.** Based on the success and versatility of lipid-based carriers for delivery of anti-cancer drugs, liposomes were utilized for the studies performed within the thesis for the delivery of anticancer drug e.ggemcitabine hydrochloride. Here, a brief review of this technology is provided with the aim of establishing a general understanding of the field as it relates to the research included in this thesis.

TABLE 1 2 List of FDA approved liposomal agents

Product Name	Therapeutic Agent	FDA	Disease Treated	Company Name
Ambelcet®	AmphotericinB	1995/ 1996	Systemic fungal Infections	Enzon
Ambisome®	AmphotericinB	1997	Systemic fungal Infections	Gilead Sciences
Amphotec®	AmphotericinB	1997	Systemic fungal Infections	Alza Corp.
DaunoXome®	Daunorubicin	1996	AIDS-related	Gilead Science
DepoCyt®	Cytarabine	1999	Lymphomatoous Meningitis	SkyePharma / Enzon
Doxil® / Caelyx®	Doxorubicin	1995/ 1999	AIDS-related Kaposi's sarcoma/ovarian and breast cancer	Alza Corp. (Sequus) / Schering-Plough
Myocet®	Doxorubicin	2000	Metastatic breastcancer	Elan Corp
Visudyne®	Verteporfm	2000	Age-relatedmacular Degeneration	QLT / Novartis Opthalmics

1.2.1 Liposomes

Liposomes are microscopic or sub-microscopic bilayer vesicles with size ranging from 10 nm to 20µm. They are composed of one or several bilayers enclosing aqueous compartments. When phospholipids are hydrated, they spontaneously form lipid spheres (liposomes) enclosing the aqueous medium and the solute. Kulkarni *et al.* (1995)[2] have explained the mechanism of liposome formation upon hydration of phospholipids. Phospholipids are amphipathic molecules containing a hydrophobic tail and a hydrophilic or polar head. Because of this amphipathic nature, phospholipids form closed bilayers in the presence of water. When phospholipids are exposed to water the fatty acid tails align towards each other, excluding water from this hydrophobic domain in that process. Conversely, the polar head groups orient themselves towards the bulk aqueous phase, leading to a bilayer configuration. The large free energy difference between the aqueous and

the hydrophobic environment promotes the formation of bilayer structures in order to achieve the lowest free energy level. Bilayer structures do not exist in the absence of water, because it is the water that provides the driving force for lipid molecules to assume a bilayer configuration [2-4]. Depending on the number of bilayers formed and diameter of the vesicles, liposomes are broadly classified into small unilamellar vesicles (SUVs; single bilayer, size 10 to 100 nm), large unilamellar vesicles (LUVs; single bilayer, size 100 to 1000 nm), and multilamellar vesicles (MLVs; several bilayers, size 100 nm to 20 µm). Liposomes have a wide range of therapeutic application ranging from topical cosmetics to the intracellular delivery of genetic materials. An array of compounds can be encapsulated in liposomes, including small molecules, proteins, and nucleic acids [5-9]. The therapeutic applications of liposomes have been extensively reviewed in the literature. A major advantage of liposome carriers is their ability to alter the pharmacokinetics of the free drug. The particulate nature of the liposomes causes them to be distributed within the body in a pattern significantly different from that of the free drug. Drugs with varying lipophilicities can be encapsulated in liposomes, either in the phospholipid bilayer, in the entrapped aqueous volume or at the bilayer interface.

1.2.1.1 Methods of Preparation of Liposomes.

Numerous procedures have been developed to prepare liposomes. There are at least fourteen major published methods for making liposomes. The seven, most commonly employed methods are, Lipid film hydration method [10], Ethanol injection method [11] Ether infusion method [12], Detergent dialysis method [13], French press method [14], Rehydration-dehydration techniques [15] and Reverse phase evaporation method [16].

1.2.1.2 Characterization of Liposomes

The behavior of liposomes in both physical and biological systems is determined to a large extent by factors such as physical size, chemical composition, quantity of entrapped solutes etc. Hence, liposomes are characterized with respect to the following parameters:

a. Size and Size Distribution

There are number of methods reported in the literature to determine size and its distribution of the vesicles [10, 17]. The most commonly used ones are light microscopy preferably using electron microscope, laser light scattering or cryoelectron microscopy.

b. Lamellarity

The lamellarity, the average number of bilayers present in liposomes, can be determined either by 31P-NMR spectroscopy or freeze fracture electron microscopy.

c. Determination of Entrapment Efficiency

The quantity of material entrapped inside liposomes can be determined more commonly by mini column centrifugation method, protamine aggregation method, dialysis technique or by gel chromatography.

1.2.2 Liposomes as Drug Carrier

The use of liposomes as drug delivery agents has evolved from a line of research originating over 40 years ago, based on the ability of these unilamellar vesicles to entrap material in an aqueous compartment [10]. It was then known that most amphipathic membrane lipids form multilamellar vesicles (MLV) consisting of concentric bilayers when they are dispersed in aqueous media. MLV are relatively large (micron) sized structures, however they can be extruded through 100 nm pore size polycarbonate filters to produce unilamellar vesicles with a homogeneous size distribution [18-21]. Typically, the resulting liposomes are 100 nm in diameter and each particle contains numerous lipid molecules [4]. Liposomes have been widely used as models of biological membranes to study membrane permeability and transport across the bilayer [22]. In addition to their utility as model membranes, drugs may be encapsulated within their interior aqueous compartment. The ability of liposomes to deliver drugs preferentially to disease sites, such as solid tumors, can result in considerable improvements in efficacy; therefore, liposomes are widely studied for use in therapeutic applications **(Figure 1.2).**

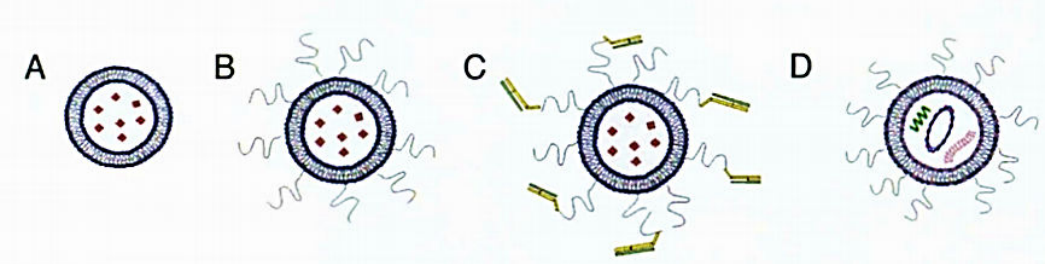


FIGURE 1 2 Types of liposomes employed for drug delivery

A) Liposomes encapsulating drugs **B)** "Stealth®" liposomes with hydrophilic surface coating (usually polyethylene glycol (PEG)) that increases their circulatory time *in vivo* **C)** Surface-modified targeted liposomes with a targeting ligand to increase specificity in target cells **D)** Liposomes encapsulating biological molecules such a plasmid, siRNA.

The first preparation of a liposome with entrapped solute was characterized in 1965 by A. D. Bangham in Cambridge, UK [10]. The evolution of liposomes as drug delivery systems was subsequently accelerated in the 1980,s by the development of techniques to rapidly generate well defined liposomal systems and to efficiently load them with drugs [19, 23, 24]. The

observation that long-circulating liposomespreferentially accumulate at sites of disease, including sites of infection, inflammation and tumors, due to the leaky nature of the vasculature in these regions [25-27], gave a solid rationale for delivering drugs in liposomal systems. Liposomes have several features that have contributed to their success as a drug delivery system. Encapsulation within the aqueous cavity of an liposomescan enhance the *in vivo* activity of drugs by protecting them from breakdown in the body and can reduce the toxic effects of drugs, such as anti-cancer drugs, by reducing delivery to sensitive tissue [28]. In addition, liposomes are biocompatible, so that they may be used in vivo, and their physical properties can be readily manipulated. Liposome production and drug-loading techniques have been optimized and standardized such that liposomes can be manufactured on a large scale. Liposomal technology also offers flexibility such that the lipid composition may be varied to match the desired characteristics of the drug that is being delivered, and can be used to optimize the halftime of release of the drug from the liposomes. These features are summarized by Maurer *et al.* [6] and more recently by Allen and Martin, [29] noting examples where liposomal encapsulation gave the associated drug long circulation lifetimes, enhanced accumulation at disease sites and increased efficacy for a variety of drugs. The range of material that may be encapsulated within the liposomes. Liposomes have been used for the encapsulation of small molecule drugs including anti-cancer [30] and antifungal drugs [31] and nucleic acid-based drugs such as plasmids for gene therapy [32], immunogenic DNA oligonucleotides [33] and siRNA oligonucleotides [34] **(Figure 1.2).** A review of the current status of modern drug delivery systems and their *in vivo* application has been published, noting six Liposomal formulations that are clinically approved drugs and many others in advanced clinical trials [35]. The application of LIPOSOMESs as carriers of nucleic acids has required the development of a sophisticated class of nanoparticles for *in vivo* Liposomal delivery. These particles must entrap high amounts of nucleic acid [36] survive for prolonged amounts of time in the circulatory system [37] and release their contents in the cytoplasm of cells that internalize the particle [38, 39].

1.2.3 Liposomes for Targeted Drug Therapy

The concept of site specific drug delivery for treatment of localized disease in the body to improve therapeutic index of the drug is considered as perennial challenge to the formulator in modern formulation design. Constant efforts have been pursued in designing such an ideal drug delivery system which can effectively overcome dose related toxicity and adverse side effects and thus improve patient compliance [40]. One such area which has attracted ever growing attention of pharmaceutical scientist and has shown tremendous potential and promise is colloidal drug carrier system [41]. The idea of drug carrier with targeted

specificity has fascinated scientists for number of years and in the last decade successful efforts have been made to achieve this goal. The ultimate form of targeted drug delivery system should be realization of Paul Ehrlichs “magic bullet concept” [42] which documents the delivery of drug exclusively to a preselected targeted cell type.

Amongst all targeted drug delivery systems, Liposomes are recently gaining popularity because of their biological inert nature, freedom from antigenic, pyrogenic or allergic reaction and their enhanced stability [43]. Vastly improved technology in terms of drug capture, vesicle stability on storage, scale-up production and the design of formulations for special tasks has facilitated the application of a wide range of drugs in the treatment and prevention of diseases in experimental animals and clinically.Liposomes are micro-particulate or colloidal carriers which form spontaneously when certain lipids are hydrated in aqueous media [44]. Liposomes are composed of relatively biocompatible and biodegradable material and they consists of aqueous volume trapped by one or more bilayers of natural or synthetic lipids. Generally hydrophobic molecules are incorporated into the lipid bilayers whereas hydrophilic compounds are entrapped in the internal aqueous volume.

The delivery of liposomes at the appropriate site, however, is still not achieved. For this purpose, both active targeting and passive targeting are considered. Conventional liposomes, however, tend to be trapped by the reticuloendothelial system (RES) such as liver and spleen before encountering the target. On the contrary, passive targeting, especially targeting to tumor tissues, could be achieved by reducing the RES trapping, since the vasculature in the tumor tissues is leaky enough to extravasate liposomes and circulating liposomes may accumulate passively in tumor tissues **(Figure1.3)**[45]. The development of liposomes containing lipid derivatives of PEG or saturated phospholipids such as DSPC with cholesterol has made targeted liposomal therapy more feasible by reducing the uptake by the RES system and there by prolonging the circulation time.Particularly, PEG is useful because of its ease of preparation, relatively low cost, controllability of molecular weight and linking ability to lipids or peptide including RGD peptide by a variety of methods. The presence of PEG reduces binding of serum protein, i.e. opsonins marking the liposome for clearance by macrophages.

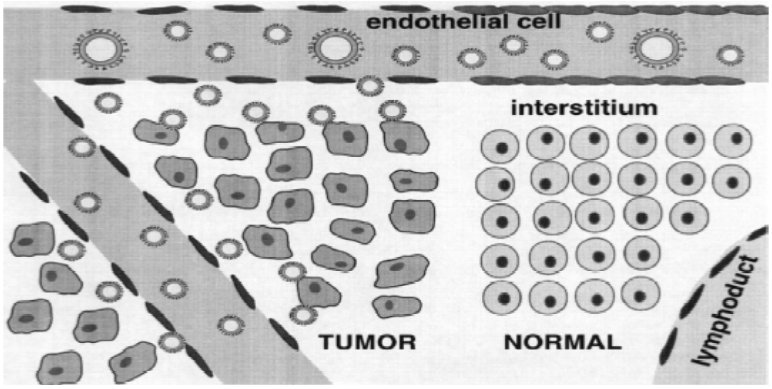


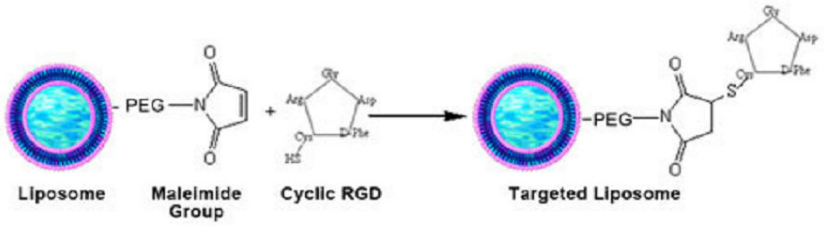
FIGURE 1 3 Passive accumulation of liposomes at tumour through leaky tumour endothelium

Active targeting of liposomes to tumor cells is generally attempted by conjugating ligands to the liposomal surface which allow a specific interaction with the tumor cells. Several types of ligands have been used for this purpose, including antibodies or antibody fragments, vitamins, glycoproteins, peptides (RGD-sequences), and oligonucleotide aptamers. Among the different approaches of active targeting, RGD grafted liposomes using RGD motif as a targeting ligand and a lipid vesicle as a carrier for both hydrophilic and hydrophobic drugs, is a fascinating prospect in cancer therapy **(Figure1.4).**

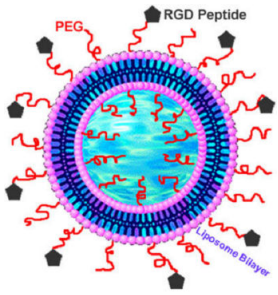
Targeting using small peptides like RGD has certain advantages over the use of conventional protein macromolecules. These include ease of preparation, lower antigenicity, and increased stability [46]. RGD peptides have reportedly been used to deliver cytotoxic molecules to the tumor vasculature [47-50]. Tumor vasculature is a suitable target for targeted cancer therapy because it is composed of nonmalignant endothelial cells that are genetically stable and therefore unlikely to mutate into drug-resistant variants. In addition these cells are more accessible to drugs and have an intrinsic amplification mechanism. It has been estimated that elimination of a single endothelial cell can inhibit the growth of 100 tumor cells. Tumor vasculature undergoes continuous angiogenesis and express molecular markers that characterize these vessels. These markers in angiogenic endothelium include certain receptors for vascular growth factors, such as the receptor for vascular endothelial growth factor, and the $\alpha_v\beta_3$ integrin [51, 52]. Preventing the $\alpha_v\beta_3$ integrin from binding to their ligands triggers apoptosis in the endothelial cells of newly formed blood vessels. Peptides that mimic ligands of these integrins and anti-integrin antibodies are capable of inhibiting their ligand-binding. In the last decade, many molecules specifically expressed on to the tumor endothelium cells have been proposed as target molecules for tumor vasculature targeting. The $\alpha_v\beta_3$ integrins are overexpressed on actively proliferating endothelium on and around tumor tissue [53, 54]and identified as a promising determinant on angiogenic endothelium. It can interact with various RGD (Arg-Gly-Asp) sequence containing

extracellular matrix components. Synthetic cyclic RGD peptides have been shown to bind selectively to $\alpha_v\beta_3$ integrin inhibiting the angiogenesis [55].

The process of targeted drug delivery with ligand targeted liposomes can be roughly divided into two phases: the transport phase, in which the ligand targeted liposomes travel from the site of administration (often i.v. administration) to the target cells, and the effector phase that includes the specific binding of ligand targeted liposomes to the target cells and the subsequent delivery of entrapped drugs [56]. Ligand targeted liposomes for the treatment of tumor should satisfy a number of requirements aimed at maximum targeting effect of ligand targeted liposomes administered systemically in the bloodstream. The blood clearance of ligand targeted liposomes must be minimized in comparison with rate of extravasation in the tumor. Ligand targeted liposomes must allow efficient loading and retention of a selected anticancer drug. And finally, the drug and ligand incorporation must be stable enough to permit liposomal entry into the tumor tissue without the loss of either of these agents [57].



(a)



(b)

FIGURE 1 4 Schematic representation (a) coupling reaction between functionalized PEG chain and cyclic RGD peptide (b) targeted liposome delivery system

RGD peptides coupled to the distal end functionalized PEG chain in the liposome[58]

Antineoplastic agents used in the treatment of lung cancer, solid tumor, testicular cancer, breast cancer, several types of leukemia, lymphoma and etc. have often associated with number of severe toxicities such as bone marrow depression results in granulocytopenia,

agranulocytosis, throbocytopenia, and aplastic anaemia, lymphocytopenia and inhibition of lymphocyte function results in suppression of host immunity [59].

Cancer chemotherapy is generally accompanied by side effects. If an anticancer drug could deliver only the right site in the right concentration at the right time, cancer could be cured without side effects. For such delivering system, liposomal formulation is thought to be useful since liposomes are essentially non-toxic and biodegradable, their size, components, and modifications with various molecules are easily controlled, and they could deliver the large amount of either hydrophilic or hydrophobic agents [45].

1.2.4 Critical Parameters in Development of Targeted Liposomal Delivery System

A comprehensive review of these critical engineering considerations and varying perspectives were presented recently [60-62]. In general, important critical design characteristics include: 1) liposome formulation stability while in the general circulation; 2) enablement of long circulating properties for the carrier; 3) proper linkage of the targeting ligand to the liposome carrier; 4) selection of a stable, specific, and non-immunogenic targeting ligand; and, 5) the choice, as well as compatibility, of the active agent for liposome encapsulation. Other important considerations include chemical conjugation strategies and the methods used for assembly of the targeted liposomal construct. Each of these design considerations has a critical role in determining the success of a targeted liposome delivery system.

1.2.4.1 Liposome Formulation

Use of long chain acyl chains such as DSPC or HSPC and the presence of an optimum level of cholesterol in the membrane was shown to minimize membrane defects and reduce drug leakage[60] . In addition to improving membrane rigidity, cholesterol also dries the lipid/water interface, thereby enhancing close contact and vander Waals interactions between adjacent lipid molecules [63]. This drying effect contributes to the increased chemical stability of the liposomal membrane against peroxidation and acyl ester hydrolysis. Presence of a-tocopherol in the formulation was shown to reduce auto-oxidation of lipid components and prolong the shelf lives of liposomes [64, 65]. Incorporation of PEG conjugated lipids in the formulation, in addition to providing steric stabilization, was shown to induce a drying effect at the lipid/water interface [66] especially when present in an appropriate levels. The mechanism by which PEG induces dehydration of the head group region of lipids is related to the fact that PEG, chemically attached to the lipid head group, undergoes steric exclusion from the liposome surface in a similar way to free PEG. This results in greater density of the grafted PEG further from the surface. Thus, the local

concentration gradient of PEG chains from the liposome surface leads to an osmotic imbalance, changes in thermodynamic properties and hydration of the lipids [67]. Depending on the level of PEG-conjugated lipid in the bilayer, different configurations were proposed by these authors. At concentrations less than 4 mol % PEG chains were shown to be in non-overlapped mushroom configuration. At higher concentrations (>10 mol %) PEG chains will be in highly overlapped brush configuration. Due to repulsion of PEG chains, at these high concentrations of PEG lipids, weakening of the bilayer packing was observed.

1.2.4.2 Ligand Selection

Selection of an appropriate ligand for targeted liposome carrier depends on several factors including specificity, lack of immunogenicity and ready access to the target receptor. A wide array of targeting ligands has been utilized in the construction of targeted liposomes. These include antibodies or antibody fragments[62, 68], low molecular weight ligands [69], protein ligands such as transferrin , sugars [70], peptides [71, 72], and RNA aptamers[46, 73]. Naturally occurring ligands such as folic acid and transferrin, while non-immunogenic, may be relatively non-specific and may cause considerable toxicities [62]. Low molecular weight ligands, such a RGD peptide, offer the advantage of increased chemical stability, ease of manufacturing and simple targeted formulation assembly when compared to larger biomolecules such as antibodies. Peptides are commonly incorporated at 300- to 1000-molecules per liposome, compared to 20- to 40-copies of carefully optimized antibody fragments [50]. However, a very high ligand density can also lead to non-specific interactions and result in an increased clearance [7].

1.2.4.3 Conjugation Methodologies

The conjugation methodologies for attaching the targeting ligand to the liposome surface are important to the stability and reactivity of the targeted formulation, as well as the scalability of the targeted liposomal product. A variety of functionalized lipids are available for attaching ligands. A lipophilic moiety merged within the lipid portion of the bilayer typically serves as an anchor for ligand attachment. The linkage between the anchor and the ligand should be stable, non-immunogenic, and should not affect the reactivity of the ligand or stability of the liposomal drug. The anchor bears a functional group that forms a covalent or strong non-covalent bond with the ligand. A wide variety of covalent and non-covalent chemical linkages have been used to attach ligands to the surface of liposomes [71, 74].Hansen, C.B *et al.* (1995) [75] have evaluated different conjugation techniques for attaching antibodies to sterically stabilized liposomes. Among these, conjugation of ligands through a maleimide functional groups has been shown to be effective. The reaction between maleimide and thiol groups is rapid and proceeds close to completion [76]. The resulting thio-ether bond is stable

under physiological conditions. Hence, the ligand will not dissociate from the liposome in the systemic circulation physiologically. Unstable bonds, such as esters and disulfides, as well as lipid anchors of insufficient affinity to bilayers have been shown to be suboptimal if the targeting ligand is to remain intact on the liposome surface while in the circulation [74, 76, 77]. A polymer spacer between lipid bilayer and ligand is also shown to be important in retaining access of the ligand to the receptor. Using anti-HER2 Fab' fragments, Kirpotin, *et al.* (1997) [68] have demonstrated strong binding and internalization of targeted liposomes when Fab' was conjugated to the distal end of PEG, as opposed to directly to the bilayer membrane surface. Similar improvements in targetability were reported upon ligand coupling through a polymer linker [78, 79]. The length of the spacer was also found to influence target recognition and binding in liposomes that already contained PEG-derivatized lipids [80]. A novel method of preparing targeted liposomes has been reported by Ishida *et al*. [81]. The method, called post-insertion technique, involves incorporation of ligand lipid conjugates from micelles into preformed liposomes. Water soluble, micellar conjugates of the ligand and an amphiphilic lipid anchor, co-incubated with preformed liposomes, spontaneously insert themselves into the liposome bilayers without loss of the liposome integrity. This post-insertion technique, also called the micellar insertion technique, has been shown to be a rapid and relatively simple method for transforming non-targeted liposomal drugs into ligand-targeted liposomal drugs [7, 60]. The targeted liposomes made by this new post-insertion technique have shown comparable in vitro binding, in vivo pharmacokinetic profiles and therapeutic efficacy to targeted liposomes made by conventional coupling methods [82]. However, this post-insertion technique is usually performed at elevated temperatures (55 to 60°C) to accommodate lipid bilayers with higher melting temperatures. Therefore, the denaturation of protein ligands under these conditions is a concern.

1.2.5 Drug Loading in Liposomes

In the early 1970s, it was proposed that liposomes could potentially hold entrappedpharmaceuticals for treatment of diseases. Within this thesis two anti-cancer drugs, were encapsulated in liposomes.The advantages and disadvantagesof different loading methods used to encapsulate these drugs will be discussed below.

1.2.5.1. Passive Loading

Hydrophobic drugs (e.g., taxol and amphotericin B) or water soluble drugs (e.g., cytarabine and gemcitabine) may be passively entrapped within liposomes during hydration of lipid and liposome formation. Encapsulation efficiencies up to 100% may be achieved for hydrophobic drugs when exhibiting favorable drug-lipid interactions and drug solubility.

Passive loading of water soluble drugs is typically very low (<30%) and is dependent on the trapped volume of the liposome and liposomal lipid concentration. If drugs have to be encapsulated using passive loading methods it is more difficult to control parameters such as drug-to-lipid ratios and trapping efficiency. In the case of cytotoxic drugs, passive trapping also means that careful methods must be in place during liposome preparation and following preparation to remove the unencapsulated drug.

1.2.5.2. Remote Loading

Drugs, such as anthracycline antibiotics, can alternatively be loaded into preformed liposomes containing a pH gradient (pH 4.0 inside, pH 7.4 outside). This method is limited to drugs having an ionizable amine function, and results in encapsulation efficiencies of less than 98%. For anthracyclines, the encapsulation efficiencies are much higher than predicted by the Henderson-Hasselbach equation, and may be explained by the formation of drug micro precipitates and/or drug association with or partitioning into the lipid membrane. Drug retention by this method is dependent on liposome composition including surface charge, phospholipid acyl chain length, cholesterol content, internal buffering capacity, drug-to-lipid ratio, pH gradient, and liposome size parameters that can all be independently altered. Other active loading methodologies include the ammonium sulfate gradient methodand metal complexation. The latter is of potential interest since drug loading may not be dependent on maintenance of an established pH gradient.

1.2.6 Drugs Suitable for Liposome Formulations

Not all drugs are suitable for delivery via liposome carriers [83]. The drug must be efficiently loaded into the liposomal carrier. The drug must be compatible with the carrier, it must be stably transported in the circulation but still released at the tumor site. Highly hydrophobic drugs tend to associate mainly with the bilayer compartment of the liposome: This leads to lower entrapment stability due to faster redistribution of the drug to plasma components. With this class of drugs, liposomes may be used simply as the means to formulate them for intravenous administration rather than using liposome encapsulation to achieve enhanced tumor delivery. For example, Sharma *et al.* (1997) [44] has formulated paclitaxel into liposomes. But it is equally effective when formulated as microemulsion [84]. As suggested by Drummond, *et al.* (1999) [83] amphipathic drugs are most suitable for liposomal carriers. These authors also indicated that liposome formulations should be optimized for each drug. There is no universal liposome formulation to suite for all classes of drugs. Factors Influencing Encapsulation of Drugs in Liposomes Kulkarni *et al.* (1995) [2] have reviewed various factors influencing encapsulation of drugs in liposomes. These include:

1. Characteristics of liposomes

    A. Type of vesicle

    B. Selection of phospholipids

    C. Presence of charge

    D. Bilayer rigidity

2. Characteristics of drugs to be encapsulated

3. Method of preparation

4. Addition of ion-pairing and complexing agents

1.2.6.1 Type of vesicle

MLVs contain more than one lipid bilayer and have a low aqueous encapsulation volume. Therefore, they are suitable for the encapsulation of bilayer-interacting hydrophobic drugs, and less appropriate for hydrophilic drugs. For hydrophilic drugs LUVs are preferred because of their large entrapped aqueous volume [19, 85]. However, encapsulation of highly hydrophobic drugs is affected to a lesser extent by vesicle type, since they remain entrapped within the phospholipid bilayers [2]. Selection of phospholipids although a variety of phospholipids (PLs) are available for the preparation of liposomes, the choice of PL is often limited to the family of the phosphatidylcholines (PC) and phosphatidylglycerols (PG), mainly because of toxicological considerations, the availability of pure compounds, and the cost [2]. PLs undergo a characteristic gel to liquid crystalline phase transition at a temperature range known as phase transition temperature (Tm). This Tm is a function of the acyl chains, branched chains or those carrying bulky side groups. At temperatures below Tm, bilayers are considered to be in a solid (gel) state, and at temperatures above Tm, they are in a fluid state. Length of alkyl chains and degree of saturation affect the encapsulation of lipid soluble drugs and the stability of liposomes. Ma *et al.* (1991) [86] have reported that increasing the alkyl chain length of PL increased partitioning of hydrophobic drugs into the bilayers. The partitioning was also influenced by the fluidity of the membrane structures; due to the high surface density of the bilayer at low temperatures, very small amounts of drug were found to be encapsulated. An increase in temperature improved fluidity of the bilayers and improved drug encapsulation. In many cases, liposomes are prepared rom mixtures of different PLs. When selecting PLs precautions should be taken since non-ideal mixing may result in phase separation, if the chain lengths differ by four or more methylene groups [19-21]. Presence of charge on bilayer anionic lipids like phosphatidic acid, or phosphatidyl serine have been used to impart a negative charge. Instead of adding charged PL, charge on

the bilayers can also be induced by incorporation of stearyl amine (for positive charge), or dicetyl phosphate (for negative charge). In such types of liposomes, due to the presence of a charged interface, there is an electrostatic repulsion between adjacent bilayers; this leads to a rise in the volume of the internal aqueous compartment of MLVs [2]. Presence of charge may also prevent aggregation of liposomes [87, 88]. Effect of bilayer rigidity Liposomes prepared with only PL are not sufficiently rigid. They are permeable and often leak encapsulated drugs during storage. To overcome these problems, cholesterol is often incorporated into the lipid bilayers to impart rigidity. There are conflicting reports in the literature regarding the effect of cholesterol on liposome drug loading. Encapsulation of a hydrophobic, membrane-interacting drug, Adriamycin decreased as the concentration of cholesterol in negatively charged liposomes increased [89]. However, itsreported that partitioning of salbutamol (a hydrophilic drug) was independent of the addition of cholesterol in MLVs. An increase in the encapsulation of sodium cromoglycate in MLVs due to the inclusion of cholesterol has been reported by Taylor *et al.* (1990) Ma *et al.* (1991) [86, 90] observed an increase in the encapsulation of hydrophobic compounds when cholesterol was incorporated into bilayers. Kulkarni *et al.* (1995) [2] observed a slight increase in encapsulation of hydrophobic steroids as the concentration of cholesterol was increased, when the drug input was well below the encapsulation capacity. However, it had an opposite effect when the drug input was equal to or higher than the encapsulation capacity. Therefore, these authors concluded that cholesterol improves encapsulation of hydrophilic drugs by decreasing permeability of bilayers and for hydrophobic drugs it improves encapsulation only if the drug input is less than the encapsulation capacity for that drug.

1.2.6.2 Characteristics of The Drug to be encapsulated

Different types of drugs vary in the mechanism by which they become associated with liposomes. Depending on the interaction between the drug and the liposome bilayers, Talsma and Crommelin (1992) [91] have divided drugs into four classes:

1. Water soluble, non-bilayer interacting drugs;

2. Hydrophobic, bilayer-interacting drugs that are bound inside the hydrophobic region of the bilayer;

3. Drugs in the aqueous domain that can be associated with the bilayer via electrostatic interaction; and,

4. Drugs that are neither water soluble, nor bilayer bound nor bilayer associated.

Since a water soluble, non-bilayer interacting drug remains within the aqueous phase, its encapsulation depends on the entrapped aqueous volume, and therefore on the size and type of liposomes and lipid concentration. Also, the encapsulation of the drug is relatively independent of the nature of phospholipid as long as the membrane provides an adequate permeability barrier. Highly water soluble drugs are entrapped by only passive encapsulation and typically exhibit low encapsulation efficiency. On the other hand, active loading techniques can be used for encapsulation of amphipathic drugs. Encapsulation of bilayer-interacting, hydrophobic drugs largely depends on the total amount of phospholipid, the chain length and other bilayer additives (e.g., cholesterol, tocopherol) present in the bilayer, and the properties (gel or fluid state) of the bilayers. Generally, these drugs tend to be incorporated more efficiently in fluid membranes where the fatty acyl side chains have considerable freedom of movement. Hydrophobic drugs, because of their direct interactions with the liposome membranes, are also likely to change the physical characteristics of liposomes when present in large amounts [92]. Drugs having intermediate hydrophilicity / lipophilicity tend to partition between aqueous and lipid compartments. For many of these drugs, their hydrophilicity depends on the pH of the aqueous medium. Any change in pH causes a change in the aqueous solubility of these drugs and this affects their partitioning. Encapsulation of a tinmesomorphin was found to be higher (about 90%) at pH 5 than at pH 7 (<10%) [93]. For bilayer associated drugs, the encapsulation of the drug depends on the mechanism of interaction. If the drug-liposome interaction is based on electrostatic forces, then, encapsulation is affected by the density of the charge inducing bilayer constituent and the ionic strength of the aqueous medium. For drugs that are covalently bound to the bilayers, the availability of binding sites determines the loading efficiency. Drugs that are neither water soluble, nor bilayer bound or associated exhibit very low encapsulation efficiency and are poor candidates for liposomal formulation [2].

1.2.6.3 Method of Preparation

The process used for preparation of liposomes also influences drug loading. When using the lipid film hydration process, the formation of a thin film of lipid with larger surface area is desirable to facilitate the efficient hydration of the bilayer and to increase drug loading. Use of pear-shaped flasks is therefore preferred because they offer a large surface area [2]. The surface area can further be increased by adding contact masses (e.g., glass beads). Kulkarni *et al.* (1995) [2] have reported 10- to 14- times increased encapsulation of colchicines in MLVs when glass beads were added and a 50 times larger flask was used. The film hydration time, quantity of water used for hydration, and conditions of agitation (speed and temperature) are also important factors for obtaining maximal encapsulation in a

reproducible manner [21]. Amselem *et al.* (1990) [94] studied encapsulation of doxorubicin in liposomes by five different hydration methods. Optimum hydration was obtained when a thin lipidfilm with a large surface area was formed.

1.2.6.4 Addition of Ion-Pairing and Complexing Agents

Encapsulation of polar drugs can also be increased by addition of ion-paring or complex-forming agents [2]. Jay and Digenis (1982) [95] successfully increased the encapsulation of quaternary ammonium compounds by ion-pairing with trichloroacetate. Similarly, Lee *et al.* (1988) [96] improved the encapsulation of isopropamide iodide by using an ion-pairing agent, sodium taurodeoxycholate. The ion-pair complex of isopropamide with sodium taurodeoxycholate was highly hydrophobic and a three times increase in encapsulation was observed.

1.2.7 Drug Release

In addition to the extent of drug loading, encapsulation stability is also important for optimum performance of the liposome formulation [97]. The release rate of the loaded molecule from liposomes was shown to be dependent on the following factors [98]:

1. Temperature;

2. Medium-related properties (medium composition, ionic strength, pH;

3. Liposome related properties (membrane lipid composition, liposome type, which includes number of lamellae, liposome size, physical state of the phospholipid membrane, i.e., liquid-disordered, liquid-ordered, solid-ordered); and,

4. Loaded -molecule related properties (lipophilicity, hydrophilicity, size).

Barenholz (2003)[99] also recently emphasized the importance of controlling the drug release rate from liposome formulations to increase their therapeutic efficacy. For an intravenously administered liposomal drug formulation, only when the drug release (Koff) is slower than the liposome clearance (Kc), will the liposome determine drug pharmacokinetics and biodistribution. When K0ff»Kc, the benefits of use of liposomes for drug delivery will be minimal or none as the performance of the liposomal drug will be similar to that of the "free" drug. This is exemplified by ciprofloxacine delivered via sterically stabilized liposomes [100]. On the other hand, when the Koff is too slow and there is no liposome uptake by the target cells, there will be no therapeutic efficacy even if the loaded liposomes will reach the target very efficiently as the free drug concentration at the target tissue will be too low, as exemplified by sterically stabilized cisplatin liposomes [101]. Therefore, as summarized by Barenholz (2003) [99] worthwhile benefits from liposome

formulations may be achieved only when liposomes with high drug-to-lipid ratio will reach the target site and the drug will be released at a level and rate sufficient to achieve efficacy, or when these liposomes will be taken up by the target cells where drug will be released. Using "leakier" liposomes will result in release of most of the drug either in storage or while in circulation after administration, reaching the target site with drug-poor liposomes. To overcome this problem one has to design a liposomal system that is stable upon storage and while circulating in vivo in the plasma, but release its contents quickly once at the target site [102]. To fulfill this goal, the liposome carrier should be designed to lose at least part of its stability once at the target site. This is the case for Doxil® formulation, where the conditions in the tumor interstitial fluid differ to a large extent from the conditions in the plasma [24, 103]. Factors leading to Doxil® release may include collapse or partial collapse of the ammonium sulfate gradient and/ or the activity of phospholipases which hydrolyze the liposome phospholipids, there by destabilizing the liposome membrane.

1.2.8 Factors Influencing In Vivo Behavior of Liposomes

Several physicochemical parameters of liposome carriers, such as size, surface charge, membrane lipid packing, and steric stabilization, affect their physical and biological performance as described below.

1.2.8.1 Liposome Size

Size is one of the main parameters that determines the in vivo fate of liposomes in terms of both circulation longevity and tumor accumulation. Small liposomes (< 0.1 (am) are opsonized less rapidly and to a lower extent than large liposomes (> 0.1 |am) and therefore the rate liposome uptake by the reticular endothelial system (RES) increases with size of the vesicle [104]. Inclusion of PEG-DSPE in the liposome composition was shown to result in clearance rates that are relatively insensitive to size in the range of 80 to 250 nm [105]. Selective accumulation of liposomes at tumor sites is also a function of their size. Macromolecular size of liposomes prevents them from passing through the 2 nm pores found in the endothelium of blood vessels in most healthy tissues or even the 6 nm pores found in post capillary venules [106]. Discontinuous tumor microvasculature where pore sizes vary between 100 to 780 nm in size [107] enables accumulation of liposomes in these areas.

1.2.8.2 Surface Charge

The nature and density of charge on the liposome surface are important parameters which influence the mechanism and extent of liposome-cell interaction. Both of these parameters can be altered by changing the lipid composition. Lack of surface charge can reduce physical

stability of small unilamellar liposomes by increasing their aggregation. Further, neutral liposomes do not interact significantly with cells and in such cases the drug may mainly enter cells after being released from liposomes extracellularly [44]. On the other hand, high electrostatic surface charge could promote their plasma clearance after systemic administration [108, 109]. Negatively charged liposomes may also release their contents in the circulation and/or extracellular interaction with blood components and tissues. It is reported that the negatively charged liposomes are predominantly taken up by cells through coated-pit endocytosis, while cationic liposomes deliver contents to cells either by fusion with cell membranes or through coated pit endocytosis [44].

1.2.8.3 Bilayer Fluidity

Lipids have a characteristic phase transition temperature (Tm), and they exist in different physical state above and below the Tm. The lipids are in a rigid, well ordered arrangement (solid gel like phase) below the Tm, and in a liquid crystalline phase (fluid phase) above the Tm. The fluidity of liposome bilayers can be altered by using phospholipids with different Tm, which in turn can vary depending upon the length and nature (saturated or unsaturated) of the fatty acid chains. Liposomes containing high phase transition temperature lipids (Tm>37 °C) are rigid at the physiological temperature and are less leaky. In contrast, liposomes composed of low Tm lipids (Tm< 37 °C) are more susceptible to leakage of drugs encapsulated in aqueous phase at physiological temperatures. The fluidity of bilayers also influences interaction of liposomes with plasma components and cell membranes. Liposomes composed of high Tm lipids were reported to have lower extent of uptake by RES, compared to those containing low Tm lipids [83]. Incorporation of cholesterol into lipid bilayer increases membrane rigidity thereby affecting their stability both in vitro and in vivo [110-112].

1.2.8.4 Steric Stabilization

Inclusion of small fractions (5 to 10 mol%) of compounds bearing hydrophilic groups, such as PEG conjugated lipid, in the bilayer membrane was shown to reduce the interaction of liposomes with plasma components and make liposomes sterically stabilized [113]. Presence of hydrophilic surface coatings offers steric hindrance to opsonin adsorption on bilayer there by reducing rate of liposome uptake by cells of the RES [114]. Plasma levels of PEG-DSPE containing liposomes were increased 2- to 2.5- fold over DSPC/cholesterol (2:1) liposomes in mice [105, 115]. The ability of PEG to elicit this affect when grated onto the surface of liposomes has been explained by its chains 'high mobility, conformational flexibility, and water-binding ability [78, 103, 116, 117]. These properties all contribute to the, so called, steric stabilization effect which results in the well-known propensity of PEG to

exclude proteins, other macromolecules and particulates from its surroundings. This steric stabilization allows the liposomes to evade RES uptake and remain in blood circulation for a longer time thereby increasing the possibility of tumor targeting [118]. The presence of PEG-DSPE may also decrease the stability of some liposomal drug formulations [119, 120]. These authors suggested that this stability problem is a consequence of the negative charge found at the membrane interface with PEG-DSPE. The substitution of PEG-DSPE with a neutral PEG-ceramide conjugate resulted in greater stability of liposomal vincristine preparation [119, 120].

1.2.9. Biological Stability of Liposomes

The success of lipid-based carriers for anti-cancer drugs is dependent on their prolonged circulation longevity. The study of pharmacokinetics ("what the body does to the drug") consists of absorption, distribution, metabolism and excretion processes. In many of the studies within the thesis, the rate of liposomal lipid and drug elimination were assessed. The elimination of liposomes was determined by measuring the liposomal lipid concentration in plasma over a defined time interval. These data, when combined with data obtained by measuring plasma drug levels, could be used together to follow changes in drug-to-lipid ratios over time, following administration. A reduction in the drug-to-lipid ratio provided an indication of drug release from the liposome in the plasma compartment. Further analysis of the drug plasma concentration versus time curves with pharmacokinetic modeling may be used to determine the plasma half-life (T1/2), clearance and volume of distribution. Pharmacokinetic models are mathematical relationships that are used to predict the behavior of a drug in the body. There are three types of modeling including physiologic, compartmental and non-compartmental. Physiologic models are based on disposition of a compound in anatomic regions within the body based on blood flow, tissue volumes, binding, and transport and elimination parameters. Physiologic models are most often used when applying small vertebrate data to larger vertebrates, such as humans. Compartmental analysis is based on dividing the body into different homogenous compartments, not based on anatomic or physiological regions. For instance, a one compartment model assumes the administered drug distributes quickly into a central compartment (consisting of the blood compartment and highly perfused organs). Sampling from the blood compartment is thus equivalent to the concentration within the central compartment from which the drug is eliminated by first-order kinetics. For multi-compartmental analysis the drug will distribute into the central compartment followed by a peripheral compartment(s) (which are less perfused tissues such as skin and muscle). Non-compartmental analysis is based on the statistical moment theory and does not have the assumptions that are present in

compartmental models. In general, changes in the pharmacokinetics of a liposome-encapsulated drug are reflected by delayed absorption, restricted bio-distribution, decreased volume of distribution, delayed clearance and retarded metabolism relative to the non-encapsulated drug. The plasma elimination of liposomes following i.v. administration is dependent on vesicle size lipid composition and lipid dose. Prolonged circulation longevity is observed with liposomes exhibiting size distributions between 80- 150 nm, prepared of neutral phospholipids, and is further improved by incorporation of ganglioside GMi or PEG-derivatized lipids for surface stabilization. The pharmacokinetic behaviour of liposomes is also influenced by interactions with plasma proteins and cells of the mononuclear phagocytic system and these factors are briefly discussed in the following sections.

1.2.9.1. Plasma Proteins

Plasma proteins have been shown to interact with liposome membranes upon intravenous administration. Interactions that are dependent on the liposome surface attributes include charge and hydrophilicity. There are three consequences of plasma protein adsorption on the liposome membrane; (i) destabilization of the lipid membrane and leakage of encapsulated contents and (ii) presentation to and subsequent endocytosis by the macrophages of the mononuclear phagocytic system (MPS), and (iii) adsorption of plasma proteins, that in turn, mediate changes in the properties of liposomes. The latter effect is less well defined but highlights the fact that physicochemical characteristics of liposomes in the absence of plasma protein may be remarkably different than the physicochemical characteristics of liposomes with adsorbed plasma proteins. There are several supporting observations that demonstrate that the interaction between plasma proteins and liposomes results in phospholipid transfer from liposomes to high density lipoproteins (HDL) particles; a process mediated in part by a phospholipid transfer protein (PLTP). Further, phospholipid transfer was not observed in lipoprotein deficient mice and when various lipoproteins were re-introduced, only HDL compromised liposome stability. Moreover, the addition of cholesterol or lipids such as sphingomyelin (SM) or 1, 2-distearoyl-sn-phosphatidylcholine (DSPC) reduced phospholipid loss. Other plasma proteins that interact with liposomes include lipoprotein [ÿ-glycoprotein I (apolipoprotein H) and complement proteins which bind to negatively charged phospholipids. Albumin, the most abundant protein in serum, does not have a detrimental effect on the integrity of liposomes, while immunoglobulins, such asIgG and C-reactive protein, mediate uptake by macrophages. Fibronectin, a protein involved in cell adhesion, phagocytosis and cytoskeletal organization, induces liposome aggregation. Even long circulating liposomes have been shown to adsorb plasma proteins, and it is unknown whether complete abrogation of liposome-plasma protein interactions

may further extend blood circulation times. It is also not well understood how protein binding ultimately affects the fate of and biological response to injected liposomes. Some have even argued that plasma protein binding actually protects liposomes in the plasma compartment.

1.2.9.2. Mononuclear phagocytic system

The mononuclear phagocytic system (MPS), also referred to as the reticuloendothelial system (RES), consists primarily of the macrophages in the liver (Kupffer cells), spleen and bone marrow as well as the circulating precursors to these cells, the blood monocytes. These cells are actively involved in the immune response to foreign matter in the body. Intravenous administration of liposomes leads to the predominant uptake of liposomes within tissues containing these phagocytic cells. Plasma protein interactions with liposomes can facilitate endocytosis of the liposomes by macrophages or monocytes of the MPS system. The addition of cholesterol to liposome membranes was shown to moderately decrease accumulation within the liver and spleen. Although delivery to macrophages has been exploited for vaccine development, it is considered not to be beneficial for liposomal antitumor agents and may be one factor that limits accumulation of drug-loaded carriers in tumor sites. The use of surface stabilizing polymers significantly delayed the rate of liposome uptake by the MPS and resulted in extended circulation lifetimes. Further investigation into strategies to reduce cell uptake have been successful in mediating improvements in the circulation longevity of liposomes. This may be achieved by preventing adsorption of proteins that facilitate (opsonins) phagocytosis and promoting adsorption of proteins that inhibit (dysopsonins) phagocytosis by macrophages of the MPS.

1.2.10 Problems Associated with Liposome Formulation Development

Some of the problems limiting the manufacturing and development of targeted liposomes have been stability issues, batch to batch reproducibility, sterilization method, low drug entrapment, particle size control and production of large batch sizes [44].

1.2.10.1 Stability

Storage stability is one of the major problems limiting the wide spread use of liposomes. The stability comprises both physical stability (colloidal stability, drug retention) as well as chemical stability. Physical instability involves drug leakage from the vesicles and/or aggregation or fusion of vesicles to form larger particles. Physical instability may also occur due to partitioning out of a hydrophobic drug from the bilayer into the solvent on standing. Chemical instability is caused by hydrolysis of ester bond and /or oxidation of unsaturated acyl chains of lipids. In addition to the lipid components, drug molecules are also subject to

chemical hydrolysis in liquid formulations [121]. All of these processes change the in vivo performance of liposome formulations and their therapeutic utility [83, 113]. Fatty acid esters are sensitive to both acid and base hydrolysis giving rise to membrane destabilizing lysolipids under certain conditions [98, 121]. Lipid peroxidation is a particular concern for unsaturated lipid components and as such incorporation of a-tocopherol is a common technique used to prevent oxidative damage to lipids [63, 65]. Some of the approaches used to avoid physical instability problems are:

1. Using saturated lipids in the bilayer composition[122].

2. Incorporation of cholesterol to increase membrane rigidity [122].

3. Putting charge on the bilayer.

4. PEG steric stabilization.

The presence of PEG on the surface provides a steric barrier that prevents liposome aggregation. PEG coated liposomes are stable with respect to both size and drug-encapsulation over the period of many months to years when stored below the phase transition temperature of the phosphatidyl choline components [60, 66]. Another promising approach to overcome most of these stability problems is lyophilization or freeze-drying of the liposome product [44]. In this approach, liposome formulation is freeze-dried with an appropriate lyoprotectant and is reconstituted with vehicle immediately prior to administration. Lyophilization increases the shelf life of the finished product by preserving it in a relatively more stable dry state.

1.2.10.2 Sterilization

Identification of a suitable method for sterilization of liposome formulations is a major challenge because phospholipids are thermolabile and sensitive to sterilization procedures involving the use of heat, radiation and /or chemical sterilizing agents [44]. A presently available method for sterilization of liposome formulations after manufacture is filtration through sterile 0.22 |iim membranes. But this method is not suitable for large vesicles (>0.2 jo,m). Additionally, 0.2 jam filtration is not able to remove viruses. Zuidam *et al.* (1995) [98] have investigated the possibility of sterilization of liposome products by y-irradiation. No changes in either size or bilayer rigidity were seen; however, changes in melting characteristics were found at irradiation of liposome formulations. Oxidative damage to lipid components was also reported. Presence of certain nitroxides (tempo, tempol) was shown to reduce this radiation induced damage to liposomes [123, 124]. It has also been shown that

under certain conditions, liposomes with thermostable, lipophilic drugs could be sterilized by autoclaving without substantial loss of contents and/or degradation of phospholipids [125].

1.2.10.3 Encapsulation Efficiencies

Obtaining the required drug encapsulation is another important problem faced by liposome formulators. Liposome formulation of a drug could only be developed if the encapsulation efficiency is such that therapeutic dose could be delivered in a reasonable amount of lipid. Although there are some new approaches (active loading techniques) to obtain high encapsulation efficiency of certain hydrophilic drugs [66, 126], these active loading techniques are not suitable for hydrophobic drugs. Hydrophobic drugs are only passively incorporated into the lipid bilayers. Further, as these hydrophobic drugs tend to associate mainly with the bilayer compartment of the liposomes, they usually exhibit lower entrapment stability due to faster redistribution of the drug to plasma components. Therefore, it remains a great challenge to achieve the good encapsulation of these drugs with necessary loading stability, especially if they have low affinity for the lipid bilayers [44].

1.2.11 Freeze-Drying of Liposomes

As indicated earlier, stability is one of the major problems limiting the widespread use of liposome formulations. In the liquid state, liposome formulations are subject to both physical and chemical instability [44]. One way to overcome most of these stability problems is through lyophilization of the liposome product [46, 73, 127]. Removal of water by lyophilization prevents hydrolysis of phospholipids. Other chemical and physical degradation processes are also retarded by low molecular mobility in the solid phase. Further, freeze-drying of liposome formulations, if performed successfully, results in a pharmaceutically elegant dry cake which can be reconstituted within seconds to obtain the original dispersion. Freeze-drying of liposomes is not so straight forward as that of single component composition. The freezing and drying stresses involved in the process can damage liposomes irreparably [127, 128]. This damage includes massive aggregation and fusion of the vesicles as well as leakage of the entrapped compounds. Freeze-drying of MLVs and subsequent recovery of the very same liposome type is relatively simple and has been reported by many investigators [127, 129, 130]. With unilamellar liposomes the situation is more complex. Unless specific precautions are taken, these small ULVs revert upon lyophilization and reconstitution to the much larger MLVs [131, 132]. Certain excipients such as disaccharides have been reported to protect liposomes during freeze-drying process [133-135]. The present status of knowledge, various problems and challenges involved in freeze-drying of liposomes has been reviewed recently by Van Winden (2003) [127]. Successful freeze-drying of liposomes depends on a variety of factors, such as the presence of a lyoprotectant,

vesicle size, lipid composition, and process parameters [129, 131]. Presence of lyoprotectants both inside and outside the liposomes was shown to maximize protection effect [136, 137]. Crowe *et al.* (1987) [138] have carried out extensive investigations on possible mechanisms by which sugars protect biological membranes during freezedrying. The mechanism involves formation of glassy state by the sugars as well as a direct interaction between sugars and the phospholipid head groups. A direct interaction between the sugar and the phospholipid head groups is pivotal to prevent leakage through the bilayers, whereas vesicle fusion can be prevented by the formation of a stable glassy state. Glucose is not effective in protecting liposomes during drying due to its low Tg of 36 °C [139, 140]. The Tg of monosaccharides such as glucose is generally too low to stabilize liposomes in the dried state, where as oligo- and poly-saccharides are able to form a stable glass, but due to their large size are unable to interact with lipid head groups. Disaccharides are small enough to be able to interact with vesicles and have a sufficiently high Tg. Peer, *et al.* (2003) [132] have reported lyophilization of targeted unilamellar liposomes without added sugars. Hyaluronan, the surface bound ligand in the targeted bioadhesive liposomes also protected liposomes during freeze-drying process. They proposed that hyaluronan, like sugars, protects liposomes by providing substitute structure-stabilizing hydrogen bonds. Recently, Ohtake, S, *et al.* (2005) [141] have studied phase behavior of freeze-dried liposomes stabilized with trehalose. Trehalose was shown to stabilize cholesterol containing DPPC and DPPE liposomes. Using differential scanning calorimetry these authors showed that cholesterol containing liposomes exhibit multiple phase transitions upon dehydration. Addition of trehalose to these systems lowered the phase transition temperature and prevented the phase separation of the lipidic components upon freeze drying. Ugwu *et al.* (2005) [142] have reported lyophilization of liposome formulations of mitoxantrone. Mitoxantrone was entrapped in small ULV composed of DOPC, cholesterol and cardiolipin. Sucrose was found to be more effective than trehalose in protecting liposomes during freeze-drying. The sucrose containing formulations retained their size after lyophilization and rehydration at sucrose-to-lipid ratios of 7.5 or higher. Less than 2% of the drug was released from the reconstituted liposomes after 72 hr of dialysis. Long-term stability studies showed that lyophilized formulation was stable for up to 13 months when stored at refrigerated condition. Dodov *et al.* (2005) [129] reported lyophilization of MLV containing 5-fluorouracil with saccharose as cryoprotectant. The process of lyophilization, without cryoprotectant, resulted in particle size increase and significant content leakage. By the addition of saccharose, the lipid bilayers become more stable and less permeable to the encapsulated drug. Particle size distribution was maintained after lyophilization and reconstitution. Stevens and Lee (2003) [143] have developed a formulation method for liposomal

doxorubicin based on lyophilized liposomes incorporating glucose and sucrose as lyoprotectants. Blank liposomes composed of egg phosphatidyl choline and cholesterol were lyophilized. Doxorubicin was loaded into liposomes after lyophilization and reconstitution by pH gradient based remote loading procedure. Cryoprotectants were effective in maintaining liposome size distribution but not drug retention during lyophilization.

1.2.12. Forthcoming Advances in Liposomal Drug Delivery

When considering the use of liposomes to improve the therapeutic potential of existing drugs, increasing selectivity of a drug carrier for a target cell population and achieving controlled release rates are two of the goals for drug delivery systems. Increased selectivity for anti-cancer agents may be achieved by using strategies that extend the blood circulation lifetimes of liposomes by incorporation of surface stabilizing agents, such as PEG. Further strategies that increase carrier selectivity for malignant cells are being pursued and include development of ligand-targeted (immuno-) liposomes. It is anticipated, for example, that conjugation of novel tumor-specific monoclonal antibodies, such as Herceptin® (binds to HER2/Neu receptor) and Rituximab® (binds to CD20), will direct liposomes to malignant cells for local and systemic disease. Related efforts include those designed to promote localized drug release following passive targeting as well as more specific intracellular delivery and include pH-sensitive [96], programmable fusogenic and thermosensitive liposomes. pH-sensitive liposomes undergo a transition from a bilayer to a non-bilayer (hexagonal) phase that can result in loss of encapsulated contents and membrane fusion with nearby cells or membranes. This transition, as the name implies, occurs when the liposomes encounter an acidic environment, such as that which may be found within tumors or within cellular endosomal compartments. Programmable fusogenic liposomes exhibit a time-dependent destabilization based on the loss/exchange of liposome-associated PEG-derivatized lipids from the membrane surface. Thermosensitive liposomes exhibit membrane phase transition temperatures a few degrees above physiological body temperature, and site-specific drug accumulation can be triggered through mild heating of a tumor site. The use of multifunctional liposomes **(Figure 1.5)** utilizing a combination of these targeting and triggered release technologies may provide a superior approach to treat cancer.

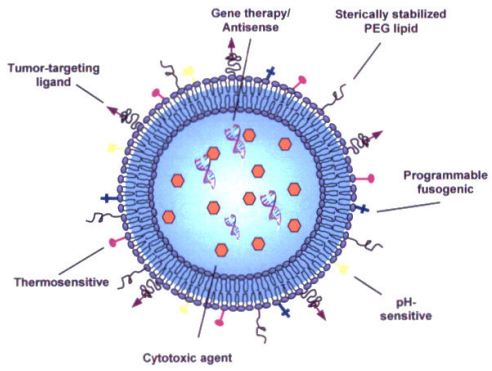


FIGURE 1 5 A multifunctional liposome with multiple targets and triggered release mechanisms

1.2.13 Drug Delivery to Solid Tumors

The term "tumor" (neoplasm) refers to a collection of abnormally growing cells which is not always synonymous with cancer. Some tumors are benign (non-cancerous) while others are malignant (cancerous). The term solid tumor is used to distinguish between a localized solid mass of tissue and leukemia. Leukemia is a type of cancer that is defined by abnormal increase in the number of leukocytes and since it affects the blood, it takes on fluid properties. Malignant tumors differ from benign ones in that they are capable of spreading into surrounding tissues (invasion) and transferring to other organs or parts of the body that are not directly in contact with the malignant tumor through blood circulation and/or lymphatic system (metastasis). Tumors tend to grow exponentially; a single tumor cell can produce a one-gram tumor (10 to 10 cells) after about 30 doublings in volume, and with another 10 doublings in volume it would produce a very large tumor of about one kilogram [144, 145]. Over 85% of the human cancers are solid tumors [146]. The effectiveness of cancer chemotherapy in solid tumors depends on adequate delivery of the therapeutic agent to tumor cells. Before a blood borne chemotherapeutic agent can begin to attack malignant cells of a solid tumor, it must accomplish three critical tasks. First, it must survive in the systemic circulation, find and reach the target tumor site. Then, it must extravasate into the tumor interstitium. And, finally, it should migrate through the tumor matrix to distribute throughout the tumor and kill all of the malignant cells. Unfortunately, solid tumors develop in such a way to hinder each of these steps [146, 147]. There are several indomitable barriers that limit effective delivery of cancer chemotherapeutic agents to solid tumors by the traditional means [148]. These include both physiological and pathological barriers.

1. Most of the existing anti-cancer drugs lack selectivity towards target tumor cells. Once in the systemic circulation, these drugs distribute throughout the body, causing many unwanted side effects.

2. Malignant tumor cells in solid tumors are relatively inaccessible to blood borne chemotherapeutic agents.

3. Limited penetration of drugs into tumor tissue. This is due to extravasation difficulty and inability to distribute in the tumor mass [148].

4. Poor perfusion of solid tumors and their heterogeneous blood supply [149].

An elevated interstitial pressure further limits drug diffusion into distant tumor cells [150]. The center of the tumor has high interstitial pressure. This elevated pressure in the inner zone can impede movement of drug molecules into the tumor matrix. 6. In addition, rapidly proliferating cancer cells easily undergo treatment resistant mutations and are known to develop, so called, 'multi drug resistance, very soon. Development of this drug resistance is probably through generation of drug exporters in these tumor cells [151]. Metastasis of these cancerous cells spreads the disease to other tissues as well. These limitations virtually restrict our ability to kill the solid tumor cancer cells per se with the available cytotoxic drugs; therefore, the actual target has to be redefined. Recently, vasculature targeting has been proposed to be a promising alternative for effective solid tumor treatment [152]. This approach involves killing tumor cells by denying them of their life blood. This strategy may involve anti-angiogenesis-inhibition of new blood vessel formation, or anti-vascular approaches that aim to cause a rapid and extensive shutdown of the established tumor vasculature leading to secondary tumor cell death. The functioning vascular network in tumors provides the tumor cells with oxygen and nutrients and enables removal of the toxic waste products of cellular metabolism and as such, it is pivotal for the survival of the tumor cells. Disruption of this vasculature results in tumor cell death and consequently tumor cures. Malignant cells, like normal mammalian cells require oxygen and nutrients for their survival and are, therefore, located within 100 to 200 (im of blood vessels – the diffusion limit for oxygen. For tumors to grow beyond this size, they must recruit new blood vessels by vasculogenesis and angiogenesis. Without blood vessels, tumors cannot grow beyond a critical size and cannot metastasize to another organ [153]. Considering the limitations of existing solid tumor cancer therapy, anti-vascular therapy using solid tumor vasculature targeting appears to be an attractive strategy.

1.2.13.1 Advantages of Tumor Vasculature Targeting

There are several major advantages to the concept of tumor vasculature targeting to treat solid tumors [152].

1. Many thousands of tumor cells depend on every blood vessel. Theoretically, damage to relatively few endothelial cells in a vessel could stop tumor blood flow and trigger a cascade of tumor cell death.

2. The cells to be targeted are next to the bloodstream and are readily accessible to intravenously administered drug delivery system. Therefore, delivery problems common with therapies that target tumor cells themselves are overcome.

3. Because endothelial cells are genetically more stable than tumor cells, treatment resistant mutations are less likely to emerge [154]. This potentially can overcome the serious problem of multi drug resistance development. And,

4. Therapeutic strategies directed against tumor vasculature also potentially reduce the tumor's ability to develop metastases [155].

1.2.13.2 Basis for Tumor Vasculature Targeting

One key issue in the development of targeted treatment strategies is identifying differences that exist between the tumor vasculature and that of normal tissues. It is well established that the blood vessels in tumors proliferate more rapidly than those in most normal tissues [156]. It is also shown that rapidly proliferating endothelial cells over express certain surface markers such as av-integrins [54, 157-159]. These surface markers discriminate tumor endothelial cells from the normal endothelial cells and can be used as a target for antiangiogenic or anti-vascular therapy. Therefore, simply targeting features of proliferating endothelium, or even newly formed vasculature, could achieve some selectivity for cancer treatment in adults. Additionally, the selectivity for solid tumor vasculature can be further enhanced by pre-irradiating tumor areas for therapeutic purposes [160, 161].

1.2.14 Effects of Irradiation on Tumor Vasculature

Radiation therapy is one of the major treatment modalities available for cancer treatment. Indeed it is uncommon for a patient with cancer not to have had radiation treatment as part of therapy [162, 163]. Chemotherapy usually follows the radiation therapy. Radiation produces its effects by localized release of high energy. Photons interact with water molecules to form hydroxyl radicals, which initiate DNA strand breaks. There are four basic types of ionizing radiation. Alpha particles (helium nuclei), beta particles (electrons), gamma rays (high frequency electromagnetic waves, X-rays are generally identical to gamma rays except for their place of origin), and neutrons [144, 145]. Ionizing radiation has enough energy to eject electrons from electrically neutral atoms, leaving behind charged atoms or ions. The amount of radiation absorbed by the tissues is called the radiation dose. Before 1985, dose was measured in a unit called "rad" (radiation absorbed dose). Now the unit is called "gray"

(abbreviated as Gy). One Gy is equal to 100 rads. The interactions leading to energy deposition in tissue occur very rapidly and generate chemically reactive free electrons and radicals. It is well accepted that DNA damage is central to the biologic effects of ionizing radiation. Baker *et al.* (1989) [164] have reviewed the effects of irradiation on microvascular system. They explained that radiation causes both structural and functional changes in the vasculature. Among other things, permeability of small blood vessels and the endothelial lining of larger blood vessels is increased by irradiation. Radiation induced increase in permeability of the vasculature is a part of the body's repair process. These permeability changes are only transient lasting for a few hours or longer depending on the dose. These authors have also indicated that rapidly dividing endothelial cells of the tumor vasculature are more sensitive to radiation damage than those of the normal vasculature. Tumor microvasculature is significantly different from that of normal tissues. Tumor vasculature has very few smooth muscle cells (sometimes none). Endothelial cells are very irregular. The basement membrane is missing. Lack of all these supporting layers makes the tumor vasculature extremely sensitive to irradiation. Further, blood vessels in tumors are tortuous with arteriovenous shunts and no defined hierarchy. One of the main effects of irradiation on tumor vasculature is the activation of inflammatory response [161]. Ionizing radiation activates inflammatory cascade through induction of cell adhesion molecules and cytokines [165]. This response of the endothelium is to maintain the homeostasis by preserving the barrier function of the blood vessels. Several adhesion molecules including ICAM-1, Eselectin, P-selectin and P3 integrins have been shown to be up-regulated in tumor vasculature in response to irradiation. These molecules have been used to target drugs and drug delivery systems to the tumor vasculature specifically [161, 166]. Irradiation was shown to cause instantaneous injury to the microvasculature, even at 2 Gy dose [166]. Repair process was also observed as early as 16-24 hr. It is this repair process that involves inflammatory response and subsequent up-regulation of adhesion molecule expression. Ionizing radiation induces oxidative injury in the endothelium [167]. The endothelium responds to maintain homeostasis by preserving the barrier function in blood vessels. This is accomplished by activation of the inflammatory response and platelet aggregation. The mechanism by which radiation activates these homeostatic responses is, in part, through the induction of cell adhesion molecules. Cell adhesion molecules such as ICAM-1, E-selectin, and P-selectin are induced by irradiation of the endothelium and bind to receptors on the circulating leukocytes to initiate inflammatory cascade. Prior to adhesion to these cell adhesion molecules, leukocytes are relatively quiescent, but mediate the inflammatory cascade in response to endothelial cell injury. Radiation, therefore, initiates the inflammatory cascade through activation of the endothelium. Irradiation also induces pro-coagulative state in the

endothelium. The mechanisms are related to the release of von Willebrand factor and interaction with leukocytes [168-170]. Radiation was shown to induce apoptosis in microvascular endothelial cells [171]. In addition to this direct cytotoxic effects, radiation induced injury to the endothelium activates homeostatic response. In this regard, platelets are activated within irradiated tissues resulting in platelet aggregation. Irradiation also induced genes encoding tumor necrosis factor and interleukin -1. Release of these inflammatory cytokines result in an acute inflammatory state. These proteins and proteoglycans activate circulating leukocytes and therefore mediate the inflammatory response to irradiation [165]. Radiation induced oxidative injury to the endothelium leads to up-regulation of endothelial cell adhesion molecules such as ICAM-1, E-selectin, and P-selectin [161] and expression of stored proteins from the endothelial cytoplasm [167]. Donnelly *et al.* (2001) [172] have used a tumor vascular window and Doppler sonography to measure changes in tumor vascularity and blood flow upon irradiation. They reported that radiation doses in the range of 2 to 3 Gy increase vascularity within tumors. In contrast, larger doses of radiation such as 6 Gy reduced tumor vascularity and hence blood flow. Dynamic contrast-enhanced magnetic resonance imaging has been used to study the radiation induced changes in tumor vasculature [173]. No significant change in vascular volume was observed after irradiation of the tumor. But irradiation increased tumor vascular permeability.

1.12.15 Targeted Drug Delivery to Irradiated Tumors

As indicated previously, irradiation of tumors for therapeutic purposes upregulates expression of a variety of cell adhesion molecules (including IC AM-1, Eselectin, P-selectin and avp3 integrins) in tumor microvasculature [166]. Over-expression of these molecules on tumor vasculature can be utilized to target drug delivery systems to tumor areas selectively [160, 161]. Hallahan *et al.* (2001) [166] have studied targeted drug delivery to radiation induced neoantigens in tumor microvasculature. RGD peptides achieved the greatest site-specific peptide binding within irradiated tumor blood vessels in tumor bearing mice when. "biodistribution" was studied via I conjugated ligands. Radiation mediated targeted drug delivery clinical trials have been initiated with RGD mimetics as targeting ligands [166]. In another study, nanoparticles have been conjugated to fibrinogen to accomplish site-specific binding to irradiated tumor blood vessels. Integrin P3 has been shown to accumulate within the lumen of blood vessels in response to radiation. The P3 integrin is associated with integrinsav or ct2b to form heterodimers ot2bP3 and avP3. The heterodimer a2bP3 is the component of a receptor on activated platelets, whereas avP3 is the vitronectinreceptor [174]. The advantage of using radiation induced cell adhesion molecules for drug targeting is

that it can be localized precisely to tumors, sparing surrounding normal tissues [161]. Ionizing radiation activates the inflammatory cascade and increases the procoagulation state with in blood vessels of both tumors and normal tissues. These responses are mediated through oxidative injury to endothelium, leading to induction of cell adhesion molecules and cytokines [167]. As indicated by Kiani *et al.* (2002) [161], radiation induced up-regulation of endothelial cell adhesion molecules provides the opportunity to target drugs to select tissues via a combination of radiation and ligand receptor drug targeting technology. These authors successfully targeted microparticles coated with a MAb to ICAM-1 to select tissue via radiation induced up-regulation of cell adhesion molecules.

1.12.16 RGD Peptide

In an attempt to reduce macromolecular ligands to small recognition sequence, the tri-peptide motif arginine-glycine-aspartic acid (RGD) was identified as the minimal essential cell adhesion peptide sequence in fibrinectin[175, 176]. Since then, cell adhesive RGD sites were identified in many other extracellular matrix proteins, including vitronectin, ibrinogen, Van Willebrand factor, collagen, laminin, osteopontin, tenascin and bone siloprotein. It has also been identified in membrane proteins of viral and bacterial origin, and in snake venoms [177]. The conformations of the RGD containing loop and its flanking amino acids in the respective proteins are mainly responsible for their different integrin affinity [177]. RGD sequence constrained in a cyclic conformation was reported to bind avP3 integrins with high affinity [174, 178].

1.12.16.1 Advantages of RGD Peptide as Targeting Ligand

Using small peptides, such as RGD, as targeting ligand has several advantages over large macromolecular ligands, such as antibodies [179]. Production of proteins is costly and time consuming. A large quantity of protein must be used because of its high molecular weight. On the other hand, small peptides, can be easily synthesized and small amounts of peptides are enough for effective targeting. 2) Proteins may elicit an undesirable immune response such allergic reactions to certain antibodies derived from murine origin. 3) Conformation and orientation of the protein effects receptor binding. 4) Small peptide motifs exhibit higher stability toward sterilization conditions, heat treatment and pH variation, storage, and conformational shifting as well as easier characterization and cost effectiveness. 5) Because of lower space requirements peptides can be packed with a higher density on to surfaces. Rapidly proliferating angiogenic endothelial cells in tumor vasculature can be selectively targeted using RGD peptides that bind to avP3 integrin on these endothelial cells [180]. The integrin avP3 is associated with angiogenesis [51] and its expression is highly up-regulated in angiogenic tumor vascular endothelial cells. Cyclic RGD peptides have been reported to

have a high affinity for the avp3 integrin and were used to target drugs to angiogenic endothelium [181-183]. It is also reported that the RGD peptide and RGD peptide mimetics bind within irradiated tumor vasculature through radiation activated receptors [166]. Kok *et al.* (2002) [180] have prepared several RGD modified proteins to target tumor vasculature endothelial cells. They reported that tumor vascular endothelial cells internalize and degrade RGD-modiied proteins [181-183]. Using pharmacokinetic and cellular distribution studies, these authors have also reported that RGD-modified proteins are suitable carriers to deliver therapeutic agents into tumor or inflammation induced angiogenic endothelial cells [181-183]. Schiffelers *et al.* (2002) [184, 185] have used this RGD peptide to target liposomes to tumor vascular endothelial cells. Although several integrin receptor subtypes recognize and interact with RGD, each of these require a distinctly different configuration of the sequence for high affinity binding [186]. Cyclic RGD peptides have been synthesized as selective ligands for the avP3 integrin receptor. A penta-peptide cyclo (Arg-Gly-Asp-D-Phe-Val) was shown to be selective for the avp3 integrin [187]. Later it was shown that the valine in this penta-peptide can be substituted without loss of affinity with any L-amino acid or other functional side chain[188]. Recently Haubner *et al.* [189, 190] have used radio labeled cyclic RGD peptides for the visualization of avP3 integrin expression. [191] have synthesized a series of RGD analogues with different side chain functional groups to be used as potential vectors for targeted drug delivery. They showed that it is possible to tether a fairly large pharmaceutical agent or carrier to such a RGD analog without significantly affecting the affinity to the intended receptor.

1.3 Gemcitabine Hydrochloride

Gemcitabine Hydrochloride (HCl) is a cytotoxic drug available on the market in the freeze-dried form of an aqueous solution known as Gemzar. After reconstitution Gemzar is used for intravenous administration as an infusion only.

1.3.1 Therapeutic Indications

Gemcitabine has numerous applications for cancer and is indicated for breast cancer, non-small cell lung cancer (NSCLC), bladder cancer, ovarian cancer and pancreatic cancer. Gemzar can also be used in patients displaying the following conditions: Cancer of the lymph system, the bile ducts, the gallbladder and germ cell tumors of the ovaries and testes.

1.3.2 Chemical Characteristics

The chemical name of gemcitabine is 2′-deoxy-2′, 2′-difluorocytidine mono-hydrochloride. Gemcitabine, or difluorodeoxycytidine (dFdC), is an antimetabolite cytotoxic. It is a chemical analogue to the natural nucleoside deoxycytidine **(Figure 1.6). (6, 11, 12)**

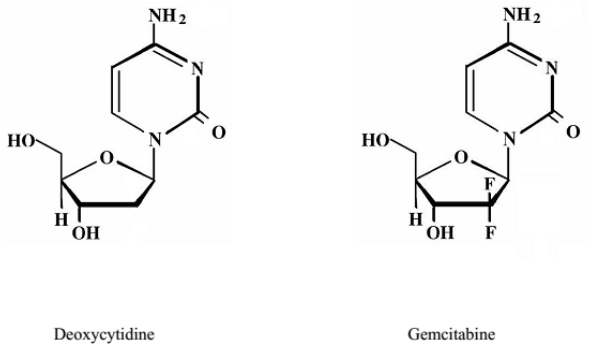


FIGURE 1 6 Chemical structure of deoxycytidine and the antimetabolite gemcitabine

From **Figure 1.6** it can be seen that two hydrogen atoms of the carbon on the second position of deoxycytidine are substituted with two fluoride atoms giving gemcitabine. The pair of fluoride atoms is contributing tothe low pKa value of 3.58 for the gemcitabine HCl salt due to the increased electronegativity. Gemcitabine has a MW of 263.199 g/mol whereas gemcitabine HCl has a MW of 299.66 g/mol dueto the presence of hydrochloride. Gemcitabine HCl is a white to off-white powder soluble in water.

1.3.3Mechanism of Action

The prodrug gemcitabine is converted intracellularly via deoxycytidine kinase to difluorodeoxycytidine monophosphate, which is further converted to two active metabolites, dFdCDP and dFdCTP, di– and triphosphate, respectively. Firstly, dFdCDP inhibits the catalysing enzyme ribonucleotidereductase resulting in a reduced amount of deoxynucleotide, deoxycytidine triphosphate (dCTP), available for DNA synthesis. Secondly, dFdCTP competes with dCTP for incorporation into DNA. Incorporating dFdCTP results in chain termination after the further addition of one more nucleotide and thus to apoptosis. Thus dFdC affects the synthesis phase of cell metabolism in two different ways and exhibits a self-potentiating effect.

1.3.4Metabolism

DFdC is rapidly metabolised in the blood, liver, kidneys and other tissues. Gemcitabine displays a short t½ ranging from8 to 17 minutes. Less than 10 % is excreted unchanged in the urine, however large amounts of its primary metabolite, difluorodeoxyuridinedFdU were detected. Only a small portion of dFdC will convert into dFdCDP and dFdCTP whereas 91-98 % of administered dFdC will turninto the inactive difluorodeoxyuridine (dFdU).

1.3.5Toxicity

The cytotoxic activity of gemcitabine in vivo is schedule dependent. This means the activity and the toxicity are related to the dose given and the dosage interval of the treatment. The

problem with Gemzar is its short plasma t½ and its quick metabolism into dFdU followed by elimination from the body. Therefore high doses of dFdC are required in order to achieve sufficient cytotoxic concentrations of dFdCTP. Due to the narrow therapeutic window, high administered doses increase the possibility of toxicities and concentration dependent side effects for patients. According to clinical studies, the primary dose limiting toxic effect is myelosuppression; neutropenia, leucopenia, anaemia and thrombocytopenia. In addition, together with other side effects, such as hepatic abnormalities, nausea and vomiting, 10 % of patients ceased treatment.

1.3.6 Properties of Gemcitabine HCl and Advantage of Liposomal Formulation

As mentioned above gemcitabine is a prodrug, which is activated to its active metabolite intracellurlaly. Its half-life in the body is relatively short, only between 42 and 94 minutes, depending on gender and age [192]. As any drug entrapped inside a liposome vesicle would be protected against metabolic breakdown and elimination, liposomes could enhance the short half-life of gemcitabine. Liposomes also serve several other beneficial properties, among other things liposomes in the smaller size range (up to a diameter of 400-600 nm) will have enhanced permeability and retention effect at the site of the tumor. This is because of the special characteristics this tissue holds that differs from that in normal healthy tissue; the blood vesicles in tumor sites are leakier due to their accelerated growth to enable rapid tumor growth, and the cells are often not as densely packed as cells in healthy tissue. In addition the lymphatic system is often less expressed in tumor tissue [193, 194].

1.3.7 Approaches for Improving Formulation

Some attempts, including the use of liposomes, have been tried in order to overcome the problems seen with Gemzar. DFdC is uncharged at physiological pH and is a low MW molecule, which will make it diffuse quickly through the liposomal membrane. Gemcitabine has successfully been entrapped within liposomes, however it leaked out of the vesicles very quickly. In addition, dFdC appears to induce degradation of the liposomal membranes. In order to avoid these problems Gemzar was encapsulated into vesicular phospholipid gel (VPG) reaching an encapsulation efficiency (EE) of 33 % and a shelf life above 14 months. A pilot study proved the formulation encapsulating dFdC into VPG had biphasic elimination (due to free dFdC and encapsulated dFdC) resulting in an increased concentration in plasma compared to Gemzar.

Despite the increased potency of the VPG formulated gemcitabine, GemLip, it also revealed increased toxicity. The increased toxicity was most likely due to the prolonged and fractioned administration of gemcitabine. However, GemLip consists of 33 % encapsulated

dFdC and 67% of extra-liposomal dFdC. This amount of free dFdC will exhibit the same rapid metabolism and the same toxicities as conventional Gemzar.

1.4 Rationale, Hypotheses and Objective

1.4.1 Rationale

In the light of the available literature to achieve success rate in cure of lung cancer having second highest incidence and mortality rate. The current cure chemotherapy for lung cancer has limitation being non-selective and manifests in toxicity.

1.4.2 Hypothesis

It is hypothesized that surface functionalized nanoconstructs of the drugs will selectively enhance cellular uptake of the selected drugs and minimize macrophages uptake and ciliary clearance in the lung. Further, the nanoconstructs will provide the sustained release of the encapsulated drug. It will improve the therapeutic benefits in treatment of lung cancer. Formulating these nanoconstructs into as a lyophilized powder formulation may be delivered systemically after reconstitution.

1.4.3 Objective

This project aims to develop a liposomal targeted delivery system for the chemotherapeutic agent gemcitabine HCl, to selectively take this drug to the tumor site. The overall hypothesis is that liposome drug carriers bearing appropriate ligands can be targeted to tumor cells via up-regulated adhesion molecules.

In order to test the aforementioned hypotheses, the present study had the following specific aims:

1. To develop and validate a HPLC method for the assay of gemcitabine hydrochloride.
2. Design a tumor targeted liposomal delivery system for Gemcitabine Hydrochloride.
3. Formulate the designed targeted liposomal delivery system.
4. Develop a process for the preparation of the targeted liposomal delivery system.
5. Characterize the prepared delivery system.
6. Optimize the liposome formulation for maximum possible drug loading
7. Evaluate functional properties of the targeted delivery system *in vitro* using human lung cancer cell lines.
8. Develop of a lyophilized formulation and process for the targeted liposome delivery system to enhance its storage stability.
9. Evaluate comparative stability of lyophilized and liquid liposome formulations at different storage temperatures.

1.5 REFERENCES

1. Aisner, J., et al.., *Intensive combination chemotherapy, concurrent chest irradiation, and warfarin for the treatment of limited-disease small-cell lung cancer: a Cancer and Leukemia Group B pilot study.* J Clin Oncol, 1992. **10**(8): p. 1230-6.
2. Kulkarni, S., G. Betageri, and M. Singh, *Factors affecting microencapsulation of drugs in liposomes.* Journal of microencapsulation, 1995. **12**(3): p. 229-246.
3. Lasic, D.D. and D. Papahadjopoulos, *Medical applications of liposomes*. 1998, Amsterdam ; New York: Elsevier. xiv, 779 p.
4. Winterhalter, M. and D.D. Lasic, *Liposome stability and formation: experimental parameters and theories on the size distribution.* Chem Phys Lipids, 1993. **64**(1-3): p. 35-43.
5. Fenske, D.B. and P.R. Cullis, *Entrapment of small molecules and nucleic acid-based drugs in liposomes.* Methods Enzymol, 2005. **391**: p. 7-40.
6. Maurer, N., D.B. Fenske, and P.R. Cullis, *Developments in liposomal drug delivery systems.* Expert Opin Biol Ther, 2001. **1**(6): p. 923-47.
7. Noble, C.O., et al.., *Development of ligand-targeted liposomes for cancer therapy.* Expert Opin Ther Targets, 2004. **8**(4): p. 335-53.
8. Schiffelers, R.M., et al.., *Liposomal targeting of angiogenic vasculature.* Curr Drug Deliv, 2005. **2**(4): p. 363-8.
9. Schiffelers, R.M., et al.., *Liposome-encapsulated prednisolone phosphate inhibits growth of established tumors in mice.* Neoplasia, 2005. **7**(2): p. 118-27.
10. Bangham, A.D., M.M. Standish, and J.C. Watkins, *Diffusion of univalent ions across the lamellae of swollen phospholipids.* J Mol Biol, 1965. **13**(1): p. 238-52.
11. Batzri, S. and E.D. Korn, *Single bilayer liposomes prepared without sonication.* Biochim Biophys Acta, 1973. **298**(4): p. 1015-9.
12. Deamer, D. and A.D. Bangham, *Large volume liposomes by an ether vaporization method.* Biochim Biophys Acta, 1976. **443**(3): p. 629-34.
13. Kagawa, Y., *[Reconstitution of oxidative phosphorylation system].* Tanpakushitsu Kakusan Koso, 1971. **16**(9): p. 775-86.
14. Barenholzt, Y., S. Amselem, and D. Lichtenberg, *A new method for preparation of phospholipid vesicles (liposomes) - French press.* FEBS Lett, 1979. **99**(1): p. 210-4.
15. Shew, R.L. and D.W. Deamer, *A novel method for encapsulation of macromolecules in liposomes.* Biochim Biophys Acta, 1985. **816**(1): p. 1-8.
16. Cortesi, R., et al.., *Preparation of liposomes by reverse-phase evaporation using alternative organic solvents.* J Microencapsul, 1999. **16**(2): p. 251-6.
17. Meeren, P.V.d., et al.., *Particle sizing of liposomal dispersions: a critical evaluation of some quasi-elastic light-scattering data-analysis software programs.* Journal of Liposome Research, 1992. **2**(1): p. 23-42.
18. Olson, F., et al.., *Preparation of liposomes of defined size distribution by extrusion through polycarbonate membranes.* Biochim Biophys Acta, 1979. **557**(1): p. 9-23.
19. Szoka, F., et al.., *Fluorescence studies on the mechanism of liposome-cell interactions in vitro.* Biochim Biophys Acta, 1980. **600**(1): p. 1-18.
20. Szoka, F., et al.., *Preparation of unilamellar liposomes of intermediate size (0.1-0.2 mumol) by a combination of reverse phase evaporation and extrusion through polycarbonate membranes.* Biochim Biophys Acta, 1980. **601**(3): p. 559-71.

21. Szoka, F., Jr. and D. Papahadjopoulos, *Comparative properties and methods of preparation of lipid vesicles (liposomes).* Annu Rev Biophys Bioeng, 1980. **9**: p. 467-508.
22. Madden, T.D., M.J. Hope, and P.R. Cullis, *Modelling the biological membrane.* Biochem Soc Trans, 1987. **15**(1): p. 75-7.
23. Mayer, L.D., et al.., *Uptake of antineoplastic agents into large unilamellar vesicles in response to a membrane potential.* Biochim Biophys Acta, 1985. **816**(2): p. 294-302.
24. Gabizon, A., et al.., *Superior therapeutic activity of liposome-associated adriamycin in a murine metastatic tumour model.* Br J Cancer, 1985. **51**(5): p. 681-9.
25. Segal, H.L., *Lysosomes and intracellular protein turnover.* Front Biol, 1975. **43**(4): p. 295-302.
26. Jain, M.K. and D. Zakim, *The spontaneous incorporation of proteins into preformed bilayers.* Biochim Biophys Acta, 1987. **906**(1): p. 33-68.
27. Jain, M.K. and W.L. Vaz, *Dehydration of the lipid-protein microinterface on binding of phospholipase A2 to lipid bilayers.* Biochim Biophys Acta, 1987. **905**(1): p. 1-8.
28. Forssen, E.A. and Z.A. Tokes, *Use of anionic liposomes for the reduction of chronic doxorubicin-induced cardiotoxicity.* Proc Natl Acad Sci U S A, 1981. **78**(3): p. 1873-7.
29. Allen, T.M., et al.., *Therapeutic liposome composition and method of preparation*. 2000, Google Patents.
30. Olson, F., et al.., *Characterization, toxicity and therapeutic efficacy of adriamycin encapsulated in liposomes.* Eur J Cancer Clin Oncol, 1982. **18**(2): p. 167-76.
31. Mehta, R., et al.., *Liposomal amphotericin B is toxic to fungal cells but not to mammalian cells.* Biochim Biophys Acta, 1984. **770**(2): p. 230-4.
32. Ambegia, E., et al.., *Stabilized plasmid-lipid particles containing PEG-diacylglycerols exhibit extended circulation lifetimes and tumor selective gene expression.* Biochim Biophys Acta, 2005. **1669**(2): p. 155-63.
33. Mui, B., et al.., *Immune stimulation by a CpG-containing oligodeoxynucleotide is enhanced when encapsulated and delivered in lipid particles.* J Pharmacol Exp Ther, 2001. **298**(3): p. 1185-92.
34. Zimmermann, R., *Médecine nucléaire la radioactivité au service du diagnostic et de la thérapie*. 2006, EDP Science,: Les Ulis. p. 1 online resource (viii, 171 p.
35. Allen, T.M. and P.R. Cullis, *Drug delivery systems: entering the mainstream.* Science, 2004. **303**(5665): p. 1818-22.
36. Jeffs, L.B., et al.., *A scalable, extrusion-free method for efficient liposomal encapsulation of plasmid DNA.* Pharm Res, 2005. **22**(3): p. 362-72.
37. Fenske, D.B., I. MacLachlan, and P.R. Cullis, *Stabilized plasmid-lipid particles: a systemic gene therapy vector.* Methods Enzymol, 2002. **346**: p. 36-71.
38. Kale, A.A. and V.P. Torchilin, *"Smart" drug carriers: PEGylated TATp-modified pH-sensitive liposomes.* J Liposome Res, 2007. **17**(3-4): p. 197-203.
39. Li, L., F.S. Braiteh, and R. Kurzrock, *Liposome-encapsulated curcumin: in vitro and in vivo effects on proliferation, apoptosis, signaling, and angiogenesis.* Cancer, 2005. **104**(6): p. 1322-31.
40. yw, C., *Novel drug delivery system* 2nd ed. 1992, New York: Marcel Dekker Inc.

41. Crommelin, D., Schreier H, *Liposomes*. Colloidal drug delivery systems., ed. K. J. 1994, New York: Marcel Dekker. pp 73-190.
42. Vyas, S.P. and R.K. Khar, *Targeted & Controlled Drug Delivery: Novel Carrier Systems*. 1997, New Delhi: CBS publishers & distributors.
43. Jain SK, J.N., *Liposomes as drug carriers*, in *Controlled and Novel drug delivery*, J. NK, Editor. 1997, CBS Publishers: New Delhi. p. 304 – 352.
44. Amarnath Sharma, U.S.S., *Liposomes in drug delivery: progress and limitations* International Journal of Pharmaceutics, 1997. **154 (1997) 123 140**
45. Oku, N., *Anticancer therapy using glucuronate modified long-circulating liposomes.* Adv Drug Deliv Rev, 1999. **40**(1-2): p. 63-73.
46. Willis, M. and E. Forssen, *Ligand-targeted liposomes.* Adv Drug Deliv Rev, 1998. **29**(3): p. 249-271.
47. Ellerby, H.M., et al.., *Anti-cancer activity of targeted pro-apoptotic peptides.* Nat Med, 1999. **5**(9): p. 1032-8.
48. Arap, W., R. Pasqualini, and E. Ruoslahti, *Cancer treatment by targeted drug delivery to tumor vasculature in a mouse model.* Science, 1998. **279**(5349): p. 377-80.
49. Arap, W., R. Pasqualini, and E. Ruoslahti, *Chemotherapy targeted to tumor vasculature.* Curr Opin Oncol, 1998. **10**(6): p. 560-5.
50. Schiffelers, R.M., et al.., *Anti-tumor efficacy of tumor vasculature-targeted liposomal doxorubicin.* J Control Release, 2003. **91**(1-2): p. 115-22.
51. Brooks, P.C., et al.., *Integrin alpha v beta 3 antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels.* Cell, 1994. **79**(7): p. 1157-64.
52. Friedlander, M., et al.., *Definition of two angiogenic pathways by distinct alpha v integrins.* Science, 1995. **270**(5241): p. 1500-2.
53. Hynes, R.O., *A reevaluation of integrins as regulators of angiogenesis.* Nat Med, 2002. **8**(9): p. 918-21.
54. Gibson, K.A., et al.., *Expression of the alphaEbeta7 integrin by mast cells in rheumatoid synovium.* J Rheumatol, 2000. **27**(12): p. 2754-60.
55. Koivunen, E., B. Wang, and E. Ruoslahti, *Phage libraries displaying cyclic peptides with different ring sizes: ligand specificities of the RGD-directed integrins.* Biotechnology (N Y), 1995. **13**(3): p. 265-70.
56. Mastrobattista, E., G.A. Koning, and G. Storm, *Immunoliposomes for the targeted delivery of antitumor drugs.* Adv Drug Deliv Rev, 1999. **40**(1-2): p. 103-127.
57. Maruyama, K., et al.., *Possibility of active targeting to tumor tissues with liposomes.* Adv Drug Deliv Rev, 1999. **40**(1-2): p. 89-102.
58. Nallamothu, R., et al.., *A targeted liposome delivery system for combretastatin A4: formulation optimization through drug loading and in vitro release studies.* PDA J Pharm Sci Technol, 2006. **60**(3): p. 144-55.
59. Tripathi, D., et al.., *Chronic administration of losartan, an angiotensin II receptor antagonist, is not effective in reducing portal pressure in patients with preascitic cirrhosis.* Am J Gastroenterol, 2004. **99**(2): p. 390-4.
60. Allen, C., et al.., *Controlling the physical behavior and biological performance of liposome formulations through use of surface grafted poly(ethylene glycol).* Biosci Rep, 2002. **22**(2): p. 225-50.

61. Sapra, P. and T.M. Allen, *Internalizing antibodies are necessary for improved therapeutic efficacy of antibody-targeted liposomal drugs.* Cancer Res, 2002. **62**(24): p. 7190-4.
62. Sapra, P., P. Tyagi, and T.M. Allen, *Ligand-targeted liposomes for cancer treatment.* Curr Drug Deliv, 2005. **2**(4): p. 369-81.
63. Samuni, A.M., A. Lipman, and Y. Barenholz, *Damage to liposomal lipids: protection by antioxidants and cholesterol-mediated dehydration.* Chem Phys Lipids, 2000. **105**(2): p. 121-34.
64. Fukuzawa, K., et al.., *Antioxidative effect of alpha-tocopherol incorporation into lecithin liposomes on ascorbic acid-Fe2+-induced lipid peroxidation.* Arch Biochem Biophys, 1981. **206**(1): p. 173-80.
65. Hunt, C.A. and S. Tsang, *α-Tocopherol retards autoxidation and prolongs the shelf-life of liposomes.* International Journal of Pharmaceutics, 1981. **8**(2): p. 101-110.
66. Tirosh, O., et al.., *Hydration of polyethylene glycol-grafted liposomes.* Biophys J, 1998. **74**(3): p. 1371-9.
67. Prieve, M.G., et al.., *Differential importin-alpha recognition and nuclear transport by nuclear localization signals within the high-mobility-group DNA binding domains of lymphoid enhancer factor 1 and T-cell factor 1.* Mol Cell Biol, 1998. **18**(8): p. 4819-32.
68. Kirpotin, D., et al.., *Sterically stabilized anti-HER2 immunoliposomes: design and targeting to human breast cancer cells in vitro.* Biochemistry, 1997. **36**(1): p. 66-75.
69. Goren, D., et al.., *Nuclear delivery of doxorubicin via folate-targeted liposomes with bypass of multidrug-resistance efflux pump.* Clin Cancer Res, 2000. **6**(5): p. 1949-57.
70. Eliaz, R.E. and F.C. Szoka, Jr., *Liposome-encapsulated doxorubicin targeted to CD44: a strategy to kill CD44-overexpressing tumor cells.* Cancer Res, 2001. **61**(6): p. 2592-601.
71. Zalipsky, S., et al.., *Peptide attachment to extremities of liposomal surface grafted PEG chains: preparation of the long-circulating form of laminin pentapeptide, YIGSR.* Bioconjug Chem, 1995. **6**(6): p. 705-8.
72. Moreira, J.N., et al.., *A growth factor antagonist as a targeting agent for sterically stabilized liposomes in human small cell lung cancer.* Biochim Biophys Acta, 2001. **1514**(2): p. 303-17.
73. Willis, M.C., et al.., *Liposome-anchored vascular endothelial growth factor aptamers.* Bioconjug Chem, 1998. **9**(5): p. 573-82.
74. Tardi, P., M.B. Bally, and T.O. Harasym, *Clearance properties of liposomes involving conjugated proteins for targeting.* Adv Drug Deliv Rev, 1998. **32**(1-2): p. 99-118.
75. Hansen, C.B., et al.., *Attachment of antibodies to sterically stabilized liposomes: evaluation, comparison and optimization of coupling procedures.* Biochim Biophys Acta, 1995. **1239**(2): p. 133-44.
76. Martin, F.J., W.L. Hubbell, and D. Papahadjopoulos, *Immunospecific targeting of liposomes to cells: a novel and efficient method for covalent attachment of Fab' fragments via disulfide bonds.* Biochemistry, 1981. **20**(14): p. 4229-38.
77. Parr, M.J., et al.., *Factors influencing the retention and chemical stability of poly(ethylene glycol)-lipid conjugates incorporated into large unilamellar vesicles.* Biochim Biophys Acta, 1994. **1195**(1): p. 21-30.

78. Blume, G., et al.., *Specific targeting with poly(ethylene glycol)-modified liposomes: coupling of homing devices to the ends of the polymeric chains combines effective target binding with long circulation times.* Biochim Biophys Acta, 1993. **1149**(1): p. 180-4.
79. Maruyama, K., et al.., *Targetability of novel immunoliposomes modified with amphipathic poly(ethylene glycol)s conjugated at their distal terminals to monoclonal antibodies.* Biochim Biophys Acta, 1995. **1234**(1): p. 74-80.
80. Gabizon, A., et al.., *Targeting folate receptor with folate linked to extremities of poly(ethylene glycol)-grafted liposomes: in vitro studies.* Bioconjug Chem, 1999. **10**(2): p. 289-98.
81. Ishida, T., D.L. Iden, and T.M. Allen, *A combinatorial approach to producing sterically stabilized (Stealth) immunoliposomal drugs.* FEBS Lett, 1999. **460**(1): p. 129-33.
82. Iden, D.L. and T.M. Allen, *In vitro and in vivo comparison of immunoliposomes made by conventional coupling techniques with those made by a new post-insertion approach.* Biochim Biophys Acta, 2001. **1513**(2): p. 207-16.
83. Drummond, D.C., et al.., *Optimizing liposomes for delivery of chemotherapeutic agents to solid tumors.* Pharmacol Rev, 1999. **51**(4): p. 691-743.
84. Wheeler, J.J., et al.., *Polyethylene glycol modified phospholipids stabilize emulsions prepared from triacylglycerol.* J Pharm Sci, 1994. **83**(11): p. 1558-64.
85. Stamp, D. and R.L. Juliano, *Factors affecting the encapsulation of drugs within liposomes.* Can J Physiol Pharmacol, 1979. **57**(5): p. 535-9.
86. Ma, L., C. Ramachandran, and N.D. Weiner, *Partitioning of a homologous series of alkyl p-aminobenzoates in dipalmitoylphosphatidylcholine liposomes: effect of liposome type.* International journal of pharmaceutics, 1991. **77**(2): p. 127-140.
87. Heurtault, B., et al.., *Physico-chemical stability of colloidal lipid particles.* Biomaterials, 2003. **24**(23): p. 4283-300.
88. Heurtault, B., et al.., *The influence of lipid nanocapsule composition on their size distribution.* Eur J Pharm Sci, 2003. **18**(1): p. 55-61.
89. Ganapathi, R. and A. Krishan, *Effect of cholesterol content of liposomes on the encapsulation, efflux and toxicity of adriamycin.* Biochem Pharmacol, 1984. **33**(4): p. 698-700.
90. Taylor, K., et al.., *Drug entrapment and release from multilamellar and reverse-phase evaporation liposomes.* International journal of pharmaceutics, 1990. **58**(1): p. 49-55.
91. Talsma, H., M.J. Van Steenbergen, and D.J. Crommelin, *The cryopreservation of liposomes. 2. Effect of particle size on crystallization behavior and marker retention.* Cryobiology, 1992. **29**(1): p. 80-6.
92. Mohammed, A.R., et al.., *Liposome formulation of poorly water soluble drugs: optimisation of drug loading and ESEM analysis of stability.* Int J Pharm, 2004. **285**(1-2): p. 23-34.
93. Cannon, J.B., et al.., *Targeted delivery of a heme oxygenase inhibitor with a lyophilized liposomal tin mesoporphyrin formulation.* Pharm Res, 1993. **10**(5): p. 715-21.
94. Amselem, S., A. Gabizon, and Y. Barenholz, *Optimization and upscaling of doxorubicin-containing liposomes for clinical use.* J Pharm Sci, 1990. **79**(12): p. 1045-52.

95. Jay, M. and G.A. Digenis, *Enhanced entrapment of a quaternary ammonium compound in liposomes by ion-pairing.* J Pharm Sci, 1982. **71**(8): p. 958-60.
96. Lee, J.H., et al.., *Enhanced Entrapment of Isopropamide Iodide in Liposomes by Ion-Pairing with Sodium Taurodeoxycholate.* Drug Development and Industrial Pharmacy, 1988. **14**(4): p. 451-463.
97. Lim, H.J., et al.., *Role of drug release and liposome-mediated drug delivery in governing the therapeutic activity of liposomal mitoxantrone used to treat human A431 and LS180 solid tumors.* J Pharmacol Exp Ther, 2000. **292**(1): p. 337-45.
98. Zuidam, N.J., et al.., *Physical (in) stability of liposomes upon chemical hydrolysis: the role of lysophospholipids and fatty acids.* Biochim Biophys Acta, 1995. **1240**(1): p. 101-10.
99. Barenholz, Y., *Relevancy of drug loading to liposomal formulation therapeutic efficacy.* J Liposome Res, 2003. **13**(1): p. 1-8.
100. Lasic, D.D. and D. Papahadjopoulos, *Liposomes revisited.* Science, 1995. **267**(5202): p. 1275-6.
101. Bandak, S., et al.., *Pharmacological studies of cisplatin encapsulated in long-circulating liposomes in mouse tumor models.* Anticancer Drugs, 1999. **10**(10): p. 911-20.
102. Barenholz, Y., *Liposome application: problems and prospects.* Current Opinion in Colloid & Interface Science, 2001. **6**(1): p. 66-77.
103. Lasic, D.D., *Doxorubicin in sterically stabilized liposomes.* Nature, 1996. **380**(6574): p. 561-2.
104. Harashima, H., et al.., *Enhanced hepatic uptake of liposomes through complement activation depending on the size of liposomes.* Pharm Res, 1994. **11**(3): p. 402-6.
105. Woodle, M.C. and D.D. Lasic, *Sterically stabilized liposomes.* Biochim Biophys Acta, 1992. **1113**(2): p. 171-99.
106. Baban, D.F. and L.W. Seymour, *Control of tumour vascular permeability.* Advanced Drug Delivery Reviews, 1998. **34**(1): p. 109-119.
107. Yuan, F., et al.., *Vascular permeability in a human tumor xenograft: molecular size dependence and cutoff size.* Cancer Res, 1995. **55**(17): p. 3752-6.
108. Gabizon, A., et al.., *Effect of liposome composition and other factors on the targeting of liposomes to experimental tumors: biodistribution and imaging studies.* Cancer Res, 1990. **50**(19): p. 6371-8.
109. Gabizon, R. and S.B. Prusiner, *Prion liposomes.* Biochem J, 1990. **266**(1): p. 1-14.
110. Kirby, C., J. Clarke, and G. Gregoriadis, *Cholesterol content of small unilamellar liposomes controls phospholipid loss to high density lipoproteins in the presence of serum.* FEBS Lett, 1980. **111**(2): p. 324-8.
111. Kirby, C., J. Clarke, and G. Gregoriadis, *Effect of the cholesterol content of small unilamellar liposomes on their stability in vivo and in vitro.* Biochem J, 1980. **186**(2): p. 591-8.
112. Kirby, C. and G. Gregoriadis, *The effect of the cholesterol content of small unilamellar liposomes on the fate of their lipid components in vitro.* Life Sci, 1980. **27**(23): p. 2223-30.
113. Senior, J., et al.., *Influence of surface hydrophilicity of liposomes on their interaction with plasma protein and clearance from the circulation: studies*

with poly(ethylene glycol)-coated vesicles. Biochim Biophys Acta, 1991. **1062**(1): p. 77-82.
114. Allen, T.M., et al.., *Liposomes containing synthetic lipid derivatives of poly(ethylene glycol) show prolonged circulation half-lives in vivo.* Biochimica et Biophysica Acta (BBA) - Biomembranes, 1991. **1066**(1): p. 29-36.
115. Woodle, M.C., et al.., *Sterically stabilized liposomes. Reduction in electrophoretic mobility but not electrostatic surface potential.* Biophys J, 1992. **61**(4): p. 902-10.
116. Blume, G. and G. Cevc, *Molecular mechanism of the lipid vesicle longevity in vivo.* Biochim Biophys Acta, 1993. **1146**(2): p. 157-68.
117. Torchilin, V.P., et al.., *Poly(ethylene glycol) on the liposome surface: on the mechanism of polymer-coated liposome longevity.* Biochim Biophys Acta, 1994. **1195**(1): p. 11-20.
118. Klibanov, A.L., et al.., *Amphipathic polyethyleneglycols effectively prolong the circulation time of liposomes.* FEBS Lett, 1990. **268**(1): p. 235-7.
119. Webb, R.J., et al.., *Hydrophobic mismatch and the incorporation of peptides into lipid bilayers: a possible mechanism for retention in the Golgi.* Biochemistry, 1998. **37**(2): p. 673-9.
120. Webb, R.J., et al.., *Transmembrane alpha-helices in phospholipid bilayers.* Biochem Soc Trans, 1998. **26**(3): p. S309.
121. Barenholz, Y., et al.., *Stability of liposomal doxorubicin formulations: problems and prospects.* Med Res Rev, 1993. **13**(4): p. 449-91.
122. Grit, M. and D.J. Crommelin, *Chemical stability of liposomes: implications for their physical stability.* Chem Phys Lipids, 1993. **64**(1-3): p. 3-18.
123. Samuni, A.M. and Y. Barenholz, *Stable nitroxide radicals protect lipid acyl chains from radiation damage.* Free Radic Biol Med, 1997. **22**(7): p. 1165-74.
124. Samuni, A.M., et al.., *Gamma-irradiation damage to liposomes differing in composition and their protection by nitroxides.* Free Radic Biol Med, 1997. **23**(7): p. 972-9.
125. Zuidam, N.J., S.S. Lee, and D.J. Crommelin, *Sterilization of liposomes by heat treatment.* Pharm Res, 1993. **10**(11): p. 1591-6.
126. Cullis, P.R., et al.., *Influence of pH gradients on the transbilayer transport of drugs, lipids, peptides and metal ions into large unilamellar vesicles.* Biochim Biophys Acta, 1997. **1331**(2): p. 187-211.
127. van Winden, E.C.A., *Freeze-Drying of Liposomes: Theory and Practice*, in *Methods in Enzymology*, D. Nejat, Editor. 2003, Academic Press. p. 99-110.
128. van Winden, E.C., W. Zhang, and D.J. Crommelin, *Effect of freezing rate on the stability of liposomes during freeze-drying and rehydration.* Pharm Res, 1997. **14**(9): p. 1151-60.
129. Glavas-Dodov, M., et al.., *The effects of lyophilization on the stability of liposomes containing 5-FU.* Int J Pharm, 2005. **291**(1-2): p. 79-86.
130. Sarbolouki, M.N. and T. Toliat, *Storage stability of stabilized MLV and REV liposomes containing sodium methotrexate (aqueous & lyophilized).* PDA J Pharm Sci Technol, 1998. **52**(1): p. 23-7.
131. Komatsu, H., et al.., *Effects of the acyl chain composition of phosphatidylcholines on the stability of freeze-dried small liposomes in the presence of maltose.* Chem Phys Lipids, 2001. **113**(1-2): p. 29-39.

132. Peer, D., A. Florentin, and R. Margalit, *Hyaluronan is a key component in cryoprotection and formulation of targeted unilamellar liposomes.* Biochim Biophys Acta, 2003. **1612**(1): p. 76-82.
133. Sun, K. and D. Mauzerall, *Charge transfer across a single lipid-water interface causes ion pumping across the bilayer.* Biophys J, 1996. **71**(1): p. 309-16.
134. Sun, K. and D. Mauzerall, *Evidence for ion chain mechanism of the nonlinear charge transport of hydrophobic ions across lipid bilayers.* Biophys J, 1996. **71**(1): p. 295-308.
135. Zhdanov, R.I., et al.., *[New cationic liposomes for transfecting eukaryotic cells].* Dokl Akad Nauk, 1998. **362**(4): p. 557-60.
136. Crowe, L.M., et al.., *Preservation of freeze-dried liposomes by trehalose.* Arch Biochem Biophys, 1985. **242**(1): p. 240-7.
137. Harrigan, P.R., T.D. Madden, and P.R. Cullis, *Protection of liposomes during dehydration or freezing.* Chem Phys Lipids, 1990. **52**(2): p. 139-49.
138. Crowe, J.H., B.J. Spargo, and L.M. Crowe, *Preservation of dry liposomes does not require retention of residual water.* Proc Natl Acad Sci U S A, 1987. **84**(6): p. 1537-40.
139. Wolkers, W.F., et al.., *Preservation of dried liposomes in the presence of sugar and phosphate.* Biochim Biophys Acta, 2004. **1661**(2): p. 125-34.
140. Suzuki, T., H. Komatsu, and K. Miyajima, *Effects of glucose and its oligomers on the stability of freeze-dried liposomes.* Biochim Biophys Acta, 1996. **1278**(2): p. 176-82.
141. Ohtake, S., et al.., *Phase behavior of freeze-dried phospholipid-cholesterol mixtures stabilized with trehalose.* Biochim Biophys Acta, 2005. **1713**(1): p. 57-64.
142. Ugwu, S., et al.., *Preparation, characterization, and stability of liposome-based formulations of mitoxantrone.* Drug Dev Ind Pharm, 2005. **31**(2): p. 223-9.
143. Stevens, P.J. and R.J. Lee, *Formulation kit for liposomal doxorubicin composed of lyophilized liposomes.* Anticancer Res, 2003. **23**(1A): p. 439-42.
144. Tannock, I.F. and D.G. Warr, *Unconventional therapies for cancer: a refuge from the rules of evidence?* CMAJ, 1998. **159**(7): p. 801-2.
145. Tannock, I.F., *Conventional cancer therapy: promise broken or promise delayed?* Lancet, 1998. **351 Suppl 2**: p. SII9-16.
146. Jang, S.H., et al.., *Drug delivery and transport to solid tumors.* Pharm Res, 2003. **20**(9): p. 1337-50.
147. Jain, R.K., *Transport of molecules, particles, and cells in solid tumors.* Annu Rev Biomed Eng, 1999. **1**: p. 241-63.
148. Jain, R.K., *Barriers to drug delivery in solid tumors.* Sci Am, 1994. **271**(1): p. 58-65.
149. Hamberg, L.M., et al.., *Spatial heterogeneity in tumor perfusion measured with functional computed tomography at 0.05 microliter resolution.* Cancer Res, 1994. **54**(23): p. 6032-6.
150. Baxter, L.T. and R.K. Jain, *Transport of fluid and macromolecules in tumors. I. Role of interstitial pressure and convection.* Microvasc Res, 1989. **37**(1): p. 77-104.
151. Doyle, L.A., et al.., *A multidrug resistance transporter from human MCF-7 breast cancer cells.* Proc Natl Acad Sci U S A, 1998. **95**(26): p. 15665-70.

152. Molema, G., *Tumor vasculature directed drug targeting: applying new technologies and knowledge to the development of clinically relevant therapies.* Pharm Res, 2002. **19**(9): p. 1251-8.
153. Carmeliet, P. and R.K. Jain, *Angiogenesis in cancer and other diseases.* Nature, 2000. **407**(6801): p. 249-57.
154. Chaplin, D.J. and G.J. Dougherty, *Tumour vasculature as a target for cancer therapy.* Br J Cancer, 1999. **80 Suppl 1**: p. 57-64.
155. Kerbel, R.S., *Tumor angiogenesis: past, present and the near future.* Carcinogenesis, 2000. **21**(3): p. 505-15.
156. Denekamp, J., *Vascular attack as a therapeutic strategy for cancer.* Cancer Metastasis Rev, 1990. **9**(3): p. 267-82.
157. Reynolds, L.E., et al.., *Enhanced pathological angiogenesis in mice lacking beta3 integrin or beta3 and beta5 integrins.* Nat Med, 2002. **8**(1): p. 27-34.
158. Max, R., et al.., *Immunohistochemical analysis of integrin alpha vbeta3 expression on tumor-associated vessels of human carcinomas.* Int J Cancer, 1997. **71**(3): p. 320-4.
159. Folkman, J., *Addressing tumor blood vessels.* Nat Biotechnol, 1997. **15**(6): p. 510.
160. Stacy, D.R., B. Lu, and D.E. Hallahan, *Radiation-guided drug delivery systems.* Expert Rev Anticancer Ther, 2004. **4**(2): p. 283-8.
161. Kiani, M.F., et al.., *Targeting microparticles to select tissue via radiation-induced upregulation of endothelial cell adhesion molecules.* Pharm Res, 2002. **19**(9): p. 1317-22.
162. Baker, D.G. and R.J. Krochak, *The response of the microvascular system to radiation: a review.* Cancer Invest, 1989. **7**(3): p. 287-94.
163. Sikora, K., *The impact of future technology on cancer care.* Clin Med, 2002. **2**(6): p. 560-8.
164. Baker, D.G., *How significant are the risks from occupational exposure to ionizing radiation?* Appl Radiol, 1989. **18**(9): p. 19-21, 24.
165. Hallahan, D., J. Kuchibhotla, and C. Wyble, *Cell adhesion molecules mediate radiation-induced leukocyte adhesion to the vascular endothelium.* Cancer Res, 1996. **56**(22): p. 5150-5.
166. Hallahan, D.E., et al.., *Targeting drug delivery to radiation-induced neoantigens in tumor microvasculature.* J Control Release, 2001. **74**(1-3): p. 183-91.
167. Hallahan, D.E., et al.., *Drug-radiation interactions in tumor blood vessels.* Oncology (Williston Park), 1999. **13**(10 Suppl 5): p. 71-7.
168. Jahroudi, N., A.M. Ardekani, and J.S. Greenberger, *Ionizing irradiation increases transcription of the von Willebrand factor gene in endothelial cells.* Blood, 1996. **88**(10): p. 3801-14.
169. Jahroudi, N., A.M. Ardekani, and J.S. Greenberger, *An NF1-like protein functions as a repressor of the von Willebrand factor promoter.* J Biol Chem, 1996. **271**(35): p. 21413-21.
170. Verheij, M., et al.., *Ionizing radiation enhances platelet adhesion to the extracellular matrix of human endothelial cells by an increase in the release of von Willebrand factor.* Radiat Res, 1994. **137**(2): p. 202-7.
171. Eissner, G., et al.., *Critical involvement of transmembrane tumor necrosis factor-alpha in endothelial programmed cell death mediated by ionizing radiation and bacterial endotoxin.* Blood, 1995. **86**(11): p. 4184-93.

172. Donnelly, E.F., et al.., *Quantified power Doppler US of tumor blood flow correlates with microscopic quantification of tumor blood vessels.* Radiology, 2001. **219**(1): p. 166-70.
173. Yu, H., et al.., *A longitudinal study of radiation-induced changes in tumor vasculature by contrast-enhanced magnetic resonance imaging.* Radiat Res, 2002. **158**(2): p. 152-8.
174. Ruoslahti, E., *RGD and other recognition sequences for integrins.* Annu Rev Cell Dev Biol, 1996. **12**: p. 697-715.
175. Pierschbacher, M.D. and E. Ruoslahti, *Variants of the cell recognition site of fibronectin that retain attachment-promoting activity.* Proc Natl Acad Sci U S A, 1984. **81**(19): p. 5985-8.
176. Pierschbacher, M.D. and E. Ruoslahti, *Cell attachment activity of fibronectin can be duplicated by small synthetic fragments of the molecule.* Nature, 1984. **309**(5963): p. 30-3.
177. Pfaff, M., *Recognition Sites of RGD-Dependent Integrins*, in *Integrin-Ligand Interaction*. 1997, Springer US. p. 101-121.
178. Ruoslahti, E., *Integrin signaling and matrix assembly.* Tumour Biol, 1996. **17**(2): p. 117-24.
179. Hersel, U., C. Dahmen, and H. Kessler, *RGD modified polymers: biomaterials for stimulated cell adhesion and beyond.* Biomaterials, 2003. **24**(24): p. 4385-415.
180. Kok, R.J., et al.., *Preparation and functional evaluation of RGD-modified proteins as alpha(v)beta(3) integrin directed therapeutics.* Bioconjug Chem, 2002. **13**(1): p. 128-35.
181. Schraa, A.J., et al.., *Development of vasculature targeting strategies for the treatment of cancer and chronic inflammatory diseases.* Biotechnol Annu Rev, 2002. **8**: p. 133-65.
182. Schraa, A.J., et al.., *Endothelial cells internalize and degrade RGD-modified proteins developed for tumor vasculature targeting.* J Control Release, 2002. **83**(2): p. 241-51.
183. Schraa, A.J., et al.., *Targeting of RGD-modified proteins to tumor vasculature: a pharmacokinetic and cellular distribution study.* Int J Cancer, 2002. **102**(5): p. 469-75.
184. Schiffelers, R.M., et al.., *Ligand-targeted liposomes directed against pathological vasculature.* J Liposome Res, 2002. **12**(1-2): p. 129-35.
185. Schiffelers, R.M., et al.., *Targeting of liposomes to angiogenic endothelial cells.* Cell Mol Biol Lett, 2002. **7**(2): p. 255.
186. Pfaff, M., et al.., *Selective recognition of cyclic RGD peptides of NMR defined conformation by alpha IIb beta 3, alpha V beta 3, and alpha 5 beta 1 integrins.* J Biol Chem, 1994. **269**(32): p. 20233-8.
187. Aumailley, M., et al.., *Arg-Gly-Asp constrained within cyclic pentapeptides. Strong and selective inhibitors of cell adhesion to vitronectin and laminin fragment P1.* FEBS Lett, 1991. **291**(1): p. 50-4.
188. Haubner, R., et al.., *Structural and Functional Aspects of RGD-Containing Cyclic Pentapeptides as Highly Potent and Selective Integrin αVβ3 Antagonists.* Journal of the American Chemical Society, 1996. **118**(32): p. 7461-7472.
189. Haubner, R. and C. Decristoforo, *Radiolabelled RGD peptides and peptidomimetics for tumour targeting.* Front Biosci (Landmark Ed), 2009. **14**: p. 872-86.

190. Haubner, R., et al.., *Synthesis and biological evaluation of a (99m)Tc-labelled cyclic RGD peptide for imaging the alphavbeta3 expression.* Nuklearmedizin, 2004. **43**(1): p. 26-32.
191. Jiang, J., et al.., *Synthesis of RGD analogs as potential vectors for targeted drug delivery.* Bioorg Chem, 2001. **29**(6): p. 357-79.
192. N.N. *Gemzar*. 2009c; Available from: http://www.cancer.gov/Templates/drugdictionary.aspx?CdrID=41213. .
193. Massing, U. and S. Fuxius, *Liposomal formulations of anticancer drugs: selectivity and effectiveness.* Drug Resist Updat, 2000. **3**(3): p. 171-177.
194. Brandl, M., *Liposomes as drug carriers: a technological approach.* Biotechnol Annu Rev, 2001. **7**: p. 59-85.