

Chapter 2

Literature Review



2.1 Cancer

Introduction:

Cancer is an English term derived from the Greek word for crab, Karkinos, believed to be first used by Hippocrates, who attributed the adversity to an excess of black bile. Cancer was known in antiquity, being described in the early writings of Greek and Romans. Next to heart disease, cancer is the major killer of mankind. Irrespective of the aetiology, cancer is basically a disease of cell characterized by the loss of normal cell growth, maturation and multiplication, and thus homeostasis is disturbed (Barar, 2000).

The main features of cancer are

- Excessive cell growth (usually in the form of tumor)
- Invasiveness (ability to grow into surrounding tissue)
- Undifferentiated cells or tissues (more similar to embryonic tissue)
- Ability to metastasize or spread to new sites and establish new growths
- A type of acquired heredity in which the progeny of the cancer cells also retain cancerous properties.
- A shift of cellular metabolism towards increased production of macromolecules from nucleosides and amino acids, with an increased catabolism of carbohydrates for cellular energy.

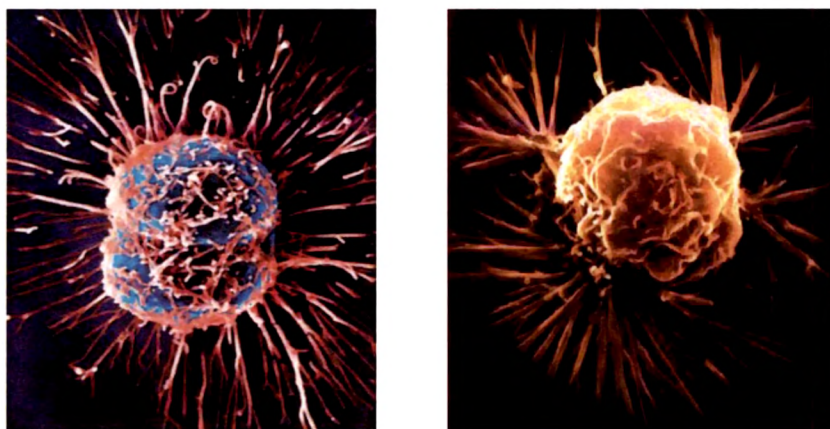


Figure 2.1. Dividing and Metastasizing Cancer cells

The magnitude of problem:

Cancer has become one of the leading causes of death for the past 50 years. Worldwide, every year more than 10 million people are diagnosed with cancer, and more than 6 million die from cancer. By 2020 WHO estimates that there will be some 20 million new cancer patients in the world each year. More than 70 % of these patients will be in developing countries.

There is no worldwide cancer registry; therefore, the incidence of cancer is estimated on the basis of the National Cancer Institute's Surveillance, Epidemiology, and End results (SEER) database which tabulate cancer incidence and death figures from the U.S. population. The *Cancer Trends Progress Report – 2007 Update*, includes key measures of progress along the cancer control continuum. In 2004, the rate of new cases of all cancers combined was 471.6 per 100,000 people per year. The death rate for all cancers was 185.7 cancer deaths per 100,000 people per year. Healthy People 2010 target reduce the overall cancer death rate to 158.6 cancer deaths per 100,000 people per year.

The treatment of cancer with drugs was started by Huggins and Hodges in 1941, with the discovery that estrogens palliate prostate cancer. Subsequently the polyfunctional alkylating agents were developed during World War II. Since then a number of chemotherapeutic agents have become available for the treatment of cancer. In the past, due to poor therapeutic response, and a high incidence of adverse reactions, chemotherapy was only considered as a resort after more successful treatments like surgery and radio therapy had failed. The main problem in cancer therapy is the lack of highly selective drugs, and the rapidly dividing normal cells of the bone marrow, gut, lymphoid tissue, spermatogenic cells, foetus and hair follicles are also killed (Barar, 2000).

According to the report a significant reduction in the rate of recurrence was observed following surgery for common solid tumors, such as breast and colorectal cancers. Overall, declining death rates have slowed. Importantly, refinements in treatment programs, thanks to several new, more effective drugs, have led to substantively better

survival rates for both of these diseases over the past five years. Based on further recent improvements in the treatment of advanced breast and colorectal cancers with antiangiogenic therapies, monoclonal antibodies, and molecularly targeted drugs, ability to significantly decrease the risk of relapse for these diseases after primary surgery should only continue to improve.

Measuring New Cancer Cases

In 2007, more than half of all new cancers were cancers of the prostate, breast, lung, and colon/rectum (Figure 2.3). It was projected that there would be 1,444,920 new cases of cancer in 2007, including 218,890 prostate cancers; 178,480 female breast cancers; 213,380 lung cancers; and 153,760 cancers of the colon/rectum.

Cancer incidence is usually measured as the number of new cases each year for every 100,000 people (for gender-specific cancers, people of the same gender serve as the denominator) and age-adjusted (to a standard population) to allow comparisons over time.

Cancer Survival

Advances in the ways cancer is diagnosed and treated have increased the number of people who live disease-free for long periods of time. This report looks at trends in 5-year survival rates for cancer, the time period traditionally associated with good prognosis. However, some people will have a recurrence of their cancer after 5 years.

In 2004 nearly 10.8 million Americans were alive who had been diagnosed with cancer. Of these, 2.4 million were diagnosed with female breast cancer, 2 million were diagnosed with prostate cancer, and 1.1 million were diagnosed with colorectal cancer. Approximately 628,339 (6 percent of the 10.8 million) Americans diagnosed with cancer were longer-term survivors diagnosed at least 29 years earlier.

Measuring Cancer Deaths

In 2004, cancers of the breast, prostate, lung, and colon/rectum accounted for more than half of all cancer deaths in the United States. Lung cancer alone claimed more than one-fourth of the lives lost to cancer. It was projected that in 2007 there would be 559,650 cancer deaths overall, including 160,390 deaths from lung cancer; 52,180 from cancers of the colon/rectum; 40,460 from female breast cancer; 33,370 deaths from cancer of the pancreas (replacing prostate cancer as the fourth leading cause) and 27,050 from prostate cancer. Cancer mortality usually is measured as the annual number of deaths from cancer for every 100,000 people, adjusted to a standard population.

Types of cancers

Benign tumors are not cancers whereas malignant tumors are cancers which divide without control and can invade nearby tissues or organs. Also, cancer cells can break away from the malignant tumor and enter the blood stream or the lymphatic system spreads from the original cancer site to form new tumor in other organs. The spread of cancer is called metastasis.

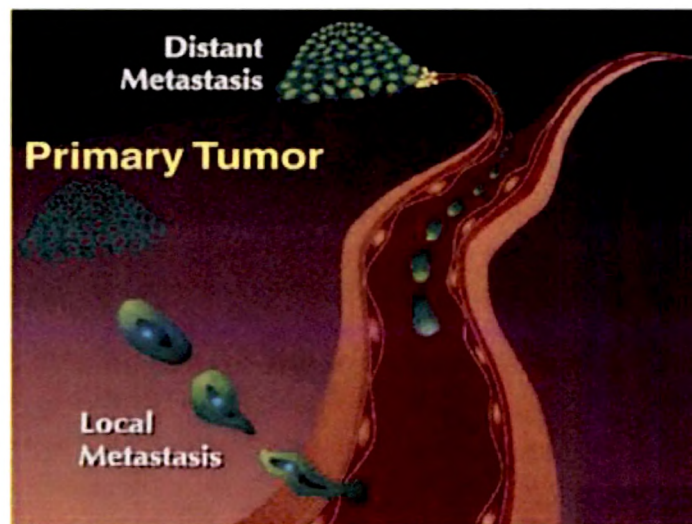


Figure 2.2. Depicting spread of cancer cells to other organs

Most cancers are named from the organ or type of cell in which they begin. Lung, breast, Bladder, colorectal, head & neck, endometrial, lymphoma, melanoma, leukemia, non-small-cell lung cancer, ovarian and prostate cancers are the most common cancers. Brain

tumor, cervical, esophageal, kidney, liver, oral, skin, small –cell lung, childhood sarcoma, chronic lymphocyte, multiple myeloma and adult non-Hodgkin's are the some of the less common cancers. Depending upon the body location or system, cancers can be divided as bone, brain, breast, childhood cancer, Digestive /Gastrointestinal, endocrine, eye, germ cell, gynaecologic, head & neck, leukemia, lung, lymphoma, neurologic, respiratory/thoracic and sarcoma/musculoskeletal (Sausville and Longo, 2001).

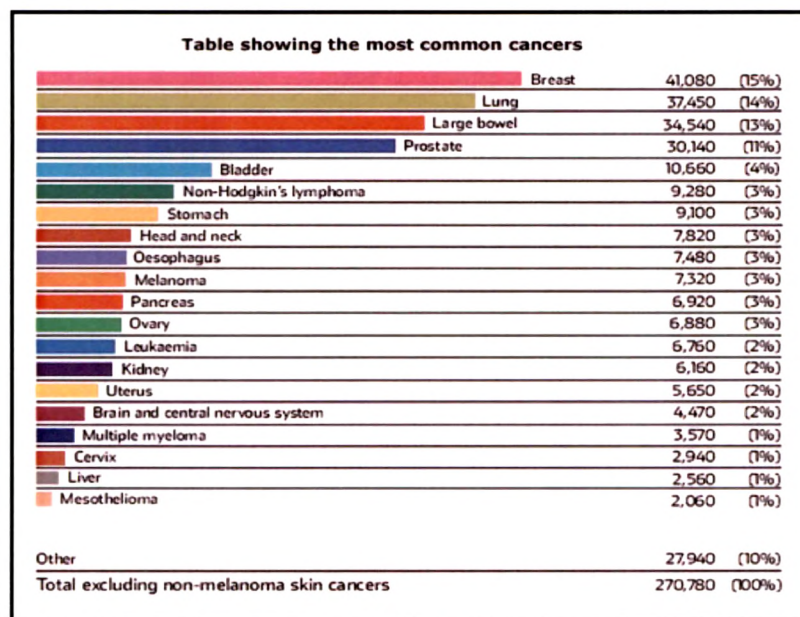


Figure 2.3. The most common cancers.

Cancer treatment

The cancer treatment aims for eradication of cancer. If this primary goal cannot be accomplished the goal of cancer treatment shifts to palliation, the betterment of the symptoms, and preservation of quality of life while attempting to extend life. Every cancer treatment has the potential to cause harm, toxicity with no benefit. The therapeutic index of many interventions is quite narrow and most treatments are given to the point of toxicity. One of the challenges of cancer treatment is to use the various treatment modalities alone and together in a fashion that maximizes the chances for patient benefit.

2.2 Methods of treatment

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

Chemotherapy

Historic background

The treatment of patients with cancer using chemicals in the hope of causing regressions of established tumors or to slow the rate of tumor growth arose by analogy to the proposition of Ehrlich that bacterial could be killed by compounds acting as “magic bullets”. Candidate compounds that might have selectivity for cancer cells were suggested by the marrow- toxic effects of sulphur and nitrogen mustards and lead, in the 1940’s, to the first notable regressions of haematopoietic tumours following use of these compounds by Gilman and colleagues. As these compounds caused covalent modifications of DNA was thereby identified as a potential target for drug design efforts.

Biochemical studies demonstrated the requirement of growing tumour cells for precursors of nucleic acid led to nearly contemporaneous studies by Farber of folate analogues. The cure of patients with advanced choriocarcinoma by methotrexate in the 1950’s provide further impetus to define the value of chemotherapeutic agents in many different tumor types. This resulted in efforts to understand unique metabolic requirements for biosynthesis of nucleic acids and led to the design, rationale for the time, of compounds that might selectively interdict DNA synthesis in proliferating cancer cells. The capacity of hormonal manipulations including oophorectomy and orchiectomy to cause regressions of breast and prostate cancers respectively provided a rationale for efforts to interdict various aspects of hormone function in hormone-dependent tumours. The serendipitous finding that certain poisons derived from bacteria or plants could affect normal DNA or mitotic spindle function allowed completion of the classic

armamentarium of “cancer chemotherapy agents” with proven safety and efficacy in the treatment of certain cancers.

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

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

Table 2.1. Current chemotherapy of various kinds of cancer

| CATEGORY | NAME OF THE DRUGS |
|---------------------|--|
| Lung cancer | Doxorubicin, Cyclophosphamide, Etoposide, Cisplatin, Methotrexate, Topotecan, Irinotecan, Paclitaxel |
| Breast cancer | Cyclophosphamide, Methotrexate, Fluorouracil, Doxorubicin, Anastrozole, Docetaxel, Paclitaxel |
| Ovarian cancer | Doxorubicin, Carboplatin, Cisplatin, Topotecan, Cyclophosphamide, Paclitaxel |
| Testicular cancer | Carboplatin, Cisplatin, Etoposide |
| GIT carcinoma | Doxorubicin, Carboplatin, Cisplatin |
| Colorectal cancer | Oxaliplatin, Fluorouracil, Irinotecan |
| Colon cancer | Fluorouracil |
| Bladder cancer | Carboplatin, Cisplatin, Etoposide |
| Pancreatic cancer | Gemcitabine, Methotrexate |
| Lymphatic Leukaemia | Doxorubicin, Methotrexate, Mercaptopurine, Cyclophosphamide, Etoposide, Daunorubicin |

The cell kill caused by cytotoxic drugs follows first order kinetics meaning that a constant percentage, rather than a constant number of cells is killed by a given dose of cytotoxic drugs. Precisely Cytotoxic drugs kill a constant fraction of the tumor cell population, but never a 100 percent cell kill. The current chemotherapy of various kinds of cancer is summarized in table 2.1.

Solid tumors

Over 85 % of human cancers are solid tumors (Jang et. al., 2003). The effectiveness of cancer therapy in solid tumors depends on adequate delivery of therapeutic agent to tumor cells. Inadequate delivery would result in residual tumor cells, which in turn would lead to regrowth of tumor and possibly development of resistant cells. Following a systemic administration, drug delivery to cells in a solid tumor involves 3 processes, i.e. transport within a vessel (e.g. blood circulation), transport across vasculature walls into surrounding tissues, and transport through interstitial space within a tumor (Jain, 1989). These process are determined by the physicochemical properties of a drug particle (e.g. molecular/particle size, diffusivity, drug binding to cellular macromolecules) and the biologic properties of a tumor (e.g. tumor vasculature, extracellular matrix components, interstitial fluid pressure (IFP), tumor cell density, tissue structure and composition).

Tumor structure and physiology

At the simplest level, the successful delivery of cytotoxic agents, whether small molecule, antibodies or liposomes to a solid tumor depends upon the relationship between the tumor cells and the blood vessels supporting their growth. Therefore the first requirement for the effective delivery is a fully functional vasculature. In solid tumor this criterion is rarely met. This neovasculature is responsible for continued growth of the tumor, through the delivery of the nutrients and removal of catabolites. The process by which the new vessels are formed, or “angiogenesis”, is a result of a complex programme of proteolytic and migratory events involving the endothelial cell (Zeman et. al., 1986).

Cell cycle

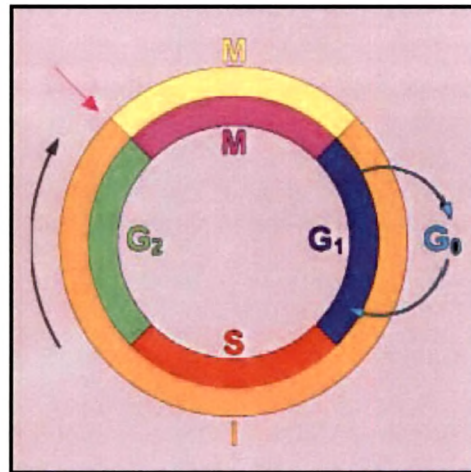


Figure 2.4. Stages of the cell cycle

Presynthetic phase (G₁): The interval following cell division to the point where DNA synthesis starts. Cells increase in size, produce RNA and synthesize protein.

S (DNA synthesis) phase: DNA replication occurs.

G₂ Phase: Cells continue to grow and produce new proteins

M (Mitosis) phase – Interphase: DNA replicates, the centrioles divide, and proteins are actively produced

M (Mitosis) phase: Each replicated chromosome comprises 2 chromatids

M (Mitosis) Phase: Mitotic spindle fibers align all chromosomes in one plane at the centre of the cell. Then spindle fibers shorten, and the chromatids (daughter chromosomes) are pulled apart and moving to the cell poles. The cell divided into 2 daughter cells.

Table 2.2. Classification of Cytotoxic drugs

| | |
|--|---|
| Alkylating agents | Nitrogen Mustards:(Chlorambucil, Chlormethine, Cyclophosphamide, Ifosfamide, Melphalan) Nitrosoureas; (Carmustine, Fotemustine, Lomustine, Streptozocin) Platinum: (Cisplatin, Carboplatin, Oxaliplatin) Busulfan, Decarbazine, Procarbazine, Temozolamide, Uramustine |
| Antimetabolites | Folic acid: (Aminopterin, Methotrexate, Pemetrexed) Purine: (Cladribine, Clofarabine, Mercaptopurine) Pyrimidines: (Capecitabine, Cytarabine, Fluorouracil, Gemcitabine) |
| Spindle poison /mitotic inhibitors (Plant alkaloids and terpenoids) | Taxanes (Paclitaxel, Docetaxel) Vinca alkaloids (Vincristine, Vinblastine, Vinorelbine) |
| Cytotoxic or Antitumor antibiotics | Anthracyclines family: (Daunorubicin, Doxorubicin, Epirubicin, Idarubicin, Mitoxantrone, Valrubicin) Streptomyces:(Actinomycin, Bleomycin, Mitomycin) Hydroxyurea. |
| Topoisomerase inhibitors | Camptothecins(Irinotecan, Topotecan) Podophyllotoxin (Etoposide, Teniposide) |
| Monoclonal Antibodies | Trastuzumab, cetuximab, Rituximab, Bevacizumab |
| Photosensitizers | Aminolevulinic acid, methyl amino levulinate, Verteporfin |
| Tyrosine kinase inhibitors | Dasatinib, Erlotinib, Gefitinib, Imatinib, Sunitinib |
| Others | Retinoids: 9 Alitretinoin, tretinoin)Amsacrine, Arsenic trioxide, BexaroteneEstramustine, Masoprocol, Mitotane. |
| Hormonal Therapy | Tamoxifen, Flutamide, Leuprolide, Finasteride |

2.3 Nanotechnology in cancer treatment

The multi disciplinary field of nanotechnology is emerging as a promising tool in the 21st century with the technological break through and is moving very fast from concept to reality. The flexibility to modify or adopt nanotechnology to meet the needs of pathological conditions either for therapeutic applications or as a diagnostic tool is the important characteristic of the technology. Pharmaceutical nanotechnology is the application of nanoscience to pharmacy. A concept of novel drug delivery approach using design of nanomedicine has been now well established in current pharmaceutical scenario.

Pharmaceutical nanotechnology has provided fine tuned diagnosis and treatment of disease at molecular level using various nanomedicines like liposomes, dendrimers, polymeric micelles, nanospheres, niosomes and more recently carbon nanotubes, quantum dots etc. Liposomal based drug delivery systems have created great impact on practically every branch of medicine including cardiology, ophthalmology, endocrinology, pulmonology, immunology and also on highly specialized areas like gene delivery, targeting to brain, tumor targeting, oral vaccine formulations and other areas. The pharmaceutical nanotechnology market especially in diagnostic and carrier for drug has been rapidly growing over last decade. Some pharmaceutical nanotechnology based products liposome, nanoparticles, polymer micelles, dendrimers, monoclonal antibody modified nanosystems have been approved by US-FDA and have entered the market (www.nano.cancer.gov).

Since the revolutionary discovery of Alec Bangham roughly 40 years ago that phospholipids in aqueous systems can form closed bilayered structures, liposomes have moved a long way from being just another interesting object of biophysical research to become a pharmaceutical carrier of choice for numerous practical applications. In the recent past, breakthrough in the application has been the selective delivery of the anticancer agent doxorubicin in polyethylene glycol (PEG) liposomes for the treatment of solid tumours in patients with breast-carcinoma metastases, which have resulted in a subsequent improvement in survival (Symon et. al., 1999; Perez et. al., 2002; O'Shaughnessy, 2003). A combination therapy comprising liposomal doxorubicin and Paclitaxel (Schwonzen et. al., 2000) or CAELYX[®] (Schering-Plough) (doxorubicin in PEG liposomes) and carboplatin (Goncalves et. al., 2003) has been used to target breast-carcinoma metastases. CAELYX[®] is also in Phase II clinical trials for patients with squamous cell cancer of the head and neck (Harrington et. al., 2001) and ovarian cancer (Johnston and Gore, 2001). Other indications targeted by liposomal formulations include amphotericin B for the treatment of visceral leishmaniasis (Sundar et. al., 2003) and long-acting analgesia with liposomal bupivacaine in healthy volunteers (Grant et. al., 2004).

This increasing number of liposomal formulations in the clinical trials as well as the markets today seems to show that these formulations have a very promising future.

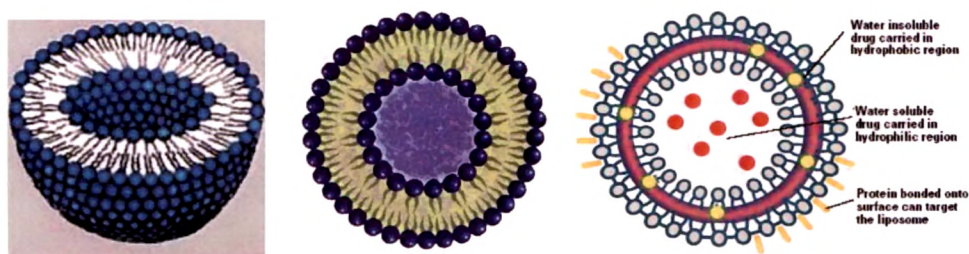


Figure 2.5. Different pictures showing bilayers structure of liposomes.

2.4 Development in Liposomal Drug Delivery

The major drawback of the use of liposomes is the fast elimination from the blood and liposomal preparations captured by the cells of the Reticulo-Endothelial System, primarily in the liver. A number of developmental methods have designed to reduce or overcome this problem.

Long-Circulating Liposomes

Different methods have been reported to achieve long circulation of liposomes *in vivo*, including coating the liposome surface with inert, biocompatible polymers, such as PEG, which form a protective layer over the liposome surface and delay the liposome recognition by opsonins and therefore subsequent clearance of liposomes (Klibanov et. al., 1990; Blume and Cevc, 1993). Long-circulating liposomes are widely used in biomedical *in vitro* and *in vivo* studies and they have also found their way into clinical practice (Gabizon, 2001). The significant feature of protective polymers is their flexibility, which allows a relatively small number of surface-grafted polymer molecules to create an impermeable layer over the liposome surface (Torchilin et. al., 1994; Torchilin and Trubetskoy, 1995). Long-circulating liposomes exhibit dose-independent, non-saturable, log-linear kinetics and increased bioavailability (Allen and Hansen, 1991).

Modern research on PEGylated liposomes focuses on attaching PEG in a removable manner to facilitate liposome capture by cells. After PEG-liposomes collect at the target site, through the enhanced permeability and retention (EPR) effect (Maeda et. al., 2001), the PEG coating is detached under the action of local pathological conditions (acidic pH in tumours).

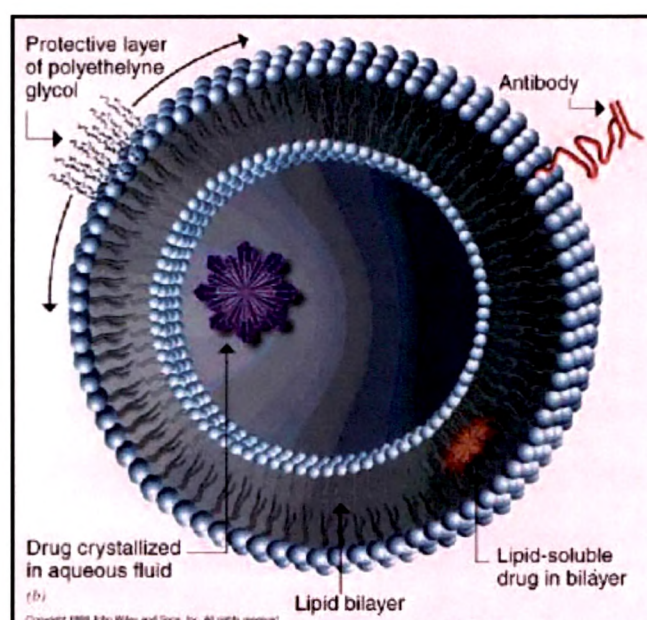


Figure 2.6. PEGylated Liposome

Although, PEG being the gold standard for the steric protection of liposomes, preparation of long-circulating liposomes have also been tried using poly[*N*-(2- hydroxypropyl) methacrylamide)](Whiteman et. al., 2001), poly-*N*-vinylpyrrolidones (Torchilin et. al., 2001), L-amino-acid-based biodegradable polymer-lipid conjugates (Metselaar et. al., 2003) and polyvinyl alcohol (Takeuchi et. al., 2001). Studies of the relative roles of the liposome charge and protective polymer molecular mass revealed that, opsonins with different molecular masses might be concerned in the clearance of liposomes containing differently charged lipids (Levchenko et. al., 2002).

Immunoliposomes.

To increase liposomal drug accumulation in the preferred tissues and organs, the use of targeted liposomes with surface-attached ligands capable of recognizing and binding to cells of interest has been recommended. Immunoglobulins (Ig) of the IgG class and their fragments are the most widely used targeting moieties for liposomes, which can be attached to liposomes, without affecting liposomal integrity or the antibody properties, by covalent binding to the liposome surface or by hydrophobic inclusion into the liposomal membrane after alteration with hydrophobic residues (Torchilin, 1985). In spite of improvements in targeting efficacy, the greater part of immunoliposomes accumulate in the liver as an outcome of insufficient time for the interaction between the target and targeted liposome, better target accumulation can be anticipated if liposomes can be made to remain in the circulation for a long time. Combinations of the properties of long-circulating liposomes and immunoliposomes in one preparation to overcome certain drawbacks of immunoliposomes have been reported (Torchilin et. al., 1992; Blume et. al., 1993; Abra et. al., 2002). Experiments have been initially performed by simple co-immobilization of an antibody and PEG on the surface of the same liposome, although the protective polymer can create steric hindrances for target recognition with the targeting moiety (Torchilin et. al., 1992). To attain better selectivity of PEG-coated liposomes, it is advantageous to attach the targeting ligand using a PEG spacer arm, so that the ligand is extended outside of the dense PEG brush, which reduces steric hindrance of binding to the target. At present, various advanced technologies are used, and the targeting moiety is usually attached above the protecting polymer layer, by coupling it with the distal water-exposed terminus of activated liposome-grafted polymer molecule (Blume et. al., 1993; Torchilin et. al., 2001).

2.5 New Ligands for Targeting Liposomes***Antibody-mediated liposome targeting***

The greater part of research in this area relates to cancer targeting, which utilizes a variety of antibodies. Internalizing antibodies are required to achieve a much-improved therapeutic efficacy with antibody-targeted liposomal drugs, as shown with B-lymphoma

cells and internalizable epitopes (CD19) (Sapra and Allen, 2002). An attractive idea was developed to target HER2-overexpressing tumours using anti-HER2 liposomes (Park et. al., 2001). The antibody CC52, which is directed against rat colon adenocarcinoma CC531 lines, was tagged to PEGylated liposomes and resulted in the restricted accumulation of liposomes in a rat model of metastatic CC531 (Kamps et. al., 2000). Nucleosome-specific antibodies capable of recognizing various tumour cells via tumour-cell-surface-bound nucleosomes enhanced Doxil (Alza) targeting to tumour cells and improved its cytotoxicity (Lukyanov et. al., 2004). Immunoliposomes containing the novel antineoplastic drug fenretinide, and targeting the ganglioside GD2, induced apoptosis in neuroblastoma and melanoma cell lines, and demonstrated strong antineuroblastoma activity both *in vitro* and *in vivo* in mice (Raffaghello et. al., 2003). The grouping of immunoliposome and endosome-disruptive peptide enhances the cytosolic delivery of the liposomal drug, increases cytotoxicity and opens up new avenues for developing targeted liposomal systems. This was revealed with the diphtheria toxin A chain, which was pooled with pH-dependent fusogenic peptide diINF-7 into integrated liposomes specifically targeted to ovarian carcinoma (Mastrobattista et. al., 2002).

Folate-Mediated Liposome Targeting

Folate-modified liposomes targeting to tumours represents a widespread approach, because folate receptors (FR) are recurrently over expressed in a range of tumor cells. Initial studies established the possibility of delivering macromolecules (Leamon et. al., 1991) and then liposomes (Lee and Low, 1994) into living cells using FR endocytosis, which could avoid multidrug resistance, interest in folate-targeted drug delivery by liposomes grew rapidly (Lu and Low, 2002; Gabizon et. al., 2004). Delivery of liposomal daunorubicin (Ni et. al., 2002) as well as doxorubicin (Pan et. al., 2003) to various tumour cells through FR, demonstrated increased cytotoxicity.

In recent time, folate-modified doxorubicin-loaded liposomes were applied for the treatment of acute myelogenous leukemia was combined with the induction of FR using all-*trans* retinoic acid (Pan et. al., 2002). The folate-targeted liposomes have been



projected as delivery vehicles for boron neutron capture therapy (Stephenson et. al., 2003) and also used for targeting tumors with haptens for tumor immunotherapy (Lu and Low 2002). In the field of gene therapy, folate-targeted liposomes have been used for both gene targeting to tumour cells (Reddy et. al., 2002) as well as for targeting tumours with antisense oligonucleotides (Leamon et. al., 2003).

Transferrin-Mediated Liposome Targeting

Transferrin (Tf) receptors (TfR) are over expressed on the surface of many tumor cells. So, antibodies against TfR, as well as Tf itself, are admired ligands for liposome targeting to tumors and inside tumor cells (Hatakeyama et. al., 2004). Modern studies have focused on the coupling of Tf to PEG on PEGylated liposomes to unite longevity and targetability for drug delivery into solid tumours (Ishida et. al., 2001). Identical approach was effective to the delivery of agents for photo-dynamic therapy, including hypericin, into tumours (Derycke et. al., 2002; Gijssens et. al., 2002), and for intracellular delivery of Cisplatin into gastric cancer (Iinuma et. al., 2002).

Increased binding and toxicity were demonstrated against C6 glioma cells (Eavarone et. al., 2000) by Tf-coupled doxorubicin-loaded liposomes. The increase in the expression of TfR was also revealed in post-ischaemic cerebral endothelium, which was used to deliver Tf-modified PEG liposomes to post-ischaemic brain in rats (Omori et. al., 2003). Tf (Joshee et. al., 2002) as well as anti-TfR antibodies (Xu et. al., 2002; Tan et. al., 2003) have also been used to support gene delivery into cells by cationic liposomes. Tf-mediated liposome delivery was also used for brain targeting effectively.

Immunoliposomes containing the OX26 monoclonal antibody, which is aimed to rat TfR, were found to assemble on brain microvascular endothelium (Huwyler et. al., 1996).

pH-sensitive liposomes

Of late, the focus of research in the area of liposomes has been the development of strategies to increase the ability of liposomes to mediate intracellular delivery of biologically active molecules. This resulted in the emergence of a modified form of

liposomes called the pH sensitive liposomes. These liposomes are stable at physiological pH (pH 7.4) but undergo destabilization, and acquire fusogenic properties under acidic conditions, thus leading to the release of their aqueous contents.

The theory of pH-sensitive liposomes emerged from the reality that certain enveloped viruses developed strategies to take benefit of the acidification of the endosomal lumen to infect cells, as well as from the survey that some pathological tissues (tumours, inflamed and infected areas) exhibit an acidic environment as compared to normal tissues (Torchilin et. al., 1993). Different classes of pH-sensitive liposomes have been proposed in the literature based on the mechanism of triggering pH-sensitivity (Torchilin et. al., 1993; Drummond et. al., 2000; Venugopalan et. al., 2002). The most commonly established hypothesis involves the blend of phosphatidylethanolamine (PE) or its derivatives with compounds containing an acidic group (e.g. carboxylic group) that act as a stabilizer at neutral pH (Ellens et. al., 1984; Liu and Huang, 1989; Duzgunes et. al., 1991; Torchilin, 2005). Contemporary studies describe the use of novel pH-sensitive lipids, synthetic fusogenic peptides/proteins either encapsulated (Mastrobattista et. al., 2002; Provoda et. al., 2003) or included in the lipid bilayer (Parente et. al., 1990; Ishiguro et. al., 1996; Bailey et. al., 1997; Nir et. al., 1999; Turk et. al., 2002), and attachment of pH-sensitive polymers with liposomes (Leroux et. al., 2001; Roux et. al., 2002; Mizoue et. al., 2002).

It is revealed that, liposomes need to be stable in biological fluids and establish long circulation times while administered intravenously, in turn to reach target cells (such as tumor cells) and mediate cytoplasmic delivery (Simoes et. al., 2004). Usage of lipids with high transition temperatures such as distearoylphosphatidylcholine (DSPC), hydrogenated soya PC (HSPC), the incorporation of cholesterol (Chol) and lipid conjugates such as phosphatidylethanolamine–poly (ethylene glycol) (PE–PEG), has led to a considerable decrease in leakage of the encapsulated drugs throughout circulation or in the extracellular environment. These lipids also diminish non-specific interactions between the liposomes and serum proteins (opsonins), thus avoiding liposome clearance by the cells of the reticuloendothelial system (RES). Furthermore, the use of liposomes of

size below 150 nm can contribute to the increase of the circulation time (Gabizon and Papahadjopoulos, 1988; Zalipsky, 1995; Woodle, 1995).

2.6 Mechanisms of Intracellular Delivery Mediated By pH-Sensitive Liposomes

Biophysical properties underlying the pH sensitivity of liposomes

In distinction to the majority of the phospholipids, PE presents a minimally hydrated and small head group which take up a lower volume as compared to the respective hydrocarbon chains, exhibiting a cone shape (as opposed to the cylinder shape of bilayer stabilizing phospholipids), thus obstructing the formation of a lamellar phase (Cullis and de Kruijff, 1979; Seddon et. al., 1983). The cone shape of PE molecules favors the formation of strong intermolecular interactions between the amine and phosphate groups of the polar head groups, illustrating the strong affinity of these molecules to acquire the inverted hexagonal phase above the phase transition temperature (T_H) (for DOPE the T_H is 10 °C). (Fig. 2.8)

Intercalation of amphiphilic molecules containing a protonatable acidic group (negatively charged at physiological pH) between PE molecules favors electrostatic repulsion and allows the formation of bilayer structures, which leads to liposome formation at physiological pH and temperature (Lai et. al., 1985; Duzgunes et. al., 1985). This successive approach constitutes the basis for the biophysical mechanisms underlying the pH-sensitivity exhibited by PE-containing liposomes. Although stable liposomes are formed at physiological pH, acidification triggers protonation of the carboxylic groups of the amphiphiles, reducing their stabilizing effect, leading to destabilization of liposomes, as under these conditions PE molecules revert into their inverted hexagonal phase (Torchilin et. al., 1993; Lasic, 1998). The selection of the amphiphilic stabilizers as well as its molar percentage with respect to the PE content are enforced by the desired properties of the liposomes, together with the extent of cellular internalization, the fusogenic ability, pH-sensitivity and stability in biological fluids. Above said properties determine the liposome efficacy to mediate cytoplasmic delivery of the encapsulated molecules (Torchilin et. al., 1993; Drummond et. al., 2000; Chu and Szoka, 1994).

Binding and Cell Internalization

It has been reported that pH-sensitive liposomes are internalized more effectively than non-pH-sensitive formulations (Schroit et. al., 1986; Chu et. al., 1990). This internalization has been attributed to the tendency of PE-containing liposomes to form aggregates, owing to the poor hydration of its head group, which can explain their high affinity to adhere to cell membranes (Chu and Szoka, 1994; Collins, 1995).

Different strategies have been explored based on receptor-mediated targeting through specific ligands coupled to the liposome surface, in an attempt to further improve their binding and cellular internalization. pH sensitive liposomes composed of DOPE/Oleic acid (OA) (4:2) or DOPE/OA/Chol (4:2:4) and targeted by the anti-H-2K^k antibody, showed a higher efficacy in mediating cytoplasmic delivery of their aqueous contents as compared to the same formulations missing the antibody (Collins and Huang, 1987; Connor and Huang, 1986; Wang and Huang, 1989).

These results concluded that: (a) the extent of liposome internalization is a vital step in the process of intracellular delivery and (b) receptor-mediated endocytosis is more effective than non-specific endocytosis. These observations suggest that fusion or destabilization of liposomes induced by acidification of the endosomal lumen represents the most important stage in the process of intracellular delivery.

Figure 2.7. demonstrates the main steps involved in the internalization and intracellular delivery mediated by pH-sensitive liposomes. After binding to cells, the liposomes are internalized by means of the endocytotic pathway, with or without the involvement of clathrin coated vesicles. Liposomes will be retained in early endosomes, independent of the internalization process, which mature into late endosomes. The capability of pH-sensitive liposomes lies in their potential to undergo destabilization at this stage, preventing their degradation at the lysosomal level, and therefore increasing entry to the cytosolic or nuclear targets (Collins, 1995; Yoshimura et. al., 1995).

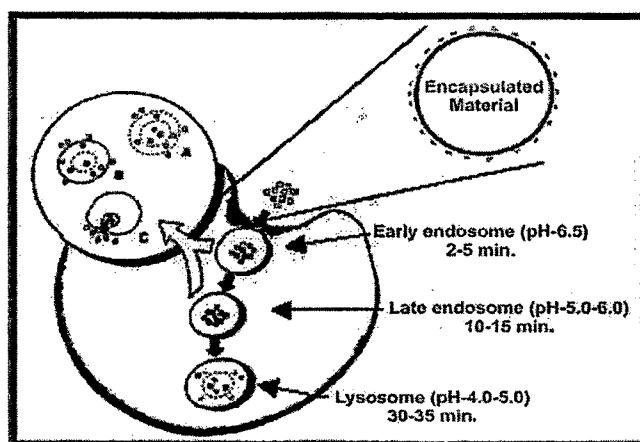


Figure 2.7. Hypothetical mechanism of internalization and intracellular delivery of pH-Sensitive liposomes.

Destabilization of Liposomes at the endosomal level

Investigations concerning the incubation of cells with lysosomotropic agents (e.g. ammonium chloride or chloroquine, which prevent endosome acidification) reveal that the efficacy of pH-sensitive liposomes depends on the drop in pH upon endosome maturation. Besides kinetic studies have shown that liposomes composed of DOPE/OA, DOPE/palmitoylthiomocysteine (PHC) or DOPE/dipalmitoylsuccinylglycerol (DSPG) (Collins et al 1989) or of DOPE/cholesteryl hemisuccinate (CHEMS) (Collins et. al., 1992) release their contents into the cytoplasm over a period of time that ranges from 5 to 15 min upon their incubation with the cells, thus signifying that cytoplasmic delivery occurs from early and late endosomes.

Three hypothetical mechanisms have been proposed (Fig. 1): (i) destabilization of pH-sensitive liposomes triggers the destabilization of the endosomal membrane, presumably through pore formation, leading to cytoplasmic delivery of their contents (ii) upon liposome destabilization, the encapsulated molecules diffuse to the cytoplasm through the endosomal membrane (iii) fusion between the liposome and the endosomal membranes, leading to cytoplasmic delivery of their contents (Collins, 1995; Ropert et. al., 1995). The fusogenic properties of PE associated with its trend to form an inverted hexagonal phase under certain conditions suggest that hypotheses (i) and (iii) are the most acceptable. At low pH (5.0) aggregation, release of contents and lipid intermixing are observed with

DOPE/CHEMS liposomes, while no intermixing of aqueous contents takes place (Ellens et. al., 1985). However, these liposomes are efficient in delivering their encapsulated contents into cultured cells (Chu et. al., 1990). Out of the involved mechanisms, the efficacy of cytoplasmic delivery mediated by pH sensitive liposomes is drastically reduced upon increase of the molecular weight of the encapsulated molecules. Investigations performed with high molecular weight proteins (e.g. DTA and BSA), shown that only 0.01–10 % of the molecules are released into the cytoplasm, in contrast to essentially 100 % release observed with low molecular weight fluorescent probes like calcein (Chu et. al., 1990).

2.7 Role of DOPE in pH Sensitive Liposomes

Dope liposomes and its mechanisms of cytosolic delivery

Liposomes composed of DOPE/phosphatidylglycerol (PG), DOPE/phosphatidylserine (PS), DOPE/PC and DOPE/CHEMS were incubated under very acidic conditions. Among these formulations, DOPE/CHEMS liposomes were those with the highest extent of cell association, while did not exhibit any pH-sensitivity (Simoes et. al., 2001). These results suggest that the processes underlying the intracellular efficacy of the different DOPE-containing liposomes involve more complicated mechanisms than the mere decrease of the endosomal pH. The combination of these results with those reported formerly (Slepishkin et. al., 1997) indicates that the presence of DOPE is the vital factor determining the ability of such liposomes to undergo destabilization upon acidification of the endosomes. Moreover, in those studies non-pH-sensitive liposomes composed of DSPC/CHEMS/DSPE-PEG were the only ones where a correlation between a lack of pH sensitivity in buffer and failure to mediate intracellular delivery was observed. Interestingly, studies on fusion between endocytotic vesicles (isolated from reticulocytes) and liposomes with different compositions showed that the presence of PE when compared with other phospholipids, namely PC, is essential to the fusion process (Vidal and Hoekstra, 1995). This exclusive effect of PE or DOPE to promote liposome–endosome interactions can be explained by the low hydration of its polar head group as compared to the significant repulsive forces associated with the hydration layer of PC or DSPC polar head groups (Schroit et. al., 1986). Hence, the presence of DOPE enhances

the hydrophobicity of the liposomal membrane, thus supporting dynamically favorable interactions between lipid bilayers. Moreover, DOPE tends to assume a hexagonal inverted phase (H_{II}) leading to the formation of non-lamellar structures (Litzinger and Huang, 1992). This may signify a key element to trigger endosomal destabilization, thus leading to cytoplasmic delivery of their contents. This idea partially explains observations on the striking similarity of the efficacy of intracellular delivery mediated by all DOPE-containing formulations. The treatment of endocytotic vesicles with trypsin strongly reduced their interaction with PE-containing liposomes (Vidal and Hoekstra, 1995), thus providing proof that endosome associated proteins play a major role in this process. Based on these investigations, it was proved that such proteins may not only be involved in the process of membrane fusion, but also promote liposome aggregation, which favors their destabilization. Similar results were obtained in lipid mixing studies involving pH-sensitive liposomes and human erythrocyte ghosts, where a decrease in the extent of lipid mixing was noticed by cleaving the sialic acid residues of the glycocalyx (Chu et. al., 1990).

A better understanding of the mechanisms underlying liposome-cell interactions were obtained from the results on studies with agents that interfere with the endocytotic pathway. The drastic reduction of the efficacy of intracellular delivery observed for all the formulations tested when their internalization was inhibited (using a mixture of antimycin A, sodium fluoride and sodium azide). This indicates that such liposomes utilize the endocytotic pathway to promote the intracellular release of their contents. In contrast, the strong inhibition observed for the calcein/rhodamine fluorescence ratio when the cells were treated with lysosomotropic agents demonstrates clearly that acidification of the endocytotic vesicles is important to the intracellular delivery mediated by the liposomes (Slepishkin et. al., 1997; Simoes et. al., 2001). Despite the similarity of the efficacy of intracellular delivery assessed in terms of calcein release, observed among the different liposomes tested, it should be noted that, such findings cannot be extrapolated directly to other types of encapsulated molecules, particularly to those with high molecular weights (Chu et. al., 1990). Additionally, it is also reported that the ability to mediate intracellular delivery of molecules with relatively high molecular weight (e.g. antisense oligonucleotides) is considerably higher for liposomes composed of

DOPE/CHEMS compared to other DOPE-containing liposomes (Duzgunes et. al., 2001). Formulations containing DOPE/PG, DOPE/PS and DOPE/PC were shown not to be pH-sensitive in buffer, proved to be less efficient in releasing large molecules into the cytoplasm.

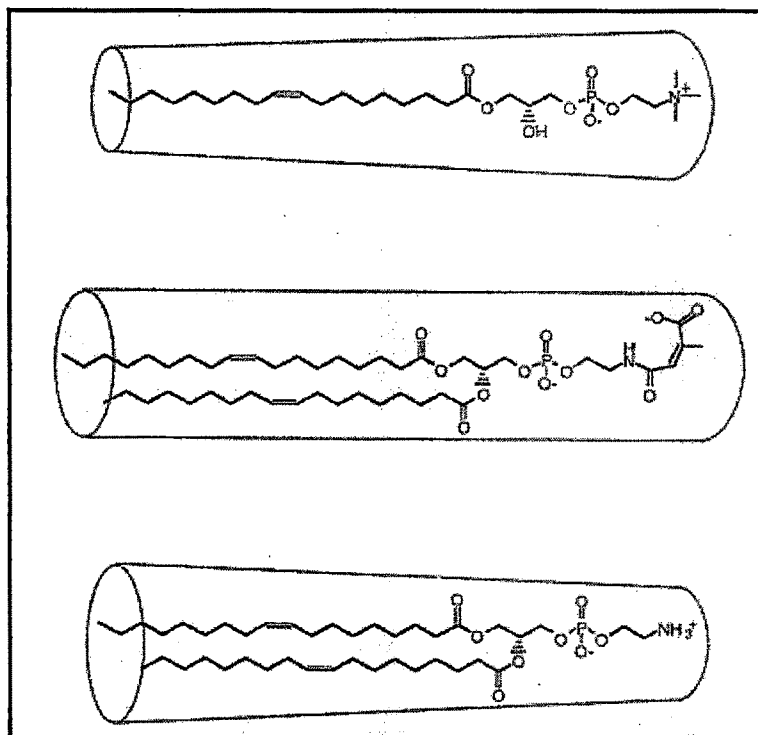


Figure 2.8. Molecular shapes of lipids -Lipids with a single acyl or alkyl chain such as lysolipids and detergents have a small molecular cross-sectional area in their hydrophobic acyl chains relative to their hydrophilic head groups, giving the lipid a conical shape (top). Most naturally occurring phospholipids such as phosphatidylcholine or sphingomyelin, or N-acylated phosphatidylethanolamines (middle) have an approximately equivalent cross sectional area in the head group and acyl chains, giving a cylindrical shape. Lipids such as unsaturated phosphatidylethanolamines (bottom) have a weakly hydrated head group and thus a small molecular area in the head group relative to the acyl chains. Lipids will form structures so as to maximize the interactions of water with both itself and hydrophilic groups on the lipids, while excluding water from hydrophobic areas (the hydrophobic effect). The types of structures formed by lipids are greatly influenced by their molecular shapes.

DOPE in long-circulating pH-sensitive liposomes

Destabilization of DOPE/OA liposomes was extensively observed upon their incubation with 90 % human plasma at physiological pH (Liu and Huang, 1994). Extraction of OA by serum albumin from the liposomes was considered to be responsible for this destabilization process. However, this effect was shown to be dependent on the size of the liposomes (Drummond et. al., 1999), since liposomes with an average size smaller than 200 nm were more stable in serum than larger liposomes, while the opposite was observed when serum was substituted with phosphate buffered saline (PBS). The stabilizing effect evolve from the fact that the high membrane curvature of the liposomes of small size may favor protein incorporation, like apoprotein A1 of the high-density lipoproteins (HDL), to replace the extracted OA (Liu and Huang, 1994). This increase in liposome stability leads to significant reduction in pH sensitivity, thus constituting a constraint for their appropriate in vivo use.

Different strategies considered for improving the biostability of pH-sensitive liposomes have been explained, including the inclusion of a third component to provide stability to the lipid bilayer. Addition of Chol in DOPE/OA liposome formulations resulted in a considerable increase in plasma stability, without decreasing their pH-sensitivity (Liu and Huang, 1989). The utilization of other amphiphilic stabilizers (such as Chol derivatives—CHEMS, or lipids with double acyl chains—DPSG) that were shown to be resistant to the extraction by albumin resulted in the formation of liposomes exhibiting higher stability in biological fluids, while maintaining their pH-sensitivity (Chu et. al., 1995; Collins et. al., 1990). The in vivo efficacy of pH sensitive liposomes depends strongly on the interactions with serum components (opsonins) that influence their pharmacokinetics and biodistribution apart from their limitations related to stability and changes in pH-sensitivity. Whereas the number of studies describing the in vivo use of pH-sensitive liposomes is limited, the acceptance is that upon their intravenous administration these liposomes are cleared quickly from blood circulation, accumulating in the liver and spleen (Torchilin et. al., 1993; Connor et. al., 1986; Slepishkin et. al., 1997; Liu and

Huang, 1990). On the contrary, the tendency of pH-sensitive liposomes to aggregate in the presence of biological fluids may justify their accumulation in the lungs (Connor et. al., 1986). Even though the fact that pH-sensitive liposomes exhibit a higher affinity to macrophages than non-pH-sensitive liposomes (Torchilin, 1993), their pharmacokinetics and biodistribution pattern are basically the same (Allen, 1992). Studies using pH-sensitive immunoliposomes (DOPE/ OA) established in vivo that the presence of an antibody at the liposomal surface neither affects their pharmacokinetics (namely the blood clearance rate) nor their biodistribution (Wang and Huang, 1987). pH-sensitivity and prolonged circulation time are highly desirable for the delivery of therapeutic macromolecules, such as nucleic acids, to cells. In this regard, pH-sensitive liposomes that can circulate in the blood for long periods and deliver encapsulated macromolecules to target cells may be useful. In vivo studies with the ganglioside GM₁ were shown to give relatively prolonged residence in circulation to pH-sensitive liposomes composed of DOPE and DSPG (Liu and Huang, 1990). The addition of lipids with covalently attached PEG in liposomes of various non-pH-sensitive compositions has been shown to conquer the problem of their rapid removal by the RES (Blume and Cevc, 1990; Klivanov et. al., 1990; Papahadjopoulos et. al., 1991; Senior et. al., 1991). One of the first compositions of liposomes with the combined properties of pH-sensitivity and prolonged circulation in vivo was developed and reported (Slepishkin et. al., 1997). The blood clearance curve of ¹¹¹In encapsulated in sterically stabilized pH-sensitive liposomes was similar to that of earlier developed liposomes with extended circulation time (Woodle et. al., 1992; Bakker-Woudenberg et. al., 1992). A sizeable percentage of liposomes (8.5%) remained after 24 h in the blood. On the contrary, the radioactive marker encapsulated in regular pH-sensitive liposomes was almost completely removed within 0.5 h from the bloodstream. The $t_{1/2}$ of control DSPC/CHEMS/PE-PEG liposomes and sterically stabilized pH-sensitive DOPE/CHEMS/PE-PEG liposomes was similar (11.8 ± 0.7 and 11.1 ± 0.6 h, respectively). The area under the curve for control liposomes was greater than that for sterically stabilized pH-sensitive liposomes ($1071 \pm 151\%$ and $629 \pm 52\%$ dose h/ml, respectively), most probably because a larger number of the latter was taken up quickly by the liver and spleen and therefore did not appear in blood samples. It is important to notice that the area under the curve for regular pH-sensitive liposomes

($6.47 \pm 1.24\%$ dose h/ml) was about 100-fold lower than that for sterically stabilized pH-sensitive liposomes. The radioactive marker encapsulated in the former liposomes was removed from the circulation moreover because it leaks out as a result of the interaction of plasma proteins with liposomes or due to phagocytosis of the liposomes by the cells of the RES. ^{111}In associated with the lipid membrane of liposomes to differentiate between these possibilities. Blood clearance curves of lipid-labeled liposomes demonstrated rapid elimination from the circulation of the lipid component of the regular pH-sensitive liposomes but not that of sterically stabilized pH-sensitive liposomes. This observation revealed that the elimination of the liposome-encapsulated water-soluble marker was mostly due to removal of the liposomes by the RES from the blood. ^{111}In encapsulated in either long circulating pH sensitive or control liposomes primarily accumulated in the spleen and liver which had similar patterns of distribution in vivo. High level of ^{111}In in the liver resulted after injection of regular pH sensitive liposomes. This observation and the rapid clearance of these liposomes from the circulation shows that liver cells take up most of the liposomes within an hour, making only a fraction of the encapsulated marker leak out and is then excreted in urine. A higher fraction of the DOPE/CHEMS/PE-PEG liposomes was localized in the liver and spleen, even after 24 h of injection compared with the non-pH-sensitive control DSPC/CHEMS/PE-PEG liposomes. Compared with the control liposomes the total urinary excretion of ^{111}In was lowered by about 2-folds. This result with the observation that the $t_{1/2}$ of both sterically stabilized liposomes were similar, suggesting that a higher fraction of the contents of pH-sensitive liposomes was accumulated and retained most likely in the liver and spleen because they were delivered into the cytoplasm and thus partially avoided the usual metabolic processing of liposome contents. In another attempt, DOPE-containing liposomes were stabilized in the bilayer form by the addition of a cleavable lipid derivative of PEG in which the polymer was attached to a lipid anchor via a disulfide linkage (PEG-S-S-DSPE) (Kirpotin et. al., 1996; Ishida et. al., 2001). An encapsulated dye was retained by liposomes stabilized with either a non-cleavable PEG (PEG-DSPE) or PEG-S-S-DSPE at pH 5.5. Though, treatment at this pH of liposomes stabilized with PEG-S-S-DSPE, with either dithiothreitol or cell-free extracts, caused contents release due to cleavage of the PEG chains and related destabilization of the DOPE liposomes. PEG-S-S-DSPE was rapidly

cleaved in circulation as suggested by the pharmacokinetic studies. Also, therapeutic studies performed in a murine model of B-cell lymphoma confirmed clearly that the developed pH-sensitive formulation targeted to the CD19 receptor was better than the stable, long-circulating, targeted non-pH-sensitive liposomes, even with the more rapid drug release and clearance of the pH-sensitive formulation (Ishida et. al., 2001). A novel category of pH-sensitive liposomes based on the incorporation of a PEG–diortho ester–distearoyl glycerol conjugate (POD) has been represented (Guo and Szoka Jr., 2001). This conjugate, comprising of a head group, an acid-labile diortho ester linker and a hydrophobic tail was shown to be stable at neutral pH for more than 3 h, but at pH 5 degraded completely within 1 h. Liposomes formulated of POD/DOPE (1:9) remained stable for up to 12 h in neutral buffer and in the presence of 75% fetal bovine serum (content release less than 25%), releasing their contents (84%) in the next 4 h. PEG head groups are cleaved off, leading to liposome aggregation at pH 5–6 releasing most of their contents in 10–100 min. A great potential for the rapid delivery of drugs/genes at therapeutic sites by these liposomes is due to the fast kinetics of acid catalyzed POD hydrolysis, where the decrease of pH is perhaps only one unit pH or less. The liver and intestine being the main sites of accumulation, the blood clearance pattern of these liposomes was monophasic, with an elimination half-life of 200 min. The incorporation of other lipids such as Chol is able to further improve the blood half-life of these liposomes remains to be seen. Liposomes composed of DOPE and Chol, together with the positively charged lipid, didodecyldimethylammonium chloride (DODAC) have been developed (Adlakha-Hutcheon et. al., 1999). The mixture was stabilized in a bilayer organization by including PE–PEG (30:45:15:10). Binding of the liposomes to the cells via electrostatic interactions was promoted by inclusion of cationic lipid. The use of different acyl chains with PE–PEG, leading to different exchange rates of this component from the lipid bilayer, provides the liposomes a time-dependent destabilization. For the delivery of mitoxantrone such an approach was evaluated. It was noted that the reduction in the length of the acyl chain of PE–PEG lipid (from DSPE to dimyristoylphosphatidylethanolamine (DMPE)) associates with an increasing rate of liposome clearance from blood, as well as with an increasing drug leakage rate. Almost 50% of the injected dose accumulated in the liver at 1 h, with only a small increase at

later times in the case of liposomes composed of PEG-DMPE, while for liposomes stabilized with PEG- DPPE liver accumulation occurred at later times (between 1 and 4 h). After 24 h less than 15% of the injected dose of PEG-DSPE-containing liposomes was observed in the liver (Adlakha-Hutcheon et. al., 1999). The therapeutic activity was tested either against a pseudometastatic leukemia murine model (L1210) or a human colon subcutaneous xenograft model (LS180) for mitoxantrone-containing liposomes. Upon intravenous administration, liposomes containing PEG-DMPE or PEG-DSPE showed a higher therapeutic activity (most animals showing disease-free survival) than the free drug or mitoxantrone-containing DSPC/Chol liposomes. Despite the different pharmacokinetic properties referred to above, both fusogenic formulations exhibited similar therapeutic activity. This can be described by the fact that in the leukemia model tumor cells are localized in readily accessible organs such as the liver and spleen (Adlakha-Hutcheon et. al., 1999). A different trend was observed, in the treatment of distal (non-RES) tumor sites, such as in the case of the subcutaneous LS180 model. Among the several treatments tested liposomes containing PEG-DSPE presented the highest activity, while the ability of liposomes containing PEG-DMPE to delay initiation of tumor growth was even lower than that observed for free drug. In this tumor model, the increased therapeutic activity is steady with the use of a moderately stable system that provides greater mitoxantrone bioavailability and delivery (Adlakha-Hutcheon et. al., 1999). In recent times, the same approach was used for both DNA and oligonucleotide delivery, except that another exchangeable PEG conjugate (PEG-ceramide) was incorporated into the lipid bilayer (Zhang et. al., 1999; Hu et. al., 2001).

2.8 Alternative Strategies under Acidic Conditions to Generate Fusogenic

Liposomes

While the development of pH-sensitive liposomes has been correlated normally with the inclusion of DOPE in the liposomal formulations, other strategies have also been investigated.

Uses of Novel pH-sensitive lipids

Inference obtained with a new type of pH-sensitive liposome formulation, composed of egg yolk phosphatidylcholine (EPC) liposomes bearing succinylated poly (glycidol), a PEG derivative having carboxyl groups, proved that under weakly acidic and acidic conditions fusion ability of the liposomes increases (Kono et. al., 1994). This has been shown to result in intensive and diffuse cytoplasmic fluorescence as a consequence of intracellular delivery of calcein. It is concluded according to the observations, that polymer-modified liposomes, upon endocytosis by CV-1 cells, transfer their content into the cytoplasm by fusing with the endosomal membrane (Kono et. al., 1997). Recently this strategy has been utilized to target anti-BCG antibody-bearing pH-sensitive liposomes to tumor cells expressing BCG antigen (Mizoue et. al., 2002). More recently, three different techniques have been engaged to generate pH-sensitive liposomes in the absence of DOPE (Guo et. al., 2002; Shi et. al., 2002; Sudimack et. al., 2002). Formulations with cationic/anionic lipid combinations were shown to be highly efficient vehicles for intracellular drug and gene delivery. Liposomes composed of EPC, dimethyldioctadecylammonium bromide (DDAB), CHEMS, and Tween-80 (25:25:49:1, mol/mol) were shown to stably entrap calcein at pH 7.4 and undergo destabilization and irreversible aggregation under acidic pH. These liposomes showed improved retention of pH-sensitivity in the presence of serum, compared to pH-sensitive liposomes containing DOPE (Shi et. al., 2002). One more hopeful strategy consisted of preparing anionic pH-sensitive liposomes composed of diolein/CHEMS (6:4). The outcome proves that these liposomes are stable at physiological pH, however undergo rapid aggregation and efficiently release encapsulated calcein at pH 5.0. Complexes formed upon association of the developed liposomes with DNA-protamine mixtures maintained their transfection activity in media containing up to 50% fetal bovine serum, on the contrary to what was observed for DOPE-containing liposomes (Guo et. al., 2002). Hopeful results in terms of pH-sensitivity and resistance to serum were also gained using a novel liposome formulation composed of PC, CHEMS, oleyl alcohol (OAlc), and Tween-80 (Sudimack et. al., 2002). The above formulation showed much better retention of its pH sensitive properties in the presence of 10 % serum, compared to DOPE-based pH-sensitive liposomes.

Liposomal co-encapsulation of synthetic fusogenic peptides and therapeutic molecules

To promote cytosolic delivery of hydrophilic molecules with limited access to sub cellular compartments the liposomal co-encapsulation of therapeutic molecules with peptides has turned out to be a promising strategy. Gelonin, a type I plant toxin, represents an example of such a compound known to inactivate ribosomes and arrest protein synthesis. The failure of this compound to permeate the plasma membrane and to escape efficiently from Endosomes into the cytosol, leads to its rapid degradation within endosomes and lysosomes, thus compromising its antitumor activity. To overcome this problem, gelonin was co-encapsulated inside pH-sensitive liposomes with listeriolysin O, the pore-forming protein that mediates escape of the intracellular pathogen *Listeria monocytogenes* from the endosome into the cytosol (Provoda et. al., 2003). This technique resulted in a significant improvement on the cytotoxicity of encapsulated gelonin against the murine B16 melanoma cell line, compared to free gelonin or gelonin encapsulated alone in pH-sensitive liposomes. The inclusion of PEG strongly decreased the cytotoxicity of the pH-sensitive formulation co-encapsulating gelonin and listeriolysin O. It is to be noticed whether the inclusion of PEG compromises the cell uptake and/ or the pH-sensitivity properties of the liposomes (Provoda et. al., 2003). A similar strategy has been explored (Mastrobattista et. al., 2002) to demonstrate that co-encapsulation of a pH-dependent fusogenic peptide (diINF-7) and diphtheria toxin A chain (DTA) in non pH- sensitive immunoliposomes promotes cytosolic delivery of the encapsulated macromolecule. This peptide (resembling the NH₂-terminal domain of influenza virus hemagglutinin HA-2 subunit) was used as functional characterization studies showed its capability to induce fusion between liposome membranes and leakage of liposome-entrapped compounds when exposed to low pH. In addition, co-encapsulation of diINF-7 into DTA-containing immunoliposomes resulted in significantly increased cytotoxicity toward ovarian carcinoma cells, indicating that this peptide can be used to obtain cytosolic delivery of liposome entrapped drugs with poor membrane permeation capacities (Mastrobattista et. al., 2002).

Synthetic fusogenic peptides/proteins with cationic liposomes

As cationic liposome/DNA complexes are believed to enter cells primarily through endocytosis, it has been theorized that the use of peptides that can destabilize endosomes or facilitate the fusion of the liposome/DNA complexes with the endosomal membrane would enhance gene delivery. Incubation of COS-7 cells with a β -gal-expressing plasmid, and anionic or cationic derivatives of the N-terminal peptide of the HA-2 subunit of the influenza virus fusion protein, hemagglutinin, in the presence of Lipofectin, resulted in the improvement of transfection activity by a factor of 2–7 over that of Lipofectin alone (Kamata et. al., 1994). The degree of Transfectam (lipopolyamine) - mediated transfection of H225 human melanoma cells could be increased by up to 1000-fold (over that obtained with a sub-optimal charge-equivalent (1.5) of Transfectam/DNA) by adding the hemagglutinin- derived peptide INF6 to the preformed lipoplexes (Kichler et. al., 1997). The correlation of the pH-sensitive peptide GALA (Subbarao et. al., 1987; Parente et. al., 1988) with DOTAP/DOPE (1:1) liposomes before complexation with plasmid DNA resulted in a significant enhancement in luciferase expression in COS-7 cells, which depended on the cationic liposome/DNA (+/-) charge ratio (Simoes et. al., 1998).

The capacity of certain peptides to cause endosomal destabilization due to their fusogenic properties may also be extended to proteins. Actually studies on the methods of gene delivery mediated by transferrin associated lipoplexes performed indicated that besides triggering internalization of the complexes, transferrin may also play a role in the cytoplasmic delivery of DNA by facilitating endosome destabilization (Aronsohn and Hughes, 1998). Indeed, transfection experiments carried out with cells pre-treated with drugs that prevent acidification of the endosomal lumen (bafilomycin A1 and chloroquine) demonstrated that a significant inhibition was observed not only in the levels of transfection activity, but also in the extent of release of complexed DNA into the cytoplasm, as assessed by fluorescence microscopy. Contradictory to the observation of diffused cytoplasmic fluorescence in the absence of the lysosomotropic drugs, treatment of cells resulted in punctate fluorescence restricted to intracellular organelles, suggesting that complexed DNA was incapable to escape from endosomes. Also, recent studies on the kinetics of the initial steps involved in lipoplex–cell interactions have suggest that

association of transferrin to lipoplexes increases the extent of fusion with endosomes. Generally, these results support the hypothesis that transferrin acquires fusogenic properties under acidic conditions by exposure of hydrophobic domains, thus facilitating endosomal disruption and intracellular release of DNA (Aronsohn and Hughes, 1998; Schenkman et. al., 1981). Alarmed by these results and by previous reports describing the ability of albumin to promote membrane fusion under acidic conditions (Zanta et. al., 1999; Chan and Jans, 1999) further studies showed that albumin could also function as a fusogenic protein that destabilizes endosomes under acidic conditions, thus enhancing intracellular gene delivery and transfection activity (Plank et. al., 1994).

pH-Sensitive polymers with liposomes

In the recent years, a number of studies have confirmed the potential of alkylated N-isopropylacrylamide (NIPAM) copolymers to provide pH-sensitivity to liposomes. Complexation of hydrophobically modified copolymers of NIPAM (either randomly or terminally alkylated) with EPC/Chol liposomes resulted in an improvement of in vitro release of both highly-water soluble markers and amphipathic drugs upon acidification (Leroux et. al., 2001; Zignani et. al., 2000; Roux et. al., 2002; Roux et. al., 2004). Randomly alkylated NIPAM anchored Liposomes containing ara-C were shown to mediate a higher cytotoxicity towards J774 macrophage-like cells as compared to non-pH-sensitive liposomes (Roux et. al., 2002). Recently, different NIPAM based copolymers were synthesized and estimated in terms of their ability to give pH-sensitivity, serum stability (Roux et. al., 2002; Roux et. al., 2003; Roux et. al., 2002) and steric stabilization to liposomes (Roux et. al., 2003; Roux et. al., 2002). Terminally alkylated NIPAM copolymer coating promotes steric stabilization of liposomes, as a result leading to prolonged blood circulation. However, as this effect was still considered insufficient for in vivo purposes, co-incorporation of PEG-lipid derivatives into the liposomal surface was evaluated. As anticipated, a considerable increase in blood circulation time was observed, blood clearance profiles being essentially analogous to that observed for stealth liposomes without NIPAM. Similar to earlier observations, the stabilizing effect of PEG noticeably reduced the liposome pH-sensitivity contributed by the copolymers (Slepushkin et. al., 1997; Roux et. al., 2003).

2.9 Applications of long circulating pH-Sensitive Liposomes for the delivery of anticancer drugs and other therapeutic molecules

The usefulness of pH-sensitive liposomes has been well exhibited in a wide variety of applications. These include: (i) the transport of fluorescent probes to estimate the efficacy of different liposome compositions and also to explain the mechanisms involved in intracellular trafficking; (ii) the effective delivery of neoplastic drugs or recombinant proteins; (iii) the intracellular transport of antigens, targeting at intracellular pathways involved in processing and presentation of antigens and enhancing the immune response to tumor cells; (iv) the intracellular transport of genetic material for application in gene and antisense therapies. Applications of pH-sensitive liposomes for the transport and intracellular delivery of anticancer drugs for cancer therapy are shown in Table 1. A large extent of work has been dedicated to improve the therapeutic efficacy of drugs entrapped in pH-sensitive liposomes, to confer tissue and cell specificity by directing the liposomes to cell surface receptors. Different ligands have been coupled covalently to the liposome surface or to the distal end of PEG-lipid conjugates for this purpose. These ligands comprise monoclonal antibodies against the H-2K^b receptor (expressed in several types of tumor cells) (Wang and Huang 1987), E-selectin (on activated vascular endothelial cells) (Spragg et. al., 1997), CD-19 (on B-lymphoma cells) (Ishida et. al., 2001), CD3 (on T-leukemia cells) (Turner et. al., 2002), P-glycoproteins (on endothelial cells) (Ng et. al., 2000) and BCG antigen (Mizoue et. al., 2002). The folate receptor targeted pH-sensitive liposomes via coupling of folic acid to the distal end of PEG molecules have been recently used to deliver neoplastic drugs (Shi et. al., 2002; Sudimack et. al., 2002) and plasmid DNA (Shi et. al., 2002; Reddy and Low, 2000; Lee and Huang, 1996). pH-sensitive liposomes have been revealed to be less effective than cationic liposomes to mediate intracellular gene delivery into mammalian cells under the same experimental conditions (Legendre and Szoka Jr., 1992). Different factors, not commonly unique, can explain this dissimilarity in transfection activity, including the lower amount of DNA encapsulated into pH-sensitive liposomes, their lower extent of cell internalization and the fact that once in the cytoplasm cationic liposomes could be more useful in protecting DNA against nucleases and in mediating its nuclear entry. Different methods have been

developed recently, to avoid the limitations of pH-sensitive liposomes for nucleic acid delivery. These approaches share a identical strategy, that basically consists of, complexing preformed pH-sensitive liposomes with plasmid DNA precondensed with a cationic polymer (Guo et. al., 2002; Shi et. al., 2002; Turner et. al., 2002; Reddy and Low, 2000; Reddy et. al., 1999). The described approaches allow the efficient condensation and protection of plasmid DNA, and targeting to a specific cell (through coupling of a PEG-lipid conjugate to a ligand), in comparison with “conventional” liposome formulations, resulting in improved transfection efficiency. These strategies were further optimized by promoting nuclear entry of DNA, which was achieved by incorporation of a nuclear targeting sequence into the plasmid DNA (Reddy et. al., 1999). While these approaches generated satisfactory results in vitro, their pharmacokinetics, biodistribution and in vivo gene delivery potential remain to be established. For nucleic acid delivery, the use of liposomes exhibiting both pH-sensitivity and steric stabilization has not been reported significantly. The approach of intracellular delivery of antisense oligonucleotides and ribozymes to inhibit virus production in HIV-infected macrophages derived from human peripheral blood monocytes has been assessed (Duzgunes et. al., 2001). 15-mer anti-Rev-responsive element (RRE) phosphorothioate oligonucleotide hindered viral p24 production by 91% when delivered by pH-sensitive liposomes, whereas the free (unencapsulated) oligonucleotide was not active against HIV infection in macrophages. The oligonucleotide was also efficient when delivered through sterically stabilized pH-sensitive DOPE/CHEMS/PE-PEG liposomes, but not when encapsulated in non-pH-sensitive liposomes. A non-specific oligonucleotide encapsulated in pH-sensitive liposomes had no effect at 1 μ M, but at 3 μ M it inhibited HIV infection by 53%. Identical trials were executed with a 38-mer chimeric ribozyme complementary to HIV 5'-LTR. Cationic liposome-mediated delivery of this ribozyme to HIV-1-infected cells could not effectively reduce virus production under conditions where the delivery method was not toxic to the cells (Konopka et. al., 1998). When this ribozyme was delivered by pH-sensitive liposomes to HIV-infected macrophages, virus production was inhibited by 88%, while the free ribozyme caused a decrease of only 10% (Duzgunes et. al., 2001). The inclusion of a low mole fraction of PE-PEG in the membrane of pH-sensitive

liposomes composed of DOPE/CHEMS results in prolonged circulation, without compromising their ability to deliver encapsulated molecules into macrophage-like cells (Slepushkin et. al., 1997). As these liposomes circulate for prolonged periods and can localize in lymph nodes after intravenous or subcutaneous injection (Allen et. al., 1993), they may be useful for the delivery of antisense molecules to lymph nodes where active HIV replication takes place (Pantaleo and Fauci, 1995; Pantaleo et. al., 1993). As reported earlier, other fusogenic liposome formulations exhibiting long circulation times have also been utilized for nucleic acid delivery. Programmable fusogenic vesicles (PFV), formerly revealed for the delivery of mitoxantrone, are liposomes composed of DOPE, Chol, DODAC and an exchangeable PEG-ceramide conjugate. When incorporating PEG-ceramide C₈ these systems were able to mediate high in vitro transfection levels of COS-7 and HepG2 cells. High levels of luciferase expression were also observed upon intraperitoneal administration into B16 intraperitoneal tumor-bearing mice. It is necessary to point out that gene expression in tumor tissue was considerably higher than that observed for cationic liposome/DNA complexes (Zhang et. al., 1999). PFV have also been assessed as carriers for the intracellular delivery of antisense oligonucleotides. PFV containing PEG-ceramide C₁₄ were shown to boost intracellular delivery of oligonucleotides relative to PFV displaying faster (with PEG-ceramide C₈) or lower (with PEG-ceramide C₂₀) rates of destabilization. A substantial drop (about 20% and 25%) in the levels of bcl-2 mRNA was found upon treatment of cells with PEG-ceramide C₁₄ liposomes containing the antisense oligonucleotide against the target proto-oncogene (Hu et. al., 2001).

Table 2.3. Applications of pH-sensitive liposomes to deliver anticancer drugs

| Liposome formulation (composition and method of preparation) | Encapsulated material (Drug) | Target disease/ therapeutic applications | Reference |
|---|--|---|---|
| DOPE/DSPG/DSPE-PEG (7:3:5) (reverse-phase evaporation) | Methotrexate | Cancer therapy | (Hong et. al., 2002) |
| PC/DDAB/CHEMS/Tween-80/folate-PE-PEG (25:25:49:1:0.1)PC/CHEMS/Tween-80/OAlc/ folate-PEG- Chol (50:50:2:80:0.5) (freeze- thawing) | Cytosine-β-D- arabinofuranoside | Cancer therapy (KB human oral cancer cells) | (Shi et. al., 2002; Sudimack et. al., 2002) |
| DOPE/CHEMS/PEG-S-S-DSPE/MAL-PEG- DSPE[anti-CD19] (6:4:0.12:0.06) (hydration method) | Doxorubicin | Cancer therapy (hematological malignancies, B- lymphoma) | (Ishida et. al., 2001) |
| DOPE/CHEMS (3:2) (freeze- thawing) | FICT-labeled antisense oligonucleotide | Cancer therapy (NG 108-15 neuroblastoma and glioma cells) | (Skalko-Basnet et. al., 2000; Skalko-Basnet et. al., 2002) |
| DOPE/N-citraconyl-DOPE/Chol/folate-PEG- DOPE (45.8:10:40:0.1) (freeze- thawing) | pDTS β-gal condensed with poly-L-lysine | Cancer therapy (transfection of KB human oral cancer cells) | (Reddy et. al., 1999) |
| DDAB/CHEMS/folate-PE-PEG (30:70:0.1) (freeze- thawing) | pCMV-Luc condensed with poly- L-lysine | | (Guo et. al., 2002) |
| Diolein/CHEMS (6:4) (ethanol injection) | pCMV-Luc condensed with poly- L-lysine | | (Shi et. al., 2002) |
| DOPE/CHEMS (2:1) (reverse-phase evaporation) | pSV2 luc, pRSV luc, pC luc and pCMV-β- Gal | Transfection of plasmid DNA into various mammalian cell lines | (Legendre and Szoka Jr., 1992) |
| DOPE/OA/Chol/DSPE-PEG-MAL [anti-CD3] (40:20:39.9:0.1) (hydration method) | pEGlacZ β-Gal condensed with poly- L-lysine | Transfection of Jurkat T-leukemia cells | (Turner et. al., 2002) |
| POPE/CHOH/MPL (7:3:0.01) (freeze- thawing) | Cytotoxic T lymphocytes (CTL) epitope peptides from Hantaan nucleocapsid protein (M6) or human papilloma virus E7; FITC- conjugated H-2K CTL epitope peptide | Tumor therapy mediated by CTL that recognize tumor- associated antigen | (Chang et. al., 2001) |
| POPE/CHOH/MPL (7:3:0.01) (freeze- thawing) | | Induction of antigen specific CTL response in vivo | (Lee et. al., 2002) |
| DOPE/HSPC/CHEMS/CHOL/ mPEG ₂₀₀₀ -DSPE (4:2:2:0.3) DOPE/HSPC/CHEMS/CHOL (4:2:2:2) (hydration method) | Doxorubicin | Cancer therapy (B lymphoma cells) | (Ishida et. al., 2006) |

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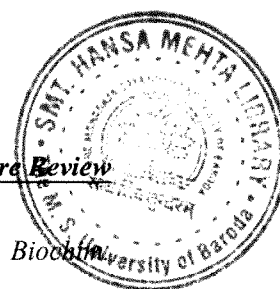
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