Chapter 2

Literature Review

Review Article: Microbubbles as an ultrasound contrast agent and drug delivering agent: Their composition, methods of preparation, characterization and mechanism of action. **Ultrasonics**. (Under communication)

2. INTRODUCTION

2.1 Cancer

2.1.1 General

Cancer is a disease characterized by the uncontrolled growth and potential spread of abnormal cells. It is an English word derived from the Greek word 'Karkinos' and first used by Hippocrates. The human body is made up of billions of cells and, normally, cells reproduce themselves 5by dividing so that growth occurs (1). Cancer cells have uncontrolled proliferation, invasiveness and capacity to metastasize. Occasionally, cells abnormally grow into a mass called a tumor. Some tumors are benign (noncancerous) and the others are malignant (cancerous). The growth of a benign tumor may interfere with body functions, such as urinating, but these tumors are seldom life threatening. Malignant tumors, on the other hand, invade and destroy normal tissue. By a process called metastasis, cells break away from a cancerous tumor and spread through the blood and lymph nodes to other parts of the body, where they form new tumors.

2.1.2 Costs for Cancers

The National Institutes of Health estimate overall annual costs for cancer at $107 billion; $37 billion for direct medical costs (total of all health expenditures), $11 billion for indirect morbidity costs (cost of loss of productivity due to illness) and $59 billion for indirect mortality costs (cost of loss of productivity due to premature death). Treatments for breast, lung, and prostate cancers account for over half of the direct medical costs. Insurance status and barriers to health care may affect the cost of treating cancer in this country. According to 2003 data, about 19% of Americans under age 65 have no health insurance, and about 26% of older persons have only Medicare coverage. During 2003, almost 18% of Americans reported not having a usual source of health care. Also, 12% of American families had members who experienced difficulty or delay in obtaining care or did not receive needed health care services (2).

Table 2.1: Carcinomas, sarcomas and mixed tissue tumors and their respective tissues (3)

Carcinomas/ Types of epithelial tissue	**Malignant tumor**
Gastrointestinal Tract	
Stomach	Gastric adenocarcinoma
Esophagus	Esophageal carcinoma
Colon	Adenocarcinoma of the colon
Liver	Hepatocellular carcinoma/ hepatoma
Glandular Tissue	
Thyroid	Carcinoma of Thyroid
Adrenal glands	Carcinoma of adrenals
Pancreas	Carcinoma of pancreas
Breast	Carcinoma of breast
Prostate	Carcinoma of prostate
Skin	
Squamous cell layer	Squamous cell carcinoma
Basel cell layer	Basal cell carcinoma
Melanocyte	Malignant melanoma
Lung	Adenocarcinoma of lung
	Small cell carcinoma
	Epidermoid carcinoma
Kidney and Bladder	Hypernephroma
	Transitional cell carcinoma of the bladder
Reproductive Organs	Cystadenocarcinoma of ovaries
	Adenocarcinoma of the uterus
	Squamous cell carcinoma of the cervix
	Seminoma and embryonal cell carcinoma
Sarcomas/ Types of connective tissue	**Malignant tumor**
Bone	Osteosarcoma
Muscle	
Smooth muscle	Lieomyosarcoma
Striated muscle	Rhabdomyosarcoma
Cartilage	Chondrosarcoma
Fat	Liposarcoma
Fibroust tissue	Fibrosarcoma
Blood vessel tissue	Angiosarcoma
Blood-forming tissue	
Leukocytes	Leukemia
Lymphocytes	Hodgkin's disease
	Non-Hodgkin's disease
	Burkitt's lymphoma
Plasma cells	Multiple myeloma
Nerve tissues	
Embryonic nerve tissue	Neuroblastoma
Neuroglial tissue	Astrocytoma
Mixed tissue tumors/ Types of tissue	**Malignant tumor**
Kidney	Wilm's tumor
Ovaries and testes	Teratoma

2.1.3 Classification of Cancer

About half of all cancer deaths are caused by malignancies that originate in lung, breast, or colon. However, in all there are more than 100 distinct types of cancer, each having a unique set of symptoms and requiring specific types of therapy. It is possible to divide these specific types of cancer in to three broad group on thee basis of histogenesis-that is, by identifying the particular tissue (hist/o) from which the tumor cells arise (-genesis). These major groups are called carcinomas, sarcomas, and mixed-tissue tumors. Carcinomas, the largest group, are solid tumors that are derived from epithelial tissue that lines external and internal body surfaces, including skin, glands, and digestive, urinary and reproductive organs. Approximately 90% of all malignant neoplasms are carcinomas. Sarcomas are less common and are derived from supportive and connective tissue, such as bone, fat, muscle, cartilage, bone marrow and from cells of immune (lymph) system. Sarcomas account for approximately 5 % of all malignant neoplasms. Mixed tissue tumors are derived from tissue that capable of differentiating into both epithelial and connective tissue.

2.2 Prostate Cancer

2.2.1 Introduction

The prostate is a gland of the male reproductive system. It is located in front of the rectum and just below the bladder, the organ that stores urine. The main purpose of the prostate is to produce fluid for semen, which transports sperm during the male orgasm. For men under 40 years of age, it is likely that the prostate has not caused any problems. But for men who are 40 and over, the prostate may become a source of problems. For instance, 1 in 10 men will develop prostate cancer. Prostate cancer is the second most common type of cancer among American men (4). Early stages of prostate cancer may not cause any symptoms; thus, yearly medical checkups are important. Other problems, such as benign prostatic hyperplasia (BPH) and prostatitis (inflammation of the prostate) may cause bothersome symptoms, such as difficulty in urinating.

2.2.2 Prostate Cancer Statistics

New Cases: An estimated 180,400 new cases in the US during 2000 (5). To put that number in perspective, there were more estimated new cases of prostate cancer diagnosed in 2000 than there were new cases of AIDS or even breast cancer. Prostate

cancer incidence rates remain significantly higher in black men than in white men. *Deaths:* An estimated 41,000 or more deaths in 1996 in United States (6), the second leading cause of cancer death in men (7). Although mortality rates are declining among white and black men, rates in black men remain more than twice as high as rates in white men. *Early Detection:* Men age 50 and older that have at least a 10-year life expectancy should talk with their health care professional about having a digital rectal exam of the prostate gland and a prostate-specific antigen (PSA) blood test every year. Men who are at high risk for prostate cancer (black men or men who have a history of prostate cancer in close family members) should consider beginning these tests at an earlier age. *Survival:* Seventy-nine percent of all prostate cancers are discovered in the local and regional stages; the 5-year relative survival rate for patients whose tumors are diagnosed at these stages is 100%. Over the past 20 years, the survival rate for all stages combined has increased from 67% to 92%. Survival after a diagnosis of prostate cancer continues to decline beyond five years. According to the most recent data, 67% of men diagnosed with prostate cancer survive 10 years and 52% survive 15 years. The incidence of prostate cancer increases with age. More than 75% of all prostate cancers are diagnosed in men over age 65. Some studies have shown an overall 2- to 3-fold increase in the risk of prostate cancer in men who have a history of this disease in their family. The incidence rate of prostate cancer is nearly two times higher in African-American men than Caucasian men.

2.2.3 Causes and Symptoms of Prostate Cancer

The exact cause of prostate cancer is unknown. Early prostate cancer usually does not have any symptoms. If left untreated, prostate cancer may spread from the prostate to nearby lymph nodes, bones, or other organs. This spread is called metastasis. As a result of metastases, many men experience aches and pains in the bones, pelvis, hips, ribs, and back. Unfortunately, there are often no early warning signs of prostate cancer and without regular screening; prostate cancer can go undetected for years. As the tumor grows, it may spread from the outer part of the prostate to the inner part of the prostate, eventually putting pressure on surrounding parts of the body, such as the urethra. This may block the flow of urine from the bladder and cause other urinary problems, which are usually the first symptoms of prostate cancer. Typical symptoms of prostate cancer are frequent urination (especially at night), weak urinary stream, inability to urinate, interruption of urinary stream (stopping and starting), pain or burning on urination and Blood in the urine.

2.2.4 Prostate cancer treatment options

Over the past 20 years, overall survival rates for all stages of prostate cancer combined have increased from 67% to 89% Some of the possible reasons for the increase in survival rates include public education, new techniques of early detection, and aggressive therapy. The major treatment options for prostate cancer include surgery, radiation, medical therapy, a combination of medical therapy and surgery or radiation, chemotherapy, and watchful waiting. A patient's treatment options will depend upon his age, the stage of the disease, and the advice of a physician. Table 2.2 and 2.3 show hormonal therapy and hormonal agents used for treatment of prostate cancer (8).

TABLE 2.2. Hormonal Agents for the treatment of Prostate Cancer

Class	Mechanism/action	Major side effects
Estrogens		
Diethylstilbestrol	Suppresses LH-RH secretion, ↓LH, ↓T	Cardiovascular events, loss of libido, impotence
LH-RH agonists		
Leuprolide Goserelin	Suppress LH-RH secretion, ↓LH, ↓T	"Flare" phenomenon, hot flashes, loss of libido, impotence
LH-RH antagonists		
Abarelix	Antagonizes LH-RH receptor, ↓LH, ↓T	Histamine release, loss of libido, impotence, hot flashes
Steroidal antiandrogens		
Cyproterone acetate Megestrol acetate	Antagonize AR in target tissues, suppress LH-RH secretion, ↓LH, ↓T	Cardiovascular events, fluid retention, gynecomastia, impotence
Non-steroidal antiandrogens		
Flutamide Nilutamide Bicalutamide	Antagonize AR in target tissues, ↑T	Gynecomastia, hepatotoxicity (flutamide), visual and respiratory disturbances and alcohol intolerance (nilutamide), GI problems
5-α-reductase inhibitors		
Finasteride Benzoquinoline	Inhibits type II 5a-reductase, ↑T, ↓DHT Inhibits types I/II 5a-reductase, ↑T, ↓DHT	Decreased libido, impotence
Adrenal androgen inhibitors		
Corticosteroids Ketoconazole	Suppresses ACTH secretion Inhibits P450 hydroxylase for adrenal and testicular steroidogenesis, ↓T	Mood changes Adrenal insufficiency, fatigue, GI problems

TABLE 2.3. Treatment Options as Hormonal Therapy for Prostate Cancer

Modality	Methodology	Advantages	Disadvantages
Surgical castration	Bilateral orchiectomy	Rapid ablation of testicular T Relative simple procedure Lower cost	Definitive castration Associated psychological problems Irreversible loss of libido and sexual potency Reduced muscle mass and energy Hot flashes Anemia and osteoporosis Unaffected adrenal androgens
Medical castration	Estrogens LH-RH agonists LH-RH antagonists	Reversible castration Ablation of testicular T More acceptable than orchiectomy	Cardiovascular events (estrogens) ''Flare'' phenomenon (LH-RH agonists) Loss of libido and sexual potency Reduced muscle mass and energy Hot flashes Anemia and osteoporosis Unaffected adrenal androgens
CAB	Castration + anti-androgen	Ablation of testicular T+ competitive inhibition of adrenal androgens	Increased side effects Antiandrogen withdrawal response
Antiandrogen monotherapy	Non-steroidal antiandrogen	Competitive inhibition of both testicular and adrenal androgens Retaining sexual potency Less severe side effects Oral administration only	Gynaecomastia Less effective
	Steroidal antiandrogen	CAB effect Oral administration only	Cardiovascular events Side effects due to lowering of serum T Gynaecomastia
IAB	Intermittent hormonal therapy	Longer androgen-sensitive period Reduced side effects and costs	Investigational may achieve continuous androgen ablation
TrAB	Intermittent CAB + 5αR inhibitor	Superior to IAB	Investigational
SAB	5αR inhibitor + anti-androgen or LH-RH agonist	Superior to monotherapy	Investigational

T, testosterone; LH-RH, luteinizing hormone-releasing hormone; CAB, complete androgen blockade; IAB, intermittent androgen blockade; TrAB, triple androgen blockade; 5aR, 5a-reductase; SAB, sequential androgen blockade.

2.3 Leukemia

2.3.1 Introduction

Leukemia is a cancer characterized by uncontrolled growth of one of the types of WBC. Normally, Blood consists of three types of cells: (a) Red blood cells (RBCs, or erythrocytes): these cells carry oxygen to all parts of the body and give the blood its red color. (b) Platelets (thrombocytes): these cells cause blood to clot when bleed. (c) White blood cells (WBCs, or leukocytes): these cells defend the body from infections. There are several different types of white blood cells Leukemia is a cancer characterized by uncontrolled growth of one of the types of WBC: (1) Granulocytes: fight bacteria by surrounding them and "eating" them. (2) Monocytes: fight germs, but aren't as specific as granulocytes. (3) B-lymphocytes: these cells attach antibodies on germs (or anything they don't think belongs) with antibodies, which in turn signal other WBCs to get the tagged germ. (4) T-lymphocytes: these cells signal orders to other WBCs to come to a germ, and they make those other WBCS stay at the battle sight.

RBCs, platelets, and WBCs are all made in the bone marrow. In fact, they all derive from one cell type, called a stem cell, which then differentiates into one of the three types. All of these products are formed in the bone marrow, a spongy area located in the center of bones. The bone marrow contains a small percentage of cells that are in development and are not yet mature. These cells are called blasts. Once the cell has matured, it moves out of the bone marrow and into the circulating blood. The body has mechanisms to know when more cells are needed and has the ability to produce them in an orderly fashion. In the case of leukemia, one blood cell goes awry (in the majority of cases this cell is a white blood cell) and the body produces large numbers of this cell. When looked at under a microscope, these abnormally produced cells look different than the healthy cells and do not function properly. The body continues to produce these abnormal, non-functional cells, leaving little space for healthy cells. This imbalance of healthy and unhealthy cells is what causes the symptoms of leukemia.

2.3.2 Classification of leukemia

There are several types of leukemia and these are classified by how quickly they progress and what cell they affect (9). The most common kinds of leukemia are: (a) Lymphocytic: uncontrolled growth of B- or T-lymphocytes (b) Myelogenous (granulocytic): uncontrolled growth of granulocytes. Leukemias are classified by two factors, how quickly the disease develops and what cells are affected. The disease is either classified as acute or chronic, referring to how quickly it develops and progresses. In acute leukemias, the white blood cells multiply very rapidly and are very immature, and therefore cannot function properly (immature cells are called blasts). Acute leukemias are most prevalent in children and are therefore often called "childhood leukemias". ALL, acute lymphocytic leukemia, means the blood contains abnormal, immature lymphocytes. These abnormal cells can resemble either a B-lymphocyte or a T-lymphocyte or neither at all. These abnormal cells are called lymphoblasts or blasts or leukemia cells. In chronic leukemia, the blasts form more slowly, allowing the body to continue to produce functional cells, causing fewer symptoms for the patient. The types are further divided by which type of white blood cell is affected - lymphoid cells or myeloid cells. These types are called lymphocytic leukemia and myelogenous leukemia, respectively. The types include: (1) Acute myeloid leukemia (also called AML) - occurs in both children and adults. (2) Acute lymphocytic leukemia (ALL) - the most common type seen in children, but also seen in adults over 65. (3) Chronic myelogenous leukemia (CML) - occurs mostly in adults. (4) Chronic lymphocytic leukemia (CLL) - most often seen in people over age 55, can affect younger adults, but almost never seen in children.

2.3.3 Causes and symptoms of leukemia

Several things have been identified as risk factors - that is, exposure to them puts a person at a higher risk of developing leukemia, but it is not a certainty that this exposure will lead to leukemia. These factors include exposure to high-energy radiation, like that released from a nuclear accident or bomb. Some genetic syndromes, such as Down's syndrome, put a person at higher risk. People who work with the chemical like benzene over a long period of time also have a greater chance of getting leukemia. Some scientists feel that exposure to electromagnetic fields, like those that come from power lines, may put a person at higher risk, but this has not been proven. The blast cells are unable to perform their normal function of fighting infection, so patients may develop fevers or infections that won't go away. As the

number of immature cells (blasts) increases, the normal cells are crowded out. This leads to low red blood cell counts and low platelet counts. A low red cell count is called anemia, which may cause the patient to feel tired or appear pale. A low platelet count affects blood clotting, causing the patient to bleed or bruise easily. In chronic leukemia, symptoms may not appear for some time, and when they first appear, they may be mild. Some common symptoms include: fever, chills, weakness and fatigue, swollen or tender lymph nodes, liver or spleen, easy bleeding or bruising, swollen or bleeding gums, night sweats, and bone / joint pain. In acute leukemia, the abnormal cells can accumulate in the brain or spinal cord, causing headaches, vomiting, confusion, or seizures.

2.3.4 Treatments for leukemia

Leukemia is treated by combination chemotherapy (10). This means that patients are given combinations of anticancer drugs. The combinations of anticancer drugs currently being used are called the standard protocol. The standard protocol is the culmination of years of investigative studies. Treatment is dependent on the type of leukemia, and may differ dependent on the patient's age, health and extent of the disease. Leukemia is a complex disease, and with about 30,800 cases per year in the United States, it is relatively rare. For this reason, it is recommended that patients receive treatment at a medical center that is experienced in treating the disease. Acute leukemias need to be treated quickly. The goal of therapy is to induce a remission, which means there is no evidence of leukemic cells, and the body returns to normal. Once this is achieved, patients often receive further therapy to prevent a relapse (return of the disease). Chronic leukemias may not need to be treated right away, depending on the symptoms at diagnosis. It has been thought in the past that chronic leukemias could never be cured, but this thinking has changed with the development of new therapies. Any child entering a treatment protocol for ALL in 1997 is given a 70% chance of cure. This means that two years after treatment has ceased, there is a 70% chance that blast cells will not be detected and this that doctor calls a cure. Certain factors lend to higher and lower risk factors. The oncologists to determine in which direction the treatment will take use these factors. Some factors count more than others.

Table 2.4: Factors considered during the treatment of leukemia

Towards low risk	Towards high risk
Under 10 years old	Over 10 years old
Girls	Boys
Low WBC (<50,000)	High WBC (>50,000)
Blasts not found in spinal fluid	Blasts found in spinal fluid
Pre B-cell	T-cell
No blasts detected in marrow day 7	Blasts detected in marrow day 7

2.3.4.1 Chemotherapy Drugs

The majority of the chemotherapy drugs used in the 1990s belong to the general group of medicines known as "antineoplastics" (2). Simply, "against neoplastics", neoplastics being cancer cells. Most antineoplastics work by stopping cell division in one or another stage of the cell cycle. They cause cell death in any dividing cell, and since most human cells are not dividing all the time, they preferentially kill cancer cells. But any human cells that divide frequently are also killed, for instance, cells in the gastrointestinal tract, bone marrow cells, and hair follicles. The chemotherapeutic agents used widely in leukemia are:

Anti-cancer agents:

- Daunorubicin, Doxorubicin, and Idarubicin
- Vincristine
- 6-Mercaptopurine and Thioguanine
- Methotrexate
- Cytoxan
- Cytarabine
- L-Asparaginase
- Steroids: Prednisone and Dexamethasone

2.3.5 Precautions during treatment

The white blood cells are monitored closely throughout the treatment. Without the right kind of white blood cells, the patients are not able to fight off an infection. The ANC, or Absolute Neutrophil Count measures the amount of "right kind" of white blood cell. The ANC is calculated by multiplying the percentage of "seg" and "bands" (neutrophil blood cells; the ones that fight infection) by the total white blood cell count. If the ANC is >1000, the patient can live a relatively normal life. If it is 500-1000, he is entering the danger zone. If it is <500, he has to avoid crowded

places. Fewer than 500 are designated "neutropenic". (These are words us parents like to throw around a lot.).

If a leukemia patient runs a fever of 101, he must immediately call the clinic to see if he has to be checked. Infections develop rapidly in these patients. Fevers are not taken lightly with leukemia patients. They usually mean the child has to be admitted to the hospital for a minimum stay of 48 hours. Infections can be fungal, bacterial, or viral. Fungal infections can be particularly nasty in a leukemia patient. Sometimes the infections are due to the medi-port lines; sometimes there is a fever and the doctors cannot find an infection.

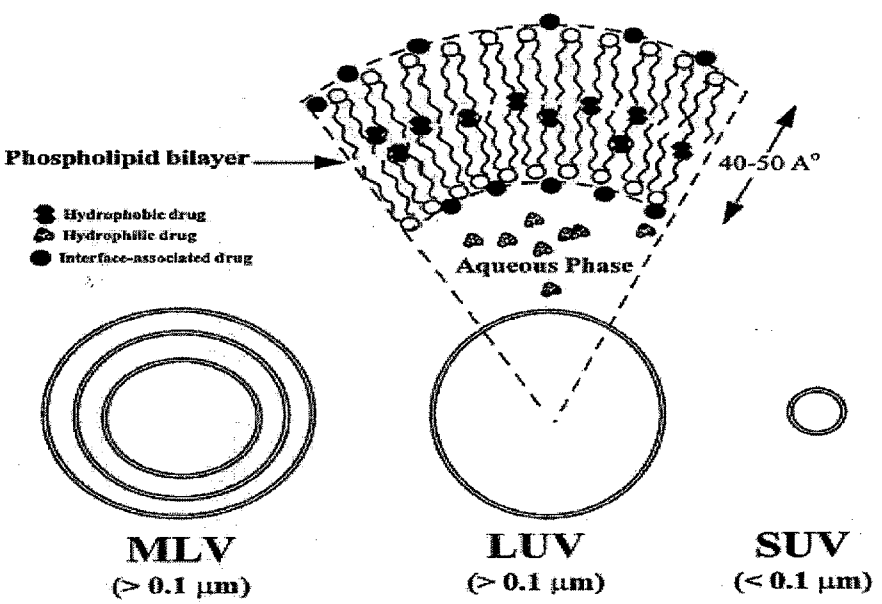
Chickenpox poses a real threat to leukemia patients. During treatment, patients cannot receive immunizations, unless directly approved by the oncologist. And, family members cannot get immunizations of live viruses during certain stages of treatment, because they could pass the infection to the cancer patient.

2.4 Liposomes

2.4.1 Introduction

At the turn of the 20th century, the German bacteriologist Paul Ehrlich coined the expression "magic bullets" in his search for chemotherapeutic agents with specific affinity for diseased tissues. Understanding target structure and function, developing drug delivery strategies to achieve controlled release, and targeting of drugs to specific tissues of the body have been a major focus of research in the last decades in an attempt to improve selectivity in cancer treatment. Shortly after they were first characterized (11), liposomes were proposed as drug carriers in cancer chemotherapy by Gregoriadis *et al.* (12). Since then, interest in liposomes as devices for drug delivery gradually increased, and they have been one of the main players in the cancer drug delivery area for the last 25 years. Liposomes are micro-particulate or colloidal carriers, usually 0.05–5.0 μm in diameter that form spontaneously when certain lipids are hydrated in aqueous media (13). Liposomes are composed of relatively biocompatible and biodegradable material, and they consist of an aqueous volume entrapped by one or more bilayers of natural and: or synthetic lipids (Fig. 2.1). Drugs with widely varying lipophilicities can be encapsulated in liposomes, either in the phospholipid bilayer, in the entrapped aqueous volume or at the bilayer interface (Fig.2.1).

Figure 2.1: Type and composition of liposomes



Liposomes have been investigated as carriers of various pharmacologically active agents such as antineoplastic and antimicrobial drugs, chelating agents, steroids, vaccines and genetic material (14). Due to recent developments in liposome technology, more effective strategies are now available for controlling the stability and reactivity of liposomes after systemic administration (15).

2.4.2 Classification of liposomes

2.4.2.1. *On the basis of size*

Table 2.5: Liposome classification by size and number of lamellae

Type	Usual size	Characteristics
MLV (Multilamellar vesicles)	\0.1 μm	More than one bilayer; moderate aqueous volume to lipid ratio (1–4 l: mole lipid); Greater encapsulation of lipophillic drugs; Mechanically stable upon long term storage; Rapidly cleared by RES; Useful for targeting the cells of RES; simplest to prepare; Prepared by thin-film hydration method
LUV (Large unilamellar vesicles)	\0.1 μm	Single bilayer high aqueous volume to lipid ratio (7 l: mole lipid); Useful for hydrophilic drugs; high capture of macro molecules; Rapidly cleared by RES; Prepared by detergent dialysis, ether injection, Reverse-phase evaporation (REV) or 'active loading' methods.
SUV (Small unilamellar vesicles)	50.1 nm	Single bilayer; homogenous in size; Thermodynamically unstable; Susceptible to aggregation and fusion at low or no charge; Limited capture of macromolecules; Low aqueous volume to lipid ratio (0.2–1.5 l: mole lipid); Long circulation half-life; Prepared by reducing the size of MLV or LUV using probe sonicator or gas extruder, or by 'active loading' or solvent injection techniques.

The liposome size can range from very small (0.025 μm) to large (2.5 μm) vesicles. Furthermore, liposomes may have single or multiple bilayer membranes (Fig. 2.1). The vesicle size is a critical parameter in determining circulation half-life of liposomes, and both size and number of bilayers influence the extent of drug encapsulation in the liposomes. On the basis of their size and number of bilayers, liposomes can also be classified into one of three categories: (i) multilamellar vesicles (MLV); (ii) large unilamellar vesicles (LUV); and (iii) small unilamellar

vesicles (SUV). The size and characteristics of these types of liposomes are listed in Table 2.5 (16).

2.4.2.2. *On the basis of composition*

Liposomes are composed of natural and: or synthetic lipids (phospho- and sphingo-lipids), and may also contain other bilayer constituents such as cholesterol and hydrophilic polymer conjugated lipids. The net physicochemical properties of the lipids composing the liposomes, such as membrane fluidity, charge density, steric hindrance, and permeability, determine liposomes' interactions with blood components and other tissues after systemic administration. The nature and extent of liposome–cell interaction in turn determines the mode of intracellular delivery of system (RES). Liposomes can be classified in terms of composition and mechanism of intracellular delivery into five types as: (i) conventional liposomes (CL); (ii) pH-sensitive liposomes; (iii) cationic liposomes; (iv) immunoliposomes; and (v) long-circulating liposomes (LCL). The typical composition and characteristics for these types of liposomes are listed in Table 2.6 (16).

Table 2.6: Liposome classification based on composition and mode of drug delivery

Type	Composition	Characteristics
Conventional liposomes (CL)	Neutral and: or negatively charged phospholipids plus Chol	Subject to coated-pit endocytosis; contents ultimately delivered to lysosomes, if they do not diffuse from the endosome; useful for RES targeting; rapid and saturable uptake by RES; short circulation half-life; dose-dependent pharmacokinetics (PK)
pH-sensitive liposomes	Phospholipid such as PE or DOPE with either CHEMS or OA	Subject to coated-pit endocytosis; at low pH, fuse with cell or endosome membranes and release their contents in cytoplasm; suitable for intracellular delivery of weak bases and macromolecules; biodistribution and PK similar to CL
Cationic liposomes	Cationic lipids: DDAB, DOGS, DOSPA, DOTAP, DOTMA, DMRIE, and DORIE with DOPE	Possibly fuse with cell or endosome membranes; suitable for delivery of negatively charged macromolecules (DNA, RNA, oligos); ease of formulation; structurally unstable; transfection activity decreases with time; toxic at high doses; mainly restricted to local administration
Long-circulating liposomes (LCL)	Neutral high *Tc* lipids, Chol. and 5–10% of PEG-DSPE, GM1 HPI; 50.1 mm in size	Hydrophilic surface coating; low opsonization and thus low rate of uptake by RES; long circulation half-life (40 h); dose-independent PK up to 10 μmol: mouse lipid dose
Immunoliposomes	CL or LCL with attached antibody or recognition sequence	Subject to receptor-mediated endocytosis; cell-specific binding (targeting); can release contents extracellularly near the target tissue and drugs may diffuse through plasma membrane to produce their effects

2.4.3 Methods of preparation

Liposomes of different sizes and characteristics usually require different methods of preparation. The most simple and widely used method for preparation of MLV is the thin-film hydration procedure in which a thin film of lipids is hydrated with an aqueous buffer at a temperature above the transition temperature of lipids. The drug to be encapsulated is included either in the aqueous hydration buffer (for hydrophilic drugs) or in the lipid film (for lipophillic drugs). Thin-film hydration method produces a heterogeneous population of MLV (1–5 mm diameter), which can be sonicated or extruded through polycarbonate filters to produce small (up to 0.025 mm) and more uniformly sized population of SUV. Although thin-film hydration is a simple technique, one of the major disadvantages of this method is its relatively poor encapsulation efficiency (5–15%) of hydrophilic drugs. Moreover, reduction of liposome size further decreases the amount of encapsulated drug. MLV with high entrapment efficiency (up to 40%) can be prepared by freeze-drying preformed SUV dispersion in an aqueous solution of the drug to be encapsulated (17). Hydration can also increase the encapsulation efficiency of MLV lipid in the presence of an organic solvent (18-19). Several methods have been developed for the preparation of large, unilamellar vesicles (LUV), including solvent (ether or ethanol) injection, detergent dialysis, calcium induced fusion, and reverse- phase evaporation (REV) techniques. SUV can be prepared from MLV or LUV by sonication (using probe sonicator) or extrusion (passage through a small orifice under high pressure). In the methods described above, an amphiphilic ionizable drug, which exhibits lipophillic and hydrophilic properties depending on the pH of the solution, may not be encapsulated with high efficiency because the drug molecules can diffuse in and out of the lipid membrane. Thus, the drug would be difficult to retain inside liposomes. However, these types of drugs can be encapsulated into preformed liposomes with high efficiency (up to 90%) using the 'active loading' technique (20). In the 'active loading' method, the pH in the liposome interior is such that the unionized drug which enters the liposome by passive diffusion is ionized inside the liposome, and ionized drug molecules accumulate in the liposome interior in high concentrations due to their inability to diffuse out through the lipid bilayer. For example, doxorubicin and epirubicin may be entrapped in preformed SUV with high efficacy using 'active loading' methods (21-22). Detailed review of methods of liposome preparation is reported elsewhere (23-26).

2.4.4 Disadvantages of conventional liposomes[27]

(1) The pronounced tendency for liposomes to localize in reticulo-endothelium system raises concerns about RE impairment and its consequences particularly during continued liposomes administration.

(2) Short circulation times severely limit the use of liposomes as micro reservoir systems for the slow release of biologically active molecules, which are normally degraded rapidly within the vasculature.

(3) Rapid uptake of liposomes into liver and spleen greatly reduces the possibility of extra vascularization of liposomes and substantially prevents targeting of liposomes to the cells within the vasculature or targeting to non-RE tissues.

2.5 Stealth liposomes or Long circulating liposomes (LCL)

2.5.1 Introduction

Liposomes that are formulated to "escape" from being recognized by RES can remain in circulation for prolonged periods and may serve as micro reservoir system and may minimize the problems associated with conventional liposomes. The term "Stealth" liposomes (28) was coined to describe this evasive property (stealth is a registered Trademark of Liposome Technology Inc, Menlopark, CA (USA) and polyethylene glycol lipids (PEG-lipids) are commonly referred to as Stealth Lipids). Liposome membranes containing bilayer-compatible species such as poly (ethylene glycol)-linked lipids (PEG-lipid) or the gangliosides (29-36) are being used to prepare stealth liposomes. These so called "Stealth" liposomes (28) have a relatively long half-life approximately 1 day (whereas the conventional liposomes have only -minutes) in blood circulation and show an altered biodistribution *in vivo.* Sterically stabilized liposomes (Stealth liposomes) can be formulated by incorporating hydrophilic long-chain polymers in the bilayer, which can form a coat on the liposome surface and repel opsonins penetration and adsorption. Reduction in 'marking' by opsonins leads to slower uptake of these liposomes. Thus, stealth liposomes exhibits extended circulation half-life compared to the so-called 'conventional liposomes' (CL) because of their reduced recognition and uptake by the RES. Furthermore; LCL can be designed to exhibit specific interaction with target cells by attaching target specific ligands.

2.5.2 Composition of stealth liposomes

Several workers have recently tried to prolong the circulation times of the liposomes by grafting the polymers like PEGs and gangliosides into the lipid vesicles. In addition, the attachments of similar polymers are being investigated in order to minimize or prevent the adsorption of proteins and cells from the blood stream (37-42) and as a model polymer covered surfaces (43). A brief account of the composition and performance of few stealth liposomal systems is given in Table 2.7.

2.5.2.1 Polyethylene glycols

Hershfield et al (29) prepared stealth liposomes of doxorubicin composed of hydrogenated soya phosphatidylcholine/ cholesterol/ polyethylene glycol-distearoyl phosphatidylethanolamine and the therapeutic efficacy was compared in mice with conventional liposomes composed of egg phosphatidyl glycerol/egg phosphotidylcholine/

cholesterol/ dl-alpha tocopherol. Senior et al prepared liposomes which quantitatively retain aqueous markers, were covalently coupled via dipalmitoyl phosphatidylethanolamine (DPPE), to the hydrophilic polymer, monoethoxy poly (ethylene glycol) (MPEG 5000) (33). These liposomes retained the coating in the presence of plasma, and appeared to adsorb plasma components more slowly than liposomes without the polymer. New lipid carriers consisting of phosphatidylcholine (PC) liposomes casted with up to 10 mol% phosphatidylethanolamine (PE) with a covalently attached PEG 5000 head group PE-PEG were prepared by Blume et al (28). The improvement over pure PC liposomes within the first 24 hours exceeded 8000%, at this point nearly 25% of the applied PE-PEG liposome being still in the circulation.

2.5.2.2 Gangliosides and glycolipids:

Moghmi et al (36) prepared liposomes from sphingomyelin and saturated phospholipids and their affinity to different serum opsonins (liver and spleen) was characterized. Neither liver nor spleen specific opsonins have affinity for sphingomyelin saturated phospholipid liposomes since serum fails to enhance their uptake. Allen et al (45) and Gabizon (46) used gangliosides like GMI, GMz, GMs, G&s, GDla and glycolipids like sulfatides, globosides, glycosyl ceramide and several others for the preparation of stealth liposomes. But GMI, only has shown the ability to prolong circulation half-life and other negatively charged glycolipids with bulky head groups i.e., sulfatides and phosphotidylinositol, had some effect in prolonging circulation half-life. The negative charge of GM1 is shielded from the surface by the presence of two neutral sugars; while with GD, GT, GM2, and GM3 this is not the case. At the critical concentration of GM1 (7mol%) used, it imparts minimum charge density and sufficient surface hydrophilicity to the liposomes to prevent opsonization.

2.5.2.3 Synthetic phospholipids:

Park et al (47) worked on dioleoyl phosphotidyl ethanolamine (DOPE) derivatives (negatively charged phospholipids). A series of negatively charged phospholipid derivatives has been synthesized by coupling aliphatic dicarboxylic acids to DOPE. Allen et al (48) incorporated a carbamate derivative of PEG 1900 with distearoyl phosphotidyl ethanolamine (DSPE) (PEG DSPE) into liposomes (sphingomyelin/ cholesterol/egg PC 1: 1: 1) in concentrations of 5-7mol % and compared for circulation half-life with liposomes bearing 10% monosiaganglioside GM, (PEG-DSPE) liposomes had the greatest ability to decrease the mononuclear phagocyte system uptake of liposomes. Altering vesicle size

for liposomes containing PEG-DSPE resulted in only minor changes in blood levels of liposomes. Gabizon and papahadjopoulos have divided various negatively charged lipids into two categories (45). (i) A diacetyl phosphate type of lipid has negatively charged groups (ii) The other type of lipid is some glycolipids such as GMT, phosphotidyl inositol or sulfatides, which have a negative charge shielded by surrounding bulky, neutral, hydrophilic groups. It is suggested that this "Shielded negative charge" was responsible for prolonged circulation of liposomes. But Park et a/ 26 reported that, negatively charged phospholipids with the exposed and unshielded carboxylic group such as N-glutaryl DOPE (NGPE) and Nadipyl DOPE (NAPE) show considerable activity to prolong the circulation time of liposomes

Table 2.7: Composition of few stealth liposomal systems and their performance in *vivo*.

Sr. No.	Composition of the system	Performance in vivo	Reference
Polyethylene glycols			
1.	Hydrogenated SOY Phosphotidylcholine/ cholesterol/polyethylene glycol-distearoyl phosphatidylethanolamine.	Stealth liposome formulation was significantly more effective than conventional liposome formulation (Egg phosphatidylcholine/ cholesterol/d-alpha phosphotidylglycerol/Egg tocopherol) in reducing the incidence of metastases from intramammory implants of tumor MC2A, tumor MC2B and tumor MC65.	29
2.	Phosphotidylcholine liposomes casted with up to 10-mol% PE with a covalently attached PEG 5000 head group.	Circulation time of stealth liposomes within first 24 hrs exceeded 8000% over pure phosphotidylcholine liposomes.	28
3.	DSPE/cholesterol grafted with PEG (2:1) liposomes were 1900 mg/ml.	Membrane bound PEGs can exert a significant interbilayer repulsion thereby polymer chain extends to 50°A from lipid bilayer surface, which may reduce interactions with plasma proteins and phagocytic cells.	44
Gangliosides and glycolipids			
1.	Liposomes were prepared with saturated phospholipids and sphingomyelin.	These liposomes attracted serum dysopsonins, which inhibit their uptake by liver cells. Inclusion of cholesterol in these liposome preparations enhanced this uptake in splenic cells but not liver cells.	45

2.	Egg PC/sphingomyelin /cholesterol/ ganglioside GM1 in molar ratio 1:1:1 :0. 14	The ability of GM1 to reduce leakage of aqueous contents from liposomes there by reducing opsonization was due to (i) Molecular confirmation. (ii) Location of negative charge relative to phospholipid bilayer and carbohydrate back bone (iii) Packing characteristics of GM1 in phospholipid bilayers.	46
Synthetic phospholipids			
1.	Sphingomyelin/cholesterol/ egg PC (1: l: l) liposomes were prepared and a caraamate derivative of PEG 1900 with distearyl phosphatidylethanolamine (DSPE) was incorporated into these liposomes.	(PEG-DSPE) liposomes had the greatest ability to decrease the uptake by mononuclear phagocyte system.	47

2.5.3 Effect of PEG grafting on protein adsorption and cell adhesion

Recently, various PEG derivatives have been used to stabilize liposomes for increasing efficiency in drug or gene delivery. Most 'stabilized' liposomes, e.g., the so-called 'stealth' liposomes (49), or 'cryptosomes' (50), contain a certain percentage of PEG-derivatized phospholipids, which reduce the uptake by mononuclear phagocytic system (MPS), thereof prolonging the circulation times. Unlike conventional liposomes, PEG-liposomes do not show dose-dependent blood clearance kinetics (51). Vesicles containing PEG-conjugated lipids at various concentrations, molecular weights MW, or various sizes of PEG containing vesicles were reported to have different circulation times (50,52–55). It was shown that the circulation time and the organ distribution of the stabilized lipid vesicles in vivo are very sensitive to the surface density of the sterically active head-groups (50). In vitro, by incubating the vesicles with the components from the mononuclear phagocytotic system MPS, it was found that the existence of PEG on the surface of the vesicle prevents the adsorption of serum albumin. Furthermore, both the resistance to plasma component adsorption and the resulting longevity in vivo show a maximum at the same lipid stabilizer molar ratio (50). In comparison, a large number of plasma components, including serum albumin, immunoglobulin, fibronectin and various apolipoproteins, can bind to the surface of conventional liposomes, affecting their longevity (56). Because PEG has been shown to have protein-repelling activity when immobilized on a surface, due to its hydrophilicity, chain mobility, and lack of ionic

charge (51), several groups have attempted to create a biocompatible but cell repelling surface using PEG as a modifier. A solid support coated with PEG-grafted lipids may provide a biocompatible surface for biosensing and prosthetic devices. It has been recognized that the protection effect of PEG conjugated lipids is dependent on the MW of PEG moieties. In order to produce a significant inhibition of the uptake by macrophages, the ethylene oxide EO chains used in block-copolymer-coated polystyrene particles must have at least 50 subunits (57). A similar result was observed in the study of protection on protein adsorption: the degree of polymerization of PEG on the solid substrate must approach about 100 (58). A closer simulation of PEG-liposome surfaces is a lipid monolayer or bilayer on a solid support, with PEG only on the water-facing surface. These surfaces have well-defined compositions and structures. The grafted PEG moieties protrude from the surface, while the hydrophobic tails of these molecules are anchored into the surface monolayer. Compared to vesicles, the experimental conditions for supported monolayer or bilayer are easier to control. For example, it is easier to separate free proteins or cells in solution from those deposited on the polymer grafted surfaces; the properties of the polymer-coated surfaces can be determined more accurately, and the system is a convenient one for investigating hypotheses concerning protein adsorption and cell adhesion to the polymer coated surfaces (59).

2.5.4 Characterization of stealth liposomes

A detailed characterization of structure of stealth liposomes including particle size distribution, lamellarity, bilayer repeat distance and encapsulated volume has to be performed, since it gives information about differences in structure caused by changes in method of preparation and lipid composition (60). These differences in structure affect the behavior of the vesicles in vitro (stability) as well as in vivo (disposition).

Morphology: Small angle X-ray scattering (SAXS), P-NMR, freeze fracture electron microscopy (FFEM) and UV/VIS spectroscopy are used. These techniques provide information regarding mean size, size distribution, number of bilayers and encapsulated volume.

Leakage study: Allen and Clelanda developed a technique to study the leakage rates of the drugs or entrapped substances (fluorescent) from liposomes. Fluorescence increase accompanying leakage was studied using Perkin Elmer MPF-A spectrofluorimeter at $37^{O}C$ in 25% human plasma.

Exchange/Transfer with High-density lipo-proteins (HDL) (61): The radiolabelled phospholipids are used to prepare the stealth liposomes. Liposomes are incubated for 2-16 hrs at 37^{O}C with HDL followed by chromatography over a Sepharose CL-4B column and quantitation of the radiolabel associated with the HDL peak.

Localization/Targeting (62): Stealth liposomes prepared using radiolabelled phospholipids are injected (known quantity) into mice and after approximate 7 hrs, anaesthetized and radioactivity of each internal organ is counted in a gamma counter.

2.5.5 Applications of liposomes in drug delivery

New drug delivery systems such as liposomes are developed when an existing formulation is not satisfactory and reformulation offers superior therapeutic efficacy and safety over the existing formulation. Indeed, liposome formulations of some drugs have shown a significant increase in therapeutic efficacy and: or therapeutic indices in preclinical models and in humans, compared to their non-liposomal formulations. The therapeutic applications of liposomes generally fall into several categories briefly described below.

2.5.5.1. Formulation aid

Hydrophobic drugs such as cyclosporin and paclitaxel are usually formulated in surfactants and organic co-solvents for systemic administration in humans. These solubilizers may cause toxicity at the doses needed to deliver the drug. In contrast, liposomes are made up of lipids, which are relatively non-toxic, non-immunogenic, biocompatible and biodegradable molecules, and can encapsulate a broad range of water-insoluble (lipophillic) drugs. Currently, liposomes or phospholipid mixtures are being used as excipients for preparing better-tolerated preclinical and clinical formulations of several lipophillic, poorly water-soluble drugs such as amphotericin B. In preclinical studies, liposomes have been evaluated as a vehicle for the delivery of paclitaxel and its analogs as an alternative to the cremophor: ethanol vehicle (63-65). Paclitaxel liposomes were able to deliver the drug systemically and increase the therapeutic index of paclitaxel in human ovarian tumor models (66).

Table 2.8: Liposome and lipid-based products in clinical trials in USA

Product	Drug	Formulation	Company	Status	Indication: Target
Allovectin-7	HLA-B7 plasmid	DNA-lipid complex)	Vical Inc., San Diego, CA	Phase II	Gene therapy of metastatic cancers
				Phase I	Gene therapy of metastatic renal cancer with concurrent IL-2
AmBisome™	AmB(a)	Liposomes	NeXstar Pharmaceutical, Inc., Boulder, CO	Pending approval(b)	Life-threatening systemic fungal infection; visceral leishmaniasis
Amphotec™	AmB	Lipid complex	Sequus Pharmaceutical, Inc, Menlo Park, CA	Phase III	Life-threatening systemic fungal infection in immunocompromised patients
Annamycin	Annamycin	Liposomes	Aronex Pharmaceuticals, The Woodlands, TX	Phase I:II	Breast cancer
Atragen™	Tretinoin	Liposomes	Aronex Pharmaceuticals, The Woodlands, TX	Phase II:III	Kaposi's sarcoma in AIDS
				Phase II	Recurrent acute promyelocytic leukemia
				Phase I	Cancer of blood
Doxil™	Doxorubicin	Liposomes	Sequus Pharmaceuticals, Inc., Menlo Park, CA	Phase III	Refractory ovarian, recurrent breast and prostate cancers
Nyotran™	Nystatin	Liposomes	Aronex Pharmaceuticals, The Woodlands, TX	Phase II:III	Candidemia
				Phase I	Comparative study against AmB in suspected fungal infection
TLC-D99	Doxorubicin	Liposomes	The Liposome Company, Princeton, NJ	Phase III	Metastatic breast cancer
Ventus™	Prostaglandin E1	Liposomes	The Liposome Company, Princeton, NJ	Phase III	Acute Respiratory Distress Syndrome

[a] Amphotericin B; [b]NDA filed with United State Food and Drug Administration.

2.5.5.2 Intracellular drug delivery

Drugs with intracellular targets: receptors are required to cross the plasma membrane for pharmacological activity. Liposomes can be used to increase cytosolic delivery of certain drugs such as *N*-(phosphonacetyl)-L-aspartate (PALA) which are normally poorly taken up into cells (67). PALA is taken up into the tumor cells through fluid-phase endocytosis (pinocytosis) and it diffuses out into the cytoplasm as the endosome pH drops (68). However, pinocytosis is very limited in its efficiency. Liposomal delivery of drugs, which normally enter the cells by pinocytosis, can be very effective (64) because liposomes can contain greater concentrations of drug compared to the extra cellular fluid and the endocytosis process by which negatively charged liposomes are predominantly taken up by the cells, is more efficient than pinocytosis. For example, the potency of PALA encapsulated liposomes was up to 500-fold greater against human ovarian tumor cell lines than that of free PALA (69).

2.5.5.3 Sustained release drug delivery

Sustained release systems are required for drugs such as cytosine arabinoside (Ara-C) that are rapidly cleared in vivo and require plasma concentrations at therapeutic levels for a prolonged period for optimum pharmacological effects. It is now possible to design sustained release liposome formulations with an extended circulation half-life and an optimized drug release rate in vivo. For example, Ara-C encapsulated in LCL is effective as a prolonged release system in the treatment of murine L1210:C2 leukemia (70). Conventional liposomes that localize by phagocytosis in the cells of RES may also act as a sustained release depot by slowly leaking drugs from RES into the general circulation.

2.5.5.4 Gene therapy

A number of systemic diseases are caused by lack of enzymes: factors, which are due to missing or defective genes. In recent years, several attempts have been made to restore gene expression by delivery of the relevant exogenous DNA or genes to cells (71). Cationic liposomes (Table 2.7) have been considered as potential non-viral human gene delivery system (72-75). They are usually composed of a cationic lipid derivative and a neutral phospholipid (DOPE). The latter is required by certain cationic lipids to form stable liposomes. Some of the widely used cationic liposome formulations are: lipofectin (DOTMA: DOPE, 1:1); lipofectamine (DOSPA: DOPE, 3:1); transfectase (DDAB: DOPE, 1:3); cytofectin (DMRIE: DOPE); transfectam (DOGS) and DC-cholesterol. The negatively charged genetic material (e.g., plasmid) is not encapsulated in liposomes but

complexed with cationic lipids by electrostatic interactions. Allovectin-7, a gene transfer product is currently in clinical trials (phase I:II) as an immuno therapeutic agent for the treatment of metastatic melanoma, renal cell and colorectal carcinoma (Table 2.8). Allovectin-7 is composed of a plasmid containing the gene for the major histocompatibility complex antigen HLA-B7 with b-2 microglobulin formulated with the cytofectin (DMRIE: DOPE). The ongoing clinical trials have indicated that intralesional injection of Allovectin-7 can be performed safely and have demonstrated antitumor activity in some patients (76). Plasmid–liposome complexes have many advantages as gene transfer vehicles over viral-based vectors (71): (i) these complexes are relatively nonimmunogenic because they lack proteins; (ii) liposomes or lipid complexes can be used for transfection of large-sized genetic material; and (iii) viruses, unlike plasmid–liposome complexes, may replicate and cause infections.

2.5.5.5. Site-avoidance delivery

Drugs used in the treatment of diseases like cancer usually have a narrow therapeutic index (TI) and can be highly toxic to normal tissues. Decreasing delivery to critical normal organs may minimize the toxicity of these drugs. It has been shown that even a small reduction in distribution of the drug to critical organs by encapsulation in liposomes cans significantly reduce the drug toxicity (77). Liposomes are taken up poorly by tissues such as heart, kidney, and GI tract, which are major sites for toxic side effects of a variety of antineoplastic drugs. Thus, liposome formulation may improve the TI by altering the biodistribution of drug away from drug sensitive normal tissues. For instance, free amphotericin B and doxorubicin produce severe dose-limiting nephrotoxicity and cardiac toxicity, respectively. Reformulation of these drugs in liposomes results in reduced toxicity with no change in therapeutic efficacy.

2.5.5.6. Site-specific targeting

Site-specific delivery, the concept first proposed by Paul Ehrlich (78) involves the delivery of a larger fraction of drug to the target site and therefore, reducing exposure to normal tissues. Liposomes have been employed for accomplishing both passive and active targeting of drugs.

2.5.5.6.1. Passive targeting

This approach for liposome drug delivery exploits the natural tendency of certain cells such as Kupffer cells in the liver, and circulating macrophages of RES to phagocytose

foreign microparticulates such as liposomes. Conventional liposome (CL) formulations of drugs and immunostimulators have been successfully used for targeting the cells of RES, and exhibit significant improvement in the TI of the drugs (79). In clinical trials, systemic administration of CL containing muramyl peptide derivatives caused enhancement in the tumoricidal properties of monocytes in patients with recurrent osteosarcoma (80). Furthermore, CLs have also been employed for targeting of immunosuppressive drugs to lymphatic tissues such as the spleen. In a preclinical model, an increase in immunosuppressive activity, i.e. a delay in heart transplant rejection was observed with CL-encapsulated methylprednisolone (81).

2.5.5.6.2. Active targeting

Active targeting of liposome-encapsulated drugs may be accomplished by coupling specific antibodies to vesicles (immunoliposomes). Immunoliposomes containing diphtherin toxin (DT) have been shown to provide protection against the non-specific toxicity of DT during cancer chemotherapy (82). Long circulating immunoliposomes (hydrophilic polymer- coated vesicles bound to antibodies and 50.15 mm in size) can now be designed which may be able to recognize and bind with greater specificity to target cells following systemic administration (83,84). It has been shown that long-circulating immunoliposomes (LCI) enhanced therapeutic efficacy of encapsulated doxorubicin in a murine lung tumor model (85). The effect of size on biodistribution of LCI has been studied in a rabbit model of myocardial infarction (86). Small sized (0.12–0.15 mm) LCI containing infarct-specific antimyosin antibodies (AM) exhibited significantly lower accumulation in RES compared to CL with or without AM. However, the accumulation of LCI-AM was higher in kidneys and lungs compared to CL-AM. The accumulation of large sized (0.35–0.4 mm) LCI in spleen was 2-fold higher than small sized LCI (86). Active targeting using immunoliposomes has several advantages over that of antibody-drug conjugates (84): (i) immunoliposomes can carry a significantly larger number of drug molecules compared to simple conjugates; (ii) immunoliposomes can encapsulate drugs with widely varying physicochemical properties; and (iii) drugs can also reach their intracellular target by diffusion after release from immunoliposomes associated with target tissue (Fig. 2). Therefore, unlike antibodies-drug conjugates, in some cases immunoliposomes may not have to undergo receptor mediated-endocytosis to deliver their contents intracellularly.

2.5.5.7 Intraperitoneal administration

Direct administration of antineoplastic agents into the intraperitoneal (i.p.) cavity has been proposed to be therapeutically advantageous for cancers that develop in or metastasize to the peritoneal cavity (87). Intraperitoneal chemotherapy has been somewhat unsuccessful for free drugs because of relatively fast clearance of the drugs from the i.p. cavity resulting in lowered concentrations at the site of action (88). However, the clearance of liposomes from the peritoneal cavity is significantly slower than that of free drug and therefore, higher drug concentrations can be achieved in the proximity of the target site for extended periods of time with the use of liposome formulations. Furthermore, reformulation of erosive drugs in liposomes has been shown to reduce local drug toxicity such as dermal toxicity of doxorubicin (89). An increase in therapeutic index of paclitaxel in liposomes after i.p. administration (90) may also be due to a reduction in local (abdominal) toxicity of the drug.

2.5.5.8 Immunological adjuvants in vaccines

Liposomes can encapsulate antigens in their aqueous space or incorporate in the bilayer depending on the lipophilicity of the antigen. Liposomes were first used as immunological adjuvants in order to enhance the immune response to encapsulated diphtheria toxoid (91). Since then, liposomes have been used as nontoxic adjuvants with bacterial, viral, protozoan, tumor and other antigens (92,93). The mechanism by which liposomes cause increases in antigens' immune response is not fully understood. However, augmentation of liposomal adjuvanticity can be achieved by co-administration of liposome-encapsulated antigen with other adjuvants such as lipid A, lipopolysaccharides, muramyldipeptide and interleukin (IL-2) (94). Furthermore, antibody-mediated targeting of liposomal to antigen presenting cells may also improve immunostimulatory effects (92). The influence of physicochemical properties of the liposomes such as charge density, membrane fluidity and epitope density, on the immune response of the antigen has been extensively studied. For instance, liposome formulations of inactivated encephalomyocarditis and Semliki Forest viruses were significantly more immunogenic when charged phospholipids were used compared to neutral lipids (95). The phase transition temperature (*Tc*) of the lipids also appears to influence immunogenicity. For example, immunogenicity of haptens was higher in liposomes composed of lipids with a high *Tc* than in those with a low *Tc* (96). Recently, the first liposome-based vaccine (liposomes containing inactivated hepatitis A virions) was

approved for human use in Switzerland and currently, several other liposome-based vaccines are in clinical trials.

2.5.6 Limitations of liposome technology

As described above, liposomes have a great potential in the area of drug delivery. However, liposome based drug formulations have not entered the market in great numbers so far. Some of the problems limiting the manufacture and development of liposomes have been stability issues, batch-to-batch reproducibility, sterilization method, low drug entrapment, particle size control, production of large batch sizes and short circulation half-life of vesicles. Some of these issues such as short half-life have been resolved resulting in increased numbers of clinical trials (Table 2.8). Some of the remaining problems are discussed in detail below.

2.5.6.1 Stability

One of the major problems limiting the widespread use of liposomes is stability—both physical and chemical. Depending on their composition, the final liposome formulations may have short shelf lives partly due to chemical and physical instability. Chemical instability may be caused by hydrolysis of ester bond and: or oxidation of unsaturated acyl chains of lipids. Physical instability may be caused by drug leakage from the vesicles and: or aggregation or fusion of vesicles to form larger particles. Both of these processes (drug leakage and change in liposome size) influence the in vivo performance of the drug formulation, and therefore may affect the therapeutic index of the drug. For instance, large liposomes may be rapidly cleared by RES leading to subtherapeutic plasma concentrations of the drug and reduced AUCs (area under the plasma concentration –time curve). Physical instability may also occur due to partitioning out of a hydrophobic drug from the bilayer into the solvent on standing (or long term storage). Some of the stability problems may be overcome by lyophilization in which the final liposome product is freeze-dried with a cryoprotectant (mostly a sugar like trehalose) 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. Some liposome products on market or in clinical trials are provided as a lyophilized powder. For example, AmBisome™, the first liposome product to be marketed in several countries is supplied as a lyophilized powder to be reconstituted with sterile water for injection. Recently, lyophilized paclitaxel –liposome formulations have been developed which show good stability (97-98).

2.5.6.2 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. The method available for sterilization of liposome formulations after manufacture is filtration through sterile 0.22 μm membranes. However, filtration is not suitable for large vesicles (>0.2 μm) and also is not able to remove viruses. Sterilization by other approaches such as g irradiation and exposure to chemical sterilizing agents are not recommended because they can cause degradation of liposome components and may leave toxic contaminants (99). Several groups are exploring suitable methods for sterilization of liposome formulations. For instance, it has been shown that under certain conditions, liposomes with thermostable, lipophillic drugs could be sterilized by autoclaving without substantial loss of contents and: or degradation of phospholipids (100).

2.5.6.3 Encapsulation efficiency

Liposome formulation of a drug could only be developed if the encapsulation efficiency is such that therapeutic doses could be delivered in a reasonable amount of lipid, since lipids in high doses may be toxic and also cause non-linear (saturable) pharmacokinetics of liposomal drug formulation. Some new approaches that provide high encapsulation efficiencies for hydrophilic drugs have been developed. For instance, active loading of the amphipathic weak acidic or basic drugs in empty liposomes can be used to increase the encapsulation efficiency (101,102). However, active loading is not suitable for hydrophobic drugs such as paclitaxel for which encapsulation efficiency is ≤ 3 mole% mainly due to the low affinity of drug for the lipid bilayers (97).

2.5.6.4 Active targeting

One of the major limitations of active targeting using ligand-directed immunoliposomes has been their rapid clearance due to non-specific uptake by the cells of RES. The development of LCL conjugated with ligands has revived interest in this field since RES does not as rapidly clear LCL. However, many problems still remain to be overcome. For instance, foreign immunoglobulin–ligands conjugated to immunoliposomes may induce immunogenicity and increase clearance on subsequent exposure. The ligand (antibodies) conjugated with liposomes may increase the liposome size and reduce extravasation and

thus could limit targeting to intravascular targets (84). It has been shown that size of LCI may be increased in the blood circulation by interaction of the antibodies with serum components, which in turn can increase their size-dependent uptake by spleen (103).

2.5.6.5 Gene therapy

A number of technical problems have to be overcome before cationic liposome-mediated transfection can be fully exploited. For instance, liposomes are significantly less efficient than viral vectors in their transfection ability. Furthermore, the DNA–lipid complexes are not stable in terms of particle size (75,59) for long periods of time. In addition, there is lack of in vivo targeting after systemic administration, and the toxicity of cationic lipids limits the administered dose of the DNA–lipid complex. Plasmid–liposomes complexes may be more suited to delivery of genetic material by local administration.

2.5.6.6 Lysosomal degradation

Once the liposome has reached the target cell, the efficacy is determined not only by the amount of drug associated with the cell, but also by the amount of drug reaching the 'target molecule' inside the cells. Immunoliposomes may deliver the drug to the cells selectively but the pharmacological activity depends on the ability of intact drug to diffuse into cytoplasm from the endosomes in sufficient amounts.

2.6 Microbubbles

2.6.1 Introduction

Microbubbles consist of a gas (air or perfluorocarbon), which is stabilized by shell (denatured albumin, phospholipids or surfactant or cyanoacrylate) having diameter of from 1 to 10 μm (104). The evolution of science and technology of microbubbles as a drug carrier or for diagnostic purpose has passed through number of distinct phases. The tiny gas-filled microbubbles come in to limelight as a result of approval by FDA (105). It is found to be long-lived, lasting over 6 months in-vitro (106). Protein coated microbubbles are approved for medical use (107). Stabilization of microbubbles against dissolution may be achieved by creating a shell around it of protein, gelatin, surfactants or lipids (108). Lipid coated microbubbles containing polyethylene glycol mixture shows to evade the reticulo-endothelial system and exhibit prolonged blood circulation time (109). It is most widely used as ultrasound contrast agents because of specific acoustic and biological properties, which also make it a promising, tool as a vehicle for drug and gene delivery (110). It has been recently used as vascular tracers in evaluation of patients with liver pathologies (111). It is also used to increase vascular signals at Doppler ultrasound and has been shown to improve color Doppler ultrasound assessment of tumoral vessels (112,113). Gas-filled bubbles, together with ultrasound, can assist into non-invasive assessment of angiogenesis or new blood vessel growth (114). It helps to image delineation of organs, including chambers of the heart, assists in imaging blood flow, including flow deficiencies (organ perfusion) and applied for evaluation of patency of anatomical structures (e.g. fallopian tubes). It passes through heart and capillary walls of tumor-tissue microcirculation and has an affinity for tumor cells. It has fewer tendencies to stick to the walls of normal blood vessels as compared to tumorous tissue (because the walls of blood vessels are rougher and abnormal). It is small enough to pass through pulmonary circulation and sufficient pressure stable to allow passage through the left ventricle after intravenous injection.

2.6.2 Background

For the past decades ultrasound has been an imaging modality with multiple application and the development of microbubbles has increased the possibilities for diagnostic imaging. Dijkmans et al have deeply studied simultaneous application of microbubbles and ultrasound for diagnosis to therapy (115). An efficient ultrasound

contrast agent should enhance backscatter; an effect seen for example after injecting liquids as saline and indocyanine green (110,116) into aorta and heart, due to the gas bubble formed. Cavitation with microbubbles can be used to dissolve blood clots or deliver drugs. Targeting ligands and drugs can be incorporated in to microbubbles to make highly specific diagnostic and therapeutic agent for activation with ultrasound. Unger et al have reviewed some of these applications and experimental results using such agents for thrombolysis, drug and gene delivery (117). Alexander has found biodistribution, mechanism of action, targeting, sensitivity and stability of microbubbles in vitro and in vivo (118). In case of gene therapy, delivery is more problematic than for the most of other drugs. The active material must be delivered to the target cells and integrated into the cell's nuclei. Unger has also discussed the potential roles of microbubbles and ultrasound for drug delivery, particularly gene delivery (119).

2.6.3 Composition of microbubbles: -

Table 2.9: microbubble shell materials, gases and aqueous carriers

	Microbubble shell
References (US patent number/ Researcher)	**Type of materials**
4,684,479/ D'Arrigo et al	Surfactant mixture containing (A) Glycerol monoesters of saturated carboxylic acids containing 10-18 carbon atoms and aliphatic alcohols containing 10-18 carbon atoms (B) Sterol-aromatic acid esters (C) Sterols, terpenes, bile acids and alkali metal salts of bile acids (D) Sterol esters of aliphatic acids containing 10-18 carbon atoms, sterol esters of sugar acid, esters of sugar acids and aliphatic alcohols containing 10-18 carbon atoms, esters of sugar and aliphatic acids containing 10-18 carbon atoms, sugar acids, saponins, and sapogenins (E) Glycerol, glycerol di or tri-esters of aliphatic acids containing 10-18 carbon atoms and aliphatic acids containing 10-18 carbon atoms In weight ratio of 2-4:0.5-1.5: 0.5-1.5: 0-1.5:0-1.5 of A: B: C: D: E
6,033,646/Unger et al	Lipid mixture containing - 1,2 Dipalmitoyl-sn-glycero-3-phosphotidylcholine (DPPC) (70-90 mole%) - 1,2 Dipalmitoyl-phosphotidylethonolamine-polyethylene glycol (DPPE-PEG) (5-15 mole %) - 1,2 Dipalmitoylphosphotidic acid (5-15 mole %)
5,352,436/Wheatley et al	(A) First surfactant comprising of - Polyoxyethylene sorbitan monolaurate, Polyoxyethylene sorbitan monopalmitate, Polyoxyethylene sorbitan sterarte, Polyoxyethylene sorbitan tristerate, Polyoxyethylene sorbitan monooleate and mixture - TWEEN 20,40,60,65,80 (B) Second dispersible surfactant comprising of - Sorbitan monosterates, Sorbitan monopalmitate and mixture there of - SPAN 40, SPAN 60
4,276,885/Tickner et al	Gelatin
4,572,204/ Feinstein et al	Renografin-76 or 70% sorbitol or dextrose
4,957,656/Cerry et al	Human serum albumin, Hemoglobin, Collagen

6,210,611/ Needham et al	Lipid mixture consists of - 1,2 Dipalmitoyl-sn-glycero-3-phosphotidylcholine (DPPC) - 1,2 Disteroyl-sn-glycero-3-phosphotidylcholine (DSPC) - 1,2 Diarachidoyl-sn-glycero-3-phosphotidylcholine (DAPC) - 1,2 Dibehenoyl-sn-glycero-3-phosphotidylcholine (DBPC) - 1,2 Dilignoceroyl-sn-glycero-3-phosphotidylcholine (DLgPC) - 1,2 Dipalmitoyl-sn-glycero-3—[phosphoric-(1-glycerol)] (DPPG) - Phosphotidylcholine (PC) - Phosphotidylglycerol (PG) - Phosphotidic acid (PA) - Phosphotidylethanolamine (PE) - Phosphotidylinositol (PI) - Sphingolipids and Glycolipids - Polyethylene glycol (PEG) grafted polymers, PEG-40-sterate and PEG-PE
5,985,247/Soetanto et al	Mixture of (A) Surfactants consisting of Sodium stearate, Sodium Myristate, Sodium Palmitate, Sodium Laurate, and Sodium Oleate (B) Stabilizers consisting of Saponin, Steric acid, Phloxine, Crystal Violet, Polyvinyl alcohol and Sodium Laurate
	Microbubble gases
6,033,646/Unger et al	Carbon dioxide, Oxygen, Air, Nitrogen, Neon, Helium, fluorocarbon gases like sulfur hexafloride, perfluorobutane, perfluorocyclobutane, perfluoromethane, perfluoroethane, perfluoropropane, perfluoropentane, perfluorohexane, 1,11,2,3,3,3, heptafluoropropane and 1,1,2,2,3,3,3, heptafluoropropane and mixture of perfluorocarbon gas and other gases such as air, oxygen and nitrogen
	Aqueous Carriers
6,033,646/Unger et al	Water, phosphate buffer saline, normal saline, physiological saline, a mixture of water, glycerol and propylene glycol or mixture of saline, glycerol and propylene glycol in ratio of 8:1:1 or 9:1:1, v/v/v or a mixture of saline and propylene glycol in ratio of 9:1, v/v

2.6.4 Types or classification of microbubbles: -

Table 2.10: Classification of microbubbles

Types of Microbubbles	Shell materials	Gas composition	Company/University	References
Echogen emulsion	No shell	Perflenapent liquid emulsion	Sonus Pharmaceuticals Inc.	www.rad.uab.edu (120)
Echovist	Galactose granules	Air	Schering Berlin	William et al (121)
ST-68	Span 60 and Tween 80	Octafluoropropane	Drexel Univerisity	Wheatley et al (122)
Levovist	Palmitic acid with galactose matrix	Room air	Schering AG	Schlief et al (123)
Optison	Albumin	Perfluoropropane	Molecular Biosystems, San Diego, CA	DeMaria et al (124)
Albunex	Albumin	Air	Molecular Biosystems, San Diego, CA	DeMaria et al (124)
Quantison	Human Serum Albumin	Air	Quandrant Healthcare, Nottingham	Shoros et al (125)
PESDA	Albumin and dextrose	Decafluorobutane	University of Nebraska Medical Center	Porter et al (126)
Definity	Phospholipids	Octafluropropane	Bristol-Mayers-Squibbs Inc, Waltham	Dirver et al (127)
SonoVue	Phospholipids	Sulfur hexafluoride	Bracco Diagnostics, Geneva	Nanda et al (128)
Imagent	Phospholipids	Perfluorohexane	Alliance Pharmaceuticals Corp.,	Halpern et al (129)
Sonazoid	Phospholipids in sucrose matrix	Perfluorobutane	Nycomed Amersham Healthcare Inc.	Sontum et al (130)
Liposomal microbubbles	Phospholipids	Perfluoropropane	University of Texas Southwestern Medical School	Chen et al (131)
MRX-552	Phospholipids and Soyabean oil	Perfluorobutane	ImaRx Therapeutics	Evan et al (132)
Cavisome	Acrylate polymer	Air	Schering, Berlin	William et al (121)
A1-700	PLGA	Perfluorocarbon	Acusphere	www.edgaronline.com (133)
Polymeric microbubbles	Polymer/(-) Camphene in human serum albumin	Air	Nycomed Imaging AS, Norway	Bjerknes et al (134)
PB 127	Polymer (firm rubber)	Nitrogen	POINT Biomedical Corp.	Cheirif et al (135)

2.6.5 Methods of preparation of microbubbles

In the recent years, much effort was made to manufacture microbubbles. They consist of minute bubbles of gas (mostly air or perfluorocarbons), which is dispersed, in a carrier liquid for parenteral injection stabilized by shell materials. Initially, microbubbles were produced from a mixture of Renegrafin-76 (a relative non-toxic, biocompatible, radio-opaque dye) and saline in 1:1 ratio. This mixture was sonicated (subjected to high frequency energy) (136). Using viscous aqueous solution, such as 70% sorbitol or dextrose, Dr. Feinstein produced a dispersion of microbubbles by high-energy sonication. The persistence of microbubbles prepared by both methods was of few minutes, permitted the imaging agent to be prepared and administered intravenously for heart imaging (137). It is generally known that free microbubbles with an interfacial tension are prone to dissolution (138). Stabilization of microbubbles against such dissolution may be achieved by creation of a shell, typically composed of either a protein (denatured albumin) or a surfactant such as phospholipids (139-141).

2.6.5.1 Protein coated microbubbles

One of the techniques to increase stability of microbubbles is the use of proteins such as human serum albumin, collagen or hemoglobin. Subsequently, Dr. Feinstein prepared sonicated heat sensitive proteins such as albumin microbubbles. It provides stability of 24-48 hr, which is not sufficient stable for commercial manufacturing. To achieve stabilities of the order of weeks or months, proteins should be denatured. Finally, a dilute aqueous solution of heat-denaturable water-soluble, sterile 5% human serum albumin was passed through heat exchanger at temperature at which protein solution on the verge of denaturation but should not contain denatured protein. The gas (sterile air) is introduced concurrently with the introduction of heated solution to sonicated chamber. The gas containing heated solution passed through a sonication. The sonication produces gas microbubbles in the solution while the protein, which has been already heated to temperature of denaturation, obtained few degrees of additional heating during sonication to produce the insolubilized protein, forms the walls of the microbubbles (142). Protein coated microbubbles that are air-filled (Albunex) or perfluorocarbon filled (Optison) are currently approved for medical use.

2.6.5.2 Surfactant based microbubbles

Notwithstanding any potential advantages of albumin materials, the use of surfactants may be very desirable. The stabilized surfactant based microbubbles may be prepared from the mixture (Table 2.9, 143) by a variety of methods. The invention was related to a mixture including solvent, a first surfactant and a second dispersible surfactant and microbubble preparation from this mixture by sonication technique. Generally, mixing the first surfactant and second, dispersible surfactant with solvent and exposing this mixture to sonication forms microbubbles. Alternatively, the fist surfactant, second surfactant and solvent may be mixed by variety of methods. The two most easiest and suitable methods are mentioned in Figure 2.2.

Figure 2.2: Steps involved in preparation of surfactant based microbubbles

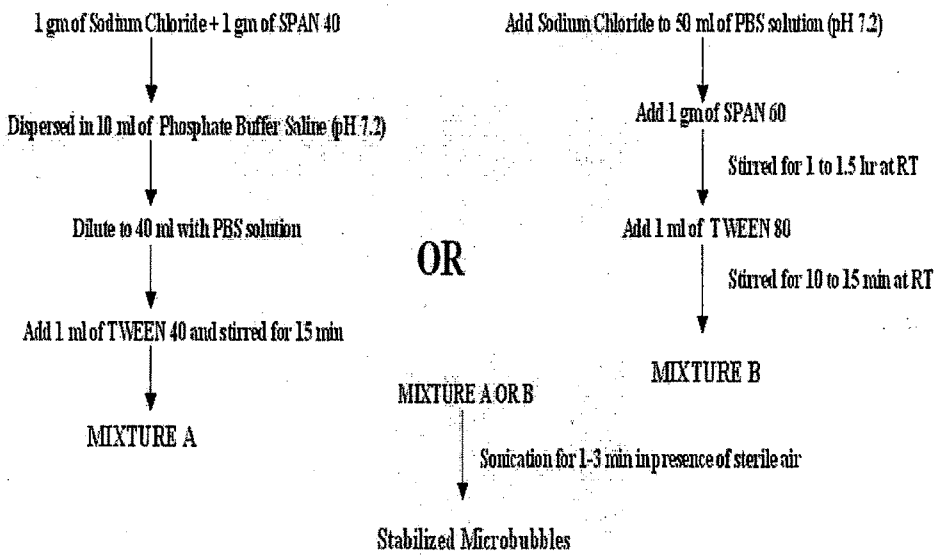
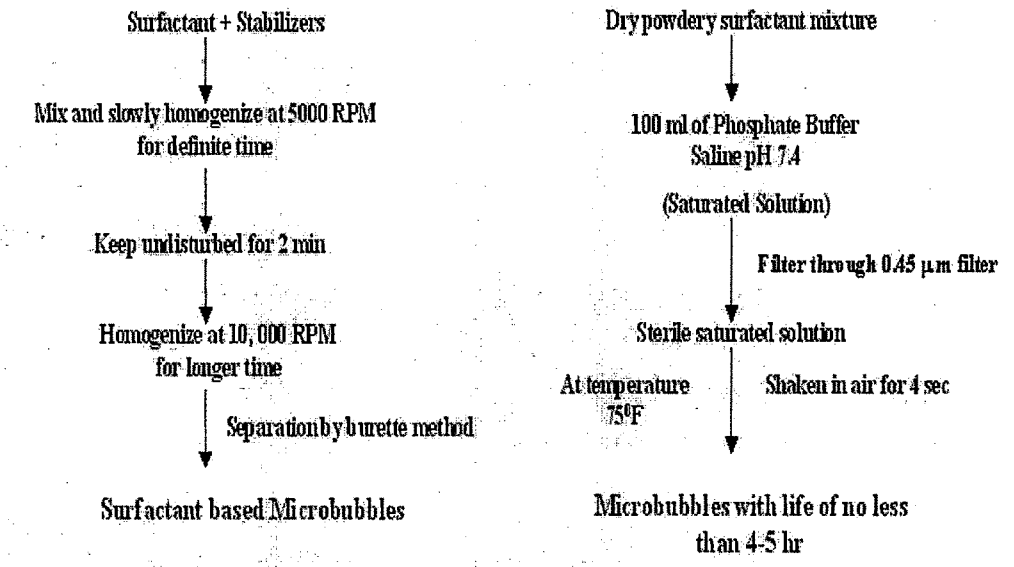


Figure 2.3 represents alternative methods for preparation of microbubbles stabilized by mixture of surfactants mentioned in Table 2.9 (144,145). The surfactant mixtures of the present method are best obtained in dry powder form. The stable microbubbles are obtained by forming at lease a substantially saturated solution of the surfactant mixture in an aqueous or oil-based media. High concentration of gas microbubbles formed by this method remains stable for 2 hr to 2-3 days under normal ambient conditions. The use of components (D) and (E) has been found to be advantageous in obtaining long-term stability and uniform microbubble size.

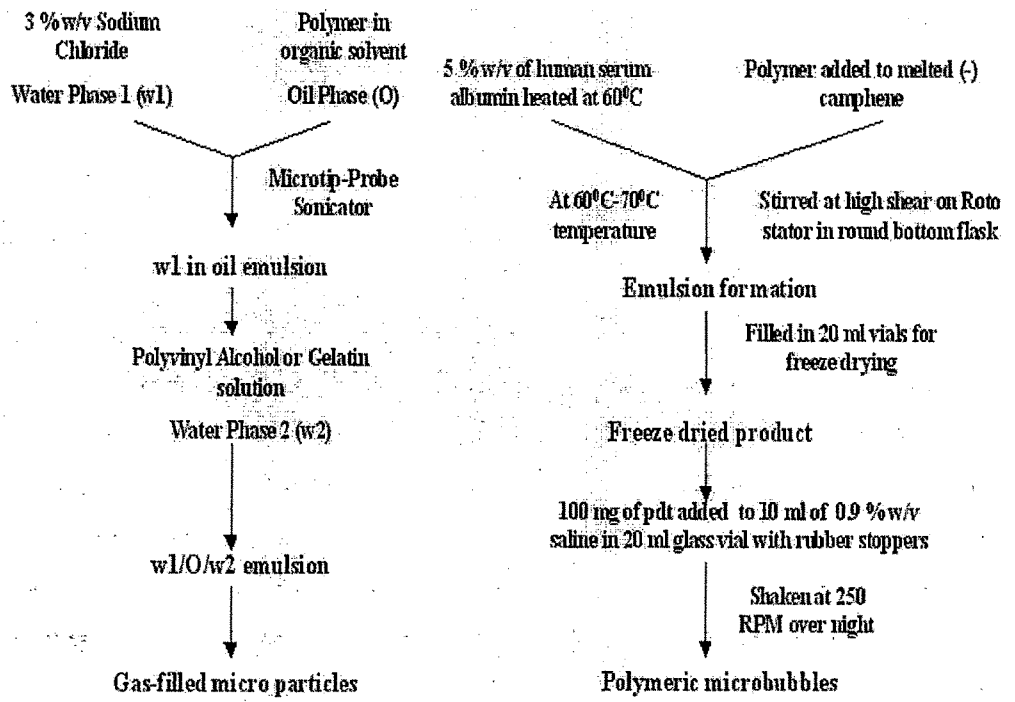
Figure 2.3: Steps involved in preparation of surfactant based microbubbles



2.6.5.3 Polymer based gas-filled micro particles

Polymer based gas-filled micro particles useful in targeting of active substances at particular site as well as for gene delivery prepared by double-emulsion solvent evaporation technique as mentioned in Figure 2.4 (146).

Figure 2.4: Steps involved in preparation of polymer based gas-filled micro particles



More stable microbubbles can be prepared by dissolving film forming polymer in a core which can be sublimed and subsequently removing the core as vapor (147-149). For parenteral uses the film-forming polymer should be biodegradable to enable elimination of the microbubbles from the body. Nycomed Imaging Inc, has produced the investigated polymer (biodegradable double-ester polymer with ethylidene units) by developed procedure (Patent No.: WO 96/07434) and used further in preparation of polymeric microbubbles (56). Here, 5 % human serum albumin solution and polymer/(-) camphene mixture was used as shell material and stabilized microbubbles were prepared by method as described in Figure 2.4.

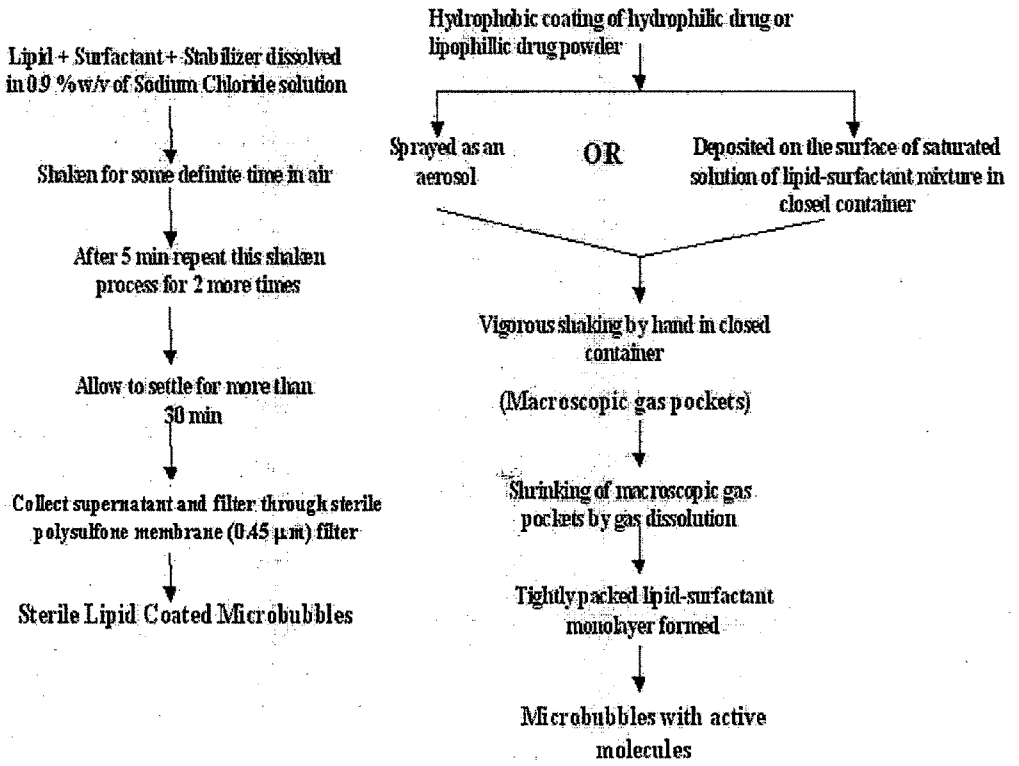
2.6.5.4 Perfluorocarbon-exposed dextrose albumin (PESDA) microbubbles

Porter et al (150) demonstrated that perfluorocarbon exposed sonicated dextrose albumin (PESDA) microbubbles, unlike room air-containing sonicated dextrose albumin microbubbles, have bioactive albumin on their surface. These can avidly bind synthetic antisense oligonucleotides, and then release them in the presence of ultrasound. They were prepared by taking 3 parts of 5 % dextrose an 1 part of 5 % human serum albumin (total 16 ml) in 35 ml syringe and mixing it with 8±2 ml of decafluorobutane gas. This solution was hand mixed by injection back and forth with stopcock in to 35 ml syringe 40 times and sonicated. The resultant microbubbles have concentration of 1.5×10^9 microbubbles/ml and size of 4.5 to 5.1 μm (126). Since PESDA microbubbles adhere to sites of endothelial injury even in the absence of ultrasound, the efficacy of this therapy in inhibiting coronary restenosis has been evaluated in animals.

2.6.5.5 Lipid coated microbubbles

The coated microbubbles can be targeted to specific in vivo sites depending upon attached ligands. Shafer et al proposed that denatured albumin based nano or microspheres were phagocytized with in 2 hr (151). The stealthy phospholipid vesicles composed of lipid/PEG mixture have shown to evade RES and exhibit prolonged blood circulation times with the lipid coated microbubbles is believed to be advantageous over the simple albumin microbubbles (109). Figure 2.5 represents method for preparation of lipid coated microbubbles (LCM) and entrapment of drug in to LCM.

Figure 2.5: Steps involved in preparation of polymer based lipid coated microbubbles



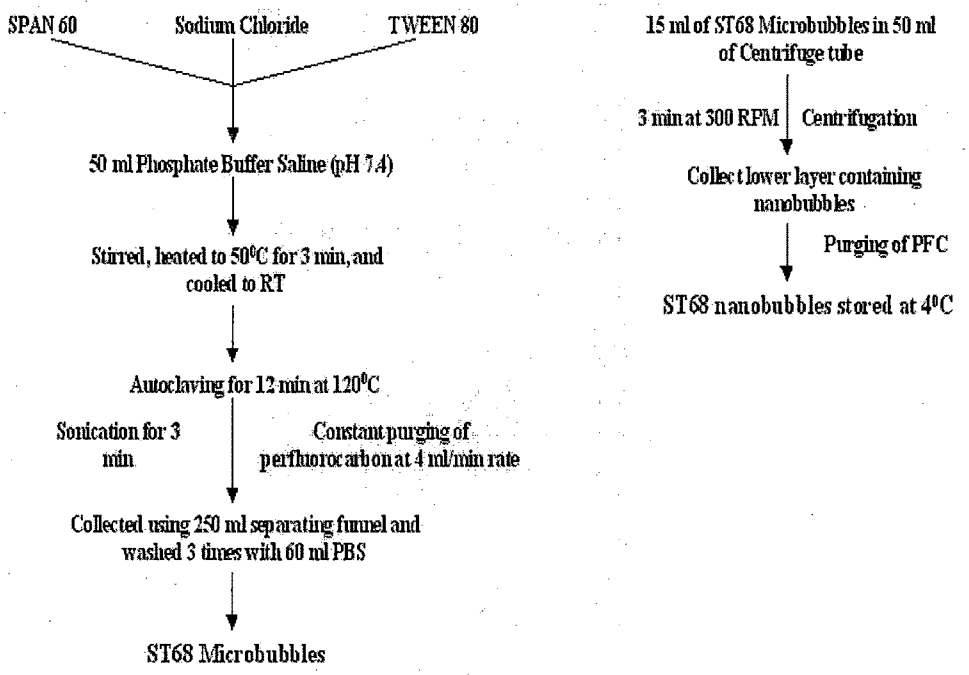
2.6.5.6 Adenovirus Microbubbles

Chen et al demonstrated successful transfection of rat myocardium in vivo by ultrasound-targeted microbubbles destruction of microbubbles containing an adenovirus encoding a beta-galactosidase reporter gene (152). The liposome microbubbles were prepared using a modification of a previously described method (153). A solution of 0.4 % DPPC, 0.1 % DPPE and 10 % glycerol was mixed with AdCMV-luc (recombinant, replica-defective adenovirus containing free fly luciferous DNA) in a 2:1 ratio. Aliquots of 0.5 ml were placed in 1.5 ml clear vials; the remaining headspace was filled with the perfluoropropane gas. Each vial was then mechanically shaken for 20 second in dental amalgamator. The microbubbles were washed three times with phosphate buffer saline to remove unattached AdCMV-luc. They concluded that high levels of transgene expression could be achieved in the heart using UTMD containing adenoviral or plasmid DNA.

2.6.5.7 Preparation of ST68 microbubbles

ST 68 microbubbles composed of surfactant based shell (SPAN 60 and TWEEN 80) filled with a perfluorocarbon gas (122). Targeting of cells outside the capillaries requires agent diameters of less that 400 nm to escape through larger pores present in leaky vasculature of a tumor. Microbubbles have the potential to be used to directly target cancer cells if the agent can be made small enough to pass through the vessels feeding the tumor and be modified to attaché to specific sites in the tumor cells. Figure 2.6 represents preparation of ST68 microbubbles and their separation from nanobubbles, which can pass through the irregular vasculature of tumor angiogenesis.

Figure 2.6: Steps involved in preparation of ST68 microbubbles



2.6.5.8 Acoustically active lipospheres (AALs)

Unger et al described uses of AALs for delivery of drugs with ultrasound (154). The proposed method for preparation of AALs is mentioned in Figure 2.7. AALs represent a new class of acoustically active drug delivery vehicles. AALs represent a new potential drug delivery system that may be useful for a variety of lipophillic drugs such as paclitaxel, steroid hormone, and amphotericin B. Peptide based drugs such as urokinase, interleukins can also be incorporated into AALs.

In vivo studies are presently in progress to determine if these new drug carriers will improve drug delivery with ultrasound.

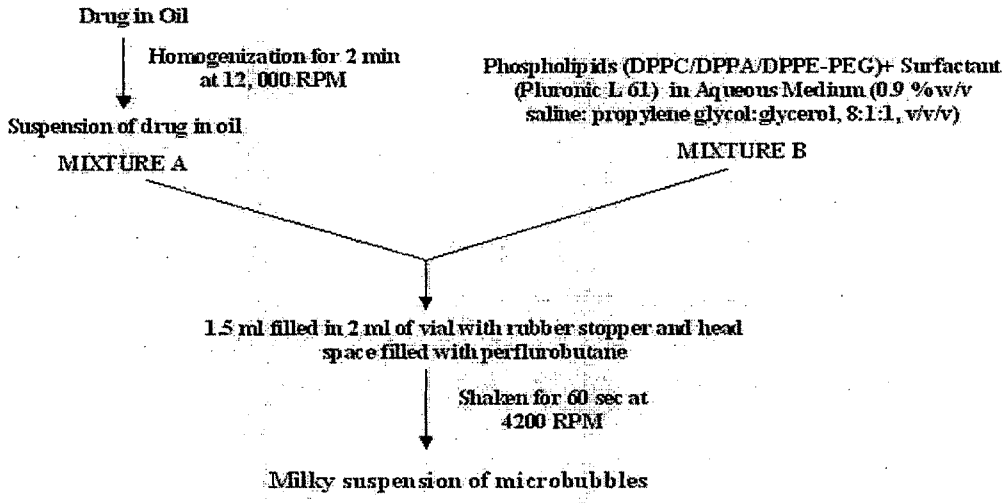


Figure 2.7: Steps involved in preparation of acoustically active lipospheres (AALs)

2.6.6 Characterization of Microbubbles

2.6.6.1 Microbubble size, size distribution and concentration

There are various techniques available for the microbubble concentration and size distribution measurements. Bjerknes et al used Coulter Multisizer for particle concentration and size distribution of the microbubble suspension (134). Particle size distribution of lyophilized gas filled microparticles as prepared by Seemann et al was measured by laser diffractometry technique using Mastersizer S (Malvern Instruments Ltd., UK) (146). Particle size was expressed as the volume mean diameter in μm. The SPAN, which describes the polydispersity of suspension, was also calculated. For the ultrasonographic contrast agents, both microbubble number concentration and volume concentration have been proposed as assay parameters (155). The microbubble number and volume can also be measured by light microscopy on a hemocytometer (156).

2.6.6.2 Air or gas content measurement

The content of air encapsulated within the microbubbles in the suspension samples was measured by oscillation U-tube densitometry with DMA-58 (Anton Paar, Austria). The instrument was calibrated with air and purified water prior to use (134).

The density of the suspension was measured before and after elimination of encapsulated air. The complete removal of encapsulated air was achieved by 5 min high-powered sonication. The air content was calculated as

$C_{air} = ((D_1-D_2)/D_2)*100$

where C_{air} is air content (% v/v) and D1 (g/ml) and D2 (g/ml) are densities of the suspension before and after elimination of encapsulated air. Bjerknes et al observed that the amount of air encapsulated within the polymeric microbubbles directly proportional to the amount of polymer used.

2.6.6.3 Surface morphology by SEM/TEM studies

The surface characteristics of microbubbles could be examined by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). For SEM studies, the microbubbles suspension is casted in a gelatin/sucrose mixture, cryo sectioned at -93^0C in a microtome set at 90 nm thickness or attached to a Thermanox grid. After 20 min incubation, the microparticles were washed twice with distilled water, air-dried and gold label was enhanced by silver coating. For TEM studies microparticles were embedded in epoxy resin and cross-sections of 70-100 nm were prepared.

2.6.6.4 Echogenic properties by ultrasound attenuation measurements

The echogenic properties means ability of microbubbles to enhance backscatters or regeneration of ultrasound contrast was determined by the frequency dependent ultrasound transmission attenuation in vitro (157). This method was used to characterize microbubbles for their ultrasound contrast efficacy in vitro, and to determine stability of freshly prepared microbubbles over the time. For the measurement, suspension is diluted and treated with ultrasound of different frequency range. The higher the attenuation, the higher the ultrasound contrast efficacy of microbubbles (158).

2.6.6.5 Microbubble ultrasound sensitivity determination

It is important to determine how much amount of microbubbles is sufficient to selectively 'mark' the target for the ultrasound examination.

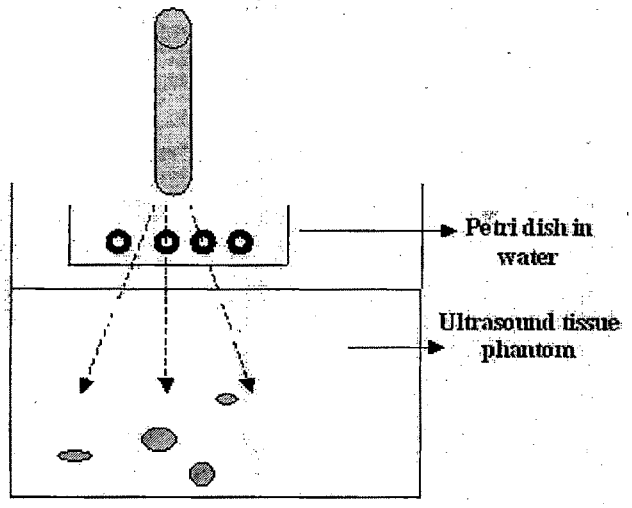


Figure 2.8: An apparatus for the measurement of microbubble ultrasound sensitivity

The amount of microbubbles necessary for successful ultrasound opacification of human blood is much smaller. Klibanov et al mentioned experimental set up for imaging of targeted microbubbles on tissue culture dish with an ultrasound diagnostic imaging apparatus (Figure 2.8) (109). Principle of biotin-avidin interaction used to measure sensitivity of microbubles for ultrasound images. Here microbubbles contain biotin on surface and tissue culture dish is covered with avidin on surface. Area of dish is covered with different concentrations of microbubbles and patches of microbubble on the surface of petri dish visualized by a medical ultrasound system as bright spots on the screen.

2.6.6.6 Inertial cavitation threshold measurements

When the pulsed ultrasound is of sufficient acoustic pressure, the microbubbles can be set into an unstable oscillation that results in collapse of the bubble, fragmentation and a release of free gas that subsequently dissolves into the medium. This violent effect is known as inertial cavitation (159). There are therapeutic applications such as thrombolysis, targeted drug delivery (160) and gene delivery (161) that rely on inertial cavitation. Hynynen K et al represented the exposure system used to monitor inertial cavitation (162). Threshold of inertial cavitation defined as the pressure amplitude value that caused a sudden increase in the wide band noise emission. Figure 2.9 shows experimental set up for the measurement of inertial cavitation threshold of microbubbles in an acoustically transparent vessel. The method of measuring inertial cavitation threshold was based on the principle that cavitating microbubbles are strong acoustic sources (163). When microbubbles cavitate, the

acoustic energy emitted by them can be detected by a passive receiving transducer that is placed at 90^0 to the axis of the active sonicating transducer. Both the sonicating and passive transducers are placed so that they focus in the center of the sample vessel.

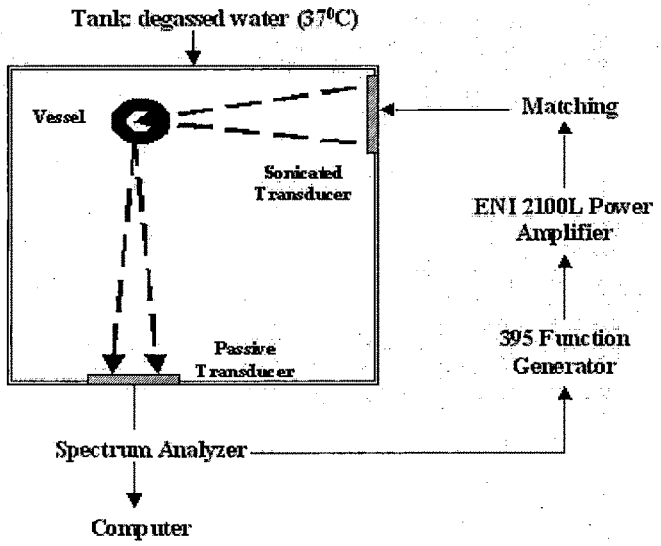


Figure 2.9: Schematic diagram of apparatus used to measure inertial cavitation threshold

2.6.6.7 In vitro measurement of acoustic properties

Unger et al have prepared an experimental set up for measuring in vitro acoustic properties and the acoustic activation ability of acoustically active lipospheres (Figure 2.10) (154). Microbubbles is injected in to chamber filled with degassed water. A 2.5 MHz transducer, permanently attached to the sidewall of the chamber, was driven by pulser/receiver. Reflected echo from the back wall of the chamber was received by the same transducer and displayed on the oscilloscope. The transducer operated in a short-burst mode with a pulse repetition frequency of 1 KHz. The amplitude of the reflected echo from the back wall was recorded. Change of attenuation with time was calculated by

Attenuation coefficient (dB/cm) = [$10*\log_{10}$ (Preamplitude / Postamplitude)/diameter of the chamber].

The same setup can be used to measure ability of AALs to be acoustically and controllably activated (burst effect) required to release encapsulated drug/gene. A 100-kHz therapeutic ultrasound transducer was selected to provide another acoustic field to activate the lipospheres. The transducer was operated at either continuous-wave (CW) or pulsed-wave (PW) mode, at a 7% duty cycle and effect of different

experimental conditions (frequencies) on acoustic activation of AALs can also be studied. They found that the most dramatic lipospheres activation occurred when PW ultrasound was applied at time of 2 min. Around 62 % of the lipospheres were burst and after time of 6 min all lipospheres were burst.

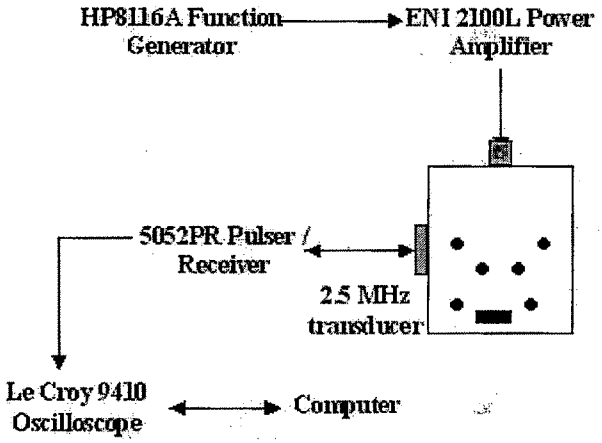


Figure 2.10: Apparatus used to measure acoustic properties of microbubbles

2.6.6.8 In vitro drug partitioning measurements

If the microbubbles are prepared by method as described by Unger et al, partitioning of drug in to the AALs and surrounding aqueous medium can be studied spectrophotometrically. Indeed, AALs are layered on 10% to 90% sucrose gradient in a conical tube and centrifuged at 3000 RPM to force the AALs to the top and to remove oil and other materials not trapped in the AALs. Aliquots of both layers are taken and amount of drug has been measured spectrophotometrically to confirm that the drug remained with the lipospheres layer and had not been pulled into the gradient with the other free materials (154).

2.6.7 Mechanisms for Target Drug Delivery Using Microbubbles

Two possible strategies for delivering drugs and genes with microbubbles are emerging. The first consists on the ultrasound-mediated microbubble destruction, which is based on the cavitation of microbubbles induced by ultrasound application, and the second is the direct delivery of substances bound to microbubbles in the absence of ultrasound. Microbubbles can directly take up genetic material, such as plasmids and adenovirus (164). Phospholipid-coated microbubbles have a high affinity for chemotherapeutic drugs. (165). The mechanisms by which ultrasound facilitates the delivery of drugs and genes depends on type of the therapeutic agent,

the microbubble characteristics, the target tissue, and the nature of ultrasound energy. The presence of microbubbles in the insonified field reduces the peak negative pressure needed to enhance drug delivery with ultrasound. This occurs because the microbubbles act as nuclei for cavitation, decreasing the threshold of ultrasound energy necessary to cause this phenomenon. The mechanisms for microbubble destruction by ultrasound are: (a) gradual diffusion of gas at low acoustic power, (b) formation of a shell defect with diffusion of gas, (c) immediate expulsion of the microbubble shell at high acoustic power, and (d) dispersion of the microbubble into several smaller bubbles. The formation of pores in the membranes of cells as a result of ultrasound-induced microbubble cavitation has been proposed as a mechanism for facilitating the drug deposition.

2.6.8 Mechanism for Microbubble application in diagnosis

Ultrasound is non-invasive medical imaging technique that relies on high-frequency sound waves to produce an image. Since most tissues are heterogeneous in structure, a sound wave sent via transducer acting as both transmitter and a receiver will be scattered or reflected (166) It is this scattered energy, or backscatter, that returns to the transducer and from which an image can be produced.

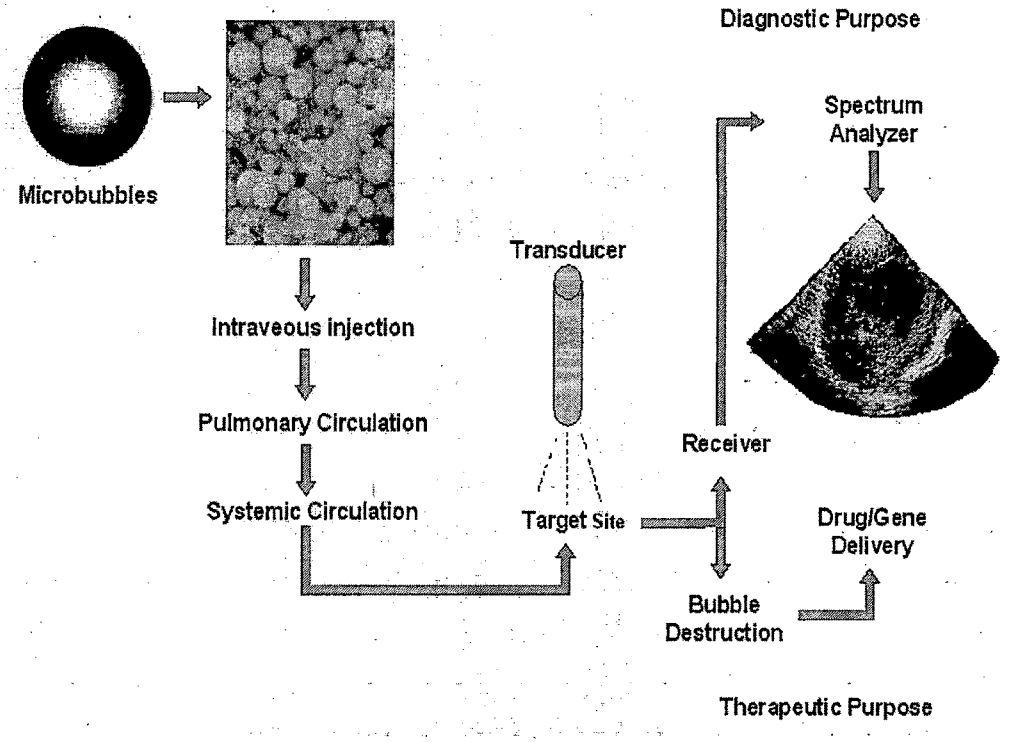


Figure 2.11: Mechanism of microbubbles used in diagnosis

Ultrasound depends on the interface between two different types of tissues to produce an image. At the interface of the two different tissues, there is difference in properties, which is known as impedance mismatch. Impedance is related to product density and the speed of sound through the material. Thus, skin and bone have been different impedances and an image of bone can be easily produced. In the situation where the tissue is homogenous and there are no different impedances, such as the imaging of breast tumor, an ultrasound contrast agent is needed, which provides a difference in impedance and makes imaging possible (167). Mechanisms for diagnosis and therapeutic purpose for microbubbles can be represented as Figure 2.11.

2.6.9 Application of microbubbles

2.6.9.1 Diagnostic applications

Restriction of the microbubbles to the vascular space makes them ideal for contrast echocardiography and vascular imaging. Contrast-enhanced ultrasound improves visualization of the cavities of the heart, the lumen of arteries and veins and small vessels within solid organs and perfused tissues. Echocardiography is used extensively to assess ischemic heart disease; however, in the absence of contrast agent, a significant proportion of examinations do not allow accurate diagnosis of ventricular dysfunction. Effective endocardial border delineation helps assessment of global heart function and detection of coronary insufficiency (168). Harmonic ultrasound imaging at the bedside with a PFC microbubble agent allowed determination of left ventricular wall motion and ejection fraction, hence ventricular function, in 91% of patients, as compared to 56% with standard imaging, and prevented misinterpretations in 44% of studies (169). This technique may, therefore, have a significant impact on patient management in the intensive care setting. Vascular imaging is intended to determine supply vessel occlusions, the presence of clots and wall abnormalities, such as atherosclerotic plaques and possible plaque ulceration and, if they exist, to assess their effects on blood flow (170). Assessment of myocardial perfusion, hence of microvascular integrity, is key to diagnosing coronary artery disease and myocardial infarction, quantitatively assessing the area at risk of necrosis and infarct area during coronary occlusion, evaluating stenoses and

the success of thrombolytic treatment and providing guidance for patient management (171-181). Assessment of perfusion differentiated stunning from necrosis and accurately predicted recovery of left ventricular function in patients after acute myocardial infarction (182). Accuracy of myocardial perfusion quantification was similar or superior to that provided by single-photon emission computed tomography (183,184). The practical methodological issues of myocardial perfusion imaging have also been reviewed (185). Microbubble destruction/refilling techniques allowed improved visualization and quantification of kidney (186,187), liver and tumor (188-190) microcirculation. Display of contrast agent refill kinetics in cerebral microcirculation after bubble destruction by transcranial ultrasound allowed quantitative cerebral blood flow analysis (191,192). Because of differences in vasculature between malignant and normal tissue, sonographic contrast may help identify and characterize prostate cancer (193), tumors and metastasis in the liver (194,195). The potential for ultrasound to evaluate angiogenesis in breast, prostate and liver tumors as well as the response to treatment has been reviewed (196). Contrast-enhanced ultrasound may help monitor the microvascular changes associated with the development of malignant tumors and with tumor response to treatment more easily than CT or MR imaging.

2.6.9.2 Microbubbles in therapy—ultrasound-assisted drug and gene delivery

Microbubbles have potential for delivering or helping deliver drugs and genes to selected tissues. Microvessel ruptures created by ultrasound-triggered microbubble destruction (likely due to cavitation) provided focal delivery of colloidal particles and red blood cells in a given tissue (197). Incorporation of a fluorescent dye in cultured cells was also reported (198). The acoustic power required to induce sonoporation, i.e. generate transient ultrasound-induced perforations in cell membranes, was significantly reduced when microbubbles were present (199). In vitro experiments on lymphocytes indicated that sonoporation was directly related to microbubble-to-cell ratio and to bubble-to-cell spacing (199). Close bubble cell spacing increased the probability of lethal (irreversible) sonoporation. The microbubbles themselves can be loaded with bioactive material. Cargo space is, however, limited, as it usually only concerns the bubble shell. Microcapsules with thicker shells, allowing larger drug cargo, can be considered; however, thicker shells tend to reduce backscatter and susceptibility to acoustic disruption. Finally,

ultrasound can help trace the delivery of an active substance to a specific tissue and trigger the release of the active cargo. Dextrose albumin-coated *F*-butane microbubbles, unlike dextrose albumin-coated air microbubbles, preserved albumin's ability to bind synthetic antisense oligonucleotides and allowed their deposition in a specific organ upon insonation (200). Soybean oil containing *F*-butane microbubbles, suggested to comprise an oil layer inside a phospholipids shell have been used to deliver paclitaxel (201). A 10-fold reduction in toxicity was observed in mice as compared to free paclitaxel. Delivery of oxygen to tissues using PFC-stabilized microbubbles is also being investigated (202,203). Animal experiments provided convincing proof of concept, including survival of normovolemic erythrocyte-depleted rats and of pigs with potentially lethal hemorrhagic shock (203). Targeted microbubbles can allow site-specific delivery and release of material by insonation (204). Gene delivery is another much coveted goal for microbubble/ultrasound combination strategies. The presence of microbubbles can significantly enhance acoustically induced cell transfection, probably due to enhanced sonoporation (205,206). A recombinant adenoviral vector, containing the reporter gene b-galactosidase, has been combined with albumin-coated *F*-propane microbubbles and administered to rats. Transgene expression was directed to the heart by acoustic destruction of the gene-carrying microbubbles in that organ (207). Ultrasound exposure in the presence of microbubbles was reported to achieve 300-fold higher transgene expression in vascular cells in vitro than with naked DNA alone; efficacy of a polyamide transfection agent was also enhanced (208). Intrauterine injection of naked DNA-expressing proteins, in combination with PFC microbubble-enhanced ultrasound, produced protein expression in fetal mice (209). Luciferase expression increased 10 -fold in comparison with expression after 3 injection of naked DNA alone or naked DNA with ultrasound. In vitro ultrasound-mediated gene expression and transfection efficiency were enhanced when DNA was incorporated into albumin-coated *F*-propane microbubbles, as compared to unloaded bubbles mixed with plasmid (210). Adenovirus delivery in rats has been promoted using an albumin-coated PFC microbubble; ultrasound was used to image the delivery and disrupt the microbubbles, resulting in increased myocardial gene expression (211). Positively charged microbubbles that bind DNA has also been produced (206). The mechanism of plasmid delivery and transfection by microbubbles remains, however, controversial (209,212,213).