CHAPTER II

LITERATURE REVIEW

Liposomes (New, 1997; Sharma and Sharma, 1997)

Liposomes are simple vesicles in which an aqueous volume is entirely enclosed by a membrane composed of amphiphilic lipid molecules (usually phospholipids). They form spontaneously when these lipids are dispersed in aqueous media. Phospholipids are biodegradable and biocompatible so liposomes can be employed as safe and efficacious vehicles for medical applications. The ability to encapsulate a variety of agents is an advantage of liposomes as drug carriers. Both hydrophobic and hydrophilic agents can be applied because liposomes can encapsulate these agents in the lipid bilayer and in the aqueous compartment, respectively. The size of liposomes can be in the range of 0.025 μm to 2.5 μm, and it is a critical parameter that determines circulation half-life of liposomes in biological systems.

Liposomes can be arbitrarily classified, based on composition and other properties, into at least five types: conventional liposomes, pH-sensitive liposomes, cationic liposomes, immunoliposomes, and long circulating liposomes. These are shown in Table 1. In addition, liposomes can also be classified by their sizes and number of bilayers as shown in Table 2.

Liposomes have been investigated as carriers of various pharmacologically active agents such as antimicrobial drugs, antineoplastics, steroids, vaccines, and genetic materials. Many studies demonstrate that liposomes, compared to their non-liposomal formulations, can effectively increase therapeutic efficacy and therapeutic indices of drugs in preclinical models and in humans.

Table 1: Classification of liposome based on composition and mode of drug delivery. (From Sharma and Sharma, 1997)

Type	Composition	Characteristics
Conventional liposomes (CL)	Neutral and/ or negatively charged phospholipids plus cholesterol	Subject to coated-pit endocytosis; contents ultimately delivered to lysosomes, if they do not diffuse from the endosome; useful for reticuloendothelial system (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 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, cholesterol plus 5-10% of PEG-DSPE, GM1 or HPI; ≤ 0.1 μm in size	Hydrophilic surface coating; low opsonization and thus low rate of uptake by RES; long circulation half-life (≈ 40 h); dose-independent PK upto 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

Table 2: Classification of liposome by size and number of lamellae. (From Sharma and Sharma, 1997)

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 lipophilic 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 or hydration of lipids in presence of an organic solvent.
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 macromolecules; rapidly cleared by RES; prepared by detergent dialysis, ether injection, reverse-phase evaporation (REV) or active loading method
SUV (Small unilamellar vesicles)	≤ 0.1 μm	Single bilayer; homogeneous in size; thermodynamically unstable to aggreagation 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.

1. Materials used in the preparation of liposomes (New, 1997)

1.1 Phosphatidylcholines

Phosphatidylcholines are the major structural components of liposomal bilayers. They are amphipathic molecules in which a glycerol bridge links a pair of hydrophobic acyl hydrocarbon chains with a hydrophilic polar head group. Phosphatidylcholine molecules are not soluble in water in the accepted sense. In aqueous media, they align themselves closely in planar bilayer sheets in order to minimize the unfavorable interactions between the bulk aqueous phase and the hydrocarbon fatty acid chains. Such interactions are completely eliminated when the sheets fold on themselves to form closed sealed vesicles. In contrast to the other amphipathic molecules (detergents, lysolecithin), the bilayer sheets are formed in preference to micellar structures. The double fatty acid chains give the molecule an overall tubular shape. This geometric structure is more suitable for aggregation into planar sheets. On the contrary, detergents, with a polar head and single chain assuming conical shape, fit nicely into a spherical micellar structure. The structures of phospholipids are shown in Figure 1.

Phosphatidylcholines, also known as lecithin, can be derived from both natural and synthetic sources. They are readily extracted from egg yolk and soya bean but less readily from bovine heart and spinal cord. They are often used as the principal phospholipid in liposomes for a wide range of applications because of their relatively low cost and their neutral charge as well as chemical inertness. Lecithin from natural sources is in fact a mixture of phosphatidylcholines, each with chains of different lengths and varying degrees of unsaturation. Lecithin from plant sources has a high level of polyunsaturation in the fatty acyl chains, while that from mammalian sources contains a higher proportion of fully saturated chains.

1.2 Negatively charged lipids

Charged lipids may be included in the membrane to increase the surface charge density to prevent close approach of liposome vesicles and, hence, prevent aggregation and fusion. Also, they are included in liposomal structure to push adjacent membranes

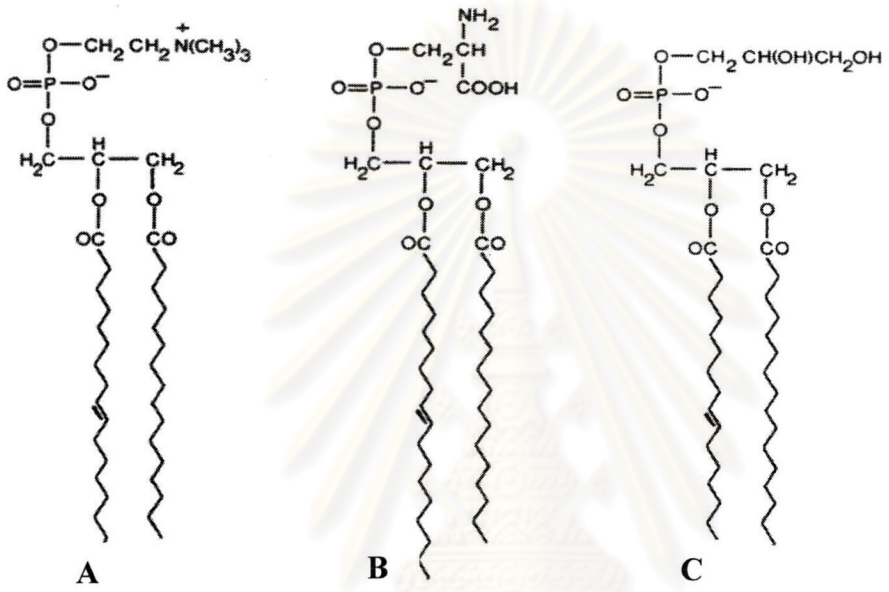

Figure 1: Molecular structure of phospholipids. A, phosphatidylcholine (PC); B, phosphatidylserine (PS); C, phosphatidylglycerol (PG). (From Graham and Higgins,1997)

apart within a multilamellar liposome vesicle to increase the internal aqueous volume. In negatively charged (acidic) phospholipids such as phosphatidylserine (PS), phosphatidylglycerol (PG), and cardiolipin, three possible forces regulate headgroup interactions of bilayer membranes. These are steric hindrance, hydrogen bonding, and electrostatic charge. Dicetylphosphate (DCP) and diacylglycerol have been incorporated into liposomes in order to impart a negative surface charge. Membranes composed of acidic phospholipids can bind strongly to cations. The addition of cations can induce a phase change from liquid-crystalline to gel phase. Total charged lipids are normally used between 10-20 mol%.

A high electrostatic surface charge may enhance liposome-cell interactions. Negatively charged liposomes are predominantly taken up by cell via coated-pit endocytosis (Straubinger et al., 1983).

1.3 Cholesterol

Cholesterol (CH) can not form bilayer structure by itself, but it can be incorporated into phospholipid membranes at various concentrations. CH inserts into the membrane with its hydroxyl group oriented towards the aqueous surface and the aliphatic chain aligned parallel to the acyl chains in the centre of the bilayer. CH reduces the fluidity of membranes above the phase transition temperature (T_c), with a reduction in permeability to aqueous solutes. Thus, incorporation of CH into lipid bilayers can also reduce the leakage rate of aqueous solutes from liposomes. Consequently, inclusion of CH into unsaturated membranes is often essential for sufficient liposome stability. On the other hand, CH increases the fluidity of membranes below T_c, so that its inclusion in saturated membranes, which are usually in the gel phase at ambient temperature, may result in reduction in stability.

Chang and Flanagan (1994) found that the incorporation of 30-50% CH into liposomes reduced the percentage of suramin entrapment. This study suggested that a significant portion of the entrapped suramin resulted from binding of suramin to the surface of the liposomes or intercalation into the liposomal bilayers. On the contrary, Miyajima et al. (1993) found that the addition of cholesterol to the membrane of positively charged liposomes enhanced the entrapment of superoxide dismutase

(SOD). The author suggested that CH allowed better mixing of the positively charged lipid with lecithin. SOD could then bind to the positively charged membrane via electrostatic attraction. Thus, the effects of cholesterol in the entrapment of liposomes also depend on physicochemical properties of drugs as well as membrane components.

2. Liposome-cell interactions (New, 1997)

There are several mechanisms by which liposomes interact with biological membranes. Some of these mechanisms can lead to specific intracellular delivery of active compounds by liposomes.

2.1 Intermembrane transfer

Intermembrane transfer of lipid components can take place upon close approach of the phospholipid bilayers without disruption of the liposomes. However such transfer may occur with complete retention of the content of the liposomal aqueous compartment.

2.2 Contact release

Liposome contact with the cell causes an increase in permeability of the liposomal membrane. This leads to release of water soluble solutes in high concentration in the close vicinity of the cell membrane. Cell-induced leakage of solutes has been observed to be greater in membranes with CH concentrations above 30 mol%. This process is very effective under conditions where flow and turbulence of the medium surrounding the cells are reduced. In addition, the physical interactions between liposomes and cells can be strengthened by means of receptor/ligand binding between the two membranes.

The above two phenomena can be very effective for introducing materials, either membrane lipids or liposomal contents, into specific cells without the need for ingestion of the whole liposomes. These mechanisms are insignificant in cells with phagocytic activity.

2.3 Adsorption

The physical attractive forces of liposomes with the cell surface or the binding of specific receptors to ligands on the vesicle membrane may result in the adsorption of liposomes to cell surface. Adsorption of liposomes to cell surface can often occur with little or no internalization of either aqueous or lipid components. At below Tc the uptake is usually enhanced because the binding sites are more stable at low temperature. The attachment of liposomes to cell membranes via surface proteins, however, can result in rapid uptake into the cell (Leserman et al., 1980).

2.4 Fusion

Fusion can occur when liposomes have close encounter with cell membranes. Fusion can result in complete mixing of liposomal lipids with the plasma membrane of the cells and the release of liposomal contents into the cytoplasm. However, these introduced materials may be quite toxic to cells presumably through nonspecific membrane perturbing effects after incorporation within the cells. Fusion was once considered the dominant mechanism of cell interaction with liposomes in a fluid state. This process now takes second place to phagocytosis as a means of inducing cellular uptake.

2.5 Phagocytosis/endocytosis

Cells with endocytosis take liposomes into endosomes, which have a pH of 5-5.5. The early endosomes then fuse with lysosomes to form secondary lysosomes where cellular digestion occurs in a milieu of approximately pH 4.5 (Figure 2). Lysosomal enzymes break open the liposomes, the phospholipids being hydrolyzed to fatty acids, which can be recycled and reincorporated into host phospholipids. Anionic lipids and drugs can inhibit lysosomal breakdown and produce a pseudo-storage disease in the lysosomes. During the liposomal breakdown, the contents of aqueous compartment of liposomes are released. These contents will either remain sequestered in the lysosomes until exocytosis, or they will slowly leak out of the lysosomes into the cells. The mechanism of phagocytosis is discussed later under *Macrophages.*

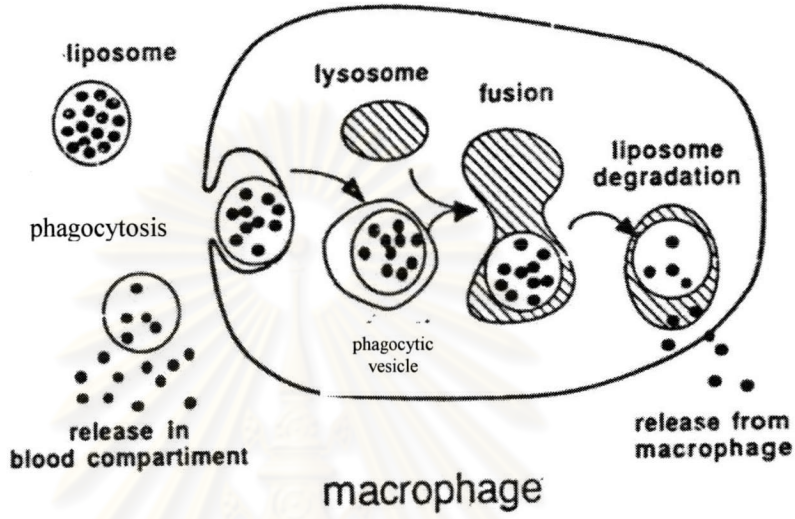

Figure 2: Mechanism of phagocytosis/endocytosis. (From Crommelin et al., 1990)

3. Determination of liposome-cell interactions (New, 1997)

Liposomes can interact with the cells by several mechanisms. In order to discriminate between the different types of interaction that may occur, many of the assay techniques can be used. In any event, differentiation of uptake mechanism generally requires the use of at least two separate and distinct markers. One is for accurate assessment of liposome lipid association with the cell, and the other is an aqueous space marker (Schroit, Madsen, and Nayar, 1986).

3.1 Differentiation between lipid exchange and association

Radiolabelled lipid markers are used. This technique is one of the simplest ways of monitoring uptake of liposomes by cells. However, many common membrane phospholipids are able to transfer from one membrane to another by intermembrane contact, without any strong or long term interaction between the two membranes. Thus incubation of liposomes with cells in vitro may well overestimate the percentage uptake if phospholipids or cholesterol are used as markers.

3.2 Differentiation between adsorption and internalization

Electron microscopy of thin sections is used to compare adsorption with internalization. The appropriate markers for inclusion in liposomes are ferritin, horseradish peroxidase, and colloidal gold. The method is cumbersome and requires highly skilled personnel. An alternative approach for assessing the degree of internalization of liposomes is to measure the extent of degradation of metabolizable markers incorporated in liposomes. The disadvantage of using the metabolism of entrapped markers as an indicator of internalization is that there may be routes of internalization which do not involve lysosomal or other degradation. In this case, measurement of the degree of metabolism may underestimate the amount of material digested. Also, not everything that is taken up is necessarily metabolized immediately.

3.3 Differentiation between fusion and phagocytosis/endocytosis

Fluorescence dequenching is the classic method for monitoring the fusion of liposomes with cells. This technique relies on the fact that at high concentrations fluorescent substances, being self-quenched, display very little fluorescence until the solution is diluted, with a consequent increase in fluorescence. The liposomal contents are delivered to the cytoplasm and are diluted many hundred-fold when the fusion of fluorescence-containing liposomes with the cell plasma membrane occur. The cell will thus display a strong diffuse fluorescence throughout the whole body, with a dark area in the region of the nucleus (Figure 3). In contrast, liposomes which have been endocytosed will display punctuate fluorescence, restricted to the secondary lysosomal and endocytic vacuoles. Adsorbed liposomes will not fluoresce at all, unless the fluorescence concentration has been reduced by extensive leakage, in which case the appearance of the cell after washing should theoretically be dully fluorescent, with a bright rim.

Carboxyfluorescein (CF) and calcein are water soluble fluorophores that can be entrapped within liposomes at different concentrations. At low concentrations (<1 mM), both probes exhibit fluorescence directly proportional to their concentration. But as the concentration is increased, the quantum yield of the fluorophores decreases dramatically. Thus, under ideal conditions, liposomes containing high concentrations of fluorophore are only marginally fluorescent. However, upon dilution of the dyes by detergent lysis or release of the liposome contents into the cell cytoplasm, a dramatic increase in fluorescence is observed. Thus, at least theoretically, liposome-cell fusion can be readily distinguished from liposome-cell binding. The use of carboxyfluorescein, however, seems to be limited by cell-induced and cell-free culture supernatant-induced leakage, and its sensitivity to acidic pH which dramatically decreases the probe's quantum yield. In contrast, calcein is not pH-sensitive in the range of pH 6-8. It has been employed as a marker for the delivery of pH-sensitive liposomes to cells where its quantum yield is apparently unaltered, whether the probe is localized in the cell cytoplasm or in acidic lysosomes. Cells incubated for periods of time (several hours) can leak fluorescence into the surrounding medium if CF is used. CF can also leak from the lysosome into the cytoplasm. Use of calcein instead of CF could overcome some of these problems.

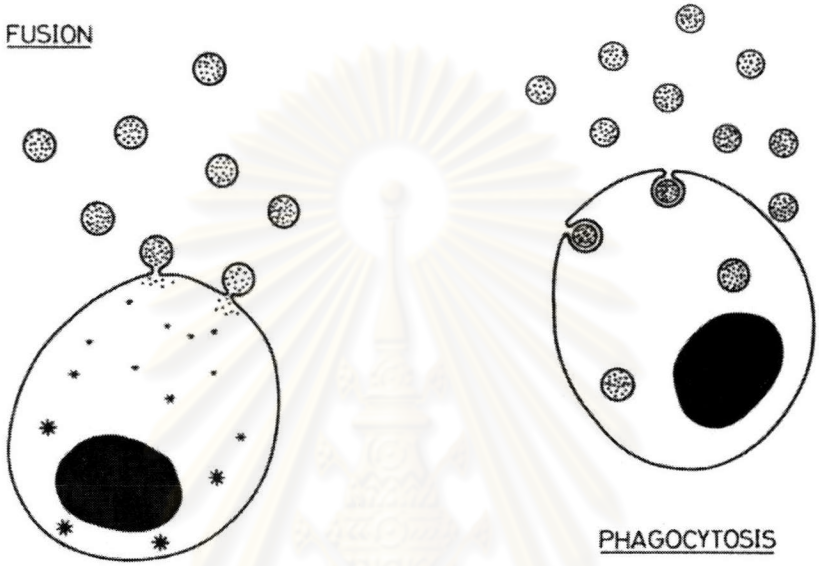

Figure 3: Cellular uptake of liposomal carboxyfluorescein. The use of carboxyfluorescein (CF) to distinguish between fusion and phagocytosis. CF, which is self-quenched when contained at high concentrations inside the liposome, displays a marked increase in fluorescence as it spreads throughout the cytoplasm and becomes unquenched. In contrast, uptake of liposomes by phagocytosis results in practically no dilution of the CF (and hence no fluorescence increase) since the liposomal contents are initially retained inside secondary lysosomes. (From New, 1997)

Another evidence indicative of one mechanism in preference to another is the influence on uptake of treatment of cells with metabolic inhibitors such as cytochalasin B or sodium azide and deoxyglucose, or with ammonium chloride or chloroquine, which are known to inhibit fusion of lysosomes with the phagosome. These agents would be expected to interfere with processing by phagocytosis but not with uptake by fusion with the plasma membrane.

In addition, the fluorescent phospholipid analogues may be used where punctuate lysosomal localization can be differentiated visually from diffuse plasma membrane fluorescence. However, adsorption will be difficult to distinguish from fusion. Such complications may be resolved by photobleaching studies, where the mobility of adsorbed lipids is lower than that of lipids incorporated into the membrane by fusion.

4. Applications of liposomes in drug targeting

Liposomes have been employed to accomplish both passive and active targeting of drugs (Figure 4).

4.1 Passive targeting

Liposomes are naturally considered foreign substances by the body. Conventional liposome formulations of drugs and immunostimulators have been successfully used for targeting cells of the reticuloendothelial system (RES). They exhibit significant improvement in the therapeutic index of the drugs (Aliving, 1991). Liposomes have also been used to enhance the antigenicity of certain molecules for new vaccine formulations (Allison and Gregoriadis, 1974). Furthermore, conventional liposomes have also been employed for targeting of immunosuppressive drugs to lymphatic tissues such as the spleen (Binder et al., 1994).

4.2 Active targeting

Active targeting of liposomal drugs may be accomplished by coupling specific antibodies to vesicles (Van Rooijen, 1995; Khaw et al., 1998). These liposomes are

termed immunoliposomes. Active targeting using immunoliposomes has several advantages over those of antibody-drug conjugates. Immunoliposomes can carry a significantly larger number of drug molecules compared to simple conjugates (Lasic and Martin, 1995). In addition, immunoliposomes can encapsulate drugs with widely varying physiochemical properties (Lasic and Martin, 1995). Finally, the drug, once released from immunoliposomes, can reach their intracellular target by diffusion without having to undergo receptor-mediated endocytosis (Figure 4).

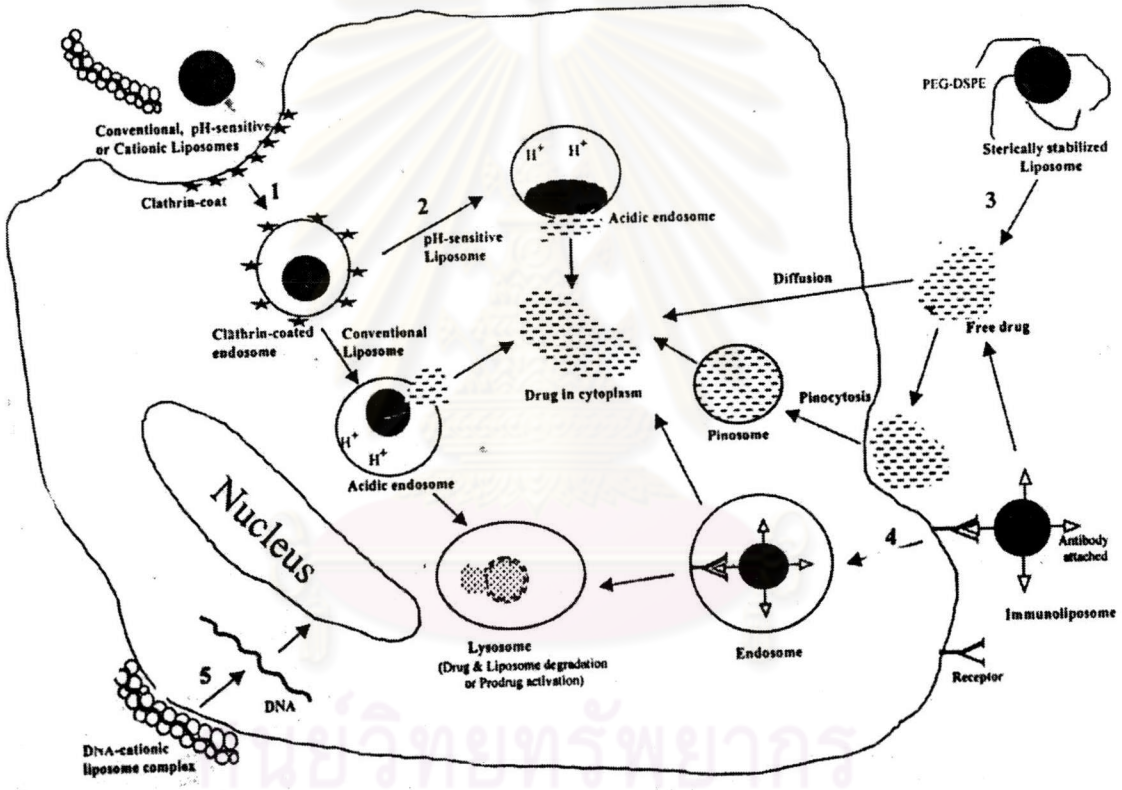

Figure 4: Predominant mechanisms of intracellular drug delivery by liposomes. The different pathways are indicated by numbers: 1. coated pit endocytosis of conventional, pH-sensitive and cationic liposomes; 2. release of drug in the acidic endosome by pH-sensitive liposomes; 3. intravascular and/or extracellular drug release from long circulating liposomes; 4. receptor-mediated endocytosis of immunoliposomes; 5. fusion of cationic liposomes with plasma membrane. (From Sharma and Sharma, 1997)

Macrophages as a host/reservoir

Macrophages have many functions. Some of these biological functions are related to their phagocytic capacity. One of the most important functions of macrophages is the host defense against microorganisms, particularly intracellular parasites. Consequently, macrophages can also act as the reservoir for a variety of bacteria and obligate intracellular parasites. Current studies have described the important role of monocytes (immature macrophages) in viral infectious diseases such as HIV-1 infection. The virus can replicate using macrophages as convenient host (Sigal and Ron, 1994). Bacteria such as *Mycobacterium avium-M. intracellulare* complex (MAC), a common complication in AIDS, can replicate within macrophages (Armstrong et al., 1985; Horsburgh, 1991). Therefore, intracellular targeting of drugs into macrophages will be of therapeutic value.

Macrophages (Sigal and Ron, 1994)

Monocytes and macrophages originate from monoblasts which are the precursors in the bone marrow. In the bone marrow, monoblasts divide and differentiate into promonocytes, which later become monocytes. Monocytes remain in the bone marrow for only a short time after which they enter the circulation. This process is illustrated in Figure 5. Peripheral blood monocytes vary in size between 20-25 μm. They have a large, oval, indented nucleus which can be either horseshoe- or kidney-shaped. Monocytes may exit the circulation and migrate into the tissues where they differentiate into macrophages. The mature macrophages are terminally differentiated cells which do not divide. They have a half-life of 3 months, after which they die and are cleared from the body. During maturation, these cells acquire many functions. These include host defense against microorganisms or, particularly, intracellular parasites, the ability to initiate immune responses through stimulation of and/or antigen presentation to immunocompetent T cells, secretion of various mediators, and control of tumor growth. Some of these functions are related to their phagocytic activity.

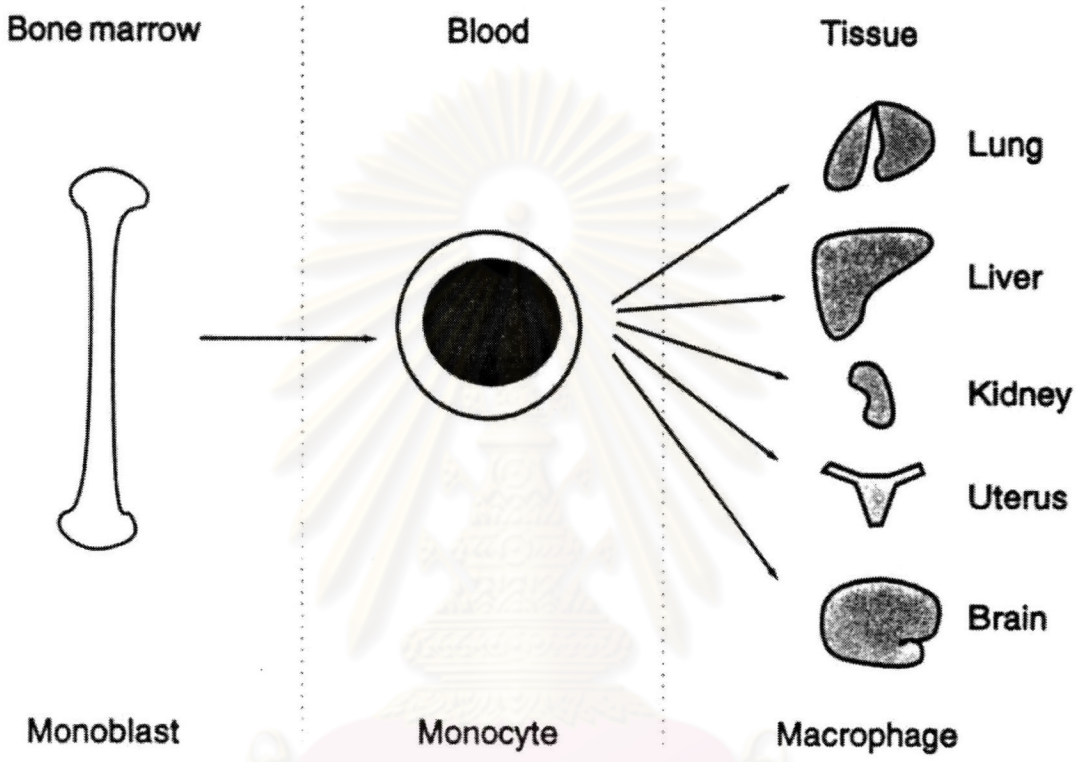

Figure 5: Differentiation of macrophages. Monocytic cells exist in different forms in three separate tissue compartments: bone marrow precursors, peripheral blood monocytes, and tissue macrophages. (From Sigal and Ron, 1994)

1. Phagocytosis (Sigal and Ron, 1994)

Phagocytosis is an important function of monocytes and macrophages. Phagocytosis by macrophages is critical for the uptake and degradation of infectious agents and senescent cells. It also participates in the immune response and inflammation. Phagocytosis is often enhanced by interaction of the phagocyte with opsonins. Macrophages discriminate between infectious agents and self by phagocytic receptors. Abnormality in phagocytic functions of the macrophages can also lead to several diseases .

1.1 Mechanism of phagocytosis (Aderem and Underhill, 1999)

There are various mechanisms by which macrophages recognize the foreign bodies and discriminate between the foreign bodies and self. This is often done via specific receptors.

1.1.1 Fc receptor-mediated phagocytosis

Fc receptors (FcRs) are a group of surface membrane molecules that specifically recognize and bind the Fc portion of the immunoglobulin molecule and then presumably mediate biologic functions. Human phagocytic cells have at least three separate protein receptors for IgG on their surface: huFcRI, huFcRII, and huFcRIII.

Several studies have documented a decreased rate of immune complex clearance in autoimmune diseases such as systemic lupus erythematosus, Sjögren's syndrome, and dermatitis herpetiformis. Additionally, there is decreased Fc-mediated phagotosis by normal individuals with the HLA haplotypes DR2 and DR3, which are associated with autoimmune disaeses.

1.1.2 Complement receptor-mediated phagocytosis

Complement proteins, present in serum, opsonize bacteria for phagocytosis by the C3b or C3bi receptors (CRs) on macrophages. Several receptors that participate in phagocytosis of complement-opsonized particles are expressed on

macrophages. While FcRs are constitutively active for phagocytosis, the CRs of resident peritoneal macrophages bind but do not internalize particles in the absence of additional stimuli. Particle ingestion by CRs can be induced by protein kinase C activators such as phorbol myristate acetate (PMA), as well as by tumor necrosis factor-alpha (TNF-α), granulocyte/macrophage colony stimulating factor granulocyte/macrophage colony-stimulating factor (GM-CSF), or attachment to laminin- or fibronectin-coated substrata.

1.1.3 Mannose receptor-mediated phagocytosis

The mannose receptor (MR) on macrophages recognizes mannose and fucose on the surface of pathogens and mediates phagocytosis of the organisms. The high affinity of this receptor for branched mannose and fucose oligosaccharides makes the MR a phagocytic receptor with broad pathogen specificity. In addition to the phagocytic signals mediating particle internalization, proinflammatory signals are generated upon MR ligation.

1.2 Maturation of the phagosomal vacuole

After internalization, F-actin is depolymerized from the phagosome, and the newly denuded vacuole membrane becomes accessible to early endosomes. Through a series of fusion and fission events, the vacuolar membrane and its contents mature, fusing with late endosomes and ultimately lysosomes, to form a phagolysosome. The rate of phagosome-lysosome fusion may be related to the nature of the interaction between the particle surface and the phagosomal membrane. De Chastellier and Thilo (1997) proposed that the nature of particle surface modified the rate of vacuole maturation and that the hydrophobic surface of a Mycobacterium inhibited recycling or maturation of phagosomal membranes, an event required for the fusion of phagosomes and lysosomes.

Conclusively for phagocytosis, particle internalization is initiated by the interaction of specific receptors on the surface of the phagocyte with ligands on the surface of the particle. The polymerization of actin at the site of ingestion and the internalization of the particle via an actin-based mechanism occur. The particle is

engulfed by the macrophage cytoplasm, and a vacuole, called a phagosome, is formed. Intracellular events involving interaction between lysosomes and phagosomes described above then occur. Lysosomes are minute bodies in the cell cytoplasm which contain various hydrolytic enzymes. The phagosomes are found near the cell membrane, but in time they migrate toward the perinuclear area where they fuse with lysosomes to form phagolysosomes. This fusion process discharges lysosomal contents, including lysosomal enzymes (e.g. acid hydrolases and peptidases), into the phagolysosomes. The lysosomal enzymes are subsequently activated by the low pH and can digest the engulfed substances, either storing them as "dense bodies" or eliminating them by exocytosis. (Giese, 1979; Sigal and Ron, 1994).

Liposomes as drug carriers for monocytes and macrophages

Liposomes have been widely used as drug carriers of various pharmacologically active agents. Many studies demonstrated that liposomes were effectively phagocytosed by macrophages or phagocytic cells. Monkkonen et al. (1995) proposed that the potency of liposomal clodronate was greater than that of free drug on a murine macrophage cell line, RAW 264. Furthermore, Duzgunes et al. (1996) reported that liposome-encapsulated sparfloxacin was more effective than unencapsulated sparfloxacin in treating MAC infections in the murine macrophage-like cell line J 774. Liposomes have been used for passive targeting of macrophages in several intracellular diseases such as leishmaniasis (Gilbreath et al., 1985).

Factors influencing the uptake of liposomes into cells

1. Liposome composition

The nature of and the charge density on the liposome surface are important parameters that influence the mechanism and extent of liposome-cell interaction. Both of these parameters can be altered by changing the lipid composition. Neutral liposomes do not interact significantly with cells, and in such cases, the drug may enter cells after being released from liposomes extracellularly. On the other hand, high

electrostatic surface charge can promote liposome-cell interaction. Negatively charged liposomes are predominantly taken up by cells through coated-pit endocytosis. They may also release their contents in the circulation and/or extracellularly after interaction with blood components and tissues. Unlike negatively charged liposomes, cationic liposomes have been proposed to deliver contents to cells by fusion with cell membranes (Sharma and Sharma, 1997).

The effect of variations in liposome composition on the uptake by bone marrow macrophages was observed to be similar both in vivo and in vitro (Allen et al., 1991; Foong and Green, 1988; Moghimi and Patel, 1992). Liposome uptake increased with liposome concentration and, for liposomes composed of egg PC, the uptake plateaued at 40 nmol lipid per mg cell protein in one study (Allen et al., 1991). Many studies indicated that negatively charged liposomes containing PS, PG, or PA were taken up faster and at greater extent than neutral liposomes or positive liposomes by phagocytic cells (Allen et al., 1991; Lee, Hong, and Papahadjopoulos, 1992; Lee, Nir, and Papahadjopoulos, 1993). Fidler (1988) found that inclusion of negatively charged phospholipids such as PS and PG in multilamellar vesicles (MLV) consisting of PC greatly enhanced their binding to and phagocytosis by macrophages.

In addition, Papahadjopoulos, Poste, and Schaeffer (1973) proposed that liposomes of certain phospholipid composition could induce the fusion of cultured cells. Liposomes composed of pure PS or its 1:9 mixture with PC caused extensive cell-cell fusion in cultures of 3T3, L929, and BHK 21 cells, while pure PC liposomes did not induce any fusion. Several studies also proposed that negative surface charge could be recognized by receptors found on a variety of cells, including macrophages (Allen et al., 1990; Allen et al., 1988; Lee, Pitas, and Papahadjopoulos, 1992).

2. Cell type

Liposome uptake differs considerably in different cell types. In an in vitro study, Lee, Hong, and Papahadjopoulos (1992) investigated the interaction of liposomes of different surface properties with two mammalian cell lines, CV1 and J 774. Inclusion of 9 mol% of PS, PG, or PA increased the extent of CV1 uptake of PC/CH liposomes by 20-fold, whereas 33 mol% of these anionic lipids was required to

reach this same level of liposome uptake in J 774 cells. In contrast, liposome uptake by CV1 cells was not promoted when monosialoganglioside G_{M1}, phosphatidylinositol, or phosphatidylethanolamine conjugated to polyethylene glycol was incorporated into PC/CH liposomes.

3. Presence of cholesterol

The fluidity of bilayer may also influence the interaction of liposomes with cells. The uptake by the RES of liposomes containing high Tc lipid is lower in extent than that of liposomes containing low Tc lipid (Gabizon and Papahadjopoulos, 1988).

Addition of cholesterol into lipid bilayer is known to affect the fluidity of membranes. Many researchers found that inclusion of increasing amounts of CH and sphingomyelin, which increased bilayer rigidity, decreased the uptake of liposomes by macrophages both in vitro and in vivo (Allen et al., 1991; Foong and Green, 1988; Moghimi and Patel, 1992). Furthermore, Moghimi and Patel (1992) showed that the presence of serum in incubation medium increased the uptake of cholesterol-rich but not cholesterol-poor liposomes in bone marrow cells, whereas serum enhanced phagocytosis of both liposome preparations in peritoneal phagocytes.

Yoshioka et al. (1990) studied the effects of liposomes on cell viability. This study showed that CH content in liposomes also affected the viability of cells. Charged lipid-containing liposomes with high cholesterol (egg PC:CH:charged lipid (R), 4: 5: 1) were more suppressive to the cells than those with lower cholesterol content (egg PC:CH:R, 7: 2: 1). On the other hand, no conclusion regarding the effect of CH on cellular uptake of liposomes could be drawn in the human macrophage U-937 cell line (Katragadda et al., 2000).

4. Size of liposomes

Uptake of liposomes will depend on physical parameters such as their size relative to the size of endocytic vesicles of target cells. Recently, a study investigated uptake of two different types of liposomes, MLV and reverse evaporation vesicles (REV), in normal rat alveolar macrophages using tumoricidal activity as the end point

(Fidler et al., 1980). The extent of tumoricidal activity by MLV-entrapped muramyl diphosphate was higher than that of REV-entrapped drug (Maruyama, 1998; Weissig, Whiteman, and Torchilin, 1998). Liposomes larger than 0.4 μm may not be able to deliver entrapped substances into the cell cytoplasm (Betageri, Jenkins, and Parsons, 1993).