

CHAPTER II

LITERATURE REVIEW

The use of nanoparticulate systems, such as liposomes, to delivery therapeutic agents is gaining attention for medical and pharmaceutical applications. This comes from the fact that the systems can be modified to meet specific physicochemical requirements, have low toxicity, and high compatibility with the body. Nanoparticles are rapidly recognized and cleared from the bloodstream within minutes upon intravenous injection by the reticuloendothelial system (RES), in which macrophages are predominant. Therefore, macrophages are considered potential cellular targets of the nanoparticulate system for several therapeutic agents because the cells play a central role in inflammation and act as reservoirs for microorganisms that cause deadly infectious diseases.

1. Activated macrophages in the immune response

Monocytes are derived from granulocyte-monocytes progenitor cells in the bone marrow during hematopoiesis. These cells circulate in the bloodstream for about 8 hours, migrate into various tissues throughout the body and maturation into macrophages. Macrophages is classified into two major groups; fixed macrophages, which reside in particular tissues, and free macrophages, which are motile by amoeboid movement throughout the tissues. Macrophages are named according to their specific locations such as alveolar macrophages in the lung, Kupffer cells in the liver and osteoclasts in bone (Goldsby et al., 2003).

A variety of stimuli in the sequence of an immune response can activate macrophages. These are particulate antigens, components of bacterial cell walls such as lipopolysaccharide (LPS), and cytokines from activated T-helper (T_H) cells. Interferon gamma ($IFN-\gamma$) is one of the most potent cytokines that can activate macrophage activity. Activated macrophages are more effective than the resting cells in elimination of potential pathogens. They exhibit greater phagocytic activity by increase ability to secrete various cytotoxic proteins for eliminating a broad range of

pathogens. They also increase secretion of inflammatory mediators to activate T cells (Goldsby et al., 2003).

1.1 Phagocytosis (Aderem and Underhill, 1999; Stuart and Ezekowitz, 2005)

Phagocytosis is the process that macrophages utilize to internalize variety of large particles, including whole microorganisms, injured or dead host cells, cellular debris, and activated clotting factors. This process requires phagocytic receptors, which are classified into two major groups. The first ones are cell-associated pattern recognition receptors that directly recognize their surface ligands on pathogens and the receptors that recognize opsonin or pathogens coated in opsonic molecules. Examples of these receptors include the mannose receptors that recognize mannose and fucose oligosaccharides on the surface of yeast and the scavenger receptors that recognize surface components including LPS and lipoteichoic acids (LTA) on the surface of Gram-negative and Gram-positive, respectively. Surface ligands on pathogens of these receptors are called pathogen-associated molecular patterns (PAMPs). PAMPs are commonly expressed by microbial but not mammalian cells (Janeway, 1992). The second type of receptors recognizes opsonin or pathogens coated in opsonic molecules. The opsonization of pathogens results in increasing efficiency and recognition diversity of phagocytic receptors. The opsonic molecules include the mannose-binding protein, the complement proteins, and antibodies. Usually pathogens are recognized and phagocytosed by more than one type of phagocytic receptors, and these receptors act in cooperative action and synergistic ways (Figure 1).

Toll-like receptors (TLRs) are also the cell-associated pattern recognition receptors on macrophages. They are membrane-signaling receptors that play essential roles in innate immune response against pathogens. Their ligands are invariant and necessary components of pathogens include bacterial derivatives (such as LTA, LPS, flagellin, and peptidoglycan) and components associated with viral replication. TLR signaling activates expression of many genes related to innate immunity and inflammation. Both TLR signaling and phagocytosis function in a more complex interplay in innate and inflammatory responses. For example, pathogens are recognized by low-specificity but high-affinity receptors such as scavenger receptors,

which in turn deliver the ligand to high-specific but low-affinity receptors as TLRs, to trigger signaling inflammatory responses.

Interestingly, the majorities of phagocytosed material are derived not only from invading pathogens but also from self-cells. Multiple receptors and opsonins, most of which recognize pathogens, are involved in recognize apoptotic cells and their debris (Figure 1). In contrast to pathogens, phagocytosis of apoptotic cells promotes anti-inflammatory signals such as transforming growth factor- β 1 (TGF- β 1) after phosphatidylserine (PS) that has translocated from the inside to the outside of the apoptotic cell membrane are exposed.

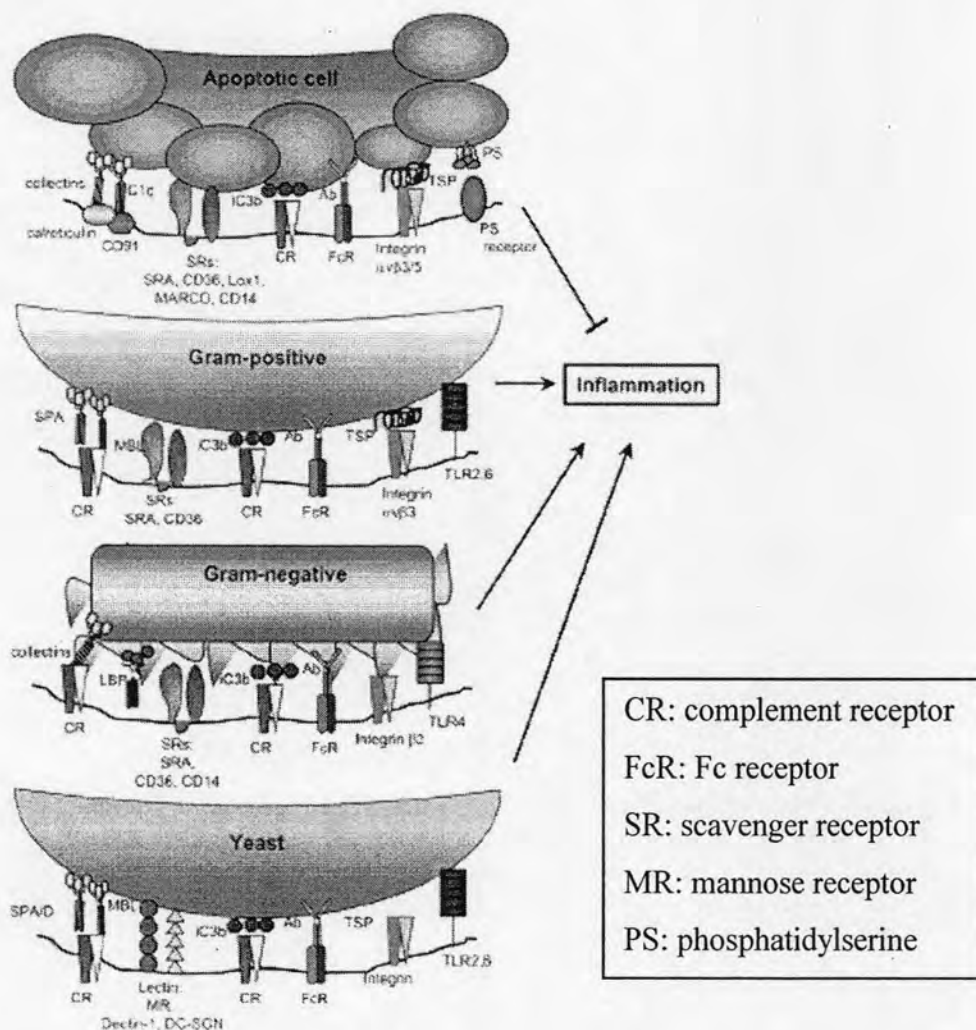


Figure 1: Multiple receptors in phagocytic recognition of apoptotic cells and pathogens (Stuart and Ezekowitz, 2005)

After internalization, particles are delivered to a membrane-bound structure called a phagosome. The phagosome then enters the endocytic processing pathway. In this pathway, a phagosome fuses with a lysosome containing lysozyme and a variety of other hydrolytic enzymes, to form a phagolysosome. Phagolysosomes possess a number of complementary degradative properties including a very low pH, hydrolytic enzymes for particle digestion, defensins and other bactericidal peptides, and ability to generate toxic oxidative compounds. If the particle is a protein antigen, it is digested into antigenic peptides. These peptides intracellularly associate with major histocompatibility complex (MHC) class II molecules. The peptide-MHC class II complexes then move to the cell membrane to present these antigenic peptides to T_H cell. The antigen processing and antigenic peptide presentation are critical to T_H cell activation, a central event in the development of both humoral and cell-mediated immune responses.

1.2 Cytokines and mediators (Alberts et al., 2002; Victor et al., 2004)

Cytokines are a low-molecular weight proteins or glycoproteins which mediate the complex interaction among cells and play roles in cell-to-cell communication. They are produced and secreted by immune cells such as macrophages and lymphocytes, and non-immune cells such as endothelial cells and fibroblasts. These molecules act on many different cell types in a complicated pattern. For example, when macrophages are activated by cytokines from T_H cell such as $IFN-\gamma$, the activated macrophages can, in turn, secrete cytokines to activated T_H cell or other neighbor macrophages. Some cytokines secreted by activated macrophages are listed in Table 1.

Table 1: Some cytokine secreted by activated macrophages (Goldby et al., 2003)

Factor	Function
Interleukin (IL-1)	Promotes inflammatory responses and fever
Interleukin (IL-6)	Promotes innate immunity and elimination of pathogens
Tumor necrosis factor (TNF- α)	Promotes innate immunity and elimination of pathogens and kills tumor cells
Interferon alpha (IFN- α)	Activates cellular genes, resulting in the production of proteins that confer an antiviral state on the cell
GM-CSF, G-CSF, M-CSF	Promote inducible hematopoiesis

Signaling pathways that induce macrophages to secrete cytokines is initiated by either TLRs recognize bacterial products such as LPS or cytokine receptors that are engaged with their cytokines (Figure 2). This engagement activates a number of intracellular signaling pathways including the I κ B kinase-nuclear factor κ B (IKK-NF- κ B) pathway and three mitogen-activated protein kinase (MAP kinase) pathways. These pathways phosphorylate and activate various transcription factors, including activator protein 1 and nuclear factor-interleukin 6 and NF- κ B. NF- κ B is the transcription factor that increases the expression of cytokines (such as TNF- α and IL-12), chemokines, inducible nitric oxide synthase (iNOS), adhesion molecules, apoptotic factors and other inflammatory mediators. It is activated by not only endotoxins and cytokines but also other variety stimuli such as ROS (especially H₂O₂), protein kinase C activators, viruses, UV light and ionizing radiation.

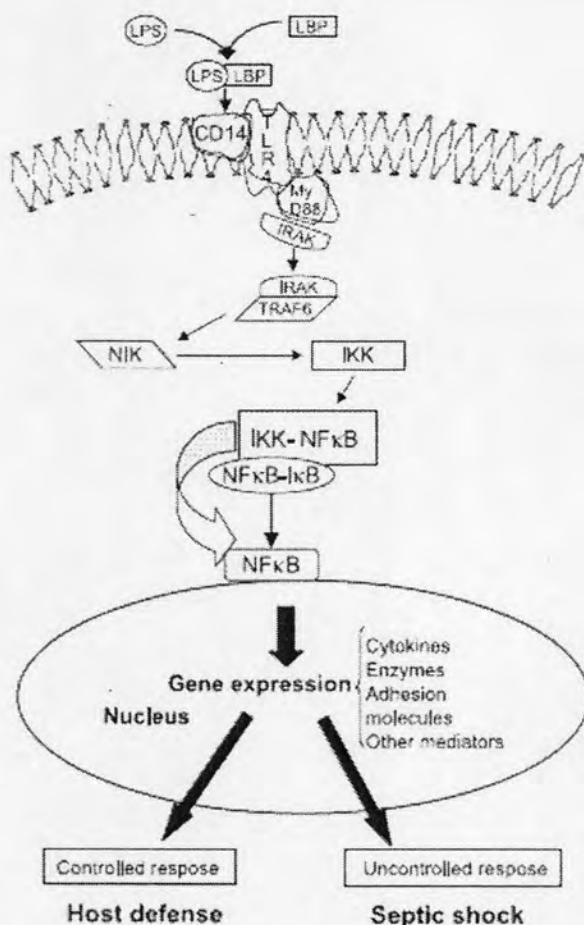


Figure 2: Schematic diagram of LPS-induced signaling pathway of the host inflammatory response in macrophages. (Victor et al., 2004)

1.3 Nitric oxide (NO) and reactive oxygen species (ROS) (Garcia and Stein, 2006; Coleman, 2001; Forman and Torres, 2001; Dedon and Tannenbaum, 2004)

During phagocytosis, activated macrophages convert molecular oxygen into reactive oxygen species (ROS) by phagolysosomal membrane-bound NADPH oxidase. This enzyme is induced and activated by many stimuli, including IFN- γ and LPS. It reduces molecular oxygen into superoxide radicals (O_2^-). The O_2^- is converted into other powerful oxidizing agents, including hydroxyl radicals (OH^\cdot), hydrogen peroxide (H_2O_2), and hypochlorite anion (ClO^-). These ROS is toxic against microbial pathogens and plays role as an intracellular messenger in protein phosphorylation and transcription of inflammatory cytokines.

In addition, activated macrophages also produce the reactive nitrogen intermediate, nitric oxide (NO). Within phagolysosome, NO combines with O_2^- to generate the highly reactive peroxynitrite radicals ($OONO^-$) that can kill microbial pathogens. NO is a soluble gas that has been involved in many physiological processes such as the vasodilatation of smooth muscle, neurotransmission, and nonspecific immune responses to infection, host defense, and cell death. It is synthesized from L-arginine by the nitric oxide synthase (NOS). This enzyme is expressed in two patterns either constitutive (cNOS) or inducible (iNOS) forms. The cNOS (neuronal NOS and endothelial NOS) are activated in response to physiological stimuli by influx of calcium into the cell, leading to a rapid, transient, and low-level production of NO. NO at low level (nanomolar level) has relatively long half-life and is mainly involved in homeostatic processes including neurotransmission, peristalsis, and blood pressure regulation. In contrast, iNOS is not expressed in resting cells, but it is induced upon macrophage activation by several stimuli, such as IFN- γ , TNF- α , IL-1 β , platelet activating factor (PAF) or LPS, either alone or combination. There are several hours between cell activation and NO production corresponding to mRNA and protein synthesis. iNOS produces high levels of NO that sustains for hours or longer, depending on the presentation of enzyme in the cells or tissue.

NO plays many roles in the immune system. It is a toxic agent against infectious organisms and cancer cells. It also regulates several immune cells in both innate and specific immunity (Bogdan, 2001). At low concentrations NO acts as pro-inflammatory agent by inducing vasodilatation and neutrophil recruitment. At high concentrations, it down-regulates expression of several adhesion molecules, suppresses activation and induces apoptosis of inflammatory cells. Moreover, it is a negative feedback inhibitor of many cytokine genes expression, including iNOS, IL-1, IL-6, IL-8, IFN- γ , and TNF- α . Moreover, NO at high levels is very rapidly oxidized to $OONO^-$, that induce neighboring cell toxicity by nitrosating DNA and tyrosine residues, and inducing lipid peroxidation.

2. Antioxidants

2.1 Definition and importance (Ratnam et al., 2006; Victor et al., 2004)

The majority of ROS are physiologically formed during the respiratory chain in mitochondria. They also are generated by activated phagocytic cells. Meanwhile, aerobic organisms have defense mechanism against ROS damage by antioxidants. Antioxidants are substances that counteract free radicals before they react with biologic targets by inhibiting the respiratory chain reactions, or preventing the activation of oxygen to highly reactive products.

There are two major groups of antioxidants namely enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants are endogenous enzymes including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). They have high affinity to ROS and act rapidly to prevent oxygen radical formation either by removing free radical precursors or by inhibiting catalysis. SOD catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide. CAT and GPx then convert hydrogen peroxide to molecular oxygen and water with no free radical production. GPx requires reduced glutathione (GSH) which is a direct antioxidant. It donates hydrogen ion to hydrogen peroxide and becomes oxidized glutathione (GSSG). Moreover, the modulation on GSH/GSSG (oxidized GSH) levels also up-regulates gene expression of several other antioxidant proteins. Non-enzymatic antioxidants are mainly from dietary sources. They remove or inhibit free radicals, which have already formed. Common non-enzymatic antioxidants include vitamin C and vitamin E.

2.2 Role of free radicals in various diseases (Ratnam et al., 2006)

Under physiological condition, there is a homeostatic balance between ROS generation and antioxidant defenses in cells. When this balance is disrupted by excessive generation of ROS and/or inadequate antioxidant defenses, oxidative stress occurs. Oxidative stress can damage biological membranes by lipid peroxidation, leading to change in membrane permeability. Oxidation of DNA and proteins by fragmentation or random cross-linking of molecules modifies protein structure and

causes functional changes in cells and tissues. The result of this process has been associated with pathogenesis of various disorders, including cancers, diabetes, cardiovascular diseases, autoimmune diseases, neurodegenerative disorders and implicated in aging.

ROS not only directly react with macromolecules, but also act as secondary messenger in cellular signal transduction and gene activation. This can ultimately lead to prolonged activation of NF- κ B and overexpression of various inflammatory cytokines and mediator proteins. The chronic stimulation of macrophages can induce various diseases such as autoimmune neuropathy (Kiefer et al., 2001), atherosclerosis (Jara et al., 2006), rheumatoid arthritis (Paulos et al., 2004), and sepsis (Victor et al., 2004).

2.3 TOC and NAC in protection of cells from oxidative stress

The protection role of antioxidants can occur both extracellularly and intracellularly. Without entering the cells, antioxidants can exert their protective effects by scavenging toxic ROS. In intracellular protection, antioxidants can also interrupt lipid peroxidation within membrane and interfere early in the process of inflammatory responses by blocking or modifying the signal transduction of inflammatory cytokines. Modulation of cellular activation can occur as presented in Figure 3.

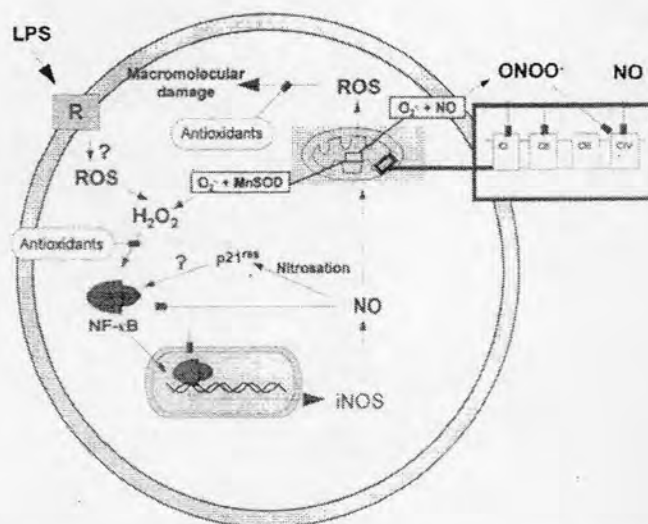


Figure 3: Mechanisms of antioxidants in protection of cellular oxidative stress (Cadenas and Cadenas, 2002)

The hydrophobic lipid localizes in the bilayer cell membrane requires a different spectrum of antioxidants defenses. TOC, a potent peroxy radical scavenger, is the major chain-breaking antioxidant in preventing membrane polyunsaturated fatty acids from undergoing lipid peroxidation, which leads to loss of membrane integrity (Traber, and Packer, 1995). The effects of TOC in signal transduction pathway depend on its antioxidant function. Pretreatment of vitamin E succinate to smokeless tobacco-activated macrophages resulted in decreased NO production (Hassoun et al., 1995). In addition, it was reported that TOC inhibits TNF- α production, TNF- α mRNA accumulation, procoagulant expression, and prostaglandin E₂ production on endotoxin-induced activation of alveolar macrophages (Mendez et al., 1995).

NAC is a sulphhydryl donor, which can replenish the intracellular cysteine required to produce GSH. NAC also reacts directly with ROS and has been used safely to treat acetaminophen overdose. The efficiency of NAC was demonstrated in several studies. Victor et al., (2003) demonstrated the decreases in the superoxide anion production, GSSG/GSH ratio, and TNF- α level in response to administration of NAC in mice with oxidative stress. Pretreatment with NAC decreased NO production in LPS-activated macrophage astrocytes and C₆ glial cells. This decrease is due to the down-regulation iNOS and TNF- α by inhibiting activation of NF- κ B (Pahan et al., 1998). Moreover, NAC treatment protected J774.2 macrophage cells against the lipotoxic effect of triacylglycerols (Aronis et al., 2005).

2.4 The role of liposomal antioxidants in oxidative stress

Antioxidants have some major problems in terms of drug delivery due to their poor availability to the target tissue. The poor bioavailability of antioxidants may be due to one or many of the several factors such as poor solubility, instability and inefficient permeability. Drug delivery systems such as liposomes have been shown to be useful in delivery antioxidants to their therapeutic targets. The protective effects against intracellular oxidant-mediated damages of liposomal antioxidants have also been shown in several studies (Minko, Stefanov and Pozharov, 2002; Yao, Degli and Huang, 1994; Suntres and Shek, 1994).

Antioxidants that have been incorporated into liposomes include lipid-soluble antioxidants, water-soluble antioxidants and combinations of antioxidants. The lipid-soluble antioxidants that were incorporated into liposomes are TOC and carotenoids. TOC can readily be incorporated into liposomes at a level as high as 30 mol% (Suntres and Shek, 1994). It was reported that TOC liposomes, administered intratracheally, had potential to correct hypoxia lung injury in the hypoxic rats (Minko et al., 2002). Moreover, intravenous administration of liposomes containing TOC is effective in decreasing mortality in mice given a lethal dose of carbon tetrachloride (Yao et al., 1994). β -Carotene can be incorporated into liposomes to a maximum of about 0.5 M (base on phospholipids). Liebler, Stratton and Kaysen (1997) found that β -carotene in liposomes can inhibit free radical-mediated lipid peroxidation.

The water-soluble antioxidants that have been incorporated into liposomes include NAC, GSH and vitamin C. NAC liposomes were potentially more effective as prophylactic pharmacological agent in decreasing LPS-induced liver injuries in rats (Alipour et al., 2007). Liposomal encapsulated NAC delivered intratracheally was demonstrated to be more effective than free NAC against oxidative stress-related lung diseases in rat model (Fan et al., 2000). Antioxidants have also been used in combination between water-soluble and lipid-soluble antioxidants in liposomes. Sinha and Basu (2001) reported that liposomes of vitamin C and E when used either in combination or individually were able to prevent the ischemia and reperfusion in rats where the free forms of these antioxidants showed little effect. In addition, Suntres and Shek (1994) found that liposomes with coexisting both TOC and GSH were more effective in protection against paraquat-induced lipid peroxidation than liposomes containing GSH alone in a rat model.

3. Liposomes for macrophage targeting

Liposomes are potential carrier systems for drug delivery because of the ability to encapsulate a variety of substances both hydrophilic and hydrophobic into their structure. Properties of liposomes are very versatile, depending on lipid composition and method of preparation, and vary with size and surface charge. The main advantage of using liposome for macrophage targeting resides in their rapid

recognition by phagocytes, primarily macrophages, in the reticuloendothelial system (RES). Generally, liposomes are cleared from the blood circulation within minutes upon intravenous injection, depending on liposome size and surface characteristics (Chellat et al., 2005; Ratnam et al., 2006).

3.1 Factors influencing the uptake of liposomes into macrophage cells

To enhance liposome-macrophage interaction, liposomes can be specifically designed to optimize their targeting capabilities to macrophage cells. This approach involves construction of liposomes with different physiochemical properties dependent on liposome composition and size.

3.1.1 Cholesterol content

Liposome composition and cholesterol concentration are parameters potentially influencing fluidity of membrane and the mechanism of liposome-cell interaction. Allen et al. (1991) reported that inclusion of increasing amounts of cholesterol (0-50 mol%) in the phosphatidylcholine (PC) liposomes resulted in a decrease in uptake by bone marrow macrophages. On the other hand, another studies demonstrated that the uptake of liposomes by macrophages in lymph node were independent of the presence of cholesterol and bilayer fluidity (Oussoren and Storm, 2001). Thus, sources of macrophages also play some role in liposome-macrophage interaction. However, the presence of cholesterol can increase stability of PC liposomes in blood circulation (Hunt, 1981). Thus, cholesterol is often included in liposome preparations intended for intravenous injection (Roerdink et al., 1989; Wilson et al., 2007).

3.1.2 Surface charge

The charge density on the liposome surface is an important parameter that influences the rate and extent of phagocytosis. Many studies indicated that negatively charged phospholipids such as phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidylinositol (PI) or phosphatidic acid (PA) are phagocytosed better than vesicles composed exclusively of neutral PC (Schroit, Madsen and Nayar, 1986; Lee

et al., 1992). Especially, incorporation of PS in PC liposomes resulted in significantly increased uptake of liposomes by macrophages in lymph node (Oussoren and Storm, 2001) in a concentration dependent manner (Allen et al., 1991).

3.1.3 Size of liposome

The size-dependency of phagocytosis is likely related to the administration routes and the process of particle transport to target cells. For example, small (< 0.1 μm), neutral liposomes can be absorbed by lymph node macrophages up to 70% of the subcutaneous injection dose. The absorption was related to the process of particle transport through the interstitium (Oussoren and Storm, 2001). Intravenously injected liposomes will target only to macrophages that directly contact with the blood circulation including the Kupffer cells in the liver, the red pulp macrophages in the spleen and macrophage in bone marrow (Rooijen and Sanders, 1998). Uptake of liposomes by bone marrow macrophages decreased when liposome size was increased from 0.05 to 0.8 μm (Allen et al., 1991). On the contrary, uptake of liposomes by Kupffer cells decreased when liposome size was reduced (Liu, 1997).

3.2 Liposomes in macrophage-mediated inflammatory diseases

Macrophages play a critical role in the initiation and maintenance of inflammation that leads to tissue destruction. The therapeutic rationale in some inflammatory diseases is based on reduction of macrophage activity. Therefore, liposomes were used to delivery therapeutic substances directly to macrophage cells. Liposomal dexamethasone-21-palmitate (Lipotalon[®]) is a liposomal formulation available for intra-articular injection treatment of osteoarthritis (Gerwin, Hops and Lucke, 2006). Cortisol palmitate has increased anti-inflammatory activity and prolonged duration of action when incorporated into liposomes (Gerwin et al., 2006). Methotrexate liposomes were found to accumulate in the synovial membrane and to slowly release the active compound (Foong and Green, 1988). Liposomes containing the drug dichloromethylene diphosphonate were used successfully to eliminate macrophages to reduce the severity of allergic encephalomyelitis diseases (Brosnan, Bornstein and Bloom, 1981).