THE USE OF POLY(ETHYLENE GLYCOL)-MODIFIED LIPIDS IN LIPOSOMES: AN IMMUNOLOGICAL PERSPECTIVE

By

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We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

May 2002

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Date July 24, 02
ABSTRACT

The potential of liposomes as delivery vehicles has long been recognized. In recent years, liposome research was further advanced by the invention of a hydrophilic polymer poly(ethylene glycol) (PEG) which stabilizes liposome surface. This second generation of liposomes exhibit long circulation lifetimes, attributed to the ability of PEG to interfere with liposome removal by the defense mechanism of the host. The long circulation property of PEGylated liposomes has been, in general, claimed to be due to the ability of these liposomes to evade the immune system. However, the finding that PEGylated liposomes can generate immune responses suggests that PEG does not actually limit liposome interaction with the immune system. To date, there is insufficient information in the literature with regard to the role of grafted PEG to alter liposome interaction with the immune system.

The research summarized in this thesis has resulted in a better understanding of the implications of using PEG-lipids in liposomes from an immunological perspective. In Chapter 2 and 3 of this thesis, the use of PEG-lipids is discussed in terms of interaction with mechanisms of the immune system for liposome removal. This concerns the development of liposomes with functional groups which are potentially immunogenic. Using biotin as a model ligand, it was found that liposomes can be protected from immune recognition by specific antibodies and the subsequent rapid removal from the circulation. In Chapter 4 and 5, from the perspective of the impact on the immune system, the ability of PEGylated-liposomes to induce immune responses was examined to test the hypothesis that incorporation of PEG-lipids re-directs liposomes from macrophages to more relevant antigen
presenting cells. This has important implications for the development of liposomes as vaccine carriers. Using two different types of antigens, namely a T-independent antigen and a tumor antigen, that stimulate different effector functions of the immune system, it is demonstrated that surface-grafted PEG enhances antibody response in both cases. The results presented in this thesis provide a better understanding of the pros and cons of using PEG-lipids in liposomes for various applications.
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<tr>
<td>Alum</td>
<td>Aluminum hydroxide gel adjuvant</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BSA-biotin</td>
<td>Bovine serum albumin conjugated-biotin</td>
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<td>Bx-liposomes</td>
<td>Biotinylated liposomes</td>
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<td>Biotin-X-DSPE (Bx-DSPE)</td>
<td>N-((6-biotinoyl)amino)hexanoyl)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine</td>
</tr>
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<td>BM-DC</td>
<td>Bone marrow-derived dendritic cell</td>
</tr>
<tr>
<td>C_{14}-Cer-PEG_{2000}</td>
<td>C_{14}-ceramide-poly(ethylene glycol)</td>
</tr>
<tr>
<td>CFA</td>
<td>Complete Freund’s adjuvant</td>
</tr>
<tr>
<td>Chol</td>
<td>Cholesterol</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T-lymphocyte</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<td>DC-Chol</td>
<td>3β[N-(n',N'-dimethylaminoethane)-carbamoyl]cholesterol, dioleoyl)</td>
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<td>DMPE-PEG_{2000}</td>
<td>1,2-Dimyristoyl-sn-glycero-3-phosphoethanolamine-n-[methoxy(polyethylene glycol)-2000]</td>
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<td>DOPC</td>
<td>1,2 Dioleoyl-sn-glycero-3-phosphocholine</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>Enzyme-linked immunospot assay</td>
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<td>IFA</td>
<td>Incomplete Freund’s adjuvant</td>
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<td>IgG</td>
<td>Immunoglobulin G</td>
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<td>IgM</td>
<td>Immunoglobulin M</td>
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<td>G_{M1}</td>
<td>Monosialoganglioside G_{M1}</td>
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<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N-2-ethane-sulphonic acid</td>
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<td>HER-2/neu</td>
<td>Human epidermal growth receptor-2</td>
</tr>
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<td>HBS</td>
<td>HEPES buffered saline.</td>
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<td>HRP</td>
<td>Horseradish peroxidase</td>
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<td>i.v.</td>
<td>Intravenous</td>
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<td>Interferon-α</td>
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<td>Interferon-β</td>
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<td>Interleukin-12</td>
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<td>MHC</td>
<td>Major histocompatibility complex</td>
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<td>LUV</td>
<td>Large unilamellar vesicle</td>
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<td>Acronym</td>
<td>Description</td>
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<tr>
<td>MLV</td>
<td>Multilamellar vesicle</td>
</tr>
<tr>
<td>MPS</td>
<td>Mononuclear phagocyte system</td>
</tr>
<tr>
<td>ODN</td>
<td>Oligodeoxynucleotide</td>
</tr>
<tr>
<td>PA</td>
<td>Phosphatidic acid</td>
</tr>
<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
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<td>PEG</td>
<td>Poly(ethylene glycol)</td>
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<td>Phosphatidylglycerol</td>
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<td>PI</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>QELS</td>
<td>Quasi-elastic light scattering</td>
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<tr>
<td>SA</td>
<td>Stearylamine</td>
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<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>SUV</td>
<td>Small unilamellar vesicles</td>
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<td>TNFα</td>
<td>Tumor necrosis factor α</td>
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ACKNOWLEDGEMENTS

This thesis could not be completed without the help of many people. First, I would like to sincerely thank Marcel, my supervisor, for accepting me as a graduate student into a lab where I felt most spoiled in many ways. Throughout the years, I was most inspired by Marcel with his creative ideas and I have learned that science can involve a lot of creative thinking. I am also very touched by the generosity of Marcel as a supervisor that allowed me to do whatever I desire. Last but not least are the wonderful scientific debates with Marcel which had been a very motivating experience.

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DEDICATION

This thesis is dedicated to my parents Ka Yu Li and Yin Nor Li,

Dou dou, a companion whom I will never forget,

and

the little adorable creatures who
had sacrificed their lives to be a part of this thesis.
CHAPTER 1
INTRODUCTION

1.1 PROJECT OVERVIEW

The remarkable potential of liposomes as delivery vehicles has been substantiated by
the advanced clinical development and the approval of several liposomal drugs in recent
years. Despite this fact, strategies to further improve the stability of lipid-based carriers in
biological systems are being actively pursued. In recent decades, the use of a surface-
modifying lipid, poly(ethylene glycol) (PEG)-modified lipid, has made a significant
contribution to liposome technology in this regard. Sterically stabilized liposomes, a term
describing liposomes containing PEG-lipids, are able to reduce or prevent liposome
interaction with macromolecules and other surfaces. In vivo, these liposomes exhibit long
circulating properties attributed to the ability of PEG to influence liposome interaction with
the innate immune system. However, the extent and exact role of PEG-lipid in altering
liposome interaction with the immune system remains unclear.

This thesis is aimed at studying the influence of including PEG-lipids in liposomes
from an immunological perspective. The ability of liposomes to interact with the immune
system has long been recognized. Relevant questions regarding this interaction include the
impact of the immune system on liposomes and vice versa. A major concern of this
interaction is the control of liposome circulation longevity which is believed to be under the
influence of the host immune system. In addition, stimulation of immune responses using
liposomes is an emerging field of interest in vaccine research. Thus, a dilemma emerges. Liposomes with surface-grafted PEG can be used with the objectives of minimizing interactions with the immune system. Conversely, it is known that these same liposomes can actually achieve enhanced immune responses. In this thesis, the implications of the changes introduced by PEG-lipids as a result of altered liposome interaction with the immune system will be examined.

1.2 LIPOSOMES

Liposomes are colloidal particles composed of lipid molecules assembled into bilayer membranes. Liposomes are characterized by their lamellarity, size, and lipid composition all of which govern their in vivo characteristics. The following sections will provide a brief introduction to liposome classification, lipid composition, important applications and their disposition in biological systems.

1.2.1 Liposome classification

Based on morphology and size, liposomes can be classified into multilamellar vesicles (MLVs), large unilamellar vesicles (LUVs), and small unilamellar vesicles (SUVs) (see Figure 1.1) (Ostro et al., 1989). They differ in the preparation method as well as trapping efficiency which will be discussed in more detail as follows.
Figure 1.1  Liposome classification. Freeze fracture micrographs and schematic diagrams of (A) SUV, (B) LUV, and (C) MLV are shown (adapted from Ostro and Cullis, 1989). Bar shown in the lower right corner represents 200 nm.
1.2.1.1 Multilamellar vesicles (MLVs)

MLVs can be prepared by adding an aqueous medium to a dried lipid film or by hydration from organic solvent (Bangham et al., 1965). Both of these methods yield large structures (>400 nm) with multiple membrane bilayers encapsulated within the core, where each bilayer is separated by an aqueous channel. The trapped volume (internal aqueous volume typically measured as μl volume per μmole lipid) of MLV is small due to membrane structures occupying the internal space. The inclusion of negatively charged lipids can increase the trapped volume by increasing the interbilayer separation due to electrostatic repulsion. As well, freezing and thawing the preparation can usually increase the trapped volume of MLVs due to disruption of lamellar structures and increases in interlamellar spacing (Mayer et al., 1985). From a pharmaceutical point of view, MLVs are not ideal delivery vehicles both because of the relatively small trapped volume and their large size causing rapid elimination in vivo (Cullis et al., 1987). Interestingly, when considering the design of liposomes targeting phagocytic cells of the mononuclear phagocyte system (MPS), MLVs are often considered since these large structures are more efficiently accumulated into these cell populations.

1.2.1.2 Large unilamellar vesicles (LUVs)

Unilamellar vesicles are structures with a single bilayer membrane encapsulating an aqueous space. Unilamellar structures of size 50-200 nm are referred to as LUVs which can be prepared using various methods, including injection of lipids in an organic solvent into an aqueous buffer (Deamer et al., 1976), reverse-phase evaporation (Szoka et al., 1978), detergent dialysis (Mimms et al., 1981; Madden, 1986), and extrusion (Olson et al., 1979;
Hope et al., 1985). The extrusion method is the most commonly used method because of many advantages over other methods such as ease in preparation, relatively fast procedure and better control of vesicle size (Hope et al., 1985; Cullis et al., 1987). LUVs are the most commonly used vesicles as delivery vehicles because of their ideal size for in vivo stability as well as optimal trapped volume when compared with the other classes of vesicles (Hope et al., 1986).

1.2.1.3 Small unilamellar vesicles (SUVs)

SUVs are like LUVs except they differ in size. Usually, unilamellar vesicles smaller than 50 nm are referred to as SUVs. SUVs can be prepared by sonication of dispersions of phospholipids and by the French press procedure (Szoka et al., 1981). Among the different types of vesicles, SUVs have the smallest trapped volume. Another disadvantage of SUVs is the high curvature of the membrane bilayer which makes them unstable and tend to undergo spontaneous fusion to form larger structures (Lichtenberg et al., 1981). Thus the use of SUVs as delivery vehicles is largely limited (Cullis et al., 1987).

1.2.2 Lipid composition

The major components of liposomes are phospholipids and cholesterol, resembling biomembranes which exist in nature. The properties of liposomes are often dictated by the chemical properties of these lipids alone as well as lipid-lipid interactions which occur when these lipids spontaneously assemble into liposomes. The assembly process is governed, in part, by the amphipathic nature of lipids which organize spontaneously in aqueous solutions
to maximize hydrophobic interaction. This has been reviewed in detail by Tanford et al. (1980).

1.2.2.1 Phospholipids

Phospholipids are one of the major components of liposomes. Phospholipids are composed of a hydrophilic phosphate-containing head group and hydrophobic acyl chains linked to the glycerol backbone via ester linkages (see Figure 1.2 for the structure of phospholipids). The distinct properties of each phospholipid are determined by the head groups they contain as well as the acyl chains which can differ in length and degree of saturation (see Figure 1.2). Choline-containing phospholipids (PC) are the most abundant phospholipid in nature. Other commonly found phospholipids include phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylglycerol (PG), phosphatidic acid (PA), and phosphatidylinositol (PI). In the case of PC and PE, the negative charge on phosphate is neutralized by the positive charge on the ethanolamine and choline head group. Thus, these phospholipids are zwitterionic and are neutral while PS, PG, PA, and PI have a net negative charge at physiological pHs. It is important to note that PE is often used for coupling functional groups to liposomes. This results in the loss of the neutralizing effect of ethanolamine head group on the phosphate. Thus, liposomes made with functional groups conjugated to PE will have a net negative charge. In this thesis, the liposomes used were composed of PC as the bulk lipid and PE which is conjugated to PEG.
Figure 1.2 Phospholipid and cholesterol as common components of liposomes. The general structure of a phospholipid is shown in (A) with common fatty acid chains and head groups shown in (B) and (C) respectively. (D) Structure of cholesterol.
One of the factors to consider when designing liposomes is the molecular shape of the lipid components which will determine the tendency of the lipid mixture to assemble into a bilayer configuration. Table 1.1 illustrates the molecular shape of different phospholipids which determines their phase behavior. As reviewed in detail by Tilcock (1986), the phase behavior of mixed lipid systems depends on many factors, including the composition of component lipids of different molecular shapes. For example, although PEG-modified lipids have a tendency to form micelles when used at high concentrations, the lipid mixtures used throughout this thesis (PEG-modified PE and PC as bulk lipid) result in formation of bilayers (see Chapter 2).

Phospholipids are said to undergo phase transition from gel phase to liquid-crystalline phase when the transition temperature ($T_c$) is reached. Each individual lipid has its distinct $T_c$ which is determined by both the properties of the lipid head group and the acyl chains. In general, lipids with longer and more saturated acyl chains have higher $T_c$. Lipids in the liquid-crystalline phase are more fluid and less ordered, making the lipid bilayer more permeable. The distinctive $T_c$ of each phospholipid has important implications in the design of liposomes as a drug carrier for drug release. The $T_c$ of lipids also has relevance in liposome preparation. In the initial step, rehydration of a lipid film at temperatures above the highest $T_c$ of the lipid components is desirable to facilitate efficient hydration of the bilayer (Szoka et al., 1981). As well, in the later step of liposome preparation, extrusion of MLVs to make LUVs can be assisted by increasing the temperature to above the $T_c$ of the lipid components (Cullis et al., 1987).
Table 1.1  Molecular shape of individual lipids and their phase behavior
(adapted from Cullis et al., 1986).

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Phase</th>
<th>Molecular Shape</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysophospholipids</td>
<td>Micellar</td>
<td>Inverted cone</td>
</tr>
<tr>
<td>Detergents</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEG-conjugated lipids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>Bilayer</td>
<td>Cylindrical</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatidylinositol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatidylglycerol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatidic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardiolipin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Digalactosyldiglyceride</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatidylethanolamine (unsaturated)</td>
<td>Hexagonal (HII)</td>
<td>Cone</td>
</tr>
<tr>
<td>Cardiolipin-Ca$^{2+}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatidic acid-Ca$^{2+}$ (pH&lt;6.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatidic acid (pH&lt;3.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphatidylserine (pH&lt;4.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monogalactosyldiglyceride</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.2.2.2 Cholesterol

Cholesterol (structure shown in Figure 1.2) is another amphipathic molecule commonly found in bilayer membranes. Cholesterol is believed to have a stabilizing effect on membranes (reviewed by Cullis et al., 1987). It is incorporated into the lipid bilayer with the hydroxyl end oriented toward the aqueous surface and the rigid ring structure lying parallel to the acyl chains of phospholipids. The insertion of cholesterol into a lipid bilayer increases the molecular packing of phospholipids and affects membrane fluidity and permeability. At temperatures below $T_c$, interaction between head groups is weakened due to phospholipids being “pushed apart” by cholesterol. The net effect is higher membrane fluidity and permeability. Above $T_c$, acyl chain fluidity is reduced by the presence of cholesterol. Thus, the membrane fluidity and permeability is reduced. The membrane-stabilizing effect of cholesterol was perhaps best illustrated by the work of Scherphof which showed that destabilization of liposomes due to interaction with lipoproteins can be eliminated by the addition of cholesterol into liposomes (Scherphof et al., 1979). Interestingly, these early studies demonstrating the benefits of using cholesterol for liposomes designed for in vivo use occurred before the discovery of surface-stabilizing polymers such as PEG or the ganglioside GM1. Recent studies by Dos Santos et al. (2002) suggest that cholesterol is not required when these stabilizing lipids such as PEG-modified PE are incorporated into the liposomal formulation.
1.2.3 Medical applications of liposomes

Liposomes as drug delivery vehicles have come a long way since its first discovery in the 1960’s. It has been recognized for a long time that liposomes have great potential in drug delivery due to their ability to alter the pharmacokinetics and biodistribution of drugs (Juliano et al., 1978; Allen et al., 1999). Liposomes are recognized as the most advanced drug carriers in terms of clinical development as indicated by approval of several formulations of liposome/lipid based drugs for human use (see Table 1.2). At the present time, the development of liposomes as drug carriers is extended to the newer classes of therapeutic agents including antisense molecules and DNA (Loke et al., 1988; reviewed by Nicolau et al., 1998). Another important medical application of liposomes under development concerns their use for vaccine delivery, an application originally conceived due, in part, to the particulate nature of the carriers to be taken up non-specifically by cells of the immune system (see section 1.4.2). In this regard, many clinical trials of liposomal vaccines are underway and some are already in advanced clinical development (see Table 1.2). It is worth mentioning that the promising future of liposomes is due, in part, to the invention of novel features to improve liposomes for better targeting and content release. However, such features often enhance the potential for these sophisticated formulations to be recognized by the immune system. In fact, it is argued in this thesis that regardless of the different applications of liposomes, it is inevitable that liposomes will interact with the host immune system. This may have important implications in the success of the delivery vehicle designed to avoid interactions with the host immune system or to actively trigger an immune response. This thesis is aimed at obtaining a better understanding of the role of a special lipid, PEG-modified lipid, in controlling this interaction.
Table 1.2 Clinical development of liposome/lipid based carrier

<table>
<thead>
<tr>
<th>Product Name</th>
<th>Drug</th>
<th>Indication</th>
<th>Company</th>
<th>Development Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxil® *</td>
<td>Doxorubicin</td>
<td>Ovarian cancer</td>
<td>ALZA/Johnson &amp; Johnson</td>
<td>Approved</td>
</tr>
<tr>
<td>Daunoxome®</td>
<td>Daunorubicin</td>
<td>HIV-associated Kaposi’s sarcoma</td>
<td>Gilead</td>
<td>Approved</td>
</tr>
<tr>
<td>AmBisome®</td>
<td>Amphotericin B</td>
<td>Systemic fungal infections</td>
<td>Gilead</td>
<td>Approved</td>
</tr>
<tr>
<td>ABELCET®</td>
<td>Amphotericin B</td>
<td>Systemic fungal infections</td>
<td>Elan</td>
<td>Approved</td>
</tr>
<tr>
<td>Myocet™</td>
<td>Doxorubicin</td>
<td>Metastatic breast cancer</td>
<td>Elan</td>
<td>Approved</td>
</tr>
<tr>
<td>Liposomal-</td>
<td>Paclitaxel</td>
<td>Breast, ovarian, non-small cell lung cancers</td>
<td>NeoPharm</td>
<td>Phase II/III</td>
</tr>
<tr>
<td>encapsulated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>paclitaxel</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Onco TCS</td>
<td>Vincristine</td>
<td>Relapsed non-Hodgkin’s lymphoma, small cell lung cancer, pediatric malignancies</td>
<td>INEX</td>
<td>Phase II/III</td>
</tr>
<tr>
<td>NX211</td>
<td>Lurtotecan</td>
<td>Solid tumors</td>
<td>Gilead</td>
<td>Phase II</td>
</tr>
<tr>
<td>Epaxal Berna</td>
<td>Inactivated hepatitis A</td>
<td>Hepatitis A</td>
<td>Berna Biotech</td>
<td>Approved</td>
</tr>
<tr>
<td>Vaccine</td>
<td>peptide, IL-2</td>
<td>Non-small cell lung cancer</td>
<td>Biomira</td>
<td>Phase II</td>
</tr>
</tbody>
</table>

Note: * Formulation with PEG-modified lipids
1.2.4 Biological fate of liposomes

After the parenteral administration of liposomes, the biological fate of liposomes is governed by their interaction with many components within the blood compartment which can either cause liposome destabilization or binding of specific proteins leading to elimination by the macrophages of the mononuclear phagocyte system (MPS). Lipoproteins are a major component of the blood which can destabilize liposomes (Scherphof et al., 1978; Scherphof et al., 1979). Many apolipoproteins, namely apo A-I, A-II, A-IV, B, C, and E, have all been shown to interact with liposome membranes and many of which can cause liposome disruption (reviewed by Williams et al., 1998). Despite the identification of the class B scavenger receptors (SR-B) that can recognize liposomes containing PS (Sambrano et al., 1995) and the recent demonstration that apoE can mediate neutral liposome uptake by hepatocytes (Kamps et al., 1998), it is generally accepted that lipoproteins play a more important role in liposome destabilization (Williams et al., 1998). In addition to lipoproteins, serum albumin is another liposome-bound protein found in abundance and may be implicated in liposome destabilization (Chonn et al., 1992).

Liposomes which can stay intact in the circulation are not necessarily available for distribution to peripheral tissues because protein binding to liposomes can enhance their recognition (opsonization) and elimination by the immune system as discussed in section 1.4.1.1. Alternatively, some investigators have suggested that bound proteins actually act as dysopsonins possibly causing the liposomes to evade recognition by cells of the immune system (see section 1.4.1.1). The availability of liposomes for distribution to peripheral
tissues can be translated to the ability of the carrier to prevent and/or alter these interactions to prolong their circulation longevity. As discussed in the next section, the invention of PEG-modified lipids has had a tremendous impact on liposome development due to the success of this special lipid in controlling the surface reactivity of liposomes.

1.3 POLY(ETHYLENE GLYCOL)-MODIFIED LIPIDS

Poly(ethylene glycol) is a hydrophilic polymer with repeating units of (CH$_2$-CH$_2$-O)$_n$ as illustrated in Figure 1.3. The ability of PEG to reduce immunogenicity has been shown for various proteins (reviewed by Harris et al., 2001). After the initial discovery of the beneficial effects of ganglioside GM1 on liposome circulation longevity (Allen et al., 1989), PEG conjugated to liposome surface was also found to improve liposome stability in vivo (Allen et al., 1991b; Allen et al., 1991c; Woodle et al., 1992). One important factor to consider when designing liposomes with prolonged circulation longevity is the choice of lipid anchor for the attachment of PEG. PEG can be chemically conjugated to various lipids, including cholesterol, monostearate, and phosphatidylethanolamine (PE). Since PEG-cholesterol and PEG-monostearate are less effective in prolonging liposome circulation longevity (Allen et al., 1991c), presumably due to the ability of these PEG-lipids to exchange out of the lipid bilayer readily, PE is the most commonly used lipid anchor. There are various methods to chemically conjugate PEG to PE, including succinate, carbamate, amide, and direct linkage (see Figure 1.3). Due to detachment of the PEG moiety from the lipid in serum, the carbamate linkage was identified to be a better method when compared to the succinate linkage (Parr et al., 1994). This is to insure that the grafted PEG remains
associated with the liposome for maximum time periods following intravenous administration.

Another useful application of PEG-lipid which appealed to many scientists in liposome research is the ability of the PEG moiety or the entire PEG-lipid to leave liposomes. Although this property of PEG-lipids may be viewed as a disadvantage when designing liposomes with prolonged circulation longevity, the leaving of PEG-lipids from the lipid bilayer has the potential of triggering the transformation of liposomes to fully reveal their function(s). In this regard, PEG-PE with disulfide linkages which are sensitive to thiolytic cleavage were developed in an attempt to have better control of liposome surface protection but had shown little success (Kirpotin et al., 1996). Programmable fusogenic liposomes with the use of exchangeable PEG-PE, on the other hand, is a successful example which was able to achieve better drug delivery (Holland et al., 1996b; Adlakha-Hutcheon et al., 1999). The exchange rate of PEG-PE has been shown to depend on the size of the hydrophilic PEG polymer as well as the acyl chain length and degree of saturation of the lipid anchor (Silvius et al., 1993a; Silvius et al., 1993b). Thus, through the selection of appropriate lipid anchor and PEG size, it is possible to design formulations with the desired periods of liposome surface protection. In Chapter 2 of this thesis, the ability of these PEG-lipids to shield and de-shield liposome surface to control liposomal ligand exposure will be discussed in greater detail.
Figure 1.3 Chemical structure of methoxy-PEG (A) and its various linkages to phosphatidylethanolamine (PE) (B)
1.3.1 Physical properties of PEGylated liposomes

To study the physical properties of PEGylated liposomes, the "mushroom and brush" model (illustrated in Figure 1.4) has been often used to describe the PEG polymer grafted on liposome surface (de Gennes, 1980). Polymers grafted on a surface are said to be in either the "mushroom" or "brush" conformation depending on both the grafting density as well as the size of the random coil of the polymer in solution, defined by the Flory radius ($R_F$) (de Gennes, 1980). When polymers are at a low grafting density, the distance between grafting points ($D$) is larger than $R_F$ of the polymer. The polymer is then allowed to "coil up" into the "mushroom" conformation. Conversely, when the grafting density is high ($D<R_F$), the polymer is limited in space and is extended into the "brush" conformation.

Research on the physical properties of sterically stabilized liposomes has focused on understanding properties like bilayer repulsive pressure, thickness of the polymer barrier, surface coverage and ability of the barrier to prevent molecule penetration onto liposome surface (Woodle et al., 1994; Needham et al., 1999). Using various techniques, including X-ray diffraction, differential scanning calorimetry, NMR, and analysis of electron density profiles, it was shown that both the thickness of the steric barrier and repulsive pressure between bilayers increase as a function of the polymer size as well as PEG-lipid content (Kuhl et al., 1994; Kenworthy et al., 1995a; Needham et al., 1999). For example, at a grafting density of 4 mol% PEG2000, it was estimated that the polymer moiety extends ~5 nm from the lipid surface (Needham et al., 1992; Kuhl et al., 1994). Increasing the grafting density to 9-10 mol% causes an increase in the polymer thickness to ~7nm (Needham et al.,
Figure 1.4  “Mushroom and brush” model of surface-grafted PEG. PEG polymer attached onto a surface is proposed to be in the mushroom conformation (A) when the distance between grafting points (D) is larger than the Flory radius ($R_F$). When $D<R_F$, the polymer is said to be in the brush conformation (B). Schematic diagrams are adapted from de Gennes, 1988.
The increase in total repulsive pressure of the bilayer due to PEG can be due to steric pressure imparted by the polymer, electrostatic pressure due to the charged PE head group, and hydration due to attraction of water molecules to the hydrophilic polymer. However, electrostatic repulsion probably plays a minor role when compared with steric pressure which plays a predominant role in liposome stabilization (Needham et al., 1992; Kuhl et al., 1994). Another theory for the thermodynamic stabilization of liposomes by PEG is the removal of water molecules from the lipid bilayer to enhance lateral packing of the acyl chains and reduce bilayer defects (Tirosh et al., 1998).

In terms of surface coverage by PEG, different estimations have been derived from various groups (Kuhl et al., 1994; Torchilin et al., 1994b; Rex et al., 1998). Using the average distance between the fixed and free ends of a polymer molecule as the radius of the protected area, Torchilin and Papisov estimated a surface coverage of 35 nm² for each PEG2000 polymer (Torchilin et al., 1994b). In another study, by measuring the repulsive pressures between two lipid bilayers as a function of distance between bilayers containing various concentrations of PEG-lipids, it was deduced that PEG2000 in the mushroom conformation has a surface coverage of 9.6 nm² (Kuhl et al., 1994). An even smaller coverage area (2.6 nm²) for the PEG2000 polymer was estimated using Monte Carlo simulations and experiments measuring the binding of acylated PEGs to lipid vesicles (Rex et al., 1998). When considering the exclusion of macromolecules from binding to liposome surface, these estimations may not be adequate as the volume occupied by the PEG polymer can be envisioned as a statistical cloud (Torchilin et al., 1994b) and that conformational entropy of the polymer plays an important role in the exclusion of proteins (reviewed by
Allen et al., 2002). Moreover, due to lateral mobility of lipids in the bilayer, phospholipids can phase separate (Noppl-Simson et al., 1996), which may allow incoming molecules to bind to bare surface. Another view of how surface-grafted PEG excludes macromolecules is the slowing down of the rate of macromolecule crossing the polymer layer which acts like an “energy trap” (Needham et al., 1999). In section 1.3.3, the effectiveness of PEG to exclude proteins from liposome surface will be discussed in more detail.

Another important biophysical parameter to consider is the amount of PEG-lipids that can be incorporated into lipid bilayers. Due to the large hydrophilic head group of PEG-lipid, the molecule is cone shaped, facilitating the formation of micelles (see Table 1.1). Phase diagram studies of liposomes showed that micelles are formed when PEG2000-lipid is incorporated at a density larger than 15-20 mol% as shown by Kenworthy et al. (Kenworthy et al., 1995b). Using other techniques such as cryo-transmission electron microscopy, it was shown that atypical liposome structures are formed at a density as low as 8 mol% (Edwards et al., 1997), very similar to the prediction of Tirosh et al. (1998).

1.3.2 Biodistribution of PEGylated liposomes

With the use of different liposome markers, it has been consistently shown by many groups that incorporation of PEG-lipids increases liposome levels in the blood and reduces uptake by the mononuclear phagocyte system (MPS) (Allen et al., 1991c; Lasic et al., 1991; Mori et al., 1991; Woodle et al., 1992; Ishiwata et al., 1995). Liposome uptake by macrophages of the MPS is a major mechanism of liposome disposition in vivo as discussed in more detail in section 1.4.1. It has been proposed that the altered biodistribution of
PEGylated liposomes is attributed to decreased rate of liposome removal by the MPS. This can prolong the resident time of liposomes in the circulation for extravasation and higher liposome accumulation in various other tissues including skin, tail, bone marrow, and the carcass (Allen et al., 1991b; Allen et al., 1991c). Enhanced liposome accumulation in tumors has been shown as well (Huang et al., 1992; Litzinger et al., 1994; Ishida et al., 1999). Unlike conventional liposomes, the rate of PEGylated liposome removal from the blood compartment is not dependent on the liposome dose given to mice (Allen et al., 1991b), but is dependent on the liposome size (Litzinger et al., 1994; Ishida et al., 1999). Smaller stealth liposomes (<200 nm) have longer circulation times whereas larger vesicles (400 nm) are more rapidly removed from the circulation and accumulate predominantly in the spleen (Litzinger et al., 1994; Ishida et al., 1999).

1.3.3 Mechanism of PEG-lipids in prolonging liposome circulation longevity

Up to date, the exact mechanism of PEG in prolonging liposome circulation longevity remains unclear. However, the proposed mechanism of PEG to reduce serum protein binding to liposome surface which mediates liposome uptake by phagocytes of the MPS is commonly accepted. Reduced cellular uptake of liposomes by PEG-lipid has been shown in vitro by many studies using various cell types including HeLa cells, macrophage cell line J774, and macrophages derived from bone marrow or isolated from the peritoneal cavity (Allen et al., 1991a; Vertut-Doi et al., 1996; Zeisig et al., 1996; Miller et al., 1998; Johnstone et al., 2001). The ability of PEG to reduce liposome uptake by the MPS was further substantiated by findings from in situ liver perfusion studies (Liu et al., 1996; Liu, 1997). It was shown
that the liver uptake of neutral liposomes were decreased by more than 70% when PEG-lipid was incorporated at a concentration of 6.25 mol% (Liu et al., 1996; Liu, 1997).

While there is sufficient evidence both in vivo and in vitro indicating the effect of PEG-lipid to reduce liposome disposition by the MPS as mentioned above, the mechanism by which PEG-lipids reduce MPS uptake remains an area of confusion. Using partitioning techniques, it was shown by Senior et al (1991) and later by Moribe et al. (1997) that PEG-lipids can slow down the interaction of plasma proteins with liposomes. However, direct measurement of protein adsorption to liposome surface after incubation with plasma yielded controversial results (Blume et al., 1993a; Litzinger et al., 1994; Cullis et al., 1998; Johnstone et al., 2001). For neutral liposomes, PEG-lipid incorporation was shown to either decrease (Blume et al., 1993a; Cullis et al., 1998) or increase (Johnstone et al., 2001; Price et al., 2001) total serum protein binding. Different techniques used between these studies are likely the source of the controversial results obtained. As indicated by partitioning experiments, liposome bound proteins can be removed during the process of liposome-plasma separation (Senior et al., 1991).

Despite the lack of consensus from serum protein binding studies, the ability of PEG to exclude individual proteins have been consistently shown. By measuring the direct binding of several proteins, Du et al. showed a PEG-lipid concentration-dependent decrease in the adsorption of BSA, laminin, and fibronectin to a DPPE lipid bilayer (Du et al., 1997). The ability of PEG-lipid to reduce the complement protein C1q binding to anionic liposomes was demonstrated as well (Bradley et al., 1998b). Using a complement depletion assay to
measure C1q adsorption, it was shown that inhibition of C1q binding to anionic liposomes was dependent on both the polymer chain length of PEG as well as its grafting density (Bradley et al., 1998b). Similarly, incorporation of PEG-lipid was shown in vitro to reduce antibody binding to liposomes containing surface haptens (see chapter 3) (Li et al., 2002). Prevention of antibody binding in vitro was correlated to the ability of PEG to protect these liposomes from the rapid antibody-mediated elimination in vivo (Li et al., 2002). In PS-containing liposomes, PEG-lipid incorporation was shown to reduce the amount of prothrombin binding to liposome surface as well as the clotting activity of the liposomes (Chiu et al., 2001).

When considering the shielding properties of PEG grafted on liposome surface, the effectiveness of PEG to exclude macromolecules can sometimes deviate from the predicted surface coverage derived from the physical properties of PEG as discussed in section 1.3.1. For example, the amount of PEG-lipid required to completely shield PS-containing liposomes both from rapid elimination in vivo and from clotting factor binding, was found to be 15 mol% for PEG2000 (Chiu et al., 2001). This amount is much higher than the 2 mol% predicted to have complete protection of the surface of a 100 nm liposome (Torchilin et al., 1994b), and higher than the 5 mol% shown to have optimal effects in prolonging the circulation longevity of neutral liposomes (Allen et al., 1991c; Woodle et al., 1992). Another example is the finding that the ability of PEG to exclude specific antibody binding to its ligand depends also on the ligand density (see chapter 3). Taken together, these examples highlight the complexity of PEG-mediated macromolecule exclusion from liposome surface.
A confounding factor often not considered is the possible effect of protein binding on the shielding properties of PEG. As discussed by Johnstone et al., the effect of serum protein binding to PS-containing liposomes may facilitate surface-grafted PEG, incorporated at a low percentage (5 mol%), to adopt a more extended configuration, further preventing liposome uptake by phagocytic cells (Johnstone et al., 2001). In addition, it may be possible that the selective binding of dysopsonic proteins (see section 1.4.1.1) due to the presence of PEG mediates the circulation longevity of PS-containing liposomes. In fact, it has been shown by SDS PAGE analysis that additional protein bands were found in the profile of serum proteins bound to PEGylated PS liposomes (Johnstone et al., 2001).

Another proposed mechanism for the reduced liposome elimination is the prevention of aggregation due to the presence of surface-grafted PEG. In a study using DSPC liposomes, a strong correlation was found between liposome aggregation and their elimination in vivo (Ahl et al., 1997). The incorporation of PEG-lipids in these liposomes substantially improved circulation longevity and reduced liposome turbidity which is an indication of aggregation. The ability of PEG to prevent liposome aggregation, together with its inhibitory effect on serum-independent uptake of liposomes in vitro (Zeisig et al., 1996; Liu, 1997; Miller et al., 1998), indicates that prevention of surface-surface interaction imparted by PEG is partly responsible for the prolonged circulation longevity of liposomes.

1.3.4 Immunogenicity of PEG

It is unquestionable that PEG has the ability to reduce or prevent liposome interaction with other surfaces and macromolecules as discussed above. Although direct evidence is lacking, reduced rate of liposome disposition in vivo is probably attributed to reduced
liposome interaction with the immune system. However, the role of PEG-lipid in affecting liposome immunogenicity remains unclear. Despite original claims that PEG conjugation can reduce protein immunogenicity, recent evidence indicates that PEG is not entirely non-immunogenic. In fact, IgM specific for PEG has been generated in mice when injected with PEG-modified β-glucuronidase (Cheng et al., 1999). For PEG-modified liposomes, Dams et al. recently reported accelerated clearance of PEGylated liposomes after repeated administrations in rats and rhesus monkeys (Dams et al., 2000). Enhanced liposome clearance was found to be due to a soluble serum factor which was identified to be neither IgG nor IgM (Dams et al., 2000). PEG may also have an adjuvant role in the induction of immune response. Phillips et al. (Phillips et al., 1995) showed that incorporation of PEG-lipid into liposomes with surface-linked IgG2a (immunoliposome) caused a higher antibody response against IgG2a in mice. All these findings suggest the need to revisit the benefit of incorporating PEG-lipids in liposomes, especially for liposomes with components which can be potentially immunogenic such as a surface ligand (section 1.4.1.4) and encapsulated antigens (section 1.4.2).

1.4 LIPOSOME INTERACTION WITH THE IMMUNE SYSTEM

As briefly mentioned in the previous sections, liposomes do interact with the immune system in a manner that is associated with their elimination following parenteral administration. This interaction has two major implications for the use of liposomes as delivery vehicles. The most obvious implication is the adverse effect on the circulation longevity of the delivery vehicle owing to both the innate and adaptive immunity of the host.
These unwanted immune responses will be detrimental for liposomes with functional groups such as a reactive ligand for active targeting as discussed in section 1.4.1.2. The other important implication of liposome interaction with the immune system is the passive delivery of liposomal antigens to specific cell populations of the immune system, making liposomes suitable for use as vaccine delivery vehicles. In this regard, the generation of immune response is desired as discussed in section 1.4.2.

1.4.1 Unwanted liposome interaction with the immune system

Interaction with the immune system causing liposome removal is having the greatest negative impact on these carriers. Unwanted liposome elimination can be a result of either innate or adaptive immune response. In both cases, the binding of specific proteins which assist in phagocyte recognition (opsonization) is believed to be the initial step in liposome elimination. In the following section, liposome recognition by these proteins and the elimination process are discussed in more detail.

1.4.1.1 Recognition mechanism of the host leading to liposome elimination

While elimination by an adaptive immune response requires the re-exposure of the liposomal antigen to the host, the innate immune system can recognize foreign particles upon first exposure due to readily available opsonins in the host. The phagocytes which remove opsonized liposomes belong to the mononuclear phagocyte system (MPS). The major organs of the MPS include the liver, spleen, and bone marrow which are the major sites where
liposomes are distributed after parenteral administration (Poste et al., 1982; reviewed by Allen et al., 1999). Currently, a number of classes and individual opsonic proteins have been identified to involve in liposome elimination (see Table 1.3). Factors which affect MPS elimination are liposome size, charge, bilayer fluidity, cholesterol content, liposome dose, as well as the inclusion of surface reactive groups (Allen et al., 1999).

Among the many opsonins, the role of complement proteins in liposome elimination has been most extensively studied (Devine et al., 1994; Marjan et al., 1994; Bradley et al., 1998a; Kiwada et al., 1998; Patel et al., 1998). The complement system, consisting of at least 25 proteins functioning in an integrated fashion, is involved in both the innate and adaptive immune response to remove invading pathogens (Reid, 1986). In addition to the opsonization and the subsequent phagocytosis of foreign particles, complement binding also leads to lysis of the opsonized complex. The involvement of complement in liposome elimination is evidenced by the following: 1) many liposome formulations have been shown to activate the complement system via the classical and alternative pathway (Bradley et al., 1998a; Kiwada et al., 1998); 2) specific complement proteins have been shown to bind to liposomes (Devine et al., 1994; Marjan et al., 1994; Bradley et al., 1998a); and 3) hepatic liposome uptake can be enhanced by complement (Harashima et al., 1994; Matsuo et al., 1994; Liu et al., 1995a; Liu et al., 1995b). In general, neutral liposomes are poor activators of complement while positively charged liposomes are strong activators of complement (Bradley et al., 1998a). In addition, complement binding to liposome is also determined by liposome cholesterol content (Chonn et al., 1991). Crystalline cholesterol, a strong activator
Table 1.3 Opsonins responsible for liposome elimination

<table>
<thead>
<tr>
<th>Opsonin</th>
<th>Recognition site</th>
<th>Opsonin Receptors</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complement C3b</td>
<td>Unknown</td>
<td>CR1</td>
<td>Chonn et al., 1991</td>
</tr>
<tr>
<td>Complement IC3b</td>
<td>Unknown</td>
<td>CR3</td>
<td>Szebeni et al., 1996</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Scieszka et al., 1991</td>
</tr>
<tr>
<td>Immunoglobulins</td>
<td>Phospholipids</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>surface ligands</td>
<td>Fc receptor</td>
<td>Derksen et al., 1987</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Hsu et al., 1982</td>
</tr>
<tr>
<td>β2-glycoprotein I</td>
<td>Phosphatidylserine, phosphatidic acid, cardiolipin,</td>
<td>β2-glycoprotein I receptor</td>
<td>Chonn et al., 1995</td>
</tr>
<tr>
<td></td>
<td>Putative co-factor for binding of antiphospholipid antibodies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibronectin</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Hsu et al., 1982</td>
</tr>
<tr>
<td>C-reactive protein</td>
<td>Altered bilayer organization, galactosyl residues</td>
<td>Fc receptor</td>
<td>Richards et al., 1979</td>
</tr>
</tbody>
</table>
of complement, can be formed in liposomes with high cholesterol content (Chonn et al., 1991). Complement binding to liposome surface can be mediated by direct interaction with phospholipids as well as interaction with other liposome-bound proteins such as immunoglobulins (Szebeni et al., 1996) and C-reactive protein (Richards et al., 1979).

The ability of surface-grafted PEG to prevent complement recognition of liposomes was first studied by Bradley et al. (Bradley et al., 1998b). As mentioned in section 1.3.3, it was shown that incorporation of chol-PEG and PE-PEG resulted in dose-dependent inhibition of the human complement C1q binding to anionic liposomes in vitro. Inhibition of complement activation was dependent on both the PEG chain length and PEG grafting density (Bradley et al., 1998b). These results suggest that prolonged circulation lifetimes of PEGylated liposomes may be due to reduced opsonization by complement proteins.

Immunoglobulin is another important class of opsonin responsible for liposome elimination from the blood compartment. Immunoglobulins against liposomal antigens can be generated as a result of innate or adaptive immune response (Yasuda et al., 1977; van Houte et al., 1979; Phillips et al., 1991; Phillips et al., 1992; Phillips et al., 1995; Li et al., 2001a). Surface-associated haptens (van Houte et al., 1979), protein antigens (Phillips et al., 1992), and even phospholipids (Alving, 1984) can be recognized by specific immunoglobulins including IgG and IgM. Opsonization by these circulating immunoglobulins can result in Fc-receptor mediated phagocytosis (Derksen et al., 1987) as well as complement activation (Szebeni et al., 1996). The elicitation of antibody response against liposomal ligands is a significant concern for the development of liposomes for active
targeting as discussed in the following section. Up to date, there is no direct information in
the literature regarding the effect of PEG on specific antibody binding to liposomes
containing surface haptens or antigens. In chapter 3 of this thesis, the ability of PEG to
protect liposomes from antibody-mediated elimination is discussed.

It is worth mentioning that while opsonization is important for liposome removal in vivo, binding of other proteins may also regulate opsonin-mediated liposome elimination. Many proteins in the plasma compartment are capable of binding to liposomes non-specifically. Proteins which are capable of reducing the number of opsonin binding sites on liposomes and/or interfering with opsonin recognition by phagocytes are referred to as dysponsonins. The demonstration of the dysopsonic effect of protein binding was perhaps first provided by Juliano and Lin (Juliano et al., 1980) who studied the protein binding profiles of different liposome formulations. It was found that a group of high molecular weight proteins (>200,000 Da) were bound to neutral and positively charged liposomes but not on negatively charged liposomes. These unidentified proteins may play a role in prolonging the circulation lifetime of neutral and positively charged liposomes. Similarly, Moghimi et al. also reported a serum factor of molecular mass >100 kDa which may be responsible for the dysopsonic activity of serum on polystyrene latex microspheres (Moghimi et al., 1993). Using the mouse liver perfusion model, Kiwada et al. reported that below a certain serum to lipid ratio, hepatic uptake of liposomes can be inhibited by increasing the amount of liposome exposure to serum, suggesting a dysopsonic effect of serum which was found to be species-dependent (Kiwada et al., 1998). The exact role of these proposed dysopsonins in regulating liposome circulation longevity awaits further investigation.
One of the primary goals of using liposomes is to improve delivery to desired target sites. Liposome interaction with the immune system is probably having the greatest negative impact on the potential of these carriers to achieve this goal. In the following section, the challenges in the development of a special class of liposomes for active targeting will be discussed.

1.4.1.2 Liposomes designed for active targeting: an overview

The coupling of molecules specific for a certain disease site onto liposome surfaces to improve liposome accumulation at the target site is referred to active targeting. The concept of actively targeted liposomes was developed more than twenty years ago and is still being identified as an important component of liposome research to improve drug delivery. Through the years, many molecules including monoclonal antibodies, peptides, receptor ligands have been coupled to liposome surface to increase their specificity to the target cell population. A list of targeting molecules is included in Table 1.4.

The success of active targeting is dependent on many factors which have been extensively reviewed (Torchilin, 1994; Vingerhoeds et al., 1994; Torchilin, 1996; Maruyama, 2000). Several key factors identified include: 1) anatomical and pathological conditions which influence access of liposomes to target site; 2) residence time of liposomes in the circulation to sufficiently allow liposomes to arrive at target site; 3) affinity of liposomes toward target cell; and 4) drug availability at target site. All of these factors are impacted if the targeting ligand used or the liposomal lipid composition selected engenders an immune response.
Table 1.4 List of macromolecules conjugated to liposomes for active targeting

<table>
<thead>
<tr>
<th>Liposomal ligand</th>
<th>Target</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Antibody and related fragments</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mab F10.2</td>
<td>ICAM-1</td>
<td>Mastrobattista <em>et al.</em>, 1999</td>
</tr>
<tr>
<td>Mab N12A5</td>
<td>HER-2/neu</td>
<td>Goren <em>et al.</em>, 1996</td>
</tr>
<tr>
<td>Mab 34A</td>
<td>Pulmonary endothelial cells</td>
<td>Maruyama <em>et al.</em>, 1995, 1997</td>
</tr>
<tr>
<td>Mab GK1.5</td>
<td>CD4+ T-cells</td>
<td>Maruyama <em>et al.</em>, 1999</td>
</tr>
<tr>
<td>Anti-laminin Ab</td>
<td>Injured vascular bed</td>
<td>Torchilin, 1994</td>
</tr>
<tr>
<td>Anti-fibronectin Ab</td>
<td>Injured vascular bed</td>
<td>Torchilin, 1994</td>
</tr>
<tr>
<td>Anti-collagen type 1 Ab</td>
<td>Injured vascular bed</td>
<td>Torchilin, 1994</td>
</tr>
<tr>
<td>Anti-myosin Ab</td>
<td>Infarcted myocardium</td>
<td>Torchilin, 1994</td>
</tr>
<tr>
<td>Chimeric IgG C225</td>
<td>Human EGRF receptor</td>
<td>Harding <em>et al.</em>, 1997</td>
</tr>
<tr>
<td>Mab Fab’ against E-selectin</td>
<td>E-selectin</td>
<td>Bendas <em>et al.</em>, 1999</td>
</tr>
<tr>
<td>Mab Y-17 Fab’</td>
<td>HLA-DR</td>
<td>Bestman-Smith <em>et al.</em>, 2000</td>
</tr>
<tr>
<td>Mab 1F11 Fab’</td>
<td>Human integrin β1 subunit</td>
<td>Sugano <em>et al.</em>, 2000</td>
</tr>
<tr>
<td>Mab Polyclonal IgG, Fab’ and F(ab’)</td>
<td>B-cell lymphoma Mouse red blood cells</td>
<td>Lopes de Menezes <em>et al.</em>, 1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Peeters <em>et al.</em>, 1988</td>
</tr>
<tr>
<td><strong>Natural receptor ligands</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Folate</td>
<td>Folate receptor</td>
<td>Gabizon <em>et al.</em>, 1999</td>
</tr>
<tr>
<td>Transferrin</td>
<td>Transferrin receptor</td>
<td>Ishida <em>et al.</em>, 2001</td>
</tr>
<tr>
<td><strong>Peptide ligand</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Laminin pentapeptide</td>
<td>Laminin receptor</td>
<td>Zalipsky <em>et al.</em>, 1995</td>
</tr>
<tr>
<td><strong>Miscellaneous ligands</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biotin</td>
<td>Streptavidin</td>
<td>Corley <em>et al.</em>, 1994; Longman <em>et al.</em>, 1995</td>
</tr>
<tr>
<td>Streptavidin/avidin</td>
<td>Biotin</td>
<td>Loughrey <em>et al.</em>, 1993; Harasym <em>et al.</em>, 1995</td>
</tr>
<tr>
<td>Plasminogen</td>
<td>Fibrin</td>
<td>Blume <em>et al.</em>, 1993b</td>
</tr>
</tbody>
</table>
Among these factors, liposome circulation lifetime has gained the most attention because it has been the most difficult problem to overcome. Liposomes with surface-associated ligands are rapidly eliminated due to enhanced MPS uptake. Due to rapid elimination from the blood compartment, these liposomes have a very low probability of reaching the target site. Importantly, the success of targeting relies on the competition between liposomes reaching the target cell population versus elimination in the circulation. The location of the target site is then a determinant factor for the success of this competition. Targeting liposomes to cells in the circulation is not considered a problem (Peeters et al., 1988; Maruyama et al., 1995; Torchilin, 1996) but due to limited access of liposomes to extravascular sites, targeting to sites outside of the blood compartment is much more difficult and depends on the pathological condition (Vingerhoeds et al., 1994). For example, in the case of cancer, the vascular endothelium is known to be leaky in the tumor vasculature and can thus facilitate liposome extravasation. Liposome accumulation in tumors has been shown to be dependent on liposome size in several tumor models (Ishida et al., 1999; Maruyama et al., 1999). The optimal liposome size for extravasation was found to be 100-200 nm (Ishida et al., 1999; Maruyama et al., 1999). Despite this added advantage which helps liposome extravasation, there is little evidence that coupling targeting ligands to liposomes improves liposome accumulation in solid tumors (Goren et al., 1996; Maruyama et al., 1997; Bestman-Smith et al., 2000; Park et al., 2001).

The choice of targeting molecule which is essential for the success of active targeting is also an important consideration (Vingerhoeds et al., 1994). With the use of highly specific molecules such as antibodies, liposomes can be more effectively targeted to the cell
population of interest. Liposome binding to specific targets in vitro has been shown by many groups and was found to be dependent on the number of targeting molecules per liposome (Kirpotin et al., 1997; Maruyama et al., 1999). In general, target binding increases with increasing numbers of targeting molecules. However, more number of copies of ligand also increases elimination by the MPS.

Among the many ligands used for active targeting, antibodies and their fragments are most commonly used to couple to liposomes. These liposomes are referred to as immunoliposomes. In general, there are two types of immunoliposomes which differ in the location antibody coupling (see Figure 1.5). The first type, referred to as conventional immunoliposome, has the antibody coupled to liposome surface. The second type, referred to as the pendant type immunoliposome, has antibody coupled to the end of the PEG polymer which serves as a spacer arm reaching out from the liposome surface (Allen et al., 1995; Hansen et al., 1995; Zalipsky et al., 1995; Kirpotin et al., 1997; Maruyama et al., 1999).

Targeting ligands such as antibodies and Fab' fragments can be coupled to liposome surface using non-covalent methods such as avidin-biotin linkage, and covalent methods using derivatized lipids and heterobifunctional reagents such as N-hydroxysuccinimidyl-3-(2-pyridyldithio)propionate (SPDP) (reviewed by Torchilin et al., 1993 and Klibanov, 1998). While many studies have shown targeting success in vitro, liposomes with coupled proteins were later found to be rapidly eliminated in vivo as a result of rapid uptake by the MPS. Incorporation of PEG-lipids can substantially prolong their circulation. However, target binding is also greatly compromised due to the steric barrier (Klibanov et al., 1991). This
A. Conventional immunoliposome

B. Pendant type immunoliposome

Figure 1.5 Types of immunoliposomes. Antibodies or their fragments can be coupled to liposome surface as in (A) or coupled to the end of PEG as in (B). Schematic diagrams are adapted from Maruyama et al., 1995.
problem led to the development of pendant type immunoliposomes with the hope that ligands coupled to the terminus of PEG can make them accessible to targets while circulation longevity can be retained. Proteins can be coupled to the PEG terminus via four different linkages including amide, hydrazone, thioether, and urethane bond (Klibanov, 1998). Various proteins, including antibodies, Fab' fragments, peptides and macromolecules have been used as targeting ligands (Blume et al., 1993b; Maruyama et al., 1995; Zalipsky et al., 1995; Goren et al., 1996; Maruyama et al., 1997; Gabizon et al., 1999; Ishida et al., 2001). In general, coupling ligands to the PEG terminus yields higher coupling efficiency than coupling to the liposome surface (Blume et al., 1993b). The usual polymer size used as the spacer arm has a molecular weight of 2000.

1.4.1.3 Enhanced elimination of ligand-targeted liposomes by the innate immune system

As mentioned previously, liposome circulation longevity is probably the most important determining factor for the success of therapeutic liposomes regardless of whether passive or active targeting is being pursued. The removal of liposomes from the blood compartment is often facilitated when ligands are attached to liposomes, whether directly on liposome surface or at the end of a PEG spacer arm (Aragnol et al., 1986; Maruyama et al., 1995; Harding et al., 1997). This faster liposome elimination is due to better recognition of liposomes by the innate immune system when ligands, including antibodies, small peptides; and other macromolecules are attached to liposomes (Blume et al., 1993b; Allen et al., 1995; Maruyama et al., 1995; Zalipsky et al., 1995; Harding et al., 1997). The addition of these ligands on liposome surface can increase liposome size as well as introduce net charges and
sites which are better recognized by opsonic proteins and macrophages. In general, the degree of MPS uptake tends to depend on the size as well as the type of ligand. The use of ligands that are endogenous and antibodies with the Fc portion deleted appear to reduce the problem (Zalipsky et al., 1995; Kirpotin et al., 1997; Gabizon et al., 1999). In general, enhanced MPS uptake appears to be less dramatic for the pendant-type immunoliposomes, possibly due to the absence of ligands on some of the PEG terminus which may interfere with the cellular uptake of liposomes. For this reason, pendant-type immunoliposomes can be rendered stable by incorporating more PEG-lipid, but targetability of these liposomes is compromised (Gabizon et al., 1999). Similarly, conventional immunoliposomes containing PEG-lipids have prolonged circulation lifetimes, but have reduced or complete loss of targeting function (Loughrey et al., 1993; Corley et al., 1994). Thus, the development of liposomes for active targeting using PEG-lipids remains a challenge due to the non-selective protection of liposome-associated ligands. In Chapter 2 of this thesis, exchangeable PEG-lipids were tested to control liposome surface shielding and de-shielding in an attempt to have better control of ligand exposure.

1.4.1.4 Immunogenicity of immunoliposomes

The generation of specific immune response is another obstacle which greatly influences circulation longevity of immunoliposomes. Protection of immunoliposomes from elimination upon first injection does not guarantee that they will survive after the second injection. This is due to the immunogenicity of the ligand-conjugated carrier which elicits a specific immune response causing rapid elimination of the same carrier when re-injected.
Many factors, such as the type and size of ligand, the number of copies of ligand per liposome, as well as the dose and number of injection, all influence the likelihood of eliciting an immune response (Phillips et al., 1991; Alving, 1998; Klibanov, 1998). Liposomes have a propensity of enhancing the immunogenicity of proteins due to its ability to deliver antigens in particulate form (Phillips et al., 1992). Elimination of the Fc portion of antibodies does not reduce the immunogenicity of the ligand. In fact, Phillips et al. (Phillips et al., 1994a) showed that a higher antibody response was obtained when coupling Fab’ fragments to liposomes surface. Despite the ability of PEG to reduce liposome uptake by the MPS, PEG cannot render immunoliposomes less immunogenic (Phillips et al., 1994a; Phillips et al., 1995). In contrast, PEGylated-immunoliposomes were even more immunogenic than immunoliposomes without PEG-lipid and induce higher antibody titer against the coupled ligand (Phillips et al., 1994a; Phillips et al., 1995). Similarly, the pendant type immunoliposome is also immunogenic. Harding et al. showed that ligand-specific humoral response was generated when rats were given ligands which were prepared in pendant type liposomes but not when the ligands were given free (Harding et al., 1997). These results strongly suggest that the use of PEG-modified lipids has no benefit in reducing immunoliposome immunogenicity, but may be used as a means to improve the efficacy of liposomal vaccines as discussed in section 1.4.2.3.

As a solution to the short circulation lifetimes of ligand-targeted liposomes due to the generation of a humoral immune response specific for the ligand, immunosuppressive methods were attempted to prevent the generation of immune responses. Doxorubicin, a cytotoxic drug known to impair the phagocytic activity of liver macrophages (Daemen et al.,
1995), was encapsulated within liposomes with surface-conjugated ovalbumin (Tardi et al., 1997; Oja et al., 2000). Indeed, repeated administration of these liposomes containing a low dose of doxorubicin resulted in prevention of humoral immune response specific for ovalbumin and substantial improvement in the circulation longevity of these liposomes (Tardi et al., 1997; Oja et al., 2000). However, the potential of this immunosuppressive method for clinical development needs to be further evaluated.

Despite discouraging results with the use of PEG-lipids for immunoliposomes in terms of prevention of immune response induction, PEG may still have a role in preventing the recognition of liposomal ligand by circulating antibodies due to the surface-shielding properties of PEG. In Chapter 3 of this thesis, the ability of PEG-lipids to prevent antibody-mediated elimination of liposomes bearing surface ligands is addressed.

1.4.2 Induction of desired immune response using liposomes

Another important implication of liposome interaction with the immune system is the impact of liposomes on the immune system to generate immune responses which may be therapeutically beneficial. The potential of liposomes for immunological applications was first discovered in the early 1970’s when an antibody response against N-dinitrophenyl (DNP) was generated by conjugating the hapten onto liposome surface (Uemura et al., 1972; Uemura et al., 1974). A few years later, it was found that liposomes can enhance the immune response against proteins which were encapsulated within the aqueous core of liposomes, demonstrating that liposomes can be used as an adjuvant (van Rooijen et al., 1977). The finding that liposomes can elicit a strong humoral response against encapsulated
proteins has led many researchers to exploit liposomes as a vaccine carrier for protein antigens derived from pathogens. Numerous viral and bacterial antigens have been tested in liposomes and were shown to be effective in inducing protective immunity against pathogens including Epstein-Barr virus and malaria (reviewed by Alving, 1987). Research on the immunological applications of liposomes has entered a different era since the discovery in the late 1980's and early 1990's that liposomes can improve cellular immune response in addition to its role in enhancing humoral response. The ability of liposomes to induce cytotoxic T lymphocytes (CTLs) has attracted many scientists to further evaluate the use of liposomes for vaccine development. CTLs play an important role in the host defense against neoplastic and virus-infected cells (Germain, 1994; Lindauer et al., 1998). They recognize and lyse target cells in an antigen- and MHC-specific manner (Germain, 1994). In recent years, the potential of liposomes to induce anti-tumor immunity and immune response against intracellular pathogen has been actively explored. To date, many antigens have been shown to induce CTLs when delivered using liposomes (see Table 1.5). The promising future of liposomes in vaccine development is also indicated by the fact that a few liposomal vaccines are already in advanced stages of clinical development (see Table 1.2).

1.4.2.1 Delivery of liposomal vaccine to antigen presenting cells

The ability of liposomes to enhance immune response is believed to be due, in part, to the protection and delivery of the immunogen to phagocytic cells of MPS (Alving, 1987). Since macrophages are the predominant cell population for liposome uptake in vivo, these cells are believed to play a significant role in antigen processing and/or presentation to mount
### Table 1.5  Induction of cytotoxic T-lymphocyte response by liposomal antigens

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Liposome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ovalbumin</td>
<td>DOPE/DOSG</td>
<td>Zhou et al., 1992; Zhou et al., 1994; Nair et al., 1995</td>
</tr>
<tr>
<td></td>
<td>DOPC/PS/Chol</td>
<td>Zhou et al., 1992</td>
</tr>
<tr>
<td></td>
<td>EPC/DMPA/Chol/Sendai virus</td>
<td>Nakanishi et al., 2000</td>
</tr>
<tr>
<td></td>
<td>PS/PC plus anti-CD40 mAb</td>
<td>Ito et al., 2000</td>
</tr>
<tr>
<td></td>
<td>POPC/Chol/PEG-PE</td>
<td>Ignatius et al., 2000</td>
</tr>
<tr>
<td></td>
<td>DOPE/Chol/MPL</td>
<td>Zhou et al., 1993</td>
</tr>
<tr>
<td></td>
<td>DOPC/PS/Chol/MPL</td>
<td>Zhou et al., 1993</td>
</tr>
<tr>
<td></td>
<td>Glycerolipids of ARCHAEA</td>
<td>Krishnan et al., 2000</td>
</tr>
<tr>
<td>LCMV antigen</td>
<td>SPC/Chol/DL-α-tocopherol plus CpG ODN</td>
<td>Ludewig et al., 2000</td>
</tr>
<tr>
<td>Malaria circumsporozoite protein</td>
<td>DMPC/DMPG/Chol/MPL</td>
<td>Richards et al., 1998</td>
</tr>
<tr>
<td>Ebola virus</td>
<td>DMPC/DMPG/Chol/Lipid A</td>
<td>Rao et al., 1999a</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>PC/DP/Chol plus</td>
<td>Tanabe et al., 1999</td>
</tr>
<tr>
<td><em>HIV-1 gp120 protein-derived peptide</em></td>
<td>DMPC/DMPG/Chol/Lipid A</td>
<td>Alving et al., 1995; White et al., 1995</td>
</tr>
<tr>
<td>(p18, CG-P18)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cw3</td>
<td>DOPC/DOTAP/Chol plus antpHD</td>
<td>Chikh et al., 2001b</td>
</tr>
</tbody>
</table>
the immune response. Indeed, macrophage depletion using various drugs before immunization abolished the humoral response as well as CTL response against the liposomal antigen (Shek et al., 1982; Zhou et al., 1992; Nair et al., 1995; Tardi et al., 1997; Wijburg et al., 1998). Since dendritic cells are professional antigen presenting cells, their role in the generation of immune response to liposomal antigens has been studied as well. Using ovalbumin as the antigen, dendritic cells stimulated \textit{in vitro} with the antigen encapsulated in liposomes were able to present the antigen to naïve T cells to cause target cell lysis (Nair et al., 1992; Chikh et al., 2001b). When compared with macrophage treated in the same manner, dendritic cells were much more superior in activating T cells \textit{in vitro} (Nair et al., 1992). As well, dendritic cell stimulation \textit{in vivo} has been shown by injection of the liposome-encapsulated ovalbumin into mice to induce T cells (Nair et al., 1992; Nair et al., 1995; Ignatius et al., 2000). A similar finding was obtained using a peptide from the lymphocytic choriomeningitis virus (GP33) encapsulated in liposomes (Ludewig et al., 2000). Immunization using the liposomal vaccine could stimulate dendritic cells to induce primary CTL response and protect the mice from virus infection (Ludewig et al., 2000).

1.4.2.2 Intracellular processing of liposomal antigen

In addition to the ability of liposomes to selectively deliver the antigen to the relevant cell population(s) for mounting the immune response, liposomes have also been shown to be useful for the intracellular delivery of antigens. To induce CTL, it is required that exogenous antigens enter the MHC class I processing pathway in order for the antigen to be presented on MHC class I molecules (Lindauer et al., 1998; Heath et al., 2001) as illustrated in Figure 1.6. Evidence from the literature strongly suggests that liposomal antigens, after being taken
Figure 1.6  Exogenous antigen processing and presentation. Exogenous antigens taken up by APCs are normally degraded in endosomes and then loaded onto MHC class II molecules for presentation to CD4+ T cells. This usually leads to induction of antibody response. Antigens delivered in liposomes can escape from endosomes into the class I processing pathway for antigen presentation by MHC class I molecules to CD8+ T cells for induction of CTL response.
up by phagocytosis, can escape from endosomes and then be processed via this pathway which finally leads to CTL activation. First, liposomal antigens which initially localize in the endosomal vacuoles after cellular uptake have been shown to later appear in the cytoplasm (Alving et al., 1995). Second, antigen presentation can be inhibited by lactacystin, a proteosome inhibitor (Nakanishi et al., 2000; Chikh et al., 2001b). Third, liposomal antigens can be visualized in the Golgi apparatus (Richards et al., 1998; Rao et al., 1999b; Rao et al., 2000) and the transport of cytoplasmic antigen to the Golgi requires the transporters associated with antigen processing (TAP) system (Nakanishi et al., 2000; Rao et al., 2000).

In an attempt to improve intracellular delivery of antigens to the cytosol, various approaches to facilitate the release of liposomal antigens from endosomes have been investigated, including the use of pH-sensitive and fusogenic liposomes. pH-sensitive liposomes are composed of dioleoylphosphatidylethanolamine (DOPE) which can form reverse hexagonal structure (see section 1.2.2.1) and an acidic amphiphile which is sensitive to low pH. It is thus proposed that liposomes made with these lipids will become destabilized when inside endosomes with an acidic environment and then fuse with the endosomal membrane to release the antigen into the cytosol (Zhou et al., 1994). To further enhance the release of antigens from the endosomal lumen to cytosolic space, listeriolyisin O (LLO) from Listeria monocytogenes, a cytolysin, can be incorporated into pH-sensitive liposomes (Lee et al., 1996; Tanabe et al., 1999). It is proposed that the low pH within endosomes will destabilize liposomes and release liposomal contents including LLO and the antigen. Antigen access to the cytosol is then facilitated by the permeabilizing ability of
LLO towards phagosomal membrane (Lee et al., 1996; Tanabe et al., 1999). Another approach to deliver liposomal antigens to the cytosol concerned the use of fusogenic liposomes made with Sendai virus particles which can directly fuse with cell membrane, bypassing the endocytosis/phagocytosis of liposomal antigen (Nakanishi et al., 2000). Very recently, it was also shown that antennapedia homeodomain (AntpHD), a peptidic vector capable of shuttling peptide sequences across cell membranes (Schutze-Redelmeier et al., 1996), can be used in liposomes to facilitate the activation of CD8+ T cells (Chikh et al., 2001b). The mechanism of enhanced activation of CD8+ T-cell response is presumably due to the ability of antennapedia to transport antigens directly into the cytosol, where the antigen can enter the class I processing pathway, resulting in MHC class I presentation of antigen.

Taken all together, strategies designed to facilitate intracellular delivery of liposomal antigens to the cytosol can improve the efficacy of liposomal vaccines to induce cellular immune responses. However, as discussed in the following section, many other factors are also important in determining the outcome of immune responses generated by liposomal vaccines.

1.4.2.3 Factors influencing the efficacy of liposomal vaccines

Co-delivery of immunoadjuvant

Another advantage associated with the use of liposomes is the co-delivery of immunoadjuvant along with the antigen within the same carrier. In general, the immunoadjuvant is more effective if co-delivered with the liposomal antigen than if given
separately (Zhou et al., 1993; Baca-Estrada et al., 1997). This may be due to: 1) protection of the immunoadjuvant from degradation in vivo; and 2) simultaneous stimulation of the same cell population responsible for antigen processing and presentation. Liposomes containing Lipid A or its monophosphoryl derivative (monophosphoryl lipid A) have been shown to be an effective intrinsic liposomal adjuvant for induction of CTLs against various antigens including malaria antigens (Richards et al., 1998), antigens from HIV (White et al., 1995) and Ebola Zaire virus (Rao et al., 1999a). The adjuvant activity of lipid A is believed to be due to the direct stimulatory effect on macrophages by recruiting them and enhancing antigen presentation (Alving et al., 1995). In addition to adjuvants, the use of cytokines can be also helpful in improving vaccine efficacy by modulating different T-helper responses. However, in vivo use of cytokines is limited because of their rapid elimination and inactivation (Baca-Estrada et al., 1997). Liposomes can be used to eliminate this problem. Co-encapsulation of cytokines such as IL-4 and IL-12 with bovine herpesvirus type-1 glycoprotein D has been shown to modulate the immune response towards a different T-helper response (Baca-Estrada et al., 1997). The immunomodulatory effect of these cytokines could not be achieved if the cytokines were given separately with the antigen encapsulated inside liposomes (Baca-Estrada et al., 1997). As well, Gursel et al. showed that IL-15 co-encapsulated in liposomes with tetanus toxoid was able to induce higher antigen-specific antibody response (Gursel et al., 1997). It was also shown in the same study that IL-15 encapsulated in separate liposomes was less effective, indicating the benefit of antigen co-delivery with the immunomodulator. IL-2 is another cytokine incorporated into liposomal vaccines and has been shown to have beneficial effects. When used in liposomes containing the MUC-1 peptide, IL-2 can reverse T-cell suppression caused by MUC-1 mucin (Morse,
The therapeutic potential of this IL-2 containing MUC-1 peptide-based liposomal vaccine, named BLP-25, is currently under clinical evaluation (see Table 1.2).

A liposomal immunoadjuvant that is considered in this thesis is CpG oligodeoxynucleotides (CpG ODN) which have shown considerable promise in vaccine development in recent years (Krieg et al., 1999; Bramson et al., 2000; Klinman et al., 2000; McCluskie et al., 2000; Weiner, 2000; Mui et al., 2001). CpG ODNs are also referred to as immunostimulatory DNA sequences due to their wide range of stimulatory effects on the immune system. The immunostimulatory effects on the innate immune system include proliferation and secretion of IL-6, IL-12, and IgM from B cells (Krieg et al., 1995; Klinman et al., 1996; Yi et al., 1996a), increased lytic activity and IFN-γ release from natural killer cells and macrophages. More recent work on the immunostimulatory effects of these motifs showed that components of the adaptive immune system are involved as well. The effects on dendritic cells are growth, maturation and activation, including secretion of IL-6, IL-12, and TNF-α, and increased expression of CD86 to activate T-cell (Jakob et al., 1998; Hartmann et al., 1999). For T cells, CpG oligonucleotides can co-stimulate primary T cells in the absence of antigen presenting cells (Bendigs et al., 1999). Previous studies have indicated that CpG ODN is much more potent if it is physically associated with the antigen (Cho et al., 2000; Shirota et al., 2000; Tighe et al., 2000a; Tighe et al., 2000b). This interesting feature of CpG ODN has led researchers to consider its delivery using liposomes as an alternative method to the chemical conjugation of the adjuvant to the antigen. Encapsulation of CpG ODN within sterically stabilized cationic liposomes has been shown to induce innate immunity to protect immunized animals from pathogen challenge (Gursel et al., 2001). Co-encapsulation of CpG
ODN with ovalbumin was also shown to substantially increase antigen-specific IFN-\(\gamma\) and IgG response (Gursel et al., 2001). Using antigenic peptides from the lymphocytic choriomeningitis virus (LCMV) and CpG ODN encapsulated in liposomes, Ludewig et al. could induce protective anti-viral and anti-tumor immunity in mice (Ludewig et al., 2000). Encapsulation of CpG ODN within liposomes was also shown to induce antibody response against a T-independent antigen (see chapter 4) (Li et al., 2001a) as well as to enhance cellular immune response against a peptide derived from the tumor antigen HER2/neu (see chapter 5). Taken together, liposomes are not only useful for antigen delivery, but are also beneficial for delivering immunoadjuvants to further improve the immune response.

**Choice of lipid**

The choice of lipid is another factor which can influence the effectiveness of liposomes as a vaccine carrier. As already mentioned in section 1.4.2.2, intracellular delivery of liposomal antigens can be improved by using fusogenic lipids so that liposomal contents can be released into the cytosol more readily. Another approach to improve the immunoadjuvant effect of liposomes is to use charged lipids. Positively charged liposomes made with cationic lipids like stearylamine (SA), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), and dimethylaminoethane-carbamol-cholesterol (DC-Chol) have all been shown to induce cellular response efficiently (Nakanishi et al., 1999; Ignatius et al., 2000; Chikh et al., 2001b; Gursel et al., 2001). In comparison to neutral and negatively charged liposomes, positively charged liposomes were far more effective in inducing CTL response (Nakanishi et al., 1999). The advantage of using positively charged lipid may be due to the much higher uptake of liposomes by macrophages as shown *in vitro* (Johnstone et al., 2001;
Chikh et al., 2001a). The higher cellular uptake of pH-sensitive liposomes made with DOPE and DOSG may also be responsible for the higher efficacy of the formulation in priming CTLs secondary to its ability to target the antigen to the cytosol (Zhou et al., 1994).

Recently, the value of PEG-lipids in modulating liposome adjuvanticity was tested. Based on the long circulating properties of PEGylated liposomes as a result of reduced elimination by the MPS, it was hypothesized that liposomal antigens may be more efficiently delivered to other antigen presenting cells, such as dendritic cells which reside in the periphery of the body. Indeed, Phillips et al. in 1994 first showed that liposomes with surface-linked antibody were more effective in eliciting humoral response if PEG-lipids were incorporated into the liposomes (Phillips et al., 1994b). More recently, PEG-lipids were also incorporated into liposomes as a new strategy to induce cellular immune response against encapsulated proteins (Ignatius et al., 2000; Gursel et al., 2001). Ignatius et al. showed that sterically stabilized liposomes were more effective in inducing antigen-specific CTLs when compared with the same formulation without PEG-lipids (Ignatius et al., 2000). In Chapter 4 and 5 of this thesis, the benefit of using PEG-lipids in combination with CpG ODN as an immunoadjuvant to generate immune response against a T-independent antigen and a tumor antigen is being evaluated.

**Mode of antigen association**

In general, antigens can be incorporated into liposomes in two ways, namely encapsulation and surface linkage. To encapsulate antigens into liposomes, the antigen is often incorporated into MLVs by reconstituting the lipid film with a buffer containing the antigen. Antigens not entrapped are then separated either by size exclusion chromatography
or centrifugation (Gregoriadis et al., 1999). In many occasions, MLVs were extruded to make LUVs before separation of free antigen. Surface linkage of antigens can be achieved by chemically coupling the antigen to liposomal lipid using heterobifunctional reagents (Leserman et al., 1984). Alternatively, the antigen can be associated to the liposome surface by adding a hydrophobic moiety to facilitate incorporation into the lipid bilayer. It has been shown by Guan et al. that the MUC1 peptide could be displayed on liposome surface by using laurylcysteine as the N-terminal amino acid (Guan et al., 1998).

For many years, the mode of antigen association was believed to affect the outcome of the immune response (Alving, 1987). Studies have been carried out to compare the effect of antigen encapsulation versus surface linkage on liposome adjuvanticity (Therien et al., 1991; Zhou et al., 1992; Shahum et al., 1995; Guan et al., 1998). Using conalbumin and BSA as antigens, it was shown that antigen delivery on liposome surface was more effective in inducing antibody response (Therien et al., 1991; Shahum et al., 1995). For induction of cellular response, on the other hand, the two modes of antigen association were equally effective (Zhou et al., 1992; Guan et al., 1998). Using the MUC1 peptide antigen, it was found that only liposomes with surface-bound peptides, but not liposome-encapsulated antigen, induced MUC1-specific antibodies (Guan et al., 1998). Thus, it appears that humoral response is favored when the antigen is exposed on liposome surface.

**Route of immunization**

As for other vaccines, the route of immunization can affect the efficacy of liposomal vaccines. Using liposomes as vaccine vehicles, many immunization routes, including intravenous (i.v.) (Zhou et al., 1992; Rao et al., 1999a; Tanabe et al., 1999); subcutaneous
(s.c.) (Ignatius et al., 2000; Ito et al., 2000; Ludewig et al., 2000), and intraperitoneal (i.p.) (Richards et al., 1998; Wijburg et al., 1998; Gursel et al., 2001) have been shown to induce CTLs. However, since many other factors such as the use of adjuvant, liposome formulation and the type of antigen all determine the effectiveness of the vaccine, it is difficult to predict which immunization route is optimal for a particular immunogen. A comparison of the different immunization routes has been reported by a few studies and the results are, so far, inconsistent. Using conalbumin as the antigen, it was found that the i.v. route was most effective in inducing antibody response (Shahum et al., 1995). A similar conclusion was made when CTL response was assessed for a liposomal vaccine containing lipid A as immunoadjuvant and ovalbumin as antigen (Zhou et al., 1993). However, Ludewig et al. found the opposite result that the i.v. route was least effective and the intradermal route was most effective in inducing CTL response against a virus-derived antigen (Ludewig et al., 2000). These results point out that each liposomal vaccine is unique and it may be necessary to compare different immunization routes to determine the best immunization method.

To fully understand the effects of PEG-lipids on liposome interaction with the immune system, it is relevant to study the impact of PEG-lipids on the immune system by assessing the immune response(s) generated by liposomes containing this lipid. In Chapter 4 and 5 of this thesis, the effects of including PEG-lipid in liposomes on the induction of humoral and cellular immune responses are discussed. Together with the known properties of PEG to interfere with liposome interaction with cells known to comprise the removal mechanisms of the immune system, the results presented in this thesis will assist in gaining a better understanding of the benefit of using PEG-lipids in liposomes from an immunological perspective.
1.5 HYPOTHESIS AND SPECIFIC OBJECTIVES

Liposomes made with PEG-lipids have substantially improved circulation longevity attributed to steric stabilization of liposome surface by the PEG polymer. Evidence from in vitro work indicates that surface-grafted PEG can reduce liposome interaction with various proteins and macrophages. Taken together, these in vivo and in vitro findings suggest a role of PEG to assist liposomes to evade the immune system. However, there is also experimental data indicating that PEGylated liposomes can be immunogenic. We hypothesize that PEG-lipids do not mask liposomes from the immune system, but rather allow liposomes to interact with cells of the immune system in a manner that promotes enhanced immune responses. If true this will have a significant impact on the further development of liposomes as drug carriers as well as their use as vaccine carriers designed to induce immune responses.

The overall aim of this thesis is to study the implications of the changes in liposome interaction with the immune system caused by the use of PEG-lipid. The specific objectives are:

1. To determine the effectiveness of exchangeable PEG-lipids in shielding and de-shielding liposome surface to control the exposure of surface-associated ligands.

2. To evaluate the ability of exchangeable PEG-lipids to prevent liposome opsonization by specific antibodies and the subsequent rapid elimination in vivo.

3. To determine the benefit of using PEG-lipid in liposomes to generate immune responses.
CHAPTER 2

INTERMEMBRANE TRANSFER OF POLY(ETHYLENE GLYCOL) MODIFIED-
PHOSPHATIDYLETHANOLAMINE AS A MEANS TO REVEAL SURFACE
ASSOCIATED BINDING LIGANDS ON LIPOSOMES *

2.1 INTRODUCTION

Intermembrane transfer of conjugated lipids is of interest due to the active roles such lipids play in controlling surface reactions. Biologically relevant exchange processes include membrane fusion (Jahn et al., 1999) and intracellular signaling mediated by the transfer of glycosylphosphatidylinositol (GPI) linked peptides (Jones et al., 1998). Lipid transfer is also becoming an important attribute in the development of liposomes as biopharmaceutical delivery systems. Two areas of particular interest concern: (i) membrane dissociation of antibody/phospholipid conjugates following interaction with a target ligand (Bally et al., 1997) and (ii) applications involving controlled loss of poly(ethylene glycol) (PEG) lipids to achieve a transformation of the liposome's surface characteristics (Holland et al., 1996b; Adlakha-Hutcheon et al., 1999). As mentioned in section 1.3, it has been established that PEG-modified lipids can transfer out of liposomal membranes, a property that is dependent on the acyl chain composition of the PEG-modified lipids as well as the size of the PEG moiety (Silvius et al., 1993b). Formulations can be designed where PEG-lipid transfer is associated with a change in the liposomal properties and this is perhaps best illustrated by the transformation of a stable liposome to one that is highly fusogenic (Holland et al., 1996a; Holland et al., 1996b; Adlakha-Hutcheon et al., 1999).

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The concept that PEG-lipid transfer could transform the properties of a liposome is novel and many practical applications of this technology can be considered. For example, despite the beneficial effect of PEG-lipid incorporation on liposome circulation lifetime (see section 1.3.2) (Woodle et al., 1992; Woodle, 1993; Allen, 1994; Torchilin et al., 1994a), the development of liposomes with surface-grafted PEG moieties and attached targeting ligands has been restricted because of PEG-mediated inhibition of binding reactions between the targeting ligand and its binding site (Loughrey et al., 1993; Corley et al., 1994). In order to address this problem, investigators have been coupling the targeting ligand to the distal end of PEG moieties which are chemically attached to lipids incorporated in the liposomes (see Figure 1.5) (Kirpotin et al., 1997; Lopes de Menezes et al., 1998; Maruyama et al., 1999; Bestman-Smith et al., 2000; Lundberg et al., 2000; Maruyama, 2000). The rationale for the latter approach can be questioned given the propensity of the resulting liposome to be immunogenic (Harding et al., 1997). In addition, the stability of the ligand-conjugated PEG-lipid in the liposomal membrane is potentially problematic given the tendency of PEG-lipids to exchange out of liposomes (Silvius et al., 1993b). This problem may be exacerbated with the additional forces that play a role in dissociation/extraction of a lipid linked to an antibody following antibody antigen interaction (Bally et al., 1997). An alternative approach to address this problem is to employ PEG-modified lipids that can be transferred from the lipid bilayer thereby slowly exposing small, surface associated, targeting ligands.

In this regard, studies on lipid transfer are of particular interest in the development of liposomes with surface-active groups, such as a targeting ligand or charged moiety. Intervesicle transfer of phospholipids has been well documented in the past (Jones et al.,
Conjugation of hydrophilic macromolecules, including PEG, to lipid anchors is known to affect the rate of transfer of the entire phospholipid out of the bilayer (Silvius et al., 1993b). In this study, a method to measure PEG-lipid transfer based on a functional assay for quantifying liposomal PEG-lipid content in the acceptor liposome is described. More specifically, well characterized biotin-streptavidin interactions were used as a tool to study (i) PEG-lipid transfer as measured by the generation of a surface shield that prevents biotinylated liposome binding to streptavidin immobilized on 1 μm superparamagnetic iron oxide particles and (ii) the effect of PEG grafted polymers on biotinylated liposome binding to a defined surface. The assay described provides a very sensitive means to assess PEG-lipid transfer.
2.2 MATERIALS AND METHODS

2.2.1 Materials

Cholesterol and streptavidin-immobilized on superparamagnetic iron oxide particles were purchased from Sigma Chemical Company (St. Louis, MO). 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-n-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000), 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-n-[methoxy(polyethylene glycol)-2000] (DMPE-PEG2000), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-n-[methoxy(polyethylene glycol)-2000] (DPPE-PEG2000), 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine-n-[methoxy(polyethylene glycol)-2000] (DOPE-PEG2000) and 1,2 dioleoyl-sn-glycero-3-phosphocholine (DOPC) were purchased from Avanti Polar Lipids (Birmingham, AL). N-((6-biotinoyl)amino)hexanoyl)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (Bx-DsPe), ^3H-DSPE-PEG2000, ^3H-DPPE-PEG2000, and ^3H-DMPE-PEG2000 were purchased from Northern Lipids (Vancouver, Canada). C_{14} cer-PEG2000 was a gift from Inex Pharmaceuticals Corporation (Vancouver, Canada). Pico-Fluor 40 scintillation cocktail was obtained from Canberra-Packard Canada (Mississauga, ON). Nuclepore® polycarbonate filters were obtained from Watman (Clifton, NJ). Biogel A15M size exclusion gel was obtained from Bio-Rad (Mississauga, ON). ^{3}H]-Cholesteryl hexadecyl ether (CHE) was obtained from NEN Life Science (Boston, MA). RAG-2 mice and BALB/c mice were bred in-house at the BC Cancer Agency animal facility (Vancouver, Canada).
2.2.2 Preparation of large unilamellar vesicles

Liposomes were prepared using the extrusion method of Mayer et al (Mayer et al., 1986). Lipid mixtures (100 to 150 μmole total lipid) were first dissolved in chloroform. \[^{3}\text{H}\]-cholesteryl hexadecyl ether, used as a liposome label, was added to the lipid mixture to obtain a specific activity of 0.1 μCi/μmole. A lipid film was formed from the lipid mixture by first drying under nitrogen gas and then under high vacuum for at least 3 hours. The lipid film was hydrated at 65°C in 1.5 ml of HEPES buffered saline (HBS; pH 7.4). The resulting multilamellar vesicles were subjected to 5 freeze-thaw cycles and then extrusion (10 times) using two 0.08 μm and one 0.1 μm polycarbonate membranes with an extrusion device (Lipex Biomembranes Inc., Vancouver, Canada) which was thermoregulated at 65°C. Liposome size was determined by quasielastic light scattering (QELS) using a Nicomp 370 submicron particle sizer operating at a wavelength of 632.8 nm. The liposomes used in this study exhibited diameters of 100 to 120 nm.

2.2.3 Biotinylated liposome binding assay

Biotinylated liposomes (DOPC/Chol/Bx-DSPE; 55/45/0.1) binding to target was determined by incubating the liposomes (100 nmole in 100 μl HBS) for 20 minutes at room temperature with 100 μl of streptavidin immobilized on iron oxide particles prepared in a suspension provided by the manufacturer (suspension in 0.85% NaCl, 0.01 M phosphate, pH 8.0, containing 0.1% bovine serum albumin, 15 mM sodium azide). Unbound liposomes in the supernatant were removed from the mixture after separation using a magnetic separator (Advanced Magnetics Inc., Cambridge, MA). SA magnetic particles with bound liposomes were then collected after several washes and the radioactivity was counted using 5 ml of
Picofluor 40 and a Beckman LS 3801 scintillation counter. The radioactivity counted was a direct measure of the amount of liposome bound to streptavidin. The radioactivity of $[^3]$H]-CHE associated with SA magnetic particles was measured in the liposomal PEG-lipid transfer assay and the radioactivity of $[^{14}C]$-CHE was measured in the micellar PEG-lipid transfer assay. The data obtained was averaged from duplicates within the same experiment unless otherwise stated.

2.2.4 PEG-lipid transfer experiments

Intervesicle transfer of PEG-lipids was determined indirectly by measuring the inhibition of biotinylated liposomes (acceptor liposomes) binding to streptavidin due to the incorporation of PEG-lipids in these liposomes. DSPC/Chol liposomes containing 5 mol% of either DSPE-PEG2000, DPPE-PEG2000, or DMPE-PEG2000 were used as donor liposomes. In the transfer assay, acceptor liposomes were incubated with donor liposomes using conditions specified in each experiment. Subsequently, 100 μl of SA magnetic particles were added to the liposome mixture to measure biotinylated liposome binding to SA magnetic particles as described above. PEG-lipid transferred to biotinylated acceptor liposomes was estimated based on the residual amount of biotinylated liposomes bound to streptavidin which was converted to liposomal PEG-lipid content using Figure 1B as a standard curve with the following assumptions: 1) PEG-lipid is transferred only into the outer membrane of acceptor liposomes; 2) reductions in biotinylated liposome binding to streptavidin is attributed to PEG-lipids in the outer leaflet; 3) PEG-lipids are symmetrically distributed on the inner and outer leaflet of liposomes if PEG-lipids were incorporated in the initial step of liposome making. With these assumptions, reductions in biotinylated liposome
binding obtained in Figure 2.1B were attributed to 50% of the total PEG-lipid content. This was considered when extrapolating PEG-lipid content in acceptor liposomes after transfer from donor liposomes. Thus, the amount of PEG-lipid transferred to acceptor biotinylated liposome was determined by extrapolating, based on the level of target binding after transfer and using Figure 2.1B, the liposomal PEG-lipid content which was then divided by two. Data points presented are averages between two individual measurements in each experiment with less than 5% standard deviation.

2.2.5 PEG-lipid transfer from micelles

PEG-lipid transfer from micelles was done using DSPE-PEG2000. PEG-lipid micelles were prepared by dissolving 4 μmoles of DSPE-PEG2000 in chloroform with trace amount of $^3$H-DSPE-PEG2000. After the lipid mixture was dried under N$_2$ gas and vacuum for at least 3 hours, micelles were formed by adding HBS to the dried lipid to make a 5 mM solution. In the transfer experiment, 500 nmoles of biotinylated acceptor liposomes (with $^{14}$C-CHE as liposome label) were incubated with the indicated amount of PEG-lipid micelles under conditions specified for each experiment. After PEG-lipid transfer, liposome binding to target was assessed using the binding assay described above. The transfer of micellar PEG-lipids into liposomes was also monitored by following the elution of the PEG-lipid label in a size exclusion column. $^3$H-DSPE-PEG micelles were incubated with non-radiolabeled biotinylated acceptor liposomes under the conditions specified in the experiment. Micelles and liposomes were then separated by size exclusion chromatography using a Biogel A15m (100-200 mesh) column (1.5x42cm) with a flow rate of 0.5 ml/min. The amount of $^3$H radioactivity in the liposomal peak would indicate the amount of DSPE-
PEG transferred to acceptor liposomes. Total amount of phospholipid in the liposomal fractions was determined by organic phosphate measurement (Chen et al., 1956).

2.2.6 *PEG-lipid exchange in vivo*

RAG-2 mice were injected i.v. with DSPC/Chol/Bx-DSPE liposomes (3 μmoles/200 μl/mouse) containing 5 mol% of either C14-Cer-PEG2000, DMPE-PEG2000, DOPE-PEG2000 or DSPE-PEG2000. At 16 hours after injection, the mice were euthanized and the plasma was collected. Since the presence of plasma did not affect the binding of Bx-liposomes to SA magnetic particles, liposome binding to target can be assessed directly using the plasma samples collected. The plasma was pooled from 3 mice in each treatment group and then diluted accordingly using untreated control mice plasma to obtain the same lipid concentration for all groups. One hundred μl of plasma (containing 42.2 nmoles of lipid) was incubated with 100 μl of SA magnetic particles for 2 hours. Liposomes bound to SA magnetic particles were then recovered using a magnetic separator and the amount of liposome bound was determined as described above. Recovery of liposome binding to streptavidin was used as an indirect measure of PEG-lipid released from liposomes. Alternatively, 3H-labeled PEG-lipids were used to monitor the retention of PEG-lipid in liposomes. BALB/c mice were injected i.v. with DSPC/Chol liposomes (3.3 μmole/mouse) containing 5 mol% of either DSPE-PEG2000, DPPE-PEG2000 or DMPE-PEG2000 with trace amounts of the respective 3H-PEG-lipid. 14C-CHE was incorporated in these liposomes as a liposome label. Circulating levels of both 3H-PEG-lipid and 14C-CHE were determined in blood samples collected at 1, 4, and 24 hours after liposome injection.
2.3 RESULTS

2.3.1 PEG-lipid mediated reductions in biotinylated liposome binding to streptavidin iron oxide particles

It is well established that incorporation of PEG-modified lipids into liposomes which contain biotinylated lipids can interfere with streptavidin binding (Loughrey et al., 1993; Corley et al., 1994; Redelmeier et al., 1995). In these studies, biotinylated liposomes binding to streptavidin immobilized on microtiter plates was much reduced when PEG-lipids were incorporated into liposomes. For this reason, it is proposed that PEG-modified lipid exchange assays could be developed on the basis of PEG-lipid mediated reductions in the interactions between biotinylated liposomes and streptavidin immobilized on magnetic particles. The effect of PEG-lipid content on the inhibition of biotin mediated binding of liposomes to streptavidin immobilized on magnetic particles was measured using DOPC/Chol liposomes containing 0.1 mol% Bx-DSPE and DMPE-PEG2000 at a concentration ranging from 0 to 5 mol%. The results (shown in Figure 2.1) indicate that the amount of biotinylated liposomes bound to SA magnetic particles decreased as the PEG grafting density increased. Instead of a proportional decrease in binding as a function of PEG-lipid content, a biphasic decrease in liposome binding was observed. Figure 2.1A was determined under conditions where 500 nmoles of liposomal lipid were incubated with 100 µl of streptavidin SA magnetic particles. When PEG-lipids were not present, 40 nmoles of liposomes were bound to the magnetic particles after incubation for 20 minutes at which time binding had reached equilibrium. Since the binding efficiency was only 8% under these
Figure 2.1 Biotinylated liposome binding to streptavidin immobilized on superparamagnetic particles (SA magnetic particles) as a function of liposomal PEG-lipid content. DOPC/Chol liposomes containing 0.1% biotin-x DSPE and 0-5% DMPE-PEG2000 were prepared as described in Materials and Methods. Liposome bound to SA magnetic particles were measured using either 500 nmoles (A) or 100 moles of liposomes (B) which were incubated at room temperature with SA magnetic particles as described in Materials and Methods. The data represents averaged results ± S.D. obtained from 2-4 liposome preparations.
conditions, the assay was repeated using lower liposome concentrations in an effort to demonstrate improved binding efficiency. When 100 nmoles of biotinylated liposomes were incubated with the SA magnetic particles, 25 nmoles were bound, as shown in Figure 2.1B, equivalent to 25% of the added liposomal lipid. Whether using 100 nmoles or 500 nmoles of liposomes, the PEG-lipid dependent inhibition of binding was comparable (Figure 2.1A and B). Inclusion of 0.5 mol% DMPE-PEG2000 caused a 30 and 33% reduction in binding to streptavidin, respectively. However, doubling the PEG-lipid content to 1 mol% only caused a further 8 to 10% decrease in binding. When a PEG-lipid content of 5 mol% was used, there was a 90% reduction in biotinylated liposomes bound to SA magnetic particles, suggesting efficient steric stabilization of the liposome surface can be achieved at this PEG grafting density, a result that is similar to previous findings (Noppl-Simson et al., 1996).

2.3.2 PEG-lipid transfer from donor liposomes to biotin labeled acceptor liposomes

A previous study using a fluorescence resonance energy transfer-based assay demonstrated that PEG-modified lipids can transfer from one liposome population (donor) to another liposome population (acceptor) (Holland et al., 1996b). The rate of transfer was dependent on the ratio of donor to acceptor liposomes as well as other parameters including the size of the PEG-moiety, the acyl chain composition of the PEG-modified lipid and incubation conditions. The transfer assay developed in this study relies on the inhibition of biotinylated liposomes binding to SA magnetic particles. Therefore, it was important to determine whether the presence of donor (DSPC/Chol) liposomes changed the binding attributes of these liposomes. As shown in Figure 2.2, at all acceptor to donor liposome ratios tested there was little effect on the amount of biotinylated (acceptor) liposomes bound
to SA magnetic particles. Since the data suggested that at an acceptor to donor liposome ratio of 1:10 (mol:mol) caused a 10% reduction in binding, subsequent experiments studying the transfer of PEG-modified lipids were completed using an acceptor to donor ratio of 1:4.

To measure PEG-lipid transfer from DSPC/Chol liposomes to biotinylated DOPC/Chol liposomes, reductions in biotinylated liposome binding to SA magnetic particles were measured following incubation of these acceptor liposomes with donor liposomes containing 5 mol% of either DMPE-PEG2000, DPPE-PEG2000, or DSPE-PEG2000. PEG-lipid transfer was measured at 4°C and 37°C as shown in Figure 2.3. The data presented in Figure 2.3A shows the amount of biotinylated liposomes bound to SA magnetic particles after incubation with the donor liposomes for the indicated time periods. Figure 2.3B represents the calculated amount (nmoles) of PEG-lipid transferred to acceptor liposomes, a value determined from the binding data in Figure 2.3A and the inhibition curve shown in Figure 2.1B for data conversion. The results demonstrate that when the acceptor liposomes were incubated with donor liposomes containing DMPE-PEG2000 for 30 minutes, there was a 53% and 86% reduction in acceptor liposome binding to SA magnetic particles when the incubation temperature was 4°C and 37°C, respectively. The amount of PEG-lipid in the biotinylated acceptor liposomes was calculated using the assumption that reductions in binding were due solely to PEG-lipid transfer to the liposome's outer leaflet. It was estimated that 2.2 nmoles of PEG-lipid (22% of total available PEG-lipid) was transferred to the acceptor liposomes within 30 minutes or less at 37°C. At 4°C, maximum transfer was
Figure 2.2 Effect of DSPC/Chol liposomes on the binding of biotinylated liposome to SA magnetic particles. The binding of biotinylated liposomes to streptavidin can be interfered by the presence of donor liposomes. DOPC/Chol/Bx-DSPE (49.9/45/0.1) liposomes (100 nmoles) were incubated with DSPC/Chol (55/45) liposomes at the indicated acceptor to donor liposome ratios for 24 hrs at 37°C. After the incubation, SA magnetic particles (100 µl) were added to the liposome mixture to measure the binding of biotinylated liposomes as described in Materials and Methods. Data presented are averages between two individual measurements in each experiment with less than 5% standard deviation.
Figure 2.3 Inhibition of biotinylated liposome binding to SA magnetic particles mediated by the transfer of PEG-lipids into liposomes. A) Inhibition of biotinylated liposome binding to streptavidin as a function of incubation time with donor liposomes containing DMPE-PEG2000 (■), DPPE-PEG2000 (●), or DSPE-PEG2000 (▲). To measure intervesicle PEG-lipid transfer, 100 nmoles of acceptor liposomes containing DOPC/Chol/Bx-DSPE (54.9/45/0.1) were incubated at either 4°C (top) or 37°C (bottom) with donor DSPC/Chol (50/45) liposomes (400 nmoles) containing 5 mol% of the indicated PEG-lipid. After incubation for the indicated time period, SA magnetic particles were added to the liposome mixture to measure PEG-lipid mediated inhibition of biotinylated acceptor liposome binding to streptavidin as described in Materials and Methods. B) PEG-lipid content in acceptor liposomes estimated based on the amount of liposomes bound to SA magnetic particles at the end of incubation with donor liposomes. Reduction in biotinylated liposomes binding to streptavidin was converted to liposomal PEG-lipid content using Figure 2.1B as described in Materials and Methods. The transfer of PEG-lipid at 4°C and 37°C is presented in the top and bottom panel respectively.
observed at the 2 hour time point, but the amount of transfer (1.6 nmoles of DMPE-PEG2000) was less than that observed at 37°C. The half-times of transfer for DMPE-PEG2000 at 4°C and 37°C, as determined by this assay, were less than 30 and 15 minutes, respectively.

The transfer of DPPE-PEG2000 and DSPE-PEG2000 was less efficient than DMPE-PEG2000, consistent with reports suggesting that the transfer of PEG-lipids is dependent, in part, on the acyl chain length of the chemically modified phosphatidylethanolamine used. Transfer of DPPE-PEG2000 was not detectable at 4°C (Figure 2.3B), however at 37°C the transfer of DPPE-PEG2000 was significant as judged by an 80% reduction in acceptor liposome binding to the SA magnetic particles (Figure 2.3A). The transfer of DSPE-PEG2000, on the other hand, could not be enhanced by increasing the temperature to 37°C. As shown in Figure 2.3B, DSPE-PEG lipid transfer was negligible at both 4°C and 37°C (< 0.08 nmoles of PEG-lipid). It should be noted, however, that there was a consistent 10 to 20% reduction in the amount of acceptor liposome binding to SA magnetic particles observed at the first time point following the addition of donor liposomes containing DSPE-PEG2000. Because this assay is sensitive to even very small amounts of PEG-lipids, this reduction in liposome binding, when converted to amount of PEG-lipid transferred, represents only 0.8 % of total available DSPE-PEG2000.

The amount of DMPE-PEG2000 transferred to acceptor liposomes was also measured at equilibrium as a function of acceptor to donor ratio and the results, plotted as the calculated amount of PEG-lipid transfer, are shown in Figure 2.4. With increasing quantities
Figure 2.4  PEG-lipid transfer as a function of donor liposome concentration. Biotinylated acceptor liposomes (100 nmoles) were incubated at 37°C with donor liposomes containing 5 mol% of DMPE-PEG2000 at the indicated ratios to allow PEG-lipid transfer. When the transfer has reached equilibrium after 2 hours, the amount of PEG-lipid present on acceptor liposomes at equilibrium was then determined by measuring liposome bound to SA magnetic particles as described for Figure 2.3.
of donor liposomes (up to 1:4 ratio), more PEG-lipid was transferred to acceptor liposomes. At an acceptor to donor liposome of 1:1, 1:2, and 1:4, the PEG-lipid content in the acceptor liposomes at equilibrium was determined to be 1.06, 1.6, and 2.2 nmol, respectively. These levels of DMPE-PEG2000 were comparable to the amount predicted on the basis of three assumptions: (i) the donor liposomes have 50% of the PEG-modified lipid in the outer leaflet and 50% retained in the inner leaflet of the liposome bilayer; (ii) only the DMPE-PEG2000 within the outer leaflet are available for transfer, and (iii) there is equal distribution of DMPE-PEG2000 among donor and acceptor liposomes when the transfer has reached equilibrium. On the basis of these assumptions, there should be 1.25, 1.65, and 2.0 nmol of DMPE-PEG2000 transferred to the acceptors when the donor to acceptor liposome ratios are 1:1, 1:2, and 1:4, respectively. As shown in Figure 2.4, the measured and theoretical values are comparable.

2.3.3 Transfer of PEG-modified lipids from micelles

As shown in Figure 2.3A there was a significant, 20%, reduction in the amount of acceptor liposome binding to SA magnetic particles observed even following addition of donor liposomes prepared with DSPE-PEG2000. This inhibition could be due to the presence of a low level of PEG-lipid that did not incorporate into the liposomes during their preparation. Since it has been shown by others that PEG-modified lipids which do not incorporate into the liposomal membrane will form micelles (Edwards et al., 1997), the possibility that the reduction in biotinylated liposome binding to SA magnetic particles due to the presence of PEG-lipid micelles was investigated. DSPE-PEG2000 micelles were prepared as described in Materials and Methods and then incubated with 500 nmoles of
biotinylated acceptor liposomes. Subsequently, liposome binding to SA magnetic particles was measured. Figure 2.5A shows the amount of biotinylated liposome bound to SA magnetic particles as a function of the amount of DSPE-PEG2000 added, as micelles, just before the binding assay. Following a 20-minute incubation with SA magnetic particles at room temperature, there was less than a 5% reduction in biotinylated liposome binding observed even in the presence of 30 nmoles of added DSPE-PEG2000 (Figure 2.5A). When the acceptor liposomes were incubated for 3 hours with DSPE-PEG2000 micelles prior to binding with SA magnetic particles, there was a concentration dependent reduction in binding. Similar to the results obtained with pre-formed PEGylated liposomes (Figure 2.1), the inhibition of binding appeared biphasic. When the amount of added DSPE-PEG2000 was increased from 0 to 2 nmoles, there was a 35% reduction in liposome binding to streptavidin. However, when DSPE-PEG2000 was increased from 2 to 25 nmoles (more than 10 fold increase), liposome binding to streptavidin was reduced further by only 36%. The results suggest that the added DSPE-PEG2000 was transferred into the lipid bilayer of biotinylated liposomes following incubation, consistent with previous studies demonstrating the transfer of PEG-lipids from micelles into liposomes (Yoshioka, 1991; Uster et al., 1996; Zalipsky et al., 1997).

To confirm that the inhibition in binding mediated by PEG-lipid micelles is due to the insertion of DSPE-PEG2000 into pre-formed liposomes, DSPE-PEG2000 micelles with trace amounts of $^3$H-DSPE-PEG2000 were first incubated with acceptor liposomes and subsequently fractionated on a size exclusion column to separate micellar DSPE-PEG- and liposomal PEG-lipid. Using previously published conditions (Holland et al., 1996a), $^3$H-labeled DSPE-PEG2000 micelles can be separated from $^{14}$C-labeled biotinylated liposomes
Figure 2.5 Effect of micellar PEG-lipid on biotinylated liposome binding to SA magnetic particles as a function of the amount of added DSPE-PEG2000. Binding to SA magnetic particles was measured using 500 nmoles of biotinylated liposomes immediately after adding DSPE-PEG2000 micelles (A) or after incubation for 3 hours at 37°C (B) with the indicated amounts of PEG-lipids.
by size chromatography on a Biogel A15m column. As shown in Figure 2.6, when $^3$H-
labeled DSPE-PEG micelles (250 nmoles) were added to acceptor liposomes (5 μmoles) and
then immediately chromatographed, the majority (>98%) of the PEG-lipid eluted in the
micellar peak (Figure 2.6B). However, if the DSPE-PEG micelles were incubated for 24 hrs
with acceptor liposomes before column separation, 100% of the $^3$H-labeled DSPE-PEG-lipid
was recovered in the liposomal fractions indicating complete transfer of the PEG-lipid into
the membrane bilayer of acceptor liposomes (Figure 2.6C). When the amount of DSPE-
PEG2000 micelles used was increased to 750 nmole (15% of the total liposomal lipid), it was
found that DSPE-PEG2000 was recovered in both the liposomal peak and micellar peak,
suggesting that the amount of DSPE-PEG2000 transfer into liposomes was saturable. By
measuring the total amount of phosphate in the liposomal peak, it was estimated that 7 mol% of
PEG-lipid was incorporated into liposomes, presumably in the outer leaflet of the
liposomal membrane.
0 hrs incubation
(acceptor:micelle ratio 1:0.05)

24 hrs incubation
(acceptor:micelle = 1:0.05)

24 hrs incubation
(acceptor:micelle = 1:0.15)
Figure 2.6 Time dependent transfer of PEG-lipids from micelles to liposomes. Transfer of DSPE-PEG2000 from micelles was followed using trace amounts of $^3$H-labeled PEG-lipid which was incorporated into PEG-lipid micelles. (A) Separation of liposomes from micelles by size exclusion chromatography. $^3$H-labeled DSPE-PEG2000 micelles and biotinylated liposomes containing $^{14}$C-CHE (used as a liposome label) were loaded separately onto a Biogel A15m (100-200 mesh) column (1.5x42cm) and then chromatographed at a flow rate of 0.5 ml/min. (B) Elution profile of $^3$H-labeled DSPE-PEG2000 which was chromatographed immediately after mixing the micelles (250 nmoles) with non-radiolabeled biotinylated acceptor liposomes (5 μmoles). (C) Elution profile of $^3$H-labeled DSPE-PEG2000 which was chromatographed after mixing the micelles (250 nmoles) with biotinylated liposomes (5 μmoles) and incubated at 37°C for 24 hours. (D) Elution profile of $^3$H-labeled DSPE-PEG2000 which was chromatographed after mixing excess amounts of micelles (750 nmoles) with biotinylated liposomes (5 μmoles) and incubated at 37°C for 24 hours.
2.3.4 PEG-lipid exchange in vivo

Our *in vitro* experiments demonstrate that in the presence of acceptor liposomes PEG-lipid transfer can be measured using a functional assay that relies on the binding of 0.1 μm liposomes to 1 μm magnetic particles. These experiments were conducted under conditions where an acceptor membrane was present in excess, however these conditions can be viewed as inadequate when considering the membrane and lipoprotein pools that exists *in vivo*. To assess the transfer of PEG-lipids *in vivo*, an assay based on similar principles of biotin mediated binding of liposomes to SA magnetic particles was used. DSPC/Chol/Bx-DSPE liposomes with and without PEG-lipids were injected into mice and then recovered from plasma 16 hours later. Recovered liposomes were incubated with SA magnetic particles to determine the binding attributes of these liposomes which in turn are dependent on exposure of biotin due to the loss of PEG-lipids. Recovery of target binding, which was used as an indirect measure of PEG-lipid loss, is expressed as a ratio of the binding level before and after injection for each of the liposome formulation studied. A binding ratio of 1 would indicate no change in target binding after circulation *in vivo*. As expected, liposomes without PEG-lipids had a reduction in binding to SA particles, likely a result of serum protein binding to the liposome surface, making biotin less accessible for binding to streptavidin. Similarly for liposomes containing DSPE-PEG2000, there was a reduction rather than an increase in target binding after injection into mice. This finding is consistent with the *in vitro* data indicating that DSPE-PEG2000 is retained well in the liposomal membrane. For all other formulations, a binding ratio of greater than 1 was obtained, indicating recovery of binding after circulation *in vivo*. Changing the acyl chain composition, either by adding double bonds (eg. DOPE-PEG2000), or changing the acyl chain length, could influence the
1. No PEG
2. C_{14}-Cer-PEG2000
3. DMPE-PEG2000
4. DOPE-PEG2000
5. DSPE-PEG2000

(A)

1.1 - i
1.0 - 0.9
0.8 - 0.7
0.6 - 0.5
0.4 - 0.3
0.2 - 0.1

(B)

% recovery of PEG, % recovery of liposomal lipid (ratio)

- DSPE-PEG2000
- DPPE-PEG2000
- DMPE-PEG2000

Time (hours)
Figure 2.7  PEG-lipid exchange in vivo. (A) PEG-lipid exchange as represented by recovery of target binding after circulation in vivo. Mice were injected with DSPC/Chol/Bx-DSPE liposomes with or without the indicated PEG-lipids (5 mol%). Liposomes were recovered from plasma after 16 hours to measure binding to SA magnetic particles as described in Materials and Methods. Recovery in binding is expressed as a ratio of the level of target binding before and after injection into mice. Data shown represent mean of 2 individual measurements in the binding assay using plasma pooled from 3 mice for each liposome formulation. (B) PEG-lipid exchange as measured using radiolabeled PEG-lipids. DSPC/Chol liposomes containing 5 mol% of either DSPE-PEG2000, DPPE-PEG2000, or DMPE-PEG2000 with trace amounts of the respective ³H-PEG-lipid were injected into mice to monitor the elimination of both ³H-PEG-lipid and ¹⁴C-CHE which was used as a liposome label. A ratio of the % recovery of ³H-PEG-lipid/¹⁴C-CHE was obtained at 1, 4, and 24 hours after liposome injection as described in Materials and Methods. Data shown represent averaged results obtained from 4 mice ± S.D.
exchangeability of the PEG-lipid as reflected by the different binding ratios obtained (Figure 2.7A). Liposomes made with DOPE-PEG2000 had a binding ratio of 3.7, corresponding to a 41% recovery in binding when compared to that observed for non-PEGylated formulations after injection into mice. Decreasing the acyl chain length to C14 caused an increase in the binding ratio to 8 and 11 for C14-Cer-PEG2000 and DMPE-PEG2000, respectively. When compared with non-PEGylated liposomes recovered from mice, these liposomes have recovered 72 and 63% of their biotin binding capacity, respectively.

To confirm that recovery in target binding was due to the loss of PEG-lipids, an in vivo experiment using $^3$H-PEG-lipids and $^{14}$C-CHE was conducted to monitor circulating levels of PEG-lipid and liposomes in vivo, respectively. Mice were injected (i.v.) with liposomes containing 5% of either DSPE-PEG2000, DPPE-PEG2000, or DMPE-PEG2000. The relative ratios of PEG-lipid to liposomal lipid were measured over a 24-hour time course following i.v. administration of the liposome (Figure 2.7B). Confirming the in vitro data and in vivo data (Figure 2.7A), there was not a measurable level of DSPE-PEG2000 loss from liposomes even when the liposomes were recovered from the plasma compartment 24 hours after i.v. injection. In contrast over 40% of the liposome associated DMPE-PEG2000 was lost by the first time point measured (1 hour). The loss of DPPE-PEG2000 in vivo was more gradual, with 10 and 25% loss of this lipid observed from liposomes in the plasma compartment isolated at 4 and 24 hrs after injection, respectively.
2.4 DISCUSSION

The method described here to evaluate PEG-modified lipid transfer was based on PEG-lipid mediated reductions in biotinylated liposome binding to streptavidin magnetic particles. This model was also developed in consideration of the goal to develop targeted liposome preparations in which the targeting ligand used is small enough to be shielded in a controlled manner by surface associated PEG polymers. It was demonstrated that the inclusion of PEG-lipid caused a concentration-dependent decrease in biotinylated liposome binding to the SA magnetic particles. However, the decrease was not proportional to the increase in liposomal PEG content. At low PEG grafting densities, there was an abrupt reduction in streptavidin binding while the decrease was more gradual at higher PEG-lipid concentrations. PEG mediated inhibition of non-specific protein binding to surfaces (Du et al., 1997) and avidin binding to biotin molecules conjugated to liposomal lipids (Noppl-Simson et al., 1996) has been documented previously. However, the results presented here suggest that even at low PEG concentrations, much lower than those required to influence protein binding, there is significant inhibition of surface-surface binding reactions. In the model developed here, PEG-modified lipids incorporated at concentrations less than 0.1 mol% can significantly reduce the binding of 0.1 μm liposomes to 1 μm particles. This discussion is focused on how PEG acts to limit the interactions between two particles as well as the role that PEG-lipid transfer can have in the design of targeted liposomal formulations.

Liposome surface coverage can be discussed based on the two different conformations of the polymer proposed by de Gennes (De Gennes, 1988) which are
determined by the polymer grafting density on liposomes. Du et al (Du et al., 1997) reported that protein adsorption decreases steeply at PEG5000 concentrations below 1 mol%, very close to 0.7 mol% at which the 5000 average molecular weight polymer is predicted to undergo transition to the brush conformation, supporting that the different effectiveness of PEG on surface shielding is due to changes in polymer conformation. However, for shorter chain polymers such as PEG2000, the transition between mushroom and brush conformation is not as distinct. Based on the prediction of Needham et al (Needham et al., 1999), the transition occurs at 4 mol% for PEG2000, whereas Rex et al. reported that PEG2000 would be in the mushroom configuration at concentrations as high as 9 mol% (Rex et al., 1998). Regardless, it is likely that the mushroom conformation was obtained with the PEG-lipid concentrations used in this study and a pattern of non-linear surface shielding was still observed similar to the finding of others using PEG750 (Noppl-Simson et al., 1996). Thus, the distinctive shielding properties obtained with different grafting densities of shorter PEG cannot be simply explained by the two different conformations of the polymer.

In contrast to most studies which measure the binding of free molecules to liposome surface (Harasym et al., 1995; Noppl-Simson et al., 1996; Du et al., 1997), the PEG shielding assay shown in this study is based on liposome binding to a target molecule on a larger surface, mimicking the situation of a target cell. It was surprising to note that there was a significant reduction in target binding when the content of PEG-modified lipid was less than 0.1%. This indicates that even low levels of grafted PEGs are sufficient to interfere with particle-particle binding when measuring the interactions between liposomes with target molecules on a larger surface. This observation is consistent with previous studies
indicating that PEG lipids inhibit the binding between antibody-targeted liposomes and their target cell population (Mori et al., 1991; Blume et al., 1993b). In contrast, it was noted that the binding of free biotin to avidin conjugated to liposomal surface was unaffected at PEG-lipid concentrations as high as 2% (Harasym et al., 1995). Thus, it appears that PEG-lipids can prevent specific interaction with molecules fixed on a larger surface better than with molecules free in solution. The differential effect of PEG in preventing adsorption of free vs bound target molecules may be explained by the size of the target molecule, which in turn determines its mobility. As noted by Needham et al., the hydrated PEG layer provides a barrier that acts like a molecular sieve when measuring penetration and binding of molecules to liposomes (Needham et al., 1999). Larger polymers such as PEG2000 and PEG5000 can restrict larger molecules (as in the case of molecules bound to a larger surface) while they are less effective in preventing penetration of smaller molecules. This result has implications with respect to the goal of designing ligand-targeted liposomes that rely on the use of PEG-lipid shielding of the surface associated targeting ligand. We believe that it is important to develop targeted liposomes with time-controlled release of PEG-lipids to protect liposomes while in the circulation, and to subsequently expose the shielded ligand for targeting when exiting the circulation.

Using the relationship obtained between the PEG content of biotinylated liposome and liposome binding to streptavidin immobilized on magnetic particles, PEG-mediated inhibition of biotin liposome binding was measured as a functional assay to determine PEG-lipid transfer. Consistent with other reports, it was found that the transfer of PEG lipids from DSPC/Chol donor liposomes was dependent on temperature and the acyl chain length of the
lipid anchor (Silvius et al., 1993a; Silvius et al., 1993b; Holland et al., 1996b). Using this method, PEG-lipid transfer was quantitated with reasonable accuracy. The experimental results obtained in this study compared well with theoretical predictions determined on the basis of several assumptions. These assumptions include the equal distribution of PEG-lipids in the outer and inner leaflets of the ‘donor’ liposomes which is supported by the prediction that the curvature of the inner leaflet of a bilayer of a 100 nm liposome would not be constrained sufficiently to affect the bilayer distribution of lipids with covalently linked PEG2000. It was also assumed that only PEG-lipids in the outer leaflet would be transferred and that at equilibrium the concentration of PEG-lipids in the outer leaflet of the acceptor and donor liposomes would be equal. An equally important assumption made in the development of this model concerned the mechanism of PEG-lipid transfer. Based on the conclusions of Silvius and Zuckermann (Silvius et al., 1993b) as well as studies demonstrating PEG-mediated inhibition of liposome/liposome interactions (Harasym et al., 1995), it is believed that PEG-lipid transfer occurred by monomer diffusion or via a micellar intermediate. It is demonstrated that DSPE-PEG2000 presented in micelles can facilitate DSPE-PEG2000 transfer to the ‘acceptor’ liposomes. However, the inter-liposome PEG-lipid transfer is probably not due to excess PEG-lipid in micellar form co-existing with donor liposomes. This interpretation is based, in part, on data suggesting that a maximum of 7 mol% PEG-lipids can be incorporated asymmetrically into pre-formed liposomes. Assuming the outer and inner leaflet of liposomes have an equivalent capacity for PEG-lipids, a total of approximately 14 mol% of DSPE-PEG2000 can be incorporated into liposomes before excess PEG-lipids form micelles.
The finding that DSPE-PEG2000 from micelles, but not liposomes, is readily incorporated into pre-formed liposomes indicates that desorption of phospholipid is the rate-limiting step in the transfer process, in agreement with the mechanism proposed by Jones and Thompson (Jones et al., 1990). Many factors which affect the rate of phospholipid desorption have been identified. These include the degree of unsaturation of the acyl chains of the lipid anchor (Silvius et al., 1993a), donor liposome lipid environment and size (Jones et al., 1990; Wimley et al., 1991), temperature and ionic strength buffer (Jones et al., 1990), and acyl chain length of the lipid anchor which seems to have the most prominent effect on the rate of transfer (Silvius et al., 1993a).

The time course of PEG-lipid transfer has very important implications in designing liposomes for therapeutic and diagnostic purposes. The in vitro data shown in this study indicated that PEG-lipids with short acyl chains (C14:0) exhibit the fastest rate of transfer, which occurs on a time scale of minutes at 37°C. This finding is well correlated with the in vivo results which demonstrate that liposomes made with DMPE-PEG2000 lost most of the PEG-lipid within one hour. A slower rate of PEG-lipid removal is probably required to protect liposomes in the circulation for a sufficient period of time before leaving this compartment. The rapid removal of DMPE-PEG2000 from fusogenic vesicles has been shown to result in their rapid elimination from the circulation and a reduction in stability of the drug loaded formulations prepared using this lipid (Adlakha-Hutcheon et al., 1999). DPPE-PEG2000 or DOPE-PEG2000, on the other hand, exhibit slower rates of transfer, where lipid loss occurs over a time scale of hours according to the in vitro and in vivo data. Knowing that liposome accumulation in tumors reaches maximum between 16 to 24 hours
(Gabizon, 1992; Huang et al., 1992), it is predicted that these PEG-modified lipids will provide the ideal choice for time-controlled exposure of liposomal ligand for targeting. In conclusion, the results shown in this study indicate that PEG-lipid transfer can lead to recovery of liposome binding attributes and that this recovery of binding is dependent on the attributes of the PEG-modified lipid used.
CHAPTER 3
PREVENTION OF ANTIBODY-MEDIATED ELIMINATION OF LIGAND-TARGETED LIPOSOMES USING POLY(ETHYLENE GLYCOL)-MODIFIED LIPIDS *

3.1 INTRODUCTION

In the previous chapter, by measuring the binding attributes of liposomes with biotin as a reactive ligand, it was shown that liposome surface shielding and de-shielding can be controlled using exchangeable PEG-lipids. In this chapter, the ability of exchangeable PEG-lipids to protect these biotinylated liposomes from opsonization by specific antibodies, a process critical for the rapid elimination of liposomes in vivo, is assessed. Using biotin as a model, ligand density can be easily manipulated by changing the concentration of biotinylated lipid in liposomes to study the effect of this parameter on liposome opsonization. As well, the protective effect of PEG-lipid as a function of PEG-lipid concentration in liposomes can be studied. An in vitro assay was developed to indirectly measure liposome opsonization which could then be correlated to the in vivo behavior of these liposomes. Information obtained from these experiments can help to identify important parameters which should be considered when designing liposomes with reactive ligands, such as would be required for an actively targeted liposomal carrier.

Active targeting can be achieved by conjugating macromolecules such as antibodies, peptides, and ligands of natural receptors onto liposome surfaces to improve the specificity of these drug carriers to disease sites (see section 1.4.1.2) (Vingerhoeds et al., 1994). While

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active targeting of liposomes has met with some success both in vitro and in vivo (Park et al., 1995; Kirpotin et al., 1997; Gabizon et al., 1999), further development of ligand-targeted liposomes for in vivo use remains a challenge due to the immunogenicity of the drug carriers bearing surface ligands which function as antigenic haptens (see section 1.4.1.4) (Phillips et al., 1991; Phillips et al., 1995). Repeated administration of these liposomes becomes problematic because the pharmacokinetic and biodistribution behaviors of the carrier change following the second and third injection of the drug (Shek et al., 1983; Harding et al., 1997; Tardi et al., 1997). Enhanced elimination of the liposomes is due to the generation of a humoral response and immunoglobulin binding to the liposomes in the plasma compartment. It has been shown that surface-grafted poly(ethylene glycol) cannot reduce the immunogenicity of these liposomes but can enhance the immune response to targeting molecules bound to the surface of liposomes (Phillips et al., 1995; Li et al., 2001a) or to the terminal moiety of the grafted PEG (Harding et al., 1997). However, it has not been shown whether PEG-lipids can protect liposomes once an immune response has been generated. This study was aimed at determining the benefits of using PEG-lipids in improving circulation longevity of ligand-targeted liposomes in mice with an existing immune response.

The use of PEG-lipids to prolong liposome circulation lifetime has been well documented (Allen et al., 1991c; Woodle et al., 1992; Woodle et al., 1994). As mentioned in section 1.3.3, the mechanism of improved liposome circulation longevity is proposed to be due to the steric effect of the grafted polymer which protects the liposome surface from the non-specific adsorption of proteins and the associated elimination of the carrier by the mononuclear phagocytic system (MPS) (Zeisig et al., 1996; Du et al., 1997; Miller et al.,
It has been previously shown that PEG-lipids can reduce the non-specific binding of various proteins, including BSA, laminin, and fibronectin to glass surface (Du et al., 1997). Incorporation of PEG-lipids into liposomes can also prevent complement binding to liposome surface (Bradley et al., 1998b). As well, endocytosis of liposomes in vitro by macrophages was shown to be reduced by incorporating PEG-lipids (Zeisig et al., 1996; Miller et al., 1998; Johnstone et al., 2001). In general, the protective effect of PEG-lipids depends on its polymer size as well as grafting density (Kenworthy et al., 1995a; Zeisig et al., 1996; Du et al., 1997; Bradley et al., 1998b; Miller et al., 1998; Needham et al., 1999). However, it has been recently demonstrated that this protective effect could actually be due to selective binding of proteins that is mediated, in part, by the presence of surface-grafted PEG (Johnstone et al., 2001). This study and others clearly question the dogma that PEG-lipid incorporation provides effective protection against non-specific serum protein binding. It is becoming apparent that PEG creates a selective barrier to protein binding and the amount and type of proteins bound to liposome with surface-grafted PEG is not well characterized. When considering the application of PEG-based surface protection for liposomal carriers designed to target defined in vivo targets, whether a cancer cell specific surface marker or disease generated element that is normally not expressed in healthy tissue, a further dilemma arises. It is now well established that PEG incorporation can interfere with surface-surface interactions. This effect results in decreases in binding avidity between liposomes and their intended target.

Since specific antibody binding to liposomal ligands appears to be a critical step in antigen recognition and elimination by the immune system (Geiger et al., 1981; Hsu et al.,
1982), it is proposed that PEG-lipids can prevent opsonization by specific antibodies and thereby improve liposome circulation longevity. In this chapter, the ability of PEG-lipids to reduce specific antibody binding to liposomes with surface-associated ligands is being evaluated using an *in vitro* method. As well, the effect of PEG-lipid incorporation in these liposomes on their circulation behavior is assessed in mice with a pre-existing immune response.
3.2 MATERIALS AND METHODS

3.2.1 Materials

BSA-biotin, O-phenylethylene diamine, cholesterol, complete and incomplete Freund's adjuvant were purchased from Sigma Chemical Company (St. Louis, MO). Monoclonal anti-biotin antibody was obtained from Biogenesis Ltd (Sandown, NH). Peroxidase-linked anti-mouse Ig was purchased from Amersham Life Science (Piscataway, NJ). Maxisorp 96-well plates were obtained from Nalge Nunc International (Rochester, NY). [3H]-cholesteryl hexadecyl ether was purchased from NEN Dupont Canada (Mississauga, Ont.). N-((6-biotinoyl)amino)hexanoyl)-1,2-disteraroyl-sn-glycero-3-phosphoethanolamine (Bx-DSPE) was obtained from Northern Lipids (Vancouver, Canada). 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-n-[poly(ethylene glycol) 2000] (DSPE-PEG2000), 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-n-[methoxy(polyethylene glycol)-2000] (DMPE-PEG2000), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-n-[methoxy(polyethylene glycol)-2000] (DPPE-PEG2000), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-n-[poly(ethylene glycol) 5000] (DSPE-PEG5000) were purchased from Avanti Polar Lipids (Birmingham, AL).

3.2.2 Preparation of large unilamellar vesicles

Liposomes were prepared using the extrusion method of Mayer et al (Mayer et al., 1986) as described in section 2.2.2. Haptenized liposomes were prepared by incorporating Bx-DSPE (1% or 0.1%) into DSPC/Chol liposomes, with a molar ratio of DSPC:Chol:Bx-
DSPE = 55-X:45:X where X=1 or 0.1. PEG-lipids were incorporated into Bx-liposomes at a concentration of 2 to 10 mol% as specified in each experiment with liposomal cholesterol content remaining constant. The liposomes used in this study exhibited mean diameters of 100 to 120 nm, and a gaussian distribution with a chi-square value of less than 2.

3.2.3 Immunization

BALB/c mice 7-9 weeks of age were injected \textit{i.p} with BSA-biotin (50 \(\mu\)g/mouse), mixed with incomplete Freund’s adjuvant which was mixed with 10\% complete Freund’s adjuvant, once per week for three weeks. One week after the last immunization, the mice were injected with various liposome formulations to monitor liposome elimination. At 1 and 2 hours after liposome injection, 25 \(\mu\)l blood was collected from the tail vein by nicking the tail and using a microcapillary tube pre-rinsed with an EDTA solution (200 mM). The blood collected was added to 200 \(\mu\)l of 200 mM EDTA solution and then centrifuged to separate the cellular components from plasma. The radioactivity in the supernatant was then determined with 5 ml of Pico-Fluor 40 scintillation cocktail and using a Beckman LS 3801 scintillation counter. At 4 hours, mice were terminated by \(\text{CO}_2\) asphyxiation and whole blood was collected by cardiac puncture. Since there is no difference in the method of blood collection for the determination of blood liposome levels, 25 \(\mu\)l blood collected from cardiac puncture was used to determine plasma liposome levels at the 4-hour time point. The remaining blood was used for the determination of antibody levels using ELISA. Importantly, all animal studies were conducted according to the protocols approved by the University of British Columbia’s animal care committee which follows the current guidelines established by the Canadian Council on Animal Care.
3.2.4 *Anti-biotin antibody quantification using enzyme-linked immunosorbant assay*

96-well plates were coated with BSA-biotin (0.02 μg/ml) at 4°C overnight and then blocked with 10% calf serum for 1 hr at 37°C. Plasma samples diluted in PBS containing 0.05% Tween 20 and 1% BSA (1:50 to 1:500 dilution, 50 μl) were added to wells in the ELISA plate and incubated for 1 hr at 37°C. Subsequently, secondary antibody (HRP-conjugated anti-mouse Ig; 1:1000, 100 μl) was added and incubated for an additional 1 hour. After each incubation step, the plate was washed three times with wash buffer (PBS with 0.05% Tween 20). For color development, o-phenylethylene diamine (OPD) was used as substrate. OPD diluted in phosphate-carbonate buffer (pH 5.0; 0.4 mg/ml; 150 μl) was added to each well and incubated for 20 min at room temperature. To stop the enzyme reaction, 40 μl of 3M H₂SO₄ was added to plate and incubated for another 10 min after which absorbance at 490 nm was read using a Dynex microplate reader (Chantilly, VA).

3.2.5 *Antibody consumption assay*

Antibody binding to liposomes was measured indirectly in an antibody consumption assay based on a competitive ELISA method. Biotinylated liposomes added to the ELISA assay described above would compete for the monoclonal anti-biotin antibody and therefore inhibit the antibody binding to immobilized antigen. The inhibition is then a measure of antibody consumption by the liposomes added to the assay. In the consumption assay, various formulations of biotinylated liposomes were incubated in the ELISA plate with the monoclonal antibody for 1 hour at 37°C. The concentration of the monoclonal anti-biotin antibody was kept low (1:8000) to ensure its concentration was not sufficient to saturate specific binding to immobilized antigen. Antibody consumption is determined by comparing
the absorbance of sample wells with the maximum absorbance obtained without liposome and is calculated as follows:

\[
\text{% Antibody consumption} = \left[ 1 - \frac{(A - A_B)}{(A_T - A_B)} \right] \times 100\%
\]

where \( A \) = Absorbance of sample well;
\( A_B \) = Background absorbance (without antibody); and
\( A_T \) = Maximum absorbance

3.2.6 **Statistical Analysis**

Two-way ANOVA was performed to detect differences among treatment groups in liposome elimination experiments. Newman-Keuls tests were preformed as post hoc analysis for two-way ANOVA. A p-value of < 0.05 was considered significant.
3.3 RESULTS

3.3.1 Antibody-mediated clearance of biotinylated liposomes

Mice were immunized using biotin-BSA conjugate as described in the Methods to induce an immune response against biotin. One week after the last immunization, biotin-specific antibody levels were measured in plasma. The results, summarized in Figure 3.1, indicate that there were significant levels of circulating anti-biotin IgG with mean values estimated to be in excess of 2400 ng/50 µl. Injection of the carrier protein (BSA) one week before the first immunization substantially reduced the biotin-specific antibody response to values less than 800 ng/50 µl. This was expected due to specific epitopic suppression by the protein conjugate as a result of clonal expansion of carrier-specific B cells thereby reducing the probability of hapten-specific B cells to react with the hapten (Schutze et al., 1989). Thus, the reduced hapten-specific response obtained by pre-immunization with the protein carrier confirmed that the immune response generated was not specific for the protein carrier.

The circulation longevity of biotinylated liposomes, our model ligand-targeted liposomes, was measured following i.v. injection into mice one week after the last immunization with BSA-biotin, a time point where the animals maintained significant anti-biotin IgG levels (see Figure 3.1). As shown in Figure 3.2, when DSPC/Chol liposomes containing 1% biotin (1% Bx-lipo) were injected i.v. into control mice previously given saline, 3.62 ± 0.38, 3.18 ± 0.17, and 2.54 ± 0.26 µmol/ml plasma remained at 1, 2 and 4 hours, respectively. The level at 1 hr can be used to estimate that more than 80% of the injected dose remained in the circulation at this time point. When the same liposome formulation was injected into mice immunized with BSA-biotin, less than 0.5% of the injected dose remained in the plasma at the same 1 hr time point, indicating that these liposomes were rapidly eliminated from the circulation due
Figure 3.1  Induction of antibody response to biotin in Balb/c mice. Mice were immunized three times using BSA-biotin (50 μg/mouse, i.p.) as described in Materials and Methods. Control mice were injected with saline only. One week after the last immunization, the mice were sacrificed to determine biotin-specific antibody levels in the plasma using the ELISA method. BSA+BSA-Bio denotes mice pre-injected with BSA given in phosphate buffered saline (1 mg/mouse, i.p.) one week prior to immunization using BSA-biotin. Values represent averaged results obtained from the number of mice indicated in brackets ± S.D.
Figure 3.2  Antibody-mediated elimination of haptenized liposomes. DSPC/Chol liposomes containing 1 mol% biotin-DSPE (1% Bx-lipo) with and without 5 mol% DSPE-PEG2000 were injected into saline-treated control mice (open symbols) or mice previously immunized with BSA-biotin (solid symbols) to monitor liposome elimination. Blood was collected and processed as described in Materials and Methods at 1, 2 and 4 hours after liposome injection to determine liposomal lipid levels remaining in the circulation as estimated through the use of $^3$H-CHE. Values represent mean ± S.D. obtained using 4 mice in each group. Data was analyzed using two-way ANOVA. A p-value of <0.05 was considered significant. * denotes significantly lower than the saline-treated control (group 1). # denotes significantly higher than the non-PEGylated formulation in immunized mice (group 3) for the indicated time points.
to the existing immune response against biotin (Figure 3.2). To test the protective effect of PEG-lipids on these liposomes, 5 mol% of DSPE-PEG2000 was incorporated into the formulation and these were injected i.v. into animals with pre-existing circulating anti-biotin IgG. The inclusion of PEG-lipid significantly increased liposome levels remaining at 1 and 2 hours (Figure 3.2). However, these liposome levels (less than 20% of the injected dose) were significantly lower when compared with the levels in saline-treated mice, indicating minimal protection of the haptenized liposomes from immune recognition and elimination. When the PEGylated Bx-liposomes were injected into saline control mice, the elimination profile obtained was comparable to that of the non-PEGylated formulation, indicating that inclusion of 5 mol% DSPE-PEG2000 did not influence the rate of non-specific elimination of liposomes in the time frame measured. It should be noted that at the lipid dose used (3.3 μmole/mouse), the effect of PEG on circulation lifetime is not observed at time periods less than 4 to 6 hours after i.v. injection.

3.3.2 Protection of liposomes with low hapten density by using PEG-lipids

The minimal liposome protection by 5% PEG-lipid was probably a result of insufficient hapten shielding due to either too much hapten or insufficient PEG grafting density. To investigate the protective effect of PEG-lipids on liposomes bearing reduced levels of surface haptens, the experiment using Bx-liposomes was repeated with a formulation prepared with ten times less biotin (0.1 mol% biotin-DSPE). As shown in Figure 3.3, Bx-liposomes with 0.1 mol% biotin exhibited rapid elimination from the circulation, similar to liposomes with high hapten density (1 mol%). There was less than 0.5% of the injected 0.1% Bx-liposomes remaining in the plasma 1 hour after i.v. injection. Inclusion of
Figure 3.3  Protection of liposomes with low hapten density using PEG-lipids. Liposomes with a low hapten concentration (0.1% biotin-DSPE) can be protected from rapid elimination by an existing immune response using PEG-lipids. Mice were immunized three times using BSA-biotin as described in Materials and Methods to elicit a biotin-specific antibody response. At 1 week after the last immunization, the mice were injected with DSPC/Chol control liposomes (■), 0.1% Bx-liposomes without PEG-lipid (●), 0.1% Bx-liposomes with either 5% DSPE-PEG2000 (●), 2% DPPE-PEG2000 (▼), or 5% DPPE-PEG2000 (▲) to monitor liposome elimination as described for Figure 3.2. Values represent mean liposome levels in the blood ± S.D. of sample size n=4. Two-way ANOVA was used to analyze data and a p-value of <0.05 was considered significant. * Denotes significantly lower than DSPC/Chol control (group 1). # Denotes significantly higher than the non-PEGylated 0.1% Bx-lipo formulation (group 2).
5 mol% of DSPE-PEG2000 in 0.1% Bx-liposomes was, in contrast to the 1% Bx-liposome, able to completely protect these liposomes from rapid elimination (Figure 3.3), where the circulation lifetime of these liposomes were not significantly different from DSPC/Chol liposomes. The protective effects of DSPE-PEG2000 were comparable to that obtained with DPPE-PEG2000. This is consistent with data indicating that DSPE-PEG2000 and DPPE-PEG2000 are retained well in the membrane following i.v. administration (see chapter 2 and Li et al., 2001b). When the concentration of PEG-lipid (DPPE-PEG2000) was lowered to 2%, the protective effect of this lipid was no longer evident. The circulating levels for these liposomes were significantly higher than the non-PEGylated formulation (0.48 ± 0.22 vs 0.014 ± 0.004 μmol/ml at 1 hour after injection) but were significantly lower than those measured for non-haptenated DSPC/Chol liposomes (2.6 ± 0.21 μmol/ml). Collectively, these data indicate that significant protection of liposomes from rapid elimination can be achieved using sufficient amount of PEG-lipid; however, hapten density is an important factor in determining the effectiveness of PEG-lipids.

Previous studies have shown that DMPE-PEG2000 is a highly exchangeable PEG-lipid that is not retained well in the liposomal bilayer. Exchange rates in the order of minutes have been measured both in vitro and in vivo when this lipid is incorporated in neutral liposomes (Holland et al., 1996b; Li et al., 2001b). The protective effect of this PEG-lipid on Bx-liposomes was also investigated in this study. 0.1% Bx-liposomes containing either 5, 10 or 15 mol% DMPE-PEG2000 were prepared and injected into mice previously immunized with BSA-biotin as mentioned above. As shown in Figure 3.4, DMPE-PEG2000 had little protective effect on these liposomes (Figure 3.4). Inclusion of 15 mol% DMPE-PEG caused
a significant increase in liposome levels at 1 hour after i.v. injection when compared with the non-PEGylated formulation (0.57 ± 0.31 vs 0.020 ± 0.006 µmol/ml plasma). However, this level of liposomal lipid represents only 12% of the injected dose and is significantly less than that observed following injection of the non-haptenized formulation (DSPC/Chol). The limited effectiveness of DMPE-PEG2000 in protecting Bx-liposomes from rapid elimination in vivo is likely due to the rapid loss of this PEG-lipid out of the liposomal bilayer (Holland et al., 1996b; Li et al., 2001b).
Figure 3.4   Elimination profiles of Bx-liposomes containing DMPE-PEG lipids. The protective effect of DMPE-PEG lipids was investigated by monitoring the elimination of Bx-liposomes containing 0 % (●), 5% (▲), 10% (▼), or 15% (♦) DMPE-PEG2000 in mice previously immunized with BSA-biotin. Liposome levels in the blood were measured at 1, 2, and 4 hours post liposome injection as described in Materials and methods. Values represent mean ± S.D. of sample size n=4. The data was analyzed using two-way ANOVA and a p-value of <0.05 was considered significant. * Denotes significantly higher than all other groups.
3.3.3 Assessment of antibody binding to Bx-liposomes

The protective effect of surface grafted PEGs on biotin labeled liposomes injected into mice with an established immune response is likely due to the prevention of antibody binding to biotin on the liposome surface. To determine the ability of PEG-lipids to prevent antibody binding to Bx-liposomes, an *in vitro* assay was used to measure biotin specific antibody consumption by liposomes. In the assay, the binding of a monoclonal anti-biotin antibody to immobilized biotin (on ELISA plate) was measured as a function of liposome concentration in the assay. Bx-liposomes binding to the anti-biotin antibody was represented as a decrease in antibody binding to immobilized biotin on the ELISA plate. Antibody consumption by liposomes was calculated based on the percent decrease in the binding of monoclonal antibody in the ELISA assay. As shown in Figure 3.5A, the addition of non-haptenized DSPC/Chol liposomes to the anti-biotin antibody assay did not result in any significant loss of antibody binding as measured using the ELISA assay, even when present at concentrations as high as 25 mM. In contrast, 50% of the anti-biotin antibody was "consumed" by the addition of 10 μM 1% Bx-liposomes (Figure 3.5A). Inclusion of 5 mol% DSPE-PEG2000 in 1% Bx-liposomes did effect a significant change in antibody binding and reduced antibody consumption by more than 1000 fold when compared with the non-PEGylated formulation (ie. 1000 fold increase in liposome concentration was needed to achieve the same level of antibody consumption). This is an interesting result when considering that the presence of 5 mol% DSPE-PEG2000 had little protective impact on the circulation life time of 1 mol% Bx-liposomes injected into mice with an established anti-biotin response (see Figure 3.2). Incorporation of 10% DSPE-PEG2000 did not have a substantial effect in further reducing antibody consumption as shown by the antibody
Figure 3.5 Antibody consumption by liposomes. Biotin-specific antibody binding to Bx-liposomes was measured indirectly in a competitive ELISA assay as described in Materials and Methods. A) Antibody consumption by liposomes containing 1% biotin as a function of liposome concentration. The effect of PEG-lipids on shielding the liposomal antigen was investigated using 10% DSPE-PEG5000 (♦), as well as 5% (▲), and 10% (▼) DSPE-PEG2000. B) Antibody consumption by liposomes containing 0.1% biotin as a function of liposome concentration. The effect of incorporating 5% DSPE-PEG2000 (▲), 5% DPPE-PEG2000 (♦), and 2% DPPE-PEG2000 (▼) into 0.1% Bx-liposomes on antibody consumption was investigated.
consumption curve which is similar to that of the formulation containing 5 mol% PEG-lipid. The effectiveness of PEG-lipids having a longer PEG polymer (MW 5000) was also investigated using this assay. The results suggest that DSPE-PEG5000 was not as effective as DSPE-PEG2000 in reducing antibody consumption.

To determine the effectiveness of PEG-lipids to prevent antibody binding to liposomes bearing a low hapten density (0.1 mol% biotin), antibody consumption by these formulations was measured (Figure 3.5B). It should be noted that antibody consumption by 0.1% Bx-liposomes was less efficient when compared with the formulation containing 1% biotinylated lipid. The 10-fold reduction in biotinylated lipid concentration resulted in a greater than 2-log shift in the antibody consumption curve. When 5 mol% of either DSPE-PEG2000 or DPPE-PEG2000 was incorporated into 0.1% Bx-liposomes, antibody consumption was reduced to the same level as that of non-haptenized DSPC/Chol liposomes, suggesting complete inhibition of antibody binding to these liposomes. When 2 mol% of DPPE-PEG2000 was used, there was a shift in the antibody consumption curve, but an antibody consumption level of 25% was still observed at a liposome concentration of 50 mM (Figure 3.5B), suggesting insufficient liposome protection. As shown in Figure 3.3, these liposomes were rapidly removed from the circulation following i.v. administration into mice with an established antibody response. Thus it can be concluded that even low levels of antibody consumption provides a good predictor of increased liposome elimination in mice which have an existing humoral immune response generated against the liposomal hapten.
3.4 DISCUSSION

The objective of this study was to investigate whether surface-grafted PEG polymers can protect hapten bearing liposomes from elimination following i.v. administration in mice with an established immune response against the hapten. Surprisingly, PEG-lipid incorporation at levels considered to provide optimal steric stabilization properties (5 mol% PEG2000) was only effective when used in liposomes bearing a low concentration of the associated hapten. An antibody consumption assay was used to determine that incorporation of PEG-modified lipids engendered a significant decrease in antibody binding to haptenized liposomes. However, only under conditions where PEG completely prevented antibody binding to liposomes, there was effective protection of the liposomes in vivo. There are two important conclusions reached in this study based on the results obtained. First, at a fixed PEG-grafting density, a density which is sufficient to achieve steric stabilization, the polymer’s ability to inhibit antibody binding to haptenized liposomes is dependent on hapten density. Second, when injecting liposomes with a surface-associated antigen, the PEG grafting density sufficient to cause a two-log reduction in antibody binding is not sufficient to ensure that the liposomes are not eliminated rapidly from the plasma compartment. These two conclusions will be discussed in turn.

Steric stabilization of neutral liposomes using PEG-lipids has been extensively studied in the past (Allen et al., 1991c; Woodle et al., 1992; Woodle et al., 1994). These earlier studies indicate that PEG-lipid concentration as low as 5 mol% was enough to dramatically improve circulation longevity of neutral liposomes. The stealth properties of
these liposomes are thought to be attributed to reduced MPS uptake. This study and another recent study (Chiu et al., 2001) using liposomes with a reactive moiety suggest that the surface-shielding properties of PEG depend on the binding avidity of the penetrating molecule for the reactive surface. This, in turn, can be determined by 1) the type of interaction, 2) the valency of the interaction, and 3) the accessibility of the reactive moiety. For liposomes containing a reactive moiety, such as an antigen or charged lipid, there is additional energy to provide for the penetrating molecules to move through the polymer barrier due to the high binding affinity. In contrast to the non-specific binding of proteins to neutral liposome surface, these specific interactions are mediated by the sum of many non-covalent forces including van der Waals force, hydrophobic force, hydrogen bonds and electrostatic force which make the binding affinity much higher. For liposomes containing phosphatidylserine, it was shown that a PEG grafting density of 15 mol% is required to protect these liposomes from rapid elimination in plasma as well as from prothrombin binding to liposome surface in vitro (Chiu et al., 2001).

In the current study, the results indicate that as low as 5 mol% PEG2000 is sufficient to prevent antibody-mediated elimination of biotinylated liposomes when biotin concentration was low (0.1%). However, the data obtained also suggests that complete antigen shielding by PEG-lipids may not be achievable with a high ligand concentration. This may be attributed to the much higher binding avidity when the ligand concentration was high. The antigens on liposomes having 1% biotin would certainly be close enough to each other for bivalent binding of the antibody. This may be sufficient to ensure that the antibody is avidly bound to the liposome surface. For liposomes with 10 times less antigen (0.1%
biotin), the estimated distance between antigens is 24 nm (assuming 68,000 lipid molecules in the outer leaflet of a 100nm liposome (Hutchinson et al., 1989)), a distance still possible for bivalent binding assuming the hinge region of the antibody is flexible (Amzel et al., 1979; Alzari et al., 1988). However, considering antigen lateral mobility in the bilayer, and that not all liposomal haptens are in a conformation available for binding (Petrossian et al., 1984), bivalent binding would be less for liposomes with a much lower antigen density. This may be the reason for the large difference in antibody consumption between liposomes with 1% biotin and 0.1% biotin observed in the current study (see Figure 3.5). Alternatively, it can be explained that at a high biotinylated lipid concentration, phase separation occurs in the lipid membrane. This would create biotin-rich domains on liposomes with PEG-lipid excluded from these areas, allowing antibody molecules to have access to biotin. It should also be noted that the inability of higher mole % PEG-lipids to completely shield liposomes containing 1% biotin may be due to the geometric arrangement of the reactive moiety. The inclusion of a six-carbon linker in the biotinylated lipid which extends the biotin molecules away from the lipid surface can make them more accessible for antibodies to bind bivalently.

Our results also indicate that the use of a longer PEG polymer (MW 5000) cannot improve the shielding from antibody when the ligand concentration is high. The lack of a better protection using a longer polymer, when compared with the same mol% of PEG2000, may be explained by the high flexibility of the polymer allowing antibodies to be “trapped” in the densely packed layer of PEG in the brush conformation, thus affecting the off-rate of the antibody. In addition, the actual amount of PEG-lipids incorporated may not be as high as 10 mol% as the maximum allowable PEG-lipid contents in liposome tend to decrease with
increasing polymer size (Beugin et al., 1998). Another confounding factor not considered in these studies is the influence of enhanced non-specific protein binding to PEGylated liposomes (Johnstone et al., 2001), an effect that will be particularly relevant for in vivo studies. The additional shielding by these non-specific proteins may be enough to protect liposomes from immune recognition when biotin concentration was low, but not when biotin concentration was high.

The fact that ligand concentration is an important parameter in determining the effectiveness of PEG in shielding liposome surface has special implications in the design of liposomes for targeting. While many studies suggest the benefit of having more ligand molecules for targeting, the results shown in this study suggest that ligand concentration should be balanced between the benefit of specific targeting and rapid elimination caused by immune recognition. Previous studies using antibodies have indicated that 40 targeting molecules per liposome would be optimal for targeting (Maruyama et al., 1995; Kirpotin et al., 1997). For biotinylated liposomes containing 0.1% biotin, it is estimated that 68 biotin molecules are present on each liposome, assuming 68,000 lipid molecules are in the outer lipid monolayer (Hutchinson et al., 1989). This concentration of biotinylated lipid has been previously shown to be equally effective in targeting streptavidin in vitro when compared with the formulation containing 1% biotin. Moreover, using the biotin-streptavidin targeting approach, it has been shown that specific targeting in vivo can be achieved using liposomes with approximately 50 copies of streptavidin as targeting ligand (Longman et al., 1995).
Another important conclusion drawn from this study is that complete protection of the liposomal ligand is critical for ensuring long circulation lifetime of the carrier. It was shown that PEG-lipid can substantially decrease antibody binding. However, depending on the ligand concentration, a 600 fold decrease in antibody consumption may not be enough to sufficiently protect liposomes from rapid elimination in vivo (Table 3.1). Even at a low level of antibody binding (20% consumption), achieved with 2 mol% PEG2000 in liposomes containing 0.1 mol% biotin, there was minimal liposome protection in plasma (Figure 3.5). This finding indicates that opsonization of liposome by circulating antibodies is a critical step in antibody-mediated clearance of liposomes with targeting ligands or haptens and even low levels of antibody binding to an liposome-bound epitope will be sufficient to accelerate liposome elimination.

While it is important to evade the immune system when liposomes are in the circulation, it would be necessary to re-expose liposome-associated targeting ligands to achieve targeting after localizing in the region where the target cells are. The use of exchangeable PEG-lipids may help to address this issue. Three PEG-lipids of varying acyl chain lengths were tested and DMPE-PEG, having the shortest acyl chain (C14:0), was found to provide the least liposome protection (see chapter 2). Less than 13% of the liposomes remained in the circulation at one hour after liposome injection, a result that was similar to haptenized liposomes containing no PEG-lipids. With such rapid elimination, there would be very little liposome accumulation in the target tissue, considering liposome extravasation is the rate-limiting step. DSPE-PEG is known for its retention in the liposomal bilayer, and is thus probably not an ideal PEG-lipid for re-exposing liposomal ligands. It remains to be
Table 3.1  Correlation of antibody consumption to liposome circulation longevity

<table>
<thead>
<tr>
<th>[Biotin]$^a$ (mol%)</th>
<th>[PEG-lipid]$^b$ (mol%)</th>
<th>Lipid remaining at 1 hr after injection (μmol/ml plasma)$^c$</th>
<th>$C_{20}$ (mM)$^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>2.6 ± 0.21</td>
<td>&gt;25</td>
</tr>
<tr>
<td>0.1</td>
<td>0</td>
<td>0.0148 ± 0.0045</td>
<td>0.131</td>
</tr>
<tr>
<td>0.1</td>
<td>2</td>
<td>0.48 ± 0.23</td>
<td>20.3</td>
</tr>
<tr>
<td>0.1</td>
<td>5</td>
<td>2.53 ± 0.3</td>
<td>&gt;50</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0.019 ± 0.004</td>
<td>&lt;0.0025</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>0.909 ± 0.52</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Note:  
$^a$ Biotinylated lipid concentration in liposomes measured in mol%.  
$^b$ PEG-lipid concentration in liposomes measured in mol%.  
$^c$ Plasma lipid concentration measured in mice with an established anti-biotin antibody response obtained from Fig. 3.2 and 3.3.  
$^d$ Lipid concentration required to mediated 20% consumption obtained from Fig. 3.5.
tested whether DPPE-PEG, with an intermediate exchange rate (see chapter 2) (Li et al., 2001b), can be released from liposomes in the time frame that is suitable for maximal target tissue localization as well as optimal dissociation to reveal the surface-associated targeting ligand.

In conclusion, PEG-lipids play a significant role in improving the circulation longevity of ligand-targeted liposomes. To design liposomes for active targeting, one should pay attention to the type and the concentration of PEG-lipids. As well, the concentration of the targeting ligand should be considered as it plays a role in determining the effectiveness of PEG-lipids in protecting liposomes from immune recognition.

Results obtained in this chapter demonstrate that PEG is beneficial in terms of protection of liposome from recognition by an existing immune response. However, in regards to the prevention of eliciting unwanted immune responses, existing evidence from the literature suggests that incorporation of PEG-lipids is not useful. It was shown that PEG could not reduce, but rather enhanced the immunogenicity of the liposomes (Phillips et al., 1995; Dams et al., 2000). This finding suggests that PEG-lipids may be beneficial for the development of liposomal vaccines (see section 1.4.2.3). In chapter 4 and 5, the potential benefit of using PEG-lipids in liposomes for the induction of immune responses against two different types of antigens, namely a T-independent antigen and a tumor antigen, will be evaluated.
CHAPTER 4

ENHANCED IMMUNE RESPONSE TO T-INDEPENDENT ANTIGEN

BY USING CPG OLIGODEOXYNUCLEOTIDES

ENCAPSULATED IN LIPOSOMES *

4.1 INTRODUCTION

T-cell independent response, characterized by a rapid production of low affinity IgM, is a natural defense mechanism of the host to remove invading pathogens including virus, bacteria and parasites (see Coutinho et al., 1975 and Mond et al., 1995 for reviews). The antigens found on the surface of infectious organisms are usually polysaccharides which stimulates antibody production without MHC class II restricted T-cell help. Although a TI antigen is classically defined by a response lacking T-cell help, there is, however, great variability in terms of T-cell involvement, IgG production, B cell memory and antibody isotype switching, owing to the heterogeneity of this class of antigens (Yasuda et al., 1977; van Houte et al., 1979; Bachmann et al., 1997). Despite differences in the absolute T-cell dependence of the immune response, relative T-cell independence of these antigens can be discerned by using athymic nude mice (Mond et al., 1995).

The general characteristics of TI antigens are large molecular weight, having repeating antigenic epitopes, and poor in vivo degradability (Mond et al., 1995 for review). TI antigens are further divided into TI1 and TI2 antigens with the former group capable of causing polyclonal B cell activation and able to stimulate immature B cells (Mond et al., 1995).

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While the exact mechanism of TI response is still not clearly elucidated, there is evidence indicating epitope density, organization, as well as affinity for the B-cell receptor is important in determining the responsiveness of B cells which, in turn, are partly responsible for the outcome of the immune response (Bachmann et al., 1993; Batista et al., 1998; Kouskoff et al., 1998). Since TI response is the first line of defense against invading pathogens, young children less than two years of age are more susceptible to infections because of the inability of immature B cells to respond to TI-2 antigens (Goldblatt, 1998). Thus it is important to develop vaccines for TI-2 antigens that are effective in young children. Protein-polysaccharide conjugate vaccines are currently being developed and have shown to be protective against some pathogens (Siber, 1994; Mulholland et al., 1997) but not for others due to poor immunogenicity of some bacterial antigens and problems in conjugating proteins to polysaccharides (Paoletti et al., 1992; Stein, 1994). In addition, conjugating polysaccharides to an immunogenic carrier can result in unresponsiveness against the linked epitope because of a pre-existing immunity generated against the protein carrier (Schutze et al., 1985). Liposomes can be used as an alternative carrier without this problem.

Immunostimulatory CpG motifs, found in bacterial DNA and synthetic oligodeoxynucleotides, have direct stimulatory effects on various components of the immune system (Krieg et al., 1999). The motif active in mice is composed of unmethylated cytosine and guanine dinucleotide flanked by two 5' purines and 3' pyrimidines whereas a different motif is active in human immune cells (Hartmann et al., 2000; Hartmann et al., 2000 Jan 15). Due to the strong and wide range of immunostimulatory effects (see section 1.4.2.3), DNA
plasmids or oligodeoxynucleotides containing these motifs have been used as adjuvants for various protein-based vaccines as well as TI antigens (Chu et al., 1997; Lipford et al., 1997; Weiner et al., 1997; Brazolot Millan et al., 1998; Davis et al., 1998; Sun et al., 1998; Threadgill et al., 1998; Carpentier et al., 1999; Chelvarajan et al., 1999). For example, when CpG ODN was used together with the LPS-O polysaccharide side chain of *Pseudomonas aeruginosa*, the TI response was enhanced as indicated by increased serum IgM levels, although total antibody levels were decreased (Threadgill et al., 1998). In another study, CpG oligodeoxynucleotides could overcome the unresponsiveness of cultured neonatal B cells to the TI antigens TNP-ficoll and anti-IgM antibody by preventing B-cell apoptosis (Chelvarajan et al., 1999).

At present, the molecular mechanism of action of CpG motifs has not been fully elucidated. However, several studies have indicated the importance of the cellular uptake of DNA for its stimulatory action (Hacker et al., 1998; Krieg et al., 1999; Manzel et al., 1999). It is also worth noting that close physical association of CpG motif-containing DNA with the antigen appears to be important for its adjuvant activity (Weiner et al., 1997; Sun et al., 1998). In addition, it was demonstrated that direct association of CpG oligonucleotides (CpG ODN) to a protein antigen, either via biotin-avidin linkage or covalent linkage, could further enhance the immune response (Klinman et al., 1999; Cho et al., 2000; Shirota et al., 2000; Tighe et al., 2000a; Tighe et al., 2000b). Due to this reason, liposomes may be used as an alternative method to deliver CpG ODN in close proximity with the antigen in an attempt to improve the immune response.
Haptenized liposomes are chosen as a model of TI antigen based on previous findings that liposomes bearing surface haptens, such as dinitrophenyl (DNP), fluorescein, trinitrophenyl (TNP), and tripeptide-linked TNP, induce a TI response as indicated by the production of hapten-specific IgM in nude mice (Yasuda et al., 1977; van Houte et al., 1979). Liposomal haptens are arranged in an organized, multimeric fashion on the liposome surface, and have long circulation lifetimes, mimicking the general characteristics of TI-2 antigens. In the current study, CpG ODN encapsulated within the aqueous core of liposomes bearing surface biotin molecules are used to immunize mice. The amount of CpG ODN used in this study is less than the dose commonly used for immunization (Chu et al., 1997; Lipford et al., 1997; Weiner et al., 1997; Threadgill et al., 1998). The results shown in the following sections indicate that CpG ODN can increase the immunogenicity of biotinylated liposomes, a poorly immunogenic model. CpG ODN incorporated into these liposomes induced a strong IgM antibody response. The combination of CpG ODN and PEG-modified lipids enhances the response further. The adjuvant effect was observed only when CpG ODN was encapsulated, and not when co-administered. It is concluded that liposomes are a useful carrier system for the development of vaccines for TI antigens using CpG oligodeoxynucleotides as an immunoadjuvant.
4.2 MATERIALS AND METHODS

4.2.1. Materials

The phosphorothioated ODNs used in this study (listed in Table 4.1) were obtained from either Hybridon Inc. (Milford, MA) or UBC oligonucleotides synthesis laboratory (Vancouver, B.C.). Cholesterol, bovine serum albumin-conjugated biotin (BSA-biotin), O-phenylethylene diamine (OPD) were purchased from Sigma Chemical Company (St. Louis, MO). Peroxidase-linked rat anti-mouse IgM monoclonal antibody was purchased from ICN Pharmaceuticals (Costa Mesa, CA). Peroxidase-linked goat anti-mouse IgG (heavy chain specific) was obtained from Caltag (Burlingame, CA). Maxisorp 96-well plates were obtained from Nalge Nunc International (Rochester, NY). N-(((6-biotinoyl)amino)hexanoyl)-1,2-disteraroyl-sn-glycero-3-phosphoethanolamine (Bx-DSPE) was obtained from Northern Lipids (Vancouver, Canada). 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-n-[poly(ethylene glycol) 2000] (DSPE-PEG2000) were purchased from Avanti Polar Lipids (Birmingham, AL). Pico-Fluor 40 scintillation cocktail was obtained from Canberra-Packard Canada (Missasauga, ON). Aluminum hydroxide gel adjuvant (Alhydrogel “85”) was obtained from E.M. Sergeant Pulp & Chemical Co (Clifion, NJ).
Table 4.1 Synthetic oligodeoxynucleotides used in this study

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
<th>Known Biological Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>CpG ODN1</td>
<td>TAACGTTGAGGGGCAT</td>
<td>C-myc antisense</td>
</tr>
<tr>
<td>CpG ODN2</td>
<td>GCATGACGTTGAGCT</td>
<td>B-cell mitogen</td>
</tr>
<tr>
<td>Non-CpG ODN</td>
<td>TGCATCCCCCAGGCCACCAT</td>
<td>ICAM I antisense</td>
</tr>
<tr>
<td>CpG-dn ODN</td>
<td>GGTCGATGCCGCGT</td>
<td>NA</td>
</tr>
</tbody>
</table>
4.2.2 Animals

BALB/c mice were obtained from the animal facility of the British Columbia Cancer Research Center (Vancouver, Canada). BALB/c (nu/nu) mice were purchased from Charles River Laboratories (Montreal, Quebec). All mice were maintained in microisolators and handled using practices and operating procedures consistent with the policies of the local animal care committee and the Canadian Council of Animal Care.

4.2.3 Liposome preparation and ODN encapsulation

Liposomes were prepared using the extrusion method of Mayer et al (Mayer et al., 1986) as described in section 2.2.2 using DSPC, cholesterol, and Bx-DSPE in a molar ratio of 54:45:1. To passively encapsulate CpG ODN, the lipid film was hydrated at 65°C in 1.5 ml of HEPES buffered saline (HBS; pH 7.4) containing ODNs at a concentration of 8-13 mg/ml. After the formation of liposomes by extrusion, free ODNs were separated from liposomes by size exclusion chromatography using a Biogel A 15M column (1.5x20 cm) run at a flow rate of 50 ml/hr. The amount of ODNs encapsulated in liposomes was determined by first extracting ODN from lipids using the method of Bligh and Dyer (Bligh et al., 1959) with some modifications. For extracting ODNs, HBS was used instead of H2O as part of the aqueous phase to extract ODN from lipids which would be in the organic phase. The amount of ODN extracted was then quantitated by measuring OD at 280 nm. High extraction efficiency was obtained with this method (>95%). The amount of ODNs encapsulated ranged from 7-13 μg/μmol lipid.
4.2.4 Immunization

DSPC/Chol/Bx-DSPE (54:45:1) liposomes (Bx-liposomes) with and without encapsulated oligodeoxynucleotides were used to immunize mice. Immunocompetent BALB/c or immunodeficient BALB/c (nu/nu) mice, 7-9 weeks of age, were immunized either intravenously (i.v.) via the tail vein or subcutaneously (s.c.) on the back with liposomes at a dose of 3.3 µmol/mouse (200 µl/mouse). The mice were immunized either once or twice, 7 or 9 days apart, as stated in each experiment. The amount of CpG ODN administered (either encapsulated or co-injected) was approximately 23-40 µg/mouse. Adjuvant activity of encapsulated oligodeoxynucleotides was measured by monitoring the clearance of haptenized liposomes in immunized mice. On the day of termination, the mice were injected with Bx-liposomes to measure liposome elimination as described in section 3.2.3. At termination, whole blood was collected from the heart to prepare serum for the determination of antibody levels using ELISA.

4.2.5 Biotin-specific IgG and IgM response

Anti-biotin IgG and IgM response was measured in serum by ELISA as described in section 3.2.4 using horse radish peroxidase-conjugated anti-mouse IgG or IgM as detection antibodies.

4.2.6 Statistical Analysis

Two-way ANOVA was performed to detect differences among treatment groups in liposome elimination experiments. Newman-Keuls tests were preformed as post hoc analysis for two-way ANOVA. A p-value of < 0.01 was considered significant.
4.3 RESULTS

4.3.1 High immunogenicity of haptenized-liposomes with encapsulated CpG ODNs

To determine the immunogenicity of biotinylated liposomes (Bx-liposomes), a method was developed to test the ability of the antigen to elicit an immune response which can quickly eliminate the same antigen when re-administered. It was shown in chapter 3 that Bx-liposomes are rapidly eliminated from the blood compartment in mice with a pre-existing antibody response against biotin which was generated using BSA-biotin conjugate. Thus, elimination of re-administered liposomes can be used as a qualitative measure of an immune response against the liposomal antigen. In the current study, BALB/c mice were immunized i.v. twice, 7 days apart, with various liposome formulations (Figure 4.1). One week after the last immunization, empty liposomes (DSPC/Chol), with or without biotin (1%), were re-administered to measure liposome elimination from plasma. The liposome dose used for immunization and measurement of liposome elimination was the same (3.3 μmole/mouse). Liposome content in the blood was determined at 1, 2 and 4 hours after liposome re-administration to obtain the elimination profile as shown in Figure 4.1. Following the injection of empty Bx-liposomes in mice previously immunized with an identical formulation, liposome levels in the blood were 26.9±0.6, 23.5±1.8, and 20.2±1.6 nmol/25μl blood at 1, 2 and 4 hrs, respectively. These liposome levels were similar to those obtained in non-immunized mice. Mice immunized with empty Bx-liposomes showed no significant IgG or IgM responses (Table 4.2), suggesting that Bx-liposomes with 1% biotin are poorly immunogenic. However, when Bx-liposomes with encapsulated CpG ODN were used to immunize mice, a biotin-specific response was obtained (Figure 4.1). The
Figure 4.1  Adjuvant effect of CpG ODN encapsulated in haptenized liposomes. BALB/c mice were immunized i.v. with empty Bx-liposomes (■), Bx-liposomes containing CpG ODN1 (● and ▲), or mixed with free CpG ODN1 (▼) on days 0 and 7 with a liposome dose of 3.3 µmol/mouse. On day 14, empty liposomes, with (solid line) and without biotin (dotted line) were injected and the plasma elimination of liposomes was measured at the indicated time points. Results shown represent data averaged from 4 mice ± S.D. Two-way ANOVA was performed to analyze differences among the four treatment groups. A p-value of <0.01 was considered significantly different. * denotes value significantly lower than the Bx-lipo group for the indicated time point.
Table 4.2  Biotin-Specific Antibody Response

<table>
<thead>
<tr>
<th>Immunization</th>
<th>Primary Response</th>
<th>Secondary Response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgM</td>
<td>IgG</td>
</tr>
<tr>
<td>i.v.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bx-lipo</td>
<td>ND(^f)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bx-lipo + (CpG ODN1)</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bx-lipo + free CpG ODN1</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>s.c.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bx-lipo</td>
<td>0.44±0.03</td>
<td>0.13±0.02</td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td>(4)</td>
</tr>
<tr>
<td>Bx-lipo + (CpG ODN1)</td>
<td>1.27±0.11</td>
<td>0.17±0.01</td>
</tr>
<tr>
<td></td>
<td>(4)</td>
<td>(4)</td>
</tr>
</tbody>
</table>

Note:

- \(^a\) Results are expressed as mean OD values ± S.D. obtained from the number of mice shown in brackets.
- \(^b\) Primary response was measured 6 days after the first s.c. immunization.
- \(^c\) Secondary response was measured 7 and 5 days after the second i.v. and s.c. immunization respectively.
- \(^d\) Biotin specific IgM was measured in serum diluted 1:100 (background OD of 0.093 was obtained using serum from non-immunized mice) for i.v. immunization and diluted 1:405 for s.c. immunization.
- \(^e\) Biotin specific IgG was measured in serum diluted 1:54 (background OD of 0.153 was obtained using serum from non-immunized mice) for i.v. immunization and diluted 1:405 for s.c. immunization.
- \(^f\) ND denotes not determined.
liposome elimination profiles showed quick removal of Bx-liposomes from the blood compartment (less than 2.2% of the injected dose remained after 1 hr), but not liposomes lacking biotin (blood liposome levels were significantly higher at all time points, \( p < 0.01 \), two-way ANOVA). When serum antibodies were analyzed in these mice, it was found that IgM levels were elevated and the IgG response was low although slightly higher than the Bx-liposome group (Table 4.2).

To determine whether the same adjuvant effect can be achieved using non-encapsulated CpG ODN, another group was included which received i.v. empty Bx-liposomes mixed with CpG ODN1 at a dose equivalent to the encapsulated amount. As shown in Figure 4.1, this immunization method was not effective in inducing an immune response against re-administered Bx-liposomes. The liposome elimination profile was comparable to that of the Bx-liposome group (Figure 4.1). When the serum of these mice was analyzed for antibody response, it was found that both IgG and IgM levels were also comparable to the Bx-liposome group (Table 4.2), clearly indicating that encapsulation of CpG ODN inside liposomes is required for its adjuvant activity. Taken all together, as indicated by the elimination of re-administered liposome and antibody response, CpG ODN encapsulated in liposomes is able to convert a poorly immunogenic antigen into an immunogenic one.

4.3.2 Adjuvant Action of CpG ODN using different immunization routes

It has been shown by earlier studies that macrophages in the marginal zone of the spleen are important for mounting the responses to T-independent antigens (Humphrey,
1985; Spencer et al., 1998). More recently, it was demonstrated that the i.v. immunization route favors the generation of TI responses as a result of increased antigen recruitment to the spleen (Ochsenbein et al., 2000). To determine whether the route of administration influences the TI nature of the antigen, mice were immunized s.c. on the back twice, 9 days apart, using Bx-liposomes with or without encapsulated CpG ODN1 (3.3 μmol/mouse). Blood samples were collected from mice 6 and 5 days after the first and second immunization respectively, and serum was tested for the presence of IgM and IgG by ELISA. As observed for mice immunized i.v., a predominant IgM response was obtained in the CpG ODN encapsulated group, at similar levels for both the primary and secondary responses (Table 4.2). This demonstrates that the immunization route does not dictate the T-independence of the antigen used in this study.

4.3.3 Adjuvant activity of CpG ODN in nude mice

The induction of a predominant IgM response obtained from both the i.v. and s.c. immunization method with the absence of memory response after secondary immunization suggests that these liposomes are TI immunogens. To further characterize the immune response, the athymic BALB/c (nu/nu) mice were used. Athymic BALB/c (nu/nu) mice and immunocompetent BALB/c mice were immunized twice i.v. on day 0 and 7, and the immune response was assessed on day 6 and 20. Similar to the findings of previous sections, Bx-liposomes with encapsulated CpG ODN1 induced a predominant IgM response after the first and second immunization in nude mice. The magnitude of the IgM response was only slightly higher in the immunocompetent control mice (Figure 4.2B), indicating little T-cell involvement, if any, in the generation of the immune response. When the elimination of Bx-
A

Lipid concentration (mmol/25μl blood)

Time after injection (hrs)

B

Day 0

Day 6

Day 20

IgM

Absorbance (1:405 dilution)

Balb/c

Nude mice

C

Day 0

Day 20

IgG

Absorbance (1:405 dilution)

Balb/c

Nude
Figure 4.2  Adjuvant effect of CpG ODN in BALB/c (nu/nu) mice. The immunocompetent BALB/c (open symbols) and immunodeficient nude mice (closed symbols) were immunized as described in Fig. 4.1 with either empty Bx-liposomes (▲), CpG ODN encapsulated in Bx-liposomes (○,●), or the T-dependent antigen BSA-biotin conjugate (50 µg) mixed with aluminum hydroxide gel adjuvant (1.2 mg) (○,●).  

A) Plasma elimination profile of Bx-liposomes in immunocompetent mice and nude mice after immunization. One week after the second immunization, Bx-liposomes were injected to measure liposome elimination as described for Fig. 4.1. The number of mice in each treatment group is indicated in brackets. Immunocompetent mice immunized with BSA-biotin (○) were euthanized for ethical reasons after the first time point due to a very strong response to liposomes. 

B) Induction of IgM response in nude mice by CpG ODN. IgM response was measured before (day 0), after the first (day 6) and second immunization (day 20). 

C) IgG response measured in mice before and after the second immunization. Data represent average result obtained from the indicated number of mice in each group ± S.D.
liposomes was monitored in the nude mice, liposomes were rapidly eliminated (less than 6.9% of the injected dose remained after 1 hr), very similar to the response in the immunocompetent mice (Figure 4.2A). This is in contrast to immunization using empty Bx-liposomes in nude mice which had substantially higher liposome levels remaining in the blood (26.3±9 nmol/25μl at 1 hr, 21.6±8 nmol/25μl at 2 hrs, and 16.4±6 nmol/25 μl at 4 hrs). When a T-dependent (TD) form of the antigen, BSA-biotin, was used to immunize nude mice, no immune response was elicited. This was shown by high liposome levels remaining in the blood at all time points (29.3±7 nmol/μl at 1 hr, 28.5±9 nmol/μl at 2 hrs, and 18.7±6 at 4 hrs), low primary and secondary IgM response, and low secondary IgG levels. In contrast, BSA-biotin induced a strong secondary IgG response in the immunocompetent BALB/c mice. These data confirmed the observation with the s.c. immunization method (Table 4.2) that Bx-liposomes with encapsulated CpG ODN does not cause antibody isotype switching. The elimination of Bx-liposomes correlates with the antibody response induced against biotin, regardless of the nature of the antibody isotype produced.

4.3.4 Adjuvant effects of other ODNs

The adjuvant effect of CpG ODN studied so far was obtained using CpG ODN1, a sequence previously shown to have immunostimulatory effects which were discovered as secondary effects of the ODN. In fact, CpG ODN1 was originally designed to down-regulate c-myc expression mediated by its antisense activity (Leonetti et al., 1996). To determine whether the adjuvant effect observed in this study was truly due to the CpG motif in the ODN, the next set of experiments included the use of three other ODNs, each having the same amount encapsulated in Bx-liposomes (approximately 8 μg/μmol lipid). Mice were immunized once via the i.v. route with either empty Bx-liposomes, Bx-liposomes containing
CpG ODN1, CpG ODN2, Non-CpG ODN, or CpG-dn ODN. CpG ODN2 is another immunostimulatory ODN which contains one CpG motif (see Table 4.1) and has been well studied for its immunostimulatory effect (Krieg et al., 1995; Yi et al., 1996b; Hartmann et al., 2000). Non-CpG ODN contains no CpG motifs and was included as a negative control ODN. CpG-dn ODN is another control ODN which contains only CpG dinucleotides but not the 5' purines and 3' pyrimidines as flanking sequences (see Table 4.1). Since some studies have demonstrated that the flanking sequence is not a strict requirement for immunostimulation (Krieg et al., 1995; Yi et al., 1996a; Weiner et al., 1997; Davis et al., 1998; Krieg et al., 1999), this ODN was included to determine its effectiveness as an adjuvant for the antigen. The effect of prior immunization with these ODNs on the plasma elimination of Bx-liposomes was studied and is presented in Table 4.3. It can be seen that CpG ODN2 had similar adjuvant effect as CpG ODN1 as shown by the very rapid liposome elimination in mice (less than 11% of the injected dose remained at 1hr). In addition, both of these CpG ODNs induced an IgM response which is of similar magnitude (Table 4.3). As expected, encapsulation of Non-CpG ODN was not able to elicit a response against the liposomal antigen as indicated by an IgM response which was comparable to that of the Bx-liposome group as well as similar liposome elimination profiles. Similarly, CpG-dn ODN was not an effective adjuvant for the antigen as indicated by an IgM response similar to that of the empty Bx-liposome group. Although there was a trend of higher liposome elimination in this group, the liposome levels were not significantly different from those in the empty-Bx-liposome group (p=0.019, 0.021, and 0.037 for 1, 2, and 4 hrs respectively). Thus, the adjuvant effect of CpG ODN can be achieved by other CpG ODNs but requires the appropriate flanking sequence.
Table 4.3  Adjuvant effect of other ODNs

<table>
<thead>
<tr>
<th>Immunization</th>
<th>Liposome Elimination</th>
<th>IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 hour</td>
<td>2 hours</td>
</tr>
<tr>
<td>Bx-lipo</td>
<td>30.2 ± 2.8</td>
<td>25.6 ± 4.6</td>
</tr>
<tr>
<td>Bx-lipo + (CpG ODN1)</td>
<td>3.8 ± 1.7 *</td>
<td>2.1 ± 1.1 *</td>
</tr>
<tr>
<td>Bx-lipo + (CpG ODN2)</td>
<td>5.5 ± 3.8 *</td>
<td>0.7 ± 0.4 *</td>
</tr>
<tr>
<td>Bx-lipo + (Non-CpG ODN)</td>
<td>24.3 ± 6.4</td>
<td>21.0 ± 4.9</td>
</tr>
<tr>
<td>Bx-lipo + (CpG-dn-ODN)</td>
<td>19.5 ± 9.6</td>
<td>14.7 ± 6.3</td>
</tr>
</tbody>
</table>

Note:  

a Mice were immunized once i.v. as described in Materials and Methods. 

b Data represents lipid levels remaining in blood (nmol/25 μl blood) at the indicated time points after injection of empty Bx-liposomes (3.3 μmoles/mouse) 7 days after immunization. Results were averaged from at least 3 mice ± S.D. Two-way ANOVA was performed to analyze differences among the 5 immunization groups. A p-value of <0.01 was considered significant. * denotes significantly different from the Bx-lipo immunization group for the indicated time point. 

c Biotin-specific IgM levels were measured in serum (1:405 dilution) collected at termination and using ELISA as described in Materials and Methods. Data represent average results obtained from at least 3 mice expressed as OD ± S.D.
4.3.5 Enhanced antibody response by incorporating PEG-lipids

In an attempt to modulate the immune response further, poly(ethylene glycol)-modified lipid (DSPE-PEG), was included in the liposome formulation to decrease the rate of liposome elimination following i.v. administration. Incorporation of PEG-lipids into liposomes has been previously shown to enhance the immunogenicity of liposomal antigens (Phillips et al., 1995), probably as a result of the carrier prolonging the exposure of the antigen to the immune system. The liposomal antigen used in that study was IgG2a, a TD antigen, and a higher IgG response was obtained with the addition of PEG-lipids. To determine whether the incorporation of PEG-lipids can enhance the IgM response to Bx-liposomes, 5% DSPE-PEG2000 was incorporated into Bx-liposomes and the antibody response generated was tested in mice immunized i.v. once. It was found that empty Bx-liposomes containing PEG-lipids were not immunogenic. However, when PEG-lipids were incorporated into Bx-liposomes containing CpG ODN, the antibody response was highly enhanced. As shown in Figure 4.3, mice immunized with this formulation had an IgM response which was 64% higher than that of the non-PEGylated control. In terms of IgG induction, however, the response was minimal. Thus, the inclusion of PEG-lipids, in combination with CpG ODN, can strongly enhance the immunogenicity of the antigen.
Figure 4.3  Enhanced adjuvant activity of CpG ODN by incorporating PEG-lipids into liposomes. Mice were immunized via the i.v. route using either empty Bx-liposomes (dark bar), Bx-liposomes containing CpG ODN1 (white bar), or PEGylated Bx-liposomes containing CpG ODN1 (grey bar). Serum IgM (left) and IgG levels (right) were measured in mice at termination (7 days after immunization). Data represent average results obtained from 4 mice ± S.D.
4.4 DISCUSSION

The aim of the current study was to investigate the benefit of using liposomes to deliver CpG ODN as an immunoadjuvant for haptenized liposomes, a TI antigen. This report shows that CpG ODN co-delivered with the antigen within liposomes can have better adjuvant activity. CpG ODN simply co-injected with biotinylated liposomes could not render the haptenized liposomes immunogenic. Liposomes play several key roles in assisting the generation of an immune response and the benefit of using liposomes as vaccine carriers has been reviewed extensively (Buiting et al., 1992; Gregoriadis, 1994; Alving, 1998). Liposomes have long in vivo stability. This feature allows them to act as a depot for the slow release of antigens to prolong immune stimulation. Importantly, liposomes can act as a co-delivery vehicle to carry immunomodulators together with the antigens to further enhance the immune response. It is believed that this last aspect of liposomes is valuable for vaccine development and it is the focus of this paper to address this point.

It has been demonstrated that delivery of immunomodulators such as IL-2 (Gursel et al., 1998), IL-12 (Baca-Estrada et al., 1997), and IL-15 (Gursel et al., 1997), and plasmid DNA containing CpG motifs (Gursel et al., 1999) in liposomes with the antigen can improve the immune response over that obtained with immunomodulators administered separately. This supports the concept that co-delivery plays an important role in the better outcome of the immune response. Similarly for CpG ODN, it was recently demonstrated that delivery of the adjuvant with the antigen, achieved by covalent linkage or biotin-avidin linkage, has better adjuvant effects (Klinman et al., 1999; Shirota et al., 2000). However, it should be
noted that either covalent linkage or biotin-avidin linkage has the potential problem of altering the three-dimensional structure of the antigen and/or CpG ODN. Liposomes can be used as an alternative method to deliver the adjuvant in close proximity with the antigen without this potential problem. In this study, it was shown that the poor immunogenicity of haptenized liposomes can be reversed by encapsulating CpG ODN in liposomes. The lack of immune response in mice immunized with empty Bx-liposomes may be due to suboptimal hapten concentration used as suggested by other studies (Yasuda et al., 1977; van Houte et al., 1979). Alternatively, the epitope density may be too high, causing B-cells to become anergic (Nossal, 1996). However, a strong IgM response was obtained when CpG ODN was encapsulated, eliminating the latter possibility.

The mechanism by which CpG ODN encapsulation enhanced the immunogenicity of biotinylated liposomes is not completely clear from this study. Using the passive encapsulation method, the possibility of surface bound ODN or ODN trapped in liposomal membrane thereby exposing segments of ODN cannot be ruled out. Surface associated ODN would create a membrane exhibiting a net negative charge which may enhance cellular uptake of liposomes. However, this charge would have also affected the elimination rate of the liposomes containing non-CpG ODNs and it was not the case in this study. Further, free CpG ODN administered at the same time with haptenized liposomes cannot achieve the same adjuvant effect. This can be explained by the more rapid elimination of free ODN administered without a protective carrier or less efficient ODN delivery to B-cells when administered free. It has been documented that liposomes accumulate in the marginal zone of the spleen (Alving, 1998). Thus it is possible that liposomes can selectively deliver
encapsulated contents to B cells. It is speculated that the delivery of antigen and adjuvant simultaneously to the same cell is important for optimal adjuvant activity. This is supported by the finding that co-encapsulation of plasmid DNA and CpG DNA within the same liposome was more effective than encapsulation in separate liposomes in generating the immune response (Gursel et al., 1999). In addition, due to the specific effects of CpG ODN on the B cell as discussed below, it is proposed that the delivery of CpG ODN together with the model TI antigen tested is of particular advantage.

The benefit of using liposome as a vaccine carrier is further supported by this and other studies showing that the immune response generated against the antigen can be modulated by incorporating special lipids (Fries et al., 1992; Alving, 1993; Lipford et al., 1994a; Phillips et al., 1995; Chang et al., 1999). In the present study, it was shown that the adjuvant activity of encapsulated CpG ODN was enhanced by incorporating PEG-lipids into the liposomes. Using 5% DSPE-PEG lipid, the antibody response was increased by more than 50%, similar to the results obtained by others (Phillips et al., 1995). The mechanisms of action of CpG ODN and PEG-lipids in modulating the immune response are probably different. CpG ODN plays a permissive role in stimulating the B cells to proliferate and secrete IgM in response to Bx-liposomes. On the other hand, PEG-lipids probably acts by ensuring that the liposomal antigens remain in the circulation for extended time periods. Thus, PEG-lipids may enhance the immune response by decreasing antigen degradability which seems to be an important characteristic for this class of antigen. Although speculative, it is known that PEG-modified lipids have the potential to dissociate from the liposomal carrier (Silvius et al., 1993a; Parr et al., 1994; Holland et al., 1996b) and it is suggested that
PEG-lipid loss will be required to regenerate a liposome surface capable of interacting with a defined target cell population, such as B cells localized in the spleen. Regardless, the presence of surface grafted PEG increases the potential for the liposomal antigen and associated adjuvant to interact with the B cells.

The adjuvant effect of CpG ODN has been well documented by many studies. In general, immunostimulatory DNA containing CpG motifs promote Th1 humoral responses for protein-based vaccines (Chu et al., 1997; Weiner et al., 1997; Brazolot Millan et al., 1998; Davis et al., 1998; Jakob et al., 1998). In this study, a strong IgM response can be induced with the use of CpG ODN encapsulated within biotinylated liposomes regardless of the immunization route. This is in contrast to a recent report showing that the s.c. route favors the generation of TD response while the same antigen can induce a TI response if given i.v. (Ochsenbein et al., 2000). In that study, intra-lymph node injection of the antigen could induce a TI response, indicating that the differences between the two immunization routes resided in the delivery of antigen to secondary lymphoid organs (Ochsenbein et al., 2000). The finding that the TI antigen used in this study can induce a TI response using both immunization routes suggests that there is sufficient delivery to the regional lymph nodes to mount the immune response when the antigen is given s.c. or i.v. This may be attributed to the use of liposomes as a vehicle to deliver the antigen to the lymph nodes as previously shown (Oussoren et al., 1997). Alternatively, it may be due to the immunostimulatory action of CpG ODN. The induction of IgM response obtained in this study may be a direct effect of the CpG ODN on B cells causing IL-6 release which in turn promotes IgM secretion in an autocrine manner (Klinman et al., 1996). Alternatively, it may be possible that the CpG
ODN stimulates other immune cells such as macrophages, dendritic cells and NK cells to release cytokines which can stimulate B-cells (Ballas et al., 1996; Cowdery et al., 1996). It is worth noting that CpG ODN is a potent B-cell mitogen and can increase B-cell survival by preventing apoptosis (Yi et al., 1996b; Yi et al., 1998). Moreover, B-cell activation by CpG ODN, is greatly enhanced when the B-cell receptor is crosslinked (Krieg et al., 1995; Goeckeritz et al., 1999). The hapten-specific IgM response obtained in this study is likely a synergistic effect of the CpG ODN on B-cell proliferation and B-cell receptor occupancy by liposomes bearing multiple haptens. Antigens in repeating units, especially in highly organized form, are able to induce a prompt T cell-independent IgM response (Bachmann et al., 1993; Mond et al., 1995) as a result of B-cell receptor cross-linking. For the liposomes used in this study, it is estimated that 680 molecules of biotin are on each liposome. Thus these liposomes are capable of causing cross-linking of the B-cell receptor. Despite previous experimental results which demonstrate the ability of CpG ODN to cause antibody isotype switching in isolated B cells (Davis et al., 1998), the in vivo data shown in this study does not support this finding, suggesting that the influence of CpG ODN on isotype switching depends on the nature of the antigen.

In conclusion, it is demonstrated that CpG ODN encapsulated in liposomes can enhance the immunogenicity of a liposomal TI antigen. This finding supports the view that CpG ODNs are potentially useful for preventing infections since they can improve the defense mechanism of the innate immune system to quickly remove invading pathogens.
CHAPTER 5
INDUCTION OF CD8+ T-CELL RESPONSE USING A HER-2/NEU-DERIVED PEPTIDE IN LIPOSOMES CO-ENCAPSULATED WITH CPG ODN: EFFECT OF PEG-LIPID INCORPORATION

5.1 INTRODUCTION

Our results in Chapter 4 using a T-independent antigen indicate that CpG ODN encapsulated in liposomes is required for its adjuvant activity. The inclusion of PEG-lipids in the formulation enhanced the T-independent immune response even more, suggesting that prolonged circulation of the formulation can improve antigen delivery to antigen presenting cells. The aim of this chapter is to test the hypothesis that PEGylated liposomes, evading MPS uptake, are better able to deliver antigens to other more relevant antigen presenting cells (APCs) such as dendritic cells which are important for inducing cellular immune responses, specifically cytotoxic T-lymphocyte (CTL) induction. To test this hypothesis, CTL responses generated by a peptide antigen encapsulated in liposomes were compared with and without PEG-lipids.

Cytolytic CD8+ T-cell response is an important effector function of the immune system against virus infection and tumor formation owing to the ability of these cells to recognize and lyse target cells in an antigen and MHC-specific manner. Induction of CD8+ T-cell response involves presentation of fragments of the tumor or viral antigen, termed CTL epitopes, to naïve T-cells on MHC class I molecules of antigen presenting cells which can
provide co-stimulation at the same time as antigen presentation. Understanding of these MHC-restricted CTL epitopes has made possible the idea of peptide vaccines which attracted many scientists to design methods to induce tumor-specific CTL responses using these peptides. However, one major challenge is that immunization using these peptides alone is ineffective due to lack of \textit{in vivo} targeting to dendritic cells and may require the use of adjuvants which are largely limited for use in animals due to safety reasons. In order for these peptides to be effective, they need to be loaded onto dendritic cells before injection back into the host (Nouri-Shirazi \textit{et al.}, 2000). Immunization using dendritic cells loaded with these peptides has proven to be effective but routine clinical applications are still challenging from a practical standpoint. As one of the alternative solutions, liposomes can be used to deliver CTL epitopes more efficiently to antigen presenting cells to induce CTL response (Lipford \textit{et al.}, 1994a; Ludewig \textit{et al.}, 2000; Chang \textit{et al.}, 2001; Chikh \textit{et al.}, 2001b). In this chapter, the efficacy of a CTL epitope encapsulated within PEGylated liposomes is being tested in terms of CTL activation.

The cellular immune response tested in this study is generated against HER-2/neu, a tumor antigen over-expressed in various types of cancers, including breast, ovarian, endometrial, gastric, pancreatic and prostate. HER-2/neu (also named c-erbB-2) is a 185 kDa cell surface receptor expressed on a variety of epithelial cells (Brandt-Rauf \textit{et al.}, 1994). A functional cause of malignancy by HER-2/neu overexpression, which is a result of gene amplification, has been proposed (Brandt-Rauf \textit{et al.}, 1994). In breast cancer, HER-2/neu is over-expressed in 30\% of all cases and is correlated to the poor prognosis and shortened survival in cancer patients (Ross \textit{et al.}, 1999). Immunotherapy using a humanized
monoclonal antibody against HER-2/neu (Herceptin®) has been approved for use in the clinic for the treatment of metastatic breast cancer. Herceptin therapy in combination with chemotherapy could increase the response of patients to anti-cancer drugs such as doxorubicin and cyclophosphamide. However, the overall response rate is only 15% when given alone in metastatic breast cancer (Cobleigh et al., 1999). Thus, it is worthwhile to develop other treatment strategies for better control of HER-2/neu positive breast cancers.

The HER-2/neu derived peptide used in this study, p63-71, is a CTL epitope previously identified in BALB/c mice by Shiku’s group (Nagata et al., 1997). Immunization using the truncated HER-2/neu protein containing the CTL epitope complexed with hydrophobized polysaccharides induced CTLs capable of recognizing target cells pulsed with p63-71 as well as syngeneic tumor cells expressing the native protein (Gu et al., 1998). Using a different approach by the same group, immunization using dendritic cells pulsed with p63-71 completely suppressed the growth of syngeneic HER-2/neu-expressing tumors in mice (Okugawa et al., 2000). Moreover, it was shown that HER-2/neu-specific CTLs can be established by presenting p63-71 on dendritic cells to peripheral blood mononuclear cells in vitro. These findings indicate that CD8+ T-cell response can be induced if p63-71 is presented appropriately. In this chapter, p63-71 is used together with CpG ODN as an immunoadjuvant in liposomes in an attempt to improve the cellular immune response to this weak immunogen.
5.2 MATERIALS AND METHODS

5.2.1 Materials

GolgiPlug™, Cytofix/Cytoperm™ kit for intracellular flow cytometry, anti-CD8-PE antibody, anti-mouse IFN-γ-FITC antibody, rat anti-mouse IFN-γ antibody, biotin-rat anti-mouse IFN-γ, and HRP-streptavidin were obtained from PharMingen (Missasauga, ON). Opti-4CN™ substrate kit was obtained from Bio-Rad (Hercules, CA). 96-well MultiScreen plate was purchased from Millipore (Nepean, ON). Recombinant murine granulocyte macrophage colony stimulating factor (GM-CSF) and interleukin-4 (IL-4) was obtained either from PeproTech Inc. (Rocky Hill, NJ). Anti-mouse DEC-205-FITC antibody was obtained from Cedarlane Laboratories Ltd. (Hornby, ON). Peptide p63-71 (TYLPTNASL) was synthesized by the Protein Service Laboratory of the University of British Columbia (Vancouver, BC). Phosphorothioated CpG ODN 1645 (GCATGACGTTGAGCT) was synthesized by Hybridon Inc. (Milford, MA).

5.2.2 Co-encapsulation of peptide and CpG ODN

Peptide p63-71 and CpG ODN 1645 were passively encapsulated in DSPC/Chol liposomes with and without 5 mol% DSPE-PEG2000. Lipid film was rehydrated at 65°C at a concentration of 100 μmole lipid per ml HBS containing p63-71 (2 mg/ml) and CpG ODN (12-17 mg/ml). Rehydrated lipid was subjected to 5 freeze-thaw cycles and then extruded as described in section 2.2.2. Peptide and ODN not encapsulated within liposomes was removed by size exclusion chromatography using a Biogel A 15M column (1.5x20 cm) run at a flow rate of 50 ml/hr. Peptide and ODN encapsulated in liposomes was quantitated after
stain buffer (1% FBS in PBS) and then transferred to 96-well plate for staining. Splenocytes were blocked (4°C, 15 min) using 100 μl block buffer containing rat serum and anti-mouse Fc receptor antibody 2.4G2, and then stained with 5 μl anti-CD8-PE antibody for 30 min at 4°C in the dark. After washing 2 times with stain buffer, the cells were fixed using the Cytofix/Cytoperm™ kit according to the manufacturer’s instruction. Fixed cells were then washed 2 times with Perm/Wash™ buffer and then stained with 1 μl anti-IFN-γ-FITC antibody in 50 μl of Perm/Wash™ buffer for 30 min at 4°C in the dark. The splenocytes were washed 2 more times in Perm/Wash™ buffer and then finally resuspended in 300 μl stain buffer for analysis by flow cytometry. Ten thousand events were collected in CD8+ gates to determine the frequency of IFN-γ positive CD8+ lymphocytes for each individual mouse.

5.2.5 Enzyme-linked immunospot (ELISPOT) assay

One day before mouse termination, a 96-well MultiScreen plate was coated overnight at 4°C with rat anti-mouse IFN-γ (2 μg/ml sodium carbonate buffer, pH 9.0, 50 μl/well). Splenocytes (7.5x10⁴-1.5x10⁵ cells) in 100 μl RPMI complete medium (with and without 10 μg/ml p63-71) was plated in duplicate wells after the plate was blocked with PBS with 1% BSA and 0.05% Tween 20 (200 μl/well) for 2 hrs at room temperature. Splenocytes were incubated for 24 hrs in a 37°C tissue culture incubator after which biotin-rat anti-mouse IFN-γ was added to ELISPOT plate (100 μl/well) at a concentration of 1 μg/ml in blocking buffer and incubated for 2.5 hrs at room temperature. HRP-Streptavidin diluted 1:2000 was added to plate (100 μl/well) and incubated for 2 hrs at room temperature. 100 μl Opti-4CN™ substrate was added to plate and incubated for 25 min at room temperature until spots appear.
stain buffer (1% FBS in PBS) and then transferred to 96-well plate for staining. Splenocytes were blocked (4°C, 15 min) using 100 μl block buffer containing rat serum and anti-mouse Fc receptor antibody 2.4G2, and then stained with 5 μl anti-CD8-PE antibody for 30 min at 4°C in the dark. After washing 2 times with stain buffer, the cells were fixed using the Cytofix/Cytoperm™ kit according to the manufacturer’s instruction. Fixed cells were then washed 2 times with Perm/Wash™ buffer and then stained with 1 μl anti-IFN-γ-FITC antibody in 50 μl of Perm/Wash™ buffer for 30 min at 4°C in the dark. The splenocytes were washed 2 more times in Perm/Wash™ buffer and then finally resuspended in 300 μl stain buffer for analysis by flow cytometry. Ten thousand events were collected in CD8+ gates to determine the frequency of IFN-γ positive CD8+ lymphocytes for each individual mouse.

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The plate was then washed 3 times with distilled H\textsubscript{2}O and then air dried before counting under a dissecting microscope or magnifying glass. Background count obtained in the absence of peptide stimulation was subtracted from results obtained with peptide stimulation for each individual mouse. Results presented are averaged dot counts obtained from 4 mice in each group.

5.2.6 *Antibody quantitation using ELISA*

Total IgG and IgM was quantitated using ELISA kits obtained from Bethyl Laboratories Inc. (Montgomery, TX). Maxisorp 96-Well plates (Nalge Nunc International, Rochester, NY) was coated with affinity-purified goat anti-mouse IgM or IgG-Fc as capture antibodies (10 μg/ml). Mouse serum was diluted 1:24000 and 1:1600 for IgG and IgM quantitation respectively. Horse-radish peroxidase-linked goat anti-mouse IgG and IgM antibodies were used as detection antibodies (1:90,000 and 1:120,000 dilution respectively). A mouse reference serum provided was used as standards for serum IgG and IgM quantitation. All incubation steps were the same as described in section 3.2.4.

5.2.7 *Liposome uptake by dendritic cells*

Bone marrow-derived dendritic cells (BM-DCs) were prepared as described above. After culture for 7 days, BM-DCs were harvested and transferred to 12-well plates with 1x10\textsuperscript{6} cells/well in 0.75 ml RPMI complete medium. Liposomes (1.5 μmole) were added to each well and incubated for either 4, 24, or 40 hrs at 37°C in a cell culture incubator. At the end of the incubation period, the adherent cells were washed 2 times after removing the supernatant. The supernatant was combined with the washes and then centrifuged to pellet
down non-adherent cells which were then added back to the adherent cells in the plate. The dendritic cells were then solubilized with Triton-X 100 (0.9%) and the radioactivity was counted.

5.2.7 Statistical analysis

One-way ANOVA was performed to determine differences in CD8+ T-cell response among the various immunization groups. Newman-Keuls tests were preformed as post hoc analysis for two-way ANOVA. A p-value of < 0.05 was considered significant.
5.3 RESULTS

5.3.1 Peptide and CpG ODN encapsulation

The CpG ODN chosen for use in this study contains the immunostimulatory motif **GACGTT** which has been shown reproducibly to have stimulatory activity by many groups (Liang et al., 1996; Yi et al., 1996a; Chu et al., 1997; Lipford et al., 1997; reviewed by Van Uden et al., 2000). Table 5.1 shows the amount of p63-71 and CpG ODN encapsulated in each of the liposome formulations used in this study. The inclusion of 5 mol% PEG-lipid in the formulation did not affect the encapsulation of both molecules. Thus the amount of p63-71 and CpG ODN encapsulated were comparable for formulations with and without PEG-lipid (formulation 1 and 2, Table 5.1).

5.3.2 Induction of cellular immune response by p63-71 and CpG ODN co-encapsulated in liposomes

Cellular immune response was assessed in mice immunized twice (on day 0 and 7) with p63-71 alone in saline, CpG ODN mixed with or co-encapsulated in liposomes containing p63-71. Immunization with dendritic cells pulsed with p63-71 was included as a positive control. Splenocytes were isolated from mice 7 days after the second immunization (day 14) and peptide-specific IFN-γ response was measured using two assay methods, namely ELISPOT and intracellular flow cytometry as shown in Figure 5.1 and 5.2 respectively. As shown by ELISPOT assay in Figure 5.1, co-encapsulation of p63-71 and CpG ODN within DSPC/Chol liposomes, either given i.v. or s.c., induced a much higher frequency of IFN-γ producing cells than p63-71 alone given in saline (140 fold).
Table 5.1 Encapsulation of peptide and CpG ODN in liposomes

<table>
<thead>
<tr>
<th>Formulation</th>
<th>P63-71 content $^a$ (µg/µmole lipid)</th>
<th>CpG ODN content $^b$ (µg/µmole lipid)</th>
<th>Peptide dose $^c$ (µg/mouse)</th>
<th>CpG ODN dose $^d$ (µg/mouse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Lipo + (P + CpG)</td>
<td>2.44</td>
<td>9.92</td>
<td>24.4</td>
<td>99.2</td>
</tr>
<tr>
<td>2. PEG-lipo + (P + CpG)</td>
<td>1.88</td>
<td>8.49</td>
<td>18.8</td>
<td>84.9</td>
</tr>
<tr>
<td>3. Lipo + P</td>
<td>2.03</td>
<td>---</td>
<td>20.3</td>
<td>---</td>
</tr>
<tr>
<td>4. Lipo + (P) + Lipo + (CpG) $^e$</td>
<td>3.58</td>
<td>16.7</td>
<td>17.9</td>
<td>83.5</td>
</tr>
</tbody>
</table>

Note:  

$^a$ Amount of peptide encapsulated in liposomes  

$^b$ Amount of ODN encapsulated in liposomes  

$^c$ Peptide dose was determined based on a lipid dose of 10 µmole given per mouse  

$^d$ ODN dose was determined based on a lipid dose of 10 µmole given per mouse  

$^e$ Peptide and CpG ODN was encapsulated in separate liposomes. 5 µmole of each of the singly encapsulated liposomes was given to each mouse. To maintain the peptide and ODN dose relatively the same as other formulations, the encapsulated amount in liposomes was adjusted.
Figure 5.1 Induction of p63-71-specific IFN-γ response in splenocytes as determined by the ELISPOT method. BALB/c mice were immunized twice (day 0 and 7) with the indicated formulations using the i.v. route except otherwise stated. Mice were terminated on day 14 and splenocytes were isolated. Isolated splenocytes were cultured for 24 hours with and without p63-71 in the ELISPOT plate as described in Materials and Methods. The p63-71-specific response was obtained by subtracting the frequency of IFN-γ positive cells obtained without p63-71 stimulation as background. The results shown are presented as averaged results obtained from 4 mice ± S.E.M. One-way ANOVA was performed for statistical analysis. P<0.05 was considered significant. * denotes significantly different from peptide alone (P) and peptide encapsulated in liposomes plus free CpG ODN (Lipo+(P)+free CpG ODN).
**A**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CD8</th>
<th>IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipo+(P+CpG)</td>
<td>0.15%</td>
<td>5.41%</td>
</tr>
<tr>
<td>Lipo+(P) + free CpG</td>
<td>0.22%</td>
<td>0.29%</td>
</tr>
<tr>
<td>P</td>
<td>0.03%</td>
<td>0.03%</td>
</tr>
<tr>
<td>DC+P</td>
<td>0.14%</td>
<td>0.66%</td>
</tr>
<tr>
<td>Lipo+(P+CpG) s.c.</td>
<td>0.19%</td>
<td>0.87%</td>
</tr>
</tbody>
</table>

**B**

- % IFN-γ positive CD8+ lymphocytes

- Lipo+ (p+CpG) 
- Lipo+(p) + free CpG
- P
- DC+P
- Lipo+ (p+CpG) s.c.

* Indicates statistical significance.
**Figure 5.2** Induction of p63-71-specific IFN-γ response in splenocytes as determined by intracellular flow cytometry. BALB/c mice were immunized twice (day 0 and 7) with the indicated formulations using the i.v. route except otherwise stated. Splenocytes were isolated on day 14 and re-stimulated *in vitro* with and without p63-71 and in the presence of Golgi Plug for 5 hours as described in Materials and Methods. Cells were gated on the CD8+ population and analyzed for intracellular IFN-γ staining. **A)** A representative FACS analysis is shown for one mouse from each immunization group. Numbers shown in the upper right quadrant represent the percentage of CD8+ cells stained positive for intracellular IFN-γ. The IFN-γ response obtained without p63-71 re-stimulation (shown in top panels) was obtained for each individual mouse and subtracted from the response obtained with p63-71 (shown in bottom panels) as background. **B)** The frequency of IFN-γ producing cells within the CD8+ population (with background subtracted) was averaged for each immunization group (4 mice) and presented as mean ± S.E.M. One-way ANOVA was performed for statistical analysis. P<0.05 was considered significant. * denotes significantly different from all other groups.
Interestingly, the response was of the same magnitude as that of the control group immunized using dendritic cells pulsed with p63-71. In comparison, immunization with free CpG ODN mixed with liposomes containing p63-71 yielded a very low response, indicating that immune stimulation requires CpG ODN to be encapsulated.

An intracellular flow cytometric assay using a second marker was performed to better identify the IFN-γ producing cells since other cell types such as natural killer cells, and CD4+ T-lymphocytes can contribute to the IFN-γ producing population. Using CD8 as the second marker, the frequency of IFN-γ producing cells within the CD8+ T-cell population which represents the CTL population can be measured. As shown in Figure 5.2, a slightly different pattern of results was obtained. Nevertheless, p63-71 and CpG co-encapsulated in liposomes was superior to all other immunization groups. It is interesting to note that the i.v. immunization method was better than the s.c. method for the induction of CTL response (Figure 5.2).

5.3.3 Liposomes containing PEG-lipids were less effective in inducing peptide-specific IFN-γ response

When the PEGylated formulation was tested, it was found that the mice were acutely sick upon second injection in which case the mice were terminated immediately due to ethical reasons. As a result, comparison of formulations with and without PEG-lipids in terms of CTL response could not be performed with two immunizations. Consequently, a second immunization study was performed with only one immunization. IFN-γ response in isolated splenocytes was again measured by ELISPOT and intracellular flow cytometry 8
days after the immunization. As shown in Figure 5.3 and 5.4, both methods yielded a similar pattern of results. Confirming the results obtained with two immunizations, the formulation containing p63-71 co-encapsulated with CpG ODN induced a significantly higher response than peptide alone (144 fold) although the response was of lower magnitude when compared with the response obtained after two immunizations (Figure 5.1 and 5.2). When compared to another control group which included both p63-71 and CpG ODN but encapsulated in separate liposomes, the co-encapsulated formulation was much more effective in stimulating the immune response (Figure 5.3 and 5.4). When the formulations with and without PEG-lipids were compared, however, the PEGylated formulation was found to be less effective, as shown by a significantly lower IFN-\( \gamma \) response (less than 50% of the non-PEGylated formulation) (Figure 5.3 and 5.4).
Figure 5.3 Effect of PEG-lipid incorporation on the induction of p63-71-specific IFN-γ response as determined by the ELISPOT method. BALB/c mice were immunized once (day 0) via the i.v. route with the indicated formulations and were terminated on day 8 to monitor the IFN-γ response of isolated splenocytes by ELISPOT. Isolated splenocytes were cultured for 24 hours with and without p63-71 in the ELISPOT plate as described in Materials and Methods. P63-71-specific response was obtained by subtracting the frequency of IFN-γ positive cells obtained without p63-71 stimulation as background. Data presented are averaged results obtained from 4 mice ± S.E.M. One-way ANOVA was performed for statistical analysis. P<0.05 was considered significant. * denotes significantly different from all other groups.
A

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CD8</th>
<th>IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipo+(P+CpG)</td>
<td>0.07%</td>
<td></td>
</tr>
<tr>
<td>Lipo+(P)+(P+CpG)</td>
<td>0.09%</td>
<td></td>
</tr>
<tr>
<td>Lipo+(P) + free CpG</td>
<td>0.04%</td>
<td>0.45%</td>
</tr>
<tr>
<td>Lipo+(P)</td>
<td>0.08%</td>
<td>0.08%</td>
</tr>
<tr>
<td>Lipo+(P)+Lipo+(CpG)</td>
<td>0.03%</td>
<td>0.27%</td>
</tr>
</tbody>
</table>

B

% IFN-γ positive CD8+ lymphocytes

- Lipo + (P+CpG) + P + Lipo+(P)+

Bar graph:

- Lipo + (P+CpG) + P + Lipo+(P)+

* indicates a statistically significant difference.
Figure 5.4 Effect of PEG-lipid incorporation on the induction of p63-71-specific IFN-γ response as determined by intracellular flow cytometry. BALB/c mice were immunized once (day 0) via the i.v. route with the indicated formulations and were terminated on day 8 to monitor the IFN-γ response of isolated splenocytes by intracellular flow cytometry. Isolated splenocytes were re-stimulated in vitro with and without p63-71 and in the presence of Golgi Plug for 5 hours as described in Materials and Methods. Cells were gated on the CD8+ population and analyzed for intracellular IFN-γ staining. A) A representative FACS analysis is shown for one mouse from each immunization group. Numbers shown in the upper right quadrant represent the percentage of CD8+ cells stained positive for intracellular IFN-γ. The IFN-γ response obtained without p63-71 re-stimulation (shown in top panels) was obtained for each individual mouse and subtracted from the response obtained with p63-71 (shown in bottom panels) as background. B) The frequency of IFN-γ producing cells within the CD8+ population (with background subtracted) was averaged for each immunization group (4 mice) and presented as mean ± S.E.M. One-way ANOVA was performed for statistical analysis. P<0.05 was considered significant. * denotes significantly different from all other groups.
5.3.4 Induction of humoral immune response

The observation that the mice were acutely sick upon second injection of the PEGylated formulation suggested the induction of a strong immune reaction to the immunogen as a result of antibody production. To confirm this, serum antibody levels were determined after one immunization. Shown in Figure 5.5 are serum total IgG and IgM levels. As expected, markedly higher total IgG levels were detected in mice immunized with the PEGylated formulation when compared with all other groups. Serum total IgM levels were found to be similar among all immunization groups.

5.3.5 In vitro liposome uptake by dendritic cells

There are now several pieces of evidence indicating that CpG ODN closely associated with the antigen can bring stronger adjuvant effects. In a recent paper, it was reported that CpG ODN has the capability of enhancing the uptake of chemically linked antigens into antigen presenting cells by many folds (Shirota et al., 2001), probably due to uptake mediated by the toll-like receptor 9 (Bauer et al., 2001). In this study, CpG ODN is not physically-linked with the peptide antigen, but is encased together with it inside the same liposomes. In the encapsulation procedure, the possibility that some CpG ODN is exposed on liposome surface cannot be ruled out. If this is in fact true, the strong adjuvant effect of co-encapsulated CpG ODN may be explained by better uptake of liposomal antigen into antigen presenting cells. To test this possibility, liposome uptake experiments were performed using dendritic cells. Shown in Figure 5.6 is the amount of liposome uptake at 37°C with the various liposome formulations as a function of time. As shown, there was no marked difference in liposome uptake among the different formulations with and without encapsulated CpG ODN.
**Figure 5.5** Induction of IgG response in mice using liposomes containing PEG-lipids. BALB/c mice were immunized once with the formulations indicated as described in Figure 5.3. At termination (day 8), whole blood was collected from cardiac puncture for preparing serum. Serum total IgG and IgM levels were measured using ELISA as described in section 5.2.6. Data shown represent averaged results obtained from 4 mice ± S.E.M. Dotted lines indicate serum antibody levels in naïve BALB/c mice.
Figure 5.6  Liposome uptake by dendritic cells as a function of time. Bone marrow-derived dendritic cells (BM-DC) were harvested as described in Materials and Methods and were harvested on day 7 for liposome uptake experiments. One million BM-DC were incubated at 37°C with the various liposome formulations (2 mM) for the indicated time periods. Data shown represent averaged results obtained from duplicate experiments ± S.D.
5.4 DISCUSSION

The rationale for using liposomes to deliver peptide-based vaccines is perhaps best illustrated by a recent finding that T cell-tolerizing effects obtained by immunization using CTL epitopes is associated with the pharmacokinetic of the peptide after injection into the host (Weijzen et al., 2001). It was shown that rapid distribution of peptides from the injection site to other organs caused deletion of antigen-specific CTLs within a short period of time. Liposomes can allow the slow release of peptide as well as deliver the peptide to the appropriate cell population for antigen processing and/or presentation. Induction of CD8+ T-cell response using liposomes to deliver CTL epitopes has been demonstrated for various antigens including ovalbumin, listeriolysin, vesicular stomatitis virus nucleoprotein, Hantaan nucleocapsid protein M6, human papilloma virus E7, simian immunodeficiency virus (SIV) gag protein, lymphocytic choriomeningitis virus (LCMV) glycoprotein GP33, and HLA-cw3 (Miller et al., 1992; Lipford et al., 1994b; Ludewig et al., 2000; Chang et al., 2001; Chikh et al., 2001b). The use of CpG ODN as an immunoadjuvant either together with or encapsulated in liposomes containing peptide antigens has been recently reported by two studies (Ludewig et al., 2000; Chikh et al., 2001b). However, in these prior studies, CpG ODN was not required to induce CD8+ T-cell response, probably due to the intrinsic immunogenicity of the peptide. In this study, it was shown that encapsulation of CpG ODN was required for inducing CD8+ T-cell response. In fact, the adjuvant effect of CpG ODN was shown to be much greater when encapsulated in the same liposomes with the HER-2/neu peptide. Several other studies also showed that the adjuvant activity of CpG ODN can be enhanced by chemically linking CpG DNA to the antigen (Cho et al., 2000; Shirota et al., 2000; Tighe et al., 2000a; Tighe et al., 2000b). Thus, the results obtained in the current
study show that liposomes can be used as an alternative method to construct a CpG-antigen complex to improve the immunogenicity of the antigen.

The exact mechanism for the synergistic effect of CpG ODN co-encapsulated in liposomes with the peptide antigen remains unknown at the present time. A recent report showed that the uptake of CpG ODN-linked antigen by dendritic cells was enhanced by many fold presumably due to CpG-guided binding and/or uptake of the antigen (Shirota et al., 2001). It was further demonstrated that dendritic cell activation, as shown by increased expression of IL-12, CD40 and CD86, was much higher if CpG was linked to the antigen. This effect could not be achieved using a mixture of CpG and antigen (Shirota et al., 2001). In an attempt to investigate the possibility of CpG ODN-facilitated uptake of liposomal antigen, liposome uptake by dendritic cells in vitro was measured and no apparent differences among formulations with and without CpG ODN were found. This can be explained by the lack of CpG ODN exposed on liposome surface. Thus, the adjuvant activity of liposome-encapsulated CpG ODN cannot be simply explained by antigen uptake. It may be possible that the simultaneous delivery of both agents within the same intracellular compartment of antigen presenting cells is important for the synergistic effect.

An important finding of this study is that inclusion of PEG-lipids could not improve CTL activation, disproving the hypothesis that reduced liposome uptake by macrophages could improve the outcome of the immune response. This may be explained by the fact that delivery of the liposomal antigen to macrophages may be important for mounting the immune response. Alternatively, it may be argued that lower CD8+ T-cell response obtained with the use of PEG-lipid is due to inhibited liposome uptake by dendritic cells which are
important for mounting the response. However, this possibility is not likely the reason since it was shown that the inclusion of PEG-lipid does not reduce liposome uptake by dendritic cells \textit{in vitro} (Figure 5.4).

The role of dendritic cells in the presentation of antigens given in liposomes has been substantiated by several studies both \textit{in vitro} (Nair et al., 1992; Chikh et al., 2001b) and \textit{in vivo} (Nair et al., 1992; Nair et al., 1995; Ludewig et al., 2000; Ignatius et al., 2000). However, the role of dendritic cells in liposomal antigen uptake and processing has not been clearly defined. In contrast, the importance of macrophages for the induction of CTL responses using liposomal antigens has been shown by several studies (Zhou et al., 1992; Nair et al., 1995; Wijburg et al., 1998). In these studies, macrophages were depleted \textit{in vivo} using liposomal Cl$_2$MDP which was shown to affect only the macrophage population but not dendritic cells (Nair et al., 1995) nor T-cells (Zhou et al., 1992). Macrophage depletion prior to immunization abolished CTL induction \textit{in vivo} (Zhou et al., 1992; Wijburg et a., 1998) and resulted in decreased activity of dendritic cells to present the antigen to T-cells (Nair et al., 1995). In the same study, it was further shown that the supernatant from macrophages treated with liposomal antigen could stimulate dendritic cells to induce T-cells (Nair et al., 1995). Collectively, these previous findings suggest that dendritic cells may be involved in antigen presentation after the majority of liposomal antigen is taken up and processed by macrophages and then transferred to dendritic cells.

Our result is in contrast to the finding of Ignatius et al. (2000) which showed that ovalbumin encapsulated in PEGylated liposomes were more effective in generating CD8+ T-cell response than non-PEGylated liposomes. Furthermore, it was shown that dendritic cells
were involved in antigen presentation \textit{in vivo} after subcutaneous injection of the immunogen (Ignatius \textit{et al.}, 2000). The controversial results cannot be explained by the different routes of immunization used since PEGylated-liposome delivery to the skin, where Langherhans cells are located, would be similar for i.v. and s.c. route of administration (Allen \textit{et al.}, 1993). Rather, differences in the immunogen used may be a contributing factor to the dissimilar results obtained. In this study, a strong immunoadjuvant, CpG ODN, was included as part of the immunogen. It has been shown by previous studies that CpG DNA can activate macrophages to secrete various cytokines, including TNF\(\alpha\), IL-12, IL-6, IL-1\(\beta\), IFN-\(\alpha\), and IFN-\(\beta\) (Stacey \textit{et al.}, 1996; Sparwasser \textit{et al.}, 1997; Pisetsky, 2000), although expression of MHC class II molecule and antigen processing was shown to be reduced \textit{in vitro} (Chu \textit{et al.}, 1999). Stimulation of cytokine production by macrophages could facilitate the cascade of events which lead to CD8\(^+\) T-cell activation. Alternatively, it may be possible that macrophages are stimulated indirectly by other immune cells which are activated by the CpG-containing immunogen to secrete various cytokines.

The disadvantage of using PEG-lipids in the immunogen tested is also indicated by the finding that a humoral response was induced and an anaphylactic reaction was obtained in mice upon re-injection of the immunogen. Together with the finding that a reduced CD8\(^+\) T-cell response was obtained, the effect of PEG-lipid incorporation may be explained by better delivery of the immunogen to B-cells, although direct evidence is lacking. Since CpG ODN is a strong stimulator of B-cells as discussed in chapter 4, it is possible that improved delivery of the immunogen to B-cells causes direct stimulation of antibody production. Alternatively, it may be possible that the liposomes are re-directed to other antigen presenting cells which in turn lead to activation of B-cells for antibody production.
CHAPTER 6
SUMMARIZING DISCUSSION

This thesis is aimed at obtaining a better understanding of the benefit of using PEG-lipids in liposomes, emphasizing interactions with the immune system. Liposome interaction with the immune system can be viewed as both undesirable and desirable. The function of the immune system to destroy and eliminate liposomes is, in general, viewed as an adverse aspect of liposome interaction with the immune system. On the other hand, the triggering of an immune response which may be of therapeutic value is considered favorable. Liposomes have the superior ability to deliver antigens to cells of the immune system for eliciting immune responses. To have a more complete understanding of the role of PEG in altering liposome interaction with the immune system, it is necessary to study this interaction from both perspectives. Namely, how liposome behavior changes under the influence of the immune system and how the immune system is affected by liposomes.

There is indisputable evidence that PEG can prevent or reduce liposome interaction with selected macromolecules and surfaces due to steric stabilization. A plethora of evidence from the literature also indicate that liposomes containing PEG-lipids have extended circulation lifetimes due to reduced elimination by the macrophages of MPS, probably a result of reduced surface-surface interaction as well as interaction with specific proteins which facilitate phagocyte recognition. This surface-stabilizing property of PEG is especially important for the development of liposomes for active targeting. Liposomes with surface-conjugated ligands are more susceptible to recognition and elimination by the innate
immune system which limits their potential as an effective targeted delivery system. Inclusion of PEG-lipids can substantially prolong the circulation longevity of these liposomes but at the same time abolish the target binding function of the liposomes. As a proof of principle, this dilemma was addressed in chapter 2 of this thesis by studying the shielding and de-shielding properties of exchangeable PEG-lipids with the hope of better controlling ligand exposure. Using both in vitro and in vivo methods, it was shown that time-controlled release of PEG-lipids can be achieved using PEG-lipids with shorter acyl chains. PEG-lipid loss was associated with recovery in the target-binding function of liposomes. The practical benefit of using these exchangeable PEG-lipids to initially protect liposomes from immune elimination and slowly expose ligands for targeting remains to be tested.

In chapter 3 of this thesis, the ability of PEG-lipids to modify liposome interaction with an existing antibody response and to protect the vehicles from rapid elimination was evaluated. Using biotin as a model ligand, it was found using an in vitro method that PEG-lipids can substantially reduce liposome opsonization by specific antibodies. A significant finding of this study is that complete prevention of antibody opsonization is required to ensure liposome protection from rapid elimination mediated by immune recognition. It was also shown that complete liposome shielding is determined by ligand concentration as well as PEG-lipid content. High ligand concentration (1 mol%) limits the ability of PEG-lipid to completely shield liposomes from antibody. Thus, it is important to consider both of these parameters when designing liposomes for active targeting, especially if the formulation is potentially immunogenic.
The role of PEG-lipids in altering liposome immunogenicity was also addressed, with the aim to generate immune responses which may be useful for immunotherapy applications. Considering the effect of PEG to reduce liposome uptake by macrophages, we hypothesize that incorporation of PEG-lipid can re-direct liposomes to other relevant antigen presenting cells. Using two different types of antigen, namely a T-independent antigen, biotinylated liposomes, and a tumor antigen, HER-2/neu, the immune response generated by liposomal vaccines containing CpG ODN as an immunoadjuvant with and without PEG-lipids was compared. It was found that, due to the different nature of each type of antigen which in turn activates different effector functions of the immune system, PEG-lipids have variable effects. When PEG-lipid was incorporated into a formulation containing a T-independent antigen which primarily stimulates B-cells, the antibody response was enhanced. In terms of cellular immune response, however, PEG-lipid decreased the CD8+ T-cell response generated by a HER-2/neu peptide delivered in liposomes while the humoral immune response was augmented by PEG-lipid. Collectively, these findings indicate that PEG-lipids are more effective in augmenting humoral immune response for liposomal vaccines containing CpG ODN. These results may be explained by the possibility that antigens delivered in PEGylated liposomes are re-directed to other antigen presenting cells such as B-cells for stimulation of antibody response for both types of antigen tested.

When considering the implications of these results, one also has to keep in mind that a strong immunoadjuvant was used in the immunogens. An important highlight of this thesis is the finding that co-delivery of CpG ODN in liposomes much enhanced its adjuvant activity regardless of the type of immune response. In cases where antibody response is an important
effector function for host protection, such as the case for T-independent antigens including many bacteria and viruses, the use of PEG-lipid, in combination with CpG ODN, may be useful for enhancing the immune response. The therapeutic potential of PEGylated liposomal vaccines to induce T-independent response needs to be verified by performing protection studies using a T-independent antigen derived from a true pathogen.

An obvious conclusion drawn from this thesis is that PEG-lipid is not capable of completely hiding liposomes from the immune system. The demonstration in this thesis that PEG-lipid can even enhance the humoral response verified the hypothesis that PEG-lipid is probably altering liposome interaction with immune system in ways which allow better exposure of the liposomal antigen to the immune system. From the perspective of decreasing liposome immunogenicity, the use of PEG-lipid is not beneficial. However, incorporation of PEG-lipids is indeed having an impact on liposome interaction with the immune system. This is shown by the results that PEG-lipids could protect liposomes from interacting with soluble components of the immune system which mediate their rapid removal from the circulation. But it is also important to keep in mind that, at the same time, the use of PEG-lipids in these liposomes has the potential of inducing an even higher antibody response which may still pose a challenge for subsequent injections. Thus, the use of PEG-lipids in liposomes which are potentially immunogenic needs to be carefully evaluated.
REFERENCES


