# SYSTEMIC ADMINISTRATION OF LIPOSOMAL ENCAPSULATED β-GALACTOSIDASE: A MODEL TO INVESTIGATE THE DEVELOPMENT OF THERAPEUTIC PROTEIN DRUG

by

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# ABSTRACT

With advances in recombinant protein technology, a growing number of therapeutic protein products have become available for clinical applications. But their wide use is limited by poor biodistribution, limited circulation time and the generation of immune responses to the drug by the patient. The specific aim of the work described in this thesis is to characterize the pharmacokinetic behaviour of an immunogenic protein encapsulated in a liposomal delivery system.

Liposomes have been demonstrated to increase the therapeutic index of drugs by ameliorating toxicity and enhancing biodistribution to sites of disease. The therapeutic index of a broad spectrum of drugs has been improved following encapsulation, including antibiotics, antimalarials, antifungals, antineoplastics, cytokines and antisense oligonucleotides. However, many studies have shown that liposomes can enhance the immune response against an encapsulated protein because they naturally target the antigen to phagocytic, antigen presenting cells such as macrophages and dendritic cells. This raises the question as to whether liposomal delivery of therapeutic proteins is feasible, particularly if repeated systemic administration is being considered. In order to test this we employed the enzyme β-galactosidase (β-gal), which was encapsulated in liposomes and administered intravenously to mice. The generation of β-gal antibodies and the pharmacokinetics of both protein and lipid vehicle were monitored following multiple, weekly injections.

An overview of liposomes and their role as drug delivery systems and vaccine adjuvants is given in **Chapter 1**. **Chapter 2** describes the protein encapsulation procedures employed and the physical characterization of the resulting liposomes. An assay was developed to measure β-gal latency and the stability of free and encapsulated β-gal was measured *in vitro* in buffer and plasma prior to initiating *in vivo* studies. It is demonstrated that vesicles can be made reproducibly with a latency > 95% and that encapsulated β-gal is protected from serum induced inactivation at 37 °C.

In the first part of **Chapter 3**, the pharmacokinetics of free protein and protein encapsulated in phospholipid/cholesterol vesicles ± polyethylene glycol (PEG) are investigated in normal mice and mice pre-immunized against β-gal protein. These experiments demonstrate that in the absence of β-gal antibodies, free protein and protein encapsulated in PEG-free vesicles are cleared from the circulation at similar rates, whereas protein formulated in PEG-coated vesicles is cleared much more slowly. The data are consistent with the known ability of PEG to protect the vesicle surface from opsonization. In the presence of β-gal antibodies (immunized mice), free protein was cleared from the blood immediately but the rate of clearance for protein protected inside PEG-free vesicles was unchanged from that measured in non-immunized animals. However, protein encapsulated inside PEG-coated vesicles was removed from the circulation as fast as free protein, indicating the formation of antigen-antibody complexes.

The difference in biodistribution between normal and PEG-coated vesicles to macrophages, and other antigen presenting cells, may influence the nature or magnitude of an immune response to repeated i.v. administrations to normal mice. This was also investigated in **Chapter 3**. Normal mice were subjected to five weekly injections of encapsulated β-gal in vesicles ± PEG. The results show that both preparations elicit β-gal antibodies at the same rate and to the same level during the course of administration. Despite this, the rate of clearance for β-gal-containing, PEG-coated liposomes is increased dramatically by the second injection compared to the naked vesicles. After the full course of five weekly injections, both types of protein delivery system exhibit similar blood clearance kinetics. These results suggest that a progressive immune response is mounted by these animals, which can recognize the protein carrier system and that PEG-coated vesicles are recognized more readily than naked vesicles. Possible reasons for the pharmacokinetic differences observed are discussed in **Chapter 4**. These include the physical characteristics of the vesicles and the exposure of protein epitopes at the surface of the vesicles, as well as the nature of the immune response and the possibility that antibodies are raised against the PEG anchor.

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# ABBREVIATIONS

| | |
|---|---|
| aa | amino acid |
| Ab | antibody |
| Ag | antigen |
| β-gal | β-galactosidase |
| BSA | bovine serum albumin |
| $CaCl_2$ | calcium chloride |
| Chol | cholesterol |
| $^3$H-CHE | [$^3$H]-cholesterylhexadecylether |
| Da | Dalton |
| DSPE-PEG 2000 | 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[poly(ethylene glycol)-2000] |
| EDTA | ethylenediaminetetraacetic acid |
| ELISA | enzyme-linked immunosorbent assay |
| $G_{M1}$ | Monosialo-ganglioside |
| HEPES | [4-(2-hydroxyethyl)]-piperazine ethane sulfonic acid |
| $H_2SO_4$ | sulfuric acid |
| HPI | Hydrogenated soy phosphatidylinositol |
| IgG | immunoglobulin G |
| i.v. | intravascular |
| LSC | liquid scintillation counting |
| LUV | large unilamellar vesicles |
| MPS | mononuclear phagocyte system |
| $MgCl_2$ | magnesium chloride |
| MLV | multilamellar vesicles |
| MW | molecular weight |
| NaOH | sodium hydroxide |
| $NaHCO_3$ | sodium bicarbonate |
| $Na_2CO_3$ | sodium carbonate |
| $NaN_3$ | sodium azide |
| POPC | 1-palmitoyl-2-Oleyl-sn-Glycero-3-Phosphocholine |
| PNPG | para-nitrophenyl-β-D-galactoside |
| RES | reticuloendothelial system |
| SM | sphingomyelin |
| s.c. | subcutaneous |
| S.D. | standard deviation |
| SUV | small unilamellar vesicles |
| Tris | tris(hydroxymethyl)aminomethane |
| $T_{1/2}$ | half-life |

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# DEDICATION

*To my*

*Daddy & Mommy*

*Ken & Edwin*

# CHAPTER 1

# INTRODUCTION

## 1.1 Liposomes as carriers

Liposomes have been employed as delivery vehicles for many small molecular weight drugs, as well as macromolecules. Drugs can be encapsulated in their aqueous core or solubilized in the membrane and administered by most routes including s.c., i.v., and i.p. It has been shown that the toxicity of many of these agents is reduced when they are formulated in liposomes [Chonn and Culllis, 1995; Sharma, et al., 1997]. This reduction in toxicity allows higher doses of the therapeutic agents to be administered resulting in improved efficacy [Gabizon et al., 1986; Bally et al., 1990]. It has also been suggested that liposomes can serve as circulating reservoirs for slow release of the entrapped agents in the blood compartment as well as at sites of disease [Mayer et al., 1990; Allen et al., 1992]. Furthermore, the circulation lifetime of entrapped drug is greatly enhanced over that observed for free drug. Recent applications of liposomes as carriers for pharmaceutical agents have been described in several reviews [Hope et al., 1986; Cullis et al., 1989; Wasan and Lopez-Berestein, 1995].

## 1.2 Liposomes

### 1.2.1 Classification and preparation of liposomes

When bilayer forming lipids are dispersed in aqueous media, they spontaneously form structures called liposomes where multiple bilayers are usually configured in an onion skin arrangement of concentric lamellae (Figure 1.1). The bilayer structure arises as a result of the amphipathic nature of lipids. The combination of a hydrophilic head group and hydrophobic tail within the same lipid molecule results in an orientation of the lipid head group toward the aqueous environment and the acyl tails facing each other so that they are sequestered from water (Figure 1.1a). Liposome is a generic term that is commonly used to describe many different types of model membrane systems formed by lipids. Throughout this thesis more descriptive terminology is used to describe the three most common types of liposome used: Multilamellar vesicles (MLV), large unilamellar vesicles (LUV) and small unilamellar vesicles (SUV).

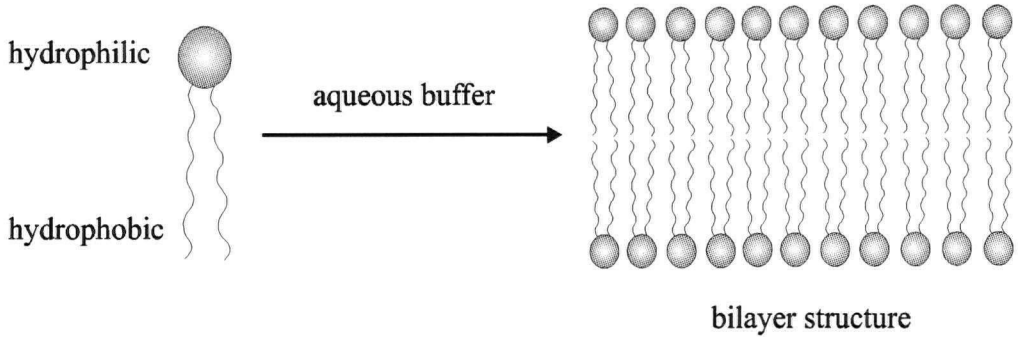
#### *1.2.1.1 Multimellar vesicles (MLV)*

Model membranes that exhibit the classical liposome structure with morphology of alternating concentric spheres of lipid bilayers and aqueous compartments are referred to as MLV. They are formed spontaneously by mechanical dispersion of a lipid film in an aqueous solution as first described by Bangham et al. [1965]. Typical diameters of MLV are on the order of 1000 nm but preparations are heterogeneous in size. In a typical structure of this type of liposome, the majority of the lipid is present as internal lamellae and only 10% or less of the total lipid is present in the outermost bilayer. MLV

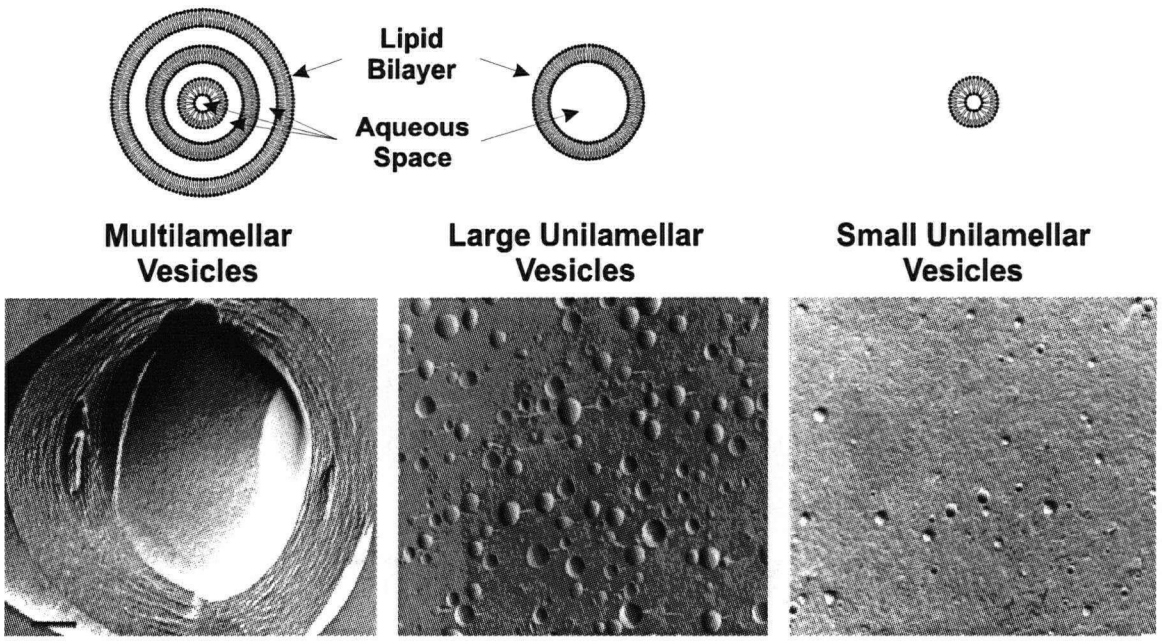
containing neutral lipids usually have an aqueous trapped volume of about 0.5 μl / μmol lipid. This can be improved by a freezing and thawing procedure [Mayer et al., 1985], which generate frozen and thawed MLV (FATMLV). This technique increases the interbilayer spacing and trapped volumes in excess of 2 μl / μmol lipid can be achieved. Another method to increase the trapped volume of MLV is to incorporate charged lipids, which cause charge repulsion between the internal lamellae, and increase the interbilayer spacing [Hope et al., 1986]. MLV are relatively large, heterogenous structures, and are rapidly cleared from the circulation by the reticuloendothelial (RE) organs and mononuclear phagocyte system (MPS), consequently these vesicles are rarely used for drug delivery.

**Figure 1.1** **(a) Amphipathic lipids in a bilayer configuration. (b) Freeze-fracture electron microscopy of multimellar vesicles (MLV), large unilamellar vesicles (LUV), and small unilamellar vesicles (SUV). The bar represents 200 nm.**

**a.**




**b.**



#### *1.2.1.2 Small unilamellar vesicles (SUV)*

SUV contain a small internal aqueous compartment (< 0.5 μl / μmol) surrounded by a single lipid bilayer 20 to 50 nm in diameter. They represent the lower physical limit of liposome size and are generally produced by sonicating MLV [Huang, 1969] or by utilizing the French press or another type of high pressure homogenization technique [Barenholz et al., 1979]. Since SUV are unilamellar and uniform in size, they have been used extensively for *in vitro* and *in vivo* studies. However, because of their small size, the bilayer is highly curved [Lichtenberg et al., 1981], resulting in an unstable vesicle prone to fusion [Wong et al., 1982], as well as attack by phospholipases [Gillett et al., 1980] and high density lipoproteins (HDL) *in vivo* (see section 1.3.2.1) [Scherphof and Morselt, 1984].

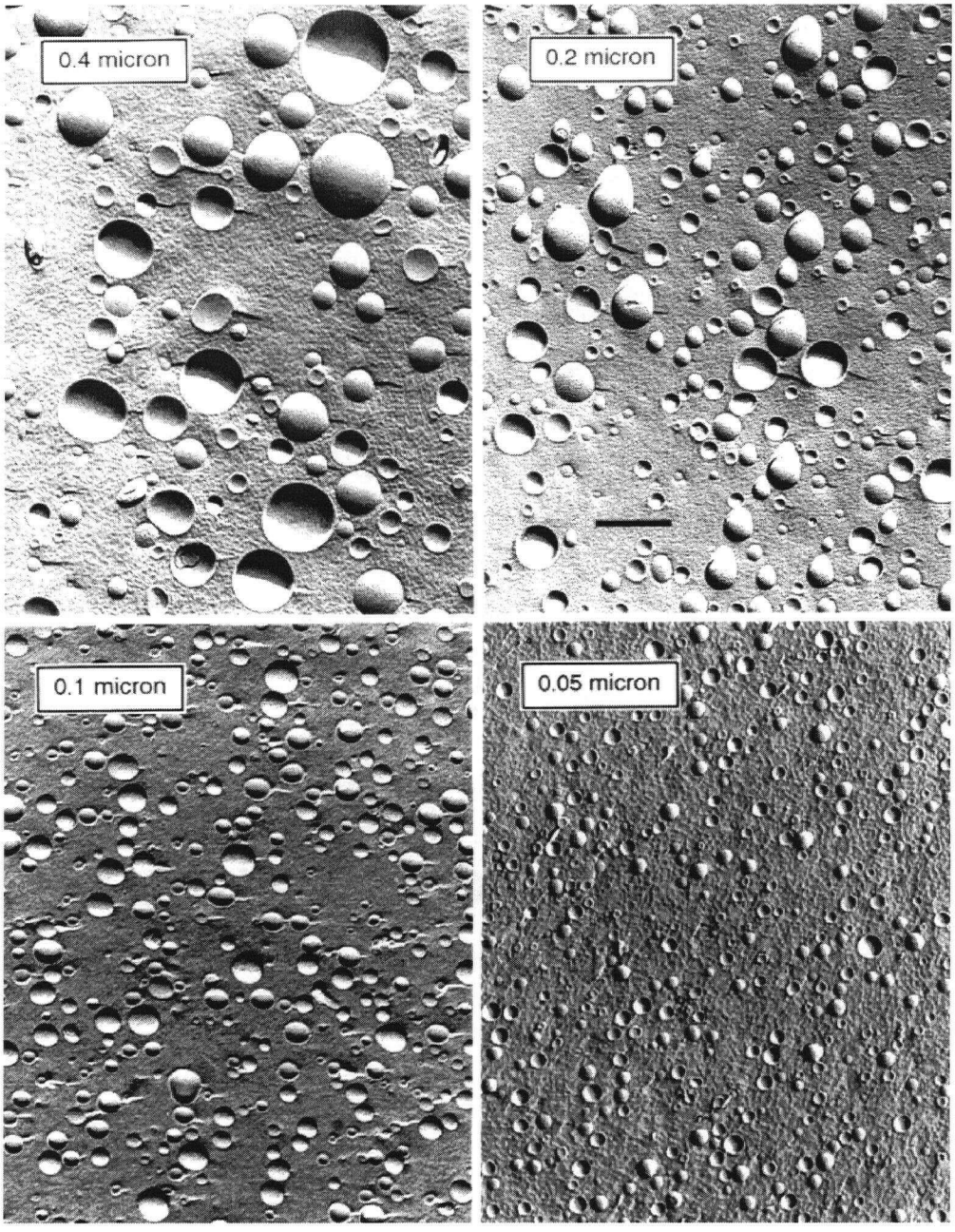
#### *1.2.1.3 Large unilamellar vesicles (LUV)*

Typical LUV range from 50 to 400 nm in diameter. They can be prepared by reverse phase evaporation [Szoka and Papahadojopoulos, 1978] or by detergent dialysis [Kagawa and Packer, 1971; Mimms et al., 1981]. Both of these techniques, however, have the disadvantage of being dependent on lipid composition, exhibit inconsistent reproducibility, and are complicated by the need to remove residual organic solvents or detergents from the final products. More recently, LUV have been produced from MLV by the extrusion technique, where MLV are forced through polycarbonate filters of

defined pore sizes under medium pressure (< 6000 kPa) [Hope et al., 1985]. Using extrusion, LUV composed from a wide variety of lipid species can be readily made at high concentration in the absence of contaminating detergents or organic solvents [Hope et al., 1985; Nayar et al., 1989]. Light scattering measurement shows that LUV produced by the extrusion method are uniform in size (Figure 1.2). Experiments reported in this thesis mostly employ LUV produced by extrusion through filters with a pore size of 200 nm.

**Figure 1.2 Freeze fracture electron micrographs of LUV produced by extrusion**

Vesicles were prepared from MLV at a concentration of 50 mg/ml by extrusion through two stacked polycarbonate filters of various sizes. The bar represents 200 nm.

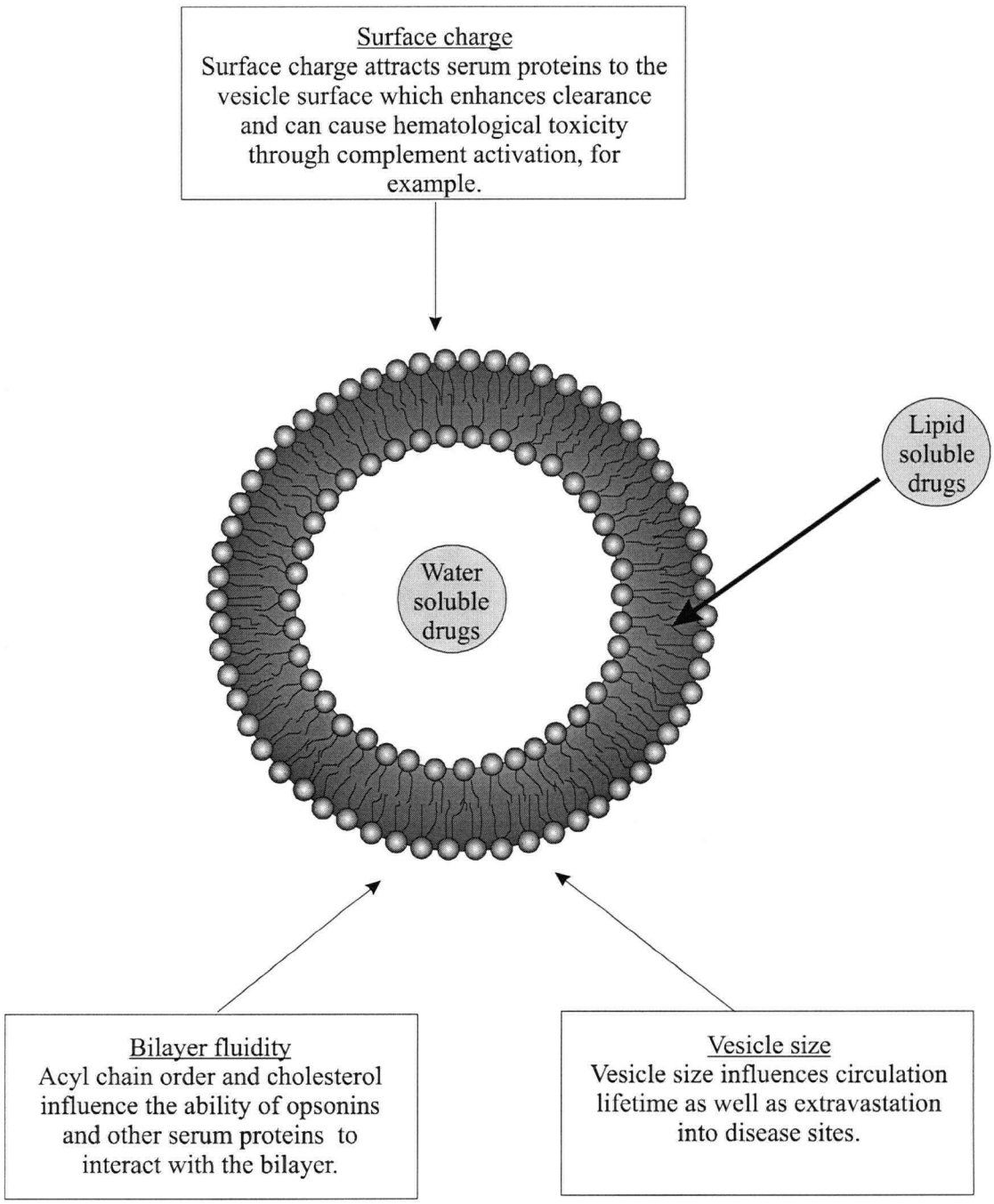


## 1.3 Properties of liposomes influencing their circulation lifetime in vivo

The use of liposomes as Ehrlich's "magic bullet" for targeting toxic drugs to sites of disease was first challenged by empirical findings that early liposomes were unstable in blood [Gregoriadis and Ryman, 1972]. In the past twenty years, significant advances have been made in understanding the factors involved in the clearance of liposomes from the circulation. Circulating blood proteins (opsonins) identify liposomes as foreign and mark them for clearance by the RE organs and elements of the host defense system known as the MPS (See section 1.3.2.2). Macrophages, which line blood vessels in organs such as liver and spleen, recognize opsonized foreign particles and remove them by receptor mediated phagocytosis.

Lipid composition is one of the most important factors contributing to the stability and clearance of liposomes from the blood. It affects vesicle permeability, surface charge, and interaction with plasma protein [Allen, 1988; Allen et al., 1990; Gabizon and Papahadjopoulos, 1988] (Figure 1.3). The two major lipid components used to make the vesicles described here are phospholipids and cholesterol.

**Figure 1.3** **Biophysical properties of liposomes that influence stability and clearance *in vivo*.**



### 1.3.1 Chemistry and physics of lipids

#### *1.3.1.1 Phospholipid*

The importance of phospholipids to living organisms is underscored by the nearly complete lack of genetic defects in the metabolism of these lipids in humans. Presumably, any such defects are lethal at early stages of development and therefore are never observed. All phospholipids are composed of various combinations of polar (hydrophilic) headgroups coupled to apolar (hydrophobic) tails via a glycerol-3-phosphate backbone (Figure 1.4). The hydroxyls on carbons 1 and 2 are usually acylated with fatty acids, and in most phospholipids the fatty acid substituent at carbon-1 is saturated, while the one at carbon-2 is unsaturated. The physical properties of the lipid bilayer are dictated by the combination of headgroup and acyl chain (Table 1.1). The acyl chain length and degree of saturation govern the temperature of the gel (rigid) to liquid-crystalline (disordered and fluid) phase transition for the lipid bilayers. In general, longer acyl chains and higher degrees of saturation will give rise to a higher phase transition temperature ($T_c$). Above the $T_c$, the acyl chains are less ordered or more "fluid" in nature (liquid-crystalline phase). Long, saturated acyl chains form extensive van der Waals interactions with each other in the bilayer thus limiting their motion. However, *cis*-double bonds, present in unsaturated phospholipid acyl chains, produce kinks, which impede nearest neighbour interactions and increase motion. In general, biological bilayers are in the fluid state at physiological temperatures and fluid model membranes

tend to be more permeable than membranes in the gel state [Papahadjopoulos et al., 1973; Bittman and Blau, 1972].

**Figure 1.4 General structure of a phospholipid showing commonly occurring headgroups and fatty acid moieties.**

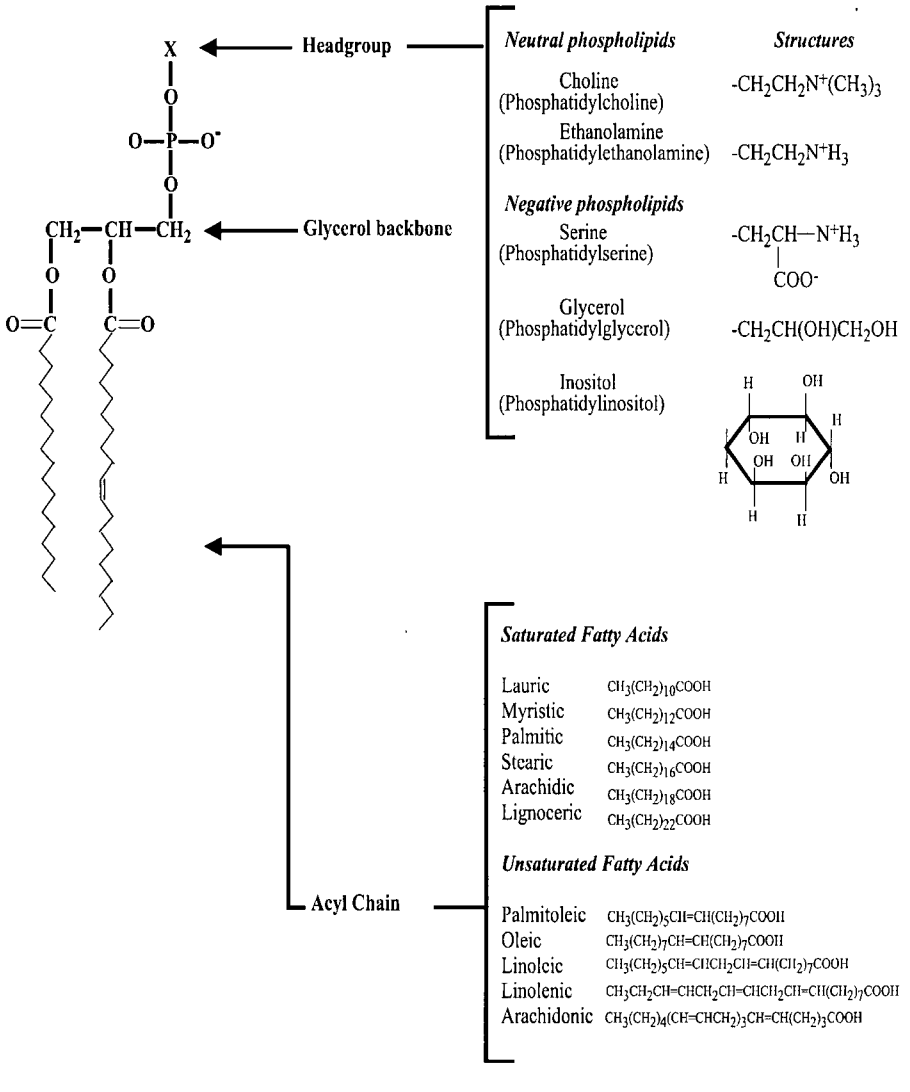


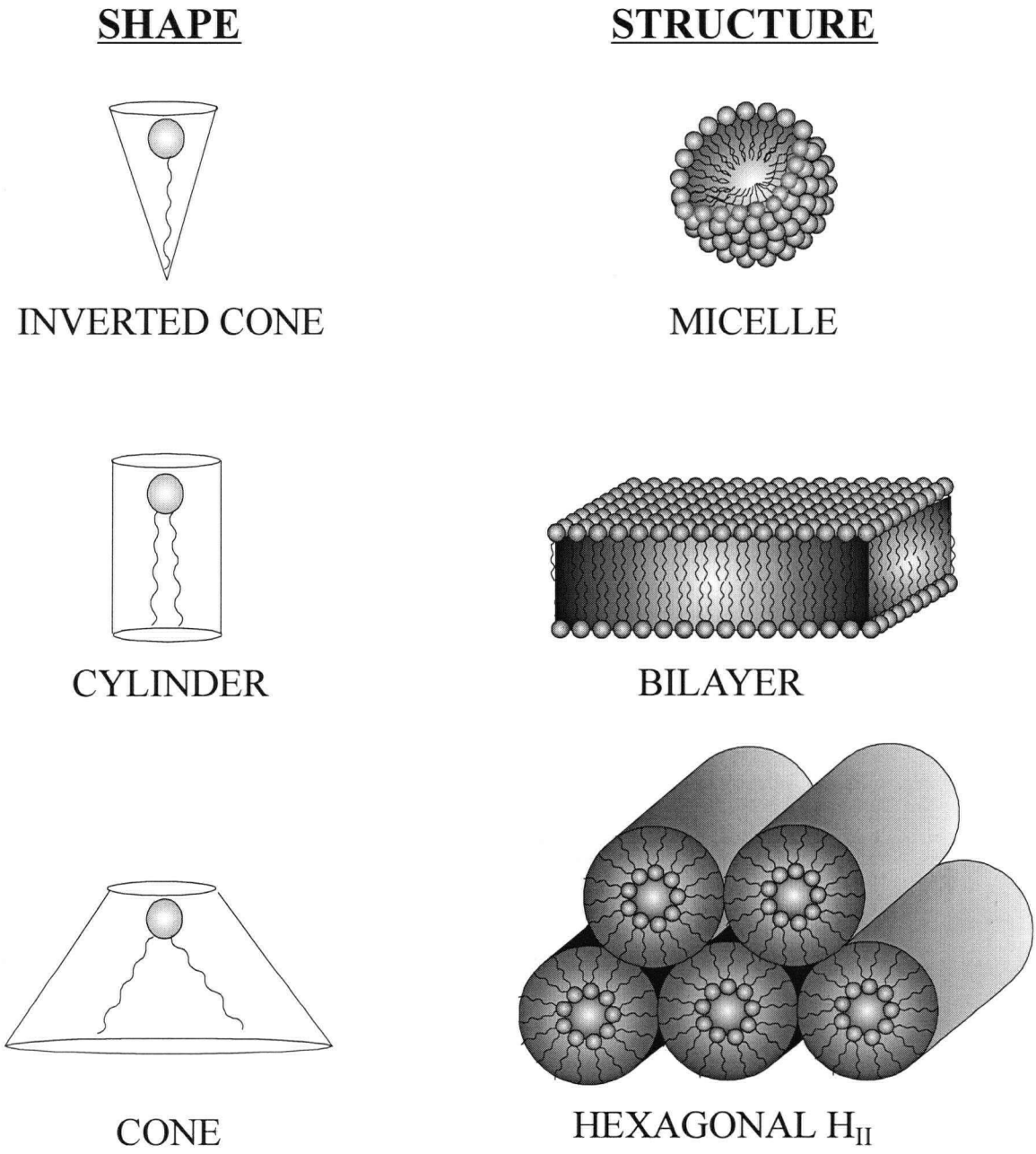
**Table 1.1** **Transition temperature (Tc) of various combinations of acyl chain length, degree of saturation, and headgroup moiety. The highlighted phospholipid, POPC was used in this thesis.**

| Lipid species (acyl chain # 1 2) | | Transition temperature (± 2 °C) |
|---|---|---|
| dilauroyl PC | (12:0, 12:0) | -1 |
| dimyristoyl PC | (14:0, 14:0) | 24 |
| dipalmitoyl PC | (16:0, 16:0) | 41 |
| distearoyl PC | (18:0, 18:0) | 55 |
| stearoyl, oleoyl PC | (18:0, 18:1) | 6 |
| stearoyl, linoleoyl PC | (18:0, 18:2) | -16 |
| stearoyl, linolenoyl PC | (18:0, 18:3) | -13 |
| stearoyl, arachidonyl PC | (18:0, 20:4) | -13 |
| dioleoyl PC | (18:1, 18:1) | -19 |
| palmitoyl, oleoyl PC | (16:0, 18:1) | -1 |
| dipalmitoyl PA | (16:0, 16:0) | 67 |
| dipalmitoyl PE | (16:0, 16:0) | 63 |
| dipalmitoyl PS | (16:0, 16:0) | 55 |
| dipalmitoyl PG | (16:0, 16:0) | 41 |

In an aqueous environment, phospholipids adopt a variety of structures. This is known as lipid polymorphism [Cullis et al., 1986] (Figure 1.5). When the cross sectional area of the hydrophobic head group is greater than that swept by the acyl tails, the phospholipid molecules can be considered to adopt a cone shape and thus will tend to pack into micelles, structures typically adopted by detergents. Unsaturated phosphatidylethanolamine (PE), does not form a bilayer when hydrated as the cross-sectional area of the relatively small, neutral headgroup is less than that occupied by the acyl chains. The dynamic shape of unsaturated PE can be conceptualized as an inverted cone. These lipids tend to form the hexagonal $H_{II}$ phase in aqueous medium [Cullis and de Kruijff, 1979], however, they will form bilayers when stabilized by bilayer forming lipids. These lipids behave as cylinders and therefore prefer to pack into a bilayer

configuration. An example is phosphatidylcholine (PC), the most common phospholipid in eukaryotic plasma membranes. PC is a zwitterion composed of a glycerol-phosphate ester with a choline headgroup and two acyl chains esterified to the sn-1 and sn-2 positions [Small, 1986]. In naturally occurring PC, the fatty acid at the sn-1 position is saturated, while the sn-2 acyl chain is usually unsaturated [Small, 1986]. The phospholipid used to form vesicles described here was 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC), a synthetic analogue of one of the most common, naturally occurring PC.

**Figure 1.5** **Lipid polymorphism – organization of lipid molecules in micelle, bilayer, and hexagonal phases.**



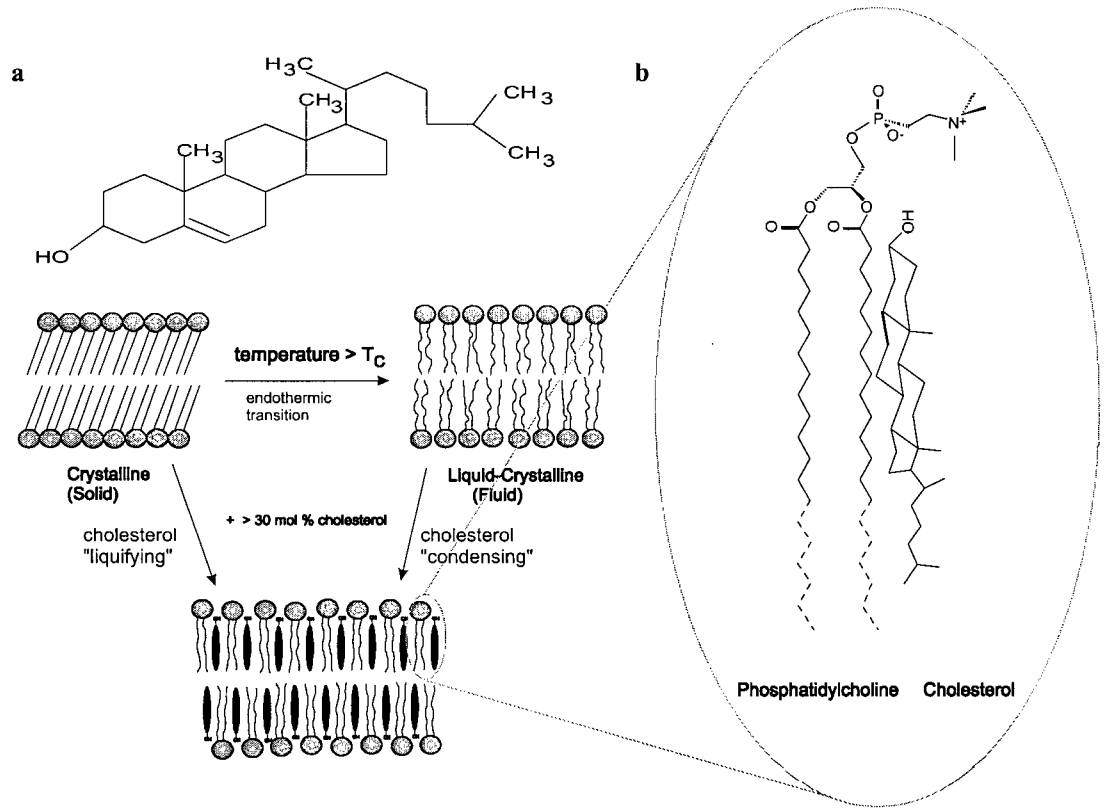
*1.3.1.2 Cholesterol*

Cholesterol is the major neutral lipid component of eukaryotic plasma membranes. It is an amphipathic molecule with a hydrophobic, rigid steroid nucleus, aliphatic side chain and 3-β-hydroxyl group. In the bilayer, cholesterol is oriented such that the hydrophobic moieties lay next to the phospholipid acyl chains and the hydroxyl group is positioned close to the carbonyl ester bond linking the acyl chain to the glycerol backbone (Figure 1.6a). Cholesterol has a very specific interaction with phospholipids and increases the "order" in the acyl chains of PCs that are in the liquid-crystalline state but decreases the order of PCs which are in the gel state [Demel and de Kruyff, 1976]. Furthermore, the incorporation of cholesterol into membranes composed of saturated PC progressively decreases the enthalpy of the gel-liquid crystalline phase transition (Figure 1.6b). At 30 mol % of cholesterol or higher, the transition can no longer be detected [Chapman, 1975]. This cholesterol-phospholipid interaction gives rise to the condensing effect measured using lipid monolayer techniques which have shown that the surface area occupied by a mixture of liquid-crystalline PC and cholesterol is actually less than the sum of the area of the two components [Hyslop et al., 1990].

The inclusion of cholesterol helps stabilize liposomes in blood [Mayhew et al., 1979; Senior 1987; Patel et al., 1983] by reducing the disruptive effects of plasma proteins, which in turn enhances solute retention in the aqueous core [Papahadjopoulos et al., 1973; Inoue, 1974]. In culture, Kupffer cells (responsible for the majority of liposomal clearance *in vivo*) exhibit decreased uptake and intracellular degradation of

cholesterol-containing liposomes [Roerdink et al, 1989]. The addition of cholesterol has also been shown to reduce the net transfer of phospholipid from liposomes to high density lipoprotein (see detail discussion on section 1.3.2.1) [Kirby et al., 1980a, b; Gregoriadis and Davis, 1979].

**Figure 1.6 The structure of cholesterol (a) and its effect on the structure of lipid bilayers (b).**




### 1.3.2 *In vivo* behavior of liposomes

The majority of a dose of liposomes administered intravenously is cleared from the circulation by the liver and spleen [Gregoriadis and Ryman, 1972; Gregoriadis, 1988]. The exact *in vivo* mechanisms responsible for this clearance and the recognition of liposomes as foreign particles are still unsolved. However, two major factors contribute to liposome clearance from the blood: (1) interaction with opsonins and plasma

lipoproteins, and (2) uptake of liposomes by cells of the MPS primarily in the liver and spleen [Gregoriadis, 1988].

### *1.3.2.1 Interaction of liposomes with plasma protein*

Liposome clearance from the blood is believed to be mediated by two different groups of plasma proteins: opsonins and lipoproteins [Patel, 1992]. Opsonins are serum proteins that adsorb onto the surface of a foreign particle, thereby rendering the particle more palatable to phagocytes. They promote phagocytosis primarily by forming a bridge between the particle and macrophage, but they are generally not involved in the internalization or digestion of particles. There are two types of opsonins: specific and nonspecific. Specific opsonins, which include immunoglobulins and components of the complement system, interact directly with receptors on macrophages, whereas nonspecific ones function by altering the surface properties of the foreign particle or the phagocyte or both, thus rendering them more adhesive to phagocytes. It has been suggested that opsonins associate with the surface of liposomes via nonspecific hydrophobic interactions [Gregoriadis, 1988]. The degree of opsonization is affected by the surface charge and molecular packing of the lipid bilayer which is dictated by phospholipid headgroups, vesicle size, acyl chain composition and cholesterol content [Scherphof et al., 1984]. Vesicles with loosely packed bilayers are more susceptible to opsonin adsorption [Scherphof et al, 1984; Moghimi and Patal, 1988a]. The surface charge of vesicles has also been demonstrated to play a role in mediating the non-specific adsorption of IgG and complement proteins, which mark vesicles for clearance via macrophages that exhibit specific receptors for IgG, the complement protein C3,

fibronectin, and other extracellular matrix components [reviewed in Patel, 1992]. The role of scavenger receptors on macrophages in mediating the uptake of liposomes has also been described [Nishikawa et al., 1990]. The circulation lifetime of liposomes has been determined to be inversely related to the affinity of liver macrophages for the liposomes. Furthermore, it has been suggested that serum may contain organ-specific opsonins that selectively enhance liposome uptake by macrophages of either the liver or spleen [Moghimi and Patel, 1988b]. Recent findings based on *in situ* liver perfusion assays demonstrated that hepatic uptake of neutral or negatively charged liposomes in mice is an opsonin independent process and does not involve serum components [Liu and Liu, 1996], whereas hepatic uptake of liposomes in rats appears to depend on serum opsonins [Liu et al., 1995].

In addition to opsonins, the interaction of liposomes with other plasma proteins plays a role in determining liposome clearance and permeability characteristics. In the absence of cholesterol in the membrane, SUV will interact with plasma lipoproteins which leads to lipid exchange, dissolution of the carrier, and premature release of the encapsulated materials [Kirby et al., 1980a]. More specifically, high-density lipoproteins (HDL), HDL-associated apolipoprotein ApoA-1 [Klausner et al., 1985], and complement proteins [Devine et al., 1994] can penetrate vesicle membranes and disrupt the permeability barrier. Some lipid compositions can activate the complement pathway inducing the formation of membrane attack complexes, which lyse the liposomal membrane [Silverman et al., 1984; Malinski and Nelsestuen, 1989]. Recent findings indicate that liposomes activate complement in a dose-dependent manner, require the

inclusion of phospholipid bearing a net negative or positive charge, and can activate both the classical and alternative pathways [for review, see Patel, 1992]. In addition, liposome size can also contribute to complement activation: for example, large LUV (400 nm) are found to be more effective in activating complement than small (50 nm) vesicles [Devine et al., 1994].

*1.3.2.2 Interaction with the mononuclear phagocyte system (MPS)*

Following i.v. injection, the majority of liposomes are removed by fixed macrophages residing in the liver and spleen, as well as in the lung, lymph nodes, and bone marrow. Macrophages patrol the circulatory system in search of unwanted particles. In the course of their travels, macrophages ingest sick and dying cells, invading pathogens and anything else that appears to be foreign. Macrophages were originally classified as part of the reticuloendothelial system (RES) [Metchnikoff, 1963] which included reticular cells, endothelial cells, fibrocytes, histocytes, and monocytes. However, since this classification did not fulfill the criteria of common morphology, function and origin of the macrophage, a new classification, the mononuclear phagocyte system (MPS), was adopted in 1969. The MPS was described on the basis of knowledge about the monocyte precursors in the bone marrow and about the monocyte-derived macrophages in various locations under normal and pathological conditions [van Furth et al., 1971]. The MPS is composed of monocytes, macrophages, and precursor cells (stem cells, monoblasts, and promocytes) in the bone marrow, as well as several other cell types found in the circulation, tissue, and body cavities under normal and inflammation conditions (Table 1.2). It is now known that the uptake of foreign particles by MPS is

dependent on the recognition of membrane associated opsonins by specific receptors on the macrophage surface [reviewed by Coleman, 1986]. As mentioned earlier, the size of liposomes and the total amount of bound protein on the liposome surface [Chonn et al., 1992] are factors which can dictate the rate of liposome clearance by macrophages.

**Table 1.2** Cells of Mononuclear Phagocytic System (MPS)

| Tissue | Cells |
|---|---|
| Bone marrow | Stem cells, monoblasts, promocytes, and monocytes |
| Blood | Monocytes |
| Liver | Kupffer cells |
| Lung | Alveolar macrophages, monocytes in the pleural fluid |
| | |
| Spleen | Free and fixed macrophages |
| Lymph nodes | |
| Thymus | |
| Lymphoid tissues | |
| Gastrointestinal tract | |
| Colostrum | |
| Endocrine organs | |
| | |
| Nervous system | Microglial cells |
| Serous cavities | Peritoneal macrophages |
| Connective tissue | Histiocytes |

### 1.3.3 Liposomes coated by Poly(ethylene glycol) (PEG)

In order to enhance the circulation lifetime of liposomes and deflect their biodistribution away from the liver and spleen attempts have been made to inhibit opsonization. Certain biological precedents suggested that this might be possible. For

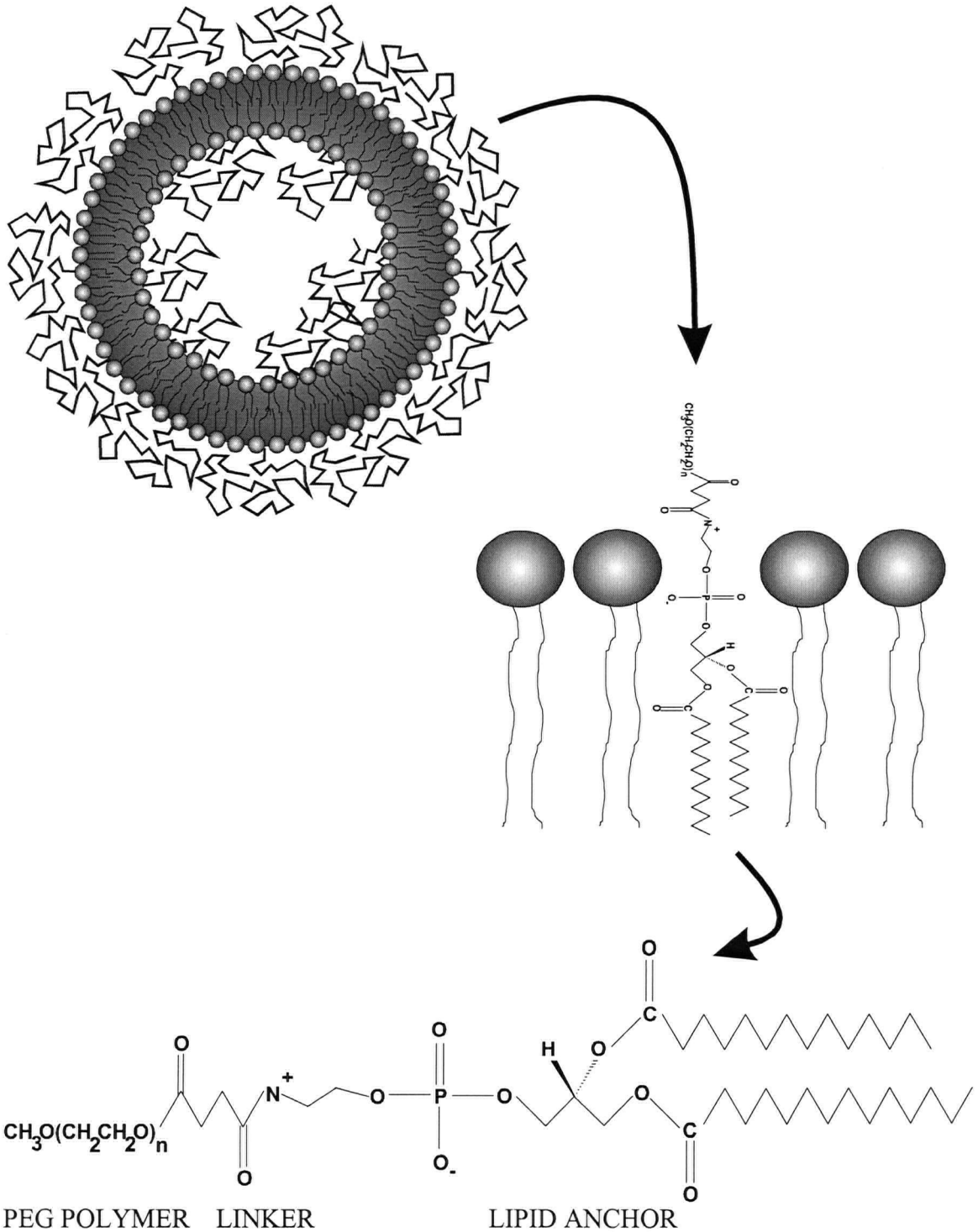
example, the basic structure of red blood cell membranes is similar to that of liposomes, and erythrocytes are able to circulate for several months before being removed by the MPS. Subsequent incorporation of specific glycolipids such as monosialo-ganglioside ($G_{M1}$) or hydrogenated soy phosphatidylinositol (HPI) have been shown to result in prolonged circulation and reduced MPS uptake [Allen and Chonn, 1987; Gabizon and Papahadjopoulos, 1988; Liu and Huang 1990]. Furthermore, coating the surface of liposomes with PEG has been shown to reduce uptake by Kupffer cells in the first few hours following i.v. administration.

PEG is a hydrophilic polymer that has previously been grafted onto proteins to prolong their lifetime in the bloodstream. Based on monitoring the structural organization, interbilayer repulsion, as well as lipid bilayer elasticity, the addition of PEG to liposome does not change the normal structure of the bilayer interior [Needham et al., 1992]. It is proposed that PEG forms a polymeric "cloud" extended ~ 5.0 nm above the liposome surface [Torchilin 1994; Torchilin et al., 1995] which reduces the hydrophobic and electrostatic interaction of lipoproteins and opsonins to the lipid surface (Figure 1.7). This phenomenon is known as surface steric stabilization and it decreases the rate at which liposomes are cleared from the circulation by macrophages [Parr et al., 1993].

Both the length of the PEG polymer and its density at the lipid bilayer surface are important factors for maximizing the stabilizing effect. It has been reported that the optimal polymer size for extending circulation time is 2000 Da, at a density of about 5 mole % of the total liposome lipid [Klibanov et al., 1991; Woodle et al., 1992; Litzinger

and Huang, 1992]. The sterically stabilized vesicles used in the work described in this thesis contained PEG-2000, which consist of an average 45 ethylene glycol monomers per PEG chain, conjugated to a distearoylphosphatidylethanolamine (DSPE) lipid anchor via a succinate linker group. This molecule has been shown to provide a stable polymer coat for 24 hours *in vivo* [Parr et al., 1994].

**Figure 1.7** PEG-coated vesicle and the chemical structure of DSPE-PEG.

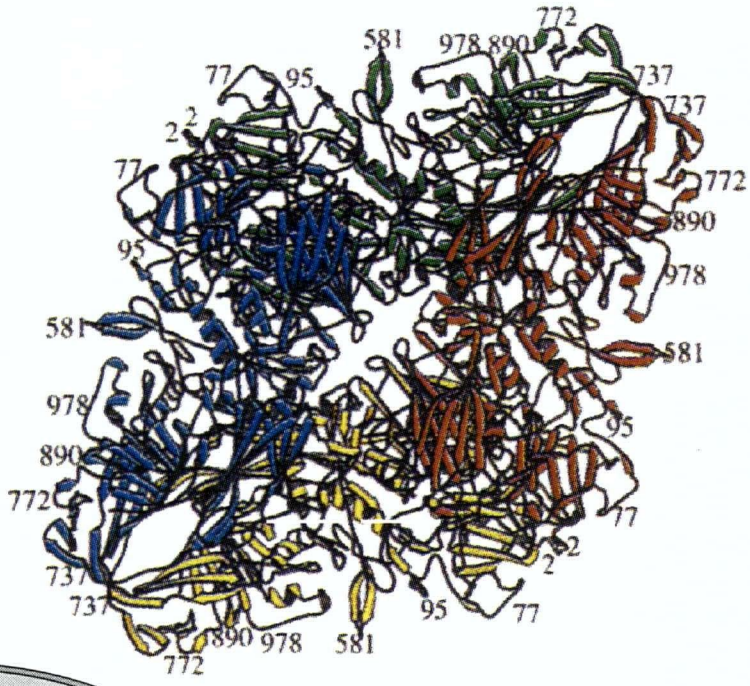


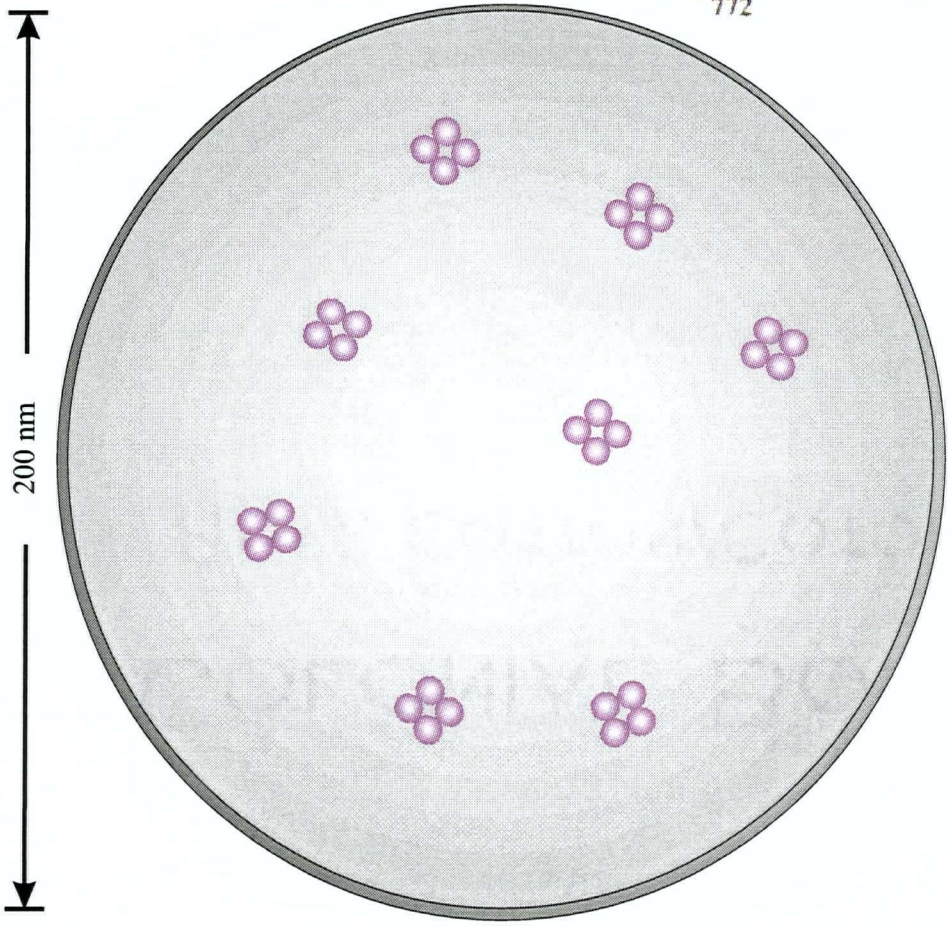
## 1.4 The β-galactosidase

The β-gal employed in this work is derived from yeast *Kluyveromyces fragilis*. It is an evolutionary conserved protein: the prokaryotic β-gal and the eukaryotic β-gal are closely related in both structure and sequence [Guiso et al., 1978; Poch et al., 1992]. The active form of β-gal is a tetramer [Edwards et al., 1988]. Each monomeric unit of β-gal is composed of 1025-aa polypeptide with MW of 117,618 Da. Electron micrographs [Karlsson et al., 1964], Fourier-transform infrared spectroscopy [Arrondo et al., 1989], and X-ray diffraction [Jacobson and Matthews, 1992; Jacobson et al., 1994] have revealed that the tetrameric β-gal from *E. coli* is roughly 17.5 x 13.5 x 9.0 nm in size and particles with three dimensions have been drawn to scale inside a 200 nm LUV to help provide some perspective (Figure 1.8). This protein is composed of 35% α-helix, 40% β-sheet (mainly parallel), 12% random coil, and 13% β-turn [Arrondo et al., 1989]. Each tetramer contains four active sites and the residues Glu 461, Met 502, Tyr 503 and Glu 537 are believed to be important for catalytic function, or reside near the active site [Jacobson et al., 1994]. The presence of $Mg^{2+}$, and monovalent cations $K^+$ and $Na^+$ is required for maximal β-gal activity [Wallenfels and Weil, 1972, Huber et al., 1994]. However, the specific role of these cations in the mechanism of action is not yet understood.

**Figure 1.8 Ribbon representation of the β-galactosidase tetramer.**

Ribbon representation of the β-galactosidase tetramer showing the largest face of the molecule. The constituent monomers form two different monomer-monomer contact that are referred to as the "activating" interface and the "long" interface. Contacts between red/green and blue/yellow dimers form the long interface. Contacts between the red/yellow and blue/green dimers form the activating interface. Formation of the tetrameric particle results in two deep clefts that run across opposite faces of the molecule and each contain two active sites (Adopted from Jacobson et al., 1994).




The dimensions of the tetrameric β-gal are shown in scale to the dimension of a 200 nm vesicle. The number of protein encapsulated inside the vesicle is estimated as outlined in table 2.1.

This protein was chosen for these studies for several reasons. (1) β-gal is a common enzyme reagent used in many biochemical, histochemical and analytical assays. Consequently, commercial kits and substrates are readily available. (2) It is important for the objectives of this study that an enzyme is employed because retention of biological activity was of interest not simply the presence of protein per se. The activity of β-gal is sensitive to protein structure and integrity which may be compromised by an immune response. (3) The β-gal enzyme employed here is available in bulk as a pure protein used in the dairy industry. This is relevant because the process of encapsulation used is inefficient, more than 85% of the starting protein is discarded, so the work had to be conducted using a cost effective protein. Finally, (4) this large protein isolated from a microorganism was expected to be immunogenic.

## 1.5 Thesis objectives

The objective of this thesis is to test the hypothesis that a biologically active protein, encapsulated in a lipid based drug delivery system, can be administered intravenously and repeatedly to mice without loss of activity or reduced bioavailability due to the immune response induced against it.

Specific objectives were:

1. Develop a well characterized formulation in which β-gal was encapsulated in a lipid carrier.

2. Develop an assay that could measure protein latency, and would work in plasma with sufficient sensitivity to detect < 10% of the injected dose in blood.
3. Measure the stability of free protein and encapsulated protein in buffer and plasma *in vitro* to confirm the feasibility of conducting *in vivo* experiments.
4. Measure the pharmacokinetics of free protein, encapsulated protein, empty liposome and liposomes containing encapsulated protein in normal mice following a single injection.
5. Develop assays to measure antibody production in mice immunized by subcutaneous injection of protein.
6. Measure the pharmacokinetics of free protein, encapsulated protein, empty liposomes and liposomes containing encapsulated protein (following a single injection) in mice that have been immunized against the delivered protein.
7. Measure the pharmacokinetics of encapsulated protein following repeat multiple weekly injections.

# CHAPTER 2

# Formulation of liposomal β-galactosidase

## 2.1 Introduction

Liposomes have been widely used as delivery vehicles for a variety of conventional, low molecular weight, synthetic drugs. However, the molecular size and structural sensitivity of large protein molecules presents formulation challenges. Fluctuations in temperature and pH outside of the normal physiological range, as well as the presence of organic solvent and/or detergent can denature protein or alter protein conformation in such a way as to have adverse effects on the biological activity.

For the purposes of this study β-galactosidase was chosen as the biologically active protein, and the reasons for this choice have been outlined in section 1.4. The encapsulation procedure employed was the mildest possible in order to minimize any loss in specific activity of the protein. This involved passive hydration of lipid mixtures in protein solutions at room temperature to form MLV, followed by sequential extrusion through filters with defined pore sizes to produce 200 nm diameter LUV. The lipid composition was chosen to form the most stable vesicle that could be produced at 37 °C. Long chain, saturated phospholipids form very stable liposomes (section 1.3.1.1), however, they must be formed at temperatures above their $T_c$. For example, vesicles composed of DSPC must be hydrated and extruded at temperatures > 65 °C, even in the

presence of cholesterol [Nayar et al., 1989], and at this temperature β-gal is rapidly denatured. Therefore the lipid mixture POPC/Chol was chosen, which readily forms vesicles at room temperature. However, it was determined that β-gal was sufficiently stable at 37 °C to allow extrusion at this temperature, thus speeding the formulation process. Polymer coated vesicles were produced by incorporating 5 mol % DSPE-PEG 2000. This amount of saturated lipid in the formulation slightly slowed the extrusion process but did not prevent vesicles from being formed.

## 2.2 Materials and methods

### 2.2.1 Materials

1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine (POPC) was obtained from Northern Lipids Inc. (Vancouver, BC, Canada). Cholesterol (Chol), para-nitrophenyl-β-D-galactoside (PNPG), and Triton X-100 (TX-100) were purchased from Sigma Chemical Co. (St. Louis, MO). 1,2-Distearoyl-sn-Glycero-3-Phosphoethanolamine-N-[Poly(ethylene glycol)-2000] (DSPE-PEG 2000) was a gift from Dr. Steven Ansell (Inex Pharmaceuticals Corp. Vancouver, BC, Canada) and [$^3$H]-cholesterylhexadecylether ($^3$H-CHE) was purchased from Dupont Canada. β-galactosidase solution (Lactozym 3000L) was a gift from Novo Nordisk Biochem North America, Inc. (Franklinton, NC). Modified Hepes buffer (HBS) optimized for β-gal activity assay consists of 0.1M Hepes, 50 mM KCl, 2 mM $MgCl_2 \bullet 6H_2O$ (pH 6.5) was used for all preparations.

### 2.2.2 Measurement of β-galactosidase activity

The lactozym 3000L (commercial β-gal product) is supplied as protein in glycerol solution. Before encapsulation and extrusion the glycerol was removed by overnight dialysis at 4 °C against HBS, typically 3 ml of β-gal solution was dialyzed against 2 L of HBS. This is because the presence of glycerol made the protein solution too viscous to extrude. Protein concentration was determined by using the standard bicinchoninic acid (BCA) assay and the β-gal concentration post dialysis was 10.3 ± 0.2 mg•ml$^{-1}$ (n=4). The activity of β-gal was measured by monitoring the cleavage of the substrate PNPG (3.0 ml of PNPG at 0.5 mg•ml$^{-1}$ in HBS) after 10 minutes incubation at room temperature. The reaction was stopped by the addition of 500 µl of 1 M NaOH, which raised the pH to above 9.0. The absorbance was measured at 420 nm using a Perkin Elmer UV/VIS spectrometer - Lambda Bio.

### 2.2.3 Encapsulation of β-galactosidase in liposomes

Encapsulation of β-gal was carried out by forming MLV in the presence of the protein solution followed by extrusion as described previously [Hope et al., 1985]. Briefly, total lipid composed of POPC:Chol (55:45, mole ratio), or POPC:Chol:DSPE-PEG 2000 (55:40:5, mole ratio) was dissolved in $CHCl_3$ at a concentration of 100 mg•ml$^{-1}$ and mixed with radiolabeled lipid marker $^3$H-CHE (20 µCi). Bulk $CHCl_3$ was removed under a stream of nitrogen gas, then trace solvent was removed by exposing the lipid films to high vacuum overnight.

The dried lipid film (100 mg) was rehydrated by the addition of one ml of dialyzed β-gal solution, and MLV were formed during vigorous vortexing. MLV were first extruded 10 times through two stacked polycarbonate filters with a pore size of 400 nm (Nuclepore, Pleasanton, CA). These were then replaced by two stacked filters with 200 nm pore size and the vesicles extruded for a further 10 passes. Extrusion was conducted at 37°C using a thermal regulated extruder (Lipex Biomembranes, Vancouver, BC, Canada). This process produced a homogeneous population of vesicles with a mean diameter of 200 ± 40 nm (n = 10). Liposome sizes were determined by Quasi-Elastic Light Scattering (QELS) (Nicomp Particle Sizing System Model 370, Santa Barbara, CA). Encapsulation efficiency was measured by using gel filtration (Sepharose CL-4B). Typically, 1.0 ml of LUV solution was loaded on a 1.5 x 11 cm Sepharose CL-4B column equilibrated in HBS. The column was eluted with HBS at a flow rate of 0.5 ml•min$^{-1}$ and 600 µl per fraction were collected. Aliquots of each fraction were assayed for lipid content by liquid scintillation counting (LSC) and β-gal activity by PNPG assay.

### 2.2.4 Latency of β-gal and *in vitro* stability study

To determine the activity of β-gal encapsulated within the liposomes the lipid vesicles must first be disrupted to release the encapsulated protein, because the vesicle membrane is impermeable to the enzyme substrate. Latent enzyme activity was measured by conducting the PNPG assay in the presence and absence of 1% TX-100 (v/v), a detergent solution capable of dissolving these vesicles.

$$\% \text{ Latency} = \frac{(\beta\text{-gal activity} + \text{TX100}) - (\beta\text{-gal activity} - \text{TX100})}{(\beta\text{-gal activity} + \text{TX100})} \times 100$$

Liposomal β-gal fractions collected from the gel filtration column, determined to be greater than 95% latent, were pooled and used for the *in vitro* stability and pharmacokinetic studies. The amount of protein encapsulated was determined from β-gal standard curves constructed with known amounts of protein in the presence and absence of detergent. The standards were prepared from a 1:5000 β-gal dilution in HBS with or without 1% TX-100 (v/v). Then, 0 to 400 µl of diluted enzyme was assayed by PNPG assay as outlined on section 2.2.3.

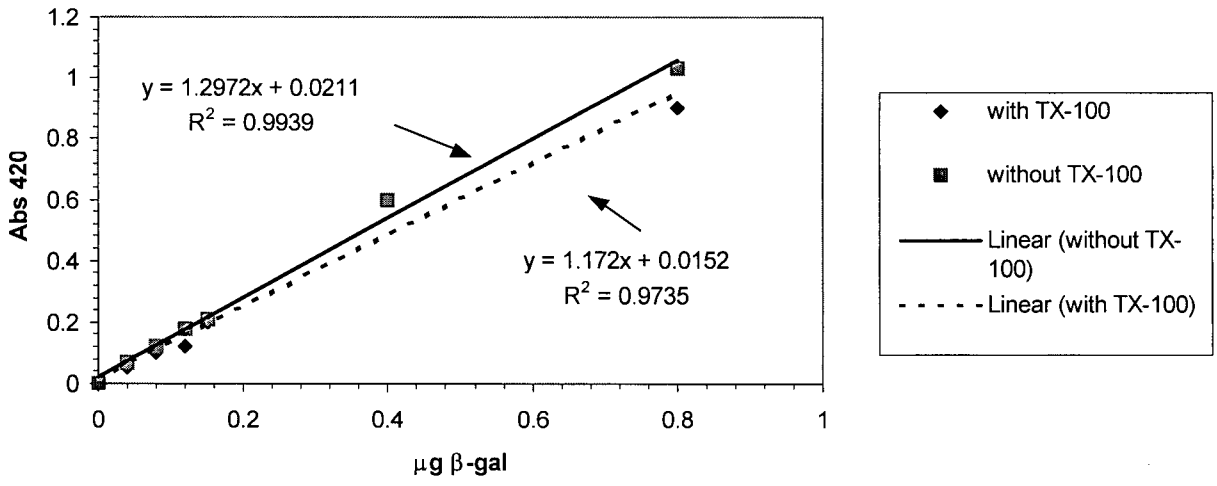
The *in vitro* stability of free β-gal and encapsulated β-gal was determined by incubating samples in 50% diluted fresh mouse (BDF-1 strain) plasma at 37°C for various lengths of time. In the presence of plasma, β-gal activity was determined as described previously using the PNPG substrate solution containing 1% TX-100 (v/v). Light scattering due to intact liposomes and plasma components was corrected by carrying out reactions in HBS containing detergent.

## 2.3 Results

### 2.3.1 Assay for measuring β-galactosidase activity

The activity of β-gal was determined using an assay based on the substrate PNPG [Hill and Huber, 1971; Huber et al., 1979]. All spectroscopic measurements were made 10 minutes after the addition of the stopping solution (NaOH). A linear relationship between absorbance at 420 nm and β-gal concentration was measured up to 0.8 μg of protein (Figure 2.1). Furthermore, the absorbance was not affected by the presence of Triton X-100.

**Figure 2.1** Standard curve for β-gal activity




β-gal was reacted with PNPG substrate in the presence or absence of 1% (v/v) Triton X-100. Lines represent linear regression by least squares fit with correlation coefficient > 0.96.

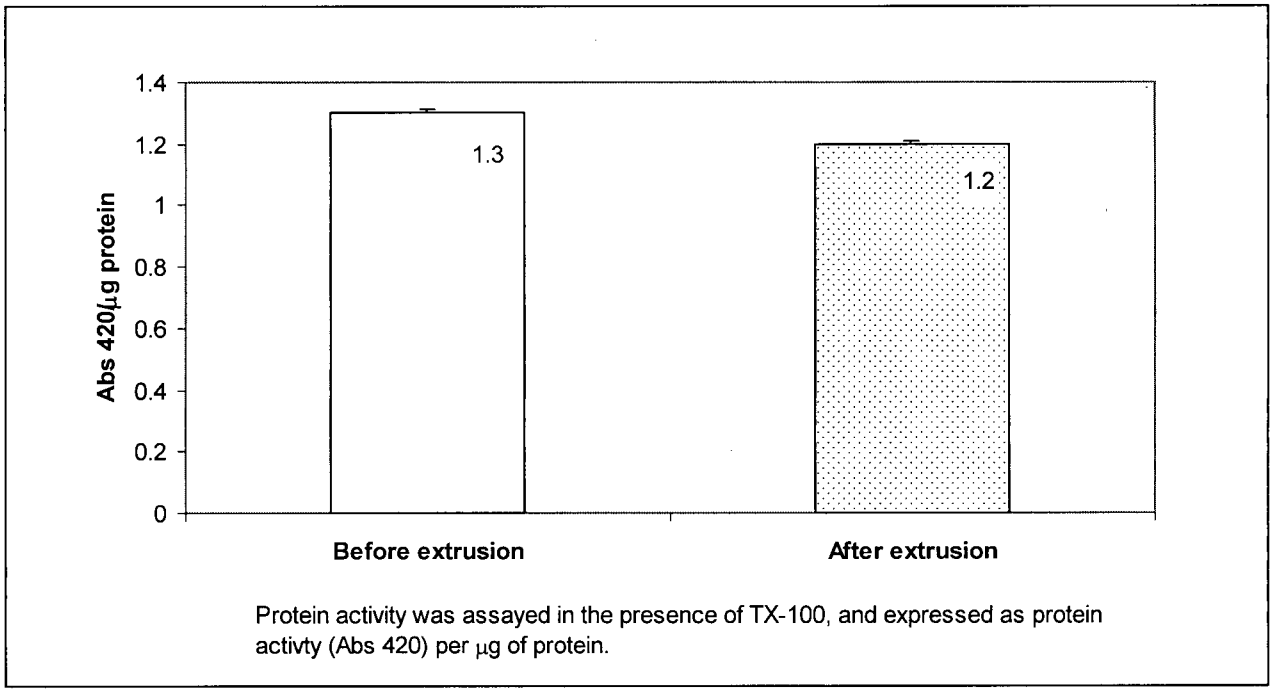
### 2.3.2 Characterization of encapsulated β-gal

Despite the fact that glycerol was removed from the β-gal solution, the protein activity throughout the formulation process remained stable with no change in the protein specific activity detected (Figure 2.2). It is common practice to employ 100 nm diameter LUV for *in vivo* applications (Hope et al., 1986). However, the 200 nm β-gal LUV could not be further extruded through a 100 nm pore size, presumably the presence of this large molecular weight protein inhibited the vesicle deformation required in order for the 200 nm LUV to extrude through the smaller pore size. The lipid free protein solution was found to pass through easily.

Figure 2.3 shows a gel filtration profile for a typical 200 nm LUV preparation. Separation of encapsulated and unencapsulated protein is good, despite the fact that the β-gal protein has a molecular weight of ~ 500,000 Da. Vesicles that elute in fractions 12 - 14 were collected for latency analysis. The latency of both POPC/Chol and POPC/Chol/DSPE-PEG β-gal LUV (Figure 2.4a and b respectively) were routinely measured at > 95%. The external activity is most likely due to small amounts of contamination from the free protein peak. This is supported by the observation that passing the vesicles down a second column produced a preparation with a latency > 99%, with the detection of unencapsulated protein at the limits of the assay procedure (Figure 2.4c). In addition, when the liposomal β-gal was briefly subjected to low pH treatment (pH 4.0) with $H_2SO_4$, vesicles with > 99 % latency were achieved with no loss in the entrapped protein activity (Figure 2.4d). The encapsulation efficiency (what proportion of the initial protein has been encapsulated) was ~ 18-20% for both formulations, which

is consistent with a vesicle trapped volume of approximately 2 μl/μmole of total lipid, expected for the size of vesicles used here.

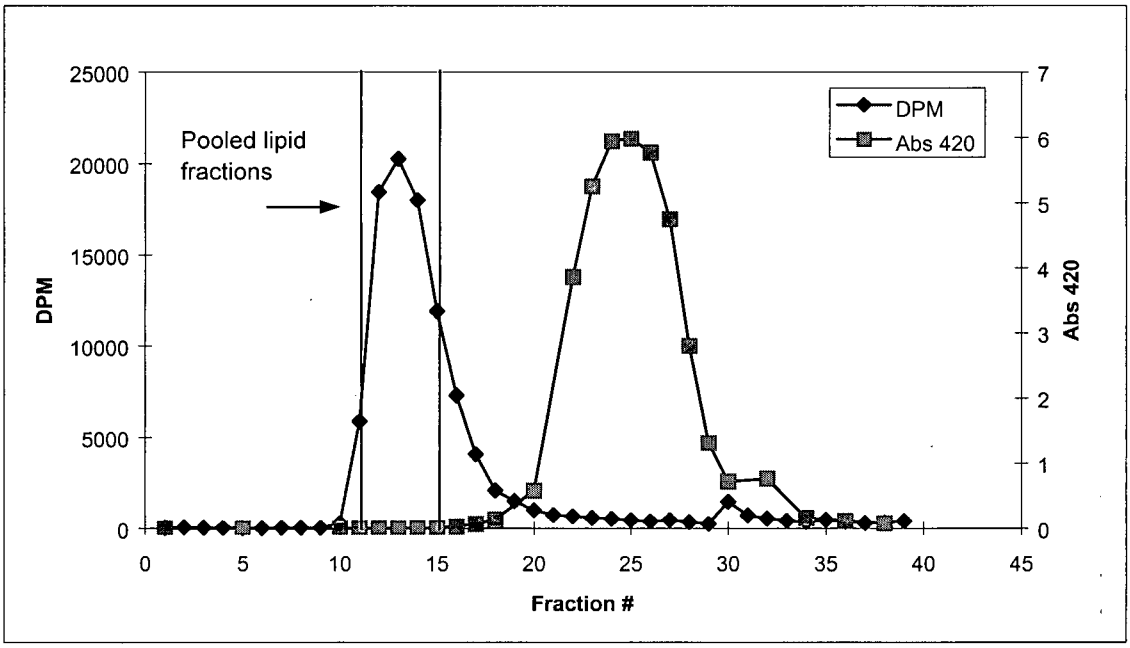
**Figure2.2 Comparison of protein specific activity before and after extrusion.**




Based on calculations which assume the LUV formulations consist of uniform spheres with a bilayer thickness of 5 nm and a lipid surface area of 0.6 $nm^2$/molecule [Deamer and Bramhall, 1986], it is estimated that there are 341,540 lipid molecules per lipid vesicle of 200 nm diameter [Harrigan, 1992]. Therefore, for a POPC:Chol (55:45) preparation, there are approximately $1.63 \times 10^{12}$ vesicles per mg of total lipid (see table 2.1). A typical formulation has 10 μg of β-gal encapsulated **inside** the liposomes per mg of total lipid (figure 2.4a for example), this corresponds to encapsulating **8** β-gal tetramers per lipid vesicle. To put this number in perspective, the internal volume of a 200 nm vesicle is $3.58 \times 10^{-21}$ $m^3$, and each β-gal tetramer is roughly 17.5 x 13.5 x 9.0 nm

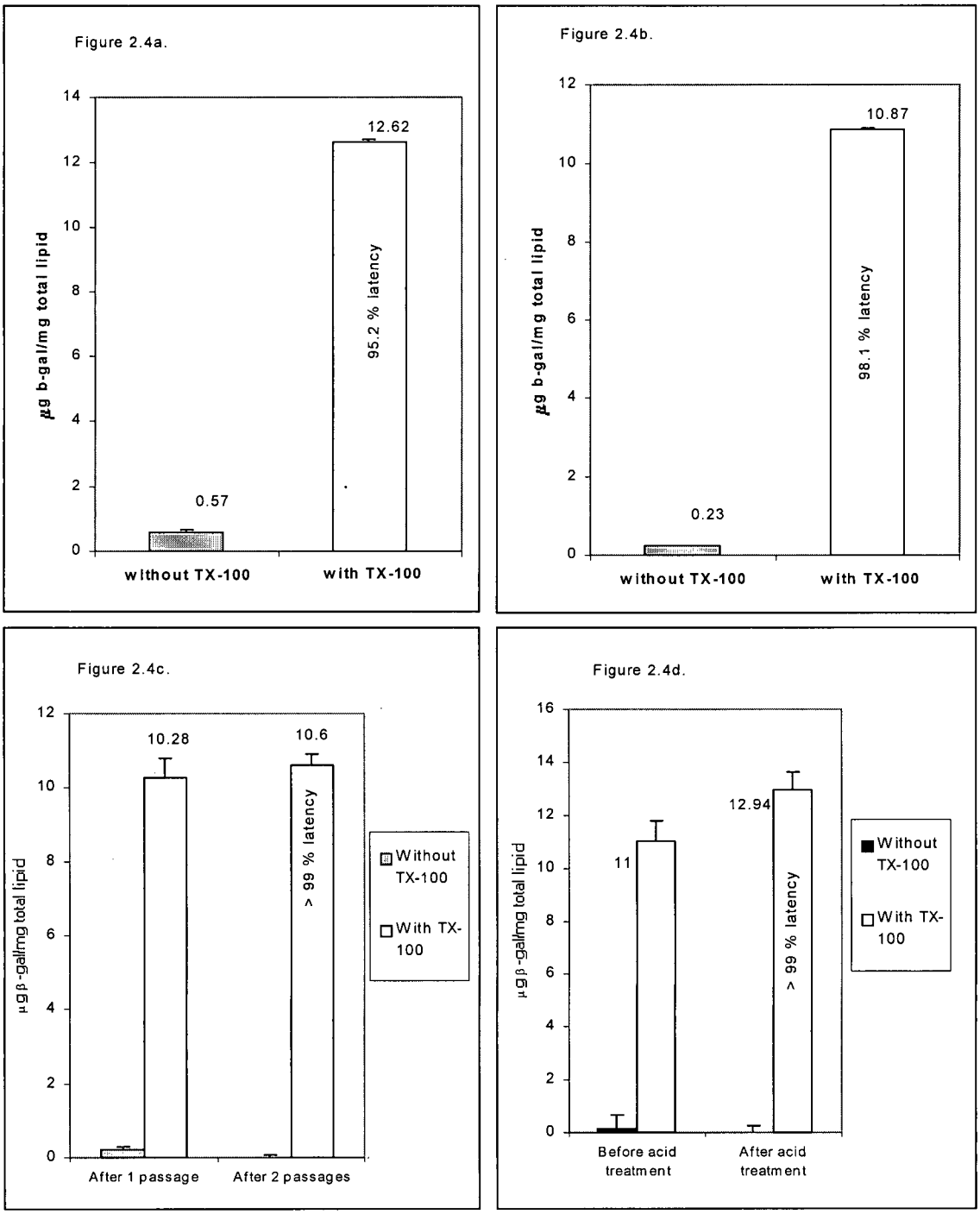
in size and occupies a volume of 2.12 x $10^{-24}$ $m^3$ (section 1.4). Consequently, the maximum number of β-gal molecules that could be packed inside a 200 nm LUV is ~1700.

**Figure 2.3** Separation of liposomal β-gal from free protein




Typical elution profile of liposomal β-gal (POPC:Chol + β-gal) expressed as DPM (lipid carrier) and Abs 420 (β-gal activity) assayed as 10ul per fraction. Similar elution profile was obtained for the PEG-coated liposomal β-gal vesicles.

**Figure 2.4** Latency measurement




Latency measurement of the (a) 200 nm POPC:Chol (55:45) liposomal β-gal, and (b) POPC:Chol:DSPE-PEG (55:40:5) liposomal β-gal. (c) Attempts to isolate vesicles with > 99 % latency was achieved by passing the PEG-coated liposomal β-gal through the sizing column twice, as well as by (d) exposing the pooled PEG-coated liposomal β-gal to pH 4.0 for two minutes. (n=3, ±SD).

**Table 2.1 Calculation of the number of β-gal molecules encapsulated inside POPC:Chol (55:45) liposomes.**

| | | |
|---|---|---|
| Vesicle diameter (nm) | | 200 |
| Total lipid molecules per vesicle | | 341540 |
| Vesicles per μmol phospholipid | | $1.76 \times 10^{12}$ |
| Vesicles per mg phospholipid[1] | | $2.31 \times 10^{12}$ |
| Vesicles per mg total lipid[2] | | $1.63 \times 10^{12}$ |
| μg β-gal per mg total lipid[3] | Outside of liposome | 0.5 |
| | Encapsulated inside liposome | 10 |
| Number of β-gal molecule per lipid vesicle[4] | Outside of liposome | 0.4 |
| | Encapsulated inside liposome | 8 |

1. MW of phospholipid = 760 g•mol$^{-1}$
2. MW of POPC:Chol (55:45) liposome = 592 g•mol$^{-1}$
3. See figure 2.4a
4. MW of β-gal = 470,472 g•mol$^{-1}$

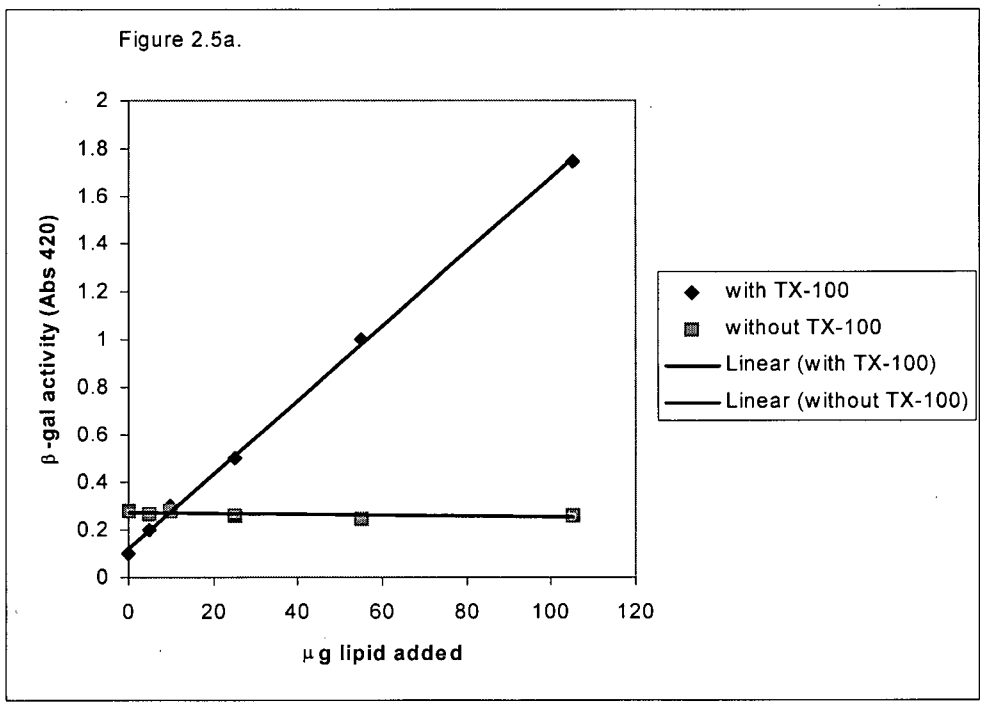
### 2.3.3 *In vitro* stability of β-gal and liposomal β-gal in buffer and plasma

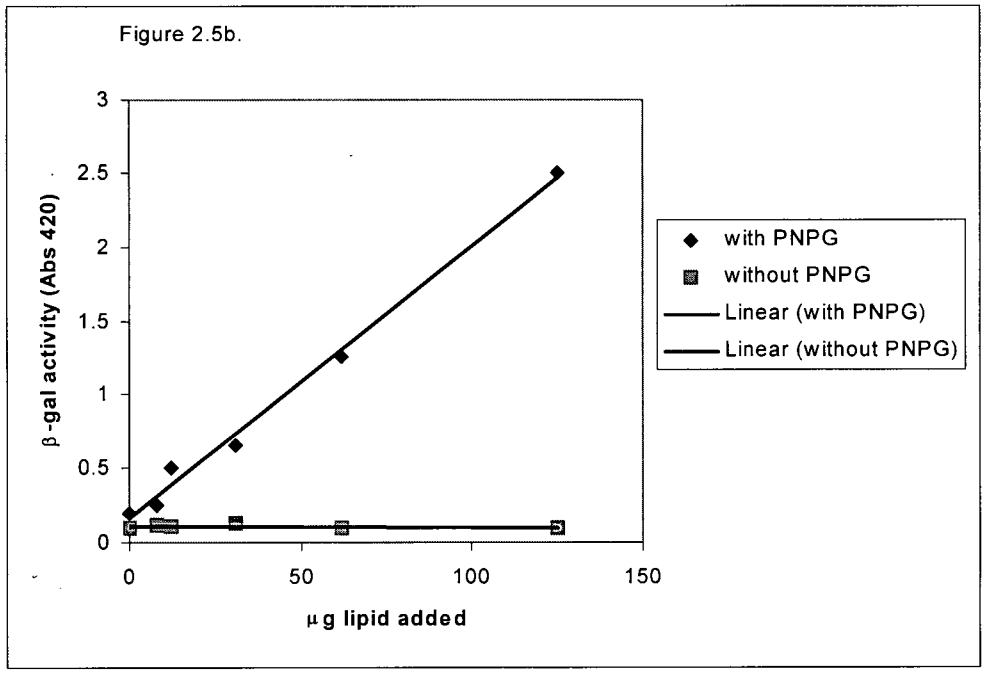
Prior to conducting the planned extensive studies *in vivo* it was important to validate the β-gal assay in the presence of serum proteins and measure the stability of both free and encapsulated protein in mouse plasma at 37 °C. From the encapsulation characteristics described above, it is possible to generate LUV that contain approximately 10 μg of β-gal per mg of lipid. After a single pass through a gel filtration column the total lipid concentration becomes diluted to approximately 20 mg•ml$^{-1}$. The maximum injectable dose (bolus) via the tail vein of a typical 20 g mouse is on the order of 200 μl, therefore the maximum amount of LUV that could be administered for the *in vivo* studies would be 4 mg of total lipid, equivalent to 40 μg of β-gal enzyme. A 20 g mouse contains 2 ml of blood that yields about 1 ml of plasma. In other words, immediately after i.v. administration of a 4 mg dose of lipid, and before any clearance from the

circulation, a 100 μl aliquot of plasma would be expected to contain ~ 400 μg of lipid. In order to adequately follow the kinetics of LUV and protein clearance from the blood it is desirable to be able to detect a circulating lipid dose that is greater than or equal to ~ 5% of the initial dose (i.e. 20 μg of lipid in a 100 μl aliquot of serum equivalent to 0.2 μg of β-gal protein). The graphs shown in figure 2.5 a and b show that there is excellent linearity in the presence of serum over the required range and that the level of sensitivity is such that the limit of detection is ~5% of the initial dose.

Detection of β-gal was dependent on the addition of TX-100, indicating that the β-gal activity being measured was encapsulated in liposomes and not accessible to the substrate even in the presence of plasma. A problem with assaying in plasma is the background scattering from lipoproteins when detergent is not present. Consequently, all samples, including blanks, were run in the presence of detergent and the reaction controlled by the presence or absence of the PNPG substrate (Fig. 2.5b). Samples from experiments with liposomal protein administered i.v. (chapter 3) were therefore assayed in the presence of TX-100 and in the presence or absence of PNPG. Absorbance measured in the absence of PNPG was taken as background scattering and subtracted from the absorbance in the presence of PNPG to obtain the true absorbance value arising from β-gal activity.

**Figure 2.5** PNPG assay validation

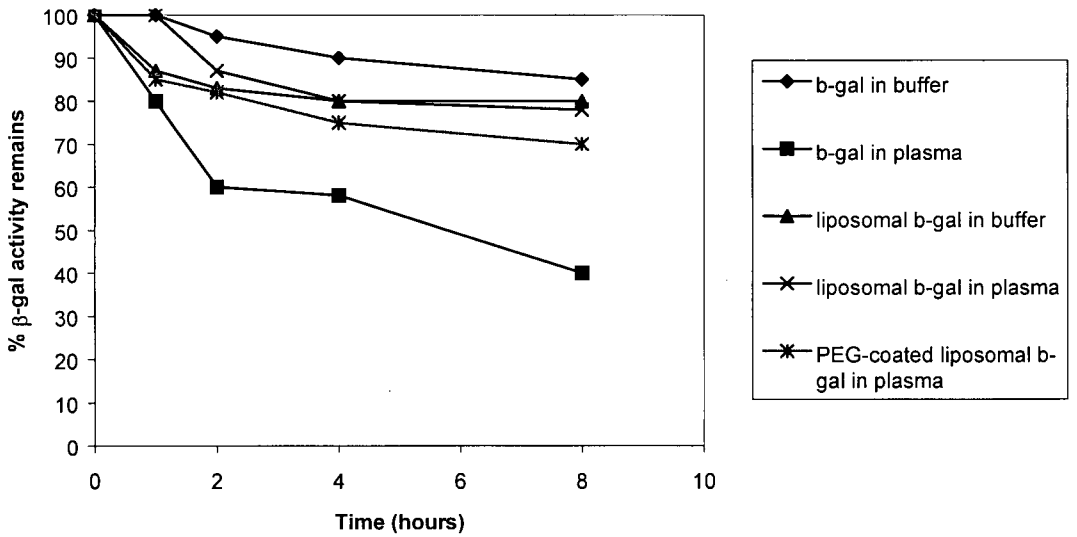



Samples were analyzed in (a) the presence or absence of 1% Trition X-100, and (b) the presence or absence of the substrate PNPG with Trition X-100.

Specific activity for free protein, liposomal β-gal and PEG-coated liposomal β-gal incubated in buffer and fresh plasma at 37°C are shown in figure 2.6. Free protein loses 60% of its activity over 8 hours when incubated in a 50 % dilution of mouse plasma. However, only a 20 % reduction in activity of the encapsulated β-gal is observed over the same period. Since both the free protein and the encapsulated β-gal exhibit the same 20% reduction in activity over 8 hours when incubated in buffer, it is likely that this loss is due to thermal destabilization of the protein at 37°C. It is not known why the loss of activity for free enzyme is greater in plasma than in buffer but it may be the result of protease degradation.

**Figure 2.6** ***In vitro*** **stability of β-gal, liposomal β-gal, and PEG-coated liposomal β-gal.**




Samples were incubated in buffer or in 50% dilution of fresh mouse plasma at 37°C. At the indicated times, β-gal activity was determined by PNPG assay as outlined in section 2.2.3. Data represent the average from two different experments.

## 2.4 Summary

Two β-gal formulations, composed of POPC:Chol and POPC:Chol:DSPE-PEG 2000, were prepared and characterized. Protein was trapped by hydrating lipid films in a protein solution at 37°C to form MLV. MLV were then sized by extrusion through polycarbonate filters with a pore size of 200 nm to form a homogeneous population of LUV with diameters of 200 ± 40 nm. Unencapsulated β-gal was removed by gel filtration. An assay was developed to measure enzyme activity in the presence of detergent and mouse serum. It exhibits a linear response over the concentration range required to conduct the *in vivo* experiments. Furthermore, the assay is sensitive enough to detect as little as 5% of the projected i.v. dose which was set as one of the criteria to be achieved before proceeding into animals.

Both formulations can be made routinely with > 95% latency after a single pass through a gel filtration column. External enzyme activity appears to arise from unencapsulated protein, which is either free in solution, or weakly associated with the vesicle surface. This external activity can be removed by a second gel filtration step or eliminated by briefly exposing the vesicles to low pH, which inactivates β-gal, without any loss of encapsulated activity.

Finally, both formulations appear to be quite stable in serum at 37°C over eight hours. Approximately 20-30% of the encapsulated proteins specific activity is lost during the incubation which is most likely due to the temperature, because a similar loss is

measured for the protein suspended in buffer alone. The free protein in serum loses 60% of its activity over the same incubation period. The data indicate that both formulations can be made reproducibly and their properties are suitable for the *in vivo* phase of this project.

# CHAPTER 3

# Pharmacokinetics of Liposomal β-Galactosidase in Normal and Immunized Mice

## 3.1 Introduction

The rationale for encapsulating therapeutic proteins in lipid carriers is to enhance their therapeutic index. This is accomplished by (1) increasing their circulation lifetime and biodistribution to sites of disease, (2) reducing their toxicity by gradual exposure of biological activity, in contrast to a bolus injection of unencapsulated protein and (3) minimizing any loss of biological potency by protecting the protein from denaturation in blood and tissue. However, it is not known whether these positive attributes of encapsulation are outweighed by the enhanced immune response expected due to the increase in delivery of therapeutic protein to antigen presenting cells such as macrophages and dendritic cells.

This chapter addresses the *in vivo* pharmacokinetic characteristics of free protein, empty vesicles and vesicles containing β-gal in normal mice, in mice that have been pre-immunized against β-gal, as well as in normal mice receiving repeated injections.

## 3.2 Materials and methods

### 3.2.1 Materials

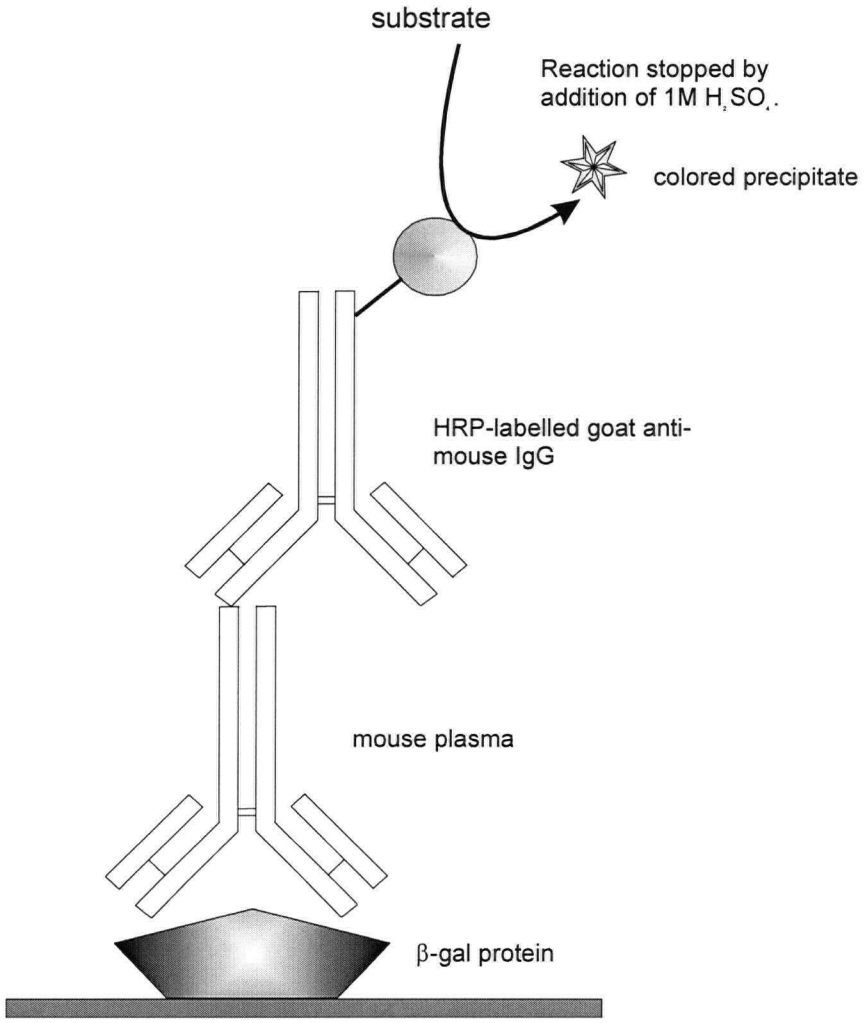
Unless otherwise specified, all lipids and chemicals used in this chapter were the same as outlined in section 2.2.1.

### 3.2.2 Measurement of anti β-gal antibodies in mouse serum

Antibody against β-gal in plasma was determined using an indirect, two-step ELISA. The following reagents: SuperBlock™ Blocking buffer in PBS, Tween 20, horseradish peroxidase conjugated ImmunoPure®Goat anti-mouse IgG ($F_c$), and 1-Step™ Turbo TMB-ELISA solution were purchased from Pierce (Rockford, Il). All other reagents were of analytical grade. Plasma samples from individual mice were assayed in triplicate as follows. Briefly, ELISA plates (Becton Dickinson Labware, Lincoln Park, NJ) were coated overnight at 4°C with 40 $\mu g \bullet ml^{-1}$ of β-gal in carbonate-bicarbonate buffer (0.015 M $Na_2CO_3$, 0.035 M $NaHCO_3$, 0.003 M $NaN_3$, pH 9.6). Plates were washed three times with 200 µl PBS-Tween 20 containing 0.1% (w/w) BSA, followed by blocking with 200 µl SuperBlock™ blocking buffer. Mouse plasma to be assayed was diluted from 10 to 78,000 x in SuperBlock™ Blocking Buffer with 0.05% (v/v) Tween 20 and applied to the plates as the primary Ab. Samples were incubated at room temperature for 1 hour, after which a 100 µl aliquot of the horseradish peroxidase conjugated goat anti-mouse IgG (diluted 1:1000) was used. After incubation for 2 hours at room

temperature, plates were washed and 125 µl of 1-Step™ Turbo TMB substrate was added for 15 minutes. The reaction was stopped by the addition of 125 µl of 1 M $H_2SO_4$. Plates were read in a Dynatech 5000 ELISA plate reader at 450 nm. Controls consisted of plasma samples obtained from untreated mice.

**Figure 3.1** **Measurement of anti β-gal IgG by indirect two-step ELISA. Primary antibody reacts with bound antigen and a labeled secondary antibody reacts with the primary antibody.**



### 3.2.3 Immunization of mice against β-galactosidase

A variation of the method described by Tardi et al. [1997] was employed to immunize mice against β-gal protein. These authors showed that PEG-coated liposomes acted as potent adjuvants in generating a humoral response against ovalbumin when mixed with the protein and administered intraperitoneally to mice. In addition, Tardi et al. (personal communication) demonstrated that repeat administration of liposomal ovalbumin subcutaneously was even more effective at eliciting an antibody response. Therefore, the PEG-coated vesicle formulation of β-gal described in chapter 2 was used as the vaccination material and was administered subcutaneously. A 100 μl aliquot in HBS containing 0.96 mg of total lipid (POPC:Chol:DSPE-PEG 2000, 55:40:5 mole ratio) and 11.7 μg of encapsulated β-gal was injected under the skin in the abdominal area of BDF-1 mice. Injections were given at intervals of 9 day for a total of 3 injections.

To investigate the therapeutic effect upon repeated administration of identical formulations, two groups of mice were established to compare PEG free and PEG-coated preparations with β-gal encapsulated. Identical dosage was used for each i.v. injection. At one hour following administration, the blood was analyzed to determine the percentage of initial dose remains in the circulation, as well as for monitoring the generation of β-gal antibodies. This was repeated for a total of five weekly injections.

### 3.2.4 Pharmacokinetic studies

The pharmacokinetic studies were carried out in female BDF-1 mice (Harlan Sprague Dawley, Prattville, Al). Mice were 8 weeks old at the beginning of the experiment and weighed approximately 20 g. A total lipid dose of 60 mg•$Kg^{-1}$ body weight was administered for this series of experiments. The liposomal formulations were diluted in HBS, such that the required dose could be administered in approximately a 200 µl injection volume via the tail vein. The β-gal content of the liposomal preparation was determined using the latency assay described, so that the same dose of free β-gal could be administered to the experimental group of mice receiving protein only. At various times following i.v. administration of the samples a group of 4 mice per time point were given an i.p. injection of a ketamine/xylazine cocktail (160/4 mg•$Kg^{-1}$) for general anesthesia. Blood was collected by cardiac puncture and transferred to Microtainer® tubes containing EDTA as anticoagulant. The blood was centrifuged at 500 g for 10 minutes and aliquots of the plasma were removed for scintillation counting to determine lipid concentrations and PNPG assay for β-gal activity.

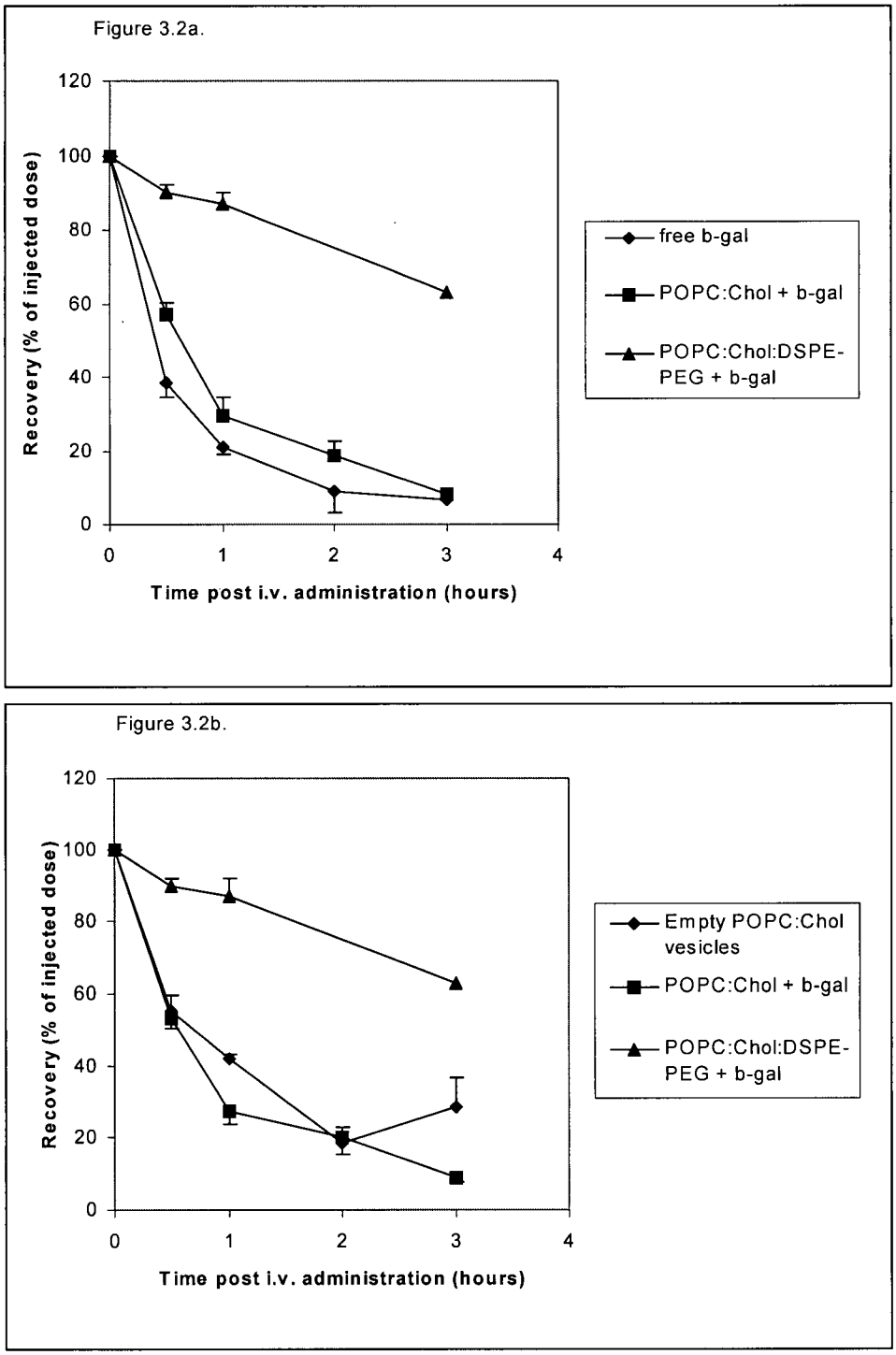
## 3.3 Results

### 3.3.1 Pharmacokinetics of free and encapsulated β-galactosidase in normal mice

In normal (non-immunized) mice free β-gal, tracked by measuring enzyme activity in the serum, is cleared from the circulation at approximately the same rate as β-gal encapsulated in PEG-free vesicles (Figure 3.2a). In contrast, the effect of PEG-

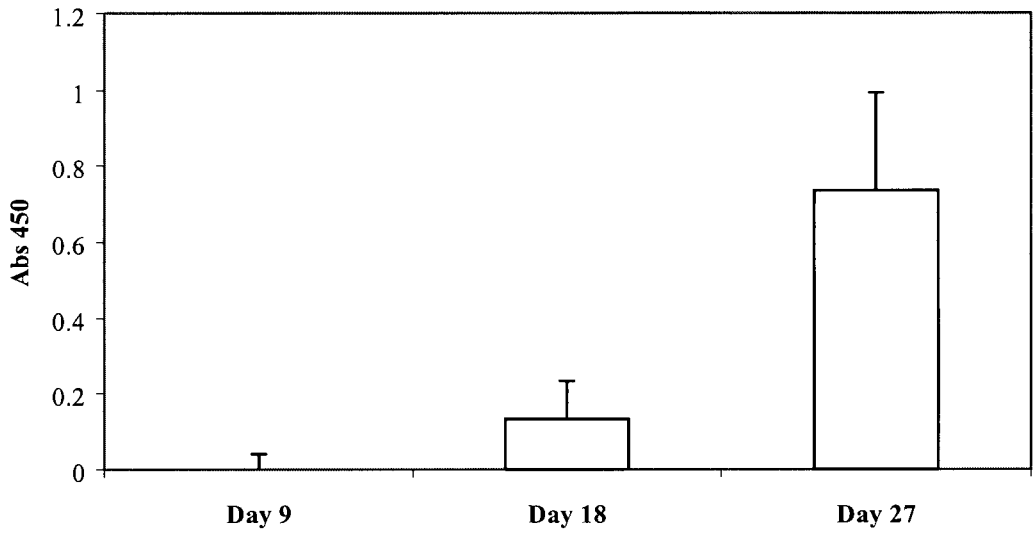
coating on opsonization and clearance of β-gal containing vesicles is striking. At 3 hours 70 % of the injected dose of β-gal is still in the blood for the PEG-coated formulation compared to < 10 % for both the naked vesicle formulation and free protein. Lipid clearance profiles are shown in Figure 3.2b, and are almost identical to the protein activity curves (Figure 3.2a). In other words, the specific activity of encapsulated β-gal per mole of lipid does not change in the blood during the 3 hour time course for either formulation. Furthermore, empty POPC:Chol vesicles exhibit the same clearance profile as POPC:Chol vesicles loaded with β-gal protein, indicating that the two preparations are treated similarly by the bodies particle clearance mechanisms. The rate of vesicle clearance is consistent with the lipid dose and vesicle diameter.

**Figure 3.2 Pharmacokinetics of free and encapsulated β-galactosidase in normal mice.**




*In vivo* clearance kinetics of (a) the encapsulated protein, and (b) the lipid carrier in normal mice. Blood was collected at various time points and the amount of β-gal and lipid carrier remaining in the circulation was determined by PNPG assay, and by LSC, respectively. The result is plotted as an average obtained from four animals. (n=4, ± S.D.)

**Figure 3.3** Generation of anti β-gal IgG upon repeat s.c. injection.



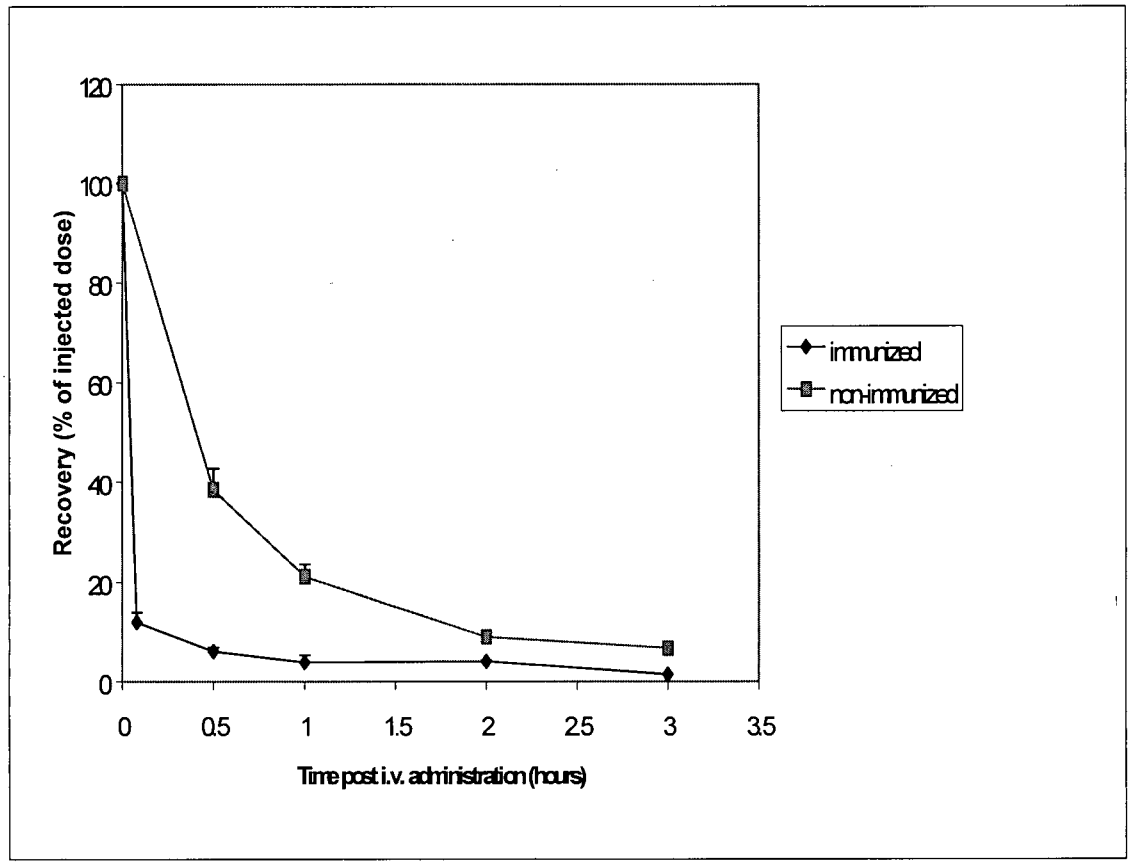

The level of anti β-gal IgG induced after three s.c. inoculations with the PEG-coated liposomal β-gal was monitored by ELISA as outlined in section 3.2.2. Measurement was taken from 0.025 dilution of mouse plasma. (n=4, ±SD)

### 3.3.2 Pharmacokinetics of free and encapsulated β-galactosidase in immunized mice

In the previous section it was shown that in normal mice there is not a large difference between the clearance of free β-gal and β-gal encapsulated in POPC:Chol vesicles. However, in mice carrying antibodies against β-gal the free protein is expected to immediately form antibody-antigen complexes that are rapidly cleared from the circulation, whereas encapsulated β-gal will be protected and therefore the circulation time should be the same as that measured in normal animals.

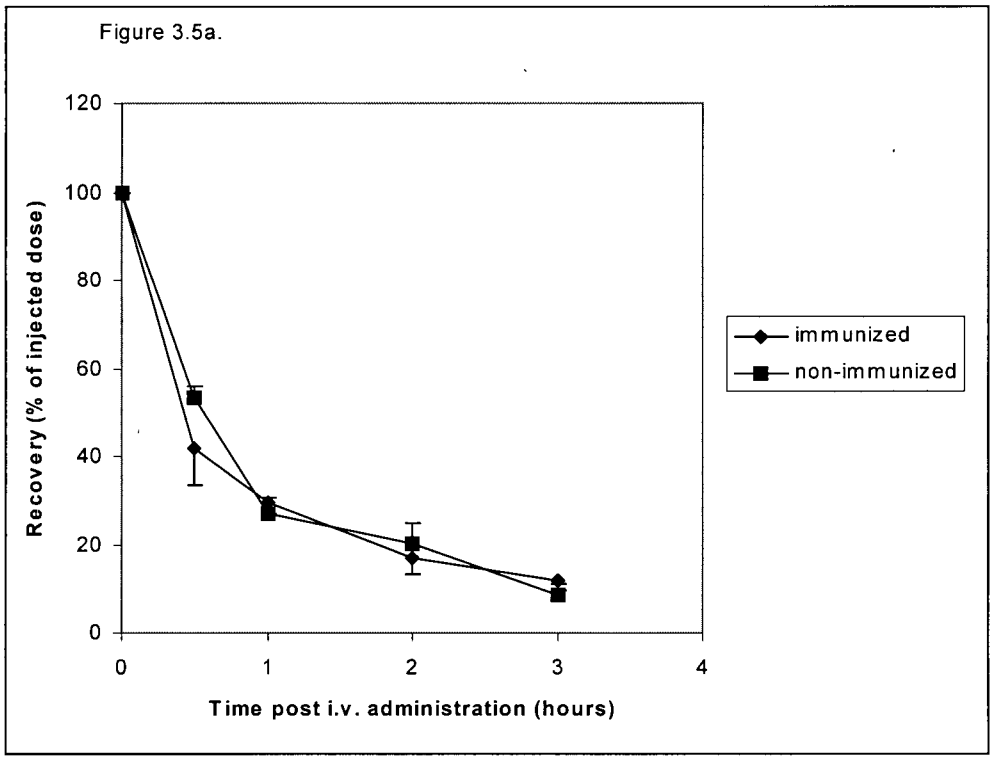
To test this hypothesis mice were immunized against β-gal protein as described in section 3.2.3. The generation of anti β-gal antibodies was monitored by ELISA (Figure 3.3), and after the third subcutaneous injection (day 27) a strong humoral response could be detected. As expected, the effect of immunization on clearance of free protein from the circulation is pronounced (Figure 3.4). At the earliest time point that can be measured (10 minutes) only 10% of the injected dose of free β-gal is detected in the blood of the immunized group of mice. This level drops to the background limits of the PNPG assay (< 5% of the injected dose) for the remainder of the 3 hour time course. However, when the same dose of protein is administered encapsulated in POPC:Chol vesicles there is no apparent effect on vesicle or protein clearance rates as the kinetics are almost identical to those observed in normal animals (Figure 3.5a and b). The same behaviour is not observed for PEG-coated, β-gal containing vesicles. Surprisingly, this formulation is cleared as rapidly as free protein when administered at the same dose to immunized animals (Figure 3.6a and b). For this experiment β-gal levels in the blood were not measured as early as 10 minutes, but at 30 minutes both β-gal activity and lipid concentration were < 10 % of the injected dose compared to 90 % in the non-immunized group.

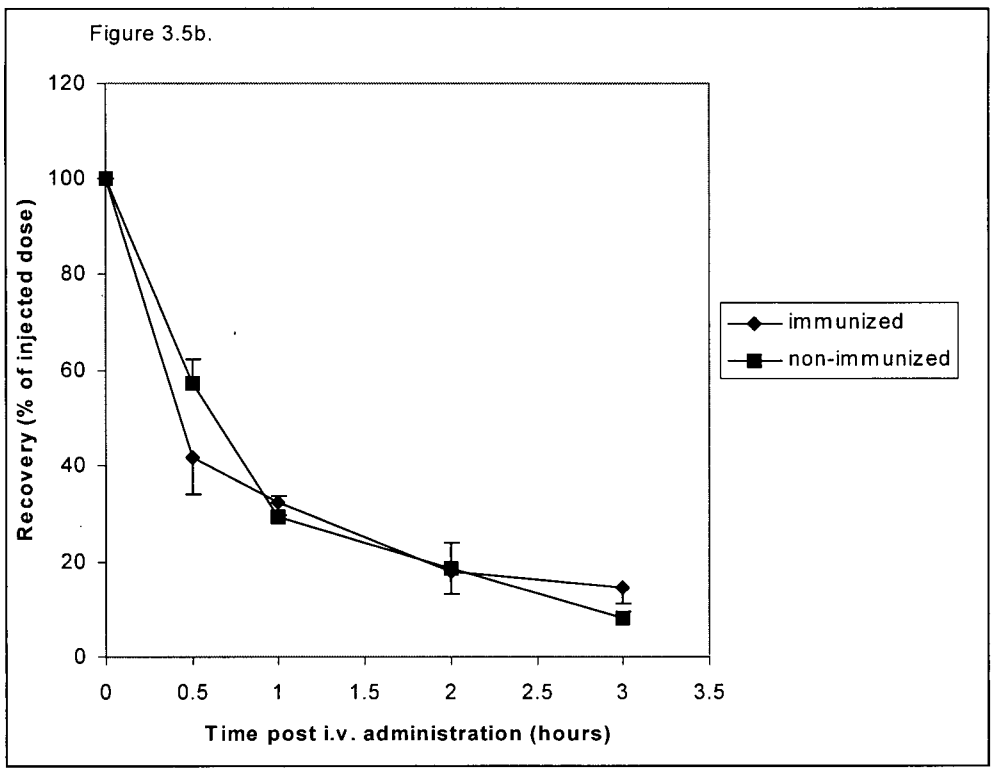
**Figure 3.4 A comparison of the circulation life-time of free β-gal in immunized and non-immunized mice.**




Mice were immunized by repeat s.c. administration with PEG-coated liposomal β-gal. Plasma was collected, and β-gal activity was determined by PNPG assay. (n=4, ±SD).

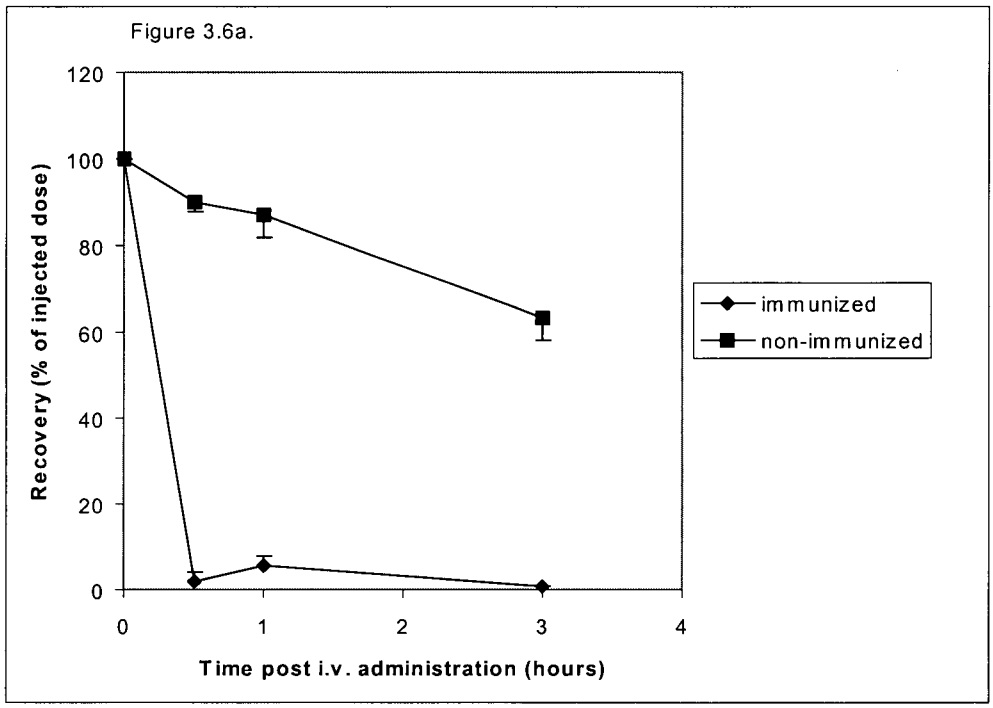
**Figure 3.5** Clearance kinetics of liposomal β-gal.

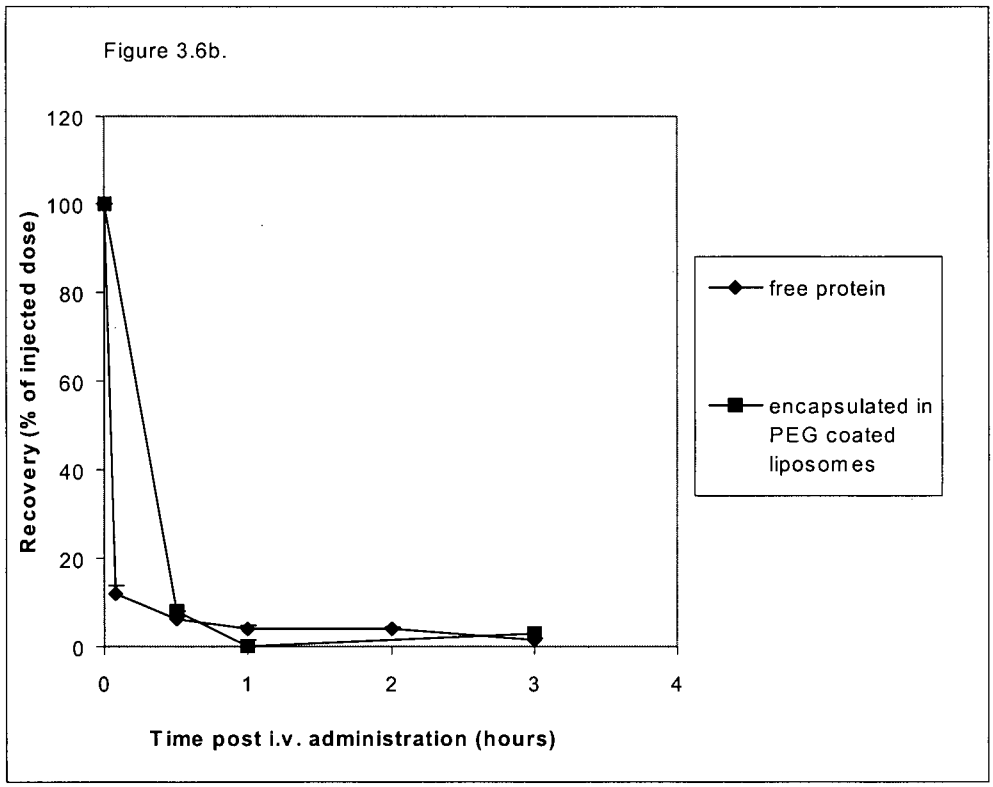



Comparison of the clearance kinetics of liposomal β-gal (POPC:Chol + β-gal) in immunized and non-immunized mice. The clearance kinetic was monitored by following the rate of clearance of (a) the lipid carrier, and (b) the protein activity. (n=4, ±SD).

**Figure 3.6** Clearance kinetics of PEG-coated liposomal β-gal.

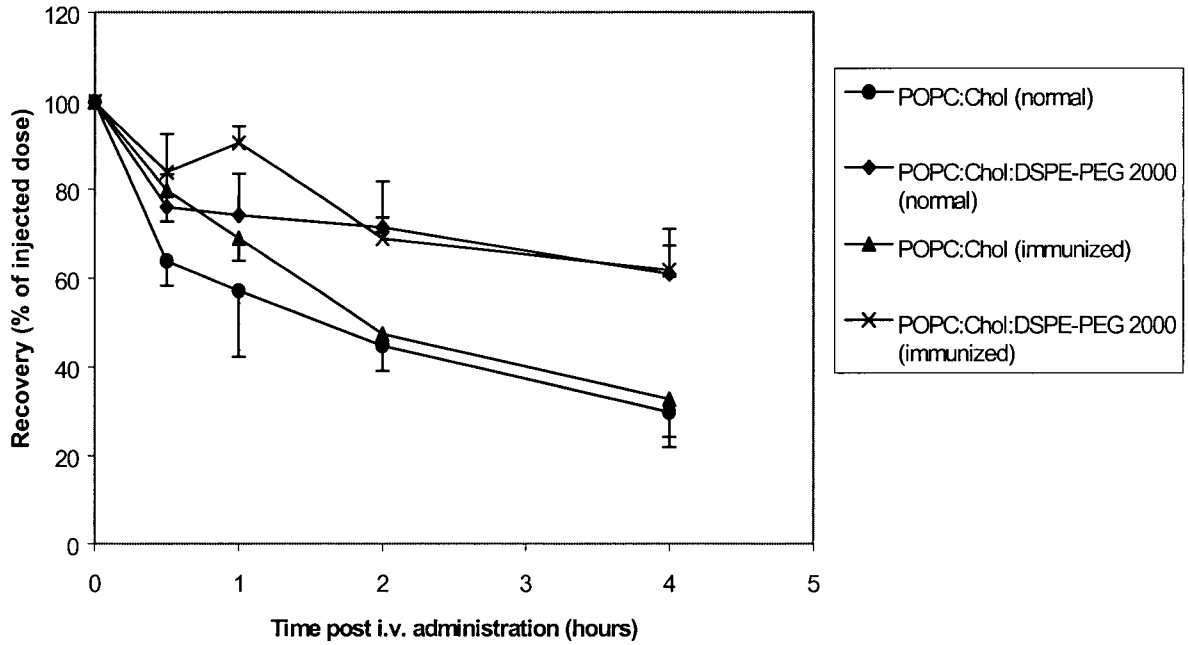



(a) Comparison of the clearance kinetics of PEG-coated liposomal β-gal (POPC:Chol:DSPE-PEG + β-gal) in immunized and non-immunized mice was monitored by following the serum concentration of the lipid carrier. (b) In immunized mice, both free protein and protein encapsulated in the PEG-coated liposomes exhibit similar rapid clearance profiles (n=4, ±SD).

This result indicates that the PEG-coated vesicles are recognized by a component of the immune system in the immunized animals whereas the naked POPC:Chol vesicles are not. The physical characterizations indicate that both formulations are similar with respect to size, protein encapsulation and latency (Figure 2.4). One important point to note is that for the purposes of this study mice were immunized using β-gal encapsulated in PEG-coated vesicles. Therefore it is possible that antibodies may also have been raised against the PEG-coating on the vesicle surface. This is unlikely as PEG has been widely used to mask the immunogenicity of proteins and is considered non-immunogenic (Ueno et al., 1996; recent review by Duncan and Spreafico, 1994). However, in order to test whether this was the case, two groups of mice were "immunized" with either empty POPC:Chol or PEG-coated POPC:Chol vesicles, following the same procedure as outlined in section 3.2.4. for immunization against β-gal. Vesicle clearance was then measured and compared to non-immunized animals. The data show no effect from immunization with lipid only on the clearance kinetics of either PEG-coated POPC:Chol or POPC:Chol vesicles (Figure 3.7).

**Figure 3.7 Immunization with protein free vesicles has no effect on the pharmacokinetics of vesicle clearance.**




Mice were immunized by three repeat s.c. injection with either empty POPC:Chol or PEG coated POPC:Chol vesicles given at 9 day intervals as oulined in section 3.2.3. Following immunization the kinetics of vesicle clearance was measured and compared to a normal, non-immunized experimental group. Plasma was collected at the indicated times and lipid concentrations determined by LSC. (n=4, +SD)

### 3.3.3 Pharmacokinetics and humoral immune response to encapsulated β-galactosidase following repeated weekly administration

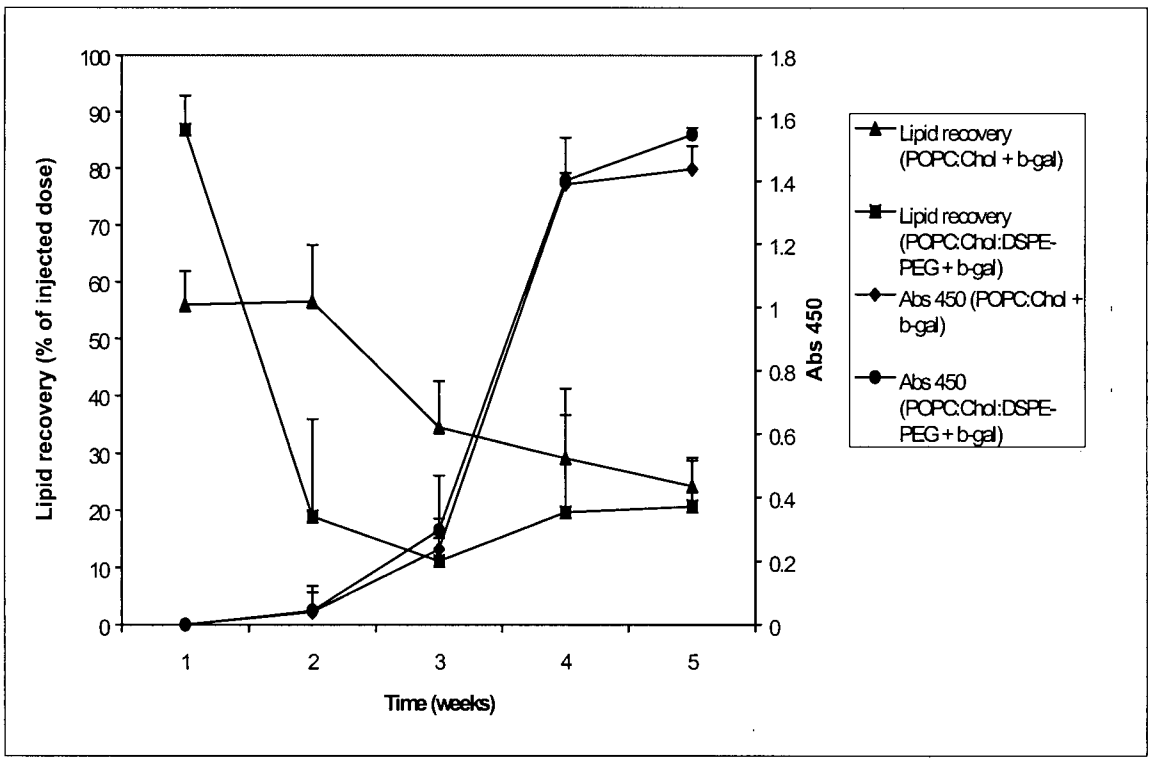
The experiments described above were conducted in mice pre-immunized against β-gal and show that both free protein and protein encapsulated in PEG-coated liposomes are sensitive to the presence of antibodies which cause them to be cleared rapidly from the circulation compared to their circulation $T_{1/2}$ in normal mice. Interestingly, naked POPC:Chol vesicles protect β-gal, and the clearance kinetics for protein in this formulation are the same in both normal and immunized mice. In the experiment

described below, the effect of repeated injection on vesicle and β-gal clearance is monitored.

Two experimental groups were established to compare PEG-free and PEG-coated preparations, each containing enough mice to allow the analysis of 5 animals per time point. All the animals received the same dose of encapsulated β-gal. One hour following the first administration five mice from each group were sacrificed and the blood analyzed for radioactivity to determine the % of initial dose still in the circulation and a sample was also analyzed for the presence of anti β-gal antibodies. This was repeated for a total of five weekly injections. After the first injection the level of lipid in the blood was within the normal range previously measured for the two formulations. Approximately 50% of the injected dose for POPC:Chol β-gal and 90% for the PEG-coated vesicles (Figure 3.8). However, after the second i.v. administration there was a dramatic drop in the circulating concentration of PEG-coated vesicles at one hour, from 90% to 20% of the injected dose. In contrast, the % injected dose remaining for the uncoated, POPC:Chol β-gal formulation was unchanged at 50%. The effect of the immune response on PEG-coated vesicle clearance does not change significantly over the next three weekly injections. However, the % dose remaining at one hour for the uncoated formulation gradually decreases between weeks two and three, so that at week five both preparations appear to be cleared at similar rates with only ~20% of the initial dose in the blood (Figure 3.8).

Interestingly there does not appear to be a direct correlation between the extent of an IgG humoral response to the repeat injections and the rate of particle clearance from the blood. The most dramatic change in clearance occurs for the second administration of PEG-coated vesicles when the antibody response can only just be detected. Moreover, the antibody response to both vesicle formulations of β-gal is identical but the effect on vesicle clearance, particularly the second injection, is very different.

**Figure 3.8 Correlation between liposomal β-gal clearance kinetics and the generation of anti β-gal IgG following weekly i.v. administration.**




All measurements were made at 1 hour post administration. The level of Ab was determined by ELISA (Abs 450) as discussed in section 3.2.2, and the clearance kinetic was assessed by determining lipid concentration by LSC and expressing the data as % of injected dose. (n=5, ±SD).

### 3.3.4 Phenotypic response of mice to β-galactosidase formulations

The work described in this thesis is designed to test the hypothesis that immunogenic proteins can be repeatedly administered in an encapsulated form without causing acute toxicity resulting from the formation of antibody-antigen complexes in plasma. The experiments described so far have quantified both the IgG humoral response and clearance of protein and carrier from the circulation. However, if an animal suffers an acute immune reaction to material being injected this can usually be observed through the animals' behaviour. In this section the gross phenotypical responses of the mice during, and for the first hour following, each administration are summarized.

**Table 3.1 Phenotypic response following bolus i.v. administration to mice pre-immunized against β-gal (section 3.2.2).**

| Experimental Group | Free β-gal | POPC/Chol β-gal | PEG-coated POPC/Chol β-gal |
|---|---|---|---|
| **Non-immunized:** | | | |
| # React/Total # Animals | 0/16 | 0/16 | 0/16 |
| Severity of Reaction | 0 | 0 | 0 |
| **Pre-immunized:** | | | |
| # React/Total # Animals | 3/16 | 2/16 | 6/12 |
| Severity of Reaction | 1 | 2 | 3 |

Scale of severity:

0 No observable effects from injection.
1 Animals motionless but recovery within minutes.
2 Some convulsions immediately upon injection and recovery takes several minutes.
3 Severe response resulting in convulsions, lack of balance, respiratory difficulty and recovery 0.5-2 hours.

**Table 3.2 Phenotypic response following five weekly bolus i.v. administrations to normal mice.**

| Time of Injection (weeks) | Free β-gal | POPC/Chol β-gal | PEG-coated POPC/Chol β-gal |
|---|---|---|---|
| **Week 1** | | | |
| # React/Total # Animals | 0/43 | 0/40 | 0/43 |
| Severity of Reaction | 0 | 0 | 0 |
| **Week 2:** | | | |
| # React/Total # Animals | 3/33 | 3/33 | 27/33 |
| Severity of Reaction | 2 | 2 | 3 |
| **Week 3:** | | | |
| # React/Total # Animals | 1/29 | 1/29 | 10/28 |
| Severity of Reaction | 1 | 2 | 2 |
| **Week 4:** | | | |
| # React/Total # Animals | 1/23 | 0/23 | 0/21 |
| Severity of Reaction | 1 | 0 | 0 |
| **Week 5:** | | | |
| # React/Total # Animals | 2/18 | 1/18 | 0/16 |
| Severity of Reaction | 1 | 1 | 0 |

Scale of severity:

0 No observable effects from injection.

1 Animals motionless but recovery within minutes.

2 Some convulsions immediately upon injection and recovery takes several minutes.

3 Severe response resulting in convulsions, lack of balance, respiratory difficulty and recovery 0.5-2 hours.

It is interesting to note (table 3.1) that 2 out of 16 animals in the pre-immunized group receiving β-gal encapsulated in POPC:Chol exhibited responses upon injection despite the fact that there was no effect seen upon the clearance kinetics of vesicles from the plasma. This is in contrast to the free protein, which produced a similar phenotypic reaction (3 out 16 mice at grade 1) but the rate of protein clearance also increased

markedly. The rate of clearance was also enhanced for the PEG-coated vesicles, but in this group 50% of the animals experienced a severe reaction to the administration.

In the multiple injection experiment (table 3.2) there was very little response seen for the free protein or POPC:Chol groups. Whereas, the second and third injections given to the group receiving PEG-coated vesicles induced a severe immune reaction. But by the 4th and 5th week the animals appeared to tolerate the injection even though the vesicles were still cleared rapidly from the circulation.

## 3.4 Summary

In this chapter the key objectives of the thesis were completed. It was demonstrated that encapsulating an antigenic protein inside a lipid vesicle can protect the protein from humoral antibodies but that this protection may be limited and vesicle dependent. The blood clearance profiles of two β-gal formulations, POPC:Chol and PEG-coated POPC:Chol, were measured in normal and immunized mice. In normal mice the two vesicle preparations behaved as expected. The 200 nm LUV vehicle was cleared from the circulation with a half-life of approximately 45 minutes, which was similar to that measured for free protein at the same protein dose. The presence of a PEG coat slowed clearance of the lipid carrier and its protein payload substantially, increasing the circulation half-life more than 5-fold. However, in the presence of β-gal antibodies PEG-coated vesicles were rapidly cleared from the blood with kinetics similar to the free protein which exhibited a half-life < 10 minutes. This contrasts to β-gal encapsulated in POPC:Chol vesicles, for which both lipid and protein circulation times remain unchanged

from that measured in normal mice. Furthermore, the PEG-coated formulation was more toxic upon injection into immunized animals than either free protein or protein encapsulated in PEG-free vesicles.

Paradoxically, PEG-coated vesicles were included in this study because it was assumed they would represent the most effective vehicle for systemic delivery of antigenic proteins. The effect observed in immunized mice, however, was also seen in normal mice receiving weekly injections. By only the second injection the amount of the administered dose remaining in the blood at one hour dropped from 90% to 20% for PEG-coated vesicles, but remained unchanged for PEG-free vesicles. By the 5th injection both formulations were cleared at similar rates. Moreover, the PEG-coated formulation of β-gal was toxic to more than 80% of the animals following the 2nd injection. These data indicate that the effect of an immune response against an antigenic protein encapsulated in a lipid carrier is complex and may limit the application of particulate carrier systems for antigenic drugs.

# CHAPTER 4

# DISCUSSION

## *4.1* Enzyme replacement and protein therapeutics

Many diseases are caused by genetic defects in which a specific protein, such as an enzyme, is either missing or inactive. Advances in sequencing the human genome mean that many of the genes responsible for producing these proteins have been or will shortly be identified. This knowledge, combined with the ability to produce pharmaceutical grade proteins on a large scale from genetically engineered microorganisms or cultured animal cells, means that protein therapy to manage these diseases is possible. Enzyme replacement strategies are an illustration of how large, biologically active proteins can be useful in controlling a disease. One example is in severe combined immune deficiency (SCID) a disorder caused by a missing or defective enzyme in the metabolic pathway responsible for the catabolism of purines. The enzyme is adenosine deaminase (ADA), and ADA-deficient SCID arises because the accumulation of a toxic metabolite (deoxyadenosine monophosphate and deoxyadenosine triphosphate) in T-cells disables the immune system [Blaese et al., 1995]. Lipid storage disorders are also caused by single enzyme deletions. Gaucher's disease is the most prevalent and is caused by an excessive accumulation of glucocerebroside in organs and tissues. Patients lack the enzyme glucocerebrosidase, which normally catalyzes the

cleavage of glucose from glucocerebroside. In the absence of this enzyme, glucocerebroside accumulates in macrophages, especially those located in liver and bone marrow. These cells are responsible for the turnover and reutilization of membrane lipids from senescent red blood cells, which are rich in glycolipids [Brady & Barton, 1996; Grabowski et al., 1995].

Both diseases are currently managed quite effectively by regular, systemic administration of purified enzymes. In the case of ADA-deficient SCID, the therapeutic enzyme is covalently linked to PEG, which slows opsonization and enhances the circulation lifetime of the protein [recent review by Burnham, 1994] by the same steric hindrance mechanism described earlier for liposomes. In Gaucher's disease the rationale is to target glucocerebrosidase to macrophages rather than develop a drug which exhibits extended circulation characteristics. Therefore, the enzyme's carbohydrate groups are modified by sequential treatment with exoglycosidases to expose mannose groups. Mannose is recognized by a lectin associated with the surface of macrophages. In both of these examples the enzyme drugs are the only therapy available for patients suffering from a fatal disease, they do not represent a cure but a means of managing their disorder. Consequently, the immune side effects resulting from long-term applications are tolerated. The most common toxicity results when patients develop antibodies against the therapeutic protein. In addition to the increased rate of clearance and resulting decrease in the drug's effectiveness, antigen-antibody immune complexes can cause kidney, joint and blood vessel damage over time.

## *4.2* Formation of antigen-antibody complexes and vesicle clearance

Encapsulation of enzymes in lipid vesicles or liposomes represents a potential means of administering long-term protein therapy whilst avoiding chronic toxicity associated with the formation of antigen-antibody complexes once patients become seropositive to the drug. However, paradoxically, the lipid vehicle is known to behave as an adjuvant and enhance the immune response against encapsulated antigen [Shek et al., 1986; Alving, 1995]. The purpose of this study was to determine whether this presented a practical problem with respect to delivering biologically active protein to a target site or not.

The assumption is that any increase in the rate of clearance of vesicles or protein from the circulation results from the formation of immune complexes with circulating antibodies, which increase particle size by multivalent cross-linking, enhance macrophage uptake through Fc receptors and accelerate opsinization through complement activation. The data indicate that lipid encapsulation prevents the formation of β-gal antigen-antibody immune complexes in pre-immunized animals, as the rate of clearance for encapsulated β-gal is almost identical to that measured in normal mice. It is reasonable to assume that the lipid membrane represents an impermeable barrier to the antibodies. This is in contrast to free protein, the majority of which is cleared immediately. Surprisingly, the rate of clearance of PEG-coated β-gal vesicles also increases dramatically. One explanation could be that there is sufficient β-gal adhered to the surface of PEG-coated vesicles to form immune complexes in the serum. However, there is little evidence to support this. The small amount of unencapsulated protein that

co-elutes with the vesicle fraction from the gel filtration column does not appear to be bound to the vesicle surface as it is readily removed by additional filtration steps. Moreover, the same amount of unencapsulated "contaminating" β-gal is administered with both types of vesicle, uncoated and PEG-coated, yet only the clearance of PEG-coated vesicles is affected. Furthermore, the PEG coat would be expected to decrease protein binding to the vesicle surface, which is why it is included in the composition. It is interesting to note that the PEG-coated formulation was used to immunize the mice. Perhaps a humoral immune response was raised against the PEG as well as the encapsulated β-gal. However, the data shown in figure 3.7 demonstrate that empty vesicles (lipid only, no β-gal) are not strongly immunogenic. Perhaps the presence of protein acts as an adjuvant, enhancing the humoral response to a degree that anti-PEG antibodies are generated (see section 4.4 for further discussion).

## *4.3* Repeated administration and vesicle clearance

A critical aspect of enzyme replacement therapy is that treatment manages the disease but does not cure it. Therefore, protein drug must be administered regularly throughout the lifetime of the patient. Repeat administration of β-gal in uncoated and PEG-coated vesicles revealed that eventually a serum reaction against both drug delivery vehicles was elicited. The data, however, support the first observation in the pre-immunized animals, that PEG-coated vesicles are either more immunogenic or more sensitive to the mounting immune response. Despite the fact that by the second administration there is barely sufficient anti-β-gal antibody to detect (figure 3.8), there is a dramatic serum reaction against PEG-coated vesicles, which causes their rapid

clearance from the circulation and severely shocks the animals upon injection. Uncoated vesicles, on the other hand, raise the same level of β-gal antibody but a serum effect is not detected, similar to what is seen in pre-immunized animals. But there is a progressive increase in the recognition of the uncoated lipid system, so that a gradual increase in vesicle clearance occurs over the course of treatment.

## *4.4* On the difference between uncoated and PEG-coated vesicles

Why are PEG-coated, β-gal containing vesicles apparently more sensitive to immune complex formation than the same formulation without PEG? Similar PEG effects have been reported by Phillips et al. [Phillips et al., 1994; Phillips et al., 1996; Phillips & Dahman, 1995]. The difference between Phillips' data and that reported in this thesis is that he used immunoliposomes, where antibodies are attached to the outside of the lipid carrier to target encapsulated drug to specific cells. Such systems are immunogenic, particularly if the vesicle associated IgG is from a different species from that of the host, however, the adjuvant activity of the liposome is sufficiently potent to generate antibodies against autologous IgG as well [Phillips et al., 1994]. PEG coating has been used in an attempt to limit the immune response against immunoliposomes [Phillips et al., 1994; Phillips et al., 1996; Phillips & Dahman, 1995]. However, the presence of this polymer has been shown to not only give rise to a powerful humoral immune response to protein antigens associated with the carrier but also to enhance the generation of antibodies to the liposomal phospholipids and the linkers used to attach ligands to their phospholipid anchors [Phillips et al., 1994]. These data suggest that PEG-coated vesicles are better adjuvants than naked vesicles when they carry a protein

payload. This may be because these vesicles are targeted to more powerful antigen presenting cells, such as dendritic cells, compared to PEG free vesicles. The latter are removed mostly by Kupfer cells in the liver and spleen based macrophages, whereas PEG-coated vesicles avoid the liver and can access distal tissue sites such as the skin [Gabizon et al., 1997] an organ rich in Langerhans cells, one of the most potent antigen presenting cells known. Different intracellular processing of the β-gal antigen delivered to antigen presenting cells may also be an important factor [Harding *et al* 1991; Rao et al., 1995].

The hypothesis that PEG-coated vesicles raise a humoral response against the surface polymer or polymer linker is readily testable. A group of mice will be immunized by weekly injection of PEG-coated β-gal vesicles then challenged with the same vesicles without β-gal. If "vesicle" antibodies are present the lipid should be cleared rapidly from the blood. If the clearance of empty vesicles is not affected then the observations reported in this thesis are likely due to β-gal epitopes exposed at the vesicle surface. The same experiment will be conducted using naked vesicles. In this instance, if empty PEG-free vesicles are cleared more quickly from the immunized animals it may indicate the presence of anti-phospholipid antibodies.

Finally, the key objective of this work was to test the hypothesis that: **A biologically active protein, encapsulated in a lipid based drug delivery system, can be administered intravenously and repeatedly to mice without loss of activity or reduced bioavailability due to the immune response induced against it**. The data

indicate that bioavailability may be severely reduced following repeat injections due to an increase in the rate of clearance of the carrier.

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