PROTEIN AND CELL INTERACTIONS WITH LIPOSOMES

SURFACE MODIFICATION OF LIPOSOMES WITH HYDROPHILIC POLYMERS: EFFECTS ON PROTEIN ADSORPTION AND CELL INTERACTIONS

By

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ABSTRACT

Liposomes have the ability to carry and deliver both hydrophilic and hydrophobic drugs and to protect them when injected into the circulatory system. They thus provide an attractive vehicle for drug delivery. However, problems of rapid clearance and inability to target liposomes to specific cells and tissues remain unresolved.

Rapid clearance has been attributed to adsorption of opsonins, and one approach to reduce such adsorption is to create sterically stabilized liposomes by modifying the surface with polyethylene glycol (PEG) or dextran.

To deliver their drug "payload" liposomes must interact with the membranes of target cells. Interactions with cellular components of the vascular walls have been observed for various sulfated polysaccharides such as heparin and functionalised dextrans.

Based on the above considerations, the purpose of this work was to investigate the ability of various polymeric modifiers on liposomes to reduce protein adsorption and promote incorporation into target cells. Liposomes of composition PC/PE/cholesterol (70/10/20 mol %) were surface modified with PEG, dextran, heparin, and functionalised dextran. Protein adsorption was studied from solutions of IgG in buffer and from plasma. Adsorption from buffer was measured by radiolabelling methods. For the plasma work, a total protein assay was used to determine the amount of protein adsorbed to the liposome surface, while gel electrophoresis and immunoblotting methods were used to examine the

profiles of protein binding. Liposome incorporation into vascular smooth muscle and endothelial cells was evaluated using fluorescent labelling and radiolabelling techniques.

The IgG adsorption studies showed reduced adsorption on all polymer-modified liposomes. Plasma adsorption data showed that adsorbed protein layer compositions on the different liposome types were similar, but different from that of the plasma itself, showing that the plasma was fractionated on the liposome surfaces. Cell interaction studies showed that liposomes modified with dextran and sulfated dextran were incorporated into both cell types. The unmodified, PEG- and heparin-modified liposomes were not incorporated to any significant extent.

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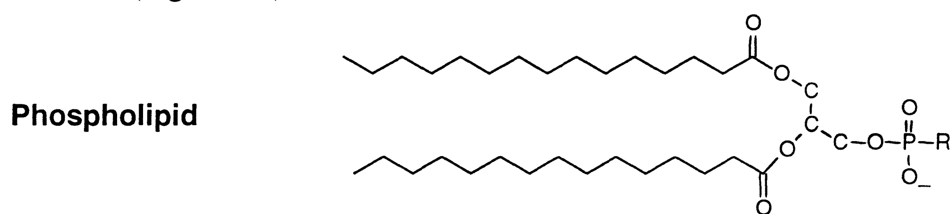
LIST OF ABBREVIATIONS

AP	**Alkaline Phosphatase**
CF	**Carboxyfluorescein**
CMC	**Critical Micellar Concentration**
CMDBS	**Carboxymethyl Dextran Benzylamide Sulfonate**
CMDSu	**Carboxymethyl Dextran Sulfate**
CPM	**Counts per Minute**
CRP	**C-Reactive Protein**
d	**Diameter**
DLS	**Dynamic Light Scattering**
DMPC	**Dimyristoyl Phosphatidylcholine**
DMPE	**Dimyristoyl Phosphatidylethanolamine**
DMPE-PEG	**Dimyristoyl Phosphatidylethanolamine-Conjugated PEG**
DPPC	**Dipalmitoyl Phosphatidylcholine**
DSPC	**Distearoyl Phosphatidylcholine**
DSPE	**Distearoyl Phosphatidylethanolamine**
EC	**Endothelial Cell**
FBS	**Foetal Bovine Serum**
FGF	**Fibroblast Growth Factor**
FTIR	**Fourier Transform Infrared Spectroscopy**
HDL	**High Density Lipoprotein**
HMWK	**High Molecular Weight Kininogen**
HUVEC	**Human Umbilical Vein Endothelial Cell**
IU	**International Units**
LDL	**Low Density Lipoprotein**
LUV	**Large Unilamellar Vesicle**
MLV	**Multilamellar Vesicle**
mPEG	**Methoxy-Terminated PEG**
MW	**Molecular Weight**
NBT	**Nitroblue Tetrazolium Chloride**
OG	**n-Octyl-β-D-Glucopyranoside or Octyl Glucoside**
PC	**Phosphatidylcholine**
PE	**Phosphatidylethanolamine**
PEG	**Polyethylene Glycol**
PG	**Phosphatidylglycerol**
PHDCA	**Polyhexadecylcyanoacrylate**
PS	**Phosphatidylseerine**
QELS	**Quasi-Elastic Light Scattering**
RES	**Reticuloendothelial System**
SDS-PAGE	**Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis**
SM	**Sphingomyelin**
SUV	**Small Unilamellar Vesicle**
SVLT	**SV40 Large T Antigen**
TCA	**Trichloroacetic Acid**
TF	**Tissue Factor**
TIRF	**Total Internal Reflection Fluorescence**
TNBS	**Trinitrobenzenesulfonic Acid**
VLDL	**Very Low Density Lipoprotein**
VSMC	**Vascular smooth muscle cell**

1. INTRODUCTION

1.1 Liposome Definition

Liposomes are lipid bilayer-based particles capable of encapsulating an aqueous solution, thus forming an internal environment which can be different from the external medium. The primary constituents of liposomes are phospholipids. These are glycerol derivatives consisting of a polar, hydrophilic "head" group attached to a nonpolar, hydrophobic "tail". The head typically consists of a phosphate group and the tail of two hydrocarbon chains (Figure 1.1).



Typical head groups (R):

Choline (PC)

Ethanolamine (PE)

Serine (PS)

Glycerol (PG)

Acid (PA)

Inositol (PI)

Figure 1.1 Phospholipid structure. Adapted from reference [1]

Because phospholipid molecules exhibit amphiphilic properties, they aggregate in polar solvents into ordered structures with typical lyotropic liquid crystalline symmetries. Figure 1.2 depicts a conventional liposome.

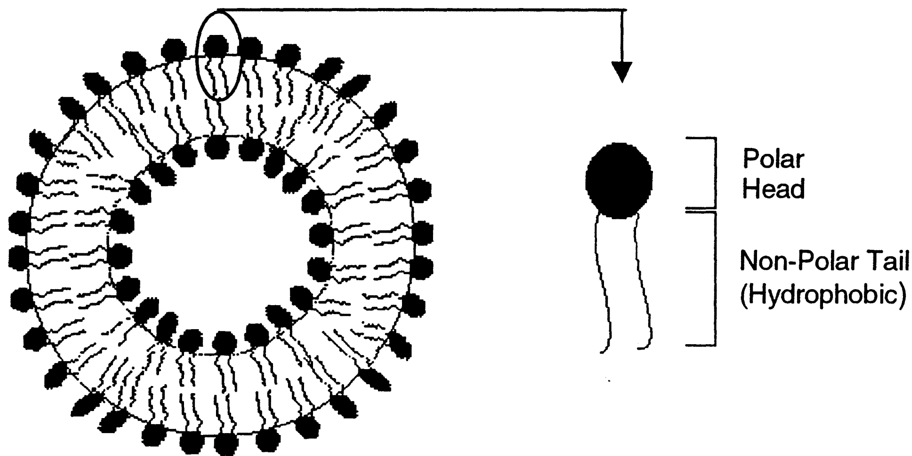


Figure 1.2 Conventional liposome structure.

Conventional liposomes are often based on mixtures of phosphatidylcholine (PC), cholesterol and non-neutral lipids such as phosphatidylserine (PS) and phosphatidylglycerol (PG). Cholesterol has been found to decrease order in crystalline phospholipids and increase order in fluid phospholipid bilayers by inserting itself between the lipid molecules [2]. Lipids tend to undergo a phase transition from a gel state (at lower temperatures) to a liquid-crystalline state (at higher temperatures). This transition occurs around a characteristic temperature (T_c) [3]. At temperatures where the lipid is normally above its T_c, the presence of cholesterol tends to increase the packing and rigidity of the bilayer, while at points where the lipid would be below its T_c, cholesterol expands and fluidizes the bilayer [4].

1.2 Liposome Formation

All methods of making liposomes involve common stages: drying down of lipids from organic solvents to form a thin lipid film, dispersion of the lipids in an aqueous medium and purification of the resultant liposomes. The main difference among the various methods of preparation of liposomes is the way in which the lipids are dispersed in the aqueous medium before forming bilayer structures.

The formation of large multilamellar vesicles (MLVs) from swelling dried lipid bilayers was explained by Lasic [5] and occurs in one of two ways. The difference between the areas of polar heads on the outer and inner monolayers, which induces curvature, causes a mismatch between the opposing surfaces of a bilayer, and can result in budding off when the excess of outer over inner monolayer surface area exceeds some critical value. A small section of the bilayer may also break off and close upon itself. Because liposomes are thermodynamically at a higher energy level than the hydrated lamellar phase in excess water, some energy input is normally required to form liposomes. Multilamellar vesicles may be further treated to produce liposomes of a desired size that consist of a single lipid bilayer. This can be achieved by forcing a suspension of MLVs through a French press [6] or by the detergent depletion method which will be discussed later.

One of the most common preparation methods is extrusion, which gives a narrow vesicle size distribution. In this method, the lipid film is hydrated in excess aqueous phase at a temperature above T_c, thus producing a heterogeneous dispersion of predominantly large multilamellar vesicles (MLV). The vesicles are then passed through

polycarbonate membranes with defined pore size under pressure. After several extrusion steps, liposomes of average size equal to the size of the pores are produced [7].

The reverse-phase evaporation technique results in a larger efficiency of entrapment of water-soluble substances. This method consists of sonication of an aqueous solution mixed with an organic liquid to form small droplets. The organic solvent is removed from the emulsion by evaporation under reduced pressure, resulting in a viscous gel-like intermediate phase. Eventually, the gel spontaneously transforms into a homogeneous liposome dispersion, which is then dialysed against buffer to remove any residual solvent. This technique yields large unilamellar vesicles (LUV) [8].

In the present work, the detergent depletion method was used. In this method, small mixed micelles of detergent-phospholipid fuse upon removal of detergent as the system minimizes its total edge energy, which is proportional to the perimeter of the disk-like micelles. This growth is opposed by entropy loss and by reduced amounts of detergent molecules which can shield the edges, thus forcing large micelles to bend and eventually self-close, eliminating the unfavourable exposure of the edges, but increasing the bending energy of the system [5]. This reasoning implies an opened bilayered fragment as an intermediate structure in the vesiculation process. The detergent depletion method is non-denaturating for the lipids compared to intense ultrasonic irradiation or use of organic solvents, and results in aggregates that are mostly unilamellar with a unimodal size distribution, and a bigger internal aqueous volume than vesicles prepared by sonication [9].

1.3 Liposomes in Contact with Blood

Liposomes have been used extensively for basic research (physical, chemical and biological), and as models of lipid bilayer membranes. They have also been used as drug carriers, vectors for gene transfer, and in cosmetics [3]. Due to their amphiphilic character, liposomal preparations have the ability to protect and carry both hydrophobic and hydrophilic molecules [10], and it is for this reason that they are used in drug delivery systems. Liposomal carriers can alter favourably the biodistribution of the encapsulated drugs, can increase their efficacy, and decrease their toxicity. Liposomes based on natural lipids are of interest for use in biological systems because they exhibit many desirable characteristics including biocompatibility, biodegradability, and interactions with biological membranes and cells.

Unfortunately, conventional liposomes are rather unstable in a biological milieu such as blood. When liposomes are exposed to plasma, they rapidly adsorb opsonins and other proteins [11-13]. In addition to causing premature clearance from the bloodstream, these interactions can affect liposome stability leading to vesicle disruption, loss of contents [14], complement activation [15] and activation of the contact phase of blood coagulation and platelets resulting in thrombus formation [16].

Opsonins, which are immune and non-immune serum factors that bind to and alter the surface of foreign particles such as liposomes [17], are recognized specifically by receptors on the macrophages of the reticuloendothelial system (RES) resulting in the rapid clearance of the liposomes from the circulation [18]. Liposomes exhibit strong interactions with blood proteins including not only opsonins (e.g. IgG [19] and

complement component C3 [11]) but others like fibronectin as well [20]. Immunoglobulin coating of liposomes is known to promote uptake by macrophages [20, 21]. The Fc portion of IgG interacts with specific receptors on macrophages, thus promoting macrophage uptake [17, 20].

Changes in liposome size and composition can alter the rate of clearance by the RES [4, 18, 22-26]. Smaller liposomes tend to be removed from the circulation more slowly than larger ones. Liposomes composed of negatively charged lipids also tend to be removed more rapidly than those of neutral or positive charge. Gabizon and Papahadjopoulos [27] found circulation times varying from 30 min in the case of phosphatidylcholine (PC) / cholesterol liposomes to 15 h for PC / cholesterol / monosialoganglioside liposomes.

Loading of therapeutic drugs into the liposomes can target pathogens that infect macrophages. For example, the disease leishmaniasis, in which a parasite infects the macrophages, can be treated by liposomal preparations containing toxic antimonial drugs [28].

The complement system consists of a group of glycoproteins in the plasma that can act in a cascading fashion to produce biologically active fragments [29]. One of the principal functions of complement is the non-specific recognition and elimination of "foreign" invaders from the body. The complement system consists of two recognition-stimulation pathways that are designated as the classical and alternative pathways, either of which may lead to the formation of a membrane attack complex (MAC) that disrupts

the cellular lipid bilayer, leading to cell death [29]. The complement system is depicted in Figure 1.3.

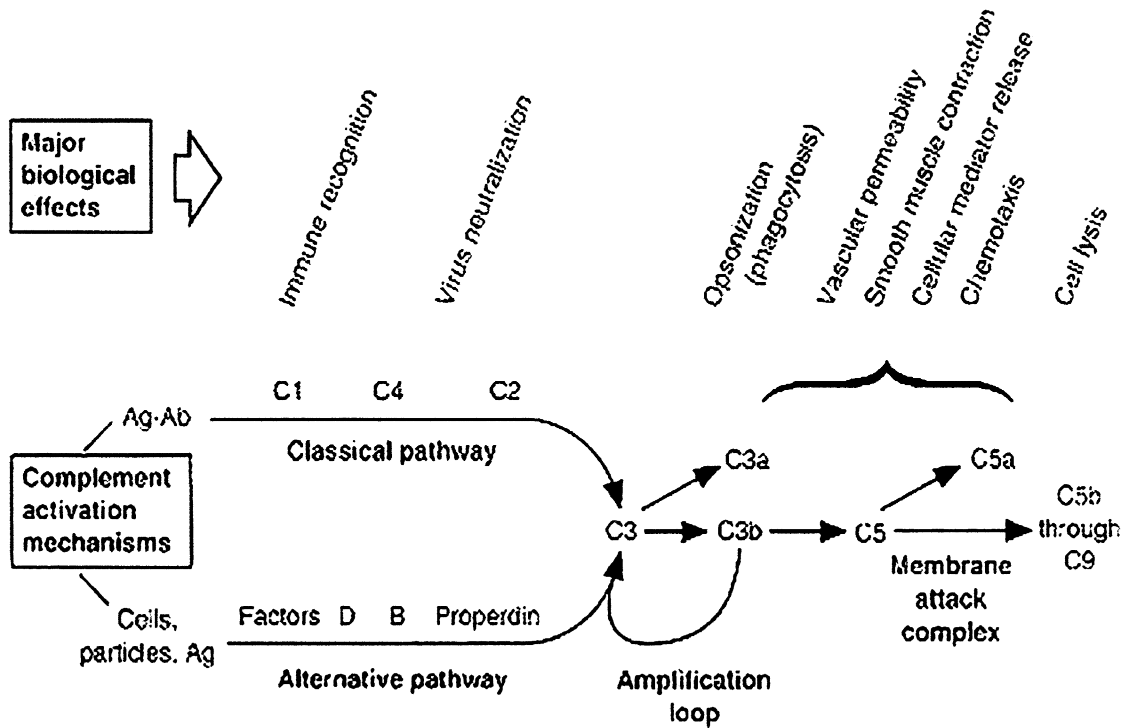


Figure 1.3 Outline of the complement system [30].

The classical pathway may be activated by antigen-antibody complexes of the IgG or IgM isotypes by their binding to the subunits of the first component of complement, C1. Consequently, the C1 subunits form an esterase that cleaves the next component, C4, into two fragments, the larger of which, C4b, binds covalently to hydroxyl or amino groups on cellular membranes. The second component C2, after binding to C4b is partially digested by a C1 subunit to form C2b. The resultant membrane-bound complex, C4b2a, is an enzyme (C3 convertase) that cleaves C3 into two biologically active fragments, C3a and C3b. The alternative pathway is activated independently of antigen-antibody complexes. The major components are the serum protein factors B, D, and P

(properdin). A small amount of C3 in the fluid phase, which normally is spontaneously activated, interacts with factor B to form C3Bb, which cleaves another C3 molecule to form C3b. C3b in turn attaches to surfaces and binds factor B. The resultant C3Bb is then cleaved by factor D to form C3bBb, the C3 convertase of the alternative pathway. This complex is stabilized by factor P. The non-specific and spontaneous nature of the alternative pathway permits activation by various biomaterial surfaces. The activation of the complement system eventually leads to the formation of the membrane attack complex (MAC) that subsequently lyses cells and possibly liposomes (Figure 1.3) [29].

IgG also triggers the classical complement pathway producing C3b, the main complement opsonin which has a high affinity for macrophages [17]. It has also been shown that negatively charged liposomes are capable of activating the classical complement pathway independent of immunoglobulins [31, 32]. Bonte et al. identified IgG as the major protein bound to conventional (including anionic) liposomes, although apolipoprotein A1, albumin and α2-macroglobulin were also found to bind, albeit to a lesser extent [33].

As indicated, liposomes in contact with blood also tend to provoke coagulation. The two coagulation pathways (the intrinsic and extrinsic pathways) lead to the formation of a fibrin clot. Although they are initiated by distinct mechanisms, the two converge on a common pathway that leads to clot formation, as depicted in Figure 1.4. The formation of a clot in response to an injured vessel wall or to a foreign surface such as a liposome, in the absence of tissue injury, is the result of intrinsic pathway activation. Clot formation in response to tissue injury is the result of extrinsic pathway activation.

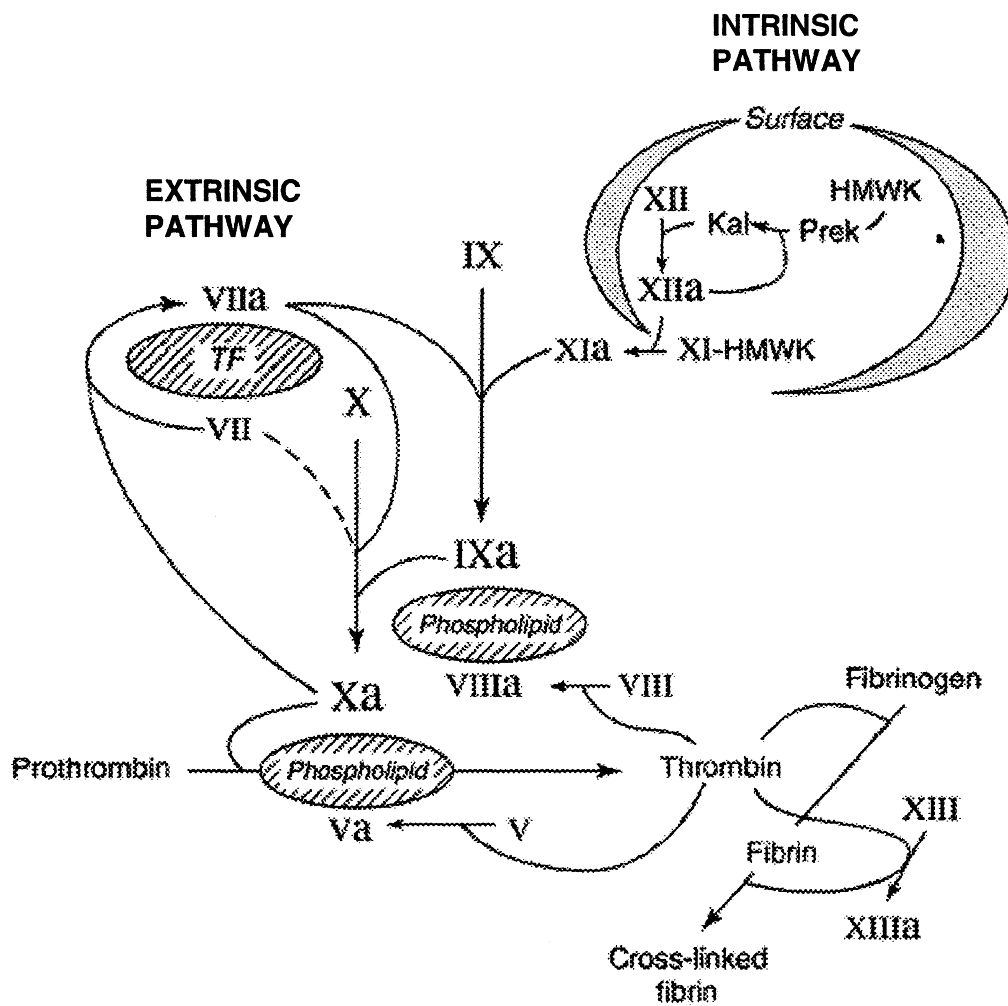


Figure 1.4 Blood coagulation pathways. HMWK = High molecular weight kininogen, Kal = Kallikrein, Prek = Prekallikrein, TF = Tissue factor. Adapted from [34, 35].

2. LITERATURE REVIEW

Drug loaded liposomes must have sufficient resistance to chemical and / or enzymatic attack to allow them to reach their target tissue and deliver the drug [36]. To increase blood lifetimes of liposomes, pre-saturation of the reticuloendothelial system with empty liposomes [37] or modification of the lipid formulation by using lipids with saturated hydrocarbon chains and containing high amounts of cholesterol have been investigated [26, 37]. The improvements achieved by these approaches were not great enough to produce much advantage over existing non-liposomal drug delivery. Other approaches involved surface modifications such as coating with proteins [38], glycolipids [39], and polysaccharides [40, 41]. Since hydrophilic polymer coatings have been shown to reduce serum protein adsorption, they are now widely used to modify liposome surfaces. A second major issue is the ability to target liposomes to specific cells or tissues.

Both issues (stability and targeting) involve liposome interactions with proteins and cells, and both are addressed in this thesis. An overview of protein and cellular interactions with conventional liposomes and with liposomes modified with polyethylene glycol, dextran, heparin, and functionalised dextran (all used in the present work) is now presented.

2.1 Conventional Liposomes

Interactions between serum proteins and liposomes are diverse, complex and difficult to predict. These interactions can lead to adverse effects on liposome stability and in vivo behaviour [4]. Not only are liposomes rapidly removed (via opsonization) by the macrophages of the reticuloendothelial system, they are also broken down by lipid exchange or depletion when they come in contact with lipoproteins and cell-surface proteins [12].

Many modes of protein-liposome interaction have been suggested [42-45]. Many proteins interact strongly with lipid vesicles if the protein and vesicle bear opposite charges [4]. Another type of interaction also involves charge interactions in the initial binding, but in addition it involves penetration of the protein into the bilayer causing expansion and alteration in bilayer permeability properties. A third mode of binding involves hydrophobic interactions, i.e. non-polar areas of the protein come in contact with the bilayer to minimize water interactions. The adsorption of both albumin and IgG depend on the surface properties of the liposome; the more hydrophobic the vesicle, the more it adsorbs these proteins [46]. Because IgG is more hydrophobic than albumin, it adsorbs more extensively onto hydrophobic liposomes [46, 47]. Effects such as protein conformational change and liposome structural changes can also affect adsorption. If the protein becomes disordered, the entropy gain that occurs can act as a driving force for adsorption [48].

The vesicle size also influences the susceptibility of phospholipids to protein attack [4]. Gupta et al. [49] and Hermetter et al. [50] showed that serum-induced disintegration

of liposomes is facilitated by high curvature of the vesicle membrane. Scherphof et al. [51] later postulated that the small radius of curvature in small unilamellar vesicles (SUVs) makes the structure susceptible to solubilization by lipoproteins .

There is also strong evidence that lipid vesicles are most susceptible to disruption by proteins, especially lipoproteins, above their critical temperature (T_c) [52, 53]. When the critical temperature is exceeded, liposomes switch to a liquid crystalline phase from a highly ordered gel state. This liquid crystalline phase is characterized by an increase in rotational freedom of the fatty acyl chains, resulting in decreased liposome stability due to the coexistence of solid and fluid domains within the bilayer [4].

The lipid composition of liposomes plays an important role in interactions with serum lipoproteins. A rigid, tightly packed bilayer reduces RES uptake due to reduced interaction with lipoproteins and leakage of internal contents [23]. Therefore liposomes based on long-chain, saturated lipids having a high transition temperature and extensive van der Waals interactions show prolonged circulation times [2]. Cholesterol also exerts a stabilizing effect when present at a level in the vicinity of 25 mol % [54]. The substitution of sphingomyelin (SM) for phosphatidylcholine (PC) at 35 mol % likewise has been found to stabilize liposomes in serum and plasma [54, 55]. Semple et al. [56] investigated the association of blood proteins with large unilamellar vesicles (LUVs) injected intravenously into mice to determine the effect of membrane fluidity and hydrocarbon chain length on liposome-plasma protein interactions and clearance. They showed that liposomes composed solely of gel state lipids having a net zero charge were very rapidly cleared from the circulation. The ability of plasma proteins to bind to PC-

based vesicles was reduced dramatically by the inclusion of cholesterol in the bilayer, with little difference in the individual types of proteins observed between the cholesterol-rich and cholesterol-poor liposomes. They concluded that it is not the liposome charge per se that triggers rapid clearance from the circulation [56]. Black and Gregoriadis [57] showed that both neutral and positively charged liposomes were rendered negatively charged in the presence of plasma proteins, whereas vesicles composed of negatively-charged lipids maintained the same overall net charge in plasma. The negative charge acquired by neutral liposomes was attributed to the adsorption of α2-macroglobulin and perhaps other plasma proteins. Ishida et al. [58] investigated the biodistribution of PC-based liposomes containing cholesterol after intravenous injection into rats. Contrary to previous reports, they found that the inclusion of cholesterol seemed to accelerate the rate of liposome uptake by the liver. They also found that the amount of bound C3 fragments was directly proportional to cholesterol content. They concluded that the complement system is responsible for the elimination of liposomes from the blood circulation, presumably as a result of opsonization by C3 fragments and assembly of the membrane attack complex (MAC) on the liposomes. By replacing cholesterol with cholesteryl methyl ether in the liposome formulation, C3 binding diminished significantly, suggesting that the hydroxyl group on cholesterol is a binding site for C3 fragments [58].

Many investigators have shown that when liposomes are pre-exposed to plasma, the rate of clearance increases in proportion to the amount of protein bound to the liposomes [11, 56, 59]. This led to the hypothesis that increasing the dose of liposomes should result in a decrease in the surface concentration of proteins critical to immune

recognition, and thus to extended circulation lifetimes [60]. Oja et al. investigated liposome-blood protein interactions by injecting LUVs into mice and monitoring the circulation lifetime and liver and spleen accumulation. They found that at higher doses of liposomes injected, longer circulation lives were observed and significantly less protein was bound per liposome. This would indicate that there is a limited pool of blood proteins able to interact with liposomes [60].

Hsu and Juliano showed that fibronectin could enhance liposome uptake by macrophages by interacting with cell surface fibronectin receptors [20]. Rossi and Wallace [61] demonstrated that binding of fibronectin to vesicles of various compositions occurred in the absence of other proteins, sugars, or divalent cations and the protein adopted a different conformation when associated with vesicles from that which it adopts in aqueous solution. This conformation was found to result from a specific interaction and not merely hydrophobic interactions, leading to the conclusion that an intermediate receptor does not need to be involved to account for the binding properties of a surface [61].

Human C-reactive protein (CRP), present in normal plasma in trace amounts, is known to increase particularly during the acute inflammation processes, has been found to activate the complement system following interaction with PC-containing liposomes [62, 63]. Richards et al. showed that for strongly charged lipid bilayers CRP-mediated complement dependent damage to liposomes was high, but negligible for neutral membranes [63].

Binding of other proteins to liposomes such as albumin [4, 33, 64, 65], fibrinogen [4, 17, 65, 66], clotting factors VIII, XII [4, 17] and V [33], α- and β- globulins [4, 17, 33, 65], vitronectin [66] and α1-antitrypsin [65] has been observed. The adsorption of human serum albumin (HSA) to liposomes has also been demonstrated and it is now well established that the binding of albumin has no detrimental effect on the structural integrity of liposomes [67].

Bonte and Juliano found that high-density lipoproteins (HDL) caused a loss of phospholipid from the vesicle bilayer and induced destabilization of the liposomes leading to their breakdown and total release of contents [4]. They also showed that low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL) interactions with liposomes were less traumatic than HDL interactions. Apolipoproteins rapidly adsorb to liposomes, possibly leading to the destruction of the vesicles to form discoidal phospholipid-protein complexes [4]. Scherphof et al. hypothesized that HDL attack is initiated by the release of apolipoprotein A1, which becomes incorporated into the lipid bilayer [66].

2.2 Modified Liposomes

It is possible to increase the repulsive forces between a liposome and a protein by modifying the liposome surface with certain natural and synthetic polymers [67]. These polymers need to possess certain qualities such as inertness, compatibility with the solvent and chain flexibility leading to the possibility of steric stabilization of the liposome [68]. Steric stabilization can be achieved by attaching the polymers to the

surface by physical adsorption (e.g. hydrophobic interactions), by electrostatic interactions, or by grafting through covalent bonding. Non-ionic, water-compatible, flexible, and well-hydrated polymers are usually preferred.

2.2.1 Polyethylene Glycol (PEG)-Modified Liposomes

The attachment of polyethylene glycol chains to the bilayer surface is the basis of "Stealth®" liposomes which exhibit longer circulation half-lives than conventional liposomes [69]. A PEG-modified liposome is schematically depicted in Figure 2.1.

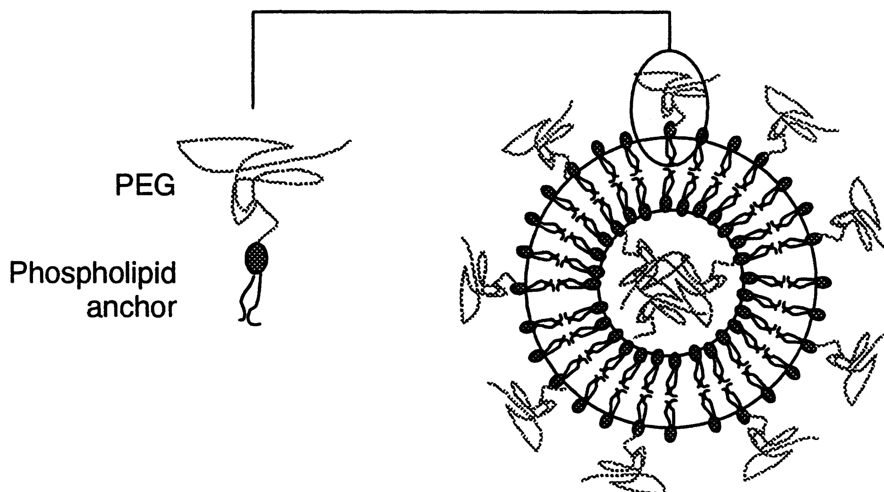


Figure 2.1 Polyethylene glycol-modified liposome. Methoxy-terminated PEG is shown covalently conjugated to PE on the inner and outer layers of the membrane.

2.2.1-1 PEG Properties

Steric stabilization by PEG prevents the natural tendency for aggregation of conventional liposomes and also appears to limit the interactions of liposomes with blood components including proteins [68]. The protein repelling effect is thought to be a result of the polymer's hydrophilicity and the mobility of its highly flexible chains, which

results in a high exclusion volume. Woodle and Lasic [2, 68] have described this stabilization as a balance of interaction forces involving mainly van der Waals and hydrophobic forces, as indicated in Equation 2.1.

$$\mathbf{F_T = -F_{vdw} - F_{hfo} + F_{est} + F_{und} + F_{hyd} + F_{steric}} \qquad \textbf{Equation 2.1}$$

Where:

F_T	=	total interaction force
F_{vdw}	=	attractive van der Waals force
F_{hfo}	=	attractive hydrophobic force
F_{est}	=	repulsive electrostatic force
F_{und}	=	repulsive undulation force
F_{hyd}	=	repulsive hydration force
F_{steric}	=	osmotic and elastic restoring force

The steric repulsion effect is thought to be due to the PEG layer on the surface of liposomes that reduces attractive forces and increases repulsive ones [68]. One explanation is in terms of osmotic effects. Water bound to PEG chains is displaced by the approaching protein, thus increasing the osmotic pressure within the polymer coil. When this pressure becomes too high, water returns to hydrate the PEG layer and the protein is repelled. De Gennes [70] described the steric repulsion mechanism in terms of entropic effects. Entropy is reduced when the polymer chains are compressed by the approaching protein. This energetically unfavourable shift results in "entropic repulsion" of the protein. At low polymer grafting densities (when the polymer is able to adopt the random coil conformation), PEG takes up a "mushroom-type" configuration. At higher densities, the relative crowding causes more extended conformations, leading to a "brush-type" configuration [70].

Prime and Whitesides [71] showed that PEG containing just a few ethylene glycol (EG) repeating units could give protein repellent structures. In this case, the surfaces

were formed by chemisorption on gold of alkane thiols terminating in oligo-ethylene glycols (self-assembled monolayers, SAMs). The density of the short PEGs was thus very high.

In an aqueous medium, PEG interacts with water through hydrogen bonds. Morra [72] stipulated that the interaction between a PEG-coated surface and water via hydrogen bonding can create an energy barrier that resists contact with an opposing surface. Morra [72] also suggested that the protein-resistant behaviour of densely packed self-assembled monolayers with short ethylene glycol chains is due to the fact that the chains do not have the conformational freedom of long-chain PEG. Since most practical PEG surfaces are in the regimes between the mushroom and the brush configurations, i.e. around 5 mol % PEG, the chains are thought to have enough freedom to adopt a conformation that maximizes interactions with water [70, 72].

Also of interest is the fact that PEG exhibits an inverse temperature-solubility relationship in aqueous solution. At low temperatures, it is soluble, whereas at higher temperatures, it flocculates and ultimately forms a gel [2]. This behaviour can be explained by the disruption of the water binding as temperature increases. With increasing temperatures, there is an increase in the disorder of water molecules leading to a decrease in water bound to the polymer. This, in turn, increases polymer-polymer interactions leading to decreased solubility [2].

2.2.1-2 Stability of PEG-Modified Liposomes in Vitro and in Vivo

Many studies have shown the importance of liposome and PEG properties with respect to improved circulation times in vivo. The liposome size and composition [4, 18, 22-26] as well as PEG molecular weight and loading [73-77] are all factors that influence the effectiveness of the steric barrier. Litzinger et al. [78] studied the biodistribution of PEG-modified liposomes of three characteristic sizes when injected into mice. The large (d > 300 nm) and small (d < 70 nm) liposomes accumulated to elevated levels in the spleen and liver. The intermediate sized liposomes were found to be the longest circulating. Maruyama et al. [79] reported a direct correlation between opsonization of PEG-coated liposomes and liposome size, and predicted that liposomes of diameter in the rage of 50 to 300 nm would show reduced accumulation in the liver and spleen [79].

By including 40 mol % cholesterol in conventional liposomes, Silvander et al. [80] observed a stabilizing effect upon exposure to 20% human serum. No such effect was noted for liposomes modified with PEG 2000. The inclusion of PEG was believed to increase the permeability of the liposomes in serum. Chiu et al. [81] investigated negatively-charged liposomes containing phosphatidylserine (PS) as a model for a biologically active membrane. They showed that modification with distearoyl phosphatidylethanolamine (DSPE)-PEG 2000 at the 15 mol % level gave plasma elimination rates, as well as spleen and liver uptake, that were comparable to those for 5 mol % DSPE-PEG 2000 incorporated into neutral liposomes. They suggested that liposomes containing surface localized PEG and reactive lipids may be appropriately designed to select for specific interactions with plasma components [81].

Liposomes coated with methoxy-terminated PEG (mPEG) 5000 were cleared up to 30% more slowly from the blood circulation than conventional liposomes after intravenous injection into mice, suggesting that PEG acts as a surface barrier to plasma factors which otherwise bind to liposomes in the blood and accelerate vesicle removal from the circulation [82]. Basanez et al. observed reduced rates of phospholipid hydrolysis in PEG-coated liposomes at PEG loadings as low as 0.1 mol % [73]. Liposome-cell interactions were reduced with as little as 0.5 mol % PE-PEG 2000 incorporation whereas inhibition of liposome binding to small macromolecules (i.e. $M_r <$ 20,000) required between 5 and 7 mol % PE-PEG 2000 [74, 75]. In the latter range of incorporation into phospholipid bilayers, PEG molecules begin to adopt a more ordered and extended "brush" configuration. The permeability of various PEG-coated liposomes to D-glucose was measured, and permeability coefficients were noted to increase with loading of PEG 5000 and with temperature. This effect was attributed to an increasing number of bilayer defects as the liposome content of PEG-grafted lipid increased [83].

2.2.1-3 Protein Adsorption onto PEG-Modified Surfaces

Many studies have focused on the effect of PEG molecular weight (MW) and PEG surface density on plasma protein interactions. While most investigators have found that protein adsorption tends to decrease with PEG loading, others have not seen such an effect.

Gref et al. showed a maximum reduction in protein binding for a PEG MW of 5000 on PEG-coated poly(lactic acid) nanoparticles [84]. It was found that a PEG content of

2-5 weight % gave optimal protein resistance. On polyhexadecylcyanoacrylate (PHDCA) particles, the amount of protein adsorbed decreased with increasing PEG loading [85]. Higher PEG contents led to a preferential adsorption of plasma proteins of low MW. In examining the adsorption of proteins onto lipid-coated glass surfaces, Du et al. [75] demonstrated that as the PEG content of these surfaces increased, the adsorption of albumin, laminin and fibronectin decreased. They also showed that the concentration of PEG-conjugated lipids required to inhibit erythrocyte adhesion decreased with increasing MW of the grafted PEG. Xu and Marchant [77] studied the adsorption of plasma proteins on PEG-grafted lipid bilayers using total internal reflection fluorescence (TIRF) spectroscopy. They observed small but increasing adsorption as the density of PEG 2000 on a DSPC bilayer increased. This result appears to be at odds with the previously-reported protein-resistant properties of liposomes modified with DSPE-PEG 2000 [74, 75].

When comparing PEG 5000 bound to aminated polystyrene surfaces in an end-on configuration to dextran in either an end-on or side-on configuration, Österberg et al. [69] found that the side-on configuration of dextran was significantly more effective than the end-on configuration in reducing fibrinogen adsorption, and marginally more effective than the PEG, although the PEG density on the surface was less than the dextran. They concluded that protein adsorption is critically dependent on the availability of bare surface that is not covered by polymer, and that packing density is more important than layer thickness in reducing protein adsorption.

Needham and Kim [86] compared small PEGs (MW 750) that inhibited the adsorption of small macromolecules (40 X 50 Å) to larger ones (MW 2000-5000) that opposed interactions with large macromolecules, lipoproteins and cells. They suggested that a combination of the two PEG species could create a molecular scale filter, which could provide either complete or selective protection of the surface to an extent dependent on the precise composition of the mixed layer.

Price et al. [76]did not observe any effect on protein adsorption by PEG modification of liposomes having a net zero charge. For negatively-charged liposomes, they found an inverse relationship between PEG loading and fibrinogen adsorption.

Pre-treatment of liposomes with serum and its effect on liposome clearance was studied by Johnstone et al [87]. Pre-incubating neutral liposomes with serum did not increase uptake by mouse bone marrow macrophages in vitro. Incubation of liposomes containing PS reduced uptake. Serum pre-treatment of PEG-containing liposomes also significantly reduced uptake of the liposomes by cultured macrophages. It was concluded that (1) the bound serum proteins can provide non-specific surface-shielding that decreases the charge-mediated interactions between liposomes and bone marrow for PS liposomes, and (2) that the incubation of PEG-bearing liposomes with serum can result in a change in the properties of the PEG, resulting in a surface that is better protected against interactions with cells.

2.2.2 Polysaccharide-Modified Liposomes

2.2.2-1 Biological Activities of Carbohydrates

Polysaccharides provide an attractive alternative to PEG for developing protein-resistance. They are also biodegradable, of low toxicity, and interact in biological recognition processes through specific sugar moieties [88, 89]. Cell surface carbohydrates are involved in numerous biological activities such as inflammation, fertilization, cell adhesion, migration and proliferation. In particular, interactions of components of the vascular walls with various sulfated polysaccharides such as heparin [90-92] and chemically-modified dextrans [93, 94] have been reported. A few polysaccharides of interest in this connection, and used in the present work, are discussed in the following section.

2.2.2-2 Heparin

Produced in mast cells, heparin is an unbranched glycosaminoglycan consisting of alternating residues of glucosamine and uronic acid, rich in N- and O- sulfate groups [95] (see Figure 2.1). Heparin accelerates the inactivation of coagulation enzymes by natural plasma inhibitors such as antithrombin [95, 96] and heparin cofactor II [97]. Its anticoagulant activity may be due to the high concentration of sulfate, sulfamate and carboxyl groups present in the molecule [98]. A specific pentasaccharide sequence containing 3,6-O and 6-O sulfated glucosamine moieties constitutes a binding site that is recognized by antithrombin [99-101]. Heparin also exhibits anticomplementary properties by interfering with the assembly and function of the classical and alternative

C3 convertase both in vitro and in vivo [102-105]. Anticomplementary activity requires intact O-sulfate groups in the molecules, whereas intact N- and O- sulfate groups are required for anticoagulant activity [105, 106]. Heparin was shown to interact with fibroblast growth factors (FGFs) and to stabilize and protect these factors, however, its anticoagulant activities forbid the use of heparin for wound healing [107, 108]. Beyond its anticoagulant and anticomplementary activities, heparin also binds to and modulates the activity of proteins, inhibits platelet function and smooth muscle cell proliferation, and increases the permeability of vessel walls [92, 98, 109]. A schematic representation of the heparin disaccharide is shown in Figure 2.2.

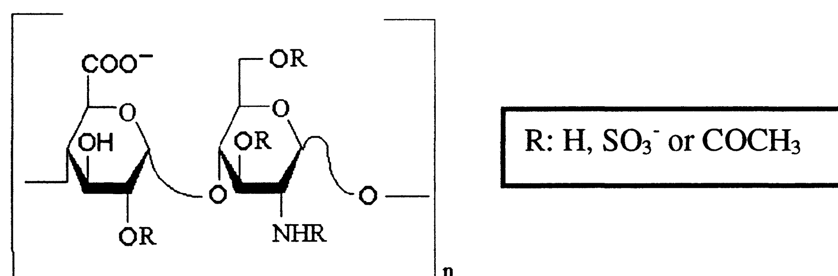


Figure 2.2 Schematic representation of the disaccharide repeat unit of heparin.

2.2.2-3 Dextran

Produced mainly from sucrose by the bacteria *Leuconostoc*, dextran molecules consist of more or less branched chains of glucose units connected by α 1-6 bonds. Dextran is a clinically acceptable plasma volume expander, although it was shown to induce hypersensitivity reactions in over 40 000 cases [110]. Dextran was also proposed as a macromolecular drug carrier [111-113]. The synthesis of different dextrans by

bacterial conversion of sucrose to a branched chain structure, which is heterogeneous in composition is well controlled, and allows its production on an industrial scale at low cost [114, 115]. Although native dextran does not exhibit by itself specific biological activities [115], the random distribution of functional groups along the polysaccharide chains of dextran makes it possible to prepare synthetic compounds having the specific structural requirements for various biological effects [116]. The structure of the dextran subunit is shown in Figure 2.3.

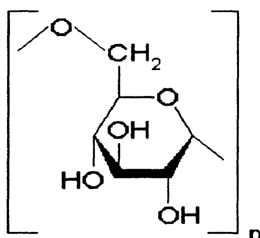


Figure 2.3 Schematic representation of the glucose subunit of dextran.

2.2.2-4 Chemically-Modified Dextrans

Functionalised dextrans, also referred to as carboxymethyl dextran benzylamide sulfonates (CMDBS), are biomimetic functional polymers possessing biospecific sites obtained by grafting of suitable chemical groups onto the polysaccharide backbone [99]. Their synthesis involves 3 sequential reactions: statistical carboxymethylation of hydroxyl groups on D-glucosyl units (CM), benzylamidation of the carboxyl groups (B) and sulfonation of phenyl rings (S) [99, 117].

Initial interest in natural and synthetic polysaccharides such as CMDBS [98] was prompted by the fact that heparin therapy has well known side effects such as allergies and thrombocytopenia. The anticoagulant properties of these functionalised dextrans are strongly dependent on the carboxymethyl and sulfonate contents and on the relative proportions of these groups. The strongest activity was obtained with dextran derivatives containing more than 50% carboxyl groups and having a high sulfonate content [98]. Huynh et al. observed anticoagulant activity about 20% that of heparin for functionalised dextrans with degrees of substitution (average number of OH-groups substituted per glucose subunit, with a total of 3 OH-groups available) of 1.3 for Su, 0.9 for CM and 0.2 for B [118]. Substituted dextrans having anticoagulant activity seem to behave like hirudin, a strong thrombin inhibitor peptide, in that binding studies of the dextrans to coagulation enzymes and inhibitors indicate these polysaccharides also have a direct inhibition effect on thrombin [119]. Like heparin, short sequences containing specific saccharide units may be responsible for the specific interactions of substituted dextrans with antithrombin and thrombin [120]. Derivatized dextrans can protect fibroblast growth factors (FGF-1 and FGF-2) from heat and pH denaturation, and against trypsin and chymotrypsin-induced degradation [121]. Some functionalised dextrans inhibit the classical and alternative complement C3 convertase in vitro, possess anticomplementary activity in vivo, and decrease iC3, C3b and C5b-9 deposition on targets of the complement system [122, 123]. Some CMDBS derivatives also exhibit very high inhibitory capacities on smooth muscle cell growth in vitro (90% inhibition at a concentration of 400 μg/ml) whereas native dextran under the same conditions is

completely inactive [98]. A schematic representation of the above-mentioned polysaccharide is found in Figure 2.4.

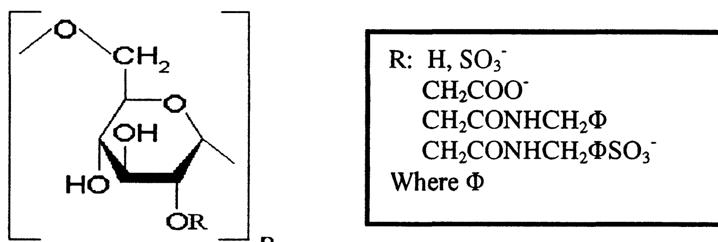


Figure 2.4 Schematic representation of chemically-modified dextran. Functionalised dextran is obtained from dextran by statistical chemical modifications of the dextran chain with carboxymethyl (CM), benzylamide (B), sulfonate (S), and sulfate (Su) groups.

2.2.2-5 Polysaccharide-Modified Liposome Studies

Few studies have examined the stability of polysaccharide-coated liposomes either in vitro or in vivo. Pullulan derivatives, polysaccharides bearing acyl chain residues, were shown to improve liposome stability by decreasing membrane permeability. Moreira et al. [120] measured carboxyfluorescein (CF) leakage from large unilamellar vesicles (based on phosphatidylcholine (PC) lipids) modified with palmitoyl-pullulan and found that leakage of CF was reduced by a factor of three for liposomes prepared with a pullulan / PC ratio of 3 compared to PC liposomes [124]. When CF-charged liposomes were prepared with modified dextran [89], there was an initial CF leakage but the liposomes stabilized, and after 15 days CF release was 20% for the modified liposomes versus 62% for the PC liposomes. The introduction of heparin into the liposome formulation also improved stability when the liposomes were stored at room temperature [125]. More stable liposomes were obtained when the surface was modified by

anchoring the heparin to the lipid bilayer via a cholesterol moiety as opposed to simple adsorption. In addition, the liposomes obtained were blood-compatible in the sense that they did not activate the coagulation cascade.

The resistance of surface-modified liposomes to enzyme attack has also been studied. Sunamoto et al. showed that modification of liposomes with polysaccharides can protect from attack by phospholipase D, which specifically destroys PC by a hydrolytic cleavage of the bond between the phosphate and choline moieties, and by pullulanase, which attacks the pullulan in the aqueous phase [41, 126].

The inclusion of dextran has been shown to stabilize liposomes [9, 89], increase their circulatory half-life [127], and also reduce protein adsorption [69, 128]. Pain et al. [127] observed that the presence of dextran at the liposome surface significantly prolonged their circulatory lifetime, with concomitant decreased levels of accumulation in the liver. The rate of clearance from the circulation was found to be dependent on the density of dextran molecules on the surface. The enhanced circulatory lifetime of dextran-modified liposomes was thought to be due to increased hydrophilicity resulting in reduced binding of plasma proteins. Other studies have shown that surface-bound dextran, which has more sites for peptide binding than PEG, reduces protein adsorption on biomaterials as effectively as surface-grafted PEG [69, 129]. Frazier et al. have demonstrated that the molecular weight of dextran affects the protein-resistant performance of the dextran layer with lower molecular weights [128].

2.3 Liposome-Cell Interactions

The immiscibility of lipid and water is apparent in cellular and intracellular membranes, most of which owe their low permeability to water-soluble materials to the high proportion of lipids in their structure. In the fluid mosaic model, described by Singer and Nicolson [130], the plasma membrane is seen as a phospholipid bilayer in which protein molecules are embedded (see Figure 2.5). Plasma membranes of most cells contain approximately 40% lipid, 0-10% carbohydrate and 50-60% protein [131]. Both phospholipids and membrane proteins diffuse laterally through the membrane, though the proteins diffuse more slowly because of their greater size. Cholesterol breaks up the van der Waals interactions and close packing of the phospholipid tails, thus increasing membrane fluidity. The cell membrane serves as the interface between the

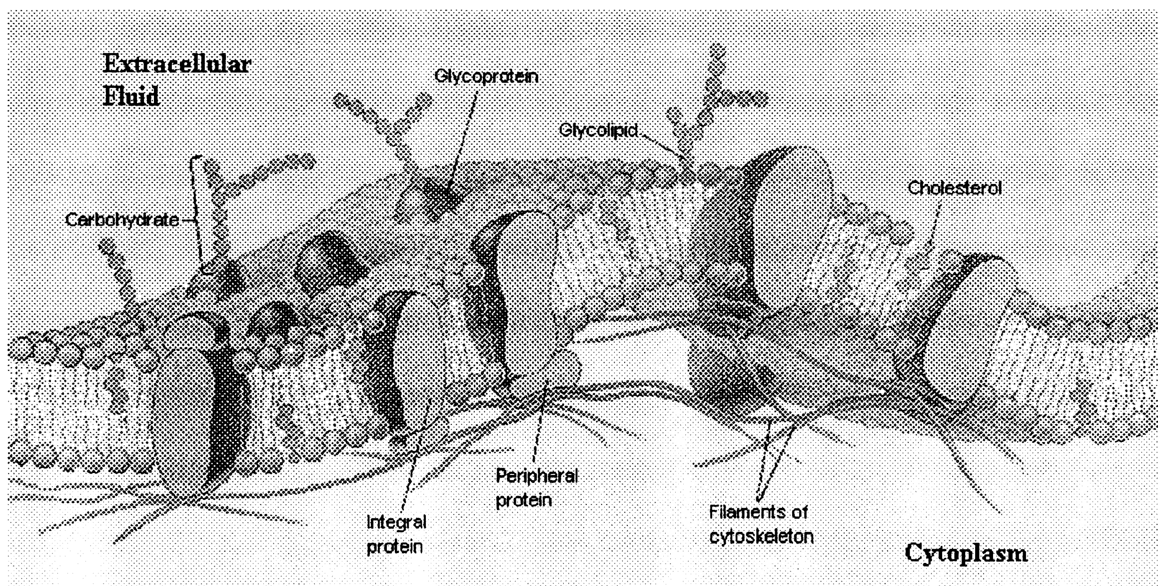


Figure 2.5 Schematic representation of the fluid mosaic model for biological membranes. Adapted from [132].

cell and its surroundings. Substances may cross the cell membrane either actively or passively. Only small molecules that are both water-soluble and lipid soluble can diffuse through the lipid portion of the membrane, such as ions, amino acids and nucleoside phosphates [133]. Bigger particles such as liposomes must find another way to cross the membrane.

One major goal of liposome pharmacology is the selective delivery of drugs to target cell populations while minimizing elimination by phagocytic macrophages and blood monocytes of the RES. In order to use liposomes as effective drug delivery agents, it is first necessary to understand the ways in which they can interact or associate with cells, and the conditions that influence these modes of interaction. The various ways in which liposomes can interact with cells are depicted in Figure 2.6. Stable adsorption, which represents the association of intact vesicles with the cell surface, could be mediated by non-specific interactions such as electrostatic and hydrophobic interactions, or by specific interactions with components present at the vesicle and / or cell surface. Endocytosis is the uptake of intact vesicles into the cell interior and may result in their delivery to the lysosomes [133]. Fusion is the merging of the vesicle bilayer with the plasma membrane bilayer, with the concomitant release of vesicle contents into the cytoplasmic space. Available data show that spontaneous fusion between liposomes and the plasma membrane is a rare event [134-136]. The fusion of liposomes with cells can be facilitated with fusogens such as PEG [137]. Lipid exchange is the transfer of individual lipid molecules between vesicles and the cell surface, without the cell-association of aqueous vesicle contents [138]. At liposome concentrations greater than 10 μM lipids,

exchange of lipids between the liposome and cell membranes has been described as a minor event [139].

When liposomes first come into contact with cells, the adsorption of the liposome to the cell surface occurs. Following adsorption, internalization of the liposomes by endocytosis or direct fusion of membranes may occur [139-142]. Endocytosis may be receptor-mediated in the case of liposomes modified with molecules that bind receptor proteins on the surface of the cell with high-specificity, or non-specific in the absence of these molecules at the surface of the liposome [133].

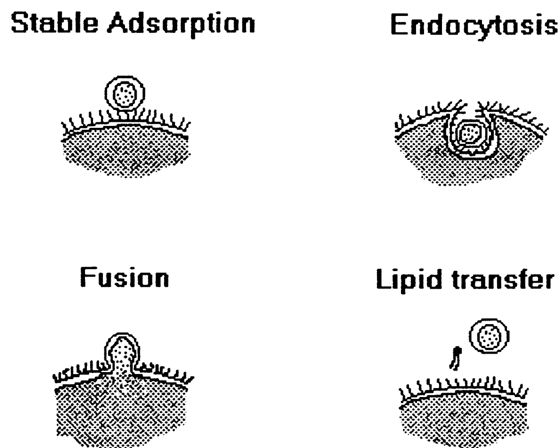


Figure 2.6. Schematic representation of possible mechanisms of interaction between small unilamellar vesicles (d < 500 nm) and the cell surface. Adapted from [143].

One must distinguish between two types of targeting, namely active and passive. When liposomes are injected in the blood stream, they tend to accumulate in the liver and spleen, regardless of lipid composition or size. Liposomes also tend to accumulate in

tumours due to "leaky" vasculature at these sites. This phenomenon is called passive targeting. Active targeting consists in modifying the liposomes to target specific cells or tissues.

Various groups studying the dose-response behaviour of cell-liposome binding have found that the association of lipid vesicles with cells is saturable. It appears that special liposome-binding sites are present on the cell surface [144-147].

Targeting of glycolipid-based liposomes to specific liver cell types was achieved both in vitro and in vivo simply by varying the sugar residues on the liposomal surface [148]. Liver accumulation depended on sugar moieties such as galactosyl residues. By incorporating various proteins, glycoproteins and glycolipids into liposomes, it was possible to modulate both the affinity of the liposomes for the cell surface and to enhance particular modes of cell-liposome interaction [149].

In order to facilitate targeting to non-phagocytic cells, the attachment to the liposome bilayer of monoclonal antibodies directed against receptors on the surface of specific cells was proposed [150, 151]. The mechanism by which antibody-conjugated liposomes deliver their contents to cells has been investigated and is thought to occur by receptor-mediated endocytosis [152]. The adjacent plasma membrane invaginates and the whole complex is internalized as a cytoplasmic vacuole. The receptor and its liposome-associated ligand separate, after which the receptor may be recycled to the cell surface or degraded. The vacuole fuses with lysosomes and the liposome is exposed to lysosomal enzymes. Liposomal contents can be degraded by these enzymes and rendered

ineffective before reaching the cytoplasm or the cell nucleus. This constitutes a serious drawback for this method of targeting.

Kamps et al. [153] studied the ability of anionized albumin-coated liposomes to target hepatic endothelial cells and found that within 30 minutes of injection into rats, the coated liposomes were completely cleared from the blood and almost exclusively taken up by the liver. On the other hand, 80% of the unmodified liposome population was still present in the blood. The uptake of the albumin-coated liposomes by liver endothelial cells was dependent on liposome size. Liver uptake decreased with increasing diameter, in favour of Kupffer cell uptake [153].

Dextran-coated surfaces have been shown to reduce cell adhesion [69, 129,154]. On the other hand, liposomes prepared with chemically modified dextrans (CMDBS) have exhibited a higher binding capacity for smooth muscle cells and endothelial cells than conventional liposomes and dextran-modified liposomes [36, 115]. These findings were assumed to be the result of high binding interactions between the CMDBS-modified liposomes and the cell membranes, but the mechanism of interaction is not yet understood. The observed CMDBS liposome-cell interactions were very rapid, occurring after only 15 minutes of incubation. For both cell types, these interactions were enhanced upon the addition of calcium to the cell culture medium. Numerous studies [155-157] have pointed to the role of calcium in the fusion of model membrane systems; however calcium was not found to induce the fusion of vesicles composed of PC and PE lipids since the ion only binds to them weakly.

3. OBJECTIVES AND HYPOTHESES

The interest surrounding liposomes is mainly due to their ability to carry both hydrophilic and hydrophobic drugs and to protect them against overly rapid clearance from the circulatory system. These properties make liposomes a vehicle of choice for drug delivery. However, problems of stability and cell targetability remain unresolved.

One approach to increase stability (i.e. blood circulation lifetime) is to reduce protein adsorption by creating sterically stabilized liposomes via polyethylene glycol (PEG) modification. The hydrophilicity and mobility of the highly flexible PEG chains results in a high exclusion volume and high chain compressibility thus preventing proteins from adhering to surfaces to which PEG chains are grafted [69].

Studies investigating the biodistribution and blood circulation lifetimes of PEG-modified liposomes have shown that the steric repulsion conferred by PEG is mainly governed by PEG molecular weight (MW) and loading. It is believed that smaller MWs (in the range of 750-5000) are most effective at repelling proteins and that loadings of 3-10 mol % are optimal, although even smaller loadings have been shown to be effective [74-76]. Liposomes have also been modified with polysaccharides such as dextran [88]. These polysaccharides also have protein-rejecting properties, in addition to being biodegradable and having low toxicity. Dextran and heparin have been shown to confer stability on the bilayer when introduced into liposomes [89, 125]. As is the case for PEG, the molecular weight of dextrans governs protein adsorption, with lower MWs giving less adsorption [128].

Targeting of liposomes to specific cells or tissues remains to be achieved. It is clear that liposomes must not only reach the intended cells, but also interact with cellular membranes in order to deliver their contents. Interactions with cellular components of the vascular walls were observed for liposomes modified with various sulphated polysaccharides such as heparin and chemically modified dextrans [93]. Knowledge of the relationship between protein adsorption and in vivo organ distribution can be utilized for drug targeting to different tissues. Carriers with different protein adsorption patterns will interact with different tissue-specific receptors or will be recognized by different macrophage subpopulations.

Given the background discussed above and in Chapter 2, the aim of the present study was to elucidate the relationship between protein adsorption and cellular interaction for liposomes modified with PEG, dextran, functionalised dextran (CMDSu) and heparin. The polymer modifiers were conjugated to an anchoring molecule to insure incorporation into the liposome surface. PEG was linked to phosphatidylethanolamine (PE), whereas a cholesterol moiety was used for the polysaccharides. Equal mass loadings of the various polymers were used. Endothelial cells (EC) and vascular smooth muscle cells (VSMC) were used for the in vitro cell interaction studies. These can be considered as models for cells in general, and in the case of endothelial cells could be targets for antithrombotic drugs.

We hypothesized that PEG-modified liposomes would adsorb less protein than unmodified liposomes, due to steric repulsion. Extensive binding of plasma opsonins and other proteins was expected for unmodified liposomes. Based on previous studies,

dextran-modified liposomes were also expected to adsorb less protein than controls [69, 128]. On the other hand, functionalised dextran and heparin-modified liposomes were predicted to adsorb more protein than controls due to the presence of charged chemical groups on the polysaccharide, which could attract proteins. Heparin also possesses a binding site for antithrombin, which could be expected to enhance binding of that protein specifically from plasma. The protein binding profiles were expected to be similar for PEG-modified and dextran-modified liposomes, due to similar steric repulsion effects. Profiles for CMDSu-modified and heparin-modified liposomes were predicted to be different from those for unmodified and PEG- or dextran-modified liposomes, due to preferential adsorption of specific proteins.

Polysaccharide-modified liposomes were expected to interact with cells more strongly than unmodified liposomes due to sugar binding sites on the cell surface. Heparin and some functionalised dextran compositions have been shown to inhibit smooth muscle cell growth [98]. This could mean that there is a specific receptor for a sequence in heparin and functionalised dextran leading to increased cellular interactions between smooth muscle cells and heparin- or CMDBS-modified liposomes. Heparin also possesses a binding site for fibronectin, a protein involved in cell-to-cell adhesion [158]. The presence of fibronectin on a liposome surface could indicate increased cell interaction. PEG-modified liposomes would probably interact only weakly with cells because of the steric barrier around the liposome.

Finally, we wished to establish a correlation between protein adsorption patterns and endothelial and vascular cell interactions. It was expected that liposomes that bind

more proteins would interact more strongly with cells due to the possibility of receptors on the cell membrane for specific proteins.

4. MATERIALS AND METHODS

4.1 Materials and Equipment

In this section, all chemicals as well as all equipment used in this work are listed in the following tables.

Buffer Preparation:

Material / Equipment	**Description**	**Supplier**
Filter unit	0.2 μm, 0.5 l unit with receiving flask	Nalgene
N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]	HEPES – free acid Purity = 99.5%	Sigma
Sodium chloride, NaCl		Caledon

Liposome Preparation:

Material / Equipment	**Description**	**Supplier**
[1α, 2α (n) – ^{3}H]-Cholesterol	48.0 Ci/mmol	Amersham Pharmacia Biotech.
Argon, Ar		Air Liquide
Chloroform		Sigma - Aldrich
Cholesterol	99+% purity	Sigma
Lyophilizer	Unitrap model 10-100	The Virtis Company
n-octyl-β-D-glucopyranoside (OG)		Sigma
Phospholipids: 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE) 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy (polyethylene glycol)-2000] (DMPE-PEG) 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (DMPE-Rh)		Avanti Polar Lipids Inc.
Rotary evaporator	Rotavapor RE111	Bucchi
Sonication bath	Model 2200	Branson
Spectra-Por cellulose tubing	12,000 – 14,000 MW cut-off	Fisher Scientific
Wheaton liquid scintillation vials	20 mL capacity, 22 mm PP cap with foamed PE	Sigma

Chromatography:

Material / Equipment	Description	Supplier
Chromatography gels: Sepharose CL-4B Sephacryl S-1000		Sigma
Size exclusion chromatography column	1.5 cm X 30 cm Econo-column	Bio-Rad

Phosphate Assay:

Material / Equipment	Description	Supplier
Ammonium molybdate tetrahydrate		Sigma
Hydrochloric acid, HCl	Reagent grade	Caledon
L-ascorbic acid, $C_6H_8O_6$		BDH
Magnesium nitrate hexahydrate, $Mg(NO_3)_2$*$6H_2O$		Sigma
Potassium dihydrogen orthophosphate (monobasic), KPO_4H_2		BDH
Spectrophotometer	DU-640 Spectrophotometer	Beckman
Test tubes	13 mm X 100 mm	Pyrex

Dynamic Light Scattering:

Material / Equipment	Description	Supplier
DLS machine	Variable angle QELS	Brookhaven Instruments

FTIR:

Material / Equipment	Description	Supplier
Spectrometer	FTS-40 FT-IR	Bio-Rad
Potassium bromide, KBr	99+ %, FT-IR grade	Sigma

TNBS Assay:

Material / Equipment	Description	Supplier
Picrylsulfonic acid hydrate (TNBS)	5% (w/v) aqueous solution	Sigma
Sodium hydrogen carbonate, $NaHCO_3$		BDH
Spectrophotometer	DU-640 Spectrophotometer	Beckman

Cholesterol Conjugation to Polysaccharides:

Material / Equipment	Description	Supplier
Absolute ethanol	100%	Aldrich
Bromopropylamine hydrobromide		Aldrich
Carboxymethyl dextran benzylamide sulfate/sulfonate (CMDBS12)	$MW_{average}$ = 55,000 g/mol determiner by HPLC, prepared from native dextran T40	Laboratoire de Recherche sur les Macromolécules (Villetaneuse, France)
Cholesteryl chloroformate		Aldrich
Dextran T40	$MW_{weight\ average}$ = 35,700 g/mol	Pharmacia
Diethyl ether	Anhydrous	Fisher Scientific
Dimethylsulfoxide, DMSO		Sigma
N-N-dimethylformamide, DMF	99,9+%, HPLC grade	Aldrich
Hydrochloric acid, HCl	Reagent grade	Caledon
Heparin H108	$MW_{average}$ = 10.7 kDa determined by HPLC, Anticoagulant activity = 173 IU/mg	Sanofi Recherche (Gentilly, France)
Lyophilizer	Unitrap model 10-100	The Virtis Company
NaOH		BDH
Pyridine		BDH
Spectra-Por cellulose tubing	12,000 – 14,000 MW cut-off	Fisher Scientific

Bio-Rad Detergent Compatible Assay for Total Protein:

Material / Equipment	Description	Supplier
Albumin	Human serum albumin	Behring
Bio-Rad DC Protein Assay kit	Reagents A (sodium hydroxide), B (Folin reagent) and S (sodium dodecyl sulfate)	Bio-Rad
Spectrophotometer	Microplate Reader II	Dupont
96-well plates		Nunc

Radiolabelling of Proteins:

Material / Equipment	Description	Supplier
Centrifuge	Centrifuge 3200	Brinkmann
Dialysis cassette	Slide-A-Lyzer cassette, 10,000 MW cut-off	Pierce Chemical
Gamma counter	Gamma 5500, Minaxiγ, Auto-Gamma® 5000 Series	Beckman, Canberra Packard Canada
Glycine	Biotechnology grade	BioShop
IgG	Human IgG purified immunoglobulin, reagent grade	Sigma
Iodo-gen® reagent	1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril	Pierce Chemical
$Na^{125}I$		ICN Radiochemicals
Phosphotungstic acid		Sigma

Ultracentrifugation :

Material / Equipment	Description	Supplier
Centrifuge	TL-100 Ultracentrifuge	Beckman
Centrifuge tubes	11 mm X 34 mm polycarbonate for TLA 100.2 rotor	Beckman
IgG	Human IgG purified immunoglobulin, reagent grade	Sigma
Plasma	Pool of 25 donors	McMaster University
Rotor	TLA 100.2	Beckman

SDS-PAGE and Western Blotting:

Material / Equipment	Description	Supplier
2-mercaptoethanol		BDH
5-bromo-4-chloro-3-indolyl phosphate, BCIP		Bio-Rad
Acrylamide	Bioultra Pure grade	BioShop
Ammonium persulfate		Millipore
Bromophenol blue		Millipore
Dimethyl formamide		Fisher Scientific
Di-sodium hydrogen orthophosphate, Na_2HPO_4		BDH
Dry skim milk		VIM
Glycerol		BDH
Glycine	Biotechnology grade	BioShop
Magnesium chloride hexahydrate, $MgCl_2*6H_2O$		BDH
Methanol	HPLC grade	Caledon
N,N'-methylenebisacrylamide		Gibco BRL
Nitroblue tetrazolium chloride, NBT		Bio-Rad
Prestained SDS-PAGE low molecular weight standards		Bio-Rad
Primary antibodies:		
Factor XI, Factor XII, Prekallikrein, HMWK, Fibrinogen, ATIII, Albumin, Vitronectin, Protein C, Thrombin		Cedarlane Laboratories Ltd.
Plasminogen, Transferrin, IgG, Beta-lipoprotein, Alpha-2-macroglobulin, Beta-2-microglobulin, Haemoglobin		Sigma
C3, Factor B, Factor H, Factor I		
Alpha-1-antitrypsin, Fibronectin		Calbiochem
Factor V		ERL

Protein S Apolipoprotein A-1		Dr. Fred Ofusu, McMaster University American Diagnostics Inc.
Protogold solution		Cedarlane Laboratories Ltd.
PVDF (polyvinylidene fluoride) membrane	Immobilon-P transfer membrane	Millipore
Pyronin Y (G)		Bio-Rad
SDS-PAGE low molecular weight standards		Bio-Rad
Secondary antibodies (alkaline phosphatase conjugate): Rabbit anti-goat IgG Goat anti-rabbit IgG Rabbit anti-sheep IgG		 Sigma Bio-Rad Bethyl Laboratories
Sodium chloride, NaCl		Caledon
Sodium dihydrogen orthophosphate (monobasic), $NaH_2PO_4*H_2O$		BDH
Sodium dodecyl sulfate, SDS	Electrophoresis grade	BioShop
Sodium hydrogen carbonate, $NaHCO_3$		BDH
TEMED	>99%	BioShop
Tris (hydroxymethyl) aminomethane	Bioultra Pure grade	BioShop
Tween 20, polyoxyethylene sorbitan monolaurate		Bio-Rad

Cell studies:

Material / Equipment	**Description**	**Supplier**
24-well cell culture plates	Sterile	Costar
Cell culture flasks	25, 75 and 162 cm^2, ventilated cap	Costar
Cell lines: EAhy 926 BLC#5	 Human umbilical vein endothelial cells (HUVEC) hybridized with human lung carcinoma cells A549/8. Rat aorta smooth muscle cells hybridized with Large T antigen of the SV-40 virus.	 Dr. C.-J. Edgell, University of North Carolina, Chapel Hill Dr. J. Castellot, Boston USA
Coulter Counter	Type ZM	Coultronics
Cryotubes	1 mL capacity	Nunc
Dimethylsulfoxide, DMSO		Sigma
Dulbecco's Mod Eagle Medium (DMEM)	Without sodium pyruvate, with 4500 mg/L glucose, with pyridine	Gibco BRL

	HCl	
Dulbecco's Phosphate Buffered Saline (PBS)	Without calcium and magnesium, without sodium bicarbonate	Gibco BRL
Foetal bovine serum (FBS)		Eurobio
Hypoxanthine-aminopterin-thymidine (HAT) supplement	50X	Gibco
Isoton		Coultronics
L-glutamine	200 mM, 100X	Gibco
Malassez cell		Poly Labo
Streptomycin –Penicillin		Gibco
Trypan blue	0.4% (v/v)	Gibco
Trypsin-ethylenediaminetetraacetic acid (EDTA)	1X in HBSS, without calcium and magnesium	Gibco

Cells in Contact With Fluorescent Liposomes:

Material / Equipment	**Description**	**Supplier**
Dulbecco's Phosphate Buffered Saline (PBS)	Without calcium and magnesium, without sodium bicarbonate	Gibco BRL
Formaldehyde	37% (v/v)	Fisher Scientific
Glycerol		BDH
Labtek slides	2 and 4-well Permanox slides, removable wells	Nunc
Microscope	Nikon Eclipse E800, rhodamine filter, Nikon FDX-35 camera	Dr. J.B. Michel, INSERM U460, Hôpital Bichat, Paris, France
n-propylgalate		Sigma

Cells in Contact With Radiolabelled Liposomes:

Material / Equipment	**Description**	**Supplier**
24-well cell culture plates	Sterile	Costar
Dulbecco's Phosphate Buffered Saline (PBS)	With calcium and magnesium	Eurobio
Liquid Scintillation counter	1214 RACKBeta model	LKB Wallac, Finland
Liquid scintillation vials		Sigma
NaOH		Sigma
Scintillation liquid	Optiphase HISafe 2, Wallac Scintillation products	Fisher Chemicals, UK
Sodium dodecyl sulfate, SDS	Electrophoresis grade	BioShop
Trichloroacetic acid, TCA	ACS reagent, ≥ 99.0%	Sigma

4.2 Methods

4.2.1 HEPES Buffer Preparation

HEPES buffer (145 mM NaCl, 10 mM HEPES) was prepared by adding 8.4738 g NaCl and 2.3831 g HEPES to a volumetric flask and bringing the volume to 1 L with distilled water. The pH of the solution was adjusted to 7.4 using concentrated NaOH. The buffer was stored at 4°C and was filtered through a 0.2 μm filter before use.

4.2.2 Liposome Preparation

Liposomes were prepared using the detergent removal technique, in which small mixed micelles of detergent-phospholipid fuse upon removal of detergent. This results in liposomes that are mostly unilamellar with a unimodal size distribution [9].

Dry phospholipids, PEG-conjugated phosphatidylethanolamine (PE-PEG) and cholesterol were weighed according to specified molar ratios. The powders were dissolved in chloroform in a 50 mL round bottom flask. Chloroform was evaporated using a rotary evaporator and a warm water bath for 30 min. The resulting lipid film was then further dried in a lyophilizer overnight. The film was then hydrated with 5 mL of HEPES buffer to obtain preparations at 10 mM total lipids ($[lipid]_{total}$ = [PC] + [PE] + [PE-PEG] + [Chol])and placed in a sonication bath for 15 min to fully release the lipid film from the sides of the flask. Following sonication, n-octyl-β-D-glucopyranoside (OG) was added to the lipid suspension at a molar ratio of detergent to total lipids (including cholesterol) of 5.4. The suspension was then stored at 4°C for a minimum of 2

days and a maximum of 4 days before further processing. For polysaccharide-modified liposomes, 1 mg/mL of polysaccharide bearing a cholesterol anchor was then added to the micellar suspension. In the case of fluorescent liposomes, rhodamine-PE was added to the initial lipid mixture at a concentration of 1 mol%. Radiolabelled liposomes were prepared by adding 0.03 mol% [^{3}H]-cholesterol to the initial lipid film. The suspension was dialysed against HEPES buffer using cellulose membrane with a molecular weight cut-off of 12,000 to 14,000 for 12 h at 4°C, changing the bath after 3 and 6 h.

The liposomes were then passed through a size exclusion chromatography (SEC) column packed with Sepharose CL-4B to remove any un-incorporated lipids and polysaccharides. The lipid content of the collected fractions was determined either by phosphate assay or by refractometry. The liposome fractions were pooled and stored at 4°C. All liposomes were used in subsequent experiments within 4 days of preparation.

4.2.2-1 Calibration of the Size Exclusion Chromatography Column

The experimental set-up of the chromatography system is depicted in Figure 4.1.

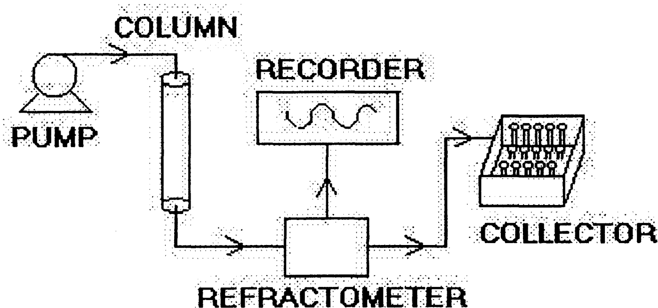


Figure 4.1 Chromatography System Set-Up

The elution profiles of all liposome types as well as all polysaccharides were determined. Liposomal suspensions were prepared using 10 mM lipid concentration and polysaccharide solutions were prepared at 25 mg/mL. Samples of 1 mL volume were injected on the column. For the liposome profiles, fractions were collected and the phospholipid content was determined by the phosphate assay. The polysaccharide (and the liposome) concentrations were monitored using a refractometer placed at the exit of the column. The elution profiles were plotted and elution times were compared.

4.2.3 Liposome Characterization

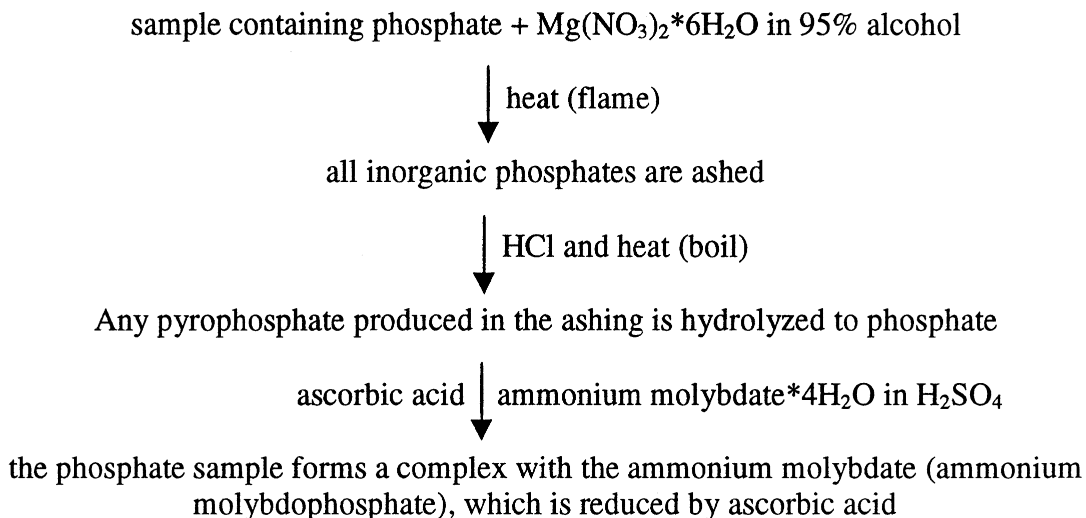
4.2.3-1 Phosphate Assay [159]

The phosphate assay was used throughout this work to determine the phospholipid content of liposome suspensions. This is a very sensitive colorimetric assay for inorganic phosphate which lends itself well to the quantification of phospholipids. The detection limit is of the order of 0.01 μmol phosphate.

Phosphate standards (0 to 75 μL of 1 mM KPO_4H_2) were placed in test tubes (in duplicate). Liposome samples (5 μL) were also placed in test tubes (in triplicate). To each tube was added 30 μL magnesium nitrate solution (10% $Mg(NO_3)_2*6H_2O$ in 95% ethanol). The contents of the tubes were then taken to dryness and ashed by shaking the tube over a strong flame until the brown fumes disappeared. Once the tubes had cooled, 300 μL of 0.5 M HCl was added. The tubes were then capped and placed in a boiling water bath for 15 min. Meanwhile, a solution containing1 part 10% ascorbic acid and 6 parts 0.42% ammonium molybdate*$4H_2O$ in 0.5 M H_2SO_4 was prepared. The tubes were

allowed to cool and 700 μL of this mixture was added. The tubes were re-capped and placed in a water bath at 45°C for 20 min. The absorbance of each tube was read at 820 nm. The standards were used to construct a calibration curve from which the phosphate concentration of the liposome samples was determined by interpolation.

The reactions occurring in this procedure are:



4.2.3-2 Dynamic Light Scattering

Dynamic light scattering or quasi-elastic light scattering (QELS) was used to determine the size distribution of the liposome suspensions. The technique consists in passing a laser beam through the diluted suspension and measuring the intensity of the scattered light produced by the particles. The basis of this method is described by Schurtenberger and Hauser [160].

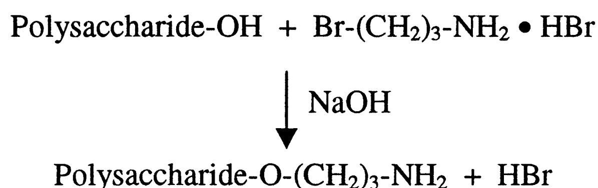
Samples were prepared by diluting 20 μL of liposomes (1 mM total lipids) in filtered HEPES buffer to a final volume of 2 mL. Parameters were set by assuming the HEPES buffer can be considered equivalent to water: $\lambda = 514$ nm, scattering angle = 90°,

temperature = 25°C, refractive index = 1.332 and viscosity = 0.8905 cP. Measurements in triplicate were taken over 90 s, with kilocounts per seconds (kcps) ranging between 100 and 200 and a baseline difference of less than 0.1%. The CONTIN software calculated the mean particle diameter and the size distribution of the vesicle suspension.

4.2.4 Polysaccharide Preparation

4.2.4-1 Amine Derivatization of Polysaccharides [116]

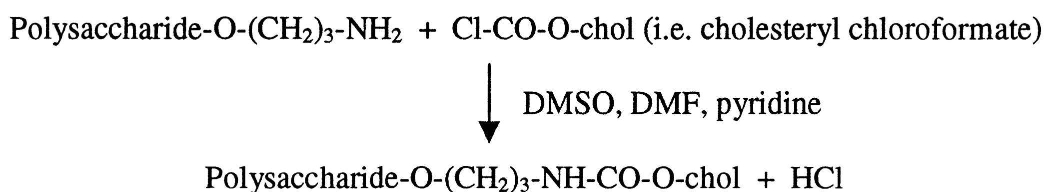
In a 50 mL round bottom flask, 1 g polysaccharide was added to 5 mL H_2O and 8 mL NaOH (6.17 N). The mixture was incubated for 20 min at 4°C. Bromopropylamine hydrobromide was added at a ratio R (mol bromopropylamine hydrobromide / mol polysaccharide subunit). The reaction was carried out with mixing for 4 h at room temperature.



Acidification of the solution with HCl to a pH between 6.5 and 7.0 was used to stop the reaction. The solution was added to 300-350 mL absolute ethanol at 4°C to precipitate the polysaccharide. The product was filtered under vacuum and then dissolved in 5 mL distilled water. The solution was dialysed against 500 mL water for 3 days with 3 to 4 bath changes using cellulose tubing (6 000-8 000 MW cut-off). The sample was then frozen and lyophilized for 24 h to yield the pure aminated polysaccharide.

4.2.4-2 Cholesterol Conjugation to Aminated Polysaccharides [161]

The aminated polysaccharide was first heated at 50°C for 2 h. In a 50 mL round bottom flask, 300 mg polysaccharide was added to 10 mL DMSO and 0.8 mL pyridine. A solution containing 0.12 g cholesteryl chloroformate in 3.5 mL DMF was then added to the flask. The reaction was carried out with mixing for 20 h in an oil bath at 75°C.



The solution was cooled and added to 150 mL diethyl ether for 12 h to precipitate the polysaccharide (unbound cholesterol is soluble in ether). The product was filtered under vacuum and then dissolved in 5 mL distilled water. The solution was dialysed against 500 mL water for 3 days with 3 to 4 bath changes using cellulose tubing (6 000-8 000 MW cut-off). The sample was then frozen and lyophilized for 24 h.

4.2.4-3 Fourier Transform Infrared Spectroscopy (FTIR)

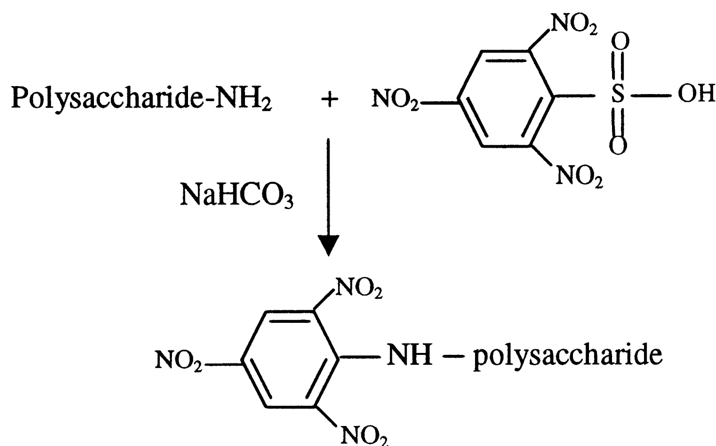
Solid samples (approx. 2 mg) were milled with approximately 200 mg potassium bromide (KBr) to form a very fine powder (KBr is transparent in the IR). This powder was then compressed into a thin pellet. Spectra were taken with a Bio-Rad FTS-40 TFT-IR spectrometer with a class II He-Ne laser operating at 632.8 nm. The sample

compartment was purged with a flow of dry nitrogen (10 psi) to remove any water vapour and CO_2 present. The data were collected and manipulated using Win-IR© Software.

4.2.4-4 TNBS Assay [162]

This assay was developed originally to determine the free amino group content of proteins. Picrylsulfonic acid (2,4,6-trinitrobenzenesulfonic acid, TNBS) was found to react specifically and under mild conditions with the free amino groups of amino acids and peptides to give trinitrophenyl (TNP) derivatives. In this work, the assay was used to determine the extent of amine conjugation to polysaccharides and to determine whether cholesterol conjugation to aminated polysaccharides occurred or not.

In a test tube, 1 mL polysaccharide solution (1 mg/mL) was added to a mixture of 1 mL 4% $NaHCO_3$, pH 8.5 and 1 mL 0.1% TNBS. The solution was allowed to react at 40°C for 5 h. 1 mL 10% SDS and 0.5 mL HCl (1N) were then added to each tube.



The absorbance of the solution was read at 340 nm against a blank treated as above but with 1 mL water instead of the polysaccharide solution.

4.2.5 Protein Adsorption Studies

4.2.5-1 Radioiodination of IgG by the Iodo-gen® Method [163]

This technique involves the introduction of radioactive iodine into proteins using a surface immobilized oxidizing agent to attach $^{125}I^-$ chemically to exposed tyrosine residues. A stock solution was prepared at a concentration of 0.1 mg/mL Iodo-gen® reagent in 100 mL chloroform. Aliquots (100 μL) of this solution were added to glass vials and the solvent was evaporated under a stream of nitrogen, thus allowing the Iodo-gen® reagent to coat the inside of the vials. The reaction vessels were then stored at -4°C.

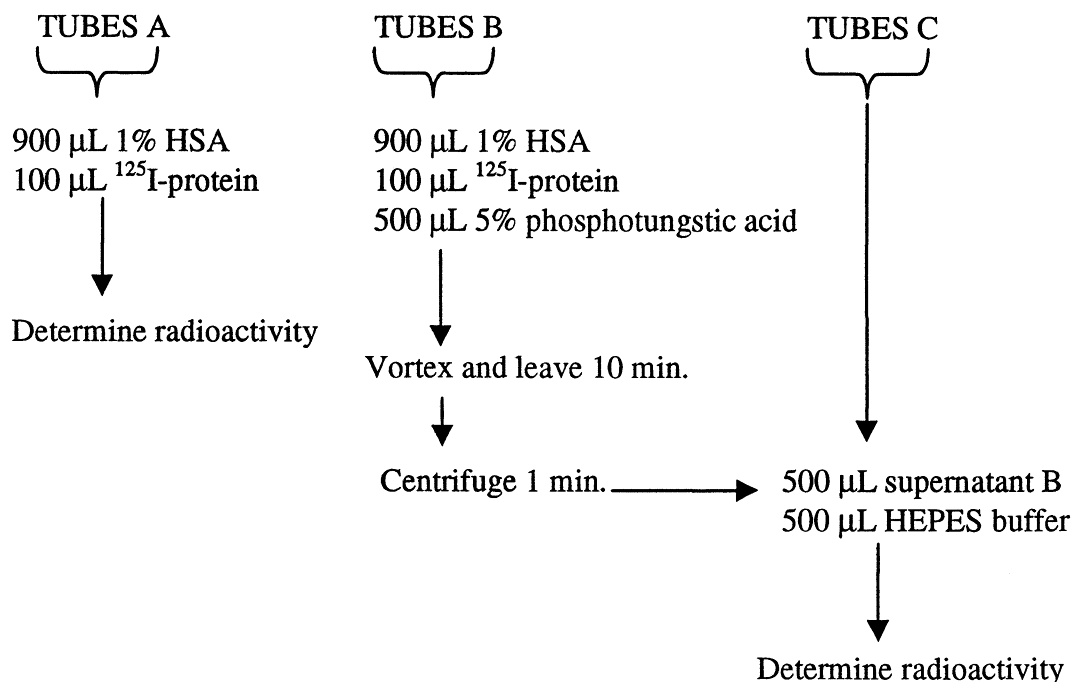
A solution of protein was prepared (typically 1-2 mg/mL) in HEPES buffer at pH 7.4 and its exact concentration was determined by reading the absorbance at 280 nm. The protein solution (typically 150-300 μL) was placed in the Iodo-gen® reaction vessel and 5 μL $Na^{125}I$ was added. The reaction was carried out with mixing for 15 min at room temperature, and the mixture was transferred to a dialysis cassette. The reaction vessel was rinsed three times with 250 μL HEPES buffer and the rinsate was added to the dialysis cassette. The solution was dialysed against 500 mL buffer for 3 h at room temperature. The buffer was then replaced and dialysis continued at 4°C overnight. The buffer was replaced again and dialysis was performed for another hour at room temperature. For adsorption experiments, labelled protein was mixed with unlabelled protein to yield a solution containing approximately 1% labelled protein.

4.2.5-2 Free $^{125}I^-$ Test

Following protein radiolabelling, a residual amount of unreacted $^{125}I^-$ remains in the solution. The quantity of free $^{125}I^-$ present is determined by this assay. Three groups of 3 centrifuge tubes were prepared and were labelled A, B and C. Human serum albumin (HSA) (1%, 900 μL) and 100 μL of the labelled protein solution were added to each of the tubes labelled A or B. To the tubes labelled B, 500 μL 5% (w/v) phosphotungstic acid was added. Phosphotungstic acid precipitates the protein, leaving only free $^{125}I^-$ in solution. The tubes were then vortexed and left for 10 min at room temperature. The tubes were then centrifuged for 1 min at 1000 rpm. 500 μL of the supernatant from tubes B and 500 μL of HEPES buffer were placed in the tubes labelled C. The radioactivity (CPM) of all tubes labelled A or C was determined and the free $^{125}I^-$ concentration was calculated as a percentage of total radioactivity using Equation 4.1:

$$\text{Free } ^{125}\text{I } (\%) = \frac{3 * (\text{average CPM of group C})}{3 * (\text{average CPM of group A})} * 100 \qquad \textbf{Equation 4.1}$$

This procedure may be summarized as follows:



4.2.5-3 Protein Adsorption from Buffer

In this study, the quantity of IgG adsorbed to each type of liposome was estimated quantitatively using the radioiodinated protein. Liposomes were prepared and characterized as previously described. Aliquots of liposomes (0.5 mL corresponding approximately to 5 µmol of total lipids) were placed in centrifuge tubes. Stock solution of radiolabelled IgG in HEPES buffer (145 mM NaCl, 10 mM HEPES, pH 7.4) was added to yield solutions of the following concentration: 0.1, 0.5 and 1.0 mg IgG/mL. A "control" tube contained 1.0 mg/mL of IgG without liposomes. The total volume was 1 mL in each tube. The tubes were vortexed briefly to ensure adequate mixing of liposomes and protein. Adsorption was carried out for 2 h at room temperature.

The liposomes were separated from the protein solution by 4 ultracentrifugation steps at 100 000 g for 30 min at 25°C. After each centrifugation, the supernatant was removed and replaced with an equal amount of fresh buffer. The tube was then vortexed to resuspend the pellet. The final liposome pellet was removed from the tube using 200 μL buffer. The tube was washed four more times with 200 μL buffer. These supernatants were then pooled and the radioactivity of the liposome sample was determined. The radioactivity of the supernatants previously removed was also recorded to determine whether unbound proteins were adequately removed from the liposome pellet. Triplicate measurements were done under each set of conditions.

4.2.5-4 Protein Adsorption from Plasma

Liposomes were prepared and characterized as previously described. Aliquots of liposomes (0.5 mL corresponding approximately to 5 μmol of total lipids) were placed in centrifuge tubes (in duplicate or triplicate). Plasma and HEPES buffer were added to each tube to yield a final plasma "concentration" of 10% normal strength. The total incubation volume was 1 mL per tube. Each tube was vortexed briefly to ensure good mixing of liposomes and plasma. Adsorption was carried out for 2 h at room temperature.

The liposomes were separated from the plasma by 7 ultracentrifugation steps at 350 000 g for 30 min at 25°C. After each centrifugation, the supernatant was removed and replaced with an equal amount of fresh buffer. The tube was then vortexed to resuspend the pellet. The final liposome pellet was removed from the tube using 100μL buffer.

The tube was washed twice more with 100 µL buffer. These aliquots were then pooled and 20 µL was removed and diluted 1:1 with HEPES buffer for the phosphate assay. The remaining 280 µL sample was solubilized in 70 µL 10% SDS and stored at -70°C for subsequent analysis by SDS-PAGE, immunoblotting and total protein assay.

4.2.5-5 Bio-Rad Detergent Compatible Assay for Total Protein [164]

The Bio-Rad DC Protein Assay is a colorimetric assay for total protein following detergent solubilization, and is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent. This assay was used to quantify the total protein bound to liposomes following exposure to 10% plasma.

Stock albumin solution was prepared in HEPES buffer containing 2% SDS and serially diluted in the range 0.1-1.0 mg/mL. In a 96-well plate, 5 µL of each standard and unknown was added to different wells (in triplicate). Reagent A' was prepared by adding 20 µL reagent S (sodium dodecyl sulfate) per 1 mL reagent A (alkaline copper tartrate solution). To each well were added 25 µL reagent A' and 200 µL reagent B (dilute Folin reagent). The samples were left at room temperature for 15 min to react. The absorbance was then read at 690 nm. The colour was stable for at least 2 h. A calibration curve was constructed based on albumin and the total protein in the unknowns was determined by interpolation.

4.2.5-6 Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis (SDS-PAGE)

This technique was used to separate the proteins bound to liposomes following exposure to 10% plasma. Briefly, a gel consisting of 4% stacking gel and 12% separating gel was cast. Liposome samples in SDS buffer were loaded onto this gel and electric current was applied. SDS treatment gives the proteins a negative charge causing them to migrate to the anode (the bottom of the gel). The speed of migration is inversely proportional to the molecular weight of the protein, i.e. smaller proteins migrate faster. The gel was then equilibrated in transfer buffer and electrophoretically transferred to a PVDF membrane. The membrane was then washed and gold stained to expose protein bands. The membranes were then rinsed and dried.

4.2.5-7 Immunoblotting

This technique allows for the identification of specific proteins bound to the liposomes by taking advantage of antibody / antigen complex formation. The protocol for Western blotting is the same as for SDS-PAGE up to and including drying of the PVDF membrane. However, only a small portion of the membrane was kept for gold staining and the rest was sliced into thin strips. Membrane areas not containing protein were blocked with 5% (w/v) skim milk. The strips were then incubated with a primary antibody, washed, and incubated with a secondary antibody (conjugated to alkaline phosphatase, AP), which was directed against the first. The strips were treated with NBT solution to develop the AP colour reactions and then air-dried. The detailed experimental protocol for SDS-PAGE and Western blotting can be found in Appendix A.

4.2.6 Cell Studies

4.2.6-1 Cell Culture

Human endothelial cells, EAhy926 line [165], were routinely cultured in medium consisting of Dulbecco's Mod Eagle Medium (DMEM) supplemented with 2 mM L-glutamine, and hypoxanthine-aminopterin-thymidine (HAT) supplement (100 μM hypoxanthine, 0.4 μM aminopterin, 16 μM thymidine) (henceforth referred to as endothelial cell growth medium), and 10% (v/v) foetal bovine serum (FBS), at 37°C in a 5% CO_2 incubator. This cell line was obtained by hybridization of human umbilical vein endothelial cells (HUVECs) and human pulmonary carcinoma cells (A549), and was kindly provided by Dr. C. Edgell. The permanent cell line maintains the characteristics of the parent HUVECs [165, 166].

Rat aorta smooth muscle cells, BLC#5 line, were routinely grown in DMEM supplemented with 2 mM L-glutamine (henceforth referred to as smooth muscle cell growth medium) and 10% (v/v) foetal bovine serum (FBS) at 37°C in a 5% CO_2 incubator. This cell line was obtained by introducing an oncogene, SV40 Large T antigen (SVLT), into Sprague-Dawley rat aorta cells, and was kindly provided by Dr. J. Castellot [167]. The BLC#5 clone retains sensitivity to heparin.

4.2.6-2 Freezing of Cells

To maintain cell culture, cells were aliquoted and frozen. It is best to freeze cells when they are in their exponential growth phase to allow optimum proliferation when they are thawed. When sufficient cells filled a flask, the growth medium was removed

and cells were washed twice with PBS (without Ca^{2+} and Mg^{2+}). Trypsin was then added (1.5 mL for 75 cm^2 flasks) and cells were incubated for 3 min at 37°C. The action of trypsin was stopped by adding growth medium supplemented with 10% FBS (approximately 3 volumes of growth medium is needed for 1 volume of trypsin). The cell suspension was then counted using a Malassez cell and centrifuged at 200 g (100 rpm) at 20°C for 5 min. The supernatant was removed and the cell pellet was resuspended in FBS with 10% DMSO (v/v) (a cryoprotecting agent) to obtain a cellular suspension containing $1X10^6$ cells/mL. This suspension was then aliquoted into cryotubes (1 mL per tube) and the tubes were progressively frozen: 20 min at -20°C, 24 h at -80°C, and then stored in liquid nitrogen (-196°C).

4.2.6-3 Thawing of Cells

Rapid thawing was performed at 37°C to maximize cell viability. The contents of a cryotube was thawed and added to a 25 cm^2 culture flask containing growth medium supplemented with 10% FBS. Cells were then incubated at 37°C and the culture medium was changed after 12 h to remove any cellular debris and residual DMSO.

4.2.6-4 Passage of Cells

To maintain cell culture, cells were re-seeded periodically in a new culture flask prior to reaching confluence. The growth medium was removed and cells were washed twice with PBS (without Ca^{2+} and Mg^{2+}). Trypsin was then added (1.5 mL for 75 cm^2 flasks) and cells were incubated for 3 min at 37°C. The sides of the flasks were then

tapped to detach all the cells from the bottom. Growth medium supplemented with 10% FBS (approximately 3 volumes of growth medium is needed for 1 volume of trypsin) was added to the cell suspension and the contents of the flask were centrifuged at 200 g (100 rpm) at 20°C for 5 min. The supernatant was then removed and replaced with fresh 10% FBS growth medium. The cells were re-distributed in new culture flasks and returned to the incubator.

4.2.6-5 Counting Cells

Once removed from their culture flask, cells were counted using two different techniques: manually using a Malassez cell, or mechanically using a Coulter counter. To count the cells manually, they were exposed to trypsin as in the passage protocol and resuspended in 10% FBS growth medium. 20 μL of this cellular suspension was pipetted into a compartment of the Malassez cell, and the cells were counted using a light microscope. The number of cells present in the counting area corresponded to the number of cells in 1 μL of cellular suspension. The Coulter counter method was simpler in that it was done automatically. A 200 μL sample of the cell suspension was mixed with 9.8 mL of Isoton. The counter counted cells in a 500 μL sample of this suspension.

4.2.6-6 Trypan Blue Assay

This assay was used to determine the viability of cells. All dead cells turn blue on contact with the Trypan blue reagent. Cellular suspension (50 μL) was added to 50 μL 0.4% Trypan blue reagent. The reaction was carried out for 5 min at room temperature.

A 20 μL volume of this suspension was pipetted into the Malassez cell and both white and blue (i.e. live and dead) cells were counted. The proportion of dead cells was compared to a control and the cytotoxicity of the products was assessed.

4.2.6-7 Cell Growth Kinetics in the Presence of Liposomes

Cells in their exponential growth phase were seeded in 24-well plates at a density of 10,000 cells/cm^2 in growth medium supplemented with 10% FBS and 1% penicillin-streptomycin. After a 24-h incubation period at 37°C in a 5% CO_2 incubator, the cells were growth-arrested by placing them in 0.4% FBS medium for 48 h. This step allows synchronization of the cells at the G0/G1 stage of the cell cycle [168]. Cells were then exposed to 2% FBS medium containing liposomes at a concentration of 0.4 mM total lipids. A control was performed with cells in 2% FBS medium only. Cells in the bottom of the flask as well as those floating in the medium were counted using the Malassez cell by using the Trypan blue assay. Cells were counted at incubation times of 6 and 24 h. The results were compared with those obtained for the control. The effect of the liposomes on cell growth was assessed.

4.2.6-8 Incorporation of Fluorescent Liposomes in Cells [92]

Cells in their exponential growth phase were seeded on Labtek slides at a density of 10,000 cells/cm^2 in growth medium supplemented with 10% FBS and 1% penicillin-streptomycin. After a 24 h incubation period at 37°C in a 5% CO_2 incubator, the cells were growth-arrested by placing them in 0.4% FBS medium for 48 h. Rhodamine-

labelled liposomes were added to the cell culture in 2% FBS medium containing 1% penicillin-streptomycin at a concentration of 0.1 mM total lipids. The slides were then wrapped in aluminium foil to shield the fluorescent marker from light. Cells were incubated for 5 min and 3 h. After the indicated times, the slides were placed on a bed of ice to stop the incorporation process. After removing the growth medium, cells were washed twice with PBS at 4°C (without Ca^{2+} and Mg^{2+}). The cells were then fixed to the slides by exposing them to 3.7% formaldehyde for 20 min. The formaldehyde was then removed, the cells washed with PBS 4 times and once with distilled water. The slide wells were removed and the slides were mounted in 80% glycerol, 20% PBS containing 0.1M n-propyl gallate. Cells were then observed under a fluorescence microscope equipped with the appropriate rhodamine filter and an automatic photographic device.

4.2.6-9 Incorporation of Radiolabelled Liposomes in Cells

Cells in their exponential growth phase were seeded in 24-well plates at a density of 10,000 cells/cm^2 in growth medium supplemented with 10% FBS and 1% penicillin-streptomycin. After a 24-h incubation period at 37°C in a 5% CO_2 incubator, the cells were growth-arrested by placing then in 0.4% FBS medium for 48 h. Tritium-labelled liposomes were added to the cell culture in 2% FBS medium containing 1% penicillin-streptomycin at a concentration of 0.2 mM total lipids. Cells were incubated for 5 min, 3, 6 and 24 h. After the indicated times, the plates were placed on a bed of ice to stop the incorporation process. The culture medium was removed and the cells were washed twice with PBS (with Ca^{2+} and Mg^{2+}) at 4°C. 0.5 mL of a 10% trichloroacetic acid

(TCA) solution at 4°C was added to each well and incubated for 30 min. The TCA solution was then removed and the cells were washed extensively with distilled water and left to dry. A 0.5 mL volume of 0.4 M NaOH, 0.5% SDS solution was added to each well and incubated for 30 min. The cellular lysates were then removed and transferred to liquid scintillation vials. The wells were rinsed with 0.5 mL PBS (with Ca^{2+} and Mg^{2+}) and the wash solution was also added to the vials. 5 mL scintillation liquid was added to each vial and the solution was counted using a beta counter for 1 min.

5. RESULTS AND DISCUSSION:

5.1 Polysaccharide Modification

Polysaccharides are attractive for vesicle coating because, in addition to possessing protein-rejecting properties, they are biodegradable and minimally toxic. In order to insert the polysaccharides into the liposome bilayer, the polysaccharides were covalently modified with a cholesterol moiety to act as a hydrophobic anchor. The synthesis of cholesterol-grafted polysaccharides was achieved by derivatizing the polysaccharides with amino groups and then conjugating cholesterol moieties to the resulting amines.

5.1.1 Cholesterol Conjugation

To attach a cholesterol anchor to the various polysaccharides, amino groups were introduced into the polysaccharides by reaction with bromopropylamine. A 1:1 molar ratio of bromopropylamine to polysaccharide was used for all the polysaccharides. At this ratio, it was shown that heparin retains its anticoagulant activity (e.g. 168 ± 5 IU/mg compared to 173 IU/mg before amination) [125]. The conditions are summarized in Table 5.1.

Table 5.1 Experimental conditions for the reaction of bromopropylamine with various polysaccharides.

Polysaccharide	Mass reacted (g)	MW subunit (g/mol)	N_o subunit (mmol)	N_o bromopropylamine hydrobromide (mmol)	Mass bromopropylamine hydrobromide (g)	R*
Dextran T40	1	162	6.17	6.17	1.3514	1
Heparin H108	0.2	Approx. 300	0.67	0.67	0.147	1
CMDSu	1	Approx. 355	7.16	7.16	1.5682	1

*R = molar ratio of bromopropylamine to polysaccharide subunit

The average molecular weight of a CMDSu subunit was calculated as follows:

$$\begin{aligned}\text{MW subunit (CMDSu)} &= MW_D \text{ subunit} + \text{meq/g CM} * MW_{CM} \text{ subunit} + \text{meq/g Su} * MW_{Su} \text{ subunit} \\ &= 162 + 1.65 * 80 + 0.6 * 101 \\ &= 354.6 \text{ g/mol}\end{aligned}$$

Conjugation of cholesterol moieties to the various aminated polysaccharides was achieved by reaction with cholesteryl chloroformate. The amount of cholesteryl chloroformate added to dextran and CMDSu was chosen in accordance with the established protocol. However, for heparin, twice as much chloroformate as was suggested in the protocol was added. This was done to improve the yield [169]. The conjugation conditions are summarized in Table 5.2.

Table 5.2 Experimental conditions for the reaction of cholesterol moieties with various aminated polysaccharides.

Polysaccharide	MW_{ave} (g/mol)	Mass used (g)	Mass cholesterol chloroformate (g)	N_o Cholesterol chloroformate (mol)
T40	35,700	0.3	120	0.267
H108	10,700	0.1	80	0.089
CMDSu	55,000	0.4	160	0.356

5.1.2 Characterization of Cholesterol-Conjugated Polysaccharides

The TNBS assay was used to determine the concentration of free amino groups in the polysaccharide. This method was previously used to determine the free amine content of proteins, and is much more sensitive than the more conventional ninhydrin assay [162]. When the original protocol was followed, solutions of polysaccharides (1 mg/mL) were tested against a blank. The data were inconclusive; no distinction could be made between the samples and the blank. In subsequent attempts, the concentration of polysaccharide was increased to 10 mg/mL. This polysaccharide concentration was a practical limit due to limited supplies. Unfortunately, even at this higher concentration, the assay remained inconclusive. The amino group concentration on the polysaccharides appeared to be below the detection limit of the method.

FTIR was used to monitor the cholesterol conjugation reaction. This method was used only qualitatively. FTIR spectra for the polysaccharides are found in Figure 5.1. Cholesterol can be detected by the characteristic peak around 1735 cm^{-1}. This peak corresponds to the presence of the carbonyl group of the amide bond (-CO-NH-) formed between the aminated polysaccharide and the activated cholesterol moiety. For cholesteryl chloroformate a peak is observed at about 1775 cm^{-1} corresponding to the (-CO-Cl) group. It appears that all cholesterol conjugation reactions were successful. However it appears that only small amounts of cholesterol were present on dextran, compared to heparin and CMDSu. This is consistent with previous experience with these reactions [169]. The presence of the amide bond suggests that the introduction of amine groups onto the polysaccharide backbone was successful, but that only small amounts

were introduced and these amounts were too small to be detected by the TNBS assay. None of the spectra showed a peak around 1775 cm^{-1} (due to cholesteryl chloroformate), which would indicate that all residual chloroformate was removed when the product was precipitated in ether. For all polysaccharides, the peak corresponding to bound water decreased after the amination and conjugation reactions.

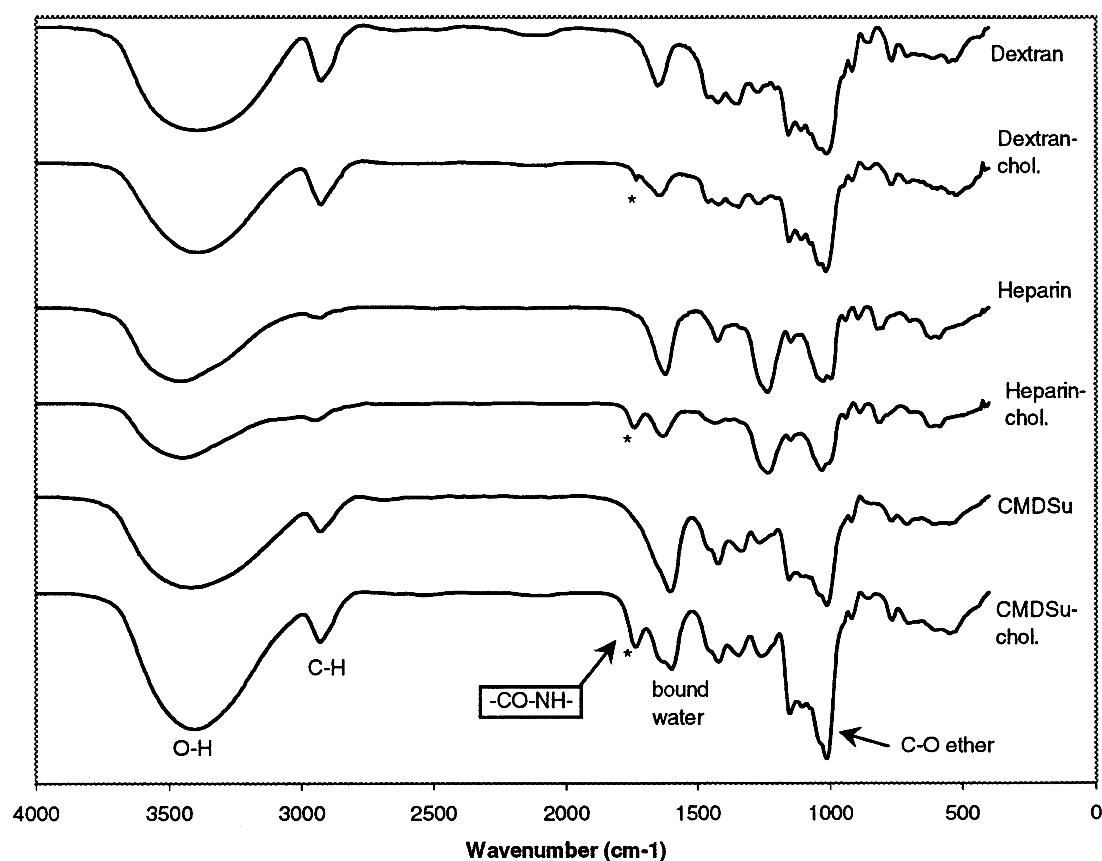


Figure 5.1 FTIR spectra of cholesterol-conjugated polysaccharides compared to the native polysaccharides. * Indicates the characteristic vibration of the amide bond formed between the polysaccharide amino groups and the activated cholesterol moiety. Other characteristic vibrations are identified on the figure.

5.2 Liposome Preparation

In this work, neutral liposomes modified with either polyethylene glycol (PEG) or different polysaccharides were studied. All liposome types were prepared with dimyristoyl phospholipids, i.e. with saturated 14-carbon fatty acid phospholipids. The various liposome compositions are summarized in Table 5.3.

Table 5.3 Liposome compositions

	Liposome composition				
Liposome type	**PC (mol%)**	**PE (mol%)**	**PE-mPEG (mol%)**	**Ch (mol%)**	**Mass polysaccharide added (mg/mL)**
Neutral (unmodified)	70	10	0	20	0
PEG-modified	70	5	5	20	0
Dextran-modified	70	10	0	20	1
Heparin-modified	70	10	0	20	1
CMDSu-modified	70	10	0	20	1

PC = phosphatidylcholine; PE = phosphatidylethanolamine; Ch = cholesterol; mPEG = methoxy-terminated PEG of MW 2000.

Saturated 14-carbon phospholipids were chosen since these are known to oxidize slowly, thus tending to increase the stability of the liposomal suspension over time. Oxidation is a radical reaction that ultimately results in the breakage of chains or, in the case of two adjacent double bonds, formation of cyclic peroxides. The composition of the "base" or unmodified liposome was chosen to mimic closely the phospholipid composition of endothelial cell membranes. It was assumed that this would minimize disruption of the cell membranes when in contact with the liposomes.

PEG was inserted into the lipid bilayer through its PE anchor. DMPE conjugated to methoxy terminated PEG (PE-mPEG) was used in this work. PE-mPEG will be referred to as PEG in the remainder of the text. PEG of molecular weight 2000 was

incorporated into the liposomes at a loading of 5 mol% of the total lipid content, including cholesterol. This loading corresponds to 1mg PEG / mL liposome suspension.

The polysaccharides studied in this work were chosen because of their biological properties. Heparin is a powerful anticoagulant and also possesses anticomplementary properties, thus making it an attractive candidate for liposome modification. Dextran is an inert and non-immunogenic hydrophilic polymer consisting of α-D-glucose chains. Although it does not possess specific biological activity, it is devoid of risk of contaminants of animal origin (unlike heparin) and lends itself well to chemical modifications rendering it biologically active. These modifications lead to the formation of biofunctionalised dextrans, polymers possessing biospecific sites obtained by grafting of suitable chemical groups onto the polysaccharide backbone. Polysaccharide loadings were 1 mg / mL liposome suspension. Table 5.4 summarizes the characteristics of the different polymer modifiers studied.

Table 5.4 Characteristics of the various polymer modifiers studied.

Polymer	**Description**	**$MW_{average}$ (g/mol)**	**$MW_{subunit}$ (g/mol)**
Polyethylene glycol (PEG)	Linear polymer.	2000	44
Dextran T40	Linear polysaccharide (α-D glucose)	37,500	162
Heparin H108	Anticoagulant activity of 173 UI/mg.	Approx. 10,700	Approx. 300
CMDSu	Carboxymethyl dextran sulfate, prepared from native dextran T40, 1.65 meq/g carboxymethyl units, 0.6 meq/g sulfate units.	Approx. 55,000	Approx. 355

Although initial modifier loading was 1mg /mL, the actual amount of modifier on the surface of the liposomes was probably lower. This was due to the presence of modifier in the internal compartment of the liposomes (thus not appearing on the outer

surface) and the possibility that not all polymer loaded was able to anchor itself into the lipid bilayer. This latter point is especially true for the polysaccharide modifiers considering the low efficiency of the anchoring reactions, as discussed previously. Each PEG chain, on the other hand, was attached to a PE molecule and was probably better able to anchor itself into the bilayer. It is also possible that the modifiers spontaneously adsorbed on the liposome surfaces through non-specific interactions, thus improving surface loadings. Unfortunately, we were not able to determine the actual amounts of modifiers on the various liposome surfaces.

Liposomes of consistent size were reproducibly prepared by detergent dialysis [9], followed by steric exclusion chromatography to remove any unincorporated polysaccharides. In contrast with most of the other vesicle formation methods where vesiculation occurs almost instantly, the intermediate structures (micelles) have greater stability with this preparation procedure. The starting point for this technique is a mixed micellar solution of phospholipids and n-octyl-β-D-glucopyranoside (or octyl glucoside, OG) detergent. Upon detergent removal, the small OG / phospholipid mixed micelles grow by fusion. Different detergents have different "shielding" abilities due to their different size, geometry, amphiphilicity, and values of the distribution coefficient between the exterior and interior of mixed micelles. Also, their critical micelle concentration (CMC) values dictate the rate of detergent removal, and thus the rate of vesicle formation [5]. In general, slower detergent removal rates produce larger vesicles because micelle fusion is not an instantaneous process. Seras-Cansell et al. [9] tested the stability in time as well as the resistance of vesicles to the actions of OG and found a

molar ratio of moles OG / moles total lipids to be of the order of 5.4. The protocol for liposome preparation used in the present experiments was adapted from this work. Seras-Cansell et al. also evaluated residual detergent levels after dialysis and found them to be less than 10^{-4} mM, which is clearly lower than the critical micelle concentration of OG at 21.6 mM [170].

Following dialysis, the liposomes were passed through a size exclusion chromatography column, which resulted in a dilution of the vesicles by a factor of 2, and a final liposome concentration of roughly 5 mM total lipids.

5.2.1 Size Exclusion Chromatography Column Calibration

Size exclusion chromatography on Sepharose CL-4B was used to separate liposomes from excess heparin, dextran, PEG or modified dextran. A refractometer was used to determine which eluted fraction contained liposomes or polysaccharides and the resulting chromatogram was produced by a recorder. The phosphate assay was used to determine the lipid concentration in each eluted fraction. Each liposome type as well as each polysaccharide was injected separately on the column to obtain chromatograms used for calibration purposes. Free polysaccharides associated with polysaccharide-modified liposomes were not detected presumably due to low concentrations. Typical chromatograms of the individual components (Figure 5.2) show that all liposome types began to elute at approximately 10-12 mL, i.e. at the exclusion volume of the column as expected for such large species. The polymers showed larger retention volumes. The polysaccharides were eluted beginning at approximately 45 mL. Liposome fractions

were therefore collected at volumes between 10 and 18 mL for the unmodified, PEG-modified and dextran-modified liposomes, and between 14 and 26 mL for the heparin-modified and CMDSu-modified liposomes. In this way the liposome content was maximized and the polysaccharide content was minimized. The fractions collected were pooled and the phospholipid concentration was determined by phosphate assay.

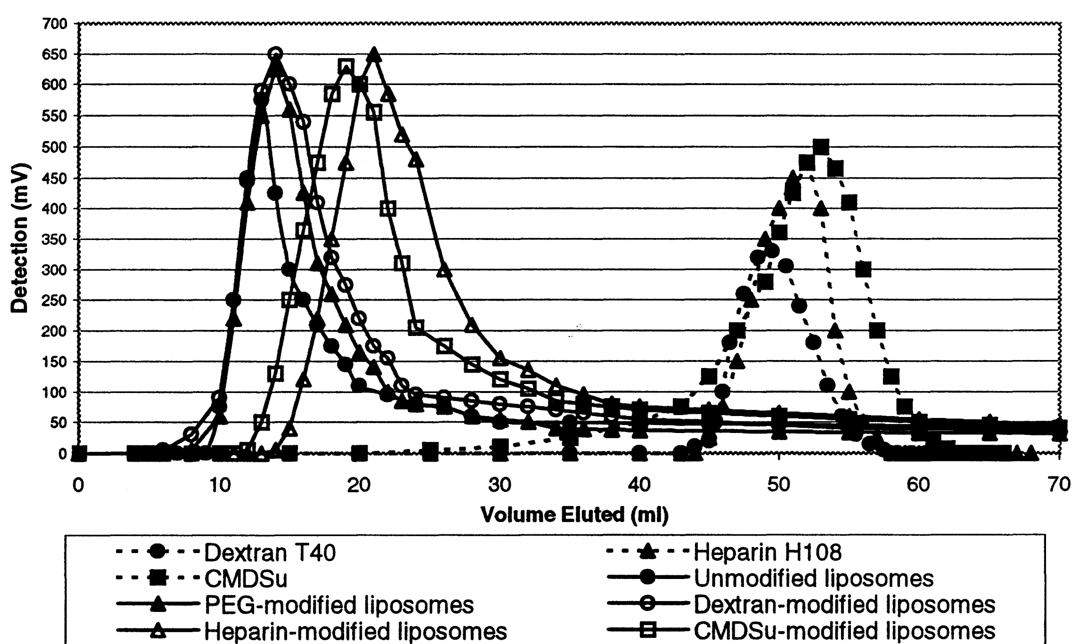


Figure 5.2 Size exclusion chromatography of each liposome type as well as each polysaccharide injected separately on the column. Modified liposomes were prepared with 1 mg modifier / mL loading and polysaccharide solutions were prepared at 25mg/ml. Column specifications: Sepharose CL-4B gel, 30 cm high, 1 cm diameter, 0.5 mL/min flow rate, HEPES buffer pH 7.4, sample volume 1mL.

5.2.2 Liposome Characterization by Phosphate Assay and Dynamic Light Scattering

The average size of the liposomes used in this work was measured by dynamic light scattering. Concentrations were determined by the phosphate assay. The size and phospholipid concentration data are summarized in Table 5.5. The size distribution was

found to be relatively constant from one liposome preparation to another. This was expected, as the liposome preparation technique is known to produce liposomes of consistent size. A larger variation was found in the concentrations, which could be due to slight variations between batches of buffer and detergent. Variability between batches of liposomes was reduced by preparing all lipid films from one batch of mixed lipids (in chloroform).

Table 5.5 Summary of liposome size and concentration.

Liposome type	**Average size ± 1 SD (nm) N* = 7**	**Average phosphate concentration ± 1SD (μM phosphate) N* = 4**
Neutral (unmodified)	176 ± 8	4.2 ± 0.4
PEG-modified	154 ± 8	7.6 ± 0.3
Dextran-modified	114 ± 9	6.4 ± 1.0
Heparin-modified	155 ± 6	5.9 ± 0.5
CMDSu-modified	181 ± 9	7.4 ± 0.1

*N = number of separate liposome preparations included in the calculation.

All liposomes except for dextran-modified ones were approximately the same size, ranging from 154 to 181 nm. Dextran-modified liposomes were consistently smaller (114 ±9 nm). This result is consistent with data of Seras-Cansell et al. [9] who found unmodified liposomes and chemically-modified liposomes in the range of 193 to 206 nm and dextran-modified liposomes with a mean diameter of 91 ± 8 nm. They used the same liposome preparation technique as in this work.

5.3 Protein Adsorption

The protein adsorption protocol followed in this work was based on the use of ultracentrifugation for separation of free protein from liposomes. A limitation on this method was the small number of samples that could be run at one time (rotor capacity of 10 samples). Damage to the liposomes during the vortexing and ultracentrifugation steps was shown by Price et al. [76] to be minimal as judged by retention of size distribution through several cycles.

5.3.1 IgG Adsorption from Buffer

A series of adsorptions from solutions of IgG in buffer were performed with all types of liposomes studied in this work. Concentrations ranged from 0 to 1 mg/mL. IgG was chosen for study because of the involvement of this protein in opsonization leading to premature clearance from the circulation and possible activation of the classical pathway of complement. IgG adsorption to conventional liposomes has been studied by others [19, 33] but there has been little or no investigation of adsorption to modified liposomes. IgG is typically found in blood at a concentration of 8 mg/ml (Table 5.9).

IgG adsorption was performed by exposing liposomes to IgG solutions containing 1% labelled protein for 2 h at room temperature. Free iodide levels were shown to be consistently below 3.5% of total radioactivity. These levels are considered acceptable and do not affect adsorption data significantly.

After the incubation period, the free protein was removed from the liposomes by ultracentrifugation. The first centrifugation step yielded soft pellets that were easily

resuspended. For this step, only 90% of the supernatant was removed to avoid breaking up the pellet and was replaced by an equal amount of fresh buffer. In subsequent centrifugation steps, the pellets became much harder and all the supernatant could be safely removed. Unfortunately, the harder pellets were more difficult to resuspend. Unmodified liposomes as well as polysaccharide-modified liposomes often required vortexing for 1 minute to resuspend the liposomes in fresh buffer.

Protein concentration in the supernatants was determined to ascertain the number of washing steps necessary to remove all free IgG from the liposome suspension. It was found that four wash / ultracentrifugation steps were sufficient to remove unbound protein. Figure 5.3 depicts the removal of free protein in a typical experiment. The initial wash removed greater than 60% of the free IgG and only 0.5% remained in the final wash.

The wash / ultracentrifugation procedures resulted in significant liposome losses. Losses were quantified and adsorption data are reported as mg protein adsorbed per mmol phospholipid (i.e. excluding cholesterol). Table 5.6 shows data on liposome losses during these IgG adsorption experiments. These data can be explained by the type of pellets obtained for each liposome type. The pellets formed by PEG-modified liposomes were always more fragile, even for the later centrifugation steps. The pellets were easily resuspended, which could account for the high losses. On the other hand, dextran-modified liposomes formed extremely hard pellets after the second centrifugation step and were very hard to resuspend, even after more than 1 min of vortexing. The extensive vortexing required may have contributed to the losses of these liposomes by mechanical

destruction and removal of membrane fragments during subsequent washing steps. The other types of liposome showed losses between these two extremes.

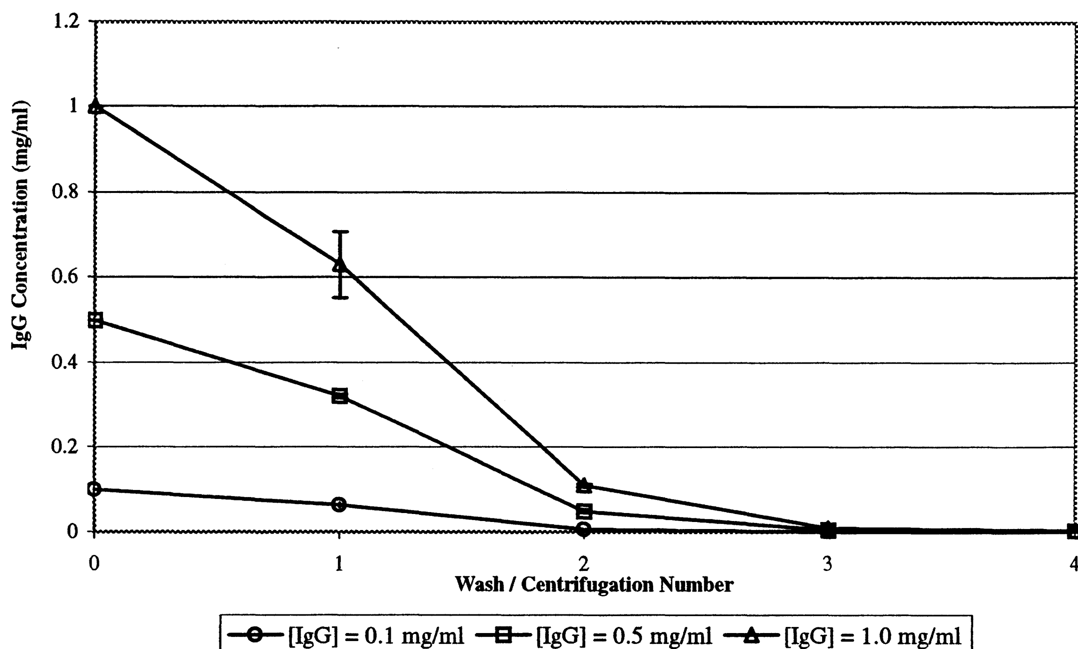


Figure 5.3 Free protein removal from an experiment on IgG adsorption to unmodified liposomes. The concentrations of IgG used for adsorption are indicated. The radioactivity of the supernatant from successive washes was determined and the IgG concentration was calculated. (Error bars = ± 1 SD, n=3; in most cases, the error bars are within the data point marker.)

Table 5.6 Average losses of liposomes in experiments on IgG adsorption from single protein solutions. The initial liposome loading was 0.5 mL corresponding approximately to 5 μmol total lipids.

Liposome Type	% Loss	N*
Neutral (unmodified)	35 ± 13	12
PEG-modified	67 ± 8	9
Dextran- modified	64 ± 7	9
Heparin-modified	33 ± 3	9
CMDSu-modified	36 ± 1	6

***N = number of samples used to calculate the average loss.**

5.3.1-1 IgG Adsorption to the Various Liposome Types

Results from the IgG adsorption experiments are presented in Figure 5.4. From these data, one can see that for all liposome types adsorption increases with concentration. It is not clear if the data at 1 mg/mL represent the limit of adsorption (isotherm plateau). Unmodified liposomes adsorbed the most protein, followed closely by dextran and CMDSu-modified liposomes. PEG- and heparin-modified liposomes adsorbed the smallest amounts of protein.

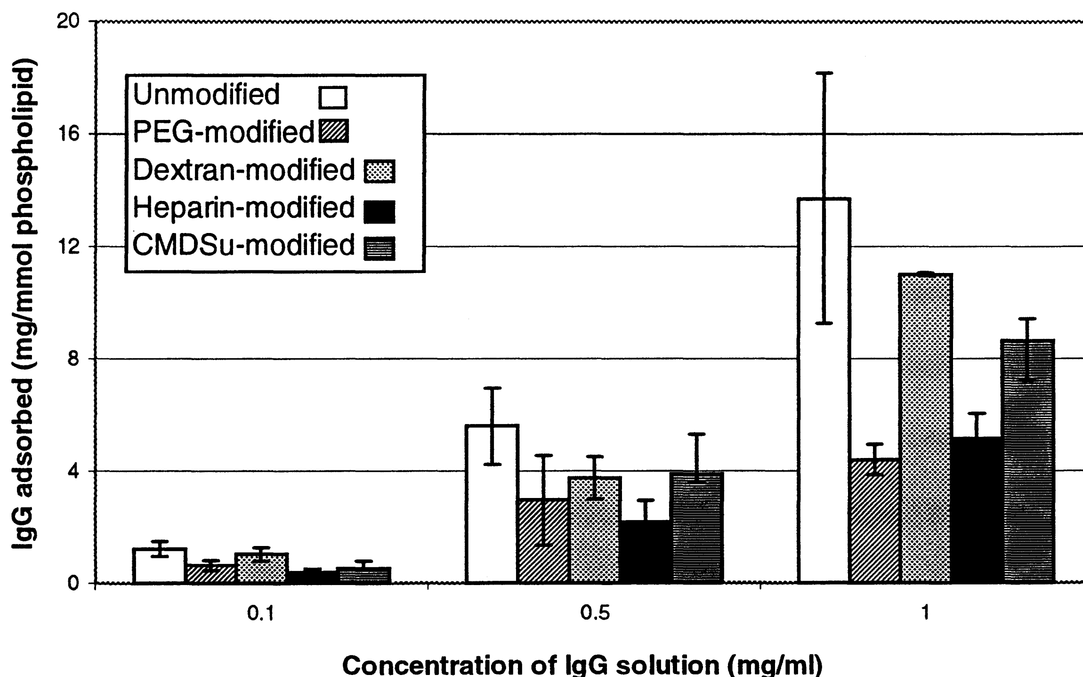


Figure 5.4 IgG adsorption to unmodified liposomes and to liposomes modified with PEG, dextran, heparin and modified dextran (2h adsorption at room temperature). Each bar represents data from 3 separate experiments with 3 replicates for each concentration. For CMDSu-modified liposomes, 2 separate experiments with 3 replicates were performed. (Error bars = ±1 SD.)

Since even at 1 mg/mL adsorption to the various liposome types was very low (range from 4 to 14 mg IgG/mmol phospholipid), the differences among the different types are difficult to assess. A two-tailed Student's t-test was used to evaluate the differences between IgG adsorption to unmodified and modified liposomes. Adsorption to dextran-modified liposomes was not statistically different ($p = 0.2270$) from that to unmodified liposomes. Significant differences were seen between the unmodified and all other modified liposomes (PEG $p = 0.0015$, heparin $p = 0.0021$ and CMDSu $p = 0.0350$). Price et al. [76] showed fibrinogen adsorption levels of about 60 mg / mmol phospholipid for neutral liposomes, and about 10 mg /mmol for PEG-coated liposomes. In terms of reducing adsorption, PEG and heparin were the most effective modifiers. Assuming that incorporation of the various modifying polymers into the liposomes was quantitative, loadings were of the order of 5 mol % of total lipid. For PEG 2000, this loading has been shown by others [74, 75, 85] to be sufficient to reduce protein adsorption. The relatively high adsorption for dextran-modified liposomes could result from low incorporation. From the FTIR data, it appears that very little cholesterol was conjugated to the dextran, thus reducing incorporation and presumably reducing any protein repelling effects of the dextran.

The error bars in Figure 5.4 indicate considerable variation among experiments, although variability within experiments was much lower ($n = 6$ for CMDSu-liposomes and $n = 9$ for the other types of liposomes). Even with this high variability, the trends remained the same from one experiment to another. To diminish the variability between experiments, a batch solution of chloroform in which the lipids had been dissolved was

distributed into separate glass vials. The films were then dried and kept in the freezer until they were needed. This procedure ensured consistent liposome compositions from one liposome preparation to another. All liposomes used in the adsorption experiments were prepared from a single batch of lipid solution and all liposomes used in the cell interaction experiments were prepared from another. This ensured lower variability from one experiment to the next than if each liposome preparation had come from different lipid batches.

5.3.2 Adsorption of Proteins from Plasma

Adsorption from 10% plasma was also studied for all the liposome types. The total amount of protein bound to the liposomes was assessed by a detergent-compatible total protein assay, and the profiles for a number of individual proteins were determined using SDS-PAGE and Western blotting.

The protocol was similar to that followed for the IgG adsorption experiments, except for the speed of the centrifugation and the number of centrifugation steps needed to completely "wash" the liposomes. When exposed to 10% plasma, the liposomes did not pellet well, especially in the initial centrifugation steps. The speed was therefore increased from 100 000 to 350 000 g and the number of wash steps was increased to 7. The liposome losses from this procedure are shown in Table 5.7. In general, the liposome losses were greater for the plasma adsorption compared to the IgG adsorption experiments. This increase may have been due to the increased speed and number of

centrifugation steps required when working with plasma. Interestingly, losses for the unmodified liposomes were about the same as for IgG experiments.

Table 5.7 Average losses of liposomes after exposure to 10% plasma. The initial liposome loading was 0.5 mL corresponding approximately to 5 μmol total lipids.

Liposome Type	% Loss	N*
Neutral (unmodified)	36 ± 3	13
PEG-modified	78 ± 4	10
Dextran- modified	74 ± 10	10
Heparin-modified	62 ± 6	7
CMDSu-modified	83 ± 2	5

***N = number of samples used to calculate the average loss.**

5.3.2-1 Total Protein Assay

The Bio-Rad DC Total Protein Assay was used to determine the quantity of protein eluted from the liposome surfaces following detergent solubilization. The data are summarized in Table 5.8. Adsorption levels range from 130 to 450 mg total protein / mmol phospholipid. This compares to values of 80 mg protein/mmol phospholipid reported by Oja et al. [60] and Semple et al. [56] for liposomes that were injected into mice. The adsorption levels for unmodified and heparin-modified liposomes are similar. Both adsorbed relatively small amounts of protein of the order of 150 mg protein/mmol phospholipid. PEG liposomes adsorbed about 250 mg /mmol. Dextran and CMDSu-modified liposomes adsorbed the most protein, with levels almost a factor of three greater than the unmodified liposomes. Compared to the amounts of protein adsorbed in the IgG experiments, these are considerably higher. The difference may be explained in part by the higher protein concentration in 10% plasma (of the order of 5 mg/mL) compared to

the maximum IgG concentration studied, i.e. 1 mg/mL. It appears that these results contradict previous published data suggesting that PEG and dextran coatings are effective in reducing protein adsorption onto surfaces [69, 84, 85], but are in accordance with other work where this effect has not been found [76, 77].

Table 5.8 Total protein adsorption after exposure to 10% plasma for 2 h. (Error bars = ±1 SD.)

Liposome Type	Protein Adsorption (mg protein per mmol phospholipid, mean ± SD)	N*
Neutral (unmodified)	127 ± 16	13
PEG-modified	248 ± 17	10
Dextran- modified	451 ± 121	10
Heparin-modified	173 ± 14	7
CMDSu-modified	438 ± 59	5

***N = number of samples used to calculate the average protein adsorption.**

5.3.2-2 SDS-PAGE

Price et al.[76] in previous work checked whether the liposomes themselves interfered with or contributed to protein bands in the gels and blots by running liposome samples not exposed to plasma on SDS-PAGE gels. They found that the liposomes did not produce any false gel bands. These tests were also run in this work and no bands appeared in the lanes containing the liposomes (data not shown).

Figure 5.5 shows a gel of the proteins adsorbed to the various liposome types from 10% plasma. Equal amounts of phospholipid (0.01 μmol) were loaded onto the gels. Differences in the amounts of protein adsorbed and the protein banding patterns for

the different types of liposomes studied were the focus of these experiments. There appeared to be more protein on the dextran- and CMDSu-modified liposomes than on the others, as indicated by the overall band intensities. In accordance with the total protein assay results, the unmodified liposomes appeared to adsorb the smallest amount of protein. The banding patterns of the five types of liposome were similar.

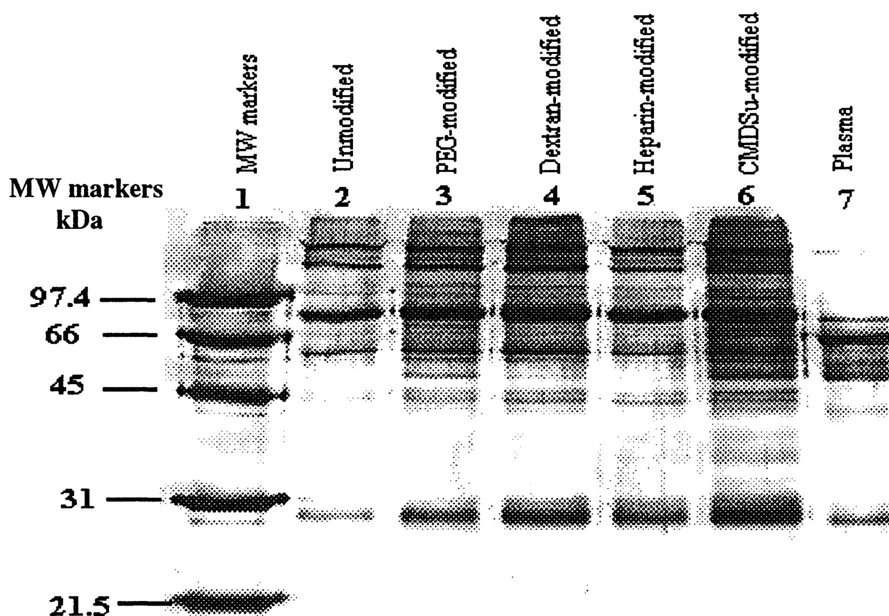


Figure 5.5 12% reduced SDS-PAGE gel for liposomes exposed to 10% plasma. In the liposome lanes (2-6), amounts corresponding to 0.01 μmol phospholipid were loaded onto each lane. In lane 7, 2 μL of 1:100 diluted plasma was loaded.

When comparing the proteins adsorbed to the liposomes to plasma itself, some proteins appear to be enriched on the liposome surface relative to plasma while others are depleted. Specifically, a number of proteins in the high molecular weight range above 97 kDa are adsorbed to all types of liposomes. A band around 75 kDa is intense in the liposome lanes, but only faint in the plasma lane. A similar band was observed by

Hernandez-Caselles et al. [64] although they were not able to identify it, however, although they were able to rule out transferrin, which normally runs near 80 kDa. Albumin at 66 kDa is the most intense band found in the plasma profile, while it is much lighter in the liposome profiles. Albumin is the most abundant protein in plasma and has previously been shown to bind extensively to liposomes [64].

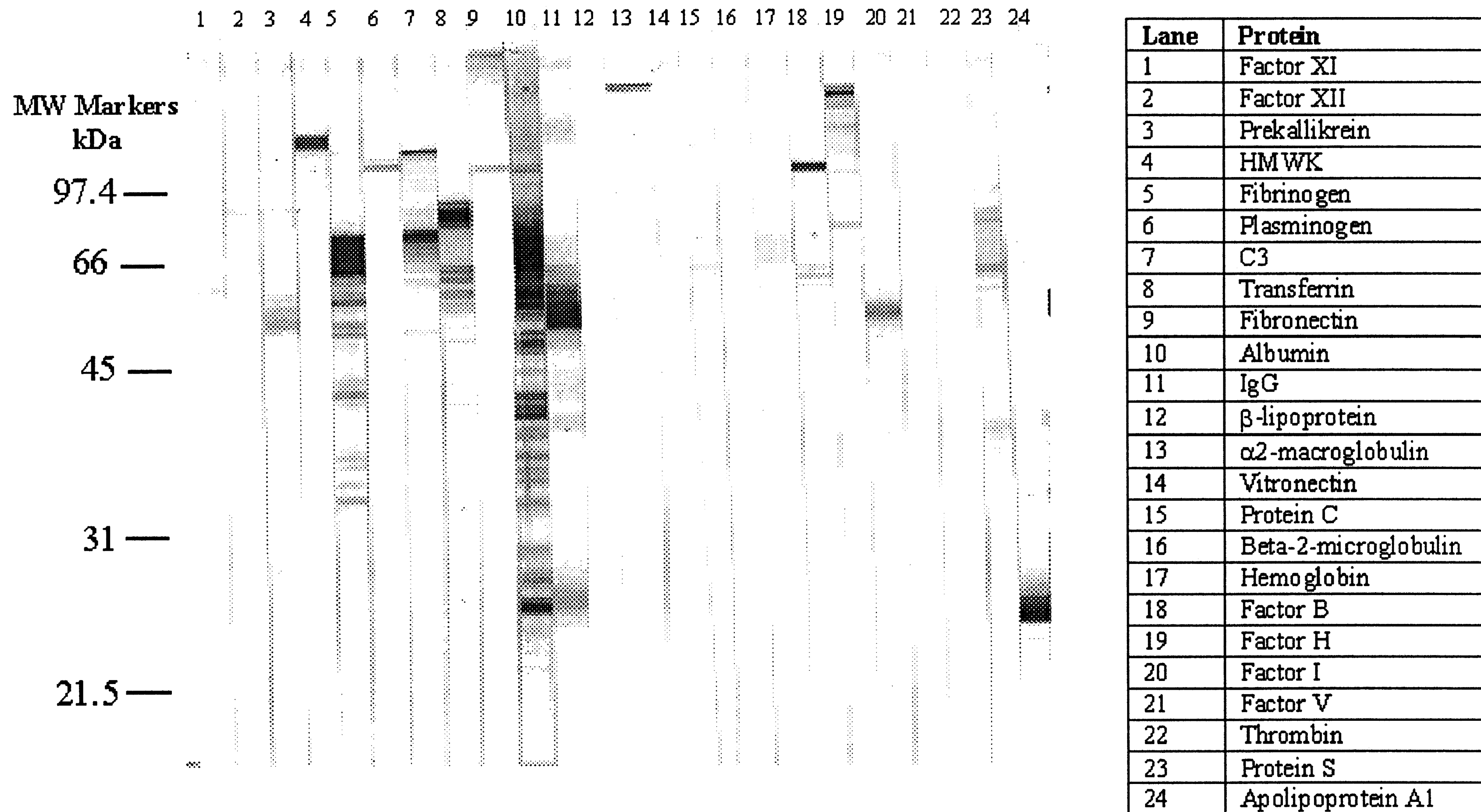
5.3.2-3 Western Blotting

Given the difficulty in identifying specific proteins from SDS-PAGE gels, Western blots were performed. Western blot data are shown in Figures 5.6-5.11. A summary of the properties of the proteins probed for in the blots is shown in table 5.9.

In the following discussion, molecular weights for band positions on the blots were determined with reference to standards using Whole Band Analysis Software (Millipore).

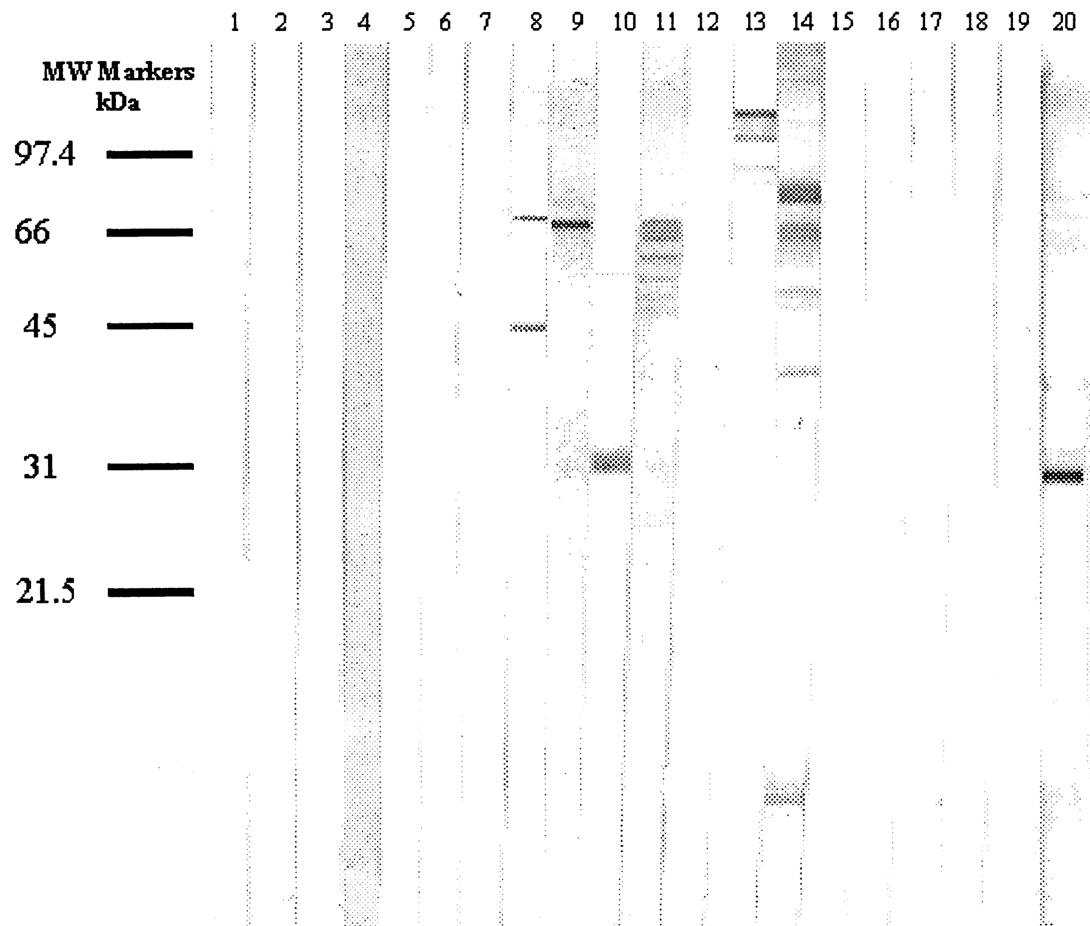
Table 5.9 Properties of the plasma proteins investigated in this work.

Plasma Protein	Molecular weight (kDa)	Molecular weights on reduced gels (kDa)	Plasma Concentration (μg/mL)
α2-macroglobulin [171, 172]	726	185	2400
β2-microglobulin [173]	11.8	11.8	2
β-lipoprotein [172]	3200	N/A	2800-4400
Albumin [174]	66.3	66.3	45,000-80,000
Antithrombin [175]	58	58	150
Apolipoprotein A1 [176]	28	28	940-1990
C3 [177]	185	75, 110	1100
Factor B [178]	93	93	200
Factor H [178]	150	150	500
Factor I [178]	88	38, 50	34
Factor XI [179]	160	83	5
Factor XII [180]	80	80	15-45
Fibrinogen [181]	340	47, 58, 67	3000-4000
Fibronectin [17]	450	200	300
Haemoglobin [171]	64	16, 32, 64	≅ 0
HMWK [180]	120	120	30-90
IgG [172]	160	27, 55	8000
Plasminogen [171]	94	94	200
Prekallikrein [180]	85	85	35-45
Transferrin [171]	80	80	2000-3200



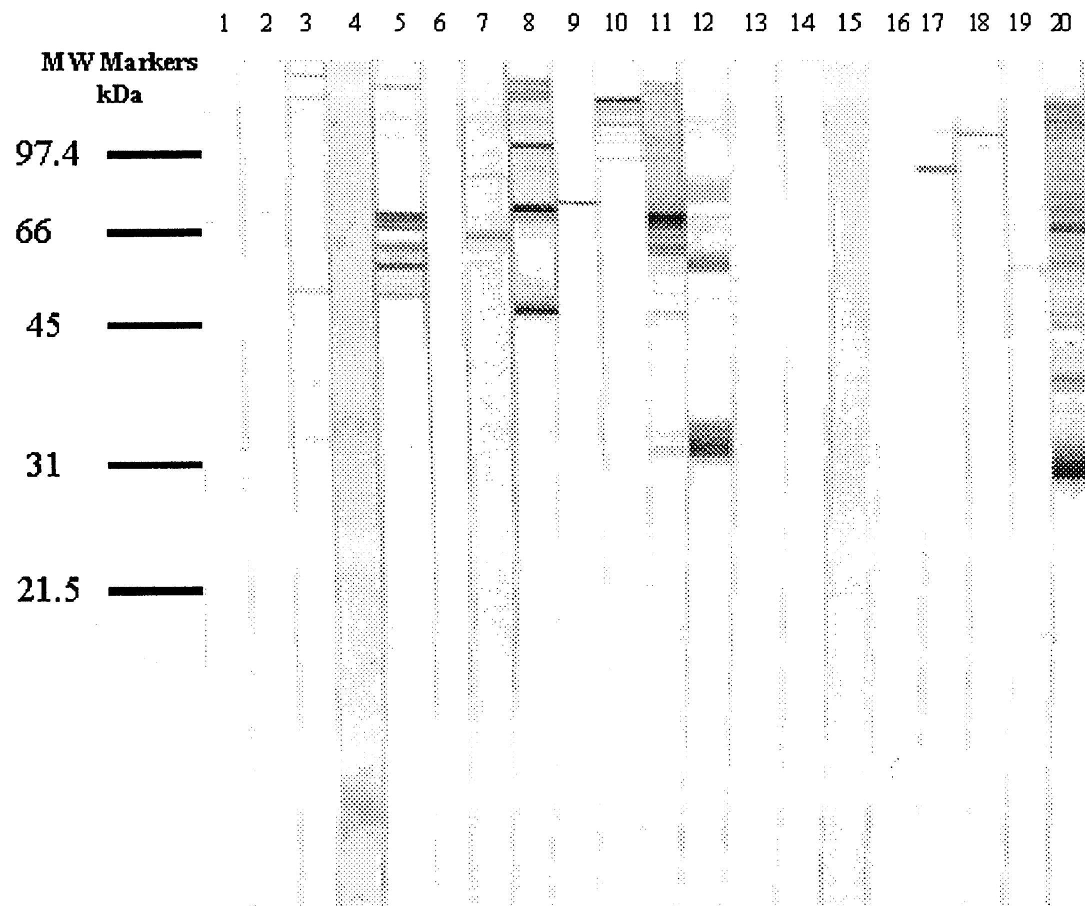
Lane	Protein
1	Factor XI
2	Factor XII
3	Prekallikrein
4	HMWK
5	Fibrinogen
6	Plasminogen
7	C3
8	Transferrin
9	Fibronectin
10	Albumin
11	IgG
12	β-lipoprotein
13	α2-macroglobulin
14	Vitronectin
15	Protein C
16	Beta-2-microglobulin
17	Hemoglobin
18	Factor B
19	Factor H
20	Factor I
21	Factor V
22	Thrombin
23	Protein S
24	Apolipoprotein A1

Figure 5.6 Western blot of plasma using antibodies to 24 plasma proteins. 5μL of 1:10 diluted plasma loaded along with 95 μL tracking dye. From reference [76].



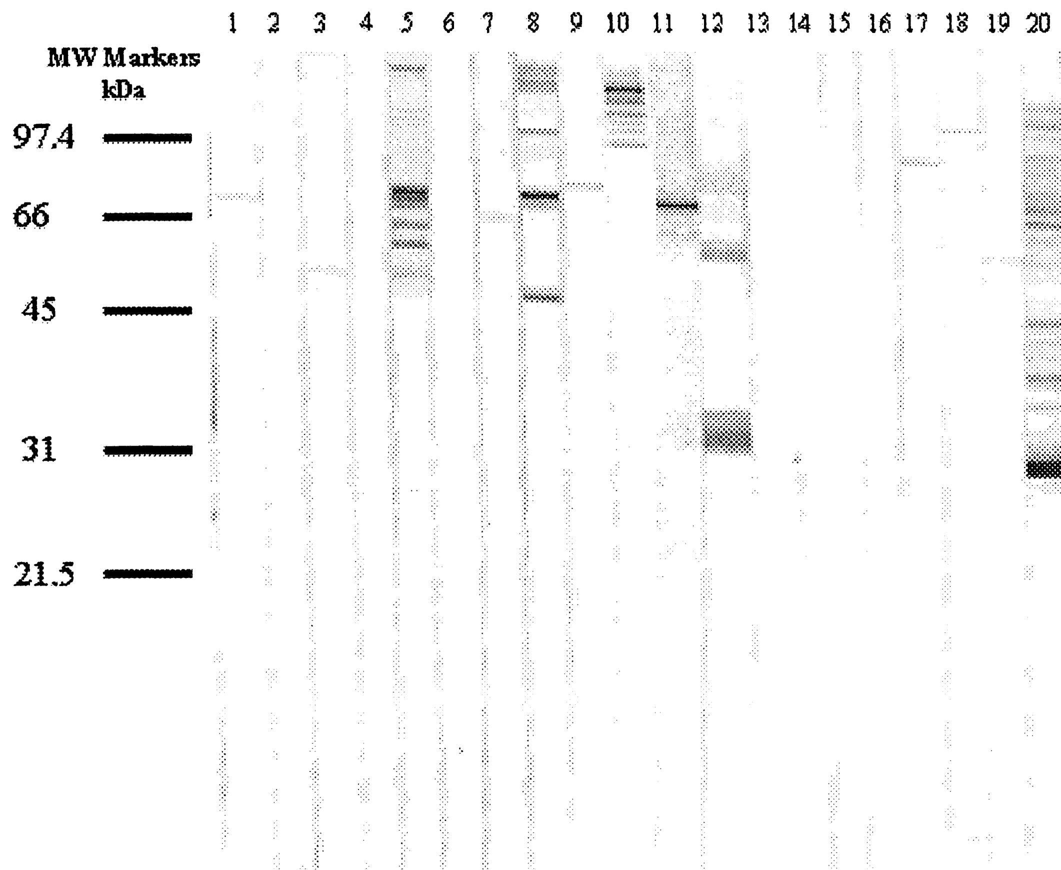
Lane	Protein
1	Factor XI
2	Factor XII
3	Prekallikrein
4	HMWK
5	ATIII
6	Plasminogen
7	Transferrin
8	C3
9	Albumin
10	IgG
11	Fibrinogen
12	Beta-lipoprotein
13	Fibronectin
14	Alpha2-macroglobulin
15	Beta2-microglobulin
16	Hemoglobin
17	Factor B
18	Factor H
19	Factor I
20	Apolipoprotein A1

Figure 5.7 Western blot of plasma proteins adsorbed to unmodified liposomes at room temperature. Adsorption time, 2 h in 10% plasma.



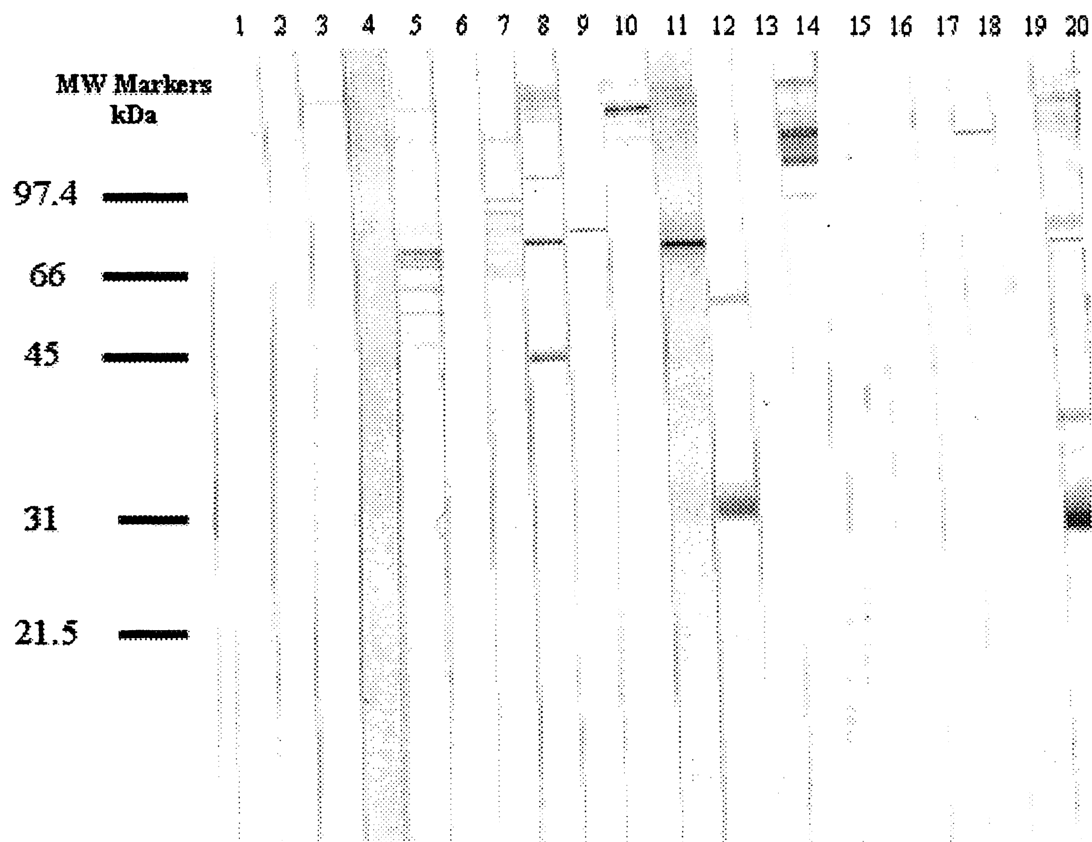
Lane	Protein
1	Factor XI
2	Factor XII
3	Prekallikrein
4	HMWK
5	Fibrinogen
6	Plasminogen
7	ATIII
8	C3
9	Transferrin
10	Fibronectin
11	Albumin
12	IgG
13	Beta-lipoprotein
14	Alpha2-macroglobulin
15	Beta2-microglobulin
16	Hemoglobin
17	Factor B
18	Factor H
19	Factor I
20	Apolipoprotein A1

Figure 5.8 Western blot of plasma proteins adsorbed to PEG-modified liposomes at room temperature. Adsorption time, 2 h in 10% plasma.



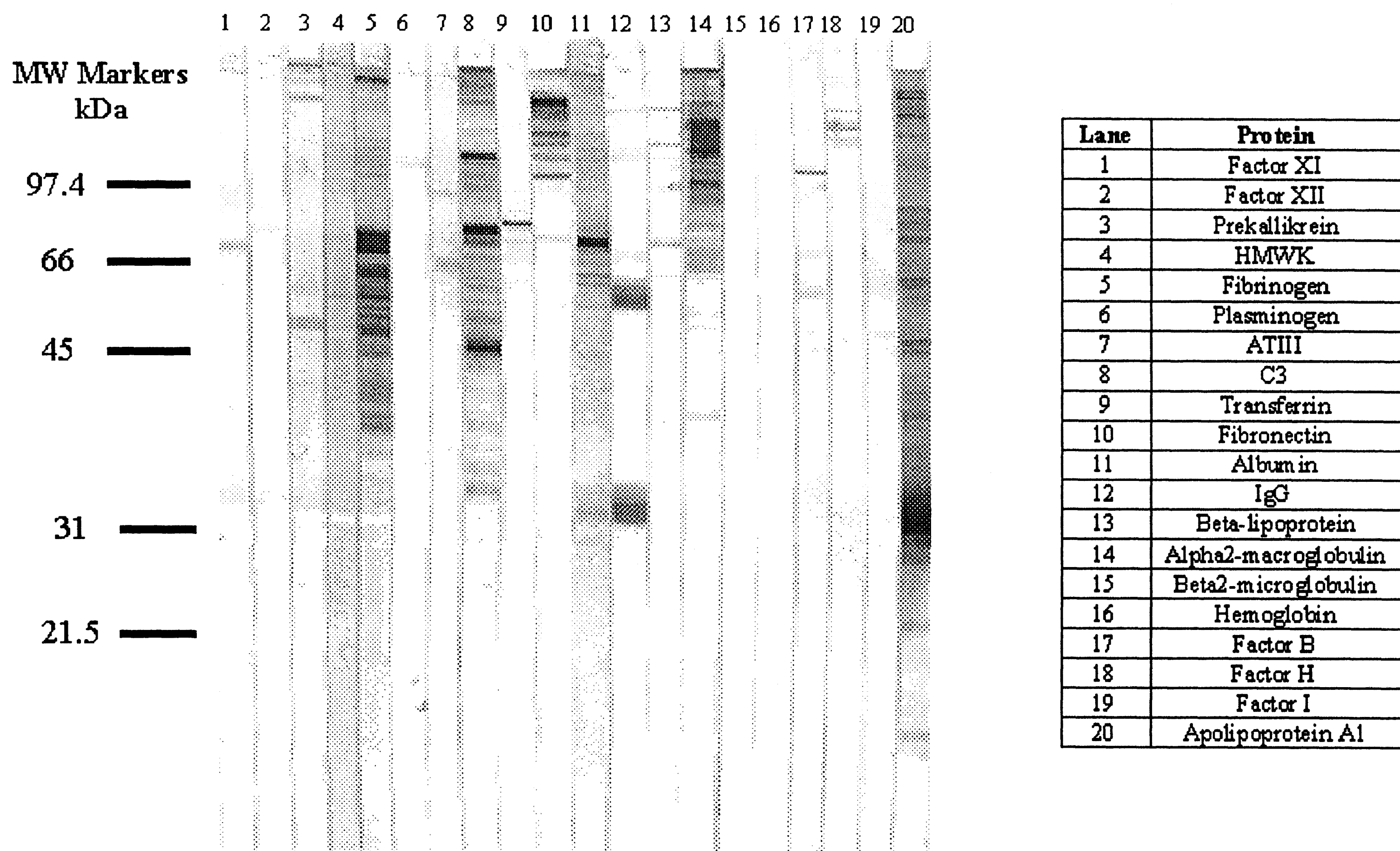
Lane	Protein
1	Factor XI
2	Factor XII
3	Prekallikrein
4	HMWK
5	Fibrinogen
6	Plasminogen
7	ATIII
8	C3
9	Transferrin
10	Fibronectin
11	Albumin
12	IgG
13	Beta-lipoprotein
14	Alpha2-macroglobulin
15	Beta2-microglobulin
16	Hemoglobin
17	Factor B
18	Factor H
19	Factor I
20	Apolipoprotein AI

Figure 5.9 Western blot of plasma proteins adsorbed to dextran-modified liposomes at room temperature. Adsorption time, 2 h in 10% plasma.



Lane	Protein
1	Factor XI
2	Factor XII
3	Prekallikrein
4	HMWK
5	Fibrinogen
6	Plasminogen
7	ATIII
8	C3
9	Transferrin
10	Fibronectin
11	Albumin
12	IgG
13	Beta-lipoprotein
14	Alpha2-macroglobulin
15	Beta2-microglobulin
16	Hemoglobin
17	Factor B
18	Factor H
19	Factor I
20	Apolipoprotein A1

Figure 5.10 Western blot of plasma proteins adsorbed to heparin-modified liposomes at room temperature. Adsorption time, 2 h in 10% plasma.



Lane	Protein
1	Factor XI
2	Factor XII
3	Prekallikrein
4	HMWK
5	Fibrinogen
6	Plasminogen
7	ATIII
8	C3
9	Transferrin
10	Fibronectin
11	Albumin
12	IgG
13	Beta-lipoprotein
14	Alpha2-macroglobulin
15	Beta2-microglobulin
16	Hemoglobin
17	Factor B
18	Factor H
19	Factor I
20	Apolipoprotein A1

Figure 5.11 Western blot of plasma proteins adsorbed to CMDSu-modified liposomes at room temperature. Adsorption time, 2 h in 10% plasma.

The Western blot data suggest differences in protein adsorption profiles between the liposomes and plasma, as well as differences among the various liposome types. In general, unmodified and heparin-modified liposomes adsorbed little protein, PEG- and dextran-modified liposomes adsorbed intermediate amounts and CMDSu-modified liposomes adsorbed the most.

Very small amounts of the contact activation factors were bound to the liposomes. These factors are involved in the preliminary stages of the intrinsic coagulation pathway, as depicted in Figure 1.3. They include factor XI, factor XII, high molecular weight kininogen (HMWK), and prekallikrein. Factor XI was detected on the dextran and CMDSu blots as a faint band near 80 kDa whereas it did not appear in any other blot, including that of the plasma itself. Factor XI usually runs at 83 kDa, corresponding to two disulfide-linked polypeptides. These are cleaved upon activation to give fragments running at 33 and 50 kDa. Factor XI was not activated on the dextran and CMDSu-modified surfaces. Factor XII was detected in plasma at 82 kDa with a very faint second band (54 kDa). These bands correspond to the native or activated forms (80 kDa) and an activation fragment (50 kDa) of the protein. Factor XII was apparently not associated with any of the liposome types, although it is possible that it would have been detected with heavier gel loadings. Of the four contact factors, prekallikrein gave the strongest blot response (albeit a faint one) for the liposome surfaces; it was not seen on the unmodified liposomes. In plasma, a band at 83 kDa corresponding to the intact protein, as well as bands at 50-55 kDa corresponding to activation fragments were observed. On the liposomal surfaces, three bands were observed: two were at molecular weights (MW)

greater than 100 kDa and the third was near 45 kDa. The high molecular weight bands indicate a complexed form of prekallikrein. HMWK was essentially absent from all liposome surfaces. It was detected in plasma mainly in its intact form at 120 kDa. These weak responses for the coagulation contact factors reflect their low concentrations in plasma (Table 5.9) but also suggest the absence of contact activation on the liposome surfaces. This was perhaps to be expected as contact activation on lipid surfaces is known to require negative charges such as a damaged vessel wall, the inner (negative) surface of the platelet membrane, or negatively charged liposomes as shown by Price et al. [76].

Fibrinogen and plasminogen are also proteins involved in blood coagulation. All immunoblots for fibrinogen displayed intense bands between 45 and 66 kDa as well as a lighter band at a high molecular weight. Fibrinogen is abundant in plasma with a concentration near 3 mg/mL, and possesses three subunits of mass 47, 55 and 67 kDa. Degradation products were also detected in plasma and on the CMDSu-modified liposomes at molecular weights below 45 kDa. It appears that the plasma and CMDSu samples contained relatively greater amounts of fibrinogen than those of the other types of liposome. The plasminogen blots showed a positive response for the plasma sample with two bands at molecular weights greater than 100 kDa, which likely correspond to Glu- and Lys- forms of plasminogen, both of which are present in normal plasma. Plasminogen molecular weights have been determined to be 94 and 84 kDa, respectively, for the Glu- and Lys- forms. It is not clear why this protein appears to run at high molecular weights in the present experiments. A faint band (higher than 100 kDa) was

also noticeable on the CMDSu blot. Plasminogen was not detected on the other liposome blots.

Antithrombin (AT) is a single chain glycoprotein of the serine protease inhibitor (serpin) super family and is considered to be the most important inhibitor of the coagulation pathways. When antithrombin is bound to heparin, its inhibition of thrombin is enhanced by three orders of magnitude [109]. Plasma concentrations of AT are near 0.15 mg/mL. Antithrombin was not probed for in plasma but normally runs at 58 kDa. Distinct bands were observed on AT immunoblots for all types of modified liposomes. No AT was detected on the unmodified surface. PEG- and CMDSu-modified liposomes gave a strong response for antithrombin with bands appearing at about 62, 91, and >100 kDa. The dextran-modified liposomes also adsorbed AT, but to a lesser extent with a single band appearing at about 62 kDa. The blots for the heparin-modified liposomes displayed multiple bands between 62 and 91 kDa. Responses at molecular weights higher than about 60 kDa presumably correspond to complexes of antithrombin with other proteins or protein fragments.

Among the complement system proteins, C3 is the most abundant. It is composed of an α chain (110 kDa) and a β chain (75 kDa) and can be cleaved to form C3a and C3b [29]. In plasma, C3 was present mainly as the intact protein with bands at 72 and greater than 100 kDa. The liposome blots exhibited the same bands; however the blots of the modified liposomes also included bands at MW >100 kDa and a band near 42 kDa. This latter band is taken as evidence of complement activation since it is probably a fragment of the α-chain as reported previously [76]. Factor B is involved in the formation of C3

convertase (see Figure 1.2). The blots for plasma and CMDSu-modified liposomes displayed a band at approximately 100 kDa corresponding to intact factor B, as well as weaker bands between 55 and 60 kDa. A band at about 100 kDa also appeared on the blots for PEG and dextran-modified liposomes. No factor B was detected on the surface of unmodified liposomes. Factors H and I are inhibitors of the complement systems and act as regulator proteins. Factor H was detected in plasma and on CMDSu-modified liposomes as multiple intense bands at a molecular weight (MW) greater than 100 kDa, and was detected on all other modified liposomes as a single band at MW > 100 kDa. It was not found on the unmodified liposome blot. Factor I was also detected in plasma and CMDSu-modified liposomes at about 53 kDa, although the band intensity was weaker on the liposome blot. Factor I was not detected on any of the other types of liposome.

Fibronectin is an adhesive protein and also plays a key role in opsonization for macrophage uptake [20]. All blots displayed multiple bands for this protein at molecular weights greater than 100 kDa. Fibronectin appeared to be relatively more abundant on the liposome surfaces than in plasma. A binding site for heparin has been identified on fibronectin [158]. Fibronectin is also known to bind to IgG and C3 [182], as well as to plasminogen [183].

Albumin is the most abundant plasma protein. It was found in intact, cleaved, and complexed forms in plasma, as seen by the intense multiple bands ranging from 27 to >100 kDa. On the liposome surfaces, it was detected only in its intact form at 66 kDa. Albumin appeared to be depleted on the liposome surfaces relative to plasma.

Immunoglobulin G (IgG) is involved in the opsonization process and in the activation of the classical pathway of complement. It is composed of two light chains and two heavy chains linked together by disulfide bonds. All immunoblots for IgG displayed bands between 28 and 30 kDa corresponding to the light chains, and bands between 48 and 61 kDa corresponding to the heavy chains of IgG. In addition to these bands, other bands at molecular weights greater than 100 kDa were seen in the plasma and CMDSu-modified liposome samples.

β-lipoprotein, also known as low-density lipoprotein (LDL) is a large particle (3200 kDa) involved in lipid transport. β-lipoprotein was detected as multiple bands only on CMDSu-modified liposomes.

α2-macroglobulin functions as a regulation protein of the coagulation, fibrinolysis, and complement systems. It was detected in plasma as a single band at a mass greater than 100 kDa. Unmodified liposomes as well as heparin and CMDSu-modified liposomes showed relatively more α2-macroglobulin on their surface, with multiple bands appearing between 57 and 100 kDa. No α2-macroglobulin was detected on the PEG and dextran liposomes.

β2-microglobulin is a small protein involved in the inflammation response. It was not detected on any of the blots, possibly because it migrated to the bottom of the gels.

Apolipoprotein A1 is the major apoprotein of high-density lipoprotein (HDL). This protein was observed as a very intense band at approximately 27 kDa on the blots for all surfaces investigated. Apolipoprotein A1 levels were enhanced on the PEG, dextran and CMDSu-modified surfaces compared to plasma.

Transferrin, is an iron transport protein and was detected in plasma in its intact and cleaved forms as multiple bands ranging from 42 to 80 kDa. It was also detected on all liposome surfaces as a single band near 73 kDa. Haemoglobin was not detected on any of the blots, as would be expected since none was present in the plasma.

5.3.2-4 Summary of Western Blots

Proteins enhanced on the liposome surfaces relative to plasma were: C3, fibronectin and α2-macroglobulin. The liposome types that adsorbed the most protein, as determined by the total protein assay, also adsorbed the most C3. The blots suggested that all modified liposomes activated complement to a significant extent. C3 binding has been linked to early clearance of liposomes from the bloodstream [59]. Fibronectin was also seen in large amounts on the liposome surfaces as was observed previously [61, 76]. The presence of significant amounts of α2-macroglobulin on liposome surfaces was also observed previously in this laboratory [76]. Black and Gregoriadis [57] also showed that this protein was adsorbed to various liposome preparations.

Proteins depleted on the liposome surfaces relative to plasma were albumin, fibrinogen, plasminogen, transferrin and factors H and I. The low adsorption of albumin was somewhat expected, since it has been reported [33, 60, 65, 76] that it adsorbs minimally to liposomes. Other studies have also shown extensive albumin binding [4, 59, 64, 75, 84, 85].

Other proteins of note bound to the liposomes were IgG, apolipoprotein A1 and antithrombin (AT). Adsorbed IgG may have been involved in complement activation, as

revealed by the C3 blots. Also fibronectin, which is known to bind IgG, appeared to be adsorbed extensively on all liposome types. Apolipoprotein A1 is known to destabilize lipid bilayers and has been observed on liposomes of various compositions [33, 65, 76, 184]. Antithrombin was also found on all the modified liposomes and maximum responses were detected on PEG, heparin and CMDSu liposomes. The strong response for heparin was not unexpected since it possesses a specific binding site for AT. It is not clear if AT was bound to this site, or if it was non-specifically adsorbed to the liposome surface. AT adsorption was previously observed on DPPC-cholesterol liposomes modified with N-substituted polyacrylamides [184].

The intense band at 75 kDa identified on the SDS-PAGE gels remained unidentified as it did not correspond to any of the proteins investigated by immunoblotting. Price et al. [76] also observed this band but were unable to identify it.

5.4 Interactions of Liposomes and Cells

The interactions of human endothelial cells (EAhy926 line) and rat aorta smooth muscle cells (BLC#5 line) with the various liposome types were studied. These cell lines were chosen because of their presence in the vasculature and their importance in interacting with circulating liposomes, and because they retain their characteristics even after numerous passages, unlike the normal non-hybridized cell lines. Growth kinetics were initially assessed for both cell types in the presence of liposomes to determine if the vesicles had any effect on cell growth. Following the preliminary checks, cells were exposed to fluorescent and radiolabelled liposomes to determine qualitatively and

quantitatively the extent of liposome-cell interactions. The fluorescence experiments were also expected to enable determining location of any bound liposomes in or on the cell.

The serum used in these experiments was heated at 56°C for 45 min to de-activate the complement system. Heat-labile opsonins of the complement system lose their opsonic activity when serum is heated at temperatures greater than 50°C for 30 min. Other opsonins, particularly immunoglobulins, retain their opsonic activity at these higher temperatures [185, 186].

5.4.1 Growth Kinetics of Cells in the Presence of Liposomes

These experiments as well as the cell-liposome interaction experiments were performed in the presence of 1% antibiotics to inhibit bacterial growth after the cells had been seeded in the wells. Twenty-four hours after seeding the cells in 10% FBS growth medium, growth was arrested by placing them in 0.4% FBS growth medium for 48 h. The cells were thus synchronized at the same stage (G0 stage) of the cell cycle for all experiments. The cells were then exposed to liposomes at a concentration of 0.4 mM total lipids in 2% FBS. This liposome concentration is four times greater than the one used in the fluorescence experiments and twice as great as the one used in radioactivity experiments. It was assumed that if the liposomes were not cytotoxic at these levels, then it would be safe to use them at lower concentrations for interaction studies. Cells were counted after 6 and 24 h and a Trypan blue assay was run to determine cell viability. The results of these experiments are shown in Figures 5.12 and 5.13.

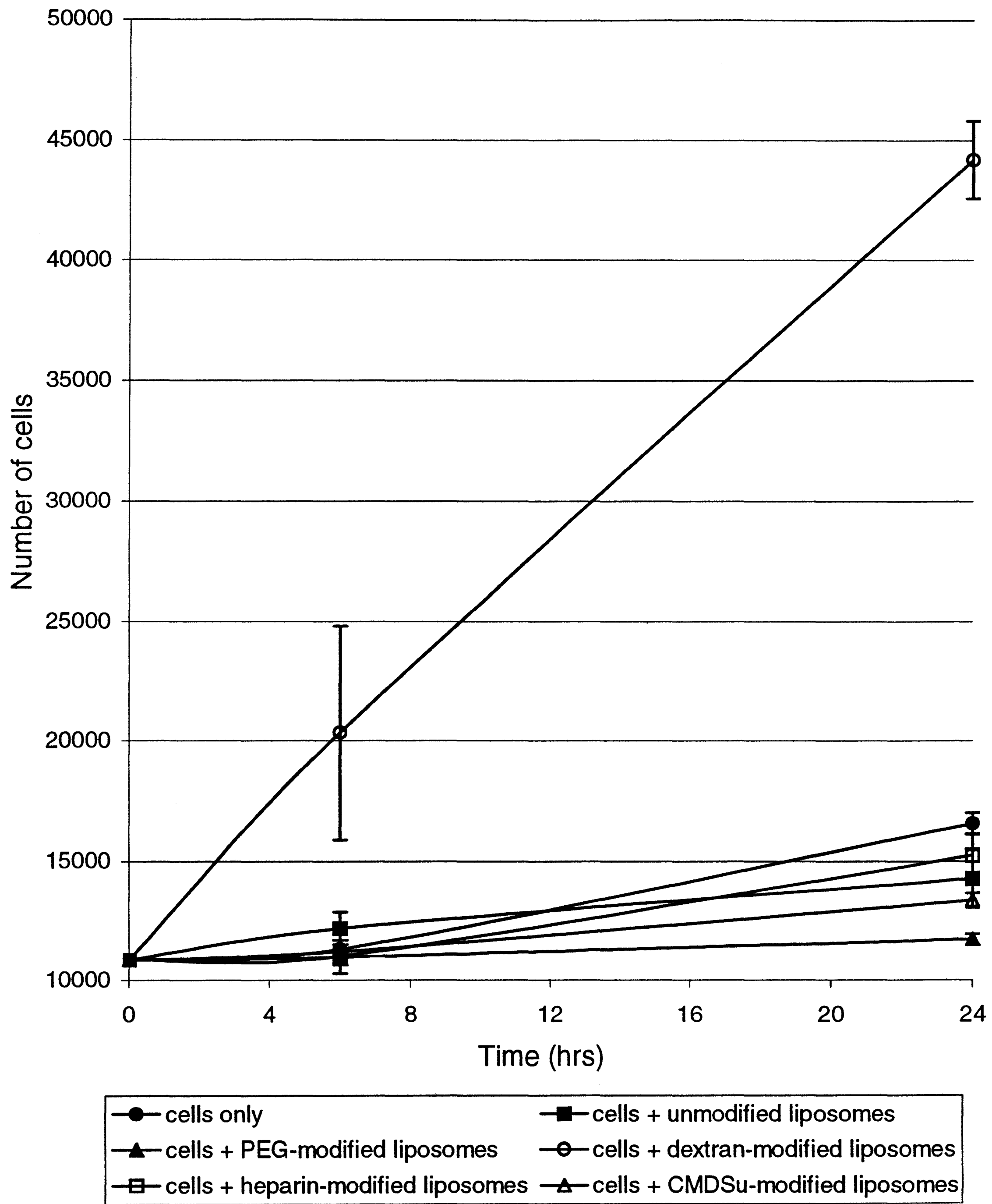


Figure 5.12 Growth kinetics of endothelial cells EAhy926 in the presence of various types of liposomes. Growth-arrested cells were released from G0 phase by addition of culture medium plus 2% FBS containing liposomes (0.4 mM total lipids). (Values are means of 3 experiments with 4 replicate wells in each experiment ±1 SD.)

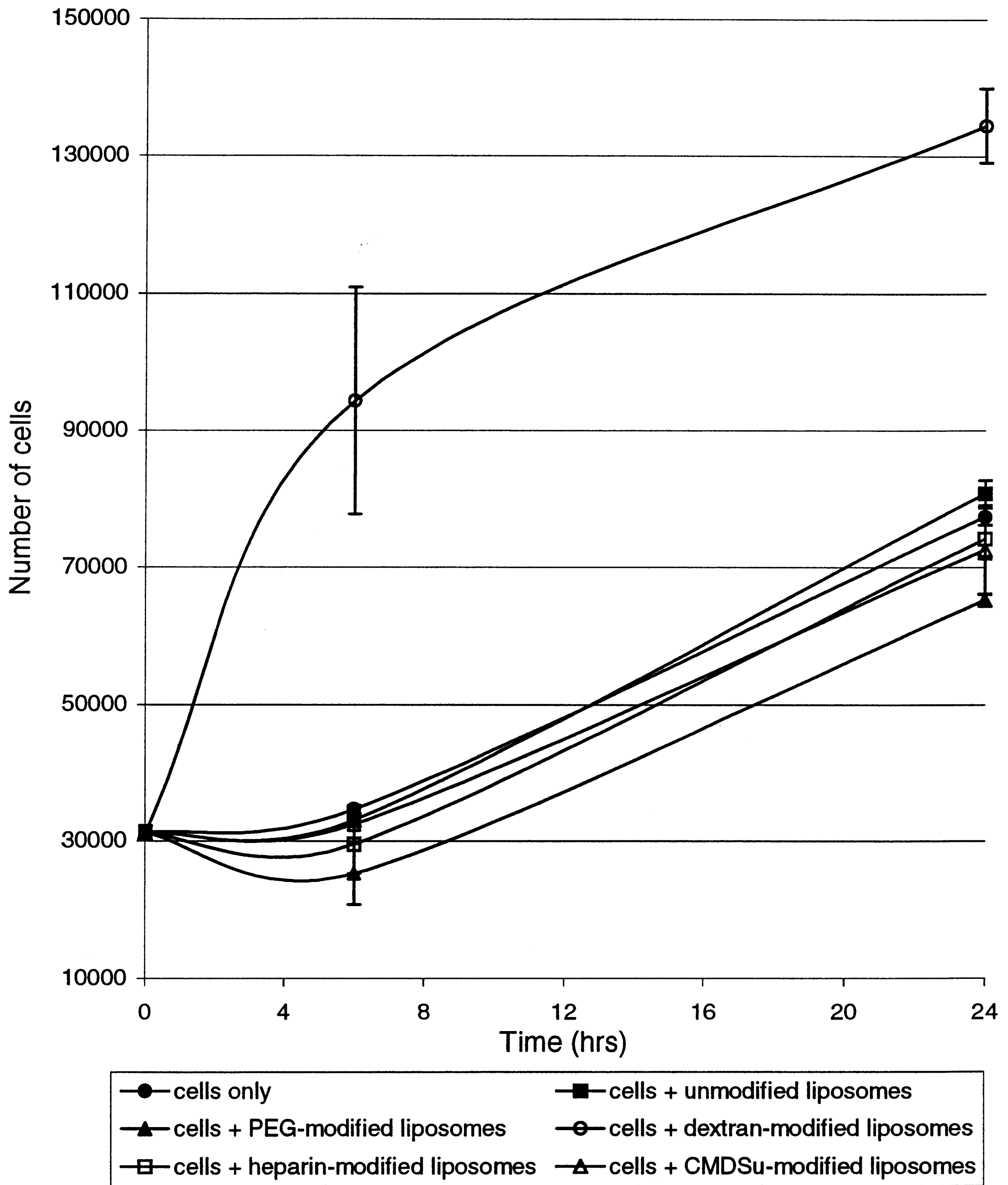


Figure 5.13 Growth kinetics of smooth muscle cells BLC#5 in the presence of various types of liposomes. Growth-arrested cells were released from G0 phase by addition of culture medium plus 2% FBS containing liposomes (0.4 mM total lipids). (Values are means of 3 experiments with 4 replicate wells measured in each experiment ±1 SD.)

Wells containing cells without any liposomes were used as a control. For both cell types, the growth rate of cells exposed to all but dextran-modified liposomes was similar to that of the control. The assessment of cell viability by Trypan blue staining indicated no cytotoxic effect of any of the liposome types at a concentration of 0.4 mM total lipids.

Heparin is known to inhibit smooth muscle cell growth [92, 98, 109], and the BLC#5 line is sensitive to heparin's actions. It appears that either the amount of heparin present on the liposomes was too small or that it was bound to the surface in a manner that did not enable it to inhibit smooth muscle cell growth. On the other hand, dextran-modified liposomes caused markedly increased cell growth, even after only 6 h incubation. These findings are puzzling since proliferation at such high rates (doubling in numbers after 6 hours for endothelial cells and tripling for smooth muscle cells) is extermely high. In previous work it was found that dextran T40 had no effect on the in vitro growth of HUVECs [187] or on 3 types of human mammary epithelial cell lines (HBL100, HH9 and MCF7) [188]. Native dextran did not affect the growth rate of either cell lines studied here when it was added to culture media at 0.1 mg/ml (data not shown). Also, Letourneur et al. [93] found that dextran was not bound or internalized by rat aorta smooth muscle cells in vitro, and Baldwin et al. [189] showed that dextran interacted only weakly with endothelial cells of rat intestinal mucosal capillaries. None of these data suggested the possibility of any stimulatory effect of dextran on the cell lines studied in this work. The experimental data for dextran-modified liposomes remain unexplained. On the other hand, derivatized dextrans containing benzylamide sulfonate groups have

been shown to exert a stimulatory effect on HUVECs [187]. The CMDSu studied in this work does not contain any benzylamide sulfonate groups and therefore was not expected to affect the growth of cells.

The results from the growth kinetics experiments showed that none of the liposome types had cytotoxic effects on either of the cell lines studied, thus indicating that further interaction studies could be validly pursued.

5.4.2 Cells in the Presence of Rhodamine-Labelled Liposomes

Approximately 10,000 cells per cm^2 were seeded on Labtek slides (with removable wells) and incubated at 37°C in 10% FBS containing 1% antibiotics and were growth-arrested 24 h later in 0.4% FBS for 48 h. The cells were then exposed to rhodamine-labelled liposomes in 2% FBS (0.1 mM total lipids). PE-rhodamine (1 mol %) was incorporated into the lipid mixture at the stage of film formation. Fluorescent liposomes were then obtained as previously described in the liposome preparation protocol. Two incubation times were studied: 5 min and 3 h. After the incubation period the cells were washed, fixed to the slides, and the slides were mounted. The slides were kept away from light at 4°C until they were ready to be viewed under a fluorescence microscope equipped with a rhodamine filter. The cells were later photographed and the fluorescence intensity was qualitatively assessed. The fluorescence intensity was taken to be proportional to the number of liposomes present on the cell surface and inside the cells. Images of cells exposed to the various types of fluorescent liposomes are shown in Figures 5.14 and 5.15.

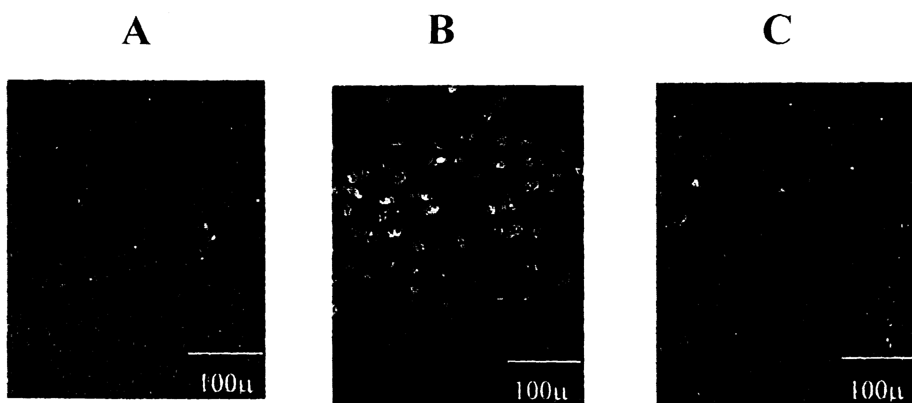


Figure 5.14 Interaction of fluorescent liposomes with endothelial cell line Eahy926. A) Dextran-modified liposomes incubated 5 min with cells. B) Dextran-modified liposomes incubated 3 h with cells. C) CMDSu-modified liposomes incubated 3 h with cells. All incubations were performed at 37°C in a 5% CO_2 incubator.

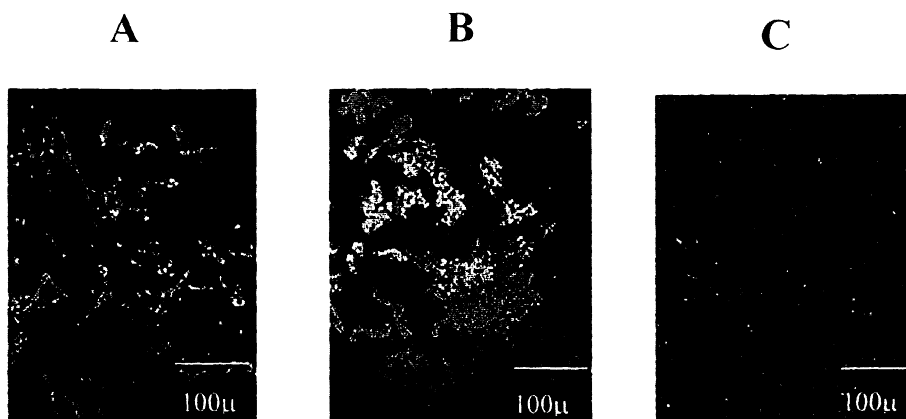


Figure 5.15 Interaction of fluorescent liposomes with smooth muscle cell line BLC#5. A) Dextran-modified liposomes incubated 5 min with cells. B) Dextran-modified liposomes incubated 3 h with cells. C) CMDSu-modified liposomes incubated 3 h with cells. All incubations were performed at 37°C in a 5% CO_2 incubator.

For both endothelial cells and smooth muscle cells, the fluorescence intensity after 3 h of incubation was strong for dextran-modified liposomes, and much weaker for CMDSu-modified liposomes. No fluorescence was detected for unmodified, PEG, or heparin-modified liposomes. After only 5 min incubation, fluorescence was observed for cells exposed to dextran-modified liposomes, indicating that interactions were very rapid for these liposomes. No fluorescence was observed after 5 min incubation for any other type of liposomes, for either endothelial or smooth muscle cells. For dextran-liposomes, fluorescent intensity increased as a function of time.

The localization of the fluorescence was similar for dextran and functionalised dextran-modified liposomes, for both endothelial and smooth muscle cells. The diffuse staining seen could indicate that the fluorescent dye was mainly localized on the cell membrane. The fluorescent response seemed more intense with smooth muscle cells than with endothelial cells.

Even though heparin is known to inhibit smooth muscle cell proliferation, the mechanism by which it acts remains unclear [167]. If this inhibition is mediated by a receptor for heparin on the cell surface, it would appear that the manner in which heparin was bound to the liposome surface rendered it incapable of binding to this receptor.

5.4.3 Cells in the Presence of [^{3}H]-Labelled Liposomes

Approximately 10,000 cells per cm^2 were seeded in 24-well plates in 10% FBS containing 1% antibiotics and were growth-arrested 24 h later by incubation in 0.4% FBS for 48 h. The cells were then exposed to tritium-labelled liposomes in 2% FBS (0.2 mM

total lipids). [^{3}H]-cholesterol (0.03 mol %) was incorporated into the lipid mixture at the stage of film formation. Radioactive liposomes were then obtained as previously described in the liposome preparation protocol. Four incubation times were studied: 5 min, 3, 6, and 24 h. After the incubation period the cells were washed, and the cells were lysed to recover any radioactivity associated with them. Cellular interactions were evaluated by comparing the recovered radioactivity to the total radioactivity from the liposome suspension initially added to the incubation medium. The percent incorporation of liposomes relative to the initial amount of liposomes present is depicted in Figure 5.16 for endothelial cells and in Figure 5.17 for smooth muscle cells.

The data collected using radiolabelled liposomes is in agreement with the data from the fluorescence experiments. For dextran- and functionalised dextran-modified liposomes, the incorporation of liposomes by both types of cells studied increased with time. Dextran-modified vesicle incorporation increased to $7.3 \pm 0.9\%$ and $13.6 \pm 0.8\%$ after 24 h incubation, for endothelial cells and smooth muscle cells respectively. These values are very different from previous data published by Cansell et al. [36] who found only approximately 0.35% incorporation for dextran T40-liposomes by human endothelial cells after 20 h incubation. These differences were unexpected as the liposome preparation and cellular interaction protocols followed in this study were similar to those used by Cansell et al.

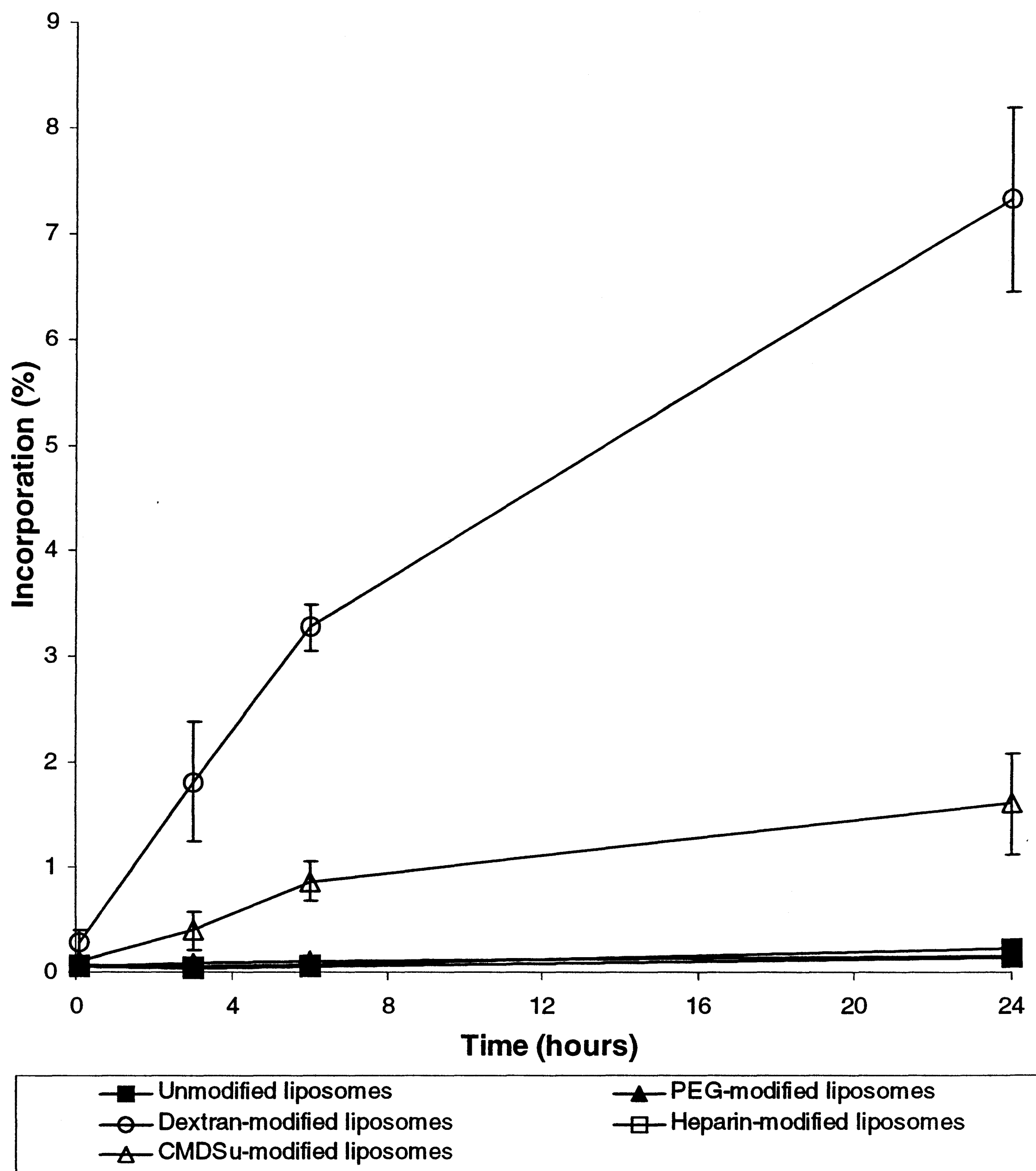


Figure 5.16 Interactions of radiolabelled liposomes with endothelial cells EAhy926. The labelled ([^{3}H]-chol) liposomes were incubated at 37°C at a concentration of 0.2 mM total lipids. Data were normalized to the total radioactivity in the incubation medium. (Values are means of 3 experiments with 4 replicate wells measured in each experiment ±1 SD.)

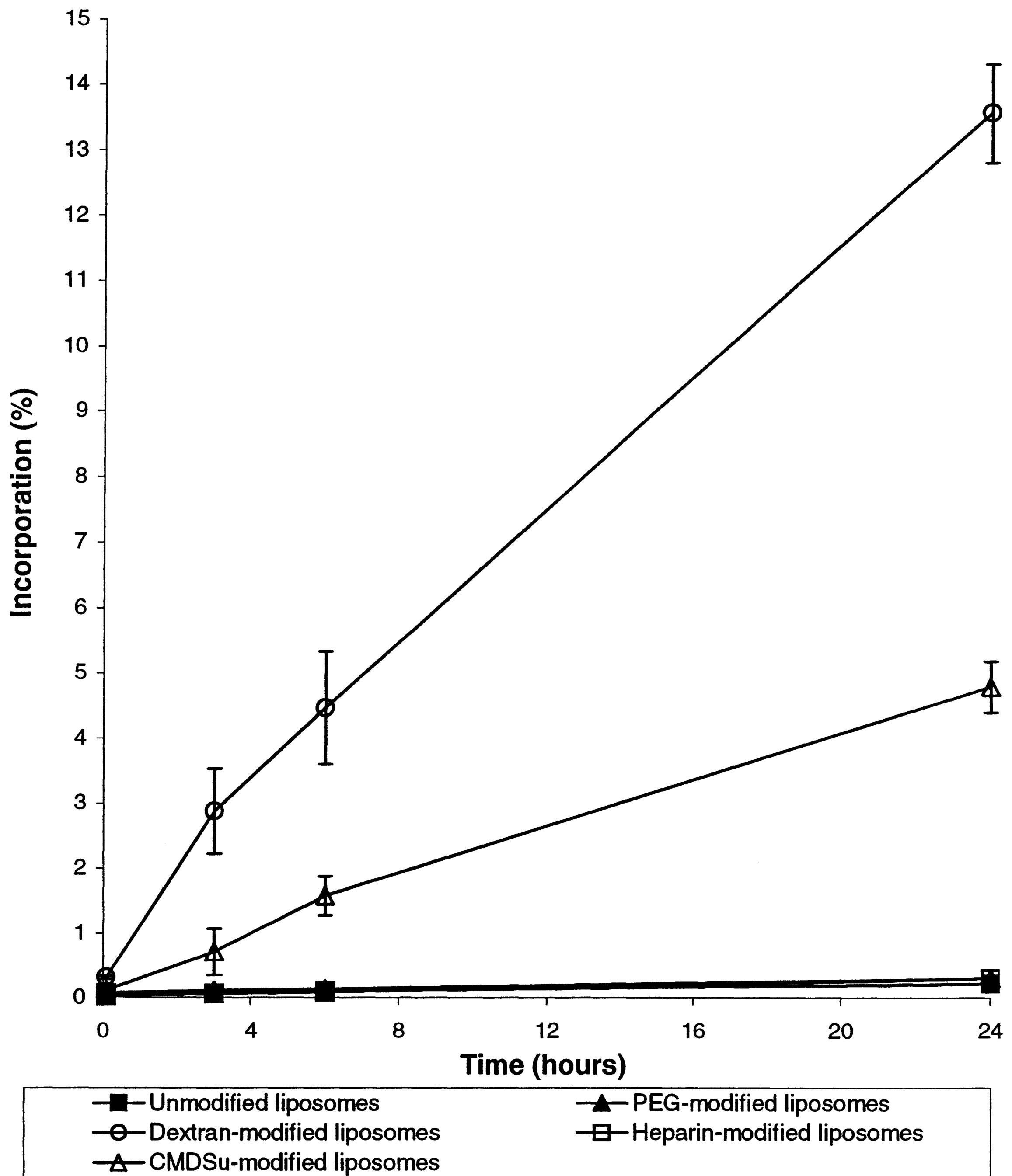


Figure 5.17 Interactions of radiolabelled liposomes with smooth muscle cells BLC#5. The labelled ([^{3}H]-chol) liposomes were incubated at 37°C at a concentration of 0.2 mM total lipids. Data were normalized to the total radioactivity in the incubation medium. (Values are means of 3 experiments with 4 replicate wells measured in each experiment ±1 SD.)

CMDSu-modified liposomes interacted with cells to a lesser extent than dextran-modified liposomes, with incorporation of 1.6 ± 0.5% for endothelial cells and 4.8 ± 0.4% for smooth muscle cells after 24 h incubation. The particular functionalised dextran studied in this work was not expected to possess any biological activity as it does not contain benzylamide sulfonate groups, which have been identified as the biologically active component in these materials [98]. For this reason, CMDSu-modified liposomes were not necessarily expected to interact with cells. On the other hand, CMDSu is derived from dextran and possesses some glucose subunits that have not been modified, and thus similar interactions to those with dextran-modified liposomes could be expected.

No significant incorporation (i.e. values less than 0.35%) was observed for unmodified, PEG, or heparin-modified liposomes, even after 24 h incubation. Cansell et al. [36] observed approximately 0.45% incorporation for unmodified liposomes by human endothelial cells. Letourneur et al. [115] observed approximately 0.12% incorporation for unmodified liposomes by rat aorta smooth muscle cells.

5.4.3-1 Addition of Dextran to the Culture Medium

As evidenced by the liposome-cell interaction data, EAhy926 and BLC#5 cell lines seem to have a high affinity for dextran-modified liposomes. We wanted to determine if this affinity was due to dextran alone, or if the free amine groups present on the polysaccharide following amine derivatization and cholesterol conjugation were also contributing to the interactions. To investigate this possibility the experiment in which cells were exposed to radiolabelled dextran- and CMDSu-modified liposomes for a

period of 6 h was repeated with the modification that a certain amount of native dextran or aminated dextran was added to the culture medium. Two concentrations of the dextrans were studied: 2 mg/mL and 20 mg/mL. These concentrations were chosen based on the amount of polysaccharide present in the liposome suspension. In the previous experiments, 20 μg dextran-cholesterol or functionalised dextran-cholesterol was present in the liposome suspension added to the culture medium. In the modified experiments the amounts of free polysaccharide present in each well was 100 and 1000 times greater than this value. The incorporation of dextran- and functionalised dextran-modified liposomes, in the presence of native dextran and aminated dextran, into endothelial cells and smooth muscle cells is depicted in Figures 5.18 and 5.19.

Figure 5.18 indicates that the incorporation of dextran and functionalised dextran-modified liposomes by EAhy926 cells remained essentially unaffected upon the addition of dextran to the culture medium. On the other hand, when 2 mg/mL aminated dextran was present, the incorporation of dextran-modified liposomes dropped by over 20%. At 20 mg/mL aminated dextran, incorporation decreased by greater than 60%. The incorporation of CMDSu-modified liposomes decreased by over 40% in the presence of 2 mg/mL dextran-NH_2, and by almost 90% at 20 mg/mL.

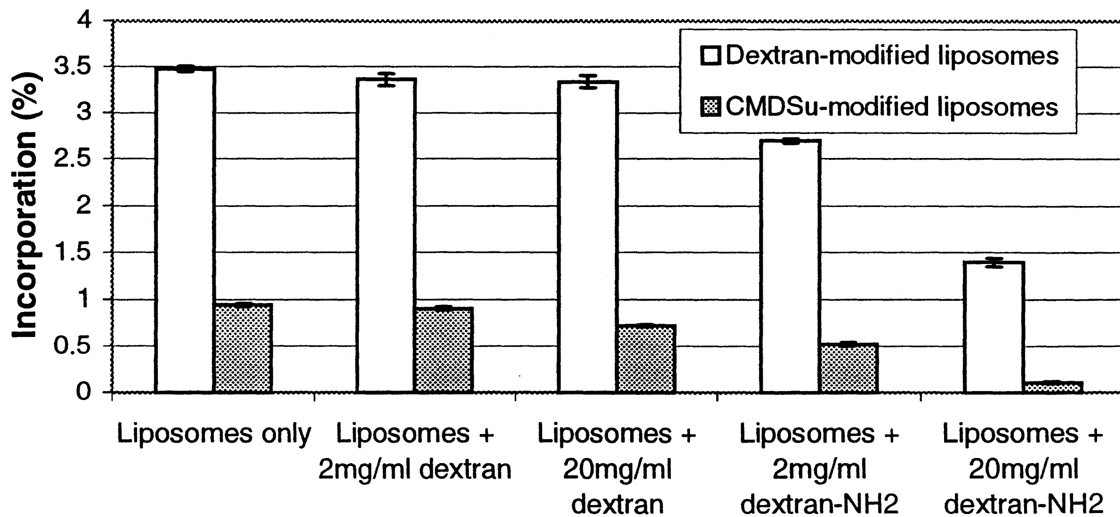


Figure 5.18 Interactions of radiolabelled dextran- or functionalised dextran-modified liposomes with endothelial cells EAhy926 in the presence of free dextran or aminated dextran. The labelled ([^{3}H]-chol) liposomes were incubated at 37°C for 6 h at a concentration of 0.2 mM total lipids with 0, 2 or 20 mg/mL free polysaccharide. Data were normalized to the total radioactivity in the incubation medium. (Values are means of 3 experiments with 4 replicate wells measured in each experiment ±1 SD.)

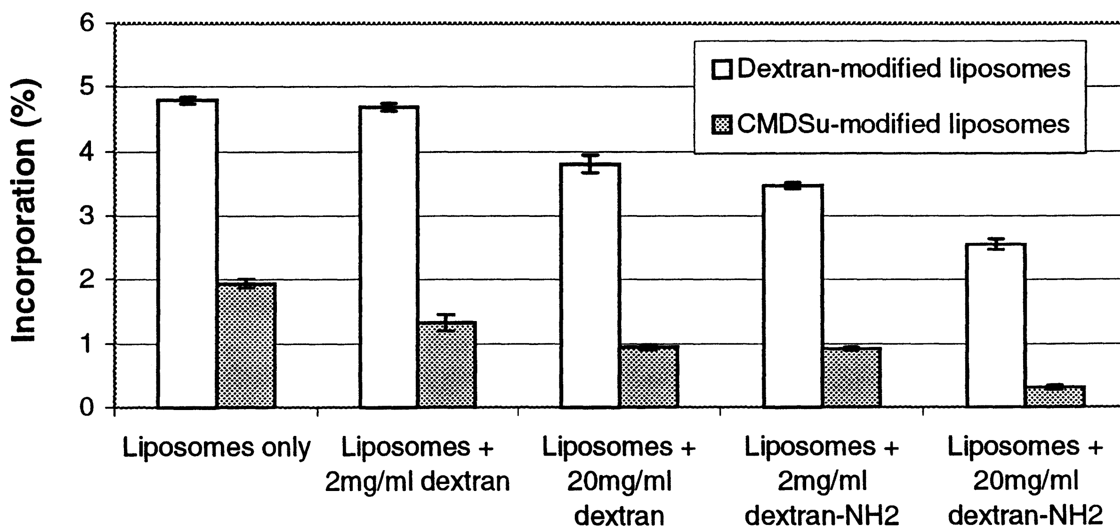


Figure 5.19 Interactions of radiolabelled dextran or functionalised dextran-modified liposomes with smooth muscle cells BLC#5 in the presence of free dextran or aminated dextran. The labelled ([^{3}H]-chol) liposomes were incubated at 37°C for 6 h at a concentration of 0.2 mM total lipids with 0, 2 or 20 mg/mL free polysaccharide. Data were normalized to the total radioactivity in the incubation medium. (Values are means of 3 experiments with 4 replicate wells measured in each experiment ±1 SD.)

Similar trends were observed for BLC#5 cells. The addition of dextran to the culture medium decreased incorporation of liposomes by the cells for both dextran and CMDSu-liposomes. The reduction of incorporation upon the addition of aminated dextran was more pronounced. When 2 mg/mL was present, the incorporation of dextran-modified liposomes decreased by almost 30%. At a concentration of 20 mg/mL, the decrease in incorporation was about 45%. The incorporation of CMDSu-modified liposomes decreased by over 50% in the presence of 2 mg/mL dextran-NH_2, and by almost 85% at 20 mg/mL.

These results suggest that the cells have a high affinity for aminated dextran. This is not surprising since sugars on the surface of cells play an important role in cell-to-cell recognition. For this reason liposomes containing natural and synthetic saccharide structures are already used as targetable drug carriers. Thus, Ghosh and Bachhawat [148] were able to target glycolipid liposomes to specific liver cell types, both in vitro and in vivo, by attaching different sugar residues to the liposomal surface.

The addition of free dextran to the medium, in the case of smooth muscle cells, and aminated dextran, for both cell types studied in this work, inhibited the uptake of dextran and CMDSu-modified liposomes in a dose-dependent manner. This could be due to free polysaccharide interacting with receptors on the cell surface, and blocking these sites for liposomes. It is also possible that the free polysaccharide is not bound to a receptor, but is simply covering the surface of the cell through non-specific hydrophobic and / or electrostatic interactions, thus rendering the cell unable to interact with liposomes.

5.4.4 General Discussion of Liposome-Cell Interactions

Both types of cells behaved in a similar manner when exposed to the various liposome types. Preferential uptake of dextran-modified liposomes was observed relative to the other liposome types. CMDSu-modified liposomes also showed some interaction with both types of cells. None of the other liposome types were incorporated by either cell line. In order for any incorporation to be detected by our methods, the interaction between the given liposome type and the cells needed to be strong enough to withstand the cell washing procedure. If the liposomes were not incorporated into the cell and were only weakly bound to the surface, they would have been washed away before being detected. Thus, only liposomes exhibiting strong interactions with the cell surface or having penetrated the cell wall were detected and considered as incorporated.

The base composition for all liposomes studied in this work was selected to mimic the endothelial cell membrane lipid composition (PC/PE/chol 70/10/20 mol%). Using fluorescently-labelled liposomes, Cansell et al. [36] observed endothelial cell uptake of unmodified liposomes having the same lipid composition as in this work after only 15 min of contact. They suggested that this showed a good affinity of the cell and liposome of membranes for each other. No fluorescence was detected in our experiments with unmodified liposomes.

Based on the fact that PEG is a known fusogen, PEG-modified liposomes were expected to interact with the cellular membrane by fusion and to release their contents once inside the cell. In PEG induced fusion, PEG causes dehydration of the cell membrane, modification of membrane fluidity and microviscosity, increases in surface

tension, aggregation of membrane proteins and lateral phase separation [190]. These effects are thought to be followed by bilayer destabilization and subsequent membrane fusion. In the work reported here none of these phenomena apparently occurred, since no fluorescence or radioactivity associated with the cells was detected, even after 24 h incubation. Okumura et a. [190] observed fusion of PEG liposomes with various cell types and found that the length of the PEG chain optimal for liposome-cell fusion may be greatly dependent on the target cells.

Dextran showed a stimulatory effect on cellular growth for both EAhy926 and BLC#5 cell lines under our experimental conditions, even after only 6 h exposure time. The interaction between dextran-modified liposomes and cells was very rapid, and both fluorescence and radioactivity associated with both types of cells were detected after only 5 min incubation. When aminated dextran was added to the culture medium along with the liposomes, the liposome uptake was significantly reduced. The effect of the addition of native dextran was less obvious. This could mean that the cell membrane has an affinity for the free amine groups present on the aminated dextran backbone. However, both heparin and CMDSu also have free amino groups, and both heparin- and CMDSu-liposome uptake was much lower. It is possible that the amine content of the dextran was higher than the heparin and CMDSu. From the FTIR data, it appeared that the efficiency of cholesterol conjugation was greater for both these polysaccharides than for dextran, as evidenced by the intensity of the amide peak. A lower cholesterol conjugation efficiency could result in a greater concentration of free amino groups, i.e. not bound to cholesterol.

It remains unclear why dextran-modified liposomes interacted more strongly with endothelial and smooth muscle cells compared to other liposome types. The hydrophilicity of the polysaccharide as well as the presence of amino groups on the dextran chains may have contributed to this effect. The small size of the dextran-modified liposomes may also have played a role in increased cell interactions. Smaller vesicles may be internalized more easily by the cellular membrane.

Heparin-modified liposomes were not incorporated by either cell type. Heparin is known to inhibit smooth muscle cell growth, but has no known effect on the growth of endothelial cells. If heparin somehow inhibits smooth muscle cell proliferation by indirect interaction with the cells, for example interaction with the surrounding extracellular matrix, then this result may not be as surprising as at first sight. On the other hand, if the inhibition of proliferation requires direct cell-heparin contact, then this would mean that the heparin on the liposome surface was not able to interact either non-specifically or with receptor sites on the cell. This limitation may be due to the conformation of heparin on the liposome. Also the manner in which the cholesterol was conjugated may block the binding site on heparin. Another possibility is that there was not a sufficient amount of heparin on the liposome surface to interact with the cells.

Liposomes modified with functionalised dextran were incorporated on the surface or into the interior of both types of cells less than for dextran-modified liposomes. Incorporation was lower by approximately 75% than for dextran-modified liposomes for endothelial cells and by 65% for smooth muscle cells. CMDSu is derived from dextran and retains approximately 50% unmodified glucose subunits while the others are

modified with carboxymethyl groups and / or sulfate groups. Both types of cells studied showed a high affinity for dextran. This could explain the significant affinity seen for CMDSu-modified liposomes which contain substantial amounts of unmodified dextran chain units.

5.5 Correlation Between Protein Adsorption and Cell Interactions

When reviewing protein adsorption data along with cell interaction data, it can be seen that the incorporation of liposomes by endothelial and smooth muscle cells was related to the amount of protein bound to the liposome surface. It would seem that liposomes to which higher concentrations of total proteins are bound tend to interact more readily with cells. Dextran- and CMDSu-modified liposomes adsorbed the most protein (adsorption levels were greater than for the other liposome types by a factor of two, as shown by the total protein data in Table 5.8) and were the only types of liposome that interacted with the cells. The steric barrier provided by PEG against protein adsorption on the liposome may also have prevented cellular interactions. Both dextran- and CMDSu-modified liposomes adsorbed significant amounts of fibrinogen, C3, fibronectin, albumin, and IgG. These proteins may have contributed to the increased cellular interaction via recognition by receptors on the cell membrane. Kamps et al. have shown preferential uptake of anionized-albumin by hepatic endothelial cells [153]. It would appear that one must choose between liposome stability and cell targetability. None of the liposomes studied here would be both stable and targetable simultaneously.

6. SUMMARY AND CONCLUSIONS

The use of liposomes as drug carriers is hindered by unresolved problems of early clearance from the bloodstream and the inability to target them to specific cell types. Natural and synthetic polymers have been used to modify the liposome surface to either improve the circulatory half-life by reducing protein adsorption, or to target a specific cell type. Little attention has been focused on resolving both issues simultaneously.

The aim of this work was to study various types of polymeric modifiers and compare them on the basis of protein adsorption and cellular interactions. It was hoped to obtain information on the relative efficiency of the various polymers in resisting protein adsorption, in particular complement components and other opsonins, while also maximizing incorporation into cells.

To attain this goal, liposomes modified with different polymers were exposed to IgG as a single protein solution in buffer, as well as to plasma for assessment of protein binding. Liposomes were also exposed to two different cell types and incorporation was studied using fluorolabelled and radiolabelled liposomes.

The liposomes studied were based on the lipid components PC, PE and cholesterol, and were prepared with a composition mimicking that of endothelial cells (70/10/20 mol% PC/PE/cholesterol). The liposomes were prepared using the detergent removal technique with n-octyl-β-D-glucopyranoside. This method yields mostly unilamellar liposomes with a monodisperse size distribution. The polymers studied were poly(ethylene glycol) (PEG), dextran, heparin, and functionalised dextran (CMDSu).

The polymer mass loading was the same (1 mg polymer / mL liposome suspension) for all liposome types. It has been suggested that PEG and dextran surface modification of liposomes increases their in vivo circulation lifetime by providing a steric barrier, which reduces the adsorption of opsonizing proteins. On the other hand, heparin is known to possess specific biological activities that could improve liposome-cell interactions. Functionalised dextrans were designed to have heparin-like properties. Such activity is believed to be linked to the benzylamide and sulfonate contents of modified dextrans.

The polysaccharides were conjugated to cholesterol, which served to anchor the molecule into the lipid bilayer. This was achieved by initially introducing amino groups into the polysaccharides and then reacting cholesterol with the amines. The amide bond formed was used to verify the incorporation of the cholesterol moieties.

Liposome size was characterized by dynamic light scattering. Size distributions of liposome preparations were found to be reproducible.

Liposomes were exposed to solutions of IgG of concentration up to 1 mg/mL for 2 h at room temperature. Non-adsorbed proteins were removed with four successive washing steps. Adsorption as a function of IgG concentration was determined using radioiodinated IgG as a tracer.

The data showed that of the various liposome types tested, the unmodified liposomes adsorbed the most protein, although the amounts were small relative to fibrinogen adsorption investigated previously in this lab [76]. PEG and heparin modification appeared to reduce protein adsorption significantly. Dextran and functionalised dextran modifications did not reduce IgG adsorption relative to the

unmodified liposomes. This result could be due to the fact that shorter polymer chains on the liposome surface are more efficient in retarding the proteins than longer chains, as suggested by Needham and Kim [86]. The PEG (MW=2000) and heparin (MW=10,700) used in these experiments were much smaller than the dextran (MW=35,700) and CMDSu (MW=55,000).

Liposomes were also exposed to 10% plasma for 2 h at room temperature. Seven successive washing steps removed unbound proteins, and the amounts of protein present on the liposome surfaces were quantitatively assessed using a total protein assay. Individual proteins present were identified by SDS-PAGE and Western blotting.

The plasma adsorption data showed trends for the various liposome types that were different from the IgG adsorption data. All four polymer-modified liposome types adsorbed more protein than the unmodified ones. However, when comparing the modified liposomes amongst themselves, the trends were similar to those for IgG adsorption. The PEG and heparin liposomes adsorbed the smallest amounts of protein. The dextran and functionalised dextran liposomes adsorbed the most. The SDS-PAGE and Western blot data showed that the bound protein profiles of all the liposome types studied were similar, but different from that of plasma. A significant reduction in the relative amounts of albumin, fibrinogen, plasminogen, transferrin and factors H and I was seen on all liposome types compared to plasma. On the other hand, an enrichment of C3 and fibronectin relative to plasma was noted. α2-macroglobulin was enriched on heparin and CMDSu liposomes, but not on PEG and dextran liposomes. Significant binding of IgG and apolipoprotein A1 was also observed. Antithrombin (AT) binding was also of

interest since it is known to bind to heparin and some functionalised dextrans. Significant AT binding was noted for PEG, heparin and CMDSu-coated liposomes. A strong band on the gels seen at 75 kDa remained unidentified.

Liposome-cell interactions were investigated by incubating endothelial cells (cell line EAhy926) and smooth muscle cells (cell line BLC#5) with the various types of liposomes. The incorporation of the liposomes by the cells was determined qualitatively using rhodamine-labelled vesicles and observing the cells in a fluorescence microscope. Incorporation was also determined quantitatively using [^{3}H]-labelled liposomes and counting the radioactivity associated with the cells after washing.

The cell interaction data showed significant liposome incorporation of dextran- and CMDSu-modified liposomes, for both cell types studied. The dextran liposomes were particularly well incorporated. This trend was seen in both the fluorescence and radioactivity experiments. None of the other liposome types exhibited significant incorporation for either cell line. Endothelial cells and smooth muscle cells showed a certain affinity for the dextran liposomes in growth kinetics experiments. When free dextran was added to the culture medium containing the dextran or functionalised dextran liposomes, the rate of incorporation by the cells decreased slightly. When aminated dextran was added, incorporation of both types of liposomes decreased significantly. Thus it would appear that aminated dextran prevented the incorporation of liposomes by either binding to the liposome receptors on the cell surface, or by simply modifying the cell surface, so as to prevent liposome-cell contact.

Overall, it appeared that the incorporation of liposomes by EAhy926 or BLC#5 cells was related to the amount of protein bound to the liposome surface. This would suggest that long-circulating liposomes such as PEG-coated liposomes have little affinity for cells and thus could not be considered a vehicle of choice for targeting specific cell types. Okumura et al. [190] suggested that surface proteins may adversely affect the susceptibility of cells to fusion with PEG liposomes. Taking all of these data and observations together leads to the conclusion that in order to increase the circulatory half-life of liposomes while increasing cellular interactions, PEG could be combined with sugars on the surface of liposomes. PEG-modified liposomes in which the PEG is conjugated to sugar moieties at the chain ends might be useful. This would combine the protein-repelling properties of PEG with the ability of some specific sugars to interact with cells. The liposomes could thus be tailored to interact with specific cells by varying the sugar moiety bound to the PEG chains.

7. SUGGESTIONS FOR IMPROVEMENTS AND FUTURE WORK

One problem encountered in this work was the difficulty in quantifying the amount of free amino groups and cholesterol moieties present on the derivatized polysaccharides, possibly due to the relatively small concentrations of these species. One possible way to increase the yield of free amines would be to increase the ratio of bromopropylamine hydrobromide to polysaccharide subunits (R). Higher concentrations of amino groups would tend to increase the efficiency of the cholesterol conjugation reaction. To further improve the protocol, the ratio of cholesteryl chloroformate to polysaccharide subunits could also be increased (as was done for heparin). A high degree of cholesterol derivatization of the polysaccharides should provide better anchoring and more extensive incorporation into the lipid bilayer.

In the protein adsorption experimental procedure, the liposomes were separated from unbound proteins by ultracentrifugation. This technique is limited because it requires vortexing to resuspend the liposomes in fresh buffer and this can damage or destroy liposomes, resulting in the loss of both liposomes and the proteins attached to them. The procedure could also result in the removal of proteins that were initially bound to the vesicles. An alternative to this technique would be to separate the liposomes from the free proteins by passing the suspension through a gel filtration column.

Plasma adsorption experiments were performed with 10% plasma. A higher concentration, say 25%, might have been more appropriate since Lelkes and Friedmann

[191] have shown that more than 90% of the effects observable with full serum were found with 25% serum.

The quantification of protein bound per unit surface area of liposome (mass of protein per mol of phospholipid) could have been determined by assessing the number of liposomes in the suspension. This could have been achieved by preparing a known concentration of liposomes, determining the average size of the liposomes by DLS, and counting the number of liposomes in a known volume using a Coulter counter.

The effect of the addition of calcium ions to the cell culture medium during liposome-cell interactions has been studied [36, 115]. Calcium has been shown to increase the incorporation of the liposomes by the cells. By adding Ca^{2+} to the culture media, the incorporation of unmodified, PEG and heparin-liposomes may increase enough to be able to show differences among the three types.

Future work should include the in vitro study of liposomes in contact with macrophages. It would be useful to determine if the liposomes that demonstrated a higher affinity for smooth muscle cells and endothelial cells also possess a high affinity for macrophages. It would also be of interest to study liposomes in vivo to determine if decreased protein binding actually decreases liposome clearance, and if long-circulating liposomes are efficient in targeting cells.

Finally, by combining PEG and dextran on the surface of liposomes, it is of interest to ask whether combination of the protein resistance and efficient cell incorporation properties would be achieved, i.e. would PEG decrease protein interactions and would dextran enhance cellular interactions simultaneously?

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APPENDIX A

SDS-PAGE and Western Blotting Procedure

SDS-PAGE and Western Blotting Procedure[192]

Polyacrylamide gel preparation (12% separating gel, 4% stacking gel)

The acrylamide/bis solution was prepared by dissolving the following reagents in distilled water, diluting to 100 mL and filtering the final solution:

Acrylamide	29.2 g
N,N'-Methylenebisacrylamide	0.8 g

The reagents for the 12% separating gel were mixed and degassed for 15 min at room temperature:

Distilled water	3.35 mL
1.5 M Tris, pH 8.8	2.5 mL
10% (w/v) SDS	0.1 mL
30% (w/v) Acrylamide/Bis	4.0 mL

Immediately prior to casting the gel, the following reagents were added to initiate polymerisation in the above mixture:

10% (w/v) ammonium persulfate (fresh)	50 µL
TEMED	5 µL

The casting plates were successively cleaned with distilled water and 95% ethanol. Once dry, the plates were inserted into the casting assembly. The assembly was then secured to the casting stand. Using a syringe, the gel plates were filled with polymerising 12% acrylamide solution, leaving enough space to pour the stacking gel. After 2 min, a small quantity of water was layered over the gel. The gel was allowed to polymerise for 1 h.

The reagents for the 4% stacking gel were mixed and degassed for 15 min at room temperature:

Distilled water	3.0 mL
0.5 M Tris, pH 6.8	1.2 mL
10% (w/v) SDS	0.1 mL
30% (w/v) Acrylamide/Bis	0.65 mL

Immediately prior to casting the gel, the following reagents were added to initiate polymerisation in the above mixture:

10% (w/v) ammonium persulfate (fresh)	25 µL
TEMED	5 µL

Using a syringe, the remainder of the gel plates was filled with polymerising 4% acrylamide solution. An appropriate comb was added and the gel allowed to polymerise for 1 h.

Sample preparation

The sample buffer used in sample preparation consists of the following reagents, mixed and stored at 4°C in 225 μL aliquots:

Distilled water	4.0 mL
0.5 M Tris, pH 6.8	1.0 mL
10% (w/v) SDS	1.6 mL
Glycerol	0.8 mL

Tracking dye (TD) was prepared by adding the following reagents to a 225 μL aliquot of sample buffer immediately prior to use:

2-Mercaptoethanol	30 μL
0.5% (w/v) Bromophenol blue	30 μL

Samples and standards used for SDS-PAGE only were prepared as follows:

0.5 μL SDS-PAGE MW Standards, Low Range, 10 μL TD
1-2 μL Protein sample, 10 μL TD
7.5 μL Prestained SDS-PAGE Standards, Low Range

Samples and standards used for immunoblotting were prepared as follows:

0.5 μL SDS-PAGE MW Standards, Low Range, 10 μL TD
10-250 μL Protein sample, 80-250 μL TD
7.5 μL Prestained SDS-PAGE Standards, Low Range

Once mixed, the samples were placed in a 95°C water bath for 7.5 min.

Electrophoresis

Once the gel polymerisation was complete, the combs were gently removed and the wells rinsed with distilled water. The gels were removed from the casting stand and placed into the clamp assembly. The assembly was then placed into the buffer chamber. A 5X stock solution of electrophoresis buffer was prepared by mixing the following reagents in distilled water and diluting to 1 L (Note: the pH of this solution should be 8.3 ± 0.3):

Tris Base	15 g
Glycine	72 g
SDS	5 g

Just prior to use, the 100 mL of the 5X stock solution was diluted with 400 mL distilled water. The upper buffer chamber was filled to a level 3 mm below the edge of the outer (long) glass plate with electrophoresis buffer. The lower buffer chamber was filled to a

level that covered the bottom 1 cm of the gel. The sample(s) was then loaded into the wells and a potential difference of 200 V applied across the gel for approximately 45 min. When performing an immunoblot, a small quantity of pyronin Y dye (dissolved in sample buffer) was layered into the well just before the tracking dye had reached the bottom of the separating gel. Electrophoresis was stopped once the pyronin Y dye had reached the top of the separating gel.

Gel equilibration

Transfer buffer was prepared by mixing the following reagents in distilled water and diluting to 1 L (Note: the pH of this solution should be 8.3 ± 0.3):

Tris Base	3.03 g
Glycine	14.4 g
Methanol (HPLC grade)	200 mL

The gels were removed from the electrophoresis assembly and equilibrated in fresh cold (4°C) transfer buffer for 30 min.

Electrophoretic transfer

Immobilon (PVDF) membranes were cut to gel-size, prewetted in methanol (1-3 s), incubated in water (1-2 min) and soaked in transfer buffer (15 min). The gels and membranes were loaded in the transfer cassettes according to specifications and placed in the transfer chamber. The chamber was then filled with transfer buffer so that the entire gel surface was covered. A potential difference of 100V (200 mA) was applied for 1 h. The membranes could then immediately be stained with colloidal gold or dried and used for immunoblot analysis.

Gold staining

The membranes were then incubated with gentle mixing in 0.3% (v/v) Tween 20 solution in PBS for 1 h at 20°C to block unbound membrane sites. PBS was prepared by mixing the following reagents in distilled water, adjusting the pH to 7.4 and diluting to 1 L:

Na_2HPO_4	1.32 g
$NaH_2PO_4 \cdot H_2O$	0.345 g
NaCl	8.5 g

The membranes were then rinsed in distilled water three times for 1 min each.

The membranes were then placed in Protogold solution and stained for 4 h or overnight. Following the staining, the membranes were rinsed extensively with distilled water and air dried.

Immunoblotting

The sections of the membrane containing MW markers lanes and a small section of the sample lane were removed to be stained with the gold staining procedure described above.

The remainder of the membrane was sliced into 2 mm strips. The strips were prewet in methanol, rinsed in distilled water and placed into plastic wells. In order to block unbound membrane sites and prevent non-specific binding, the strips were incubated for 1 h in 5% (w/v) dry skim milk in TBS, pH 7.4 with gentle agitation. This treatment was followed by three 5 min rinses in 0.1% (w/v) dry skim milk in TBS.

Each strip was then incubated for 1 h in 3 mL 1% (w/v) dry skim milk in TBS with a 1/1000 dilution of the primary antibody to the protein of interest. This treatment was followed by three 5 min rinses in 0.1% (w/v) dry skim milk in TBS. Each strip was then incubated for 1 h in 3 mL 1% (w/v) dry skim milk in TBS with a 1/1000 dilution of the alkaline phosphatase-linked secondary antibody. Again followed three 5 min rinses in 0.1% (w/v) dry skim milk in TBS. Finally, the strips were incubated for 10 to 30 min with a solution to develop the colour reaction and detect the protein bands. The buffer for this solution is prepared by dissolving the following reagents in distilled water, adjusting the pH to 9.8 and diluting to 100 mL:

$NaHCO_3$	840 mg
$MgCl_2 \cdot 6H_2O$	20 mg

The final solution was prepared by mixing 1 mL NBT stock (30 mg NBT in 1 mL 70% DMF in distilled water) and 1 mL BCIP stock (15 mg BCIP in 1 mL DMF) in 100 mL buffer. The colour development was terminated by rinsing the strips in distilled water twice for 5 min.