JONES LIPOSOME STABILITY AND FUSION M.Sc.

FUSION AND STERIC STABILIZATION OF LIPOSOMES CONTAINING MEMBRANE-ANCHORED BIOPOLYMERS

BY

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ABSTRACT

Studies examining the characteristics of membranes that facilitate and affect fusion are central to understanding the intricacies of inter- and intra-cellular fusion processes and expanding the existing knowledge of other roles membranes may have. In this thesis a model membrane system, using Sendai Virus and Egg phosphatidylcholine (EggPC) liposomes containing the receptor glycophorin A (proteoliposomes), was used in examining different fusion with proteoliposomes prepared by different methods. For the first time glycophorin A was incorporated into EggPC liposomes vectorially. This was accomplished separately with two detergents: octylglucoside and CHAPS. Fusion of Sendai Virus with the reconstituted proteoliposomes revealed that octylglucoside reconstituted proteoliposomes exhibited lower fusion compared with CHAPS reconstituted proteoliposomes. Efforts to determine the basis for this difference, using either proteinase K or O-glycosidase digestion and subsequent fragment analysis using SDS-PAGE and silver-staining, were inconclusive. A separate study examined the ability of large membrane-anchored biopolymers (chosen in virtue of their large hydrophilic domains) to sterically stabilize EggPC liposomes.

Glycophorin A, the lipophosphoglycan (LPG) from *Leishmania donovani*, and a polyethyleneglycol-conjugated phospholipid (PEG^{5000}-PE) were incorporated into Egg PC liposomes. In each case, binding of a soluble fluorescent probe, NeutrAvidin Oregon Green, to liposomes containing biotin-conjugated lipid was restricted. This supports the idea that large membrane-anchored biopolymers are able to sterically stabilize liposomes.

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To my parents and brother, God has blessed me with your insurmountable love and unwavering support throughout all my efforts. I couldn't hope for anything more. *In Cruce Vinco.* *BEATI MUNDO CORDE*

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LIST OF ABBREVIATIONS

ddH2O	double distilled water
EDTA	ethylenediaminetetraacetate (disodium salt)
Egg PC	Egg phosphatidylcholine
GD_{1a}	disialoganglioside
HEPES	4-(2_hydroxyethyl)-1-piperazineethanesulfonic acid
MES	2-[N-morpholino]ethanesulfonic acid
HN	hemagglutinin/neuraminidase protein
F	fusion protein
R18	octadecylrhodamine-B-chloride
PEG-PE	polyethyleneglycol-phosphatidylethanolamine
LPG	lipophosphoglycan
WGA	wheat germ agglutinin
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
Biotin-X-DHPE	N-((6-((biotinyl)amino)hexanoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt
SUV	small unilamellar vesicles
LUV	large unilamellar vesicles
MLV	multilamellar vesicles
ESR	electron spin resonance
DSC	differential scanning calorimetry
EM	electron microscopy
R^{sat}_{eff}	effective detergent to lipid ratio at onset of solubilization
R^{sol}_{eff}	effective detergent to lipid ratio at completion of solubilization
PEG	polyethyleneglycol
PMSF	phenylmethylsulfonylfluoride
TEMED	tetramethylethylenediamine
LIS	lithium diiodosalicylate
SDS	sodium dodecyl sulfate
BCA	bichinconinic acid

λex	excitation wavelength
λem	emission wavelength
O.D.	optical density
Glyc. A	glycophorin A
Dw	aqueous monomeric detergent concentration
K	partition coefficient

1.0 Introduction

1.1 The Biological Context of Membrane Fusion

1.1.1 Biological Membranes

The varying complexity of biological membranes reflects the myriad active and passive roles that they play in mediating physiological processes. Their most salient role, of forming physical barriers, allows the existence of membrane bound cells and organelles as physically autonomous units having specific functions. The intrinsic and extrinsic properties of biological membranes are reflective of these specific functions. The presence and release of arachadonic acid and diacylglycerol from lipids are necessary for damage response mechanisms and mitogenic processes, respectively. Maintenance of electrochemical gradients and initiation of signal transduction pathways are carried out by membrane bound ion channels and ligand receptors. The idiosyncratic functions vary with the types and proportions of lipids within the membrane, the types of intrinsic proteins within the membrane and the kinds of extrinsic membrane associated proteins.

1.1.2 Inter- and Intracellular Fusion Processes

The fusion of biological membranes is essential in many cellular processes. The fusion of germ cells during fertilization (White, 1992) and mononucleate myocytes to multinucleated cells during the formation of striated muscle (White, 1992) involve the fusion of plasma membranes. The trafficking of biological molecules between intracellular compartments within vesicular compartments requiring fusion of vesicular membranes occurs in the endosomal pathway (Hammond et al., 1997) and cycling of glucose transporters (Martin et al., 1998). Membrane internalized during receptor - mediated endocytosis forms vesicles that eventually fuse with endosomes, lysosomes or phagosomes within the cell (Palfrey et al., 1998; Xu et al., 1994). The reverse exocytotic process requires that intracellular vesicles fuse with the plasma membrane in order for their inner contents to be released into the extracellular matrix (Palfreyet al., 1998; Xu et al., 1994).

1.1.3 Virus - Cell Fusion

The infectious entry of lipid enveloped viruses into their respective host cells necessitates the fusion of the viral envelope with a host membrane in order for the contents of the virion to be released to the inside of the cell (White, 1993; Hernandez et al., 1996). Limiting this process reduces the efficiency of infectivity (Hernandez et al., 1996). Entry of lipid - enveloped viruses occurs via

two routes (Hernandez et al., 1996). The first of these is characterized by fusion of the viral envelope with the host cell plasma membrane as occurs with Sendai Virus (Hernandez et al., 1996) and HIV (Potempa et al., 1997; Litwin et al., 1996; Lawless et al., 1996). The second route utilizes the receptor mediated endocytosis pathway and fusion occurs with endosomal membranes upon acidification of the compartment. Both routes involve recognition and binding of cell - surface receptor molecules without which the virus particles would remain inert.

1.2 Viral Fusion

1.2.1 Requirements for Viral Fusion

Specific proteins must be present and specific conditions must be met in order for viral fusion with target membranes to occur. Highly specialized viral proteins recognize and bind cellular receptors (Hernandez et al., 1996; Durell et al., 1997) while other proteins cause the fusion of viral and target membranes (Hernandez et al., 1996; Durell et al., 1997). More specific requirements such as low pH (Durell et al., 1997; Gaudin et al., 1995) and proteolysis (Tomasi et al., 1998; Yamada et al., 1997) may induce the necessary fusion active conformational changes. The presence of specific lipids in the membrane also alters its propensity to undergo fusion (Chernomordik et al., 1995; Epand, 1996).

1.2.2 Cell - Surface Viral Receptors

Virions recognize their host cells through the presence of specific protein on the surface of the plasma membrane (Hernandez et al., 1996). Some viruses utilize glycolipids as receptors (Epand et al., 1995). As in the case with Semliki Forest Virus (Moesby et al., 1995) and Vesicular Stomatitis Virus (Pal et al., 1988) lipids may participate in a receptor - like interaction with viruses. This explains how some viruses may target specific organisms and how they exhibit tropisms for specific types of cells. Although the presence of particular receptors is often not the absolute determinant of infectivity or tropism, it undoubtedly limits the host cell range of viruses. Sendai virus recognizes sialylated glycoproteins (Wybenga et al, 1996; Bitzer et al., 1997) and glycolipids (Epand et al., 1995; Umeda et al., 1984) on the cell surface. Receptor binding has also been shown to activate the F protein of Sendai Virus (Dallocchio et al., 1995). Although it is a murine virus exhibiting a tropism for epithelial cells in the respiratory tract, model fusion systems have shown that Sendai virus binds sialylated glycoproteins and glycolipids isolated from human erythrocytes. Studies have utilized the ability of Sendai virus to fuse with human erythrocyte ghosts to identify factors affecting viral fusion.

1.2.3 Viral Attachment Proteins

Viral attachment proteins allow virions to bind their host cell receptors (Hernandez et al, 1996; Potempa et al., 1997). These proteins protrude from the surfaces of virus particles and often form multimers that together constitute the receptor binding pocket (Hernandez et al., 1996; Potempa et al., 1997). Viral attachment proteins are highly antigenic and are therefore targets of the host defense system. Heavy glycosylation with attachment of negatively charged sugar moieties at the glycan termini characterize the attachment proteins of many lipid enveloped viruses (Hernandez et al., 1996; Potempa et al., 1997). The overall negative surface charge imparted to the virion prevents aggregation arising from hydrophobic interactions between adjacent membranes, attractive electrostatic forces and Van der Waals forces.

1.2.4 Viral Fusion Proteins

Entry of virus particles into cells requires the attachment of virions to target membranes followed by the fusion of the viral lipid envelope with the target membrane (Hernandez et al., 1996). Some attachment proteins possess the ability to initiate and facilitate fusion (Ellens et al., 1990; Bentz et al., 1990; Carr et al., 1997; Ohuchi et al., 1997) as is the case with Influenza virus haemagglutinin. Other lipid - enveloped viruses require a distinct protein that contains this intrinsic property. The fusogenic capacity of a protein, per se, is

located in a short fusion peptide within the protein, regardless of whether the protein has several functions or whether its function is limited solely to fusion (Durrell et al., 1997). In the case of Influenza virus the HA protein carries out fusion as well as viral attachment. In the case of Sendai virus, fusion is attributed to the F - protein even though other proteins regulate fusion. The fusion peptide has been localized in close sequential proximity to the N - terminus of most fusion proteins (Durell et al., 1997). This is not the case for all viruses. The fusion peptide of Vesicular Stomatitis Virus is located near the centre of the fusion protein (Zhang and Ghosh, 1994; Durrer et al., 1995).

1.3 Membrane Properties

1.3.1 Intrinsic Properties of Lipids

There are three classes of lipids present in biological membranes: glycerophospholipids, sterols and sphingolipids (Bentz, 1993). All of these lipids are amphipathic in nature. They contain a hydrophobic, predominantly hydrocarbon domain that forms the membrane interior. They also contain a polar moiety that is positioned at the lipid/water interface. The glycerophospholipids contain a glycerol backbone with a phosphate esterified to one of the hydroxyl groups and fatty acids esterified to the other two hydroxyls. The sterols contain a fused hydrocarbon ring system, a hydrocarbon chain and a

hydroxyl group. Sphingolipids contain a ceramide moiety that has a hydrocarbon tail, a fatty acid linked to the 2 - amino position of sphingosine and a polar head group.

Lipids are capable of arranging themselves into several distinct structures. However, the lamellar phase containing a single bilayer is the most prevalent of these phases in biological membranes. Non - bilayer phases that form in vitro, such as the hexagonal phase, may have direct roles in biological systems. But direct roles, in vivo, remain largely speculative (Epand, 1996). There is evidence suggesting that cubic phases occur in biological membranes. But evidence for indirect roles of non- bilayer lipids and phases has been shown in more detail (Epand, 1996). The propensity of a membrane to form non - lamellar structures, for example, correlates with its ability to undergo fusion (Epand, 1996). Lysolipids inhibit formation of the inverted hexagonal phase and they also inhibit fusion of biological membranes (Chernomordik et al., 1993). The formation of non - bilayer phases depends on extrinsic factors such as concentration, pH, temperature and ionic strength and intrinsic factors such as the relative sizes of polar headgroup and hydrophobic tail.

The propensity of the membrane to form non - bilayer structures correlates with its stability (Chernomordik et al., 1995; Chernomordik et al., 1996) and its ability to form highly curved fusion intermediates (Chernomordik et

al., 1995; Chernomordik et al., 1996). Cone shaped lipids, in which the volume of space occupied by the polar headgroup differs from that of the acyl chains, can promote either positive or negative membrane (monolayer) curvature. Phospholipids such as phosphatidylethanolamine, whose acyl chains occupy greater volumes than the headgroup, have inverted cone shapes. Lysophospholipids such as lysolecithin, whose headgroups occupy greater volumes, have cone shapes. Lipids that have a more cylindrical shape have headgroup and acyl chain volumes that are closer. They form stable flat bilayers though bilayers containing cylindrical lipids that are highly curved (i.e. smaller vesicles) will be under strain and will consequently be less stable than membrane bilayers that exhibit less curvature.

1.3.2 Model Membranes

Information regarding the kinetics and mechanisms of membrane fusion has been gleaned from systems utilizing lipid vesicles, also known as liposomes. Liposomes can be separated into distinct classes bases on their sizes and morphologies. Small Unilamellar Vesicles (SUVs) have diameters <50nm, Large Unilamellar Vesicles (LUVs)have diameters between 50nm and 200nm, and both SUVs and LUVs contain a single lipid bilayer (Wilschut et al., 1991).

The relative simplicity of artificial membranes compared to biological

membranes and the ease with which their intrinsic properties can be manipulated to allow for fusion with respect to specific membrane characteristics. Nuclear magnetic resonance (Taraschi et al., 1982; Dorovska-Taran et al., 1996), electron spin resonance, fluorescence spectroscopy, differential scanning calorimetry and electron microscopy (Oku et al., 1982) are some of the techniques used to study aspects of membranes that relate to fusion. Due to the energetics
(Wilschut et al., 1991) of bilayer curvature and the enhancement of the potential barrier for vesicle - vesicle approach (Wilschut et al., 1991), larger vesicles have slower fusion rates than small vesicles that have the same lipid composition. Depending on the composition of the vesicles SUVs have been shown to fuse spontaneously (Wilschut et al., 1991). The fusion of SUVs composed of either zwitterionic or neutral phospholipids has been shown to occur very slowly with the fusion products of only the zwitterionic SUVs being stable.

1.3.3 Mechanism of Membrane Fusion

Although the molecular mechanism of membrane fusion is still a matter of contention there are several events in the mechanism of viral fusion that must occur (Durell et al., 1997). The viral and target membranes must undergo aggregation or adhesion. This close approach and apposition of the two

membranes would require the removal of structured water (partial dehydration) acting as a barrier to membrane contact. Secondly, since the membranes do not fuse spontaneously there must be destabilization of both lipid bilayers in the region where contact and fusion will occur. Mixing of aqueous contents marks the completion of the fusion event.

There are two general models for the mechanism of membrane fusion. These models are distinguished by their potential fusion intermediates. Fusion is thought to occur via metastable intermediates (Durell et al., 1997) because the energetic requirements of a single step fusion event would have an activation energy that would be too high for fusion to be feasible. The more widely accepted model for membrane fusion purports that fusion proceeds via stalk intermediate structures (Chernomordik et al., 1996) that are cylindrically symmetric about the axis normal to the two apposed membranes undergoing fusion (Durell et al., 1997). Both viral fusion proteins and host proteins have roles in mediating the formation of these intermediates. Proteins are thought to destabilize the membrane locally and also provide the scaffold for the formation of hemifusion and stalk intermediates.

Influenza virus fusion is a paradigm for lipid - enveloped viral fusion. Studies have shown that the fusion initiation complex involves at least three haemagglutinin trimers and an unknown number of viral receptors on the host

surface. Whether such protein complexes facilitate hemifusion, characterized by mixing of only the outer lipid monolayers, fusion pore formation, or both remains to be determined.

1.4 Proteoliposome Reconstitutions

1.4.1 Random Proteoliposome Reconstitution

The incorporation of integral membrane proteins into liposomes can be accomplished by detergent dialysis. The conditions of the reconstitution procedures determine the type of liposome that will be formed, the degree of protein incorporation into the proteoliposomes and the sizes of the liposomes (Rigaud et al., 1995). Detergent dialysis is can be used to prepare multilamellar vesicles and large unilamellar vesicles although detergent dialysis can also be used to prepare SUVs under a certain set of conditions. The most obvious drawback of this method is the heterogeneity in the types and sizes of liposomes that are generated (in both the presence and absence of protein). Relatively wide distributions of vesicle sizes are often encountered. The formation of small amounts of MLVs may also disrupt LUV and SUV preparations. Since protein and lipid both contribute to the formation of vesicles from small mixed lipid detergent aggregates in which protein is oriented randomly, the liposomes formed from these small mixed aggregates consequently contain randomly oriented protein. This, however, does not preclude the possibility that the

proteins have a propensity to orient non - randomly within the mixed lipid protein aggregates. It also does not preclude the possibility that there exists a driving force that orients the protein vectorially in the larger lipid - protein aggregates or in the proteoliposomes while they are being formed by the aggregates.

1.4.2 Vectorial Incorporation of Glycophorin A into Preformed Liposomes

The functional reconstitution of many proteins into liposomes requires that the proteins be oriented asymmetrically. Membrane bound enzymes, for example, often function in only one direction across the membrane (Anderson et al., 1983; Ivashchuk-Kienbaum, 1996). Cell surface blood group determinants and receptors such as glycophorin A (which serves both functions) are also unidirectionally oriented in biological membranes. The asymmetric reconstitution of protein into liposomes requires destabilization of the liposomal membrane by a detergent in order for the protein to be inserted (Lichtenberg, 1985; Jain et al., 1987). Since membrane proteins are often insoluble in aqueous media it is often necessary to solubilize the proteins with detergent before addition to detergent destabilized liposomes. The efficiency of protein incorporation and the degree of their asymmetry depend on factors including the type of detergent used, the rate of detergent removal, and the state of vesicle solubilization at the time of protein addition (Rigaud et al., 1995).

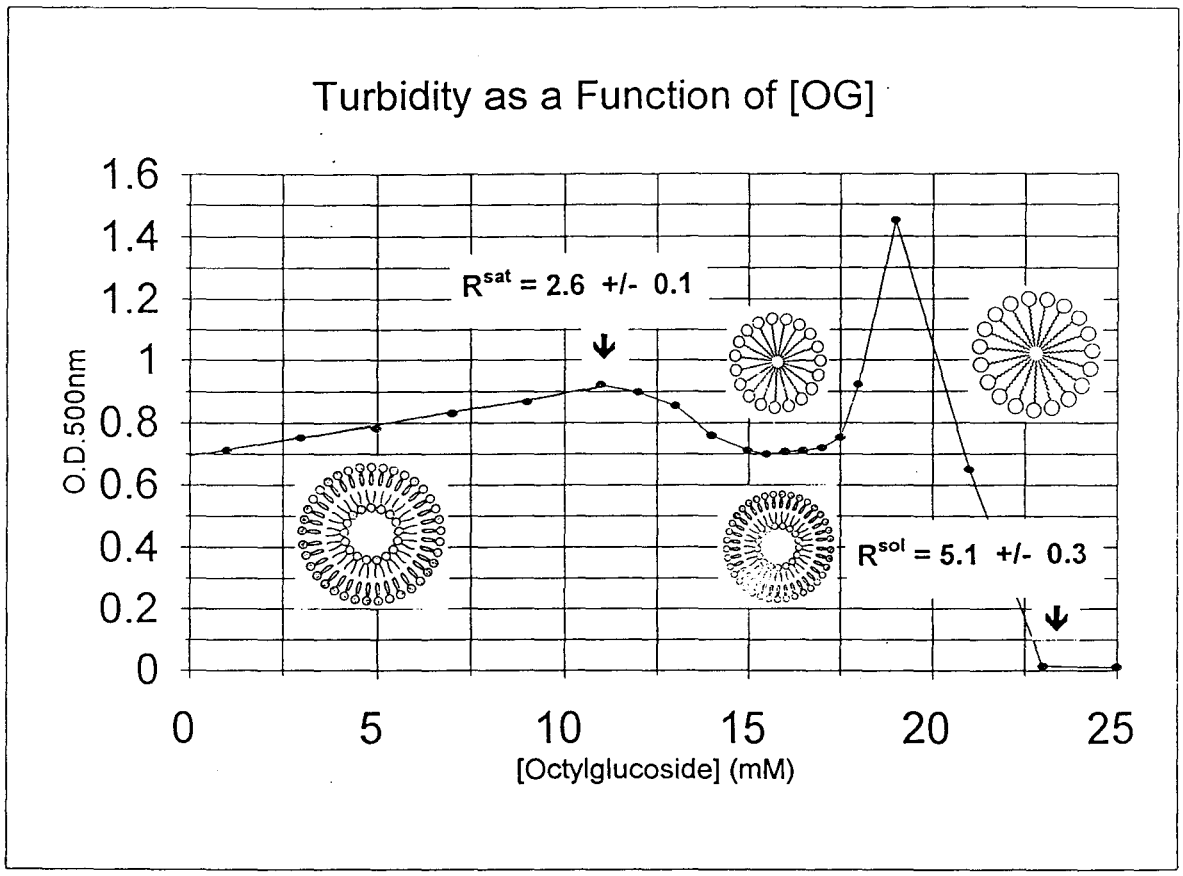
The mechanism by which vectorial insertion occurs has not been

elucidated. It is thought that proteins insert into the membrane with their most hydrophobic part first (Rigaud et al., 1995). For single transmembrane glycoproteins such as glycophorin A this should result in the large glycosylated ectodomain being oriented toward the outside of the liposome. For other proteins with multiple transmembrane spanning regions the insertion process is likely more complicated since the membrane disruption needed to insert such a large molecule would require an unfeasibly large activation energy.

1.4.3 Factors Affecting Proteoliposome Characteristics

The solubilization state of the liposomes when the protein is added during the reconstitution varies with the detergent that is used to solubilize the liposomes (Rigaud et al., 1995). Characterization of the solubilization process yields important parameters characterizing the lamellar to micellar phase transition occurring upon increase in the detergent concentration (Rigaud et al., 1995; Lichtenberg et al., 1983). In order to incorporate protein into preformed liposomes without causing a decrease in the sizes of the liposomes when the detergent is added to perturb the vesicle bilayer the protein must be added to vesicles at the onset of the lamellar to micellar phase transition (Lichtenberg et al., 1983; Levy et al., 1990; Paternostre et al., 1988; Rigaud et al., 1988). This parameter differs for each detergent and is determined through solubilization

studies (R^{sat}_{eff}). The parameter, R^{sol}_{eff}, characterized the completion of vesicle solublization. These parameters are derived from the lipid and detergent concentrations at the points in vesicle solubilization illustrated in figure 1.4.3.



A typical solubilization profile illustrating the transition of lamellar lipid vesicles to mixed lipid/detergent micelles. The effective detergent to lipid ratios within the vesicles, at the onset of solubilization (R^{sat}_{eff}) and at the completion of solubilization (R^{sol}_{eff}), are derived from lipid and detergent concentrations at the points indicated in the figure. The solubilization profile seen here is for one lipid concentration only. But this type of solubilization by the detergent, octylglucoside, is carried out at lipid concentrations between 0mM and 10mM Egg phosphatidylcholine.

The effective removal of detergent from the lipid - detergent - protein mixture relies on the free detergent monomer concentration as well as the method of detergent removal (Lichtenberg et al., 1983; Paternostre et al., 1995; Opatowski et al., 1997). Dialysis is able to remove the detergent in the monomeric form. Detergents with high critical micelle concentrations are present in the unaggregated monomeric form and are thus more easily removed by dialysis.

Hydrophobic adsorption by the use of Bio - Beads SM - 2 facilitates faster and more complete removal of detergent. These beads are composed of a hydrophobic co–polymer (styrene and divinylbenzene) to which the acyl chains of the free monomeric detergent in solution strongly adsorb. The beads facilitate the active removal of detergent compared to the passive removal that occurs by dialysis. This is desirable since the presence of residual detergent would contribute to less stable vesicles. Faster removal of detergent has also been correlated with higher degrees of protein incorporation.

The sizes of proteoliposomes are also affected by the concentration of Bio-Beads SM - 2. This effect has been documented in the literature (Levy et al., 1990). It is therefore necessary to determine the concentration of Bio-Beads SM - 2 that does not significantly affect the initial vesicle size but also results in efficient protein incorporation.

1.5 Sendai Virus

1.5.1 General

Sendai virus is a negative stranded RNA virus that belongs to the Paramyxovirus family. It is a lipid - enveloped virus that is pleomorphic with an average diameter of 150nm - 200nm and it exhibits a tropism for the respiratory tracts of mice. Entry of Sendai virus into target cells necessitates, first, the attachment of the virus to the target membrane which is followed by fusion of the viral envelope with the cell membrane. This allows entry of the virion core into the cytoplasm where protein uncoating and subsequent viral replication occur.

1.5.2 Proteins Mediating Sendai Virus Fusion

The attachment and entry of Sendai virus is mediated by two viral glycoproteins: haemagglutinin - neuraminidase (HN) which binds the receptors and the fusion protein (F) which causes fusion (Scheid et al., 1977; Hsu et al., 1981). Sialic acid containing receptors on the surfaces of the target cells are recognized by HN. The sialoglycoprotein, glycophorin A, isolated from human erythrocyte plasma membranes and the ganglioside, GD_{1a}, act as receptors for Sendai virus in reconstituted model membranes. The asialoglyprotein receptor

(which is expressed at higher levels in hepatocytes) also acts as a receptor for Sendai virus (Geffen et al., 1992). HN also facilitates the release of progeny virions from infected cells. It desialylates the cell surface after initial budding, precluding the binding of nascent virions to the cells from which they were released.

1.6 Steric Interference with Access to the Liposomes Membrane Caused by Membrane - Linked Polymers

1.6.1 Role of Membrane-Linked Biopolymers in Stabilizing Liposomes

It has been found that incorporation of polyethyleneglycol linked phospholipids (notably PEG - PEs with molecular weights between 2000 and 5000) into liposomes dramatically increases the half - lives of liposomes circulating in the blood (Blume et al., 1990). This extended circulation resulting from PEG - PE incorporation has been shown to occur regardless of membrane fluidity and surface charge (Blume et al., 1990; Lasic et al., 1991; Blume et al., 1993). It is believed that surface grafted PEG inhibits opsonization. Studies have shown that serum protein adsorption to liposomes decreases in the presence of surface grafted PEG (Du et al., 1997) suggesting that surface grafted PEG sterically interferes with the binding of serum proteins to liposomes. However, this steric effect has not been demonstrated directly.

1.6.2 Mechanism of Steric Stabilization

It has been postulated that the mechanism by which polyethyleneglycol-grafted liposomes exhibit inhibition of opsonization is through steric interference with access to the membrane surface (Lasic et al., 1991; Blume et al., 1993). When PEG - PE is incorporated into liposomes at concentrations of 5 - 10 mol% the mechanical properties of the bilayer are not affected (Needham et al., 1992). Encapsulation and permeability studies (Blume et al., 1993) have shown decreased leakage of contents from PEG - PE stabilized liposomes. This group also showed decreased adsorption of plasma components to these stabilized liposomes and demonstrated longer half - lives in vivo. Needham et al. (1992) used X - ray diffraction in combination with micropipette manipulation to show that PEG - PE did not change the structural or elastic properties of phospholipid/cholesterol liposomes (Needham et al., 1992). X - ray diffraction was also used to measure the structural organization of PEG^{1900} - PE and the interbilayer repulsion of liposomes containing 4 mol% PEG - PE. The PEG^{1900} - PE polymer was shown to extend about 50 angstroms from the surface (~43 polymer units) giving rise to a strong repulsive pressure opposing the close approach of membranes (Needham et al., 1992). This would slow the lipid exchange between adjacent vesicles that would contribute to the destabilization

of the liposomal membrane integrity.

1.6.3 Characteristics of PEG - PE, LPG and Glycophorin A

Polymers anchored to the membrane that contain large hydrophilic headgroups having the capacity to block the surface of liposomes also have the potential to exert a steric effect on access to the membrane surface (Woodle et al., 1992). PEG - linked phosphatidylethanolamine polymers contain hydrophilic (polar, but not ionic) headgroups of different chain lengths depending on the degree of oxyethylene polymerization. Depending on the concentration of PEG - PE in the membrane the PEG headgroups adopt different conformations (Du et al., 1997). The "mushroom" conformation occurs at low PEG - PE concentrations where the PEG headgroups assume a relaxed state that spreads over the membrane surface. The "brush" conformation occurs at higher PEG - PE concentrations where the PEG headgroups assume a densely packed extended conformation. PEG - PE covers and blocks access to the membrane in both the "mushroom" and "brush" conformations although high PEG - PE concentrations weaken bilayer packing because of the lateral repulsion produced by PEG chains (Tirosh et al., 1998). However, the ability of PEG - PE to cover the membrane surface at concentrations below the range where the "mushroom" to "brush" transition occurs (cloud point) varies with the

amount of "mushroom" state PEG - PE incorporated in the membrane.

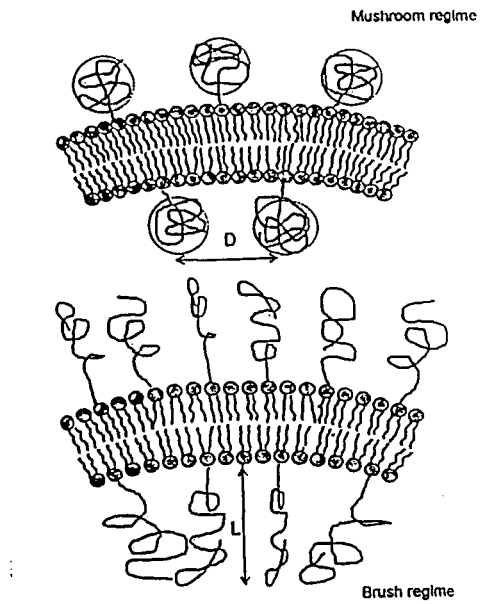


Figure 1.6.3 A schematic representation of PEG-grafted bilayers with PEG in the "mushroom" and "brush" conformations. The figure is adapted from Tirosh et al.(1988).

The lipophosphoglycan (LPG) isolated from *Leishmania donovani*, a protozoan parasite, has been shown to be a potent inhibitor of viral fusion human erythrocyte ghosts (Miao et al., 1995). It has been speculated that this inhibition of fusion may arise, at least partially , from a "steric effect " caused by LPG on the surface of target membranes (Miao et al., 1995). The natural role of LPG in the plasma membrane of *Leishmania* parasites may rely on a similar steric effect in its resistance to host degradative vacuolar enzymes upon phagocytosis. It has been shown that LPG masks recognition of the *Leishmania donovani* promastigote surface from human Kala - azar serum (Karp et al., 1991). LPG forms a dense glycocalyx on the membrane surface (Pimenta et al., 1991). Conceivably, this glycocalyx could form a steric barrier that abrogates recognition by host antibodies.

Glycophorin A is part of the dense glycocalyx covering the surface of human erythrocytes. It has been postulated that the high density of negatively charged glycoconjugates covering the surface of erythrocytes serves a functional role in preventing the aggregation of erythrocytes through electrostatic repulsion (Donath, 1978; Donath et al., 1979; Donath et al., 1980). The large glycosylated ectodomain of glycophorin A may serve an additional role in forming a steric barrier limiting access to the erythrocyte membrane.

1.6.4 Inhibition of Access to Liposomal Membranes by Membrane - Linked Polymers

Membrane - linked polymers such as glycophorin A, PEG^{5000} - PE and LPG that partially or completely cover the surfaces of liposomes in which they have been incorporated may block access of fluorescent probes to the liposomal membrane. The basis for this type of study is a detectable shift or increase of probe fluorescence upon access to the membrane. Depending on the type of probe that is used access to the hydrophobic membrane interior or, alternatively, access to the membrane surface will cause detectable changes in fluorescence. Theoretically, the presence of membrane - linked polymers could present a steric barrier that blocks access to the membrane (interior and surface).

The fluorescent probe, Nile Red, exhibits minimal fluorescence in aqueous environments (Greenspan et al., 1985). But in hydrophobic environments there is a marked increase in fluorescence. The excitation and emission maxima undergo blue shifts to shorter wavelengths in less polar environments

(Greenspan et al., 1985). Addition of Nile Red to a suspension of liposomes should be characterized by an observable blue shifted increase in fluorescence intensity as the probe enters the membrane. It would be expected that inhibition of Nile Red access to the membrane by membrane - linked polymers would be

manifest as slower increases in fluorescence intensity (Figure 1.6.4.1). If this is the case then inhibition of Nile Red access to the membrane interior, and the consequent inhibition of fluorescence increases, should display a membrane - linked polymer concentration dependent behaviour.

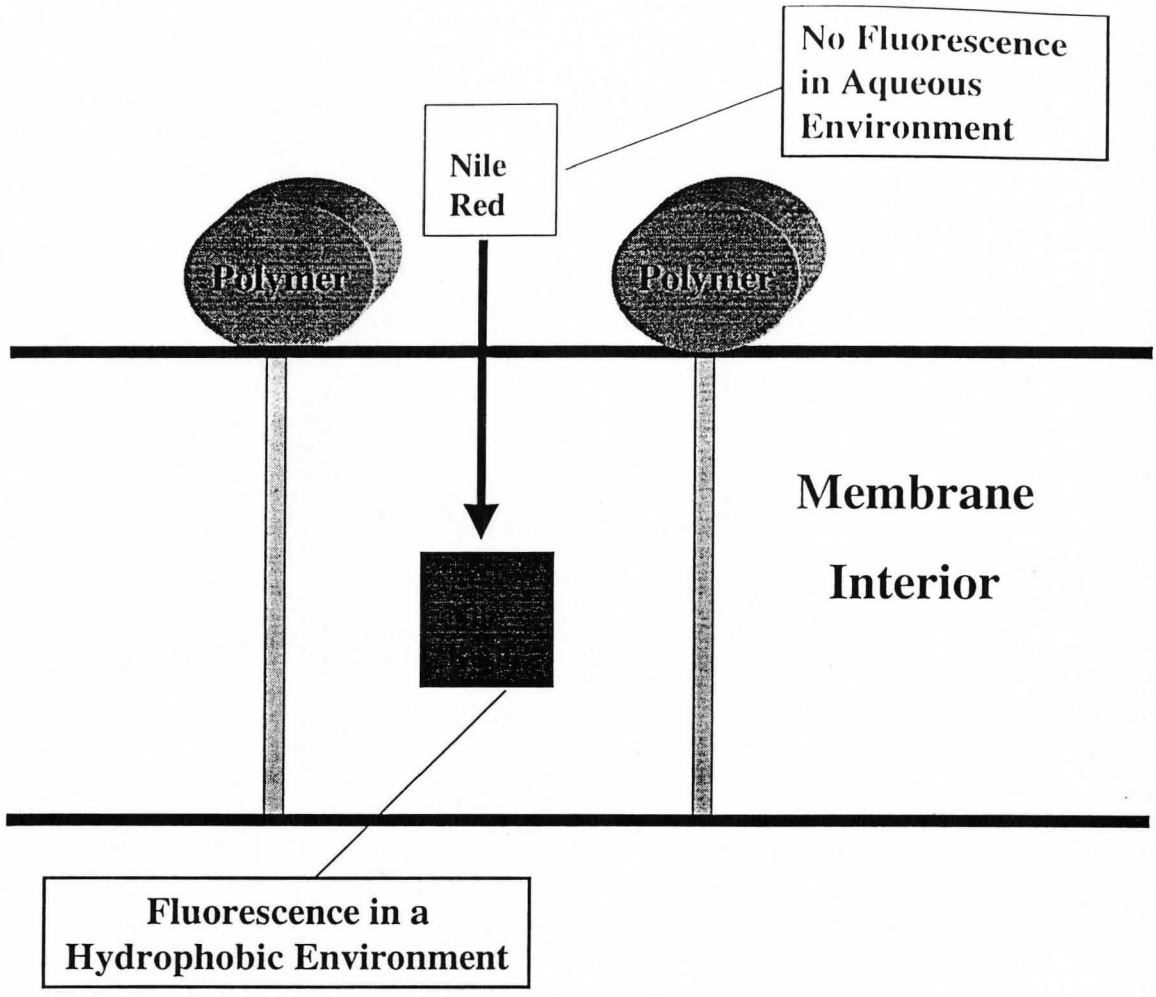


Figure 1.6.4.1 A diagram representing the scheme for the Nile Red assay of steric interference. The probe, Nile Red, fluoresces upon entering the liposomal membrane provided that the steric hindrance from the incorporated polymer does not block its access to the membrane interior. The membrane-anchored biopolymers depicted in the diagram represent either glycophorin A or LPG or PEG^{5000}-PE.

NeutrAvidin Oregon Green is a fluorescent labeled probe that has excitation and emission maxima at 496nm and 524nm, respectively (Hangland, 1992). Alone, this probe exhibits fluorescence in aqueous environments. But when it binds biotin there is an increase in fluorescence. This increase in fluorescence arises from a release of probe self - quenching caused by displacement of amino acids that interact with the fluorophores conjugated to avidin (Hangland, 1992). Lipid covalently conjugated to biotin can be incorporated into liposomal membranes. The increase in fluorescence that occurs upon NeutrAvidin Oregon Green binding to biotin - conjugated lipid is dependent on the probe's access to the membrane surface. Steric interference at the lipid/water interface would decrease the probe's access to the liposomal membrane surface and decrease the probe's access to lipid conjugated biotin (Figure 1.6.4.2). As a consequence, lower fluorescence increases should occur. Surface - grafted PEG has previously been shown to retard the binding of avidin to biotinylated lipids in the liposomal membrane (Noppl-Simson et al., 1996). But the concentration dependence remains to be demonstrated. Similar inhibition by membrane - linked polymers other than PEG - PE would demonstrate that this was not only a PEG - PE related phenomenon and would support the hypothesis that the longevity of polyethyleneglycol-grafted liposomes, *in vivo*, is due largely to steric stabilization.

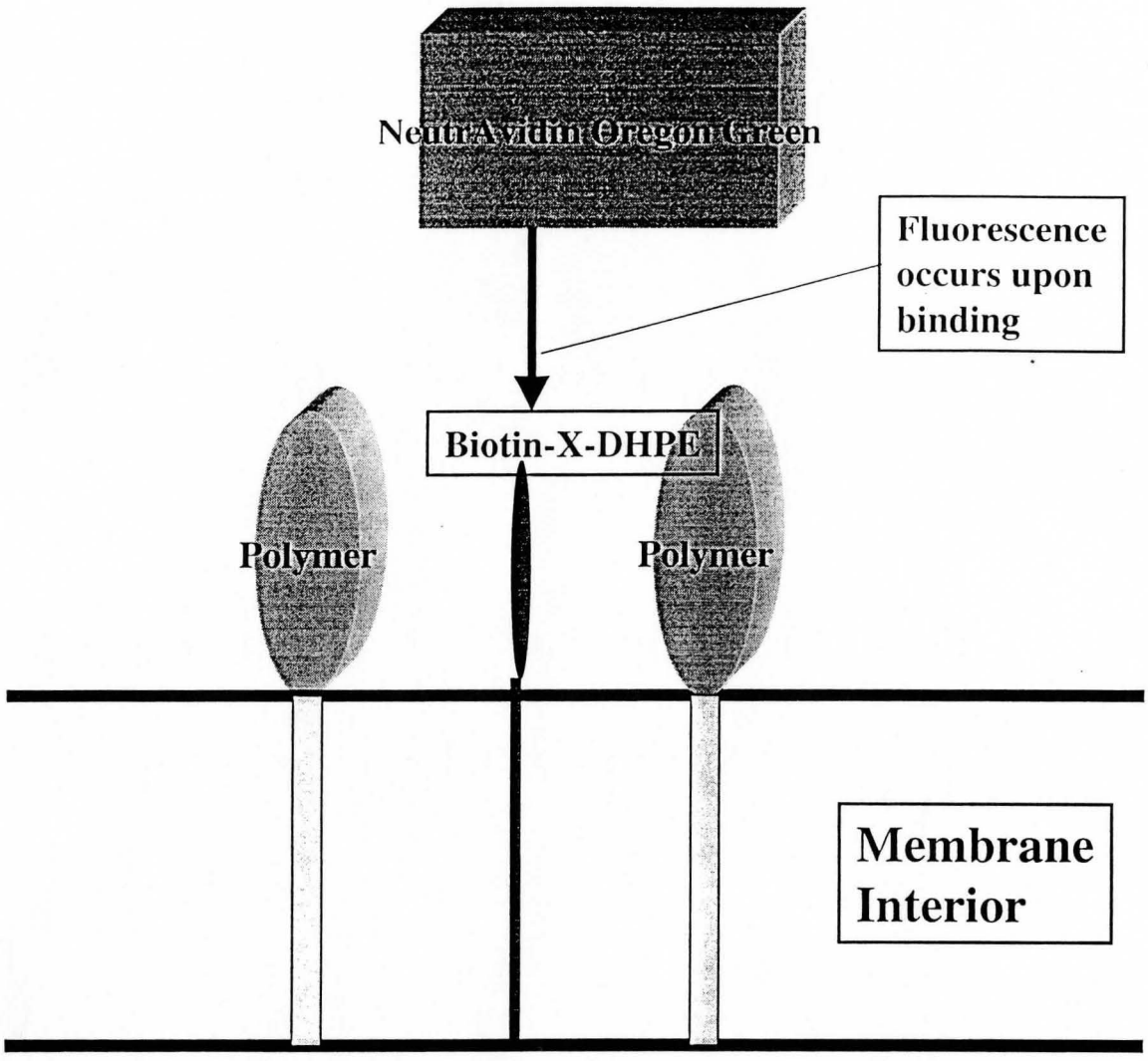


Figure 1.6.4.2 A diagram representing the scheme for the NeutrAvidin/Biotin assay for steric interference. The probe, NeutrAvidin Oregon Green, fluoresces upon binding biotin-X-DHPE, a biotin derivatized lipid incorporated into the liposomes. The membrane-anchored biopolymers (glycophorin A or LPG or PEG^{5000}-PE) sterically inhibit NeutrAvidin-biotin binding and, in this way, also inhibit the resulting fluorescence.

1.7 Objectives

The objectives of the research included and reviewed in this thesis shifted focus twice due to unexpected findings. Briefly, the initial objectives at the start of my research were three – fold. First, the membrane – anchored glycoprotein, glycophorin A, was to be isolated from human erythrocytes. Second, glycophorin A was to be incorporated into liposomes vectorially where it would act as receptor for Sendai Virus. Third, fusion of the vectorially reconstituted proteoliposomes with Sendai Virus was to be compared to fusion with randomly reconstituted proteoliposomes.

The fusion results were unexpected with fusion of the vectorially reconstituted liposomes being lower than with the randomly reconstituted liposomes. This finding warranted investigation. It was not clear whether this difference could be attributed to the detergents used in the two reconstitutions or a difference in some other factor. Experiments were designed to determine if the difference in fusion was related to the type of glycophorin A dimer present in liposomes formed using the two detergents (octylglucoside and CHAPS).

The last research done for this thesis involved a shift away from fusion related liposome work to work related to the steric stabilization of liposomes. The goal of this end work was the establishment of an assay that would demonstrate a steric effect, common to membrane – anchored biopolymers

2.0 Experimental

2.1 Materials

Materials	Source
Egg Phosphatidylcholine	Avanti Polar Lipids
PEG^{5000}-PE	Avanti Polar Lipids
Lipophosphoglycan	Gift of S.Turco
Ammonium Molybydate	BDH
Ascorbic Acid	Sigma
Chloroform	Fisher
Citric Acid	BDH
HEPES	Sigma
HCl	BDH
Magnesium Nitrate	BDH
MES	Sigma
Potassium Phosphate (Monobasic)	Fisher
Resorcinol	Sigma
Sodium Carbonate	Fisher
Sodium Citrate	BDH
Sodium Dodecyl Sulfate	Sigma
Sodium Phosphate (Monobasic)	Fisher
Sulfuric Acid	BDH
Triton X -100	Bio - Rad
Proteinase K (*Tritarachium album*)	Worthington Enzymes
O - Glycosidase (*Diplococcus pneumoniae*)	Boehringer Mannheim
Sodium Azide	BDH
EDTA	BDH
Glycine	BDH
Outdated Human Blood	Canadian Red Cross
PMSF	Sigma
Dialysis Tubing (MW Cutoff 12K - 14K)	
Acrylamide	Gibco BRL

Tris	Boehringer Mannheim
Ammonium Persulfate	Gibco BRL
TEMED	Gibco BRL
n-octyl-β-D-glucopyranoside	Sigma
CHAPS	Sigma
Sendai Virus	Collaborator
Acetone	Fisher
n - butanol	Fisher
n - butylacetate	Sigma
Octadecylrhodamine	Molecular Probes
Nile Red	Molecular Probes
NeutrAvidin Oregon Green	Molecular Probes
Biotin - X - DHPE	Molecular Probes
0.1μm Polycarbonate Filters	Nucleopore
0.2μm Polycarbonate Filters	Nucleopore
0.4μm Polycarbonate Filters	Nucleopore
Bio - Beads SM - 2	Bio - Rad
Sephadex G - 75	Pharmacia
Sephadex G - 150	Pharmacia
Sepharose 2B - 300	Sigma
Neuraminidase (Vibrio Cholerae)	Sigma
Bio - Gel A1.5	Bio - Rad
Lithium Diiodosalicylate	Sigma
Phenol	Fisher
WGA	Sigma
Cyanogen Bromide	Sigma
N - acetylglucosamine	Sigma
BCA Assay Kit	Pierce
Ammonyx-Lo (Lauryldimethlamine Oxide, 30% Solution)	Calbiochem

2.2 Isolation of Glycophorin A from Human Erythrocytes

2.2.1 Isolation of Lyophilized Erythrocyte Ghosts

Lyophilized erythrocyte ghosts were prepared form concentrated human red blood cells obtained from the Canadian Red Cross Society. Membrane glycoproteins were removed from ghost membranes using the LIS/Phenol (LIS - Lithium diiodosalicylate) extraction method previously performed (Marchesi et al., 1971). Other proteins were removed by differential centrifugation (Furthmayr et al., 1983). Glycophorin A was separated from glycophorin B and glycophorin C with a Bio - gel A1.5m gel filtration column using buffer containing the detergent Ammonyx - Lo (0.1% Ammonyx - Lo, 25mM NaCl, 5mM sodium phosphate, pH8.0, 0.01% sodium azide). The detergent was removed by extensive dialysis against double distilled water and residual lipid was removed by chloroform:methanol (2:1, v/v) extraction. The pure glycophorin A product was stored a -20°C in its lyophilized powder form.

2.2.2 Conjugation of WGA to Sepharose 2B - 300

The plant lectin Wheat Germ Agglutinin (WGA) was conjugated to Sepharose 2B - 300 beads as described by (Kahane et al., 1976). A 5ml volume of washed Sepharose beads was suspended in 5ml 0.2M $NaHCO_3$. The activated beads were mixed with a WGA solution (9.33mg/5ml 0.2M $NaHCO_3$) and shaken at 4°C overnight. Free lectin was removed by washing with 100ml 0.2M $NaHCO_3$ followed by 33ml 0.1M glycine. The WGA conjugated beads were

then incubated in phosphate buffer (0.25M NaCl, 0.025% sodium azide, 0.015M sodium phosphate, pH7.2) at 4°C for 20 hours. Finally, the beads were washed with cold phosphate buffer.

2.2.3 Verification of Glycophorin A Presence by Affinity Chromatography by WGA - Sepharose 2B - 300 Gel Filtration

Chromatographic separation of glycophorin A was carried out according to the procedure of Kahane et al. (1976). The column was washed with sodium dodecyl sulfate/phosphate buffer (0.05% SDS, 0.25mM Na Cl, 0.025% sodium azide, 0.015M sodium phosphate, pH7.2) and elution of bound glycophorin A was done with 0.1M N -acetyl - D - glucosamine in SDS/phosphate buffer. The elution was monitored by the absorbance of fractions at 280nm.

2.2.4 SDS - Polyacrylamide Gel Electrophoresis

Samples were prepared in 0.01M phosphate buffer, pH7.0 and subjected to electrophoresis on 7% polyacrylamide gels with 0.1M phosphate running buffer, pH7.0. Gels were prestained with Alcian blue following periodate oxidation (Moller et al., 1995) and were then Silver – stained according to the procedure used by Bruins et al. (1995).

2.3 Vectorial Incorporation of Glycophorin A into Preformed Liposomes

2.3.1 Preparation of Liposomes by Extrusion

Large Unilamellar Vesicles (LUVs) were formed by freeze thawing a suspension of Egg phosphatidylcholine in HEPES/MES Buffer (5mM HEPES, 5mM MES, 5mM sodium citrate, 150mM NaCl, 1mM EDTA, pH7.4) and then extruding the resulting lipid suspension through polycarbonate filters the various pore sizes (100nm, 200nm, 400nm). The lipid suspension was subjected to five freeze thaw cycles and then passed through the filters ten times to achieve a narrow distribution of vesicle sizes.

2.3.2 Detergent Solubilization of Liposomes

Detergent solubilization studies of Egg PC vesicles using octylglucoside (n-octyl-β-D-glucopyranoside) and CHAPS (3-[(3Cholamidopropyl)dimethylammonio]-1-propanesulfonate) were carried out to determine the parameter characterizing the onset of vesicle solublization according to methods previously used (Lichtenberg et al., 1983; Paternostre et al., 1988; Rigaud et al., 1988; Eytan, 1982). The solubilization profiles of Egg PC vesicles, ranging in concentration between 0mM and 10mM, were determined by monitoring the turbidity of vesicle suspensions following incremental additions of octylglucoside. Turbidity measurements, taken as the

Optical Density at 500nm, were recorded five minutes after each addition of octylglucoside. The detergent concentration at which the onset of vesicle solubilization occurred, for each respective lipid concentration, was taken as the concentration at which the turbidity first started to decrease.

2.3.3 Reconstitution Procedure

The reconstitution of glycophorin A into preformed liposomes was carried out similarly to other procedures (Jain et al., 1987 ; Rigaud et al., 1995; Levy et al., 1990; Paternostre et al., 1988; Rigaud et al., 1988). Glycophorin A was added to an amount of detergent, octylglucoside, determined to bring about the onset of vesicle solubilization. The total volume of the initial vesicle - detergent - glycophorin A suspension is 1ml with an initial lipid to protein mole ratio of 465/1. Addition of glycophorin A is followed by 20 minutes of stirring at room temperature. Removal of octylglucoside is accomplished by batchwise addition of 20mg of Bio - Beads SM - 2 to the initial suspension followed by 3 hours of stirring at room temperature and then another 20mg Bio - Bead addition followed by 2 hours stirring. The Bio - Beads are allowed to sediment to the bottom of the tubes. The suspension of vesicles then transferred to a dialysis sack and dialyzed overnight against 500ml of 20mg/ml Bio - Beads SM - 2 in HEPES/MES buffer, pH7.4 to complete detergent removal. In order to remove unincorporated glycophorin A the dialyzed vesicles are passed through a Sepharose CL 2B gel

filtration column and collected in the void volume.

2.4 Random Incorporation of Glycophorin A into Liposomes Using Detergent Dialysis

2.4.1 Incorporation Using CHAPS

The random incorporation of glycophorin A into liposomes was carried out according the method used by Wybenga et al. (1996). The stock glycophorin A solution was prepared by dissolving roughly 2mg glycophorin A in HEPES/MES buffer, pH7.4 to a concentration of 8mg/ml and then storing it in small silanized tubes. The glycophorin A solution and HEPES/MES buffer containing 25mM CHAPS were added to dried Egg PC films to give a final volume of 1ml. The lipid - protein - detergent mixture was transferred to at 0.5 - 3 ml dialysis cassette with a MW cutoff of 10000 (Pierce). The sample was dialyzed against five changes of HEPES/MES buffer, pH7.4 over a period of 48 hours to remove the detergent. The unincorporated glycophorin A was removed from the resulting proteoliposome suspension by gel filtration chromatography on a Sepharose CL2B - 300 matrix equilibrated with HEPES/MES buffer, pH7.4.

The proteoliposomes were collected in the void volume.

2.4.2 Incorporation Using Octylglucoside

The same procedure that was used for the random CHAPS incorporation was used with octylglucoside. The concentration of octylglucoside used at the start of the reconstitution procedure was 1% octylglucoside. The same amount of lipid and protein were used in order to compare the two methods.

2.5 Fusion of Sendai Virus with Proteoliposomes

2.5.1 Labeling of Sendai Virus with Octadecylrhodamine (R18)

Labeling of Sendai virus with R18 was carried out according to published procedures (Hoekstra et al., 1984). R18 was dissolved in methanol to give a concentration of 1mg/ml. 10ml of 1mg/ml R18 was added to 1ml of Sendai virus warmed to room temperature. The R18/Sendai virus mixture was incubated at room temperature light protected with aluminum foil for a period of 1 hour. The unincorporated R18 was removed by gel filtration chromatography by passing the R18/Sendai virus mix through a Sephadex G - 75 column equilibrated with HEPES/MES buffer, pH7.4. The virus was collected in the void volume. Virus containing fractions were pooled and stored in silanized glass tubes. The viral protein concentration was determined using the BCA assay (Pierce Chemical Co.). 50 µl of 0.5% SDS was added to each of the samples and standards to

disrupt the viral envelope and to correct for any interference caused by addition of 0.5% SDS.

2.5.2 R18 (Octadecylrhodamine) Lipid Mixing Assay

The R18 assay was carried out according to the procedure of Wybenga et al. (1996). LUVs were added to an appropriate amount of HEPES/MES buffer, pH7.4 to bring the final lipid assay concentration to 50 μM in a final volume of 2ml. The LUVs were brought to 37°C by pre - incubation in a thermostated cuvette holder for approximately 10 minutes. The background fluorescence of the LUVs was recorded for a period of 60 seconds at which point the recording was paused. The fusion assay was initiated by addition of 5 μg of R18 labeled Sendai virus. The fusion assays were carried out in silanized cuvettes in a thermostated cuvette holder at 37°C with continual stirring. The fluorescence was recorded using a SLM AMINCO Bowman Series 2 Luminescence Spectrometer using a Xenon arc light source. Excitation and emission monochromators were set at 565nm and 600nm respectively. A 560 filter was placed between the excitation slit and the sample and a 590nm filter was placed between the sample and the photomultiplier tube in order to minimize the contribution of light scattering. F_o is the fluorescence intensity measured upon addition of 20 μl of 10% Triton X - 100 after measuring the final extent

fluorescence. F_t is the fluorescence intensity measured at time, t (t = 10 minutes for kinetics and t = 8 hours for the final extent of fusion)

%R18 Dequenching = $100\ [(F_t - F_o)/(F_{100} - F_o)]$

For each assay the fluorescence intensity was measured for ten minutes from the addition of labeled virus to determine the parameters governing the initial rate of fusion. The cuvettes were then wrapped in foil, shaken at 37°C for 8 hours and the fluorescence measured at this point represents the final extent of fusion. Assays were done in duplicate.

2.5.3 Orientation of Glycophorin A

Reconstituted proteoliposomes were treated with neuraminidase to remove the sialic acid from glycophorin A molecules oriented toward the outside of the vesicles. 1ml of the reconstituted liposomes was treated with 1 unit of neuraminidase from *Clostridium perfringens* for 1 hour at temperature of 37°C. Following treatment with neuraminidase the proteoliposomes were passed through a Sephadex G – 75 column (fractionation range for globular proteins 3KDa – 80KDa) equilibrated with HEPES/MES buffer, pH7.4 in order to remove neuraminidase and enzymatically cleaved, free sialic acid. These proteoliposomes were collected in the void volume. The Resorcinol assay (see 2.5.7) for sialic acid was performed both on the final proteoliposome

preparation as well as on proteoliposomes treated with neuraminidase. The dilution factor was obtained by comparing phosphate concentrations of neuraminidase – treated and untreated proteoliposomes. The % glycophorin A oriented toward the inside (and outside) was obtained by comparing results of the Resorcinol assay of neuraminidase – treated proteoliposomes with those of untreated proteoliposomes.

2.5.4 Lipid to Protein Ratio

Phospholipid concentrations were measured (see section 2.5.6). Glycophorin A concentrations were determined using the Resorcinol assay (see 2.5.7) for sialic acid. Ratios were calculated using the molar concentration of phospholipid and glycophorin A

2.5.5 Determination of Vesicle Size by Quasi-Elastic Light Scattering

Vesicle sizes were determined by QELS using a Brookhaven Model B1 9000AT digital correlator according to the procedure used by Wybenga et al. (1996). HEPES/MES buffer, pH7.4 was filtered using at 0.45μM filter in order to remove dust. LUVs were diluted in diluted in filtered buffer to a concentration of 0.1mg/ml. Scattering was measured at an angle of 90° and at a wavelength of 514nm for 5 minutes. Measurements were repeated at least three times for each sample. Data were analyzed by cumulant analysis and non – negatively

constrained least squares regression using software provided with the instrument.

2.5.6 Phosphate Assay

Phospholipid concentrations were measured using an assay for inorganic phosphate (Ames, 1966). This assay is based on a colorimetric reaction involving the formation of a complex between inorganic phosphate and molybdate. All the tubes used in each individual assay were of the same type in order to minimize variation in the loose borosilicate (glass) between tubes. Furthermore, the test tubes were acid washed in 1N HCl for at least 1 hour and then rinsed in ddH_2O prior to use.

2.5.7 Resorcinol Assay for Sialic Acid

Sialic acid was assayed according to previous methods (Svennerholm, 1957; Wybenga et al., 1996). A standard curve containing up to 20µg of glycophorin A in 1 ml of ddH_2O was prepared. Samples were diluted with ddH_2O to a total volume of 1 ml.

2.5.8 Proteinase K Treatment of Proteoliposomes

Glycophorin A containing proteoliposomes were prepared randomly and

vectorially using both octylglucoside nd CHAPS. A 0.5ml volume of each proteoliposome sample was digested with 5µg proteinase K (34.5 units/mg) for 3 hours at 37°C. The digestion was stopped by placing the samples on ice and by the addition of PMSF (3mM final concentration). Proteinase K (MW27000) and proteolytic fragments were separated from the vesicles using a Sepharose 2B gel filtration column. Vesicle containing fractions were collected, pooled and then lyophilized. The dry samples were dissolved in electrophoresis sample buffer with high SDS content (2X sample buffer - 200µl 12X stacking gel buffer, 400µl 30% SDS, 214µl β-mercaptoethanol, 240µl glycerol, 146µl 0.1% bromophenol blue) and then subjected to SDS-PAGE on 15% polyacrylamide gels. Gels were then silver-stained to visualize undigested membrane bound fragments.

2.5.9 O-glycosidase and Neuraminidase Treatment of Proteoliposomes

Glycophorin A containing proteoliposomes were prepared randomly and vectorially using both octylglucoside and CHAPS. A 0.5ml volume of each proteoliposomes sample was digested with 0.3mg neuraminidase (10units/mg) for 3 hours at 37°C in order to remove sialic acid from the glycan termini. Free sialic acid and neuraminidase were removed by gel filtration using Sepahdex G-75 columns. Vesicle containing fractions were collected and pooled. The

pooled vesicle containing fractions were digested with 3µl O-glycosidase (2.5units/mg) for 24hours at 37°C. Samples were frozen in liquid nitrogen and lyophilized. The dry samples were dissolved in electrophoresis sample buffer with high SDS content (2X sample buffer - 200µl 12X stacking gel buffer, 400µl 30% SDS, 214µl β-mercaptoethanol, 240µl glycerol, 146µl 0.1% bromophenol blue) and then subjected to SDS-PAGE on 15% polyacrylamide gels. Gels were then silver-stained to visualize undigested membrane bound fragments.

2.6 Access of the Fluorescent Probe Nile Red to the Membranes of Polymer-Containing Liposomes

2.6.1 Preparation of PEG^{5000}-PE Containing EggPC Vesicles

Egg phosphatidylcholine was weighed and then dissolved in chloroform - methanol (2:1, v/v). Aliquots corresponding to 2mg were removed and placed into separate acid washed test tubes. Prior to use tubes were acid washed in 1N HCl, rinsed thoroughly with distilled deionized water and dried in an oven at 80°C. PEG^{5000}-PE was weighed and dissolved in chloroform - methanol (2:1,

v/v). Volumes of PEG^{5000}-PE giving 1mol% and 3mol% were added to the 2mg Egg PC aliquots and lipid films were prepared by placing them under a nitrogen stream. Residual organic solvent was removed by drying the lipid films in an evaporator for 1 hour 30min. Films were hydrated with HEPES/MES buffer, pH7.4 and subjected to give freeze/thaw cycles using liquid nitrogen and warm water. The resulting lipid suspension was extruded through two 100nm pore polycarbonate filters ten times. The final lipid concentration was determined using the phosphate assay.

2.6.2 Preparation of LPG Containing EggPC Vesicles

Lipid films of 2mg EggPC were prepared. LPG was dissolved in distilled deionized water and an appropriate amount of LPG was added to the lipid film. This mixture was frozen in liquid nitrogen and then placed in an evaporator for 2 hours to remove water and residual organic solvent. The dried LPG/EggPC mixture was hydrated with 2ml HEPES/MES buffer, pH7.4 and subjected to five freeze/thaw cycles. The resulting lipid suspension was extruded through two 100nm pore polycarbonate filters. The final lipid concentration was determined using the phosphate assay.

2.6.3 Preparation of EggPC Vesicles Containing 5mol% Biotin -X-DHPE and PEG^{5000}-PE

Biotin - X - DHPE obtained from Molecular Probes was dissolved in chloroform - methanol (2:1, v/v) to give a concentration of 2.5mg/ml. PEG^{5000}-PE was also dissolved in chloroform - methanol were added to 2mg EggPC aliquots (also dissolved in chloroform - methanol). Films were prepared by drying samples under a constant nitrogen stream. Films were dried in an evaporator for 1 1/2 hours, hydrated and freeze thawed five times in HEPES/MES buffer, pH7.4 and extruded through two 100nm pore polycarbonate filters ten times. Final lipid concentrations were determined by phosphate analysis.

2.6.4 Preparation of EggPC Vesicles Containing 5mol% Biotin-X-DHPE and LPG

Biotin - X - DHPE dissolved in chloroform - methanol (2.5mg/ml) was added to 2 mg Egg PC in chloroform - methanol and then dried under a constant nitrogen stream. Since it does not dissolve in methanol LPG was dissolved in distilled deionized water and then added to the lipid film. This was frozen in liquid nitrogen and the dried in an evaporator for 2 hours. The dry LPG/lipid was hydrated with 2ml HEPES/MES buffer, pH7.4 subjected to five freeze thaw cycles and then extruded through two 100nm pore polycarbonate filters ten

times. Final lipid concentrations were determined by phosphate analysis.

2.6.5 Determination of Nile Red Excitation and Emission Wavelengths in Various Environments

The fluorescent probe, Nile Red, was dissolved in either acetone similar to Greenspan et al. (1985) or in methanol such that the final concentration was 0.1mg/ml. Nile Red was added to samples with the total assay volume reaching 2ml and the final Nile Red concentration reaching 5μM. Excitation and emission scans were done in 4ml plastic cuvettes with an SLM AMINCO Bowman Series 2 Spectrofluorimeter. Excitation scans were carried out between 360nm and 600nm. Emission scans were carried out between 560nm and 700nm. All scans were made with a 0.1 second resolution and the sensitivity adjusted such that the maximum fluorescence intensity was approximately 60% of the full scale. The excitation and emission wavelengths (λ_{ex} and λ_{em}) were taken as the peak Fluorescence intensities of the excitation and emission scans, respectively. The excitation and emission wavelengths of Nile Red were determined separately in the following samples: (1) HEPES/MES buffer, pH7.4, (2) EggPC Vesicles, (3) 1mol%PEG^{5000}-PE/EggPC Vesicles, (4) 1mol% LPG/EggPC Vesicles, (5) 3mol% PEG^{5000}-PE/.EggPC Vesicles, (6) 3mol% LPG/EggPC Vesicles

2.6.6 Assay for Nile Red Access to Liposome Membranes in the Presence or Absence of Biopolymers that Cover the Surface

Time traces monitoring the fluorescence of Nile Red were made in an SLM AMINCO Bowman Series 2 Spectrofluorimeter. Excitation wavelengths, emission wavelengths and sensitivities were set to values determined for each particular sample. Samples were brought to 25°C in thermostated cuvette holders prior to initiating the assay. Measurements were recorded for 60 seconds prior to Nile Red addition in order to obtain a baseline fluorescence. The assay was initiated after approximately 60 seconds by injecting Nile Red through a light sealed septum using a Hamilton syringe. The sample was maintained at 25°C using a thermostated cuvette holder and sample homogeneity was maintained using constant magnetic stirring.

2.6.7 Variation of Light Scattering by 100nm EggPC Vesicles with Changes in Lipid Concentration

Apparent fluorescence intensities/light scattering were measured over a range of lipid concentrations: 0μM, 10μM, 25μM, 50μM, 100μM, 300μM, 500μM, 1000μM. The lipid vesicle suspensions were kept homogeneous by constant stirring. Excitation and emission monochromators were set at 496nm and 524nm, respectively. The sensitivity of the instrument was set at 745V and

the slit widths of both monochromators at 8nm. In order to minimize the contribution of light scattering a 420nm filter was placed between the light source and the sample and a 490nm filter was placed between the sample and the photomultiplier tube.

2.6.8 Variation of Background Fluorescence by 100nm EggPC Vesicles with Changes in Sensitivity

The contribution from the background fluorescence of 50μM lipid vesicles was measured using a range of Sensitivities: 704V - 930V. The sample was subjected to constant magnetic stirring. Excitation and the emission monochromators were set to 496nm and 524nm, respectively. The slit widths for both monochromators were set at 8nm. A 420nm filter was placed between the light source and the sample and a 490nm filter was placed between the sample and the photomultiplier tube. This was done in order to minimize light scattering.

2.6.9 Variation of NeutrAvidin Oregon Green Fluorescence with Changes in Concentration and Changes in Sensitivity

An amount of 1mg NeutrAvidin Oregon Green powder (Molecular Probes) was dissolved in 1ml HEPES/MES buffer, pH7.4 to give a 1mg/ml stock solution.

Dilutions of 0.1mg/ml, 0.01mg/ml and 0.001mg/ml were prepared by diluting the stock solution with HEPES/MES buffer, pH7.4. The fluorescence intensities were measured at various sensitivities for the 0.1mg/ml, 0.01mg/ml and 0.001mg/ml dilutions of NeutrAvidin Oregon Green. Excitation and emission monochromators were set at 496nm and 524nm, respectively. A 420nm filter was placed between the light source and the sample and a 490nm filter was placed between the sample and the photomultiplier tube. This was done in order to minimize light scattering.

2.6.10Assay for NeutrAvidin Oregon Green Access to the Surface of EggPC Liposomes Containing Biotinylated PE in the Presence or Absence of Biopolymers that Cover the Surface

Excitation and emission monochromators were set at 496nm and 524nm, respectively. A 420nm filter was placed between the light source and the sample and a 490nm filter was placed between the sample and the photomultiplier tube in order to minimize light scattering. The instrument sensitivity was set at 590V. 10µl of NeutrAvidin Oregon Green was added to an appropriate amount of buffer and the fluorescence was recorded for 60 seconds in order to determine the background probe fluorescence prior to the binding of lipid conjugated biotin to the liposome surfaces. The assay was initiated, after

recording the background probe fluorescence for approximately 60 seconds, by the addition of the vesicles through a light sealed septum with a Hamilton syringe. The volume of vesicles added gave a final lipid concentration of 50μM in a total assay volume of 2ml. The assay reaction was subjected to constant magnetic stirring in a thermostated cuvette holder at 25°C. Fluorescence measurements were recorded with a resolution of 0.1second for a duration of 200 seconds.

3.0 Results

3.1 Isolation of Glycophorin A from Human Erythrocytes

The isolation of glycophorin A has been accomplished by several methods. The two methods that give the highest yield of protein and at the same time produce high degrees of purity are characterized either by the use of sodium deoxycholate (Segrest et al., 1979) or by the use of lithium diiodosalicylate (Furthmayr et al., 1983; Marchesi et al., 1971). Glycophorin A is a transmembrane glycoprotein. It contains both hydrophobic and hydrophilic domains (Blanchard 1990; Brosig et al., 1998). The presence of both hydrophobic and hydrophilic domains within the same protein makes it difficult to solubilize such proteins. Detergents such as SDS can be used to release glycoprotein from membrane fragments because of their amphiphilic nature. However, detergents are difficult to remove. Salts such as sodium deoxycholate and LIS are water soluble, but at the same time exhibit a certain amount of hydrophobic character (Marchesi et al., 1971). The LIS method of isolating Glycophorin A was chosen because it was shown to give glycophorin yields almost twice as high as any other method (Segrest et al., 1979). It was reported that the main drawback of using LIS rather than sodium deoxycholate was a

higher level of LIS contamination that sodium deoxycholate contamination in the initial glycophorin preparation containing all three glycophorins (Segrest et al., 1979). But the procedure used to separate glycophorin A from glycophorin B and C was shown to remove LIS
(Furthmayr et al., 1983).

Various glycophorin A preparations were run side by side to verify the presence of glycophorin A in our preparation (Figure 3.1.1). The commercial preparation from Sigma Chemical Co. as well as a preparation from a collaborator (Dr.Sharom) were compared with two samples of our own preparation. Prestained molecular Weight markers (New England Bio-Labs) were used in order to identify the bands corresponding to the glycophorin A monomers and dimers. The gels were prestained with Alcian Blue according to the procedure of Moller et al. (1995) and then silver – stained. In the pre – staining procedure secondary alcohols of glycosyl residues are oxidized to dialdehydes which react with the dye, Alcian Blue. The dye binds silver ions or silver complexes during the sliver staining procedure resulting in a staining sensitivity similar to the silver staining of proteins that are not glycosylated.

Since glycophorin A has been shown to bind to Wheat Germ Agglutinin (WGA) samples of our glycophorin A preparation were passed through a WGA – Sepharose column and then eluted with N – acetyl - D – glucosamine. The elution profile (Results not shown) exhibited a single large peak. The peak fractions were pooled and dialyzed to remove SDS used to solublilize the protein, lyophilized to concentrate the protein, redissolved in water and then subjected to electrophoresis (Figure 3.1.2). Figure 3.1.1 shows two strong bands in the lane corresponding to purified glycophorin A (lane 7). The lower band corresponds to glycophorin A in its monomeric form and the strong upper band corresponds to the dimeric form of glycophorin A (Furthmayr et al., 1983). The bands corresponding to the monomeric and dimeric forms of glycophorin A were present for all the samples with apparent molecular weights of just greater than 47500 and 83000 respectively. A third band migrating a distance intermediate to the 25000 and 32500 molecular weight markers, corresponding to glycophorin B, is also visible in these lanes. Glycophorin C is known to migrate a distance slightly further than glycophorin A and can barely be distinguished slightly below the distance migrated by the 47500 molecular weight marker. One group has reported that the apparent molecular weights of glycophorin A, glycophorin B and glycophorin C are 37000, 24000 and 35000 respectively. However, the molecular weights of these glycoproteins vary considerably with conditions used

in electrophoresis and may differ considerably from their actual molecular weights. This is due to the presence of bulky oligosaccharide side chains containing negatively charged sialic acid moieties at the glycan termini. The actual molecular weight of the glycophorin A monomer based on its amino acid and carbohydrate composition is approximately 31000 with variations due to the heterogeneity in the glycans. Similar variations would be expected for the other glycophorins.

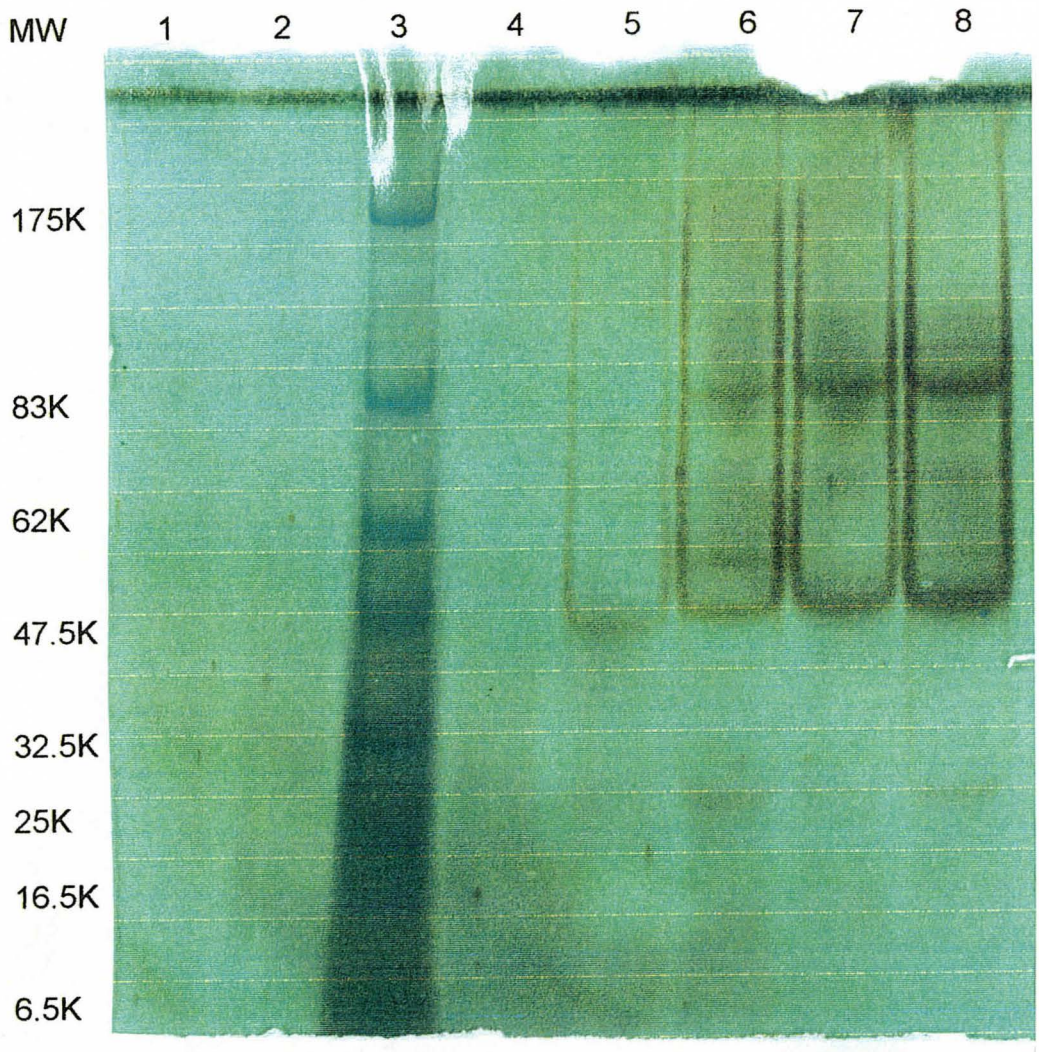


Figure 3.1.1

7.5% SDS-PAGE of Glycophorin A Samples after the Initial Isolation Procedure. Prestained with Alcian Blue following Periodate Oxidation and then Silver-Stained. Lane 1, Empty. Lane2, Empty. Lane 3, MW Markers. Lane 4, Empty. Lane 5, Sigma Glycophorin A. Lane 6, Glycophorin A Isolated by Dr. Sharom. Lane 7, Final Glycophorin A Isolated. Lane 8, Glycophorin Prior to Gel Filtration. Lanes 9 and 10, Empty.

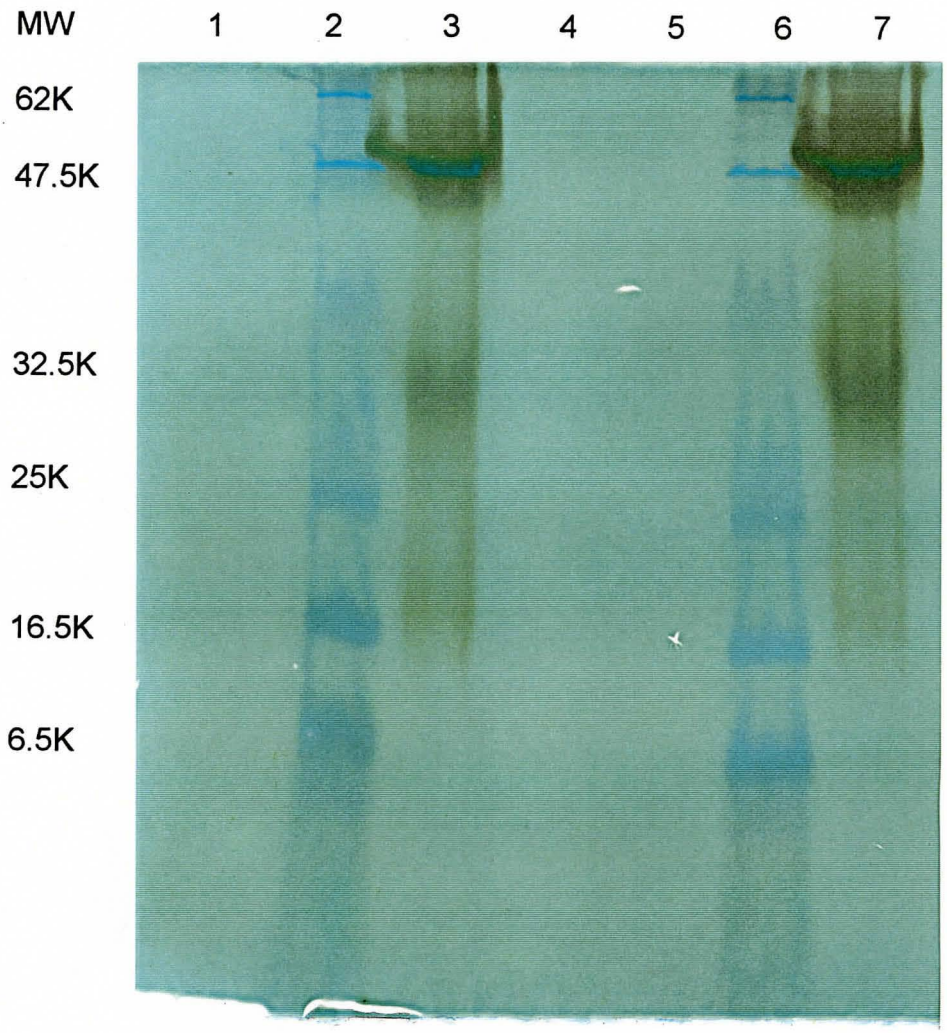


Figure 3.1.2

7.5% SDS-PAGE of Purified Glycophorin A subjected to Affinity Chromatography on a WGA-Sepharose 2B Lectin Column. Prestained with Alcian Blue following Periodate Oxidation and then Silver-Stained. Lanes 2 and 6, MW Markers. Lanes 3 and 7, Glycophorin A. All other lanes are Empty

3.2 Detergent Solubilization of Liposomes and Vectorial Reconstitutions

The solubilization studies performed with Egg PC vesicle yielded several important parameters which were used in the vectorial reconstitution of glycophorin A into these vesicles (Table 1): R^{sat}_{eff} and R^{sol}_{eff}. These are the effective detergent to lipid ratios in detergent saturated vesicles at the onset of the lamellar to micellar transition (R^{sat}_{eff}) and in mixed micelles at the completion of the lamellar to micellar transition (R^{sol}_{eff}). Values for the partition coefficient of octylglucoside and CHAPS (K) between Egg PC and the aqueous phase and for the aqueous monomeric detergent concentrations (D_w) were also determined from these studies and in particular from the phase diagrams, figures 3.2.2 and 3.2.4 (Rigaud et al., 1995; Lichtenberg et al., 1983; Opatowski et al., 1997).

The results of Egg PC vesicle solubilization with octylglucoside and CHAPS were similar to documented results (Lichtenberg et al., 1983; Levy et al., 1990; Paternostre et al., 1988; Rigaud et al., 1988). Octylglucoside solubilization profiles showed the four expected phases: first, a gradual initial increase in Optical Density; second, a decrease in O.D.; third, a large increase in O.D.; fourth, a large decrease in O.D. with nearly a complete loss of turbidity. The CHAPS solubilization profiles (Figures 3.2.4 A to F) exhibited a different pattern than the octylglucoside solubilization profiles (Figures 3.2.2 A to G): first,

a gradual increase in O.D.; second, a sudden decrease in O.D. ending in almost a complete loss of turbidity. The initial turbidity increased with increases in lipid concentration for the solubilizations using both octylglucoside and CHAPS. The observed changes in O.D. reflect the phase changes undergone by the vesicles in the transition from the lamellar state to the micellar state where all the lipid and detergent present in the suspension compose micelles. The phase diagrams illustrating the relationships between the detergent concentration and lipid concentration were derived from two critical points in the lamellar to micellar transition: the lipid and detergent concentrations at the point where a decrease in O.D. is first observed to occur and the concentrations where the turbidity first reaches a minimum. The phase transition from the lamellar to micellar vesicle states occurred at much lower concentrations with CHAPS (Figures 3.2.4 A to F) than with octylglucoside (Figures 3.2.2 A to F).

As can be seen in Table 4, the vectorial reconstitution of glycophorin A into EggPC liposomes was achieved with a high degree of asymmetry toward the outside of the vesicles. Values for the percent orientation were obtained using the resorcinol assay (see 2.5.7) and enzymatic treatment with neuraminidase (see 2.5.3). The random incorporation of glycophorin A into EggPC liposomes would be expected to yield approximately half of the incorporated glycophorin A facing the outside of the vesicles.

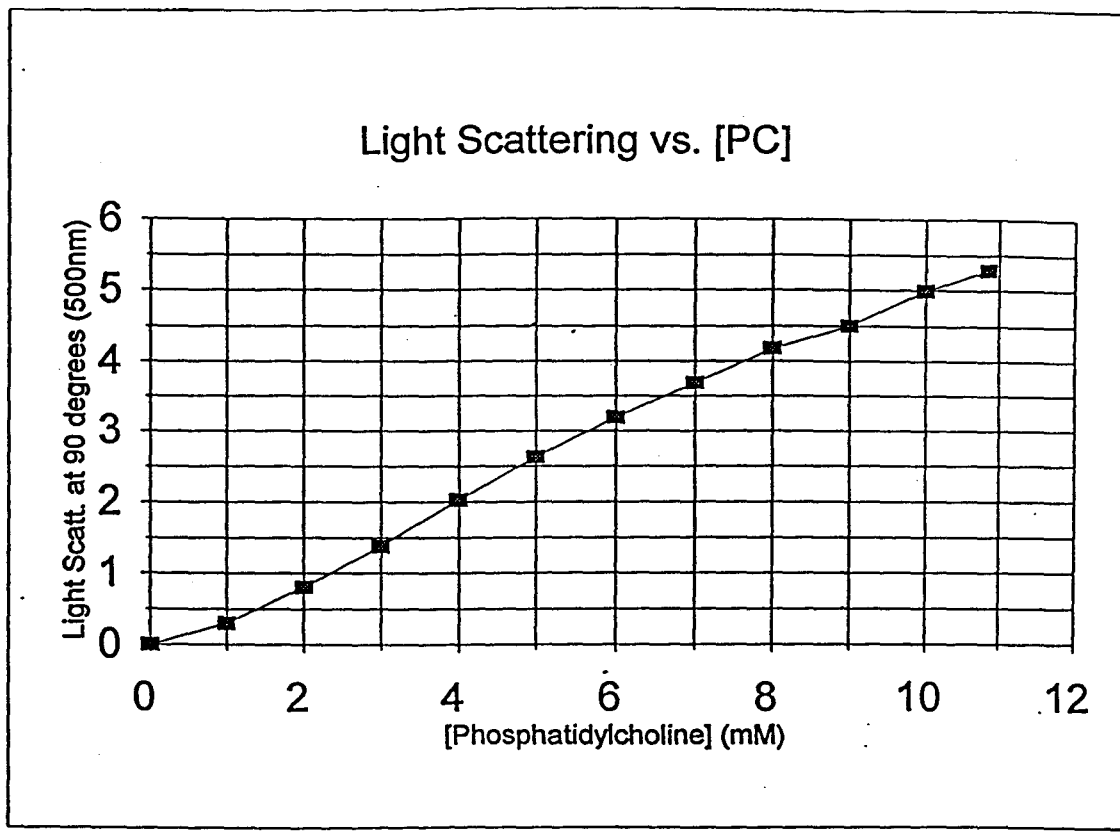
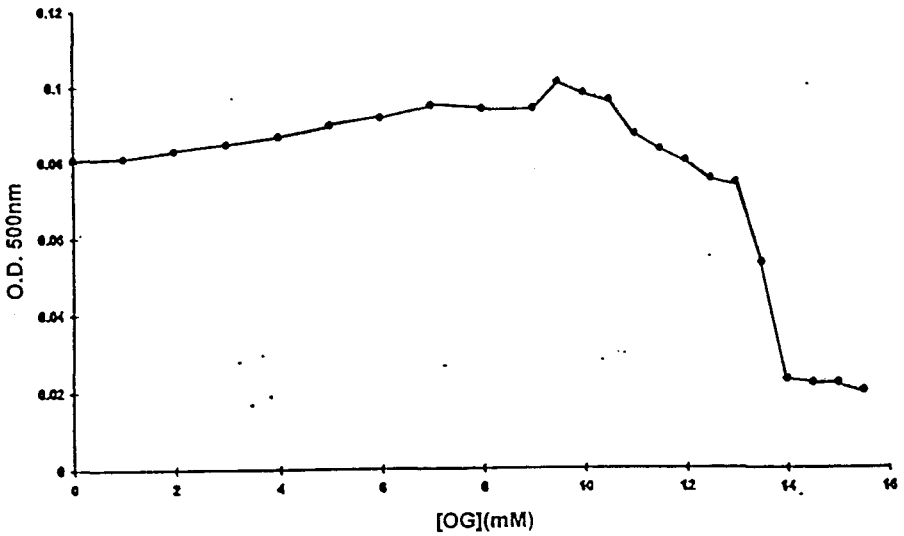


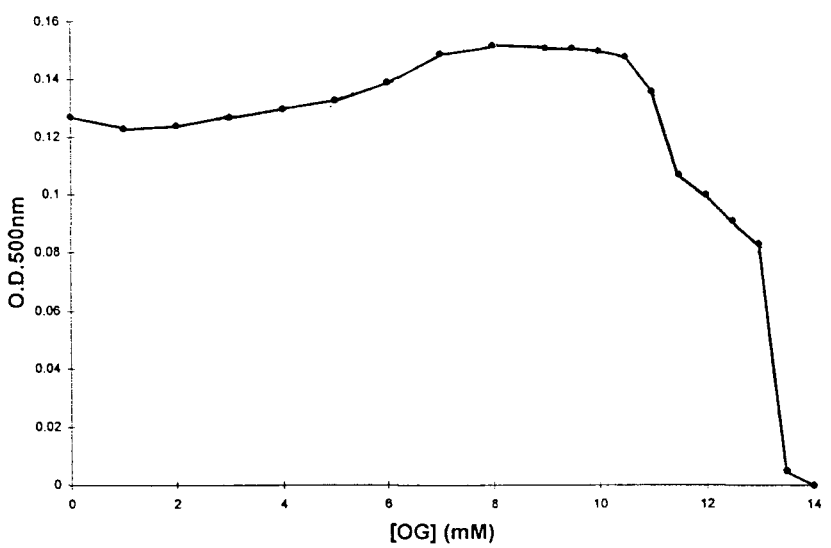
Figure 3.2.1

Right Angle Light Scattering by 200nm Egg phosphatidylcholine vesicles at varying lipid concentrations: between 1mM Egg PC and 11mM Egg PC

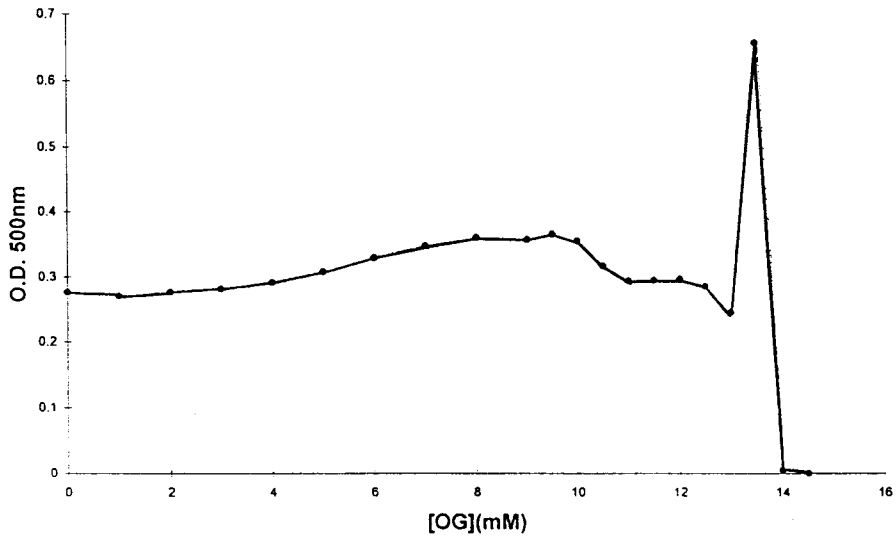
A



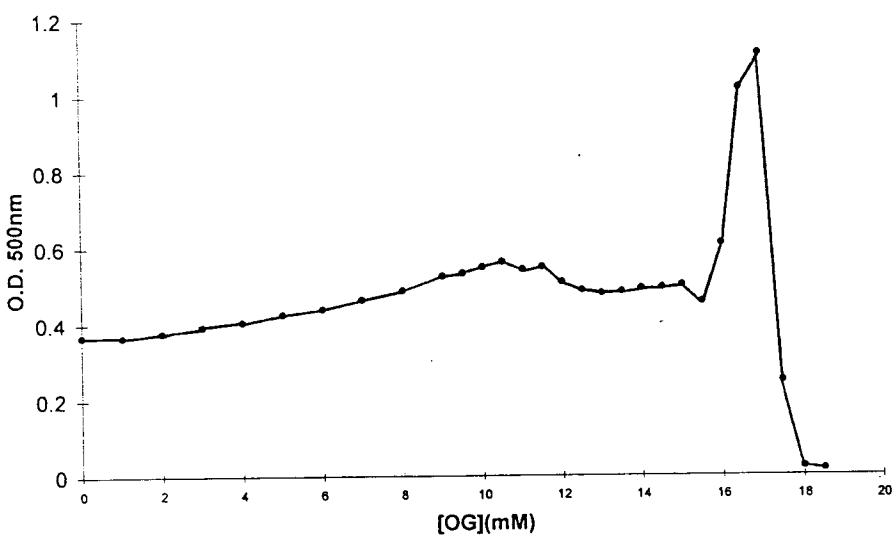
B



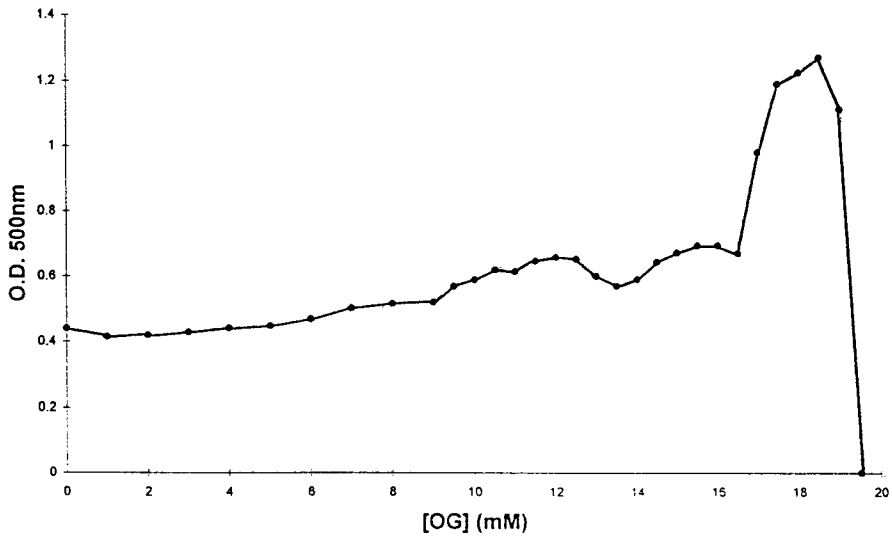
C


O.D. 500nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
2
4
6
8
10
12
14
16
[OG](mM)

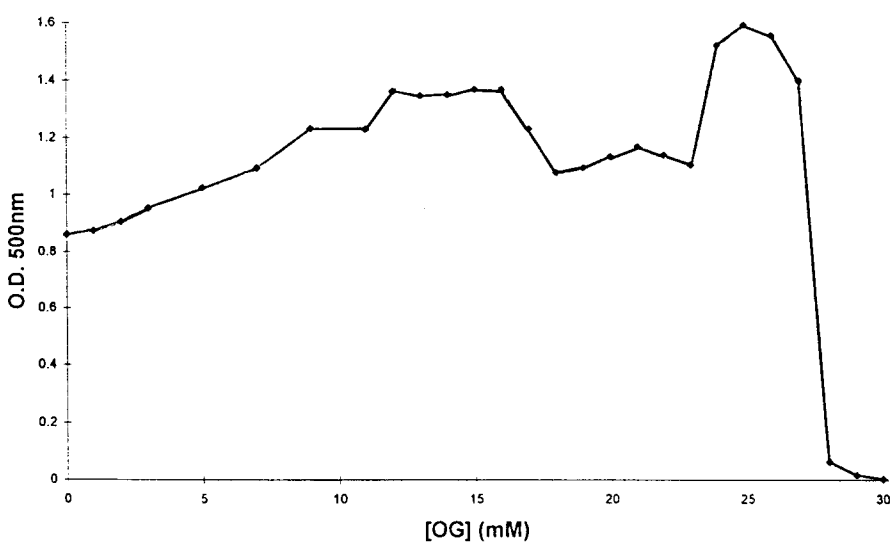
D


O.D. 500nm
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
12
14
16
18
20
[OG](mM)

E


O.D. 500nm
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
12
14
15
18
20
[OG] (mM)

F


O.D. 500nm
1.6
1.4
1.2
1
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0.6
0.4
0.2
0
0
5
10
15
20
25
30
[OG] (mM)

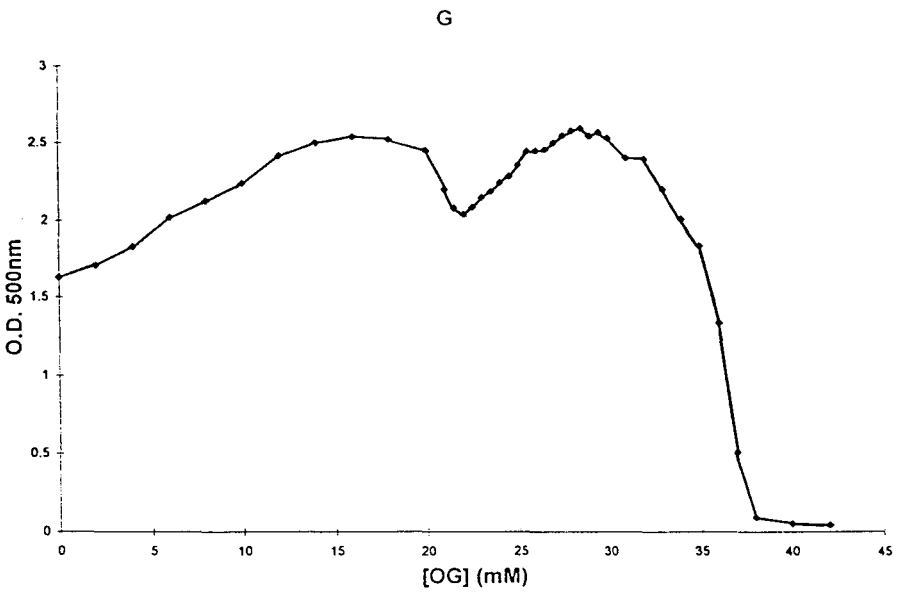


Figure 3.2.2

Solubilization of 200nm Egg PC vesicles with Octylglucoside. Vesicles were prepared by extrusion . (A) 0.5mM Egg PC LUVs (B)1.0mM Egg PC LUVs (C) 2.0mM EGG PC LUVs (D) 2.5mM Egg PC LUVs (E) 3.0mM Egg PC LUVs (F) 5.0mM Egg PC LUVs (G) 10.0mM Egg PC LUVs. At each phospholipid concentration the Octylglucoside concentration was increased by stepwise addition every 10 minutes. Measurements of the Optical Density were recorded immediately prior to Octylglucoside addition.

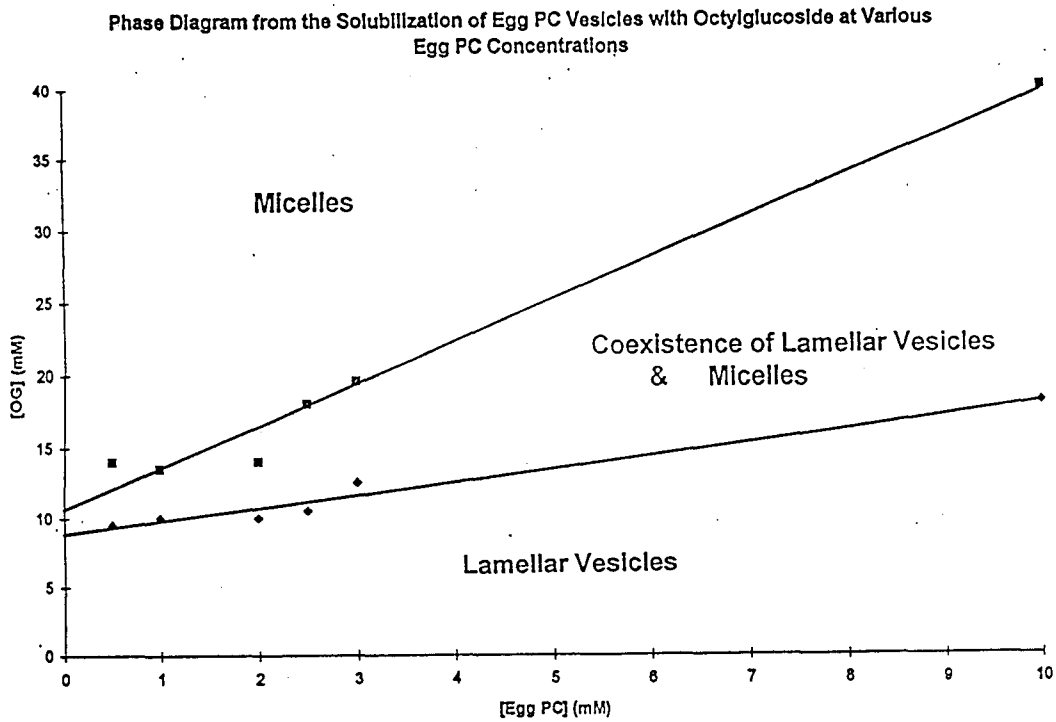
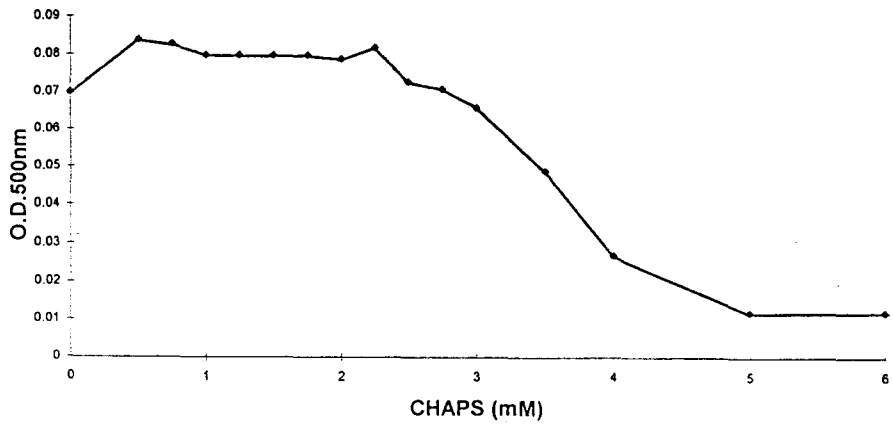


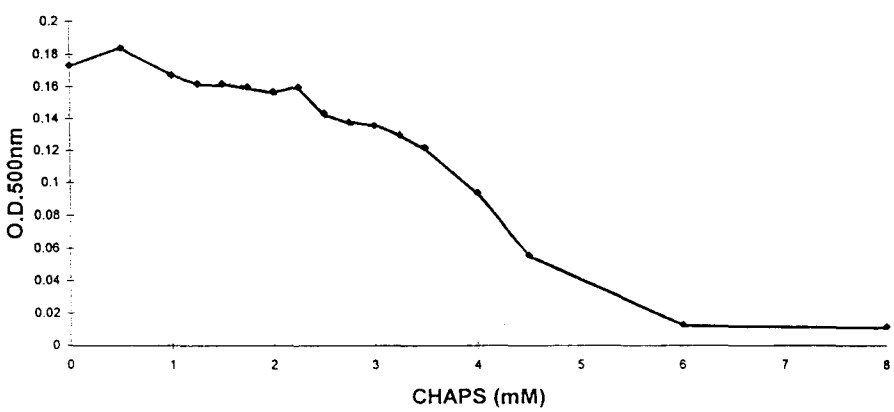
Figure 3.2.3

Phase Diagram derived from the solubilization of Egg PC LUVs with octylglucoside. The Egg PC concentrations corresponding to R^{Sat} and R^{Sol} were chosen from each of the solubilization curves. The lines marking the onset of Lamellar vesicle solubilization (Lower Line:$[OG]_{Total}=R^{Sat}_{eff}[EggPC] + [OG]_{monomeric}$) and the completion of Lamellar Vesicle solubilization (Upper Line: $[OG]_{Total}=R^{Sol}_{eff}[EggPC] + [OG]_{monomeric}$) were calculated by linear regression using Microsoft Excel. The correlation coefficients were 0.97 and 0.98, respectively.

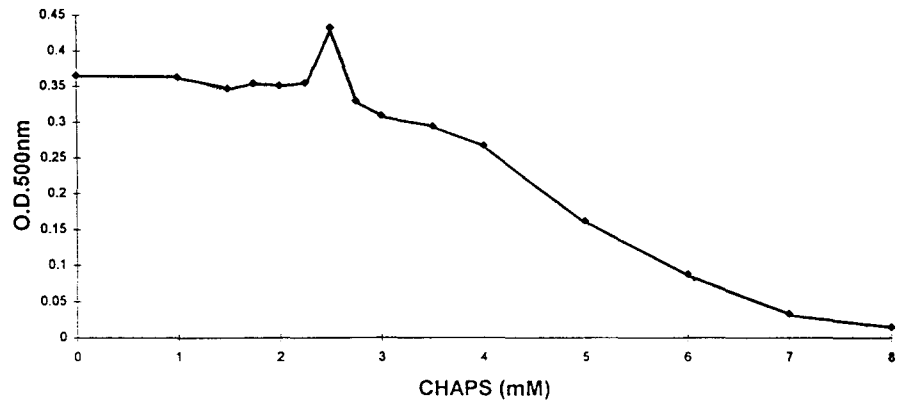
A


O.D.500nm
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
0
1
2
3
4
5
6
CHAPS (mM)

B


O.D.500nm
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
0
1
2
3
4
5
6
7
8
CHAPS (mM)

C


O.D.500nm
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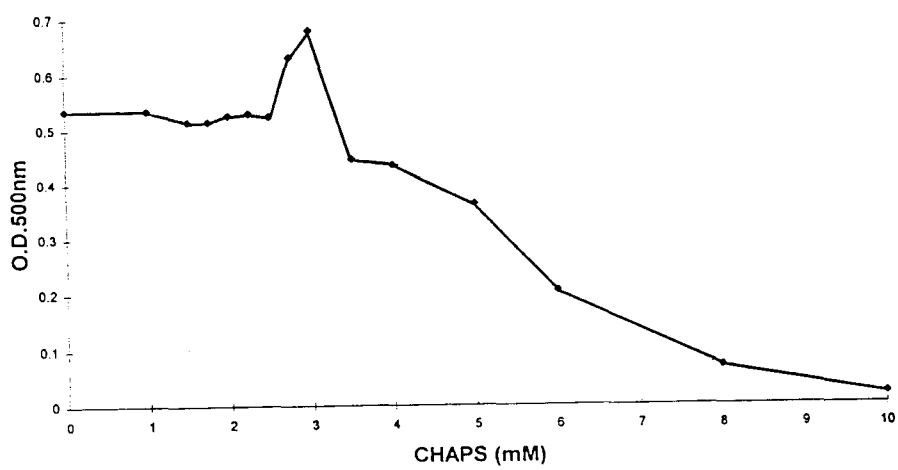
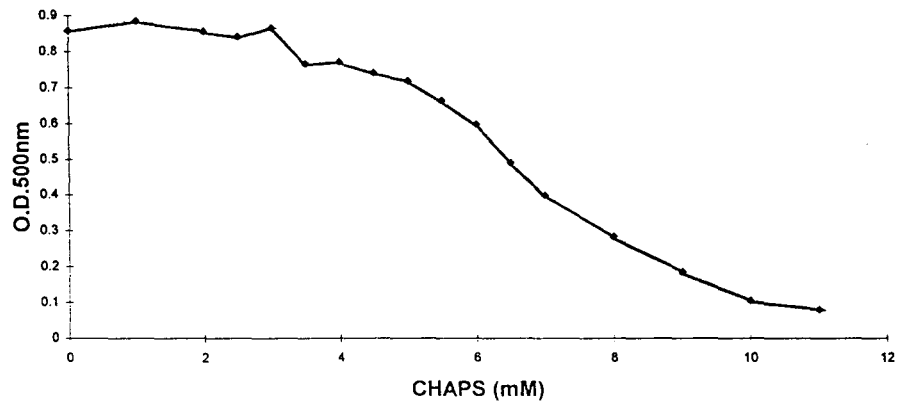
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0
1
2
3
4
5
6
7
8
CHAPS (mM)

D


O.D.500nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
4
5
6
7
8
9
10
CHAPS (mM)

E


O.D.500nm
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
2
4
6
8
10
12
CHAPS (mM)

F

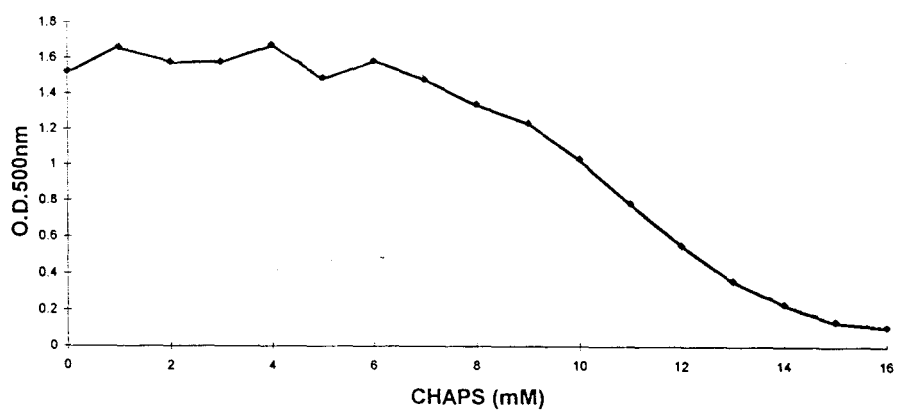

O.D.500nm
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
12
14
16
CHAPS (mM)

Figure 3.2.4
Solubilization of 200nm Egg PC Vesicles with CHAPS. LUVs were prepared by extrusion. (A) 0.5mM EggPC LUVs (B) 1.0mM Egg PC LUVs (C) 2.0mM Egg PC LUVs (D) 3.0mM Egg PC LUVs (E) 5.0mM EggPC LUVs (F) 9.4mM Egg PC LUVs At each phospholipid concentration the CHAPS concentration was increased by stepwise addition every 10 minutes. Measurements of Optical Density were recorded immediately prior to CHAPS addition.

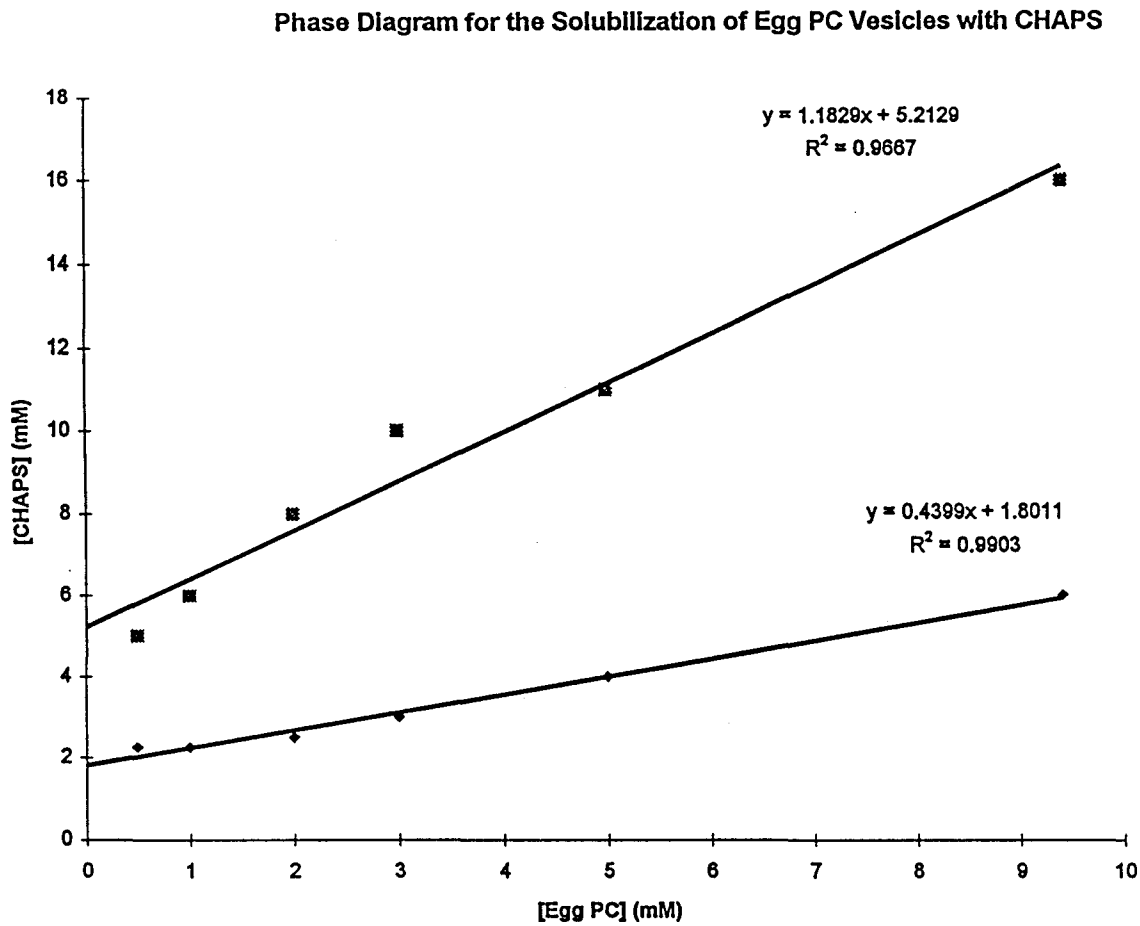


Figure 3.2.5
Phase Diagram derived from the solubilization of Egg PC LUVs with CHAPS. The Egg PC concentrations corresponding to R^{Sat}_{eff} and R^{Sol}_{eff} were chosen from each of the solubilization curves. The lines marking the onset of Lamellar vesicle solubilization (Lower Line:$[OG]_{Total}=R^{Sat}_{eff}[EggPC] + [OG]_{monomeric}$) and the completion of Lamellar Vesicle solubilization (Upper Line: $[OG]_{Total}=R^{Sol}_{eff}[EggPC] + [OG]_{monomeric}$) were calculated by linear regression using Microsoft Excel.

Parameters Derived from the Phase Diagram

Detergent	Phase	R_{eff} (mol/mol)	K (mM^{-1})	Dw (mM)
OG	Sat	0.92	0.10	8.85
OG	Sol	2.90	0.27	10.64
CHAPS	Sat	0.44	0.24	1.80
CHAPS	Sol	1.18	0.23	5.21

Table 1
Parameters Derived from the Phase Diagrams of LUV Solubilization by Octylglucoside and CHAPS. The effective detergent to lipid ratios in the LUV bilayer at the onset of solubilization (R^{sat}_{eff}) and at the completion of solubilization (R^{sol}_{eff}) correspond to the slopes in the phase diagrams. The concentration of detergent monomers present in the aqueous phase (D_w) corresponds to the intercept of the vertical axis. The partition coefficients (K), which represent partitioning of the detergent between the phospholipid vesicles and the aqueous phase in equivalent to the ratio R_{eff}/D_w.

Lipid to Protein Mole Ratios of Proteoliposomes Prepared by Vectorial Incorporation of Glycophorin A		
Initial Vesicle Size (nm)	[Bio-Beads SM-2] (mg/ml)	Final Lipid:Protein
200	20	160mol / 1mol
200	20	1mol / 0mol
200	20	260mol / 1mol
200	20	1mol / 0mol
200	20	443mol / 1mol
200	20	1mol / 0mol
200	20	172mol / 1mol
200	20	1mol / 0mol

Table 2

Lipid to Protein Mole Ratios of Proteoliposomes Prepared by Vectorial Incorporation of Glycophorin A into Egg PC Liposomes. The initial vesicle sizes in the reconstitutions were all 200nm and the Bio – Bead SM – 2 concentration used in all cases was 20mg/ml.

Lipid to Protein Mole Ratios of Proteoliposomes Prepared by Vectorial Incorporation of Glycophorin A		
Initial Vesicle Size (nm)	[Bio-Beads SM-2] (mg/ml)	Final Lipid:Protein
100	20	927mol / 1mol
100	20	1mol / 0mol
100	40	9.4mol / 1mol
100	40	1mol / 0mol
100	60	18.1mol / 1mol
100	60	1mol / 0mol

Table 3

Lipid to Protein Mole Ratios of Proteoliposomes Prepared by Vectorial Incorporation of Glycophorin A into Egg PC Liposomes. The initial vesicle sizes in the reconstitutions were all 100nm and the Bio – Bead SM – 2 concentration varied.

Asymmetry of Glycophorin A in Proteoliposomes Prepared by Vectorial Incorporation into Preformed Liposomes			
Initial Vesicle Size (nm)	[Bio-Beads SM-2] (mg/ml)	Final Lipid:Protein	% Outward Orientation
200	20	260mol / 1mol	95
200	20	1mol / 0mol	N/A
200	20	443mol / 1mol	76
200	20	1mol / 0mol	N/A

Table 4 Asymmetry of glycophorin A in reconstituted proteoliposomes. The resorcinol assay for sialic acid was used to determine the amount of total glycophorin A contained in vectorially reconstituted proteoliposomes. After enzymatic treatment with neuraminidase, to remove the sialic acid attached to glycophorin A molecules oriented toward the outside of the vesicles, the resorcinol assay was used to quantitate the remaining sialylated glycophorin A in the treated proteoliposomes. This value was used to determine the percent of glycophorin A oriented toward the outside of the reconstituted proteoliposomes.

3.3 Variation in Reconstituted Vesicle Size with the Concentration of Bio – Beads SM - 2

There was little variation in vesicle size between Bio – Bead SM – 2 concentrations of 10mg/ml and 20mg/ml for the 200nm Egg PC vesicles formed by extrusion (Figure 3.3.2). But at 80mg/ml the vesicle sizes were far smaller. These results were consistent with those of the 100nm Egg PC vesicles formed by extrusion (Figure 3.3.1).

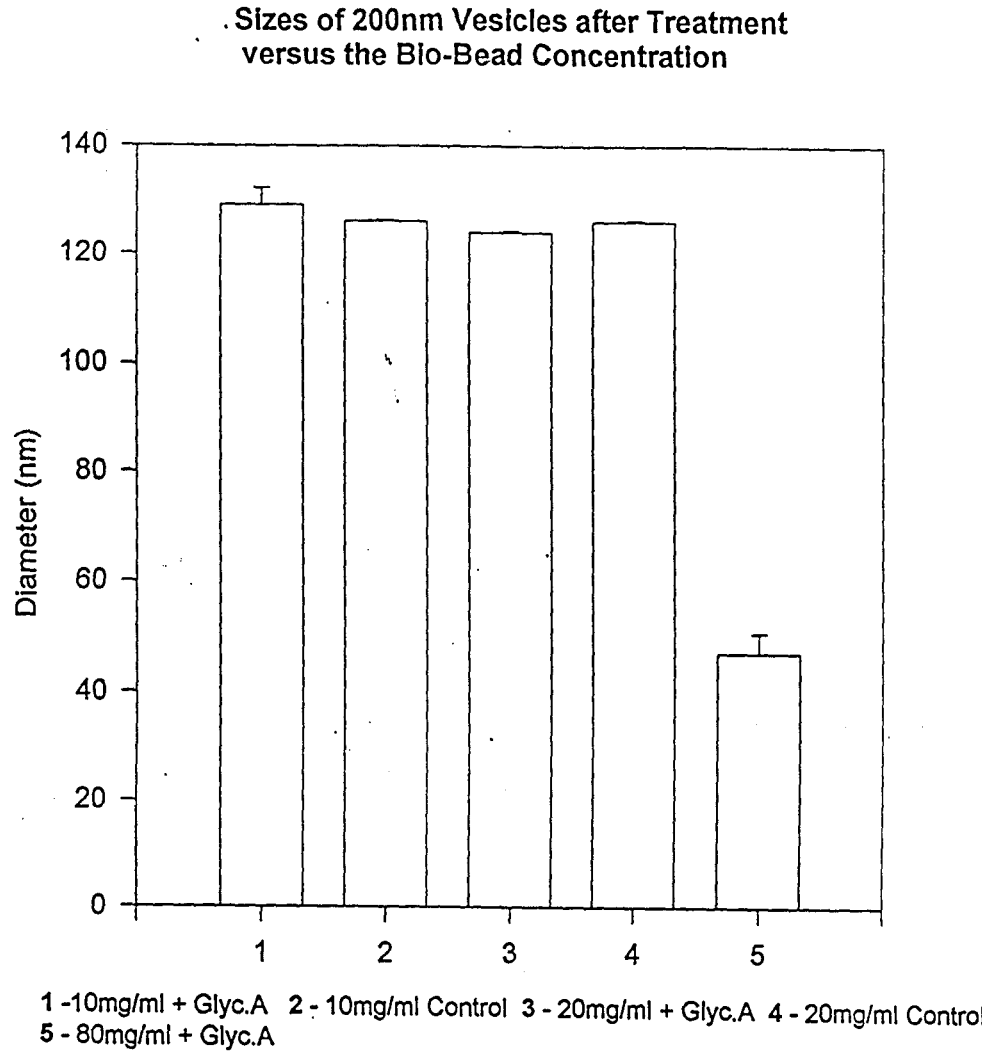


Figure 3.3.1

Sizes of 200nm Vesicles after treatment with various concentrations of Bio – Beads SM – 2. The average diameters of vesicle samples were determined using Quasi – elastic light scattering. Measurements were made at least three times for each sample.

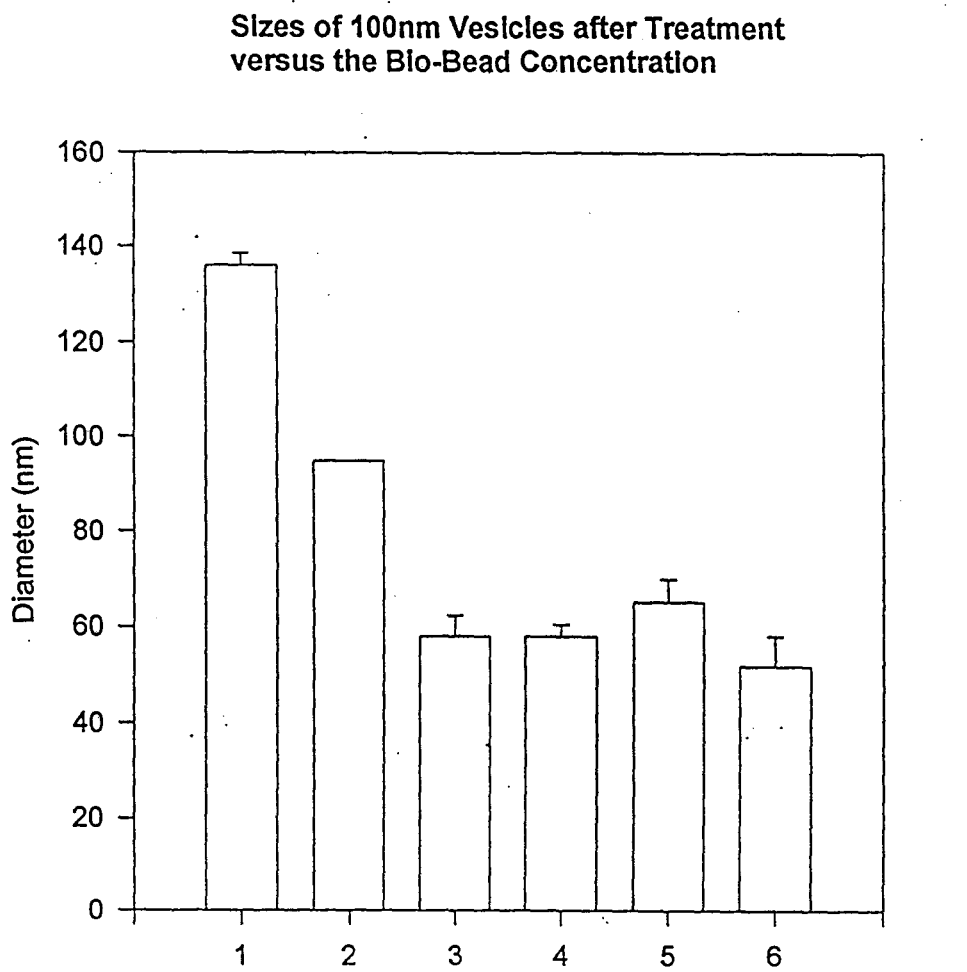


Figure 3.3.2

Sizes of 100nm Vesicles after treatment with various concentrations of Bio – Beads SM – 2. The average sizes of vesicle samples were determined using Quasi – elastic light scattering. Measurements were made at least three times for each sample.

3.4 Fusion of Sendai Virus with Proteoliposomes

The fusion of Sendai Virus with proteoliposomes containing glycophorin A was lower for those proteoliposomes reconstituted with octylglucoside than with CHAPS (Figures 3.4.1, 3.4.2 and 3.4.3). The proteoliposomes reconstituted using octylglucoside (Figures 3.4.1, 3.4.2 and 3.4.3) displayed little or no fusion enhancement over the control liposomes containing no glycophorin A. However, the CHAPS reconstituted proteoliposomes displayed an obvious enhancement of fusion over the control liposomes that contained no glycophorin A (Figures 3.4.3 and 3.4.4).

The ratio of glycophorin A to EggPC was increased four – fold to determine fusion of the vectorially reconstituted proteoliposomes compared to the random reconstituted proteoliposomes resulted from lower protein to lipid ratios. The results of Sendai Virus fusion with these proteolipomes (Figures 3.4.3C and 3.4.3D) showed that little overall fusion occurs with Sendai Virus. These results also showed no appreciable increases in fusion with proteoliposomes (Figure 3.4.3 C) compared with control Egg PC liposomes (Figure 3.4.3 D). This suggests that the fusion of Sendai Virus with the vectorially reconstituted proteoliposomes is insensitive to the amount of glycophorin A incorporated in the liposomes.

Two different detergents were used to reconstitute glycophorin A into

liposomes both randomly and vectorially. The proteoliposomes reconstituted randomly using octylglucoside exhibited lower fusion than control liposomes containing no glycophorin A as well as the liposomes reconstituted randomly using CHAPS (Figure 3.4.2). This may suggest that the low fusion of the vectorially reconstituted proteoliposomes, compared to the control liposomes and to the randomly reconstituted proteoliposomes, is a phenomenon related to the detergent, octylglucoside, used in the vectorial reconstitution.

The results displayed in Figure 3.4.2 show that the fusion of Sendai Virus with proteoliposomes reconstituted randomly using CHAPS is greater than with proteoliposomes reconstituted randomly using octylglucoside. This lends additional support to the idea that octylglucoside causes inhibition of fusion with Sendai Virus. The comparison of Sendai Virus fusion with proteoliposomes reconstituted vectorially with both CHAPS and octylglucoside supports the idea that octylglucoside causes lower fusion (Figure 3.4.3). These results further demonstrate that octylglucoside inhibits fusion since the amount of protein used in the octylglucoside reconstitution (Figure 3.4.3 C) was four – fold higher than for the CHAPS reconstitution (Figure 3.4.3 A).

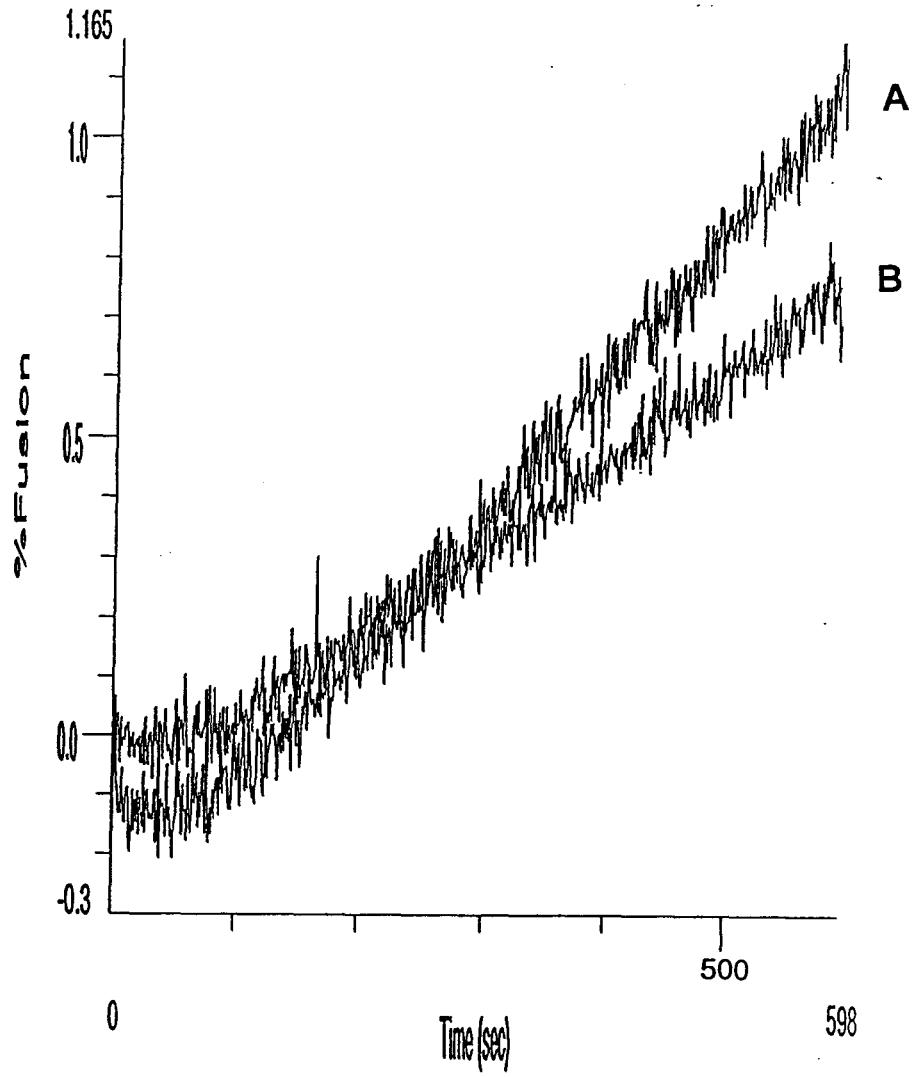


Figure 3.4.1

(A) Fusion of R18 labeled Sendai Virus with LUVs containing vectorially reconstituted Glycophorin A. The initial Lipid/Protein ratio was 465/1. (B) Fusion of Sendai Virus with Control Egg PC LUVs subjected to the same reconstitution conditions as the proteoliposome sample. Assays were carried out at 37°C in HEPES/MES buffer, pH7.4 and repeated at least three times on many separate occasions.

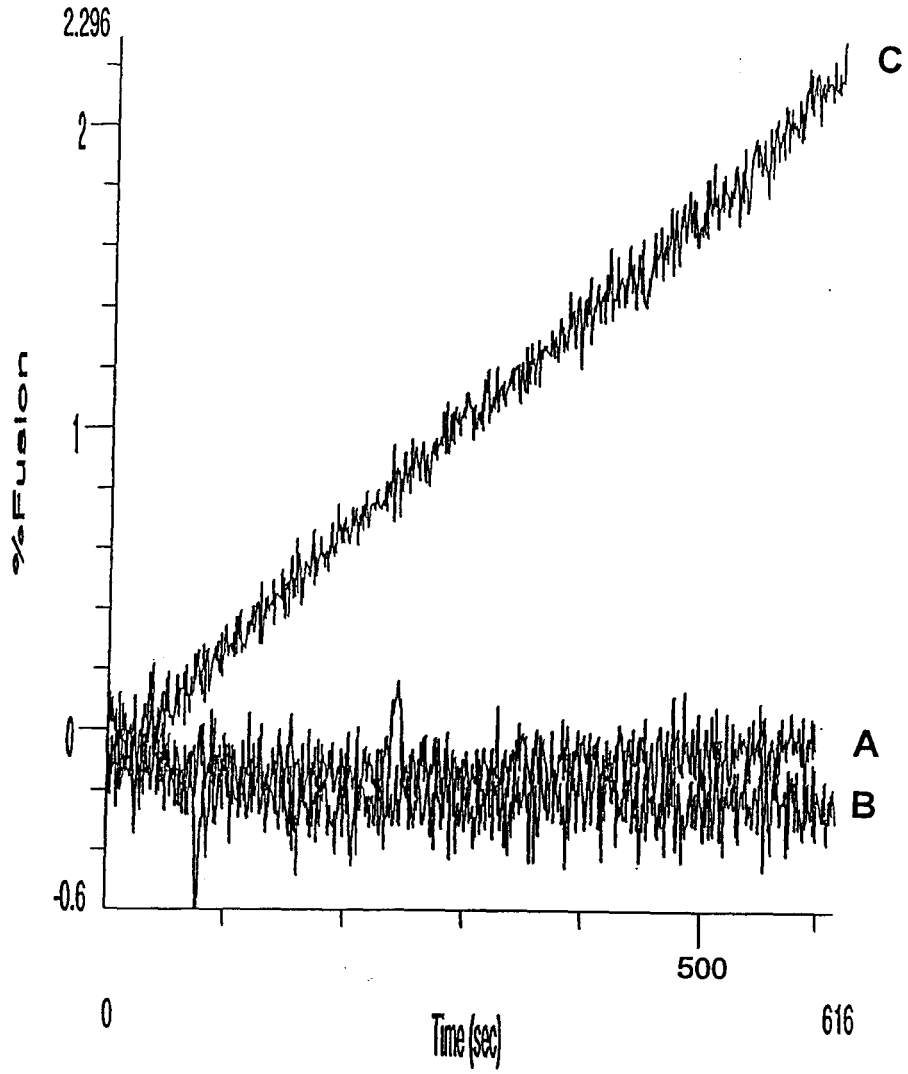


Figure 3.4.2
(A) Fusion of R18 labeled Sendai Virus with glycophorin A containing Egg PC proteoliposomes prepared by detergent dialysis with 1% Octylglucoside. (B) Fusion of R18 labeled Sendai Virus with Control Egg PC LUVs prepared by detergent dialysis with 1% Octylglucoside. (C) Fusion of R18labeled Sendai Virus with glycophorin A containing Egg PC proteoliposomes prepared by detergent dialysis with 25mM CHAPS. Fluorescence dequenching assays were carried out at 37°C in HEPES/MES buffer, pH7.4 and repeated at least three times.

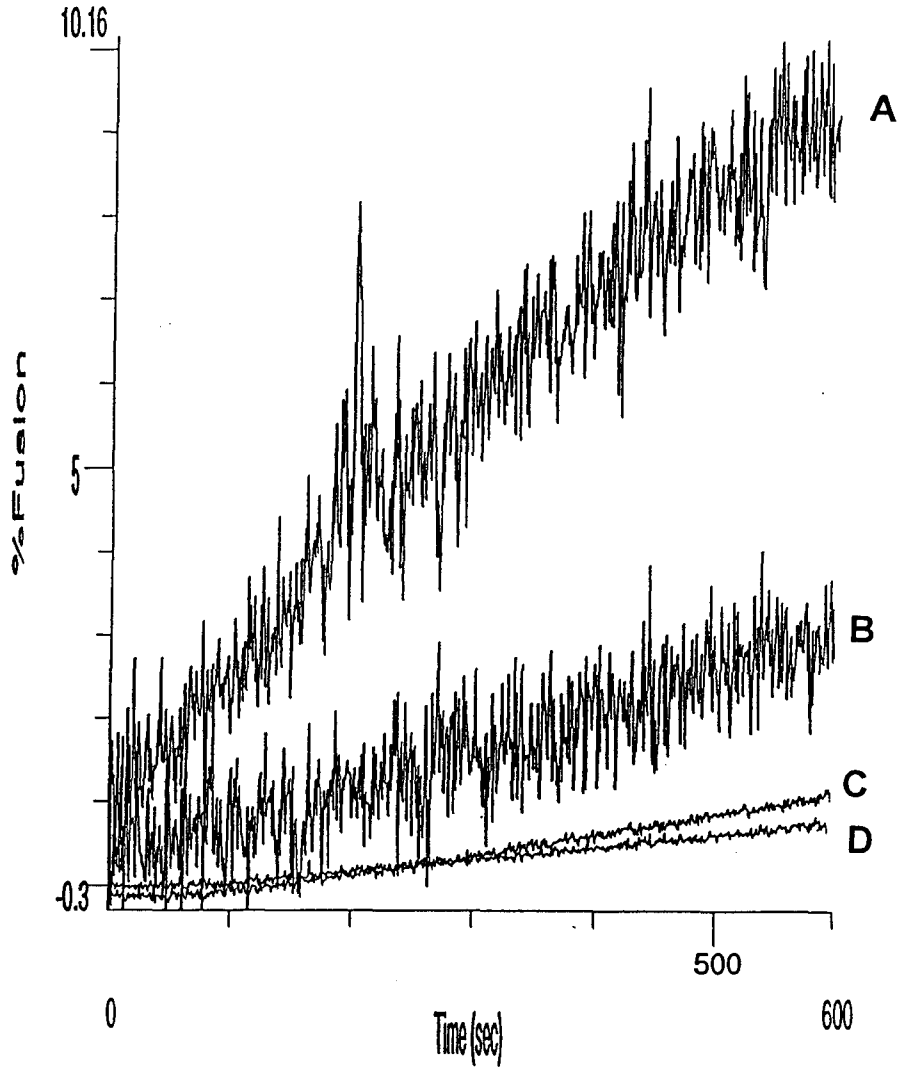


Figure 3.4.3
Fusion of R18 labeled Sendai Virus with vectorially reconstituted proteoliposomes prepared using CHAPS. (B) Fusion with Control Egg PC LUVs subjected to vectorial reconstitution conditions using CHAPS. (C) Fusion of R18 labeled Sendai Virus with vectorially reconstituted proteoliposomes prepared using Octylglucoside. (D) Fusion with Control Egg PC LUVs subjected to vectorial reconstitution conditions using Octylglucoside. Fluorescence dequenching assays were carried out at 37°C in HEPES/MES buffer, pH7.4 and repeated at least three times.

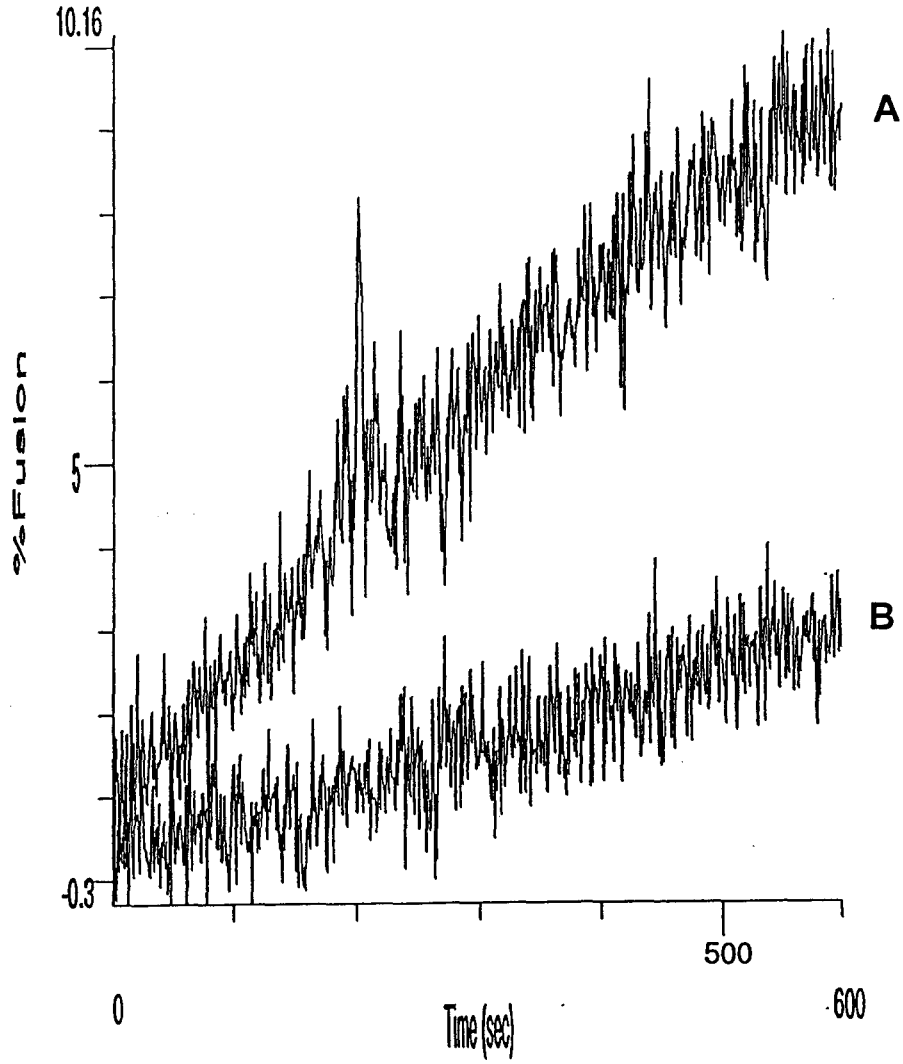


Figure 3.4.4

Fusion of R18 labeled Sendai Virus with vectorially reconstituted proteoliposomes prepared using CHAPS. (B) Fusion of R18 labeled Control Egg PC LUVs subjected to vectorial reconstitution conditions using CHAPS. Fluorescence dequenching assays were carried out at 37°C in HEPES/MES buffer, pH7.4 and repeated at least three times.

3.5 Steric Interference with NeutrAvidin Oregon Green Binding to Biotinylated Liposomes

Addition of biotin – containing liposomes to NeutrAvidin Oregon Green was followed by initial increases in NeutrAvidin fluorescence in all cases (Figures 3.5.1 – 3.5.8). This contrasts with the absence of fluorescence increases upon addition of Egg PC liposomes that did not contain biotin(Figure 3.5.9) and the absence of fluorescence increases when no liposomes were added (Figure 3.5.9).

Incorporation of PEG^{5000} – PE (Figure 3.5.1), LPG (Figure 3.5.2) and glycophorin A (Figures 3.5.3 and 3.5.4) into biotin – containing Egg PC liposomes lowered the initial increases in NeutrAvidin Fluroescence seen upon addition of the liposomes to the assay mixture. Figure 3.5.2 illustrates that higher concentrations of LPG cause greater decreases in NeutrAvidin fluorescence. However, the effect of LPG does not appear to have a strong concentration dependence between 1mol% and 5mol%.

Increasing the biotin concentration from 2.5mol% to 10mol% in the presence of either 3mol% PEG^{5000} – PE (Figure 3.5.5) or 3mol% LPG (Figure 3.5.6) revealed inconclusive results. It might have been expected that increasing the biotin concentration in the vesicles would allow greater NeutrAvidin binding and higher fluorescence. However, this pattern was not

observed for both PEG^{5000} – PE and LPG containing liposomes. Furthermore, the overall difference in fluorescence caused by increasing the biotin concentration did not mirror the large four – fold increase in biotin concentration even though the fluorescence increased in the presence of LPG (Figure 3.5.6).

A comparison of the effects caused by PEG^{5000} – PE and LPG (Figures 3.5.7 and 3.5.8) reveals that LPG has a greater effect in lowering the binding of NeutrAvidin to biotinylated liposomes than PEG^{5000} – PE. This difference is more pronounced at lower biotin concentrations (Figure 3.5.8) than at higher biotin concentrations (Figure 3.5.7). A notable difference in the fluorescence caused by PEG^{5000} – PE and LPG is the longevity of the fluorescence increase. While fluorescence of NeutrAvidin reaches and maintains a constant level during the duration of the assay when PEG^{5000} – PE is present in the liposomes this effect differs for LPG. When LPG is present the fluorescence decreases after it reaches a maximum value.

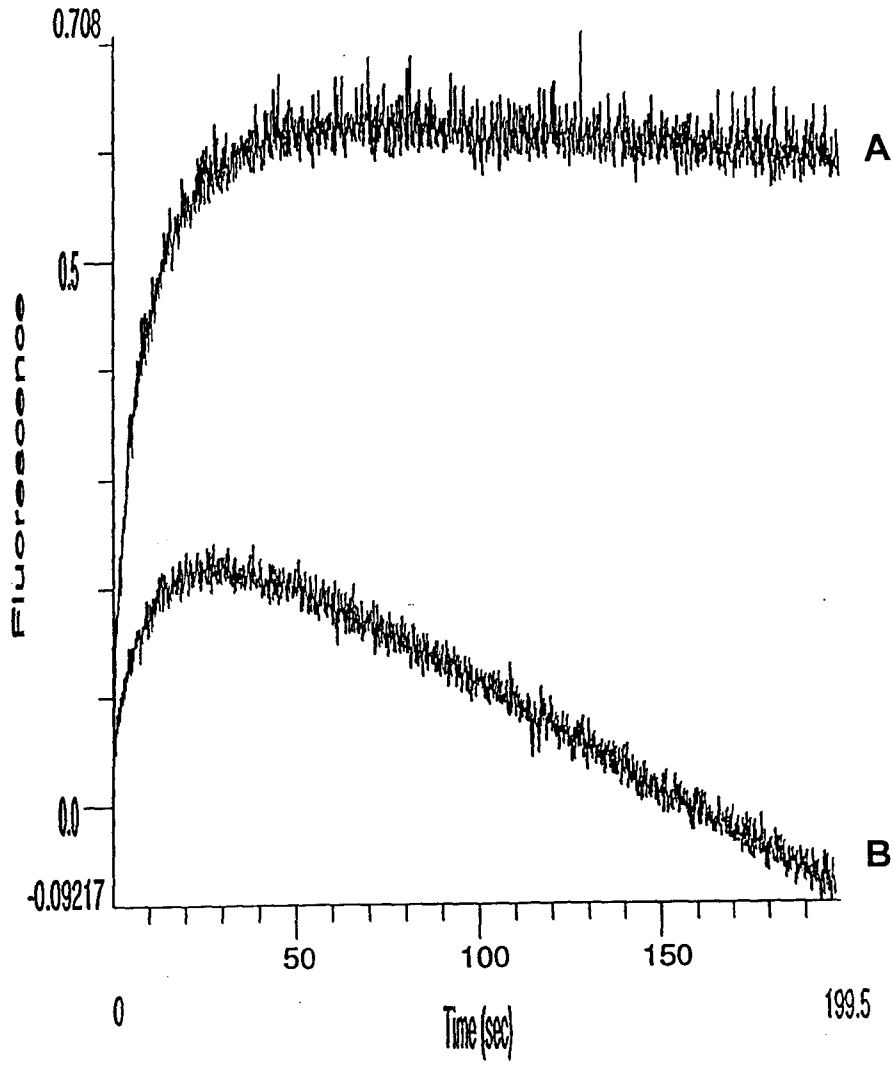


Figure 3.5.1

The effect of PEG^{5000}-PE on the binding of NeutrAvidin Oregon Green to biotinylated LUVs. Fluorescence of Neutravidin Oregon Green upon binding biotinylated Egg PC LUVs. (A) Control Egg PC LUVs contained 5mol% Biotin-X-DHPE (B) Egg PC LUVs contained 5mol% Biotin-X-DHPE and 5mol% PEG^{5000}-PE. Assays were repeated at least twice for each sample.

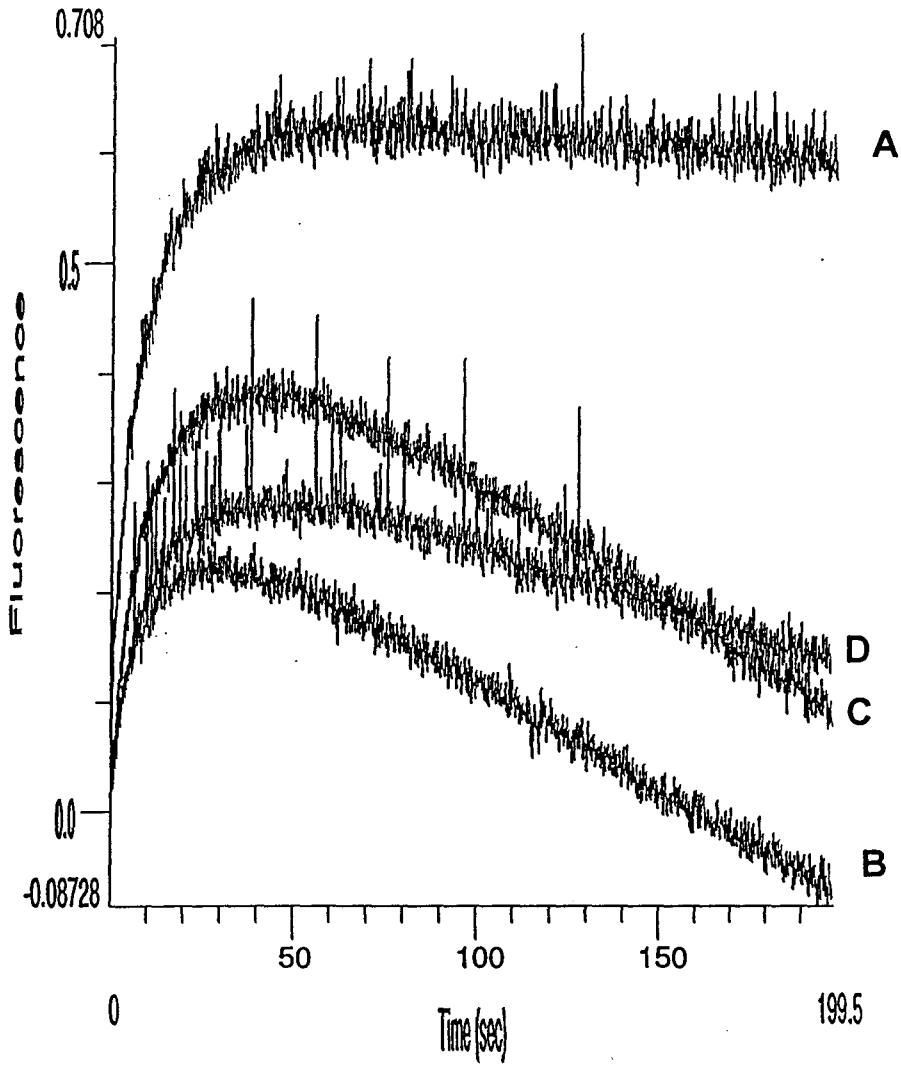


Figure 3.5.2

The Effect of LPG on the binding of NeutrAvidin Oregon Green to biotinylated LUVs. Fluorescence of NeutrAvidin Oregon Green upon binding biotinylated Egg PC LUVs. (A) Control Egg PC LUVs contained 5mol% Biotin-X-DHPE. (B) Egg PC LUVs contained 5mol% Biotin-X-DHPE and 5mol% LPG. (C) Egg PC LUVs contained 5mol% Biotin-X-DHPE and 3mol% LPG. (D) Egg PC LUVs contained 5mol% Biotin-X-DHPE and 1mol% LPG. Assays were repeated at least twice for each sample.

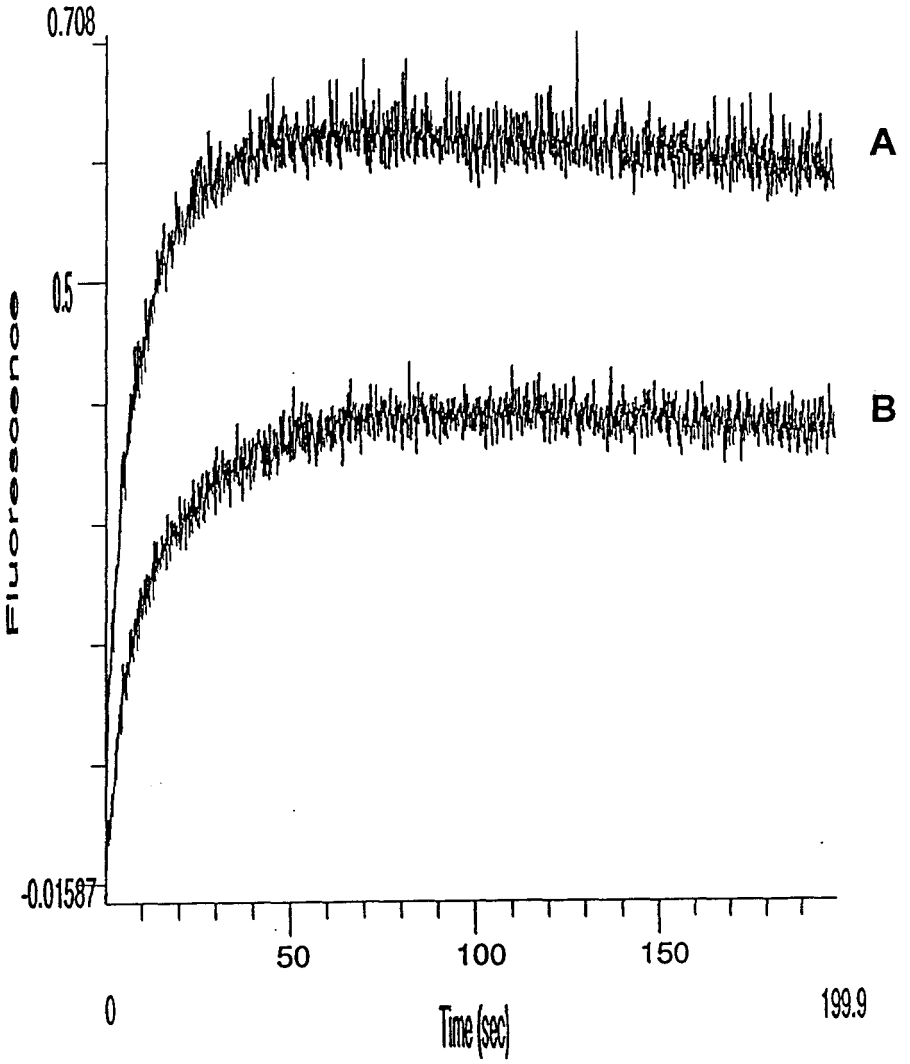


Figure 3.5.3

The effect of glycophorin A on the binding of NeutrAvidin Oregon Green to biotinylated LUVs. All of the LUVs were formed by extrusion through 100nm Pore diameter polycarbonate filters. The fluorescence of NeutrAvidin Oregon Green upon binding biotinylated Egg PC LUVs. (A) Control Egg PC LUVs contained 5mol% Biotin-X-DHPE. (B) Egg PC LUVs contained 5mol% Biotin-X-DHPE and 1mol% Glycophorin A. Assays were repeated at least twice for each sample.

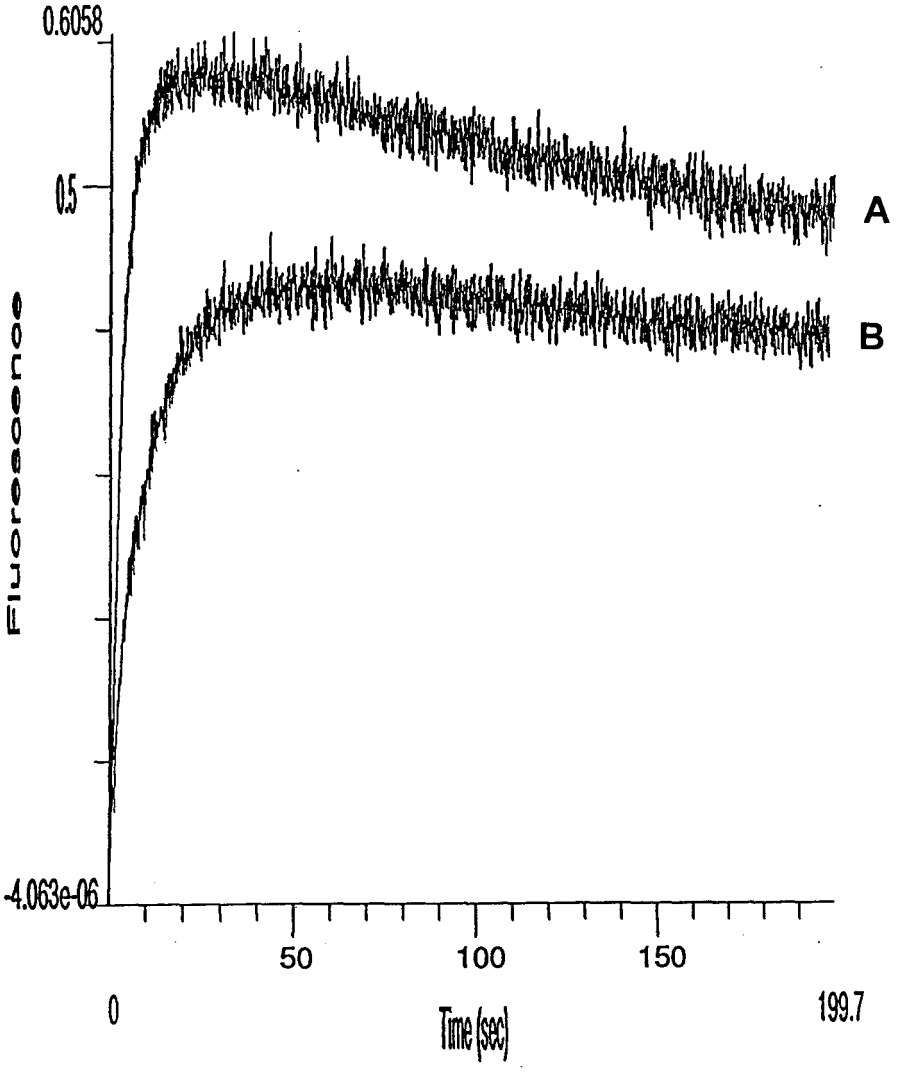


Figure 3.5.4
The effect of glycophorin A on the binding of NeutrAvidin Oregon Green to biotinylated LUVs. All of the LUVs were formed by detergent dialysis. Fluorescence of NeutrAvidin Oregon Green upon binding biotinylated Egg PC LUVs. (A) Control Egg PC LUVs contained 5mol% Biotin-X-DHPE. (B) Egg PC LUVs contained 5mol% Biotin-X-DHPE and 1mol% Glycophorin A. Assays were repeated at least twice for each sample.

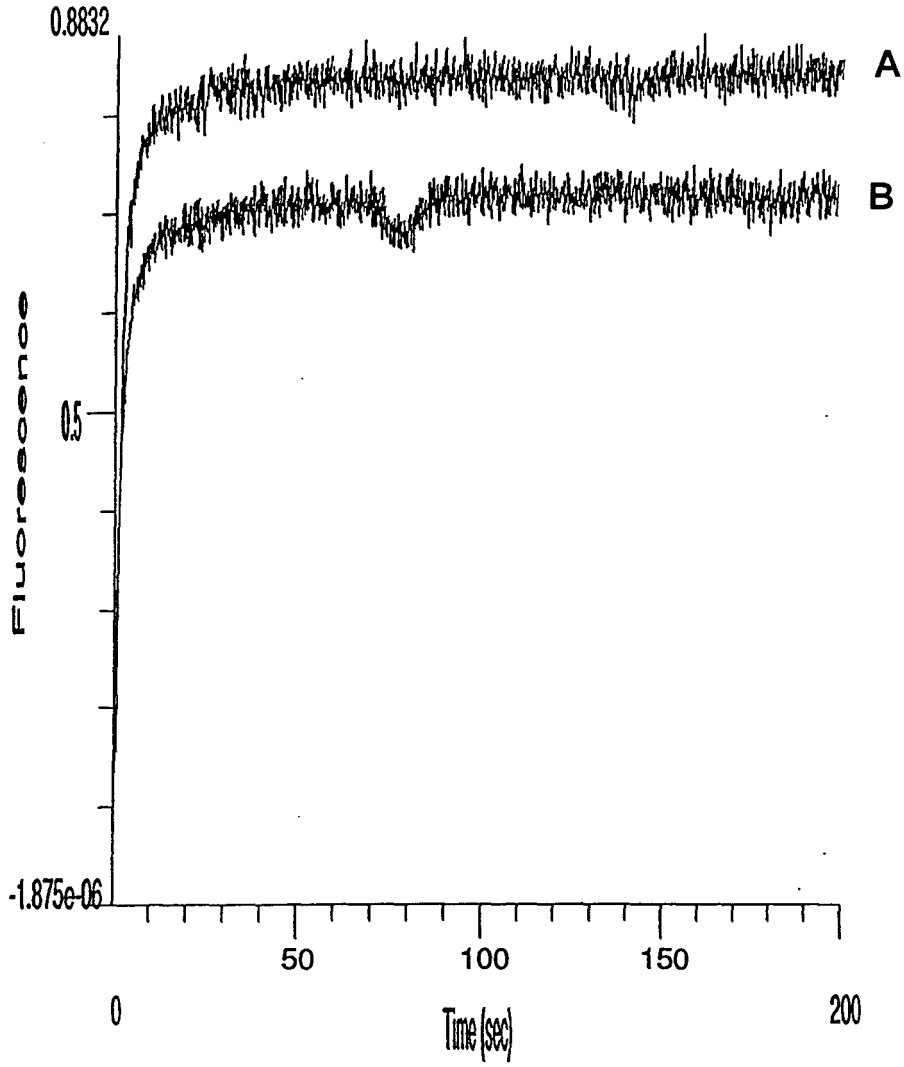


Figure 3.5.5
The effect of varying the Biotin-X-DHPE in biotinylated LUVs containing PEG^{5000}-PE. Fluorescence of NeutrAvidin Oregon Green upon binding biotinylated Egg PC LUVs. (A) Egg PC LUVs containing 2.5mol% Biotin-X-DHPE and 3mol% PEG^{5000}-PE. (B) Egg PC LUVs containing 10mol% Biotin-X-DHPE and 3mol% PEG^{5000}-PE. Assays were repeated at least twice for each sample.

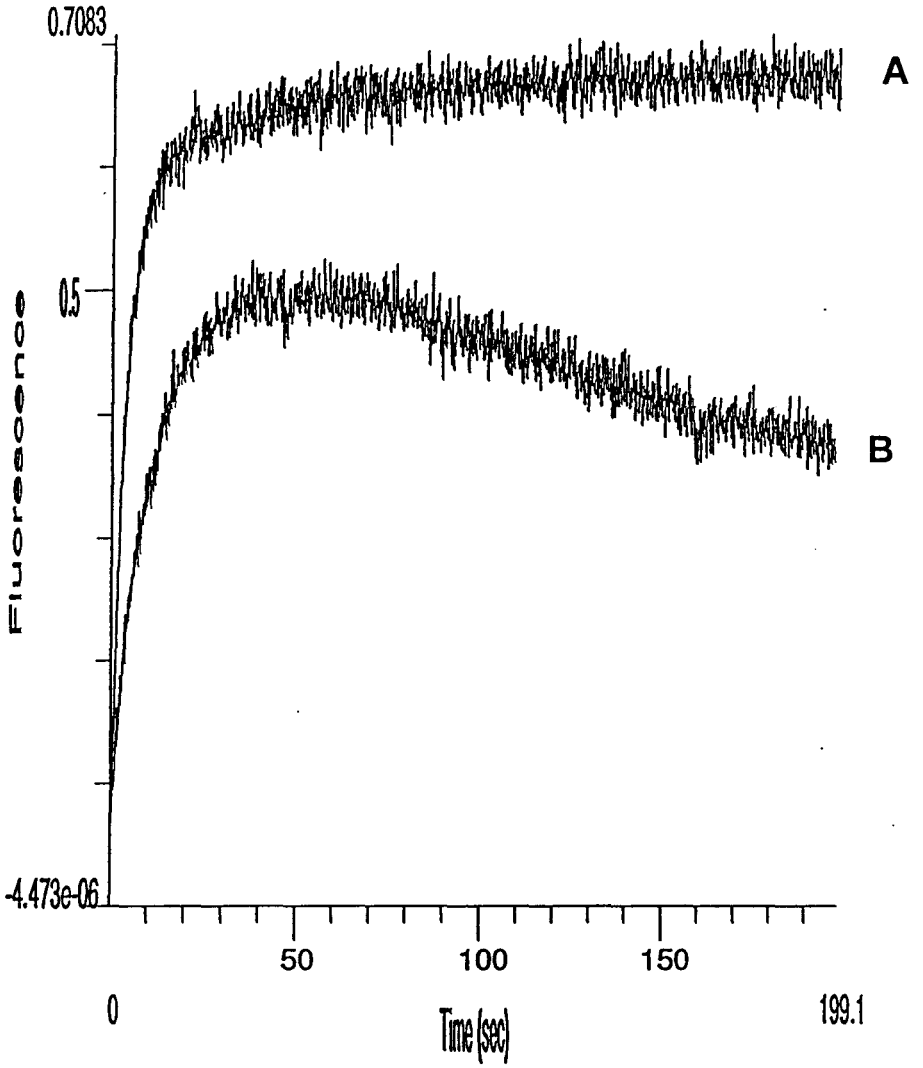


Figure 3.5.6
The effect of varying the Biotin-X-DHPE concentration in LUVs containing LPG. Fluorescence of NeutrAvidin Oregon Green upon binding biotinylated Egg PC LUVs. (A) Egg PC LUVs containing 10mol% Biotin-X-DHPE and 3mol% LPG. (B) Egg PC LUVs containing 2.5mol% Biotin-X-DHPE and 3mol% LPG. Assays were repeated at least twice for each sample.

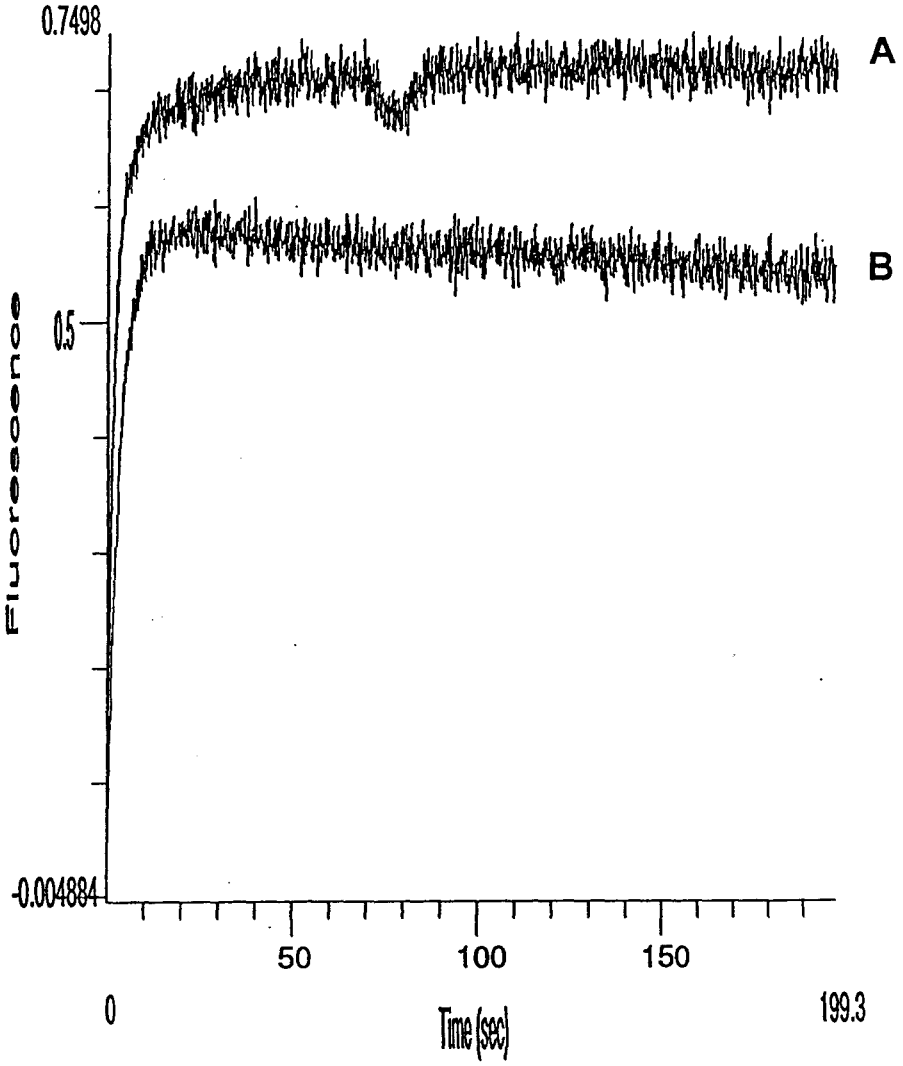


Figure 3.5.7

Comparing the effects of PEG^{5000}-PE and LPG on the binding of NeutrAvidin Oregon Green to biotinylated LUVs. Fluorescence of NeutrAvidin Oregon Green upon binding biotinylated Egg PC LUVs. (A) Egg PC LUVs containing 10mol% Biotin-X-DHPE and 3mol% PEG^{5000}-PE. (B) Egg PC LUVs containing 10mol% Biotin-X-DHPE and 3mol% LPG. Assays were repeated at least twice for each sample.

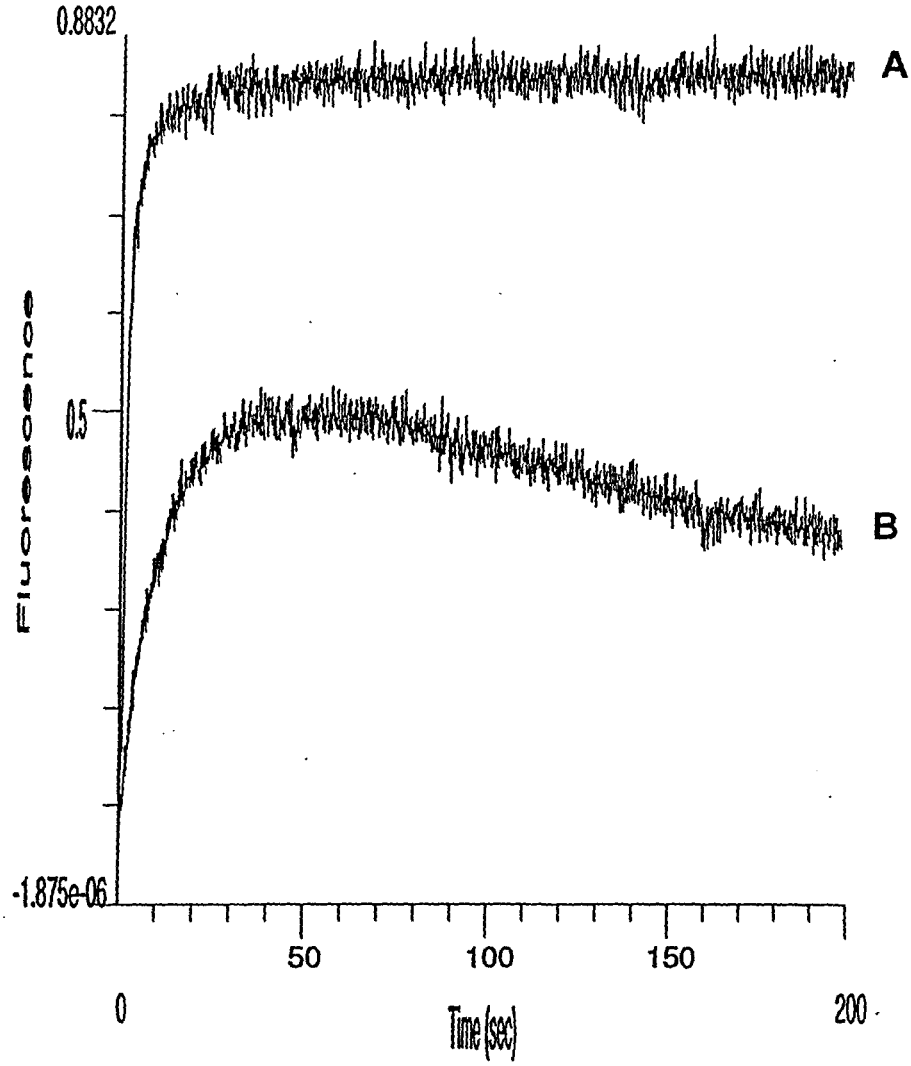


Figure 3.5.8

Comparing the effects of PEG^{5000}-PE on the binding of NeutrAvidin Oregon Green to biotinylated LUVs. Fluorescence of NeutrAvidin Oregon Green upon binding Biotinylated Egg PC LUVs. (A) Egg PC LUVs contained 2.5mol% Biotin-X-DHPE and 3mol% PEG^{5000}-PE. (B) Egg PC LUVs contained 2.5mol% Biotin-X-DHPE and 3mol% LPG. Assays were repeated at least twice for each sample.

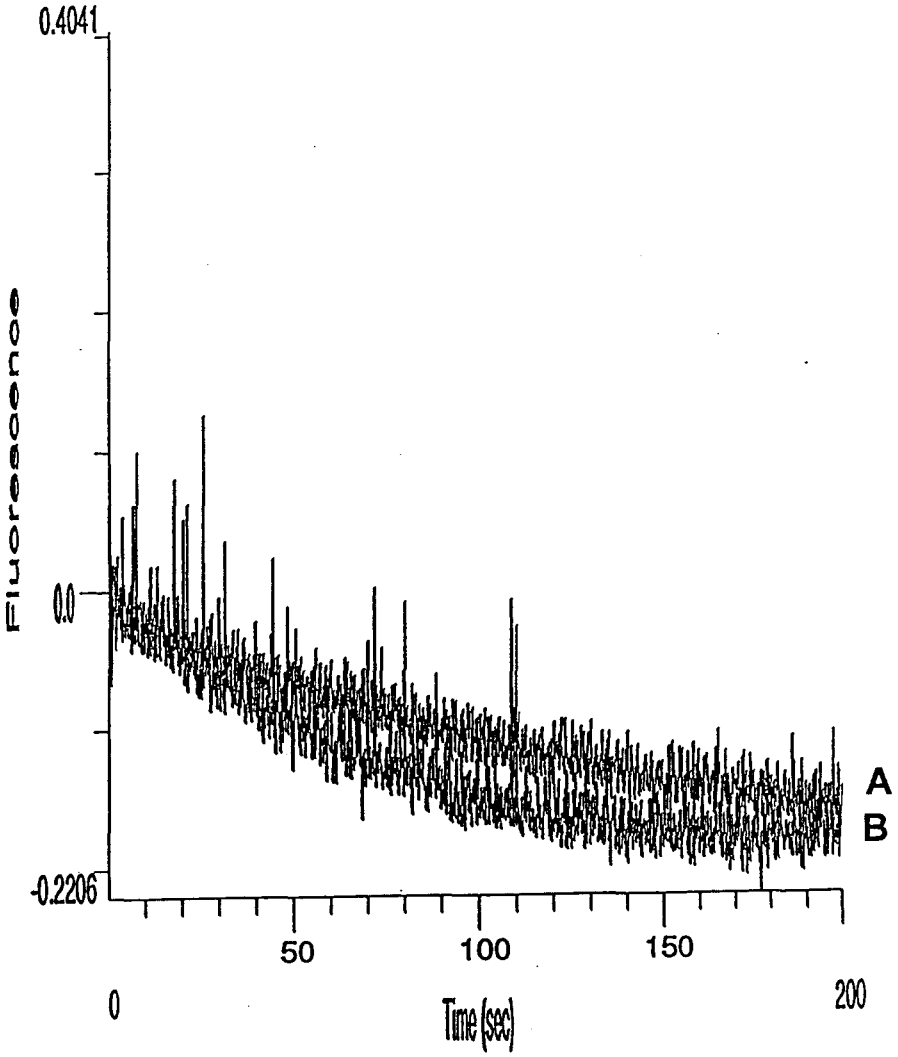


Figure 3.5.9

Fluorescence of NeutrAvidin Oregon Green (A) in the presence of bare Egg PC vesicles and (B) alone in HEPES/MES buffer, pH7.4. Assays were repeated at least twice for each sample.

4.0 Discussion

4.1 Glycophorin A Isolation

Glycophorin's mobility in SDS-PAGE is hindered (Figures 3.1.1 and 3.1.2) such that its apparent molecular weight (~47500) is much higher than its actual molecular weight (~31000). This results from the bulkiness of the carbohydrate attached to glycophorin A's ectodomain as well as SDS interacting with the hydrophobic transmembrane region. The large amounts of glycophorin A dimers present result from the strong hydrophobic interactions between transmembrane domains (Brosig et al., 1998). These interactions are strong enough to prevent monomer dissociation even under the most vigorous conditions (Brosig et al., 1998). The three dimensional structure of the dimeric transmembrane of glycophorin A has been determined by solution nuclear magnetic resonance spectroscopy (MacKenzie et al., 1997) using a combination of computational and mutagenesis methods. It is thought that the stability and specificity of this transmembrane dimer arises from van der Waals interactions between two well defined hydrophobic helices.

4.2 Vectorial Reconstitution

4.2.1 Solubilization of Egg PC Liposomes

As previously observed , the partition coefficient at the completion of

solubilization was higher than at the onset of vesicle solubilization (Table 1) (Paternostre et al., 1995). As the detergent concentrations increased during the vesicle solubilization process the amount of detergent associated with the lipid increases (Rigaud et al., 1995; Lichtenberg et al., 1983). This corresponds to the increased formation of mixed lipid/detergent micelles during the lamellar to micellar transition (Rigaud et al, 1995;Lichtenberg et al., 1983).

In order that vesicle sizes would not decrease during reconstitutions due to detergent solubilization, reconstitutions were carried out at Egg PC and detergent concentrations corresponding to those at R^{sat}_{eff}. Since micelles should not have formed at this point in the solubilization (Rigaud et al., 1995; Lichtenberg et al., 1983) of the vesicles the vesicle sizes should have increased due to the insertion of detergent monomers into the bilayer. This increase in vesicle size is manifest as increases in turbidity (Paternostre et al., 1988; Rigaud et al, 1988). The increases in turbidity with increasing detergent concentrations can be seen in Figure 3.2.2 (A - G) and Figure 3.2.4 (A – F).

The insertion of proteins into liposomes is thought to require destabilization of the lipid bilayer (Paternostre et al., 1988; Rigaud et al., 1988; Rigaud et al., 1995). This is accomplished by the insertion of detergent monomers. Destabilization without actually affecting the overall integrity of the membrane actually reaches a maximum at R^{sat}. Protein insertion takes

advantage of this destabilization with the most hydrophobic domain thought to insert first (Rigaud et al., 1995). For the reconstitution of glycophorin A into LUVs at R^{sat} concentrations of Egg PC and detergent this would mean that the transmembrane domain of glycophorin A would insert into the detergent destabilized vesicle leaving its heavily glycosylated ectodomain oriented toward the outside of the vesicles.

4.2.2 Insertion of Glycophorin A into Preformed Liposomes

It has been documented that the rate of detergent removal by Bio – Beads SM – 2 affects both the sizes of the reconstituted proteoliposomes as well as the degree of protein incorporation into the proteoliposomes (Paternostre et al., 1988; Rigaud et al., 1988; Levy et al., 1990). The results of the reconstitutions illustrated in Figure 3.3.1 and Figure 3.3.2 are in agreement with these previous findings. The rate at which the detergent is removed from the vesicles varies with the concentration of Bio – Beads SM – 2 (Paternostre et al., 1988; Rigaud et al., 1988; Levy et al., 1990). Bio – Beads are able to remove amphiphiles such as lipids and detergents by hydrophobic adsorption. But the adsorption of lipids is much less effective than the adsorption of detergents (Levy et al., 1990). Still, at high Bio – Bead concentrations significant amounts of lipids are likely removed contributing to the decreasing vesicle sizes.

It was found that the smallest decreases in vesicle size occurred when Bio – Beads SM – 2 concentrations of 10mg/ml or 20mg/ml were used to remove the detergents octylglucoside and CHAPS. At these Bio – Bead concentrations vesicle sizes were similar (Figures 3.3.1 and 3.3.20). The important difference between these proteoliposomes was in their lipid to protein ratios (Tables 2 and 3). The results in these tables also suggest that below 20mg/ml differences in Bio – Bead concentration may have very little effect on the removal of lipid and therefore on the vesicle size. This may mean that for 200nm vesicles higher rates of detergent removal within this range of Bio – Bead concentrations results in better glycophorin A incorporation (Table 2). The results with the 100nm vesicles show greater glycophorin A incorporation at higher Bio – Bead concentrations (Table 3). But at such high Bio – Bead concentration much more lipid is being removed with the effect of lowering the lipid to protein ratios.

At a Bio – Bead concentration of 20mg/ml the efficiency of incorporation appears to vary with vesicle size. In Tables 2 and 3 the lipid to protein ratios in the reconstituted proteoliposomes appear to be higher for the 100nm vesicles than for the 200nm vesicles at 20 mg/ml Bio – Beads. This may suggest that the intrinsic properties of the larger intact 200nm vesicles are more favourable for the insertion of glycophorin A than the 100nm vesicles. Smaller vesicles exhibit looser and less tightly packed lipids, faster lateral diffusion of lipids in the bilayer

and should therefore be more susceptible to the formation of packing defects (Eytan, 1982;Sheetz et al., 1972; De Kruijff et al., 1975). Contrary to the data at 20 mg/ml Bio – Beads (Tables 2 and 3 these properties would be expected to make insertion of glycophorin A into larger vesicles more difficult than into smaller ones. But the date in Table 3 also reveals that at both 40mg/ml and 60mg/ml Bio – Beads the incorporation of glycophorin A into the smaller vesicles is much greater than into the larger vesicles (Table 2). This agrees with what would be expected.

4.3 Sendai Virus Fusion with Reconstituted Proteoliposomes

The fusion of Sendai Virus with randomly incorporated glycophorin A containing proteoliposomes has been shown to occur (Wybenga et al., 1996). This method of random incorporation used the detergent CHAPS and dialysis of the detergent/lipid/protein mixture to form proteoliposomes. It was not known whether vectorial incorporation would result in greater fusion than random incorporation. The fusion of Sendai Virus with proteoliposomes containing glycophorin A incorporated vectorially using octylglucoside demonstrates a negligible increase in fusion compared with the control vesicles containing no glycophorin A (Figure 3.4.1). Glycophorin A is unidirectionally oriented in biological membranes where it serves as a receptor for viruses and as a blood group determinant (Blanchard, 1990). Asymmetrically reconstituted

glycophorin A containing proteoliposomes more closely resemble the in vivo situation. It was therefore thought that these liposomes would exhibit greater fusion with Sendai Virus than both the control liposomes which did not contain Glycophorin A and the randomly reconstituted proteoliposomes

The reason why octylglucoside inhibited fusion is unknown. We speculated that the lower fusion might have occurred as a result of different types of dimers being formed within the membrane when octylglucoside is used in the reconstitutions instead of CHAPS. In order for glycophorin A to act as a receptor for Sendai Virus its large glycosylated ectodomain must face the exterior of the liposomes. It has previously been shown that glycophorin A forms three types of dimers in proteoliposomes (Challou et al., 1994): head – head, tail – tail and head – tail. The head - head dimer has both monomer ectodomains facing the liposome exterior. The tail – tail dimer has both monomer ectodomains facing the lumen of the liposomes. The head – tail dimer has one ectodomain facing the exterior and the other facing the interior. This group used proteinase K digestion of proteoliposomes and analysis of membrane bound fragments using SDS – PAGE and amino acid sequencing to determine the types of dimers formed.

Our efforts to use similar methods to determine the types of dimers present in the liposomes were inconclusive (Results not shown). The digestion

of proteoliposomes with proteinase K would be expected to degrade any part of the glycophorin A dimers located on the exterior of the liposomes. Therefore, depending on the type of glycophorin dimer that predominates in the membrane, one would expect to find different membrane bound fragments remaining within the membrane that could be visualized and differentiated by SDS-PAGE and silver-staining. However, digestion of proteoliposomes with proteinase K yielded many more fragments than Challou et al. (1994) making it impossible to determine whether differences in membrane bound fragment sizes did exist. It is possible that the digestion of glycophorin A was incomplete thus yielding a large range of fragments between the undigested and completely digested dimeric forms.

In another set of experiments we used neuraminidase digestion followed by O – glycosidase digestion. O – glycosidase was chosen in order to remove the 15 O – glycans linked to each glycophorin A ectodomain facing the liposomal exterior. Neuraminidase pretreatment prior to O – glycosidase treatment was carried out in order to remove sialic acid which has been shown to inhibit O – glycosidase activity (Hansen et al., 1992). The results of the O – glycosidase digestions (Results not shown) were also inconclusive. Removing 15 of Glycophorin A's 16 glycans using O – glycosidase would be expected to drastically alter the molecular weight and electrophoretic mobility of glycophorin

A dimers since carbohydrate composes roughly 60% of its mass. Depending on the type of dimer present in the membrane the change in the mobility of the dimer would be expected to differ. The digestion was not efficient since enzyme treatment did not clearly alter the electrophoretic pattern of gycophorin A samples compared to untreated controls. This could be due to the interference of unremoved sialic acid with the activity of O – glycosidase or denaturation of O-glycosidase.

4.4 Steric Interference by Biopolymers Incorporated into Liposomes

Experiments attempting to demonstrate steric interference with access of Nile Red to liposomes by biopolymers yielded little information (Results not shown). Increases in the fluorescence of Nile Red upon access to bare Egg PC liposomes, liposomes containing PEG^{5000}-PE, liposomes containing LPG and liposomes containing glycophorin A occurred instantaneously. There were no delays in fluorescence increases implying easy access of the probe to the membrane and no observable steric interference by PEG^{5000}-PE, LPG or glycophorin A. Since Nile Red is a relatively small molecule it is likely that these biopolymers did not create an effective steric barrier preventing Nile Red's access to the membrane. We speculated that steric interference would be more apparent with a larger fluorophore such as cytochrome C or other protein bound

fluorophores.

The interaction between avidin conjugated fluorophores and biotinylated liposomes (Noppl – Simson et al., 1996) is interfered with by the presence of PEG^{750}-PE in the liposomes. We speculated that if PEG – PE could sterically interfere with this interaction then other large membrane – linked biopolymers such as glycophorin A and LPG coud also cause similar steric interference. The results presented in Figures 3.5.1, 3.5.2, 3.5.3 and 3.5.4 illustrate the decreases in fluorescence that result from interference with the binding of soluble NeutrAvidin Oregon Green (Molecular Probes) to Egg PC liposomes containing lipid – conjugated biotin. Noppl-Simson et al. (1996) reported a decrease in the rate at which the fluorescence decreased due to the presence of PEG^{750}-PE, but did not report a decrease in the overall fluorescence caused by PEG^{750}-PE. Our results show that PEG^{5000}-PE (Figure 3.5.1), LPG (Figure 3.5.2) and glycophorin A (Figures 3.5.3 and 3.5.4) all caused decreases in overall fluorescence of NeutrAvidin Oregon Green suggesting that decreases in overall binding to the biotinylated liposomes occurred. The fact that all three biopolymers inhibit access to the liposome surface shows that this effect is not an artifactual phenomenon resulting from the idiosyncratic properties of any one of these three biopolymers. Rather, it suggests that this effect results from an element common to all three membrane - linked biopolymers. This supports the notion

that a more general phenomenon such as steric hinderance is responsible for the decrease in biotin – NeutrAvidin interaction.

The PEG – PE, glycophorin A and LPG we incorporated into Egg PC vesicles have approximate Molecular Weights of 5400, 31000 and 9500 respectively. All of these biopolymers are significantly larger than the PEG – PE (Molecular Weight 750) used by Noppl – Simson et al. (1996) and would therefore be expected to produce a greater steric effect than PEG^{750} – PE at similar concentrations.

LPG appears to exert a greater effect than PEG^{5000} – PE in blocking the binding of NeutrAvidin to Biotin. This occurs both at 10mol% Biotin (Figure 3.5.7) and 2.5mol% Biotin (Figure 3.5.8). The larger size of LPG can account for its ability to lower the fluorescence relative to PEG^{5000} – PE. However, this does not account for secondary effects that occurred with LPG containing liposomes and only the 5mol% PEG^{5000}-PE liposomes (Figure 3.5.1). There are gradual decreases in fluorescence with these liposomes (Figures 3.5.1, 3.5.2, 3.5.7B and 3.5.8B) that do not occur with either the PEG^{5000} – PE containing liposomes (Figures 3.5.7 A and 3.5.8 A) or the glycophorin containing liposomes (Figures 3.5.3 and 3.5.4). Since the binding of avidin to biotin is very strong, with $K_a = 10^{15}\ M^{-1}$ (Wilchek et al., 1990), it is unlikely that the secondary fluorescence decrease is due to a progressive decrease in the degree of binding. The higher

concentrations of PEG^{5000}-PE (Figure 3.5.1) could have a similar effect at the liposome surface as lower concentrations of LPG (Figures 3.5.7 and 3.5.8) because of LPG's larger size. At these concentrations of LPG and at the higher concentrations of PEG^{5000}-PE quenching of the initial fluroescence by PEG^{5000}-PE and LPG may be occurring.

Increases in the amount of biotin incorporated in the liposomes would be expected to result in greater NeutrAvidin binding and, subsequently, greater fluorescence. However, the results presented in Figures 3.5.5 and 3.5.6 do not support this idea. These results appear to be inconclusive because higher biotin concentrations in liposomes containing 3mol% PEG^{5000} – PE (Figure 3.5.5) result in lower fluorescence. Only two Biotin concentrations were used in this comparison: 2.5mol% and 10mol%. It is possible that a more definitive biotin concentration dependent trend would be apparent at lower biotin concentrations. Since the biotin is conjugated to DHPE it is possible that Biotin – X – DHPE concentrations as high as 10mol% could cause localized aggregation of the PE – linked biotin and PEG^{5000} – PE conjugates in the membrane.

The possibility that the observed fluorescence increases resulted from NeutrAvidin Oregon Green alone or from the nonspecific interaction of NeutrAvidin Oregon Green with the Egg PC vesicles can be examined in Figure 3.5.9. Increases in fluorescence clearly do not occur with NeutrAvidin alone in

buffer nor as a result of interaction between NeutrAvidin and Egg PC vesicles.

4.5 Summary and Future Directions

The isolation of glycophorin A from human erythrocytes was acomplished using the LIS/phenol method and its insertion into liposomes, vectorially and randomly, was accomplished with the detergents octylglucoside and CHAPS. It was found that proteoliposomes reconstituted using the detergent octylglucoside displayed lower fusion than those reconstituted using the detergent CHAPS. An investigation of glycophorin A's dimerization state in proteoliposomes reconstituted by the two detergents did not yield information related to the difference in fusion. Further work is needed to elucidate the basis for this difference. It is possible that octylglucoside itself inhibits the fusion with Sendai Virus. Perhaps residual octylglucoside interacting directly with glycophorin A or with Sendai Virus is sufficient to inhibit fusion. One could attempt to look at this possibility by the external addition of small amounts of octylglucoside to CHAPS reconstituted proteoliposomes immediately prior to carrying out fusion with Sendai Virus.

The inhibition of NeutrAvidin Oregon Green Binding to lipos9omes containing biotin-conjugated lipid by glycophorin A, LPG and PEG^{5000} – PE is an effect common to all three biopolymers. This suggests that a phenomenon

related to all three biopolymers is responsible for the binding inhibition and it lends support to the idea of a steric effect. Other work needs to be done to substantiate these results. If indeed all three biopolymers sterically stabilize liposomes then they should also increase the resistance of liposomes to detergent solubilization. Any increase in resistance to solubilization would be mirrored by changes in the solubilization parameters as well as changes in the length of time required to solubilize liposomes with high detergent concentrations. A simple assay monitoring the turbidity (light scattering in the visible range of the electromagnetic spectrum) decrease over time could be used to demonstrate changes in liposome stability that glycophorin A, LPG and PEG^{5000} – PE might confer. It should be noted that greater incorporation of PEG – PE results in unexplained decreases in turbidity (Beugin-Deroo, 1998). However, the initial turbidity prior to solubilization should have no bearing on the length of time it takes to solubilize the liposomes.

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