Generation of a Liposome Based Platelet Substitute: Insertion of GPIb-IX into Liposomes

By

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Abstract:

The provision of platelets for transfusion is a major function of a blood transfusion service. Since platelets have a short 5-day shelf life, may carry known and theoretical risks of donor derived transfusion products, and the demand exceeds supply, it is necessary to look to the creation of alternative products. A liposome based platelet substitute is one of such alternatives.

The initial response in the formation of a platelet aggregate during a thrombotic event is the adhesion of the platelets to the damaged endothelium. This occurs via the plasma protein von Willebrand factor (vWF) that becomes activated in the blood in response to turbulent blood flow. Collagen-immobilized vWF binds to the platelet glycoprotein Ib-IX-V (GPIb-IX-V) complex, which is the initial platelet receptor involved in adhesion. Intracellular signaling linked to GPIb-IX-V binding leads to platelet activation, shape change and granule release.

During this study, GPIb-IX was purified from outdated platelet concentrates and was inserted into 100 – 200 nm unilamellar liposome vesicles containing 25 mole% cholesterol, 10 mole% 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 65 mole% 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC). From 25 platelet concentrates, 0.925 mg GPIb-IX was purified which represented 18% of the protein in that sample. Outdated platelet concentrates were lysed with Triton X-100. The lysate was first subjected to the affinity chromatography on a wheat germ agglutinin agarose (WGA-agarose) column, and in some cases, was followed by an anion exchange mono-Q-Sepharose column. The final purification after WGA-agarose was 180,000 fold from platelet concentrates.

GPIb-IX was incorporated into the liposomes using detergent exchange dialysis. Flow cytometry revealed that GPIb as well as GPIX was present on the external liposome surface. This result was confirmed by fluorescence microscopy.

To assess the function of liposomal GPIb-IX, the liposomes were exposed to vWF, ristocetin and collagen type III. Results showed that the presence of GPIb-IX in the liposomes significantly enhanced vWF binding to the liposomes; therefore, the liposome product generated may act as a platelet aggregation enhancer.
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<table>
<thead>
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<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine</td>
</tr>
<tr>
<td>A&lt;sub&gt;278&lt;/sub&gt;</td>
<td>absorbance at 278 nm</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>BSS</td>
<td>Bernard-Soulier syndrome</td>
</tr>
<tr>
<td>CHO cells</td>
<td>Chinese hamster ovary cells</td>
</tr>
<tr>
<td>CMC</td>
<td>critical micellar concentration</td>
</tr>
<tr>
<td>DOPE</td>
<td>dioleoyl phosphatidylethanolamine</td>
</tr>
<tr>
<td>DPPC</td>
<td>dipalmitoyl phosphatidylcholine</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diaminetetraacetic acid</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast Performance Liquid Chromatography</td>
</tr>
<tr>
<td>GPIb-IX-V</td>
<td>glycoprotein Ib-IX-V</td>
</tr>
<tr>
<td>GPIIb-IIIa</td>
<td>glycoprotein IIb-IIIa</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>IgG1, IgM</td>
<td>immunoglobulin γ and μ</td>
</tr>
<tr>
<td>Lipos</td>
<td>liposome</td>
</tr>
<tr>
<td>LRR</td>
<td>leucine rich repeat</td>
</tr>
<tr>
<td>MBD-PE</td>
<td>ethanolamine lipid bound to N-(7-nitro-2-1,3 benzoxadiazol-4-yl)</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PRP</td>
<td>platelet rich plasma</td>
</tr>
<tr>
<td>RGD</td>
<td>arginine-glycine-aspartate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulphate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Triton X-100, -114</td>
<td>octylphenol ethylene oxide condensed</td>
</tr>
<tr>
<td>vWD</td>
<td>von Willebrand disease</td>
</tr>
<tr>
<td>vWF</td>
<td>von Willebrand factor</td>
</tr>
<tr>
<td>WGA</td>
<td>wheat germ agglutinin</td>
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Acknowledgments:

I would like to thank all the people who have shown support and help with this project. Without them this project would not have been possible. First I would like to give a warm and grateful ‘thank you’ to my supervisor Dr. Dana Devine, for her guidance and scientific inspiration, I hope I will take many things away that I have learned from her. I would also like to thank Maria Issa who has decided to take on the challenge of this exciting project. I would like to give a ‘thank you’ to her for her encouragement and insights into the project. I would like to give a special thank you to Dr. Cedric Carter, who has been a great support and help when there were questions. Then I would like to thank the entire Devine lab ‘team’; Elena Levin, Katherine Serrano, Elisabeth Maurer, Nikky Chahal, Derek Sim, Vicky Monsalve, Jim Rupert, Susan Shinn, Amanda Bradley and Randy Dhaliwal for their kind support and friendship.

Lastly, I would like to dedicate this project to my parents and sister and thank them for their love.
Chapter I: Introduction

1.1 Project Rationale: Background

The supply of platelets and platelet products is an important component of Canada’s blood transfusion service. However, platelets have a short shelf life of 5 days after which they are out-dated. In addition, the demand for platelets is multi-fold: trauma, surgery, bone marrow malfunction due to chemotherapy or bone marrow disorders, possible platelet carrier of disease, damage by the product handling process, and sheer number (Reid et al, 1999). In addition, the current product is donor dependent and donations are not unlimited (Figure 1).

The complexity of platelets has hindered development of a platelet substitute. The platelet functions, essential to hemostasis (adhesion, secretion, activation and aggregation) have been examined by many studies, and ideas for a potential platelet substitute have been proposed.

There have been several attempts of generating platelet substitutes. Platelets frozen in 5-6% dimethyl sulfoxide have been highly effective in long-term storage but the method is time consuming and laborious (Reid et al, 1999). Some other products that have been developed include infusible platelet membrane fragments from outdated platelets (Scigliano et al, 1995), use of rehydrated lyophilized platelets (Read et al, 1995) or cryopreserved platelets (Arnaud et al, 1990). Fibrinogen coated albumin microspheres and red blood cells bearing RGD or fibrinogen protein surfaces (Agam et al, 1992 and
### Platelet Demand

<table>
<thead>
<tr>
<th>Donor Pool</th>
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<tbody>
<tr>
<td>(Public)</td>
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<table>
<thead>
<tr>
<th>Platelet Concentrate</th>
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<td>(CBS)</td>
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</table>

- **Trauma**
  - for sudden loss of blood
  - low clotting factors

- **Surgery**
  - to substitute blood loss

- **Bone Marrow**
  - megakaryocyte platelet production is low

- **Expiry**
  - 5 day maximum in storage

- **Other**
  - contaminated or otherwise unusable product

<table>
<thead>
<tr>
<th>Bone Marrow Disorder</th>
<th>Chemotherapy</th>
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<tr>
<th>Substitute</th>
<th>For</th>
<th>Against</th>
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<tr>
<td>frozed/lyophilized platelets</td>
<td>increase storage time</td>
<td>donor dependent</td>
</tr>
<tr>
<td></td>
<td>easier handling</td>
<td>disease transmission risk</td>
</tr>
<tr>
<td></td>
<td>control product amount</td>
<td>cannot replace full platelet function</td>
</tr>
<tr>
<td>frozed/lyophilized platelet fragments</td>
<td>increase storage time</td>
<td>donor dependent</td>
</tr>
<tr>
<td></td>
<td>easier handling</td>
<td>disease transmission risk</td>
</tr>
<tr>
<td></td>
<td>control product amount</td>
<td>cannot replace full platelet function</td>
</tr>
<tr>
<td>liposome based substitute</td>
<td>donor-independent</td>
<td>cannot replace full platelet function</td>
</tr>
<tr>
<td></td>
<td>increased storage time</td>
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<td></td>
<td>no matching needed</td>
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</tr>
<tr>
<td></td>
<td>ease of handling</td>
<td></td>
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<tr>
<td></td>
<td>control product amount</td>
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**Figure 1: Rationale for a Platelet Substitute.** (A) High platelet demand (trauma, surgery, bone marrow malfunction or platelet disorders) often challenges the platelet availability which is dependent on the number of donors, the platelet storage time and concentrate rejection due to unsuitable product. (B) Frozen/lyophilized whole platelets or platelet fragments still require donor compatibility and the possibility that disease is transmitted. A liposome based platelet substitute would act as a donor-independent, safe platelet “filler” which would help enhance platelet aggregation.
Coller et al, 1992 respectively) are platelet substitutes that have been shown to reduce bleeding times in thrombocytopenic animal models. A “plateletsome” was produced by Rybak et al, 1993 where this research group transferred platelet membrane proteins from a crude platelet membrane extract to liposomes. The mixture of liposomes with the platelet proteins, decreased bleeding times of thrombocytopenic rats by 67%. A plateletsome that contained only GPIIb-IIIa was not sufficient to stop bleeding.

It appears reasonable to propose that for the initial adhesion response the platelet performance is enhanced with a platelet substitute, the platelet aggregation response that follows naturally can be enhanced at low platelet count or function. When a patient has low platelet production, the number of cells that can bind exposed collagen, but once initiated by the proposed “platelet substitute”, such as a GPIb-IX liposome, the potent activation response that even a small number of platelets trigger can be sufficient to form further aggregates. Patients that will benefit from such a product include Bernard-Soulier Syndrome patients, any patients who have a low platelet count due to production defects that can be disease based (bone marrow disease) or chemotherapy or trauma induced. Such individuals have absent adhesion receptors or low platelet counts such that with an adhesion “enhancer”, their ability to aggregate would be expected to improve.

For this study, the aim was to construct a liposome carrying the GPIb-IX receptor and to show its ability to function by interacting with von Willebrand factor. This liposome model would provide a first step toward an artificial platelet substitute to control bleeding. This method of treating bleeding would address part of the problem of platelet storage (5 day maximum with a gradual decrease of platelet quality during this
time), blood transfusion related problems (donor dependent immune reactions, potential viral and bacterial infection), and platelet availability.

The GPIb-IX liposome could substitute for a part of an individual’s platelet lack of platelet function or platelet number. It will act as a “filler” to enhance the platelet response. Although the platelet substitutes developed thus far have had a very short circulating half-life *in vivo*, they can reduce bleeding time in a thrombocytopenic animal model (Rybak *et al*, 1993). Therefore, platelet substitutes may be used to replace one part of the platelet response and be used in specific clinical situations such as acute trauma as opposed to treatment of long term bleeding.

1.2 Platelet Activity: The Hemostatic Response

Platelets are small cell fragments produced in the bone marrow. They are released into the blood stream and circulate in the blood until needed to initiate the repair of a wound by the formation of a platelet plug (Wright *et al*, 1910). Platelets have been shown to be involved in various functions such as the phagocytosis of foreign particles, the activation of complement, the interaction with parasites, viruses and bacteria, the alteration of vascular tone, the enhancement of vascular permeability and the up-take, storage and metabolism of various vasoactive substances (Joseph M *et al*, 1995).

Platelets circulate in the blood at a concentration of 1.3-4.0 x 10^8 platelets/ml for 8-12 days (Aas *et al*, 1958), after which they are taken up by the spleen or liver where they are destroyed. Platelets contribute the physical plug during hemostasis (Figure 2). The granules inside the platelet, and the proteins on its membrane function to interact
with soluble coagulation factors. The coagulation factors interact to form a physical mesh (fibrin) between the platelets or they are involved in other areas of the clotting hemostatic response (complement and coagulation cascade).

1.3 Platelet Structure and Function

Platelets are produced in the bone marrow. A hormone, called thrombopoietin that is not very well characterized yet stimulates platelet production. It has been shown to be increased in thrombocytopenic patients (Tomita et al, 2000). Budding sheds small cellular fragments. The mature multinucleated megakaryocyte, produces mature anucleated discoid shaped platelets. All cellular components necessary for platelet functions are contained in this cellular fragment which makes up the mature platelet (Wright et al, 1910).

The platelet contains organelles in the cytoplasm and membrane proteins on the plasma membrane surface necessary for its function. Within the platelet are dense granules and \( \alpha \)-granules, which contain hormones and signal molecules necessary for platelet activation and aggregation. The more numerous alpha granules contain adhesive molecules (fibrinogen, fibronectin, thrombospondin), growth factors (platelet-derived growth factor) and coagulation factors (von Willebrand factor, factors V and VIII) and other proteins. Dense granules contain 5-HT, ATP, ADP, calcium and pyrophosphates. The cytoplasm contains some ribosomes and mRNA to carry out platelet protein synthesis but no nucleus is found to allow the platelet to divide. There are limited
mitochondria that allow for some of the platelet's own energy synthesis. There are also lysosomes, glycogen granules and peroxisomes randomly found in the cytoplasm.

The membrane contains various receptors, including those for coagulation and for normal platelet adhesion and aggregation: GPIb-IX-V that binds to vWF, GPIIb-IIIa that binds to vWF and fibrin/fibrinogen (McGrary et al., 1995), GPIa-IIa (Depraetere et al., 1997) and GPIV (Tandon et al., 1989) to collagen. Other transmembrane proteins that are involved in cell signaling events and other platelet functions are found on the platelet.

The platelets circulate in the blood in an inactive form until stimulated by high shear stress (Figure 2). Exposed collagen causes platelets to adhere to damaged areas of the endothelium at low shear rates through collagen receptors that directly bind the platelet to collagen (Diaz-Ricart et al., 1993 and Kainoh et al., 1992). A second mechanism involves von Willebrand factor, a multimeric protein circulating in the bloodstream. vWF becomes activated by high shear stress by undergoing a conformational change. It then appears to bind to collagen first, then GPIb-IX-V a platelet receptor expressed on the platelet membrane (Moroi et al., 1997). Glycoprotein Ib-IX-V is linked to intracellular signaling, leading to platelet shape change, platelet activation, granule release and active expression of GPIIb-IIIa on the cell surface (Yuan et al., 1999 and Andrews et al., 1998). GPIIb-IIIa binds vWF among other proteins (fibronectin and vitronectin, Thiagarajan et al., 1988) and participates in platelet aggregation after its activation (Kasirer-Friede et al., 1998). The numbers of GPIIb-IIIa (50,000 copies/platelet) molecules are more abundant than GPIb-IX-V; therefore, after the initial adhesion reaction by GPIb-IX-V (always present in an active state on the platelet), GPIIb-IIIa becomes the primary aggregation receptor after its activation (Frojmovic et al.,
1997). In addition, GPIIb-IIIa binds fibrinogen, (another clotting protein) to bind the platelets in a fibrin clot. During this process the activated platelet undergoes shape change due to several cytoplasmic protein rearrangements induced by the adhesion process (Kovacsovics et al, 1995 and Kovacsovics et al, 1996). The activation factors released by the dense and alpha granules recruit, activate and aggregate more platelets, forming the thrombus necessary to stop the blood loss from the damaged blood vessel. Platelets also exhibit an activation response to thrombin stimulation. When thrombin is generated in the coagulation reaction, platelets in the vicinity are stimulated to activate. Two receptors on the platelet function in various ways to different concentrations of thrombin: the thrombin receptor and GPIb-IX-V (Liu et al, 1997 and Greco et al, 1996).

The thrombin receptor or the ‘seven transmembrane domain receptor’ responds to moderate concentrations of thrombin (i.e., it binds thrombin with relative low affinity). Thrombin binds the receptor and cleaves a TRAP peptide, which is released from the receptor. The cleaved receptor contains a new N-terminus, which is believed to interact with the receptor to stimulate intracellular signaling. This thrombin receptor has been found to be the primary thrombin receptor of platelets (Liu et al, 1997).

GPIb-IX-V is the second thrombin receptor with a thrombin-binding site on GPIbα. This receptor is very sensitive to low concentrations of thrombin and therefore has been established as the high affinity thrombin receptor (Dong et al, 1997 and Greco et al, 1996). As for its function, it is unclear why a second receptor is required but it appears to be linked to the initial response in aggregation, because during the initial stages of aggregation, only low concentrations of thrombin are produced. Antibodies to
Figure 2: Schematic Representation of Platelet Plug Formation in Blood Vessels. (A) Represents platelets circulating in the blood until a damaged area is encountered. The blood flow becomes disrupted, stimulating platelets to aggregate. (B) GPIb-IX-V is the first receptor on the platelet to be involved in the platelet response. GPIb-IX-V binds to the endothelium via the shear-induced activation of von Willebrand factor. The platelet intracellular signaling is triggered and the platelet undergoes shape change, granule release for further activation of platelets and subsequent aggregation.
the thrombin-binding domain on GPIbα inhibit the platelet's response to low concentrations of thrombin. Once the platelet plug is under way, the thrombin receptor is the more effective receptor, leaving GPIb-IX-V free to interact with von Willebrand factor (Greco et al, 1996).

Interestingly, it has been observed that there are only approximately 50 high affinity thrombin binding sites on the platelet surface. They have functional molecular weight of 900 kDa as determined by radiation inactivation and target analysis. GPIb-IX-V appears to be found in approximately 25,000 – 30,000 copies on a single platelet. It has been suggested that several copies of GPIb-IX-V are clustered together to form a high affinity thrombin receptor and recent papers have proposed that GPV is needed to form the high affinity thrombin binding complex (Dong et al, 1997).

1.4 Properties of GPIb-IX-V

1.4.1 Overall Function of the Receptor

The platelet adhesion receptor glycoprotein Ib-IX-V (GPIb-IX-V) is necessary to initiate platelet aggregation by binding the platelet to the damaged vessel wall. A mutated GPIb-IX-V receptor (qualitative or quantitative defect) creates an autosomal recessive bleeding disorder called Bernard-Soulier syndrome. Prolonged bleeding, thrombocytopenia, and extremely large platelets characterize the bleeding disorder (Roth et al, 1991). Furthermore, it was found that the platelets could not aggregate normally when ristocetin, a peptide antibiotic known to aggregate platelets, was added to Bernard-
Soulier syndrome platelets. However, when the BSS platelets were stimulated with ADP or thrombin, they responded normally (Ruggeri et al, 1983). In the absence or mutation of GPIb-IX, but not GPV, BSS is the resultant pathology (Kenny et al 1999 and Kanaji et al, 1997).

Mutation or absence of GPV has not yet been linked to disease; therefore, the function of GPV is uncertain. However, the absence of GPV in mammalian expression systems, expressing GPIb-IX, shows reduced GPIb-IX on the surface of cells if GPV is absent versus if it is present (Calverley et al, 1995, Meyer et al, 1995). However, the relative activity remains the same for each complex. A mouse model in which a mutation of GPV was induced showed no physiological or biochemical defect (Kahn et al, 1999).

A conformational change postulated for GPIb and/or already defined for the plasma protein von Willebrand factor (vWF), will take place in the area of disrupted flow (Siedlecki et al, 1996). The conformational change is induced by the turbulent blood flow created by the damaged area. Von Willebrand factor will bind to the exposed endothelial collagen. To date it has been proposed that the conformational change occurs in von Willebrand factor alone when it is exposed to high shear rates (Siedlecki et al, 1996). vWF binds to exposed collagen which allows the collagen-dependent conformational change in vWF to then bind vWF to the N-terminal domain of GPIbα on platelets (Obert et al, 1999 and Miyata et al, 1996).
Figure 3: Schematic Representation of Glycoprotein Ib-IX-V. Each GPIb-IX-V complex consists of one GPV molecule and two of GPIbα (135 kDa), GPIbβ (27 kDa) and GPIX (22 kDa). GPIbα and GPIbβ are bound together by a disulfide bond and GPIX is associated with GPIb via ionic interactions. The presence of GPV is not required for GPIb-IX-V to be properly expressed on the surface of platelets but requires GPV for the high affinity thrombin binding function. A thrombin and von Willebrand factor binding-site (separate interactions) are found within the globular leucine rich domain of the N-terminus of GPIbα. The stem of GPIbα is highly glycosylated. GPIbβ and GPIX also contain one leucine rich repeat each. On the cytosolic domain, GPIbα contains a filamin protein linked to the cytoskeleton and a 14-3-3ζ protein linked to platelet intracellular signaling.
1.4.2 Structure of GPIb-IX-V

Each GPIb-IX-V receptor is made up of two GPIbα (145 kDa), GPIbβ (22 kDa) and GPIX (17 kDa) peptides and is complexed to one GPV (82 kDa) molecule as shown in Figure 3. GPIbα is cross-linked to GPIbβ through a disulfide linkage (Berndt et al., 1985). The GPIbαβ complex is then bound to GPIX through relatively strong non-covalent interactions forming GPIb-IX. Two GPIb-IX are bound weakly to one GPV molecule on either side of GPV forming a (GPIb-IX)2-V complex (abbreviated GPIb-IX-V in this paper).

1.4.3 Structure of GPIbα

The N-terminus of GPIbα (135 kDa, CD42b) is formed into a loop (or globular domain) that is held together partially by disulfide linkages. The loop contains 6 cysteine residues that are involved in intracellular disulfide bonds (Dong et al., 1994). Four of the cysteine molecules form disulfide bonds (CYS209-CYS248 and CYS211-CYS264) that form the loop structure of the receptor, stabilizing the vWF binding-site. The N-terminal globular domain of GPIbα also contains 7 tandem leucine rich repeats (24 amino acids in length) (LLR) that are formed into a β-α structural unit, modeled after the structure of porcine ribonuclease inhibitor, a protein that consists primarily of LRRs (Lopez et al., 1987). This LRR structure also contributes to its horseshoe-like shape. The leucine rich repeats are followed by a region of negatively charged aspartate and glutamate residues and 3 sulfated tyrosines (Murata et al, 1991 and Dong et al, 1995). The stem of GPIbα is
highly glycosylated containing O-linked sialylated hexasaccharides that are linked to the GPIb stem at an average of every 3 to 4 amino acids (Moshfegh et al 1998). The transmembrane domain consists of one transmembrane sequence, followed by a cytoplasmic tail of 96 amino acids (Lopez et al, 1987). The cytoplasmic domain is linked to an actin binding protein (ABP-280) also called filamin that binds GPIb-IX-V to the cytoskeleton and it is believed that through this interaction, GPIb-IX-V stimulation leads to platelet shape change (Takafuta et al, 1998 and Dong et al, 1997). During the purification of GPIb-IX, the interaction between filamin and GPIbα is broken with N-ethylmaleimide (Wicki et al, 1992).

Another protein interaction was found on GPIbα: the protein 14-3-3ζ is linked to intracellular signaling, and therefore suggests that GPIb-IX-V stimulation may create an intracellular signaling response (Calverley et al, 1998).

1.4.4 Structure of GPIbβ

GPIbβ (25 kDa, CD42c) has a single leucine–rich repeat on the C-terminus and is disulfide linked to GPIbα directly next to and on the outside of the platelet plasma membrane. The 34 amino acid cytoplasmic sequence contains a protein kinase A phosphorylation site at serine166 that appears to be another link to intracellular signaling propagating platelet stimulation. This phosphorylation site becomes phosphorylated with increased cellular levels of cyclic adenosine monophosphate (Wardell et al, 1989).
1.4.5 Structure of GPIX

GPIX (22 kDa, CD42a) also has a single leucine rich repeat. The interaction between GPIX and GPIbβ is through non-covalent interactions and remains associated with GPIbβ when purified in Triton X-100 (Wicki et al, 1992). GPIbβ has one transmembrane domain followed by a 5 amino acid cytoplasmic sequence (Muszbek et al, 1989).

1.4.6 Structure of GPV

GPV (82 kDa) has 15 leucine-rich repeats and a cytoplasmic tail of 16 amino acids product (Berndt et al, 1981). As mentioned, the function of GPV is uncertain but a thrombin cleavage site is present on the external side. When thrombin cleaves GPV, a GPVf₁ (69.5 kDa) fragment is created but it is unclear whether there is a function associated with this cleavage product (Berndt et al, 1981).

1.5 Properties of vWF

1.5.1 Overall Function of vWF

Von Willebrand factor is synthesized in endothelial cells and megakaryocytes, and is a plasma protein that circulates in the blood complexed to factor VIII (Rand et al, 1997). Complexed to factor VIII, it acts to prevent factor VIII from binding to
phosphatidylserine-containing membranes, where factor VIII can be degraded by activated protein C. The function of vWF therefore is twofold: 1) to prevent factor VIII from being degraded and to ensure factor VIII’s appropriate release during clotting (Vlot et al, 1998), and 2) to facilitate the interaction between platelets and the endothelium to bind the platelet plug to the damaged area (Legrand et al, 1983). Factor VIII is released from vWF by proteolytic activation by thrombin (Vlot et al, 1998).

1.5.2 Structure of Von Willebrand Factor

Von Willebrand factor (260 kDa) consists of disulfide linked monomers complexed into multimers that range from dimers to multimers with masses of up to $20 \times 10^6$ Da. The primary amino acid sequence consists of a mature protein: N-terminus-D’A1A2A3D4B1B2C1C2-C-terminus (A, B and C are homologous sequences) (Figure 4). Most cysteine residues are located at the C and N terminal ends. Two cysteine residues are found per A domain. In the A1 (CYS509 to CYS695) and A3 (CYS923 to CYS907) domains the cysteines are formed into large disulfide loops of identical size. The A2 domain contains two adjacent disulfide linked cysteines (CYS906-CYS907) (Meyer et al, 1993). The A1 domain has been found to reversibly (Fredrickson et al, 1998) bind GPIbα (amino acids 474-488, 514-542, and 694-708 appear to be involved) and collagen (amino acids 542-622) (Kroner et al, 1996 and Meyer et al, 1993). The A3 domain (amino acids 948-998 in the A3 loop) has been found to contain the primary collagen-binding domain (Pareti et al, 1987). Ristocetin has been found to interact with the proline rich, negatively charged sequence present on the outside of the A1 loop.
Figure 4: Schematic Representation of von Willebrand Factor. Von Willebrand factor is a single chain molecule with 4 D regions, 3 A regions, 2 B regions and 2 C regions. It circulates in the plasma with factor VIII complexed to the C-terminal domain to protect factor VIII from proteolysis to protein C and factor Xa. The binding site for GPIbα is in the A1 loop. Collagen binds in the A1 and A3 loops. The GPIIb-IIIa receptor binds an RGD sequence on the N-terminal domain at the sequences indicated above. (Modified from Meyer et al, 1993)
(amino acids 474-488 and 694-708) (Berndt et al, 1992 and Azuma et al, 1993). The first 1-272 amino acids (D'D3) have been found to contain the factor VIII binding site. The C1 domain contains an RGD sequence (aa1744 to 1746), which binds to activated GPIb-IIIa. The D1D2 sequence is released from vWF as the propolypeptide of the still maturing protein (Janel et al, 1997). Other binding sites to the extracellular matrix are still under discussion.

1.6 Function of the Structural Motifs Found in GPIb-IX-V

The binding sites for thrombin and von Willebrand factor of GPIb-IX-V are found in the GPIbα chain (Mazzucato et al, 1998 and Vicente et al, 1989, respectively). The extra-cellular N-terminal of GPIbα can be cleaved by calpain protease, generating the soluble peptide glycocalicin (110 kDa) which can be further cleaved by trypsin to give a 45 kDa N-terminal fragment that mimics the functional properties of the full GPIb-IX-V receptor (Lopez et al, 1987). Glycocalicin is also cleaved in platelet concentrates as a result of the storage lesion (George et al, 1992).

Recently a study by Shen et al, (2000), using canine-human chimeras of GPIbα, suggested that LRR 2, 3 and 4 in the N-terminus-7 LRR sequence are critical for vWF binding.

Yet another important feature that has been found in the vWF-binding site on GPIbα was the highly negatively charged region following the LRRs. 3 tyrosine residues (Tyr276, 278, 279) characterize the negatively charged region, which are sulfated by post-translational modifications (Lopez et al, 1994). These residues are important
because when they are eliminated in mutant GPIbα molecules, vWF binding is reduced (Dong et al, 1994). This sequence also appears to be important for the binding of thrombin (Bouton et al, 1998).

The stem of GPIbα is highly glycosylated. There are a number of serine and threonine residues that are glycosylated with O-linked carbohydrate side chains. Deglycosylation of this region has been shown to reduce the length of the stalk suggesting that the glycosylation of the stalk gives it strength to prevent it from collapsing (Moshfegh et al, 1998). The sialic acid content in this region gives the platelet surface a high negative charge and it is this negative charge that has been believed to repel the sialylated vWF and prevent it from binding a hidden positively charged binding site on vWF to the platelet in a static situation. The antibiotic ristocetin, which is often used for aggregating platelets in static conditions, functions to reduce the Zeta potential between vWF and GPIbα. Ristocetin reduces the Zeta potential with its positive charge and increases binding with its phenolic groups; the exact mechanism is unclear. The positive charge of ristocetin interacts with prolines in the A1 loop of vWF (Coller et al, 1978).

During purification procedures using an anion exchanger, the negatively charged region allows GPIb-IX-V to be purified separately from other membrane proteins. In addition, the glucosamine residues interact with the affinity column wheat germ agglutinin, which is another method used for purifying GPIb-IX-V (Wicki et al, 1992).

The GPIbα cytoplasmic domain contains binding sites for an actin binding protein that binds the α-chain to short actin filaments (Figure 3) (Takafuta et al, 1988). During activation, the cytoplasmic domain of the receptor is believed to be incorporated into the
cytoskeleton, which enables GPIb-IX-V to move through the plasma membrane more efficiently. The association with the cytoskeleton showed that later during the activation process that GPIb-IX-V is translocated to the open canicular system of the platelet (Kawano et al, 1999). As mentioned in section 1.4.3 a 14-3-3ζ molecule is bound to each GPIbα chain and has been linked to the intracellular signaling induced by GPIb-IX-V binding to vWF (Oda et al, 1995). In addition, the cytoplasmic tail of GPIbβ contains a tyrosine phosphorylation site that also has been linked to the intracellular signaling for shape change (Wardel et al, 1989). GPIbβ and GPIX have been attributed a role in the structural stabilization of the GPIbα chain and its requirement for proper expression on the platelet surface (Lopez et al, 1992).

The functional importance of GPIbαβ and GPIX is confirmed by structural mutations found in Bernard-Soulier syndrome, where their function is reduced by inadequate expression of the receptor on the platelet surface due to reduced or mutated proteins (Kanaji et al, 1997).

The physiological function, if any, of GPV has not been established to date; however, the protein has a cleavage site for thrombin to produce GPVf₁ and GPVf₂. The function of these GPV products is still unclear. There is speculation, that GPV increases the expression of GPIb-IX-V on the platelet surface but not to an extent where a pathological state has been created in its absence (Kahn et al, 1999). In addition, in recent papers it has been proposed that GPV contributes to forming the high affinity thrombin-binding site on GPIb-IX-V (Dong et al, 1997). As for the purposes of this project, GPIb-IX functions well as a receptor for vWF without the presence of GPV; therefore it is not needed when it is inserted into the liposomes.
1.7 Function of the Structural Motifs Found in vWF

Von Willebrand factor circulates in the blood complexed to factor VIII, and is also found in the subendothelium, Weibel-Palade bodies and in the α-granules of platelets and is released via several physiological stimuli. Von Willebrand factor undergoes a shape change in response to high shear stress. The shape change then allows vWF to bind to collagen, exposed by the damaged endothelium as well as to GPIb. vWF preferentially binds to collagen types I, III and VI, where as no binding is found to collagen types IV and V (Rand et al, 1991 and Sixma et al, 1997).

Von Willebrand factor is a single polypeptide of 260 kDa (mature peptide). vWF is synthesized exclusively in megakaryocytes and endothelial cells (Meyer et al, 1993). It is produced in these locations in monomers which are then dimerized and polymerized (multimers range from 500 kDa to 10,000 kDa) (Chopek et al, 1986). Structurally, vWF contains 19% carbohydrate and a high content of cysteine residues (8.3%) which are all involved in inter- and intra-chain disulfide bonds. The cysteine residues are mostly distributed to the D and B regions (Figure 4). The A-regions contain only two disulfide regions that make up the GPIb-IX-V and collagen binding sites (A1 and A3 respectively) (Titani et al, 1986). The A1 domain has binding sites for GPIb-IX-V, collagen, sulfatides and heparin. Domain A3 contains a second binding site for collagen. Also, a binding site for GPIIb-IIIa is found in the C-terminal C1 domain, overlapping an RGD sequence (arg-gly-asp), a motif found in many adhesive proteins that interact with integrin receptors (Titani et al, 1986). The D region binds factor VIII (Janel et al, 1997). Immobilization of vWF onto collagen or extra-cellular matrices or removal of sialic acid residues induces the binding to GPIb-IX-V (Crammer et al, 1999 and De Marco et al, 1981 respectively).
Addition of cofactors such as ristocetin or botrocetin induces vWF binding to GPIb-IX-V. Mutation in von Willebrand factor reduces its function, and may give rise to von Willebrand's disease (Meyer et al, 1993).

There are several types of von Willeband disease (vWD) that affect the function of von Willebrand factor or GPIb. The biochemical effect leads to a reduced (type I, IIA, and III vWD) or enhanced (most type IIB vWD) binding ability of von Willebrand factor (de Romeuf et al, 1997 and Hillery et al, 1998). The mutations in von Willebrand factor have been studied specifically as to the role of various residues on von Willebrand factor.

1.8 Liposomes in Medicine

1.8.1 Carrying Platelet Membrane Proteins

Platelet substitutes have been under development for quite some time. Platelet membrane proteins have been transferred to liposome membranes in an attempt to create liposomes carrying platelet receptors. Liposomes carrying hemostatically active agents on their surface (Rybak et al, 1993, mentioned in 1.1) or a GPIbα fragment cloned in CHO cells (Kitaguchi et al, 1999) have been produced. After I had initiated this thesis project, a group published a paper describing a liposome to which GPIbα was coupled chemically and assessed for its ability to interact with platelets (Kitaguchi et al, 1999).

The paper by Kitaguchi et al. 1999 discusses a liposome that had a fragment of the GPIbα chain, cloned in CHO cells, coupled to N-glutaryl-phosphatidylethanolamine (NGPE) lipid. The liposome was shown to aggregate alone with ristocetin and vWF in a
PA-1000 aggregometer that detected aggregation by a particle counting method using light scattering. They showed that the interaction was specific for GPIb and vWF by inhibiting the interaction using GPIb and vWF antibodies. In addition, they found that the liposomes enhanced platelet aggregation with platelets at a thrombocytopenic level of 80 x 10^6 platelets/ml.

1.8.2 Liposome Biochemistry

Liposomes are small spherical vesicles constructed of a lipid bilayer. Lipids immersed in an aqueous buffer spontaneously arrange to form liposomes, hollow spherical vesicles. Phospholipids, or lipids consisting of a polar head group and a hydrophobic tail form into a bilayer structure where the hydrophobic tails orient towards each other and the head-groups interact with the aqueous buffer. A spherical bilayer conformation is the structure favored by most lipid molecules (Lehninger et al, 1993). Bilayers rich in neutral or lipid with a size of 80 – 100 nm favor prolonged circulation in the blood (Lasic et al, 1995).

Cholesterol is a rigid molecule that is used by biological membranes to add rigidity to the membrane. Therefore, when manufacturing liposomes, cholesterol is added to add rigidity to the liposome membrane. Interestingly, liposomes containing higher amounts of cholesterol increase the circulation time of the liposome in vivo (Lasic et al, 1995).

Liposomes have been developed to a level where they are used as biomedical tools to transport membrane inserted proteins, enveloped cancer drugs or deliver DNA to
cells, plus other functions in medical treatments. Utilizing liposomes *in vivo* became of interest in the 1980's when it was discovered that including specific glycolipids and phospholipids in the liposomes, their circulation time increases *in vivo*. They have thus far been used as delivery vesicles for anti-cancer drugs and DNA targeted towards specific tissues (Lasic *et al*., 1995).

Membrane proteins were transferred from erythrocytes to small dimyristoylphosphatidylcholine (DMPC) vesicles (Bouma *et al*. 1977). Incubating liposomes with erythrocytes performed the protein transfer, allowing membrane proteins to transfer spontaneously to the liposome membrane. They reported an 80% transfer of membrane protein to liposomes.

The more widely used method at this time inserts protein into the membrane by dialysis. The protein is solubilized in detergent and mixed with the lipid in the required concentrations. The mixture is dialyzed, removing the detergent and the liposomes are formed (Helenius *et al*., 1977).
1.9 The Aim of the Project

**Hypothesis:** GPIb-IX inserted into a liposome will imitate the adhesive function of blood platelets. The specific aims were:

1. To purify GPIb-IX from outdated platelet concentrates using wheat germ agglutinin chromatography and anion exchange chromatography;

2. To determine the degree of purity of the protein;

3. To insert the protein into liposomes containing DOPE, DPPC and cholesterol lipids;

4. To assess the protein’s presence in the liposome membrane by fluorescence microscopy and flow cytometry.

5. To assess the liposome’s ability to interact with von Willebrand factor, collagen III and ristocetin and to determine whether it is likely to function with its natural ligands for possible future interaction with platelets.
Chapter II: Materials and Methods

2.1 Reagents

2.1.1 Outdated Platelet Concentrates

Outdated platelet concentrates (5-day-old) were obtained from the Canadian Blood Services center in Vancouver. The concentrates were either stored at -20°C until needed for GPIb-IX purification or they were used on the day that they were received.

2.1.2 Antibodies

Antibodies for Western blotting, fluorescence staining for microscopy, and flow cytometry were bought from Beckman-Coulter (Mississauga, Ontario, Canada). The anti-platelet antibodies purchased were CD42b-FITC (GPIbα), CD42a-FITC (GPIX), CD41-FITC (GPIIb-IIIa) and CD42b unconjugated. Control antibodies, IgM-FITC and IgG1-FITC, were also purchased from Beckman-Coulter. Monoclonal anti-vWF antibody and polyclonal goat anti-vWF were purchased from Affinity Biologicals (Hamilton, Ontario, Canada). Eight hundred micrograms per milliliter peroxidase-conjugated goat anti-mouse IgG (H+L) and FITC labeled donkey anti-goat IgG (0.3 mg/ml) were purchased from Jackson ImmunoResearch Lab. (West Grove, PA, USA).
2.1.3 Molecular Weight Standards

The broad range marker, consisted of a mixture of molecular weight ranges of 200 kDa to 6.5 kDa, was purchased from Bio-Rad (Mississauga, Ontario, Canada). The Rainbow colored protein molecular weight marker, consisted of a mixture of molecular weight ranges of 200 kDa to 14.3 kDa, came from Amersham Pharmacia (Baie d’Urfe, Quebec, Canada).

2.1.4 Aggregation Reagents

Ristocetin was purchased from Chronolog (Havertown, PA, USA) at 125mg/ml, was used at 1 mg/ml. Also, vWF was purchased from Enzyme Systems Products (Livermore, Ca, USA) and had a stock concentration of 1mg/ml. The vWF from Enzyme Systems Products consisted of vWF purified from citrated human plasma and contained monomers and multimers of vWF.

2.1.5 Column Material

Wheat Germ Agglutinin agarose column material (lectin from *Triticum vulgaris* cross-linked to 4% beaded agarose) was purchased from Sigma Chemical company (Oakville, Ontario, Canada). The mono-Q anion exchange column was purchased from Amersham Pharmacia. Extracti-gel was purchased from Pierce (Brockville, Ontario, Canada). The QAE-Sephadex was purchased Sigma.
2.1.6 Lipids for Liposomes

Freeze-dried dioleoyl phosphatidylethanolamine (DOPE), dipalmitoyl phosphatidylcholine (DPPC) and NBD labeled PE were synthetically manufactured and purchased from Avanti (Alabaster, Alabama, USA). Cholesterol was purchased from Sigma.

2.1.7 Other Reagents

Bovine lung aprotinin was purchased from Amersham Life Sciences laboratories. Human placental collagen type III (from human placenta) was purchased from Sigma. N-octyl-β-D-glucopyranoside, ULTROL grade was purchased from Calbiochem-Cedarlane Laboratories Inc. (Hornby, Ontario, Canada). The ECL Western blotting detection reagent came from Amersham, Pharmacia. The hyperfilm for Western blot detection was also purchased from Amersham Pharmacia. N-acetyl-α-D-glucosamine, for GPIb-IX elution from the WGA-agarose column was purchased from Calbiochem-Cedarlane. N-ethylmaleimide was purchased from Sigma. Bio-Rad protein assay “dye reagent concentrate” came from Bio-Rad. Albumin Standards came from Pierce.
2.2 Purification of GPIb-IX

2.2.1 Lysis of Platelets from Platelet Concentrates

The platelet lysis method used in this study was a combination of the methods published by Wicki et al (1992) and Berndt et al (1985). Ten to 25 units of platelet concentrate containing approximately 50 ml of platelets were pooled. The concentrates were obtained after 5 days of standard storage in the Vancouver blood center (CBS) under standard platelet storage conditions. The platelet concentrates are declared outdated after 5 days. The average number of platelets in a platelet unit obtained over 3 months at the CBS and represented by 50 bags/month was $74 \times 10^9$ platelets/unit and the range was $29 - 147 \times 10^9$ platelets/unit. The pooled platelet concentrate was spun at $1500 \times g$ for 15 minutes on the J2-MC centrifuge, Beckman-Coulter with a JA-20 fixed angle rotor and the platelet poor plasma was poured off. The GPIb-IX content of the supernatant and the pellet was measured by Western blot. The pellet was suspended with 1x of the plasma volume with buffer A (30 mM glucose, 120 mM NaCl, 10 mM EDTA, 30 mM sodium citrate, pH 6.5). Washing with buffer A was repeated a second time. Next, the pellet was washed with buffer B (134 mM NaCl, 10 mM EDTA, 10 mM Tris-HCl, pH 7.4) and centrifuged at 1500g for 15 minutes. For each 75 ml of platelet pellet, 100 ml of buffer C were added (124 mM NaCl, 20 mM EDTA, 2 mM N-ethylmaleimide, 10 mM Tris-HCl, pH 7.4) (Wicki et al, 1992). The solution was cooled on ice and 100 ml 2% Triton X-100 was added (Berndt et al, 1985 and Wicki et al, 1992). PMSF was added to give a final concentration of 2 mM PMSF in the lysate. The mixture was stirred
for 15 minutes and to completely disrupt the membranes, the cells were sonicated for 1 minute in the Sonic Energy Products, “Bendix” sonicator. The lysate was centrifuged at 100,000 x g for 30 minutes at 4 °C in the Beckman-Coulter Model L5-65 Ultracentrifuge using a SW41 TI rotor. The supernatant was applied to the Wheat Germ Agglutinin column for separation of glycoproteins.

The initial method used for solubilization of platelets was the procedure published by Wicki et al, (1992). This method was performed similarly to the one above except for the following changes: Two-percent Triton X-114 was added at 4°C to the washed platelets cooled to 4°C. PMSF was added to give a final concentration of 2 mM PMSF. The lysate was centrifuged once at 10,000 x g for 30 min at 4°C, followed by one centrifugation of the supernatant at 100,000 x g for 1 hour at 4°C. The supernatants (20-25 ml) from the second centrifugation were layered over 20 ml cushions of 6% sucrose, 154 mM NaCl, 1 mM EDTA, 0.06% Triton X-114, 10 mM Tris-HCl, pH 7.4. The tubes were heated to 37°C for 5 minutes in a water bath, allowing the Triton X-114 (upper) phase to become cloudy. The tubes were then centrifuged at 1000 x g for 10 minutes in a centrifuge that was not cooled. The upper phases were removed and Triton X-114 was added and stirred to give a final concentration of 1%. The sucrose centrifugation was repeated. The upper phases were pooled and Brij 98 was added to a final concentration of 0.5%. The sample was centrifuged if a precipitate formed, followed by freezing at 20°C or further purification on the WGA-agarose.
2.2.2 Wheat Germ Agglutinin (WGA) Chromatography

WGA-agarose, a lectin affinity matrix, binds proteins high in sialic acid and N-acetyl-\(\alpha\)-D-glucosamine residues. Membrane proteins often contain high amounts of glycosylation sites. GPIb-IX-V specifically contains 64% of the total sialic acid content of platelets (Moshfegh et al., 1998). This sialic acid content was exploited by Wicki et al., (1992) for the purification of GPIb-IX on a WGA-agarose column. The column was equilibrated with buffer A (0.5% Brij 98 or Triton X-100, 154 mM NaCl, 20 mM Tris-HCl, pH 7.4) applied by gravity. A five-milliliter sample of the platelet lysate was applied to a 5 ml column of WGA-agarose. The unbound material was washed through the column with 50 ml buffer A until \(A_{278}\) returned to zero and the wash was tested for GPIb-IX on Western Blot. GPIb-IX remained bound to the column via its sugar residues. Two milliliters of buffer B (0.5% Brij 98/Triton X-100, 30 mM NaCl, 20 mM TrisHCl, 2.5% N-acetyl-\(\alpha\)-D-glucosamine pH 7.4) were incubated for 10 minutes into the WGA-agarose. Then GPIb-IX was eluted from the column with 1ml fractions with buffer B until the \(A_{278}\) returned to baseline on the LKB Biochrom Ultrospec II, Fisher Scientific (Nepean, Ontario, Canada) and all the GPIb-IX eluted. The fractions were tested on Western blot. To regenerate the column, buffer B was washed off the column with 3 ml buffer A and any residual material was eluted from the WGA-agarose with 15 ml buffer C (buffer A + 2.5 M MgCl\(_2\)). The first 3 ml of buffer C were incubated in the WGA-agarose for 10 minutes followed by a remaining 12 ml of buffer C. The WGA was equilibrated with 10 ml buffer A before the next 5 ml of platelet lysate were applied.
2.2.3 Mono-Q-Sepharose Anion Exchange on FPLC

The mono-Q column is a strong anion exchanger with a positively charged resin that allows negatively charged molecules to bind to the resin at low salt concentration and allows elution of all un-charged or cationic material from the column. At high salt concentrations, the attached material is displaced and will elute.

The column was equilibrated with filtered buffer A (0.5% Brij 98, 30 mM NaCl, 20 mM TrisHCl, and pH 7.0) as published by Wicki et al 1992. One-milliliter of WGA-agarose purified GPIb-IX sample was filtered through 0.22 μm filters and applied to the column. Ten milliliters of buffer A were applied to wash all unbound protein. The flow rate was kept constant at 0.5 ml/min with a high precision solvent delivery pump supplied by the Pharmacia FPLC System, and fractions were collected into test tubes of 60 x 0.25ml volumes throughout the purification procedure. Gradually the salt concentration was increased to 1M NaCl with buffer B (filtered 0.5% Brij 98, 1M NaCl, 20 mM TrisHCl, pH 7.0) for the next 7 ml to elute the protein followed by 3 ml of decreasing gradient to re-establish buffer A conditions only. The column was equilibrated with 3 ml buffer A. In some places, where noted, this last step was omitted. The samples were stored at -20°C.

2.3 SDS-PAGE/Silver Stain

Eight- percent sodium dodecyl sulfate polyacrylamide gel electrophoresis resolving gel was used to separate proteins by their size under an electric current. The proteins
contain a uniform size to length ratio due to the presence of SDS detergent in the sample. In addition the molecules had a uniform charge, allowing the molecular weights to be the only contribution to their ability to move in the presence of an electric current. The pores of the gel contribute resistance to movement that makes it more difficult for larger proteins to move. As a result, smaller proteins travel farthest in the SDS-PAGE.

The WGA-agarose and Mono-Q fractions were run on an 8% acrylamide resolving gel (SDS-PAGE) as for the Western blot except 100 µl samples were diluted 1:1 and boiled with 2x reducing buffer (0.008% bromophenol blue, 0.125 M Tris, pH 6.8, 4% SDS, 20% glycerol, 10% β-mercaptoethanol) for 5 minutes. Forty microliters were loaded into each well. The samples were then electrophoresed as for the Western blotting procedure described in section 2.4.1, at 20 mA for 1.5 hours or until the sample reached the bottom of the gel of the Bio-Rad electrophoresis apparatus. After the electrophoresis, the gel was immersed in 100 ml fixing solution (10% acetic acid, 30% ethanol) for 2 hours. Then the gel was soaked in 30% ethanol (3 times 20 minutes) followed by two 100 ml washes in distilled, de-ionized water (dH₂O). Next, the gel was immersed in 100 ml of 0.25 mg/ml sodium dithionite for 1 minute followed by three rinses in dH₂O. The gel was then soaked in 50 ml silver stain (0.1 g AgNO₃, 3.78 µl HCHO) for 30 minutes, followed by another two rises in dH₂O. To develop the color, the gel was immersed into 100 ml developer (0.0019 g Na₂S₂O₃, 8 g K₂CO₃, 90.8 µl HCHO in 600 ml dH₂O) for 5 minutes after which the developer was changed and the development was continued until the desired color was obtained. To stop the development and for storage, the gel was placed in 50 ml of 20% acetic acid.
2.4 Preparation and Analysis of GPIb-IX Liposomes

2.4.1 Western Blot Analysis

For Western blotting, proteins were separated by SDS-PAGE. The proteins were then transferred to Bio-Rad Immobilon paper, which binds proteins, by hydrophobic interactions. An antibody that will bind the protein of choice specifically detected the protein of interest. A secondary antibody that detects the first, and is labeled with horseradish peroxidase, will give off light that can be captured on a film when activated with hydrogen peroxide.

For non-reducing conditions, 20 µl of protein sample were prepared by dilution with an equal volume of non-reducing buffer (0.008% bromophenol blue, 0.125 M TRIS, pH 6.8, 4% SDS, 20% glycerol). For reducing conditions, 50 µl of sample was diluted with 50 µl 2X reducing buffer (reducing buffer is a non-reducing buffer with 10% β-mercaptoethanol). The reduced sample was boiled for 5 minutes before loading onto the gel. In both reducing and non-reducing conditions, 20 µl or 40 µl (depending on the size of the wells) of the samples were loaded onto a 8% SDS-PAGE and the samples were electrophoresed at 20 mA/gel for 1.5-2 hours, followed by 1 hour blotting onto Immobilon paper under 300 mA. After blotting, the Immobilon paper was immersed in 5% blocking buffer (5% w/v milk powder in 20 mM Tris-HCl, 137 mM NaCl, 0.1% Tween 20, pH 7.6, or TBS-T, pH 7.6) for 1 hour. The blot was then washed by the following sequence to rinse off the blocking buffer: 2 quick rinses in TBS-T, pH 7.6 buffer, one 15 minute rinse in TBS-T, pH 7.6 followed by two 5 minute rinses in TBS-T,
pH 7.6. The membrane was then incubated in the primary antibody solution for 1 hour. The primary antibody for GPIb was an uncoupled CD42b (mouse-anti GPIbα) antibody at 1 mg/ml, which was diluted 1:5000-1:10,000 in 5ml TBS-T, pH 7.6. The blot was incubated in a sealed plastic bag and was mixed for 1 hour. After the antibody incubation, the blot was washed 5 times briefly followed by one 15-minute wash and two 5-minute washes. The secondary antibody was a goat-anti-mouse-Ig-horseradish peroxidase (0.8mg/ml) conjugated antibody (H+L), diluted 1:10,000 with TBS-T, pH 7.6. The blot was incubated as for the primary antibody for 1 hour. The blot was then washed as before with TBS-T, pH 7.6. The ECL detection was performed in the dark room with the Amersham Pharmacia ECL Western blotting detection reagent. The blot was incubated in the ECL reagent for 1 minute to activate the horse radish peroxidase. An Amersham Pharmacia hyperfilm ECL was used to detect the fluorescence emitted. The film was developed in 1:20 diluted Kodak developer. The development was stopped by dilution with tap water and the blot was fixed with 1:20 diluted Kodak Rapid Fixer solution A to visualize the presence of GPIb-IX.

2.4.2 Densitometry as a Measure of GPIb Concentration

To assay GPIb in the samples, GPIb samples were serially diluted 1:1 with reducing buffer and loaded onto a SDS-PAGE for Western blots. The intensities of the Western blot bands were measured with the Bio-Rad Gel Doc 100 densitometer and analyzed using the GEL DOC 1000/Multi-Analyst Software to determine the intensity of each band. A standard curve of GPIb was created with GPIb obtained from a platelet
lysate. Whole blood was collected in citrate tubes and was centrifuged at 140 x g for 15 minutes in the GS-6R Beckman-Coulter table top centrifuge. The concentration of platelets was measured on the Beckman-Coulter S-Plus STKR (with an average of 2 - 3 x $10^8$ platelets/ml). The PRP was diluted 1:1 with lysis buffer (120 mM NaCl, 10 mM EDTA, 2 mM N-ethylmaleimide, 2 mM PMSF, 1% Triton X-100, pH 7.4) and sonicated for 1 minute. The sample was serially diluted for preparation for Western blotting. The intensities of the platelet lysate were plotted on a graph of intensity (count x mm$^2$) versus $-\ln$(serial dilution factor). Using the published value of 25,000 GPIb-IX molecules/platelet (Coller *et al*, 1983) and a protein molecular weight of 200,000 Da for GPIb-IX, the unknown GPIb-IX concentrations were calculated. Each scan was performed 4 – 5 times and the values were averaged.

2.4.3 Bio-Rad Protein Concentration Assay

Albumin standards were diluted from 2 mg/ml to 0.0313 mg/ml, and a blank prepared from distilled water and 4 µl were pipetted into ELISA plate wells in duplicates. Four microliters of the samples to be tested were loaded in triplicates and 200 µl Bio-Rad reagent was added as instructed by the company’s protocol. The color was allowed to develop and within 2 hours the plates were read on the SLT Labinstruments 340 ATTC ELISA plate reader with the SOFT2000 SLT Labinstruments program. The protein concentrations were compared via the SOFT2000 computer program to the standard curve that was fit as a smooth curve to the $A_{620}$ of the albumin standards (see appendix 1 for sample analysis).
2.4.4 Preparation of GPIb for Dialysis

The purified protein was present in low CMC detergents; 0.5% Brij 98 (CMC = 0.1 – 0.2 mM) or Triton X-100 (CMC = 0.22 – 0.24 mM). Triton X-100 and Brij 98 are not easily removed by dialysis when forming liposomes. The protein was therefore subjected to Extracti-Gel D, a detergent removing gel, to remove the Brij 98 or Triton X-100 from GPIb-IX. The protein was then mixed with N-octyl-β-D-glucopyranoside, a high CMC detergent (CMC = 15 - 20 mM).

The column was prepared by loading two milliliters of Extracti-Gel D and the column was equilibrated with a sucrose buffer (300 mM sucrose, 10 mM HEPES, pH 7.4) which was the buffer incorporated into the liposome. Two milliliters (or equal volume to the gel bed volume) of protein was added. Then the protein passed through the Extracti-Gel but the detergent bound to the gel. Five hundred microliters of detergent-depleted protein was collected in test tubes containing 10 µl 0.144 mg/ml N-octyl-β-D-glycopyranoside. If the solution appeared cloudy, another 10 µl detergent or more were added until the sample became clear. The samples were analyzed for protein concentration using the Bio-Rad protein assay. Samples with high protein concentrations were pooled and mixed with lipid dissolved in N-octyl-β-D-glycopyranoside, ready for dialysis.
2.4.5 Lipid Preparation for Dialysis

Detergent exchange dialysis was studied extensively and is a common method for insertion of protein into liposomes (Helenius et al., 1977) with modifications as listed below. The GPIb-IX protein was inserted into 10 mol% DOPE, 65 mol% DPPC and 25 mol% cholesterol in 10 mM HEPES, 30 mM NaCl, pH 7.4. Stock solutions of DOPE, DPPC and cholesterol were each made in CHCl₃ to contain approximately 100 mg/ml lipid. The concentration of the phospholipids in the stock solution was determined using a phosphate assay (Fiske et al., 1924). The lipid phosphate was hydrolyzed at 100°C for 90 minutes by a 70% perchloric acid digestion, releasing free phosphate. The phosphate was converted to a molybdenum blue type salt with molydbate (0.22% ammonium molydbate, 0.2% sulfuric acid) solution and Fiske-Subbarow reducing reagent (15 wt% NaHSO₃, 0.5 wt% NaSO₃, 0.25 wt% 1-amino-2-naphthol-4-sulfuric acid). The samples were analyzed by A₈₁₅ measurements on a spectrophotometer.

To create the lipid mix used for dialysis, appropriate volumes of the stock solutions were added together and dried under nitrogen then residual CHCl₃ was removed under vacuum in the Labconco Freeze dry system/Freezone 4.5 L Benchtop lyophilizer, Kansas City, Missouri, USA for 4 hours. To the dried lipid, 10 mM HEPES, 30 mM NaCl, pH 7.4, was added to make a 25 mM lipid solution which was mixed with protein in the next step.
2.4.6 Dialysis of GPIb-IX into DPPC, DOPE and Cholesterol Lipids

Three hundred microliters of a 25 mM DPPC, DOPE and cholesterol mixture were mixed with 0.15 ml 1 M N-octyl-β-D-glycopyranoside to dissolve the lipid. The protein solution from 2.2.3 was mixed with the lipid/detergent solution and the mixture was injected into another dialysis cassette. For the control, 2.5 ml buffer (300 mM sucrose, 10 mM HEPES, pH 7.4) was mixed with 0.45 ml lipid/detergent solution, which was also injected into a dialysis cassette. Both samples were dialyzed at 4°C in 500 ml 300 mM sucrose, 10 mM HEPES, pH 7.4 for 2 days with 2 changes of buffer. After the dialysis, the samples were extruded with the Whitey extruder three times through 0.1 μm pore Costar Nuclepore Polycarbonate filters at 100-200 kPa N$_2$ pressure. The liposomes were then diluted in 30 mM NaCl, 20 mM TrisHCl, pH 7.4 and centrifuged in a SW41 TI swinging bucket rotor for 1.5 hours at 100,000 x g. The supernatant was removed and tested for GPIb-IX content on a Western blot. The liposome samples, contained in the pellet, were sized on the Nicomp particle sizing system (Santa Barbara, CA, USA) (Qasi-elastic Light Scattering or QELS) and analyzed with the C370 Version 12.4 - Nicomp particle sizing system program. The average particle diameter was 200 – 300 nm. The Nicomp analysis was used to obtain an average diameter of the liposomes. Also, the lipid concentration of the liposomes was assessed by phosphate assay.
2.5 Assessment of GPIb-IX on the Surface of Liposomes

2.5.1 Western Blot Analysis on Liposomes

The same Western blotting procedure was used as in 2.4.1. Ten microliters of GPIb-IX liposomes were diluted 1:1 with non-reducing buffer and run on SDS-PAGE, in an 8% acrylamide resolving gel under non-reducing conditions. The ECL detection of the incorporated protein was performed in the dark room as described in section 2.4.1 also.

2.5.2 Dot Blot Analysis

For fast analysis to confirm the presence of GPIb in samples, samples were loaded onto the Bio-Rad Bio-Dot Apparatus at concentrations calculated to be appropriate to give a detectable signal. The samples were blotted onto a nitrocellulose membrane by vacuum suction. The membrane was then immersed in 5% blocking buffer as in Western blot analysis. The subsequent steps were the same as in the Western blotting analysis in section 2.4.1. ECL was used to detect bound antibody on the nitrocellulose membrane. A positive control consisted of previously purified GPIb-IX at 23 μg/ml and a negative control consisted of a liposome control (lipid only) at approximately 2 mM lipid.
2.5.3 Flow Cytometry

Flow cytometry is a method that measures cell parameters as they pass through a laser beam one at a time in a laminar flow. In addition photomultiplier tubes detect fluorescence emission from fluorophors (FITC excitation = 460 nm, emission = 520 nm) on cells.

To prepare the liposomes for flow cytometry, GPIb-IX liposomes and the control liposome were diluted to give a 0.5 mM lipid concentration. Twenty microliters 50 μg/ml CD42b-FITC or control IgG1-FITC (50 μg/ml) were incubated with the liposomes for 30 minutes and the samples were run on the Beckman-Coulter Epics XL-MCL flow cytometer to collect 5000 to 10000 counts (1 count represents one event detected). The graphs were overlapped for comparison in the multigraph option of the Beckman-Coulter software.

2.5.4 Fluorescence Microscopy

The samples were incubated as for flow cytometry section 2.5.3. They were then viewed by microscopy using a Nikon microscope fitted with a B-2A filter to observe the fluorescence emission at 520 nm. The sample was excitated between 450-490 nm. The microscope used a Nikon super high-pressure mercury lamp power supply (EPI fluorescence). FITC has a fluorescence excitation between 465-490 nm. The samples were viewed under the 100 x oil magnification (total magnification with the ocular lense
is 1000 x). Pictures were taken with the Nikon Lab phot 2A camera and were analyzed in Adobe Photo Shop.

2.6 Functional Assessment of GPIb-IX on the Surface of Liposomes

2.6.1 Sample Aggregation and Preparation for the Flow Cytometry

Five hundred μM lipid liposomes were incubated with 20 μl 25 μg/ml vWF (Enzyme Systems Products), 10 μl 1 mg/ml ristocetin (Chronolog) and 25 μl 1 mg/ml collagen type III (Sigma) for 15 minutes. The samples were shaken on a shaker during this time. After the aggregation, 30 μl polyclonal goat-anti-von Willebrand factor antibody was added for 30 minutes. The incubation was on the same shaker to agitate the sample. A donkey anti-goat IgG antibody (FITC labeled) incubation for 30 minutes in the dark followed the antibody incubation on the shaker; the assay volume was 215 μl. Finally the volume was made up to 500 μl and the sample was analyzed on the flow cytometer.

Various controls were included in the experiment: a liposome control containing no protein aggregated with von Willebrand factor, collagen type III and ristocetin, GPIb liposome controls aggregated with various combinations of ingredients, where one or more ingredients where omitted (as indicated for each experiment in the Results section).
2.6.2 Flow Cytometer Analysis

The samples were run on the Flow Cytometer and the forward scatter (size of the vesicles) and the side scatter (granularity of the vesicles) data was collected until 5000 – 10000 counts were reached. The data was analyzed on a histogram graph where number of vesicles having a certain level of fluorescence (mean fluorescence) was compared to the number of vesicles having greater than a determined level of fluorescence (percent fluorescence). Two parameters were set to analyze the data: 1) The mean fluorescence was gated on the intensity of the control (non-aggregated liposomes) peaks and the software displayed mean fluorescence as Mnl X. The test samples were compared to the control 2) To determine the percent fluorescence of the vesicles that exceeded baseline fluorescence of the FITC positive cells. A gate was set during the control run (containing antibody + control liposome only) to give approximately 5% - 15% positive depending on the nonspecific binding in the control sample.

2.6.3 Nicomp Analysis

Liposomes were aggregated as in section 2.6.1 and their diameters were measured in the QELS system. The Nicomp distribution measures the diameter of single particles and accumulates the data into histograms.
Chapter III: Results

3.1 Purification of GPIb-IX-V

3.1.1 Lysis of the Platelets from Platelet Concentrate

The platelet concentrates were pooled and the volume measured. This volume and the purification fold of the isolation procedure are shown in Table 1B and the tabulated concentrations calculated from the densitometry analysis are shown in Table 1A (discussed later in this thesis). For the first two purifications, Triton X-114 was used to lyse the cells. Triton X-114 has an unique property compared to other detergents, in that GPIb-IX separates into the aqueous phase rather than the Triton X-114 phase, which also separates GPIIb from GPIb (Wicki et al, 1992).

Compared to the subsequent method, in which Triton X-100 was substituted for Triton X-114, the Triton X-114 method had a reduced yield because several centrifugation steps were involved that required transfer of the lysate. In particular, sample loss occurred when the supernatant was transferred from the sucrose cushion, because not all of the supernatant was transferred to avoid mixing phases. Similarly, when the platelet debris was pelleted after lysis, the entire sample was not transferred. In addition, there were several more transfer steps from test tube to test tube than was the case in the Triton X-100 purification. To reduce the loss of protein, the Triton X-100 method with less chance for loss and a simpler protocol was needed.
Table 1A: Summary of Densitometry Measurements and Average Concentrations of GPIb During Purification

<table>
<thead>
<tr>
<th>Scan Number</th>
<th>Supernatant (ng/10 μl)</th>
<th>Pellet (ng/10 μl)</th>
<th>Pure GPIb (ng/10 μl)</th>
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<tbody>
<tr>
<td>1</td>
<td>48.3</td>
<td>922.1</td>
<td>165.9</td>
</tr>
<tr>
<td>2</td>
<td>35.7</td>
<td>632</td>
<td>205.6</td>
</tr>
<tr>
<td>3</td>
<td>41.9</td>
<td>1179.9</td>
<td>133.2</td>
</tr>
<tr>
<td>4</td>
<td>29</td>
<td>449.9</td>
<td>285</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>-</td>
<td>366.9</td>
</tr>
<tr>
<td>Average</td>
<td>38.7±10</td>
<td>796.0±300</td>
<td>231±100</td>
</tr>
</tbody>
</table>

Table 1B: Protein Purification Table for GPIb from 25 Outdated Platelet Bags: 3rd Purification

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Volume (ml)</th>
<th>Tot. GPIb (mg)</th>
<th>GPIb Conc. (μg/ml)</th>
<th>Protein Conc. (mg/ml)</th>
<th>Tot. Prot. (mg)</th>
<th>Purification Fold</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet Concent.</td>
<td>1400</td>
<td>3.26</td>
<td>2.33</td>
<td>67</td>
<td>94000</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Supernatant</td>
<td>600</td>
<td>2.33</td>
<td>3.88</td>
<td>2.7</td>
<td>1600</td>
<td>96</td>
<td>71</td>
</tr>
<tr>
<td>Pellet</td>
<td>10</td>
<td>0.796</td>
<td>79.6</td>
<td>2.1</td>
<td>21</td>
<td>*</td>
<td>24</td>
</tr>
<tr>
<td>Pure GPIb</td>
<td>40</td>
<td>0.925</td>
<td>23.13</td>
<td>0.13</td>
<td>5.2</td>
<td>180,000</td>
<td>28</td>
</tr>
</tbody>
</table>

Table 1: Tabulated Densitometry Measurements of 10ul Sample Loaded into Wells on Western Blot and Protein Purification Results. (A) Calculated concentrations at each purification step are averaged and compared in (B). The final GPIbIX “pure” product recovered 28% (0.925mg) total GPIbIX with a purification fold of 180,000 times. The GPIbIX purified represents about 18% of the samples’ protein concentration.
Figure 5: Western Blot and Silver Stain of the Purification of GPIb-IX Under Reducing Conditions. Lanes 1-4 on the Western blot correspond to lane 6-9 on the silver stained gel. Lanes 1 & 6: Supernatant containing GPIb-IX of the platelet lysate. Lanes 2 & 7: Wheat germ agglutinin purified sample. Lanes 3 & 8: FPLC purified sample. Lanes 4 & 9: Broad-range molecular weight standard. Lane 5: Pellet after the lysate was centrifuged. Platelet lysate (lanes 1 & 6) shows the degree of protein diversity in a platelet lysate, which is far from a purified product. A 130 kDa band appears which corresponds to the molecular weight of GPIbα. The Western blot confirms the presence of GPIbα, which traveled the same distance in samples that were electrophoresed in parallel. Possible plasma contaminants include albumin (67 kDa), α-1-antitrypsin (45 kDa) and other platelet proteins.
The combination of theoretical approaches from Wicki et al (1992) and Berndt et al (1985) was used. The Triton X-114 used by Wicki et al, (1992) used the property of GPIb-IX that allows it to separate from other membrane proteins, by their separation into the Triton X-114 phase, whereas GPIb-IX separates into the aqueous phase. The method by Wicki et al, (1992) requires two sucrose centrifugations to separate Triton X-114 proteins from GPIb-IX (Table 2) which resulted in considerable loss of GPIb-IX. Berndt et al (1985) used Triton X-100 to solubilize the platelet membrane where GPIb-IX separates into the Triton X-100 phase. There were no extra centrifugation steps added for better separation; in addition, GPIIb-IIIa was not separated from the lysate. A test run, where only one bag was used to test each procedure showed that at the end, the Wicki method alone gave a 59% yield where as the combined method gave a 72% yield. The Triton X-100 became the lysis method of choice.

The purity and the identity of GPIb of the lysis step was analyzed on SDS-PAGE and Western blot (Figure 5) under reducing conditions. The Western blot shows a band at 130 kDa, which corresponds to the molecular weight of GPIbα. The SDS-PAGE shows a platelet lysate with a great range of different proteins.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet Wash</td>
<td>3 washes</td>
<td>3 washes</td>
</tr>
<tr>
<td>Platelet Lysis</td>
<td>Triton X-114, stirring</td>
<td>Triton X-100, sonication, stirring</td>
</tr>
<tr>
<td>Centrifugation</td>
<td>1 Sucrose centrifugations 2 additional centrifugations</td>
<td>1 centrifugation</td>
</tr>
</tbody>
</table>

Table 2: Comparison of Platelet Lysis Methods by Wicki et al, 1992 and the Method Used for this Paper. The comparison of purification methods shows that the Triton X-114 method used more purification steps, which caused protein loss.
3.1.2 The Wheat Germ Agglutinin Affinity Chromatography

The Wheat Germ agglutinin column consisted of a 5-10 ml column bed. The column was equilibrated with 0.5% Brij 98 or % Triton X-100, 154 mM NaCl, 20 mM Tris-HCl, pH 7.4. For each run 10 – 15 ml platelet lysate was added to the column, depending on the flow rate. Some debris left from the lysis affected the flow rate, clogging the column somewhat. The solution was allowed to pass through the column and the eluant was collected and assayed for GPIb on a non-reducing Western blot (shown in Figure 6A). The column was then washed with 50 ml washing buffer (0.5% Brij 98/Triton X-100 for the last purification, 154 mM NaCl, 20 mM Tris-HCl, pH 7.4), after which the baseline returned back to the original OD\textsubscript{278} of the buffer control. The wash was also assayed on a Western blot for GPIb (Figure 6A). The wash showed a negative Western blot for GPIb indicating that that protein bound to the column. Later runs of lysate on WGA showed that some GPIb-IX eluted in the wash, which was attributed to column degradation after many runs. The GPIb-IX loss reduced the total yield of protein.

After the 50 ml wash, the bound protein was displaced with 10-15 ml 2.5% N-acetyl-\(\alpha\)-D-glucosamine in 0.5% Brij 98/Triton X-100, 30 mM NaCl, 20 mM Tris-HCl, pH 7.4 or until there was no more detectable protein as monitored by \(A_{278}\) and Western blot analysis. Figure 6B shows the elution profile at this point of the purification on WGA-agarose. Figure 6A shows a Western blot and as SDS-PAGE to show the presence and the purity of the protein, respectively. The peak sample came off at about 4 – 7 ml of the elution volume which is in the range of the bed volume showing that the displacement occurred immediately upon N-acetyl-\(\alpha\)-D-glucosamine addition. No other peaks were
Figure 6: Wheat Germ Agglutinin Elution Profile. (A) Non-reducing conditions for the Western blot keep the disulfide bond between GPIbα and GPIbβ intact and the band appears at 170 kDa. Anti-GPIbα (CD42b) was used to detect the protein in each fraction. Lane 1: WGA-agarose column matrix, Lane 2: WGA wash in later experiments showing protein lost at this time Lane 9: GPIb positive control. Fractions 2-7 correspond to fraction volumes in (B). (B) WGA elution profile showing fraction volume compared to $A_{278}$ protein absorbance. A peak eluted between fraction volume 3-5 ml.
observed in the N-acetyl-α-D-glucosamine fractions, therefore it appears as if the fraction was clean of proteins that had no interaction with WGA-agarose. However, there may have been other platelet and plasma proteins present that interacted with wheat germ agglutinin that would not have been identified as different since WGA binds all carbohydrate containing proteins. As to the possible contaminants, the SDS-PAGE in Figure 5 shows additional bands at 100 kDa, 65 kDa, 55 kDa. The identity of these additional bands could be glycocalcin or GPIIIa (110kDa) (Phillips et al, 1991), or any glycoprotein.

Looking at some other blots and graphs of purification on the WGA-agarose, some samples have the elution peak at the same point as the Western blot does. Therefore, it appears on balance that the protein elution peak corresponds to the Western blot peak.

In addition to possible sources of protein loss, the WGA column material was also analyzed on Western blot after the purifications were done and a relatively intense peak appeared on the blot indicating that some GPIb-IX remained bound to the column after N-acetyl-α-D-glucosamine elution. It can be noted in figure 6, lane 1 that the column matrix contained a molecular weight band at 110 kDa which could be attributed to glycocalcin.

3.1.3 The FPLC Anion Exchange Chromatography

For the Triton X-114 preparations, following purification on WGA column, the samples containing GPIb-IX, as determined by Western blotting, were pooled and
prepared for FPLC chromatography. Wicki et al (1992) described a procedure for FPLC analysis where the sample and all buffers were adjusted to pH 7.0 to bind GPIb-IX to the positively charged column. A low salt buffer (0.5% Brij 98, 30 mM NaCl, 20 mM Tris-HCl, pH 7.0) was used to bind the proteins and subsequently to wash the column to remove unbound protein. The washing was carried out until the baseline absorbance at A$_{278}$ returned to original values after which the salt concentration was gradually increased from 30 mM NaCl to 0.6 M NaCl as shown on Figure 7B. A protein peak was eluted in fraction volume 10 – 12.5 ml, which was tested on Western blot for GPIb. The Western blots in Figure 7A show the presence of GPIb and the positive fractions were pooled for further experiments. Comparing the Western blot in Figure 7A and the elution profile in Figure 7B, the GPIb signal corresponds with elution volume 11.0 to 12.5 ml respectively.

Finally, the pooled samples were concentrated with an Amicon concentrator. In the case of the third purification (no FPLC step); the sample was concentrated to 30 ml (20x concentration) after the WGA purification. The purity of GPIb at each point in the purification is shown in Figure 5. Although protein bands are seen in the platelet lysate, after the WGA purification a great degree of purity was established as compared to the platelet lysate. After the FPLC column, even less contamination was present in the sample. As the purity of the protein sample after the WGA was sufficient to collect material for the liposome-inclusion, the FPLC step was considered optional.

The final product was stored in aliquots at -20°C until used for further experiments.
Figure 7: FPLC Anion Exchange Elution Profile: (A) Non-reducing conditions for the Western blot keep the disulfide bond between GPIbα and GPIbβ intact and the band appears at 170 kDa. Lane 2 molecular weight markers. Lane 3 positive control of purified GPIb-IX. Lanes 4-8 contain fractions 1 ml to 2.5 ml collected in 0.5 ml volumes. Lanes 9-14 contain fractions 4-9 ml (1 ml volumes). Lane 17 positive control. Lanes 18-29 contain fractions 9.5 to 15 (0.5 ml volumes). (B) The above mentioned volumes correspond to the volumes on the FPLC elution profile. The GPIb-IX sample came off after 11-13 ml elution volume between 0.5 M to 0.6 M NaCl. 1 ml sample was added for each run. A total of 15 ml volume was eluted until the program ended. The salt gradient was kept constant at 0 M NaCl until 5.5 ml volume had eluted after which it was gradually increased to 0.6 M up until 12.5 ml had eluted. Finally, the salt gradient returned back to 0 M.
3.1.4 Purification Table and Densitometry

To summarize initial experiments, GPIb was purified three times over the course of this study work to have sufficient protein stock for use in experiments. In the Triton X-114 purifications, 10 platelet concentrates were used, and for the Triton X-100 purification 25 platelet concentrates were used. Each bag contained approximately 50 to 55 ml platelet concentrates. The first two times, the method by Wicki *et al* (1992) was used to purify GPIb-IX from the lysed platelets. For the third purification a modified method was used from Wicki *et al* (1992) and Berndt *et al* (1985) and Table 1B shows the results of this purification. Both purifications used Wheat Germ Agglutinin (WGA-agarose) as a purification step after the platelet lysis. The first two purifications used a Mono-Q anion exchanger on an FPLC machine in addition to the WGA column. For the third purification, the quality of the purified product was assessed after the WGA chromatography and it was concluded that the protein was pure enough to insert into liposomes.

Table 1 shows the purification table calculated for the last GPIb-IX purification (excluding the FPLC purification). Twenty-five platelet units gave 1400 ml of platelet concentrate at 1x 10^8 platelets/ml. With an average number of platelets of Each platelet contains approximately 25,000 GPIb-IX-V molecules (Coller *et al*, 1983) and each platelet concentrate has a concentration of 1x 10^{11} platelets/L. Taking these numbers into account and the molecular weight of GPIb-IX being approximately 200 kDa and Avogadro's constant (6.0221 x 10^{23} molecules/mole), each concentrate is expected to have 1.163 mg to 1.395 mg of GPIb-IX. For each sample, the initial concentration of
Figure 8: Densitometer Estimation of GPIb-IX Concentrations during Purification. (A) Western blot of the lysate supernatant under non-reducing conditions showed a GPIb at 170 kDa. Anti-GPIbα (CD42b) was used to detect the protein in each fraction. (B) is the corresponding standard curve of the GPIb intensities obtained from the densitometry measurements. Lane 1-6: serial dilution of Platelet lysate as indicated. Lane 7-11 serial dilution of unknown GPIb sample (lysis step of purification). A positive control is found at the end.
GPIb-IX in the platelet lysate was determined using densitometry to calculate the relative intensities of the bands on a Western blot. Figure 8 shows an example of how the concentration of GPIb-IX was obtained using Western blotting and densitometry. The standard curve was derived using lysed PRP obtained from a donor. The sample was diluted 1:1 with Western blotting non-reducing buffer and the lysate was run as a standard against the unknown GPIb-IX sample on an electrophoresis gel. A Western blot for the supernatant in the lysate to be purified is shown in Figure 8A.

The blot was scanned with the Bio-Rad Gel Doc 100 densitometer and analyzed with the GEL DOC 1000/Multi-Analyst Software to determine the intensity of each band. The negative ln(dilution) of the standard was plotted as a standard curve against the intensity (count x mm²) of each band on Western blot. A concentration for each unknown was calculated by extrapolation of values from the standard curve. This procedure was performed four to five times for each blot to obtain a better average (Table 1A). The calculated results for the platelet concentrate, platelet lysate, platelet pellet and the pure GPIb-IX concentration are tabulated in Table 1B. The "platelet concentrate" GPIb-IX sample concentration tabulated in Table 1B was obtained by measuring the platelet concentration of PRP derived from a donor and taking into consideration molecular weight and concentration of GPIbIX/platelet.

The overall protein concentration was also determined for each step of the purification procedure. The Bio-Rad protein concentration assay was used to determine the concentration. For samples that contained high amounts of Triton X-100 or N-acetyl-α-D-glucosamine, the sample was dialyzed for 1-2 days or until the control buffer (containing no protein) had no color change with the Bio-Rad reagent. The samples were
diluted accordingly to fall within the range of the albumin standard curve. Table 1B shows a drastic reduction of total protein concentration with each purification step and a great increase in purification fold of GPIb-IX.

Table 1B shows that the GPIb-IX concentration increases throughout the purification as expected. Noteworthy is the GPIb-IX concentration discarded in the pellet, which constituted a relatively large proportion of GPIb-IX contained in the sample at about 0.796 mg of GPIb-IX (24% of total GPIb-IX) in a 10 ml pellet. The final yield of GPIb-IX was 28% (0.925 mg) with a purification fold of 177804 times purity (0.925 mg GPIb-IX in 5.24 mg protein for the final sample vs 3.256 mg GPIb-IX in 93.85 g of protein in platelet concentrates).

3.2 Insertion of GPIb-IX into Liposomes

3.2.1 Extracti-gel D to Remove Brij 98/Triton X-100 from GPIb-IX and Exchange it into N-octyl-β-D-glucopyranoside

Two milliliters of GPIb-IX extract was applied to 2 ml of the Extracti-Gel. The protein eluted in the void volume and was collected in 0.5 ml test tubes containing 10 µl of 0.144 mg/ml N-octyl-β-D-glucopyranoside. For most samples, the sample was cloudy and more N-octyl-β-D-glucopyranoside was needed and was added within minutes to clear the solution. The peak protein fractions, verified by Bio-Rad protein concentration assay, were pooled and were stored for dialysis. The protein containing fractions ranged in concentration between 0.2 – 0.3 mg/ml for the pooled GPIb fractions.
3.2.2 Dialysis of GPIb-IX and Control Liposomes

Phosphate was assayed to obtain a standard curve from which unknown phosphate concentrations were calculated as shown in Table 3. Each lipid molecule contains one phosphate, therefore the relationship between standard and unknown phosphate samples is direct (Table 3B). Cholesterol content was estimated from stock cholesterol concentrations, no direct assay was done to measure this. A sample calculation of the 25% cholesterol, 65% DPPC and 10% DOPE liposomes is shown in Table 3C.

The lipid, protein and detergent were mixed and dialyzed to allow liposomes to form. A cloudy precipitate-like product formed within the first two to three hours of dialysis but the sample was dialyzed for another 2 days with 3 changes of 500 ml buffer to allow the samples, N-octyl-β-D-glucopyranoside to be reduced $1.25 \times 10^8$ times (~50 nm N-octyl-β-D-glucopyranoside). The larger liposomes created a much denser and cloudier mixture than smaller liposomes. The size of the liposomes appeared to be bigger when protein was present. The control liposomes always sized between 200 – 300 nm with a narrow size and the GPIb-IX liposomes were sized to 200-300 nm by extrusion through 0.1 μm filters.

3.3 Assessment of GPIb-IX on the Liposome Surface

3.3.1 Fluorescent Microscopy

At one point, a GPIb-IX liposome sizing at 2 μm in diameter was produced. This
(A) Tabulated $A_{815}$ values obtained for the standards. (B) Standard Curve plotted from the standards and (C) unknown concentration was extrapolated from the standard curve. Final concentrations are calculated with Excel equations. Cholesterol content in liposomes was estimated from the stock concentration.

### Table 3: Phosphate Assay Result Calculation for Phospholipid Concentration Measurement

<table>
<thead>
<tr>
<th>Sample set</th>
<th>$A_{815}$</th>
<th>$PO_4$ (nmol)</th>
<th>Avg (nmol)</th>
<th>%PO$_4$</th>
<th>vol (uL)</th>
<th>[Lipid; mM/L]</th>
<th>Sample</th>
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<tr>
<td>A</td>
<td>0.061</td>
<td>16.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>control - feb14</td>
</tr>
<tr>
<td>A</td>
<td>0.063</td>
<td>17.6</td>
<td>17.3</td>
<td>75</td>
<td>10</td>
<td>2.30</td>
<td>control - feb14</td>
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<tr>
<td>B</td>
<td>0.057</td>
<td>15.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>control - feb14</td>
</tr>
<tr>
<td>B</td>
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<td>16.9</td>
<td>16.3</td>
<td>75</td>
<td>10</td>
<td>2.18</td>
<td>GPIb - feb 14</td>
</tr>
<tr>
<td>C</td>
<td>0.091</td>
<td>26.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GPIb - feb 14</td>
</tr>
<tr>
<td>C</td>
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<td>27.6</td>
<td>27.0</td>
<td>75</td>
<td>10</td>
<td>3.59</td>
<td>GPIb - feb 14</td>
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<td>D</td>
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<td>26.0</td>
<td>26.8</td>
<td>75</td>
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Figure 9: Light Microscope and Fluorescent Microscope Images of GPIb-IX Liposomes and Control Liposomes. (A) Light microscope image at 1000 x magnification of 400 nm control liposomes (lipid only). (B) Anti-CD42b-FITC stained control liposomes at 450-490 nm fluorescence excitation. (C) Light microscope image at 1000 x magnification of 2 μm GPIb-IX liposomes. (D) Anti-CD42b-FITC stained GPIb-IX liposomes at 450-490 nm fluorescence excitation.
liposome was viewed under the fluorescence microscope using anti-CD42b-FITC. Figure 9 shows the light microscope images and the fluorescent images. The GPIb-IX liposomes were about 5x larger than the control liposomes. In subsequent experiments it was not possible to photograph the liposomes because the liposomes had been extruded through 0.1μm pore-size filters and were therefore too small to photograph. The smaller 200 nm GPIb-IX liposomes did fluoresce under ultraviolet light but it was not possible to capture them on camera. Therefore, the 400 nm control liposomes in Figure 9 would have fluoresced, when viewed through the 1000 x lens, if they had had a significant degree of non-specific binding of the antibody.

3.3.2 Flow Cytometry to Assess the Presence of GPIb-IX on Liposomes

The liposomes were diluted to give a count rate of 20 – 50 events/sec (dilution to a concentration of approximately 0.2 mM) on the flow cytometer. The program was set up with a gate around the liposome population (the only population). The gate was set on the control sample to allow approximately 1% fluorescence. The test samples were run following the controls and the % fluorescence was compared. Figure 10A shows the overlaid histograms obtained for 4 samples of liposomes. The GPIb-IX liposome sample + CD42b (anti-GPIbα)-FITC was compared to 3 controls: control liposomes + IgG1-FITC (non-specific antibody), control liposomes + CD42b-FITC and GPIb-IX liposomes + IgG1-FITC. The samples that were incubated with the IgG1-FITC control antibody showed very low binding of antibody (0.39% for the GPIb-IX liposomes and 1.55% for
Figure 10: Flow Cytometry of Liposomes to Detect the GPIbα and GPIX Epitopes.
(A) Control and GPIb-IX liposomes were incubated with IgG1-FITC and monoclonal anti-CD42b-FITC. The histogram shows 84.7% fluorescence as graphed on (B) compared to the control samples with less fluorescence. Higher absorbance in the control+CD42b-FITC was attributed to non-specific binding of anti-CD42b-FITC to liposomes. (C) and (D) are control liposome and GPIb-IX liposome histograms respectively, incubated with anti-CD42a-FITC each showing the presence of GPIX on GPIb-IX liposomes. (E) Dot blot showing the presence of GPIb in the liposome sample.
the control liposomes) as compared to the control liposome incubated with CD42b-FITC (16.2%). The test sample, as expected, had the most binding at 84.1% (Figure 10B). A similar flow cytometric experiment was run for the same liposomes using CD42a-FITC (anti-GPIX) to detect GPIX on the liposome surface (Figure 10C and D). There was also increased binding on these liposomes as compared to control liposomes, confirming the presence of GPIX in the liposomes.

3.3.3 Comparison of GPIb-IX vs GPIIb-IIIa on the Surface of Liposomes

Figure 11 shows the relative amount of GPIb-IX detected on the liposomes to the relative amount of GPIIb-IIIa. GPIIb-IIIa is also a glycoprotein, which exists, in large amount on the platelet plasma membrane and it has also been found to co-purify in the GPIb-IX fraction. Figure 11 gives a measure of GPIb and GPIIb on liposomes (a GPIb-IX liposome control incubated with IgG1-FITC was used to standardize the results: GPIb-IX liposome incubated with IgG1-FITC had a fluorescence of 8.82% for the GPIIb graph and 0.27% for the GPIb graph). Comparing Figure 11A and 11B it is clear that the amount of GPIb-IX is higher than the amount of GPIIb (CD41). For the anti-GPIb antibody, 0.25 to 1.5 μg antibody was incubated in the assay, whereas the anti-GPIIb antibody ranged from 1-3 μg. The saturation for CD42b on GPIb-IX liposomes is at about 0.5 mM lipid and 30 μl antibody (data not shown). At 1 μg anti-GPIb antibody 12.3% of the GPIb-IX liposomes fluoresced, where as only 1.68% GPIb-IX liposomes fluoresced with anti-GPIIb antibody. The controls were surprisingly different for CD42b and CD41 bound to control liposomes. It would be expected that the controls are less
**Figure 11: Surface Epitope Binding of CD42b (anti-GPIbα) and CD41 (anti-GPIIb).**

(A) Increasing volume of 50μg/ml CD42b-FITC compared to (B) increasing volumes of 100μg/ml CD41-FITC on the surface of GPIb-IX liposomes shows that GPIb-IX is present in greater amounts as compared to respective control samples. IgG1-FITC was used as a standardization control for all samples.
fluorescent than any of the test samples; but the control, incubated with CD41 was surprisingly high. The trend with the GPIb-IX liposomes incubated with CD41 was as expected, and increased gradually.

3.4 Aggregation of GPIb-IX Liposomes

GPIb-IX liposomes were aggregated under static conditions by incubation with 2.33 μg/ml vWF, 46.5 μg/ml ristocetin, and 116 μg/ml collagen. A polyclonal anti-vWF antibody was chosen over a monoclonal in order to increase signal detection of vWF binding to the liposomes. A secondary antibody coupled to FITC was used to detect the goat anti-vWF antibody by the flow cytometer. Figure 12C shows that the resulting histograms overlapped for control liposomes and GPIb-IX liposomes incubated with the above reagents. Both mean fluorescence, representing the intensity of fluorescence of a population of liposomes and % fluorescence, representing the number of fluorescent vesicles in a sample, were compared. Figure 12B and 12C show the average of 4 experiments for 0.5 mM liposomes and 3 experiments for 2 mM liposomes respectively. The graph comparing control and GPIb-IX liposomes at 2 mM liposome concentration and representing % fluorescence showed statistically significant vWF binding, and possible aggregation (p<0.05 (p=0.0193), two-sample, one tailed paired t-test). Percent fluorescence of the 0.5 mM GPIb-IX liposome sample appeared to have relatively more vWF binding than the control but this result was not statistically significant (p>0.05).
Figure 12: Concentration Dependent vWF binding of Control and GPIb-IX Liposomes. (A) 0.5 mM liposomes bound vWF with 2.33 μg/ml vWF, 46.5 μg/ml ristocetin, 116 μg/ml collagen type III. An average of 4 data sets is shown normalized to the control liposomes. Error bars represent 1 SD. (B) 2 mM liposomes were incubated with 2.33 μg/ml vWF, 46.5 μg/ml ristocetin, 116 μg/ml collagen type III. An average of 3 data sets is shown. Mean and % fluorescent values are measured as shown in (C); however, % fluorescence appears to be the better measurement to detect binding. The % fluorescence of 2 mM liposomes appears to be statistically significant showing vWF binding to liposomes. (*) Represents statistical significance at (p < 0.05).
Overall, % fluorescence was a better measure of the vWF-binding event than mean fluorescence.

Figure 12 shows that the GPIb-IX liposomes had a second fluorescent peak as did all the other samples included in this average. However, this second peak appeared to be representative of liposomes bound to vWF but not aggregated. When the same liposomes were viewed under the microscope to look for aggregates, no significant change of the level of aggregates was seen which suggests that the vWF binding seen by the flow cytometer experiments was detecting single liposomes bound to vWF, not actual aggregates of liposomes. Further analyses that support this finding include the quasielastic light scattering measurements shown in Figure 13. There was no significant increase of particle size between GPIb-IX liposomes alone and GPIb-IX liposomes bound with vWF and ristocetin within a 5% error margin.

Finally, several more controls were introduced and the samples were run on the flow cytometer to assess the function of the individual components of the vWF binding experiment. Two sample runs of 0.5 mM GPIb-IX liposomes were averaged and the results are shown in Figure 14. The control sample with GPIb-IX liposomes alone was set at 1% fluorescence and all the other samples were adjusted accordingly. The results had a large error associated with them when comparing standard deviations. It can be inferred from the data that GPIb-IX liposomes alone compared to GPIb-IX liposomes induced to aggregate with vWF, ristocetin and collagen have significantly different levels of vWF binding. Also, as expected, the sample containing ristocetin alone with GPIb-IX liposomes shows that it does not bind vWF antibody. There appears to be relatively more antibody binding to the other samples but these values are not statistically significant.
Figure 13: QELS Mean Diameter Measurements of GPIb-IX Liposomes Incubated with Proteins that Promote vWF binding to GPIb. Nicomp distribution measures the individual particle’s diameter. Within 5% error, GPIb-IX liposomes alone and GPIb-IX liposomes bound vWF with 2.33 μg/ml vWF, 46.5 μg/ml ristocetin and 116 μg/ml collagen did not show significant binding.
**Figure 14: Effect of vWF, Collagen and Ristocetin on vWF Binding.** GPIb-IX liposomes incubated with antibody alone were standardized as 1% fluorescence and all other samples were adjusted accordingly. Two data sets were averaged. GPIb-IX liposomes bound vWF with 2.33 µg/ml vWF, 46.5 µg/ml ristocetin and 116 µg/ml collagen showed to be significantly different within statistical error (*statistical significance).
relative to the test sample; however, relative to the control sample, the GPIb-IX liposomes aggregated with vWF and collagen show more binding. Although the GPIb-IX liposomes aggregated with ristocetin and collagen show a relatively higher amount of fluorescence, the error associated with this result is large enough to make it difficult to draw any conclusion from this sample. More studies must be done to assess the contribution of each constituent to the aggregation.
Chapter IV: Discussion

4.1 Quality of GPIb-IX Purified from Outdated Platelet Concentrates

The aim of these studies was to purify GPIb-IX, insert the complex into liposomes and test ability of the liposome to aggregate with ristocetin, vWF and collagen. The first aim was to purify GPIb-IX from outdated platelet concentrates. The methods by Wicki et al., (1992) and Berndt et al., (1985) were followed during GPIb-IX purification using Triton X-100 for cell lysis and WGA-agarose to separate glycoproteins.

Triton X-100 and sonication of the lysate (Berndt et al., 1985) was used to lyse the cells. Wicki et al., (1992) used a purification method using Triton X-114 to separate GPIb-IX into the aqueous phase and then using a sucrose centrifugation step to separate Triton X-114. When the Triton X-114 procedure was followed a 59% yield was obtained as compared to the 72% yield for the Triton X-100 method.

The new method used fewer centrifugation steps and did not have a sucrose centrifugation step. The sucrose centrifugation entailed considerable loss of sample because not all of the supernatant was transferred to avoid a mixing of phases. The centrifugation steps contributed to additional protein loss when the supernatant was pipetted off; any GPIb-IX liberated into the supernatant was lost.

Analyzing the yield of GPIb-IX for the new method alone, several properties of the purification affected the final product. N-ethylmaleimide was added to the lysis buffer to separate actin-binding protein from the C-terminal of GPIb-IX. This was done effectively because none of the Western blots had a protein band at higher molecular
weight than the molecular weight of GPIb (170 kDa). A Western blot using antibodies to actin binding protein could verify this further. It is not known if the presence of actin binding protein would interfere with liposome formation. This would need to be investigated in other studies.

The pellet obtained after platelet lysis contained 24% of the GPIb-IX content, which was discarded. The yield could have been increased if the sample had been sonicated longer than 1 minute and/or if a second extraction had been performed on the pellet to recover more GPIb-IX.

The presence of the full complex was not determined but it would be expected that GPV be removed since it interacts with the complex through relatively weak interactions (Berndt et al, 1985). GPIb-IX remained associated because Triton X-100 is a detergent gentle enough to leave the relatively strong (but non-covalent) bonds intact. The presence of GPIX on liposomes was verified by flow cytometry (Figure 10).

4.1.2 Using Wheat Germ Agglutinin as a Purification Step for Purifying GPIb-IX

Wheat germ agglutinin chromatography was a very efficient and specific method for GPIb-IX purification. Wheat germ agglutinin interacts by binding to the O-glycans (GalNAc) on glycoproteins, making WGA a very selective purification method for membrane proteins containing sugar moieties. As tabulated in Table 1B, GPIb-IX was purified 180,000 fold from the platelet concentrate, a ~2000-fold increase is attributed to the WGA-agarose. There was 18% GPIb-IX (or 0.925 mg) in the final GPIb-IX sample. This sample was incorporated into liposomes and the protein was functional. Similarly,
the contamination by the other proteins did not inhibit GPIb-IX from being incorporated into liposomes.

A comparison was made between of the yields from this study, WGA-agarose purification, and the yields from Wicki et al, (1992), table 4 shows the amount of protein before and after GPIb-IX purification. GPIb-IX recovery was 46% by Wicki et al, (1992) and 28% for this study.

<table>
<thead>
<tr>
<th>Purification Step</th>
<th>Wicki et al, (1992): Total GPIb-IX (mg)</th>
<th>This Paper: Total GPIb-IX (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before WGA</td>
<td>63.5</td>
<td>2.33</td>
</tr>
<tr>
<td>After WGA</td>
<td>29.4</td>
<td>0.925</td>
</tr>
<tr>
<td>% Yield</td>
<td>46%</td>
<td>28%</td>
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</tbody>
</table>

Table 4: Purification Yield for Wicki et al, (1992) and This Study.

The WGA-agarose purification step also had some protein loss, as seen in the WGA fraction assayed on Western blot in Figure 6. GPIb-IX did not bind after the sample was applied to the column in the washing step, most probably due to the breakdown of the WGA-agarose column, since it was continuously used over a 2 month period. The first 75% of WGA-agarose runs had no protein elute at the wash step, which was further evidence that the column degraded. The total protein lost can only be estimated by the difference between the total protein calculated before and after the WGA-agarose separation, Table 4 (1.4 mg GPIb-IX). Finally, when some of the WGA-agarose material was tested on Western blot, some GPIb-IX was also detected, suggesting
that not all of the GPIb-IX eluted from the column. Some protein may have bound irreversibly. Also, N-acetyl-α-D-glucosamine may not be strong enough to displace all of the GPIb-IX.

Figure 6 shows that the protein eluted as measured by A_{278} at the same point as the signals appeared on Western blot. This would suggest that any protein that eluded in this peak was a carbohydrate-containing protein. Among other proteins (other than GPIb-IX) that may have eluted is GPIIb-IIIa, which has been reported to bind to wheat germ agglutinin. However, when analyzing the % fluorescence in the flow cytometer, more CD42b (anti-GPIb) than CD41 (anti-GPIIb) antibody bound to the liposomes showing that the amount of GPIb is greater than GPIIb and binding observed during aggregation can be attributed to GPIb-IX present on the liposomes rather than the GPIIb. When taking into consideration the F/P ratios and binding constants of CD41; F/P ratio 5.7, Kd = 18.8 nM (Ruan C et al, 1987) and CD42b; F/P ratio 6.8, Kd = 0.66 nM (Ruan C et al, 1987). CD42b had a 30 times stronger binding constant but a slightly lower F/P ratio. The amount of CD42b bound to the liposomes could show higher GPIb molecules compared to actual GPIIb molecules whereas the F/P ratio of GPIb on the liposomes makes the fluorescence lower compared to the GPIIb ratio. Overall, there exists the possibility that the amount of GPIIb molecules was relatively larger than indicated by the results obtained.
4.1.3 The Benefits of Using Ion Exchange Chromatography as a Purification Step for GPIb-IX

For the first two purifications, a mono-Q anion exchange column was used to purify GPIb-IX. As illustrated in Figure 7 the significant amount of protein that did not bind to the column did not contain GPIb-IX. A protein peak eluted between 0.5 M NaCl and 0.6 M NaCl, which is consistent with results obtained by Wicki et al. (1992). The protein peak contained GPIb-IX and by silver stain, looked relatively clean. It would be best to include another purification step for more optimal protein quality but due to time constraints and a large protein loss, this last step was considered optional. Other purification steps possible for GPIb-IX purification used by Wicki et al. (1992) was thrombin-sepharose affinity chromatography, taking advantage of GPIb’s thrombin binding site, and gel filtration in the presence of sodium dodecyl sulfate.

4.1.4 Purification Yield, Protein Quality Assessment of GPIb-IX

The amount of GPIb-IX expected in a platelet bag was used as a standard measurement in this study to obtain a reference measure for the yield of GPIb-IX. In Figure 8 (densitometry) it was assumed that 23.3 ng of GPIb-IX were loaded into each well on the Western blot. The value assumes that each platelet (average of 2 - 3 x 10^{11} platelets/L) contained 25,000 GPIb-IX molecules per platelet and that the platelet reader gave accurate measurements. Theoretically, it is expected that 25 concentrates of platelets (1400 ml platelet concentrate) would have 3.26 mg GPIb-IX. The following
purification steps estimated the amount of GPIb-IX from the densitometer experiments and when inspecting the concentration ranges in Table 1A one would note that they are all in the same order of magnitude (for each sample) but vary considerably within the magnitude. Therefore, there is considerable error associated with each sample. The supernatant can vary between 2.88 µg/ml to 4.88 µg/ml, which would make the total GPIb-IX 1.7 mg to 2.9 mg, which would vary the yield from 52% to 89%. The WGA-agarose purified sample can vary between 13.13 µg/ml to 33.13 µg/ml which would make the range of total protein between 0.53 mg (16%) to 1.33 mg (41%) GPIb-IX. It is therefore concluded that the values in Table 1B are significant to the order of magnitude.

When reading the intensities of the Western blot bands with the densitometer, the field to be measured was dependent on manual adjustment and carries experimental error with it.

4.2. Assessment of GPIb-IX on Liposomes after Dialysis

The protein concentration associated with the liposomes was determined after the liposomes were produced. The liposomes were separated from unincorporated protein and the lipid was removed. However, the protein concentration was too low to be determined. The procedure required a phase separation of protein partitioning into the aqueous phase. As is the case with phase separation, when one phase is transferred, to avoid mixing of phases, not the entire sample was transferred, which affects the final yield. Also, the protein was dried under N$_2$ gas flow, which may have caused some of the pellet to fly away. However, GPIb-IX was detected in the liposome prep by dot blot, which shows that GPIb was present in the liposomes.
Further, the flow cytometry results of epitope binding (Figure 10) indicate that GPIb-IX is clearly present on the surface of the liposomes since the antibody binds to GPIb-IX on the outside of the liposome. There was some non-specific CD42b-FITC antibody binding to control liposomes (36.7%) but the GPIb-IX liposomes bound significantly more CD42b-FITC (84.7%). These results varied between liposome preps depending on the GPIb-IX incorporation efficiency into liposomes and the total protein concentration that was incorporated into the liposome preparation.

The dialysis of protein into liposomes is a gentle method therefore it would not be expected that a lot of protein was denatured or damaged. The extraction of Triton X-100 may have denatured some protein because it was taken out of detergent for a brief moment, which may have put stress on the protein by unfolding or denaturation. It has been found that GPIb-IX tends to self-associate through its membrane domain in the absence of detergent (Fox et al., 1988). Because the detergent extraction was brief and GPIb-IX was present in later experiments, it is likely that not too much damage was done.

Furthermore, the fluorescence photographed in Figure 9 confirms that the antibody does not enter into the liposome but remains bound on the surface. The clear fluorescence ring observed, attributed to binding to the liposome surface illustrates this. Since the fluorochrome does not diffuse across the membrane, it can be concluded that the orientation of GPIb-IX is towards the outside as well as towards the inside and most probably the distribution is 50:50 if no alternate forces, other than chance, oriented the protein during dialysis. To come to any conclusions about this, more studies must be
done, perhaps by cleaving GPIb to glycoprotein with calpain and assessing the amount of
GPIb cleaved compared to uncleaved.

The incorporation efficiency appeared to vary and as the liposome batch became
older, the amount of antibody bound decreased. Later, the addition of aprotinin reduced
the degradation of GPIb-IX. This would indicate that some proteases were left in the
preparation. Some proteases found in plasma that may have contributed to this would be
calpain and trypsin.

4. 3 Static Binding of GPIb-IX Liposomes to vWF and Measurements by Flow
Cytometry

From Figure 12 it can be concluded that 2 mM GPIb-IX liposomes bound more anti-
vWF antibody than the control liposomes which is due to an interaction between the
components in the binding assay and GPIb-IX, since the absence of GPIb-IX did not lead
to such a great extent of binding. The binding appeared to be liposome concentration
dependent, shown by the fact that 0.5 mM liposomes do not show significant vWF
binding as compared to 2 mM liposomes. In addition it appears that the results observed
in Figure 12 represent vWF bound to liposomes without aggregates formed in most cases.
This conclusion is supported by the QELS analysis in Figure 13, where the particle size
would be expected to increase. Also, there were no aggregates seen in the microscope,
only single liposomes. Possibly, the concentration of vWF was not optimal. There was
0.5 μg vWF in the assay. If GPIb-IX is incorporated into liposomes with 50% oriented
towards the outside, all vesicles were unilamellar, 0.1 ml amount of liposome (varying
concentrations), then the amount of GPIb-IX would be below the amount of vWF by about 2 orders of magnitude. The excess vWF (present as multimers) would most probably occupy all the GPIb-IX sites. However, lower concentrations of vWF were tried and no aggregates were seen. It is difficult to say why the liposomes did not show a greater amount of aggregation but the limits of the flow cytometer assay may have contributed to the results that were seen. Also, the results do not rule out completely that aggregates formed. Small aggregates of 2 or 3 liposomes could have formed that are not very obvious under the microscope. Most probably the density of GPIb-IX on the liposome was insufficient for interactions of GPIb-IX via vWF to support aggregation.

Flow cytometry is designed for large cells (such as lymphocytes, 5-8 μm; red blood cells, 5 μm; and platelets, 2.1 μm) and flow cytometry was not designed to look at liposomes (0.2-0.3 μm) which is demonstrated by the amount of error associated with each run.

Other experiments were tried to determine the extent of aggregation. Under the microscope it was difficult to photograph the liposomes because of their small size. For aggregation measurements in the platelet aggregometer, they appeared to be too small as well. In addition, large volumes were required to have a sample concentrated enough to have aggregation take place. To bind liposomes to immobilized vWF under shear on a glass plate, again was dependent on analysis under the microscope. A paper recently published by Kitaguchi et al, (1999) described liposomes carrying a covalently linked portion of the GPIbα chain containing the vWF-binding site. The liposomes were shown, by microscopy, to aggregate. Also, liposomes were aggregated in an aggregometer and assayed by particle light scattering, which was verified under fluorescence. Furthermore,
the liposomes were shown to enhance the aggregation of platelets at low platelet count 
(20 – 80 x 10^6 platelets/ml). Their studies have a similar experimental setup as the 
experiment discussed here (static conditions with vWF and ristocetin to aggregate the 
liposomes). They were able to aggregate the liposomes with vWF and ristocetin alone 
(no collagen in the sample). However, for the current study, it was not possible to 
produce aggregates visible in the microscope. The experiments performed here were 
more static than for Kitaguchi et al, 1999. Possibly, if more shear stress were included in 
the experiment, the liposomes may have a greater chance to collide. Although this was 
tried for several of the experiments, such stirring the liposomes would be expected to 
enhance aggregation.

The ability of liposomes to aggregate with platelets may be different again. The 
ability of platelets to aggregate with the liposomes needs to be determined. 
Theoretically, the GPIb-IX liposome-bound vWF will bind GPIb-IX-V on the platelet, 
activation will be stimulated and then the GPIb-IX liposome could interact with GPIIb-
IIIa as well to help form aggregates. Results published by Kitaguchi et al, (1999) provide 
support that a GPIb-liposome can enhance aggregation of platelets. Possibly, if one were 
to work with larger liposomes that can be photographed, the microscope would provide 
useful information. Also, to couple protein to the surface chemically would be an 
advantage that may increase the amount of surface-protein, which would increase the 
amount of GPIb-IX binding sites.

The importance of collagen in the sample still needs to be determined. It appears 
from Figure 14 that more liposomes fluoresced if collagen were present but again, the 
level of error makes it difficult to conclude this. In vivo, collagen is believed to bind to
vWF first, then possibly this interaction leads to an enhanced binding of vWF to GPIb-IX. This remains to be confirmed. Wu et al, (1996) showed that anti-GPIb vWF antibodies inhibited antibody binding of collagen bound vWF to platelets. Also, the binding of platelets in whole blood to collagen coated surfaces appeared to occur in two steps (Moroi et al, 1997) suggesting that vWF bound to collagen first: initially, platelets bound a collagen surface at a lower density but with time the density. This phenomenon was not seen with platelets sheared across vWF coated plates, where the increase was linear.

Ristocetin appears to be important for aggregating liposomes in static conditions as published many times in the literature. It is known that ristocetin is important in static conditions for linking GPIb-IX molecules together via vWF. It has been well established that ristocetin reduces the Zeta potential between vWF and GPIb, allowing their binding to each other. The extent that ristocetin affects binding between GPIb and vWF for the GPIb-IX liposomes is not known. During the course of the experiments performed for this study, ristocetin created some strange artifacts such that for some aggregations, there appeared to be binding between ristocetin and the anti-vWF antibody. As to the importance of this remains to be determined. The paper by Kitaguchi et al, (1999) used ristocetin to aggregate the GPIbα-liposomes but they were able to show aggregation by microscopy and light scattering platelet aggregometry. Although they did not include controls for ristocetin, they were able to inhibit ristocetin-dependent aggregation with anti-vWF and anti-GPIb antibodies, strongly supporting that vWF binds to GPIb. Therefore, the same can be inferred for the aggregation in these results, using a similar
experimental setup to Kitaguchi et al, (1999), vWF may have likely bound to GPIb-IX liposomes in the presence of ristocetin.

Figure 15 shows the model proposed for the liposome binding to vWF using ristocetin and collagen. Collagen and ristocetin enhance the binding of vWF to GPIb-IX liposomes forming small aggregates in a static system.

4.4 Quality Assessment of the GPIb-IX Liposomes: Summary

GPIb-IX was purified from platelet concentrates with a Triton X-100 extraction and WGA-agarose chromatography. The GPIb-IX obtained was 28% pure and was incorporated into 10 mol% DOPE, 65 mol% DPPC and 25 mol% cholesterol liposomes. The GPIb-IX liposomes showed surface GPIb-IX detected by flow cytometry and fluorescence microscopy (GPIb). GPIb on the liposome was more prevalent than GPIIib, another glycoprotein which may bind vWF. Further studies showed that vWF bound to the GPIb-IX liposomes in the presence of ristocetin and collagen. The extent to which each ingredient contributed to the binding still remains to be determined.

Overall, the GPIb-IX-containing liposomes prepared during these studies were able to bind vWF but it is speculative whether aggregates formed. The sucrose-loaded liposomes were separated from the un-incorporated protein by centrifugation. This means that engulfed or membrane-inserted protein is separated in the liposome preparation. Possibly the majority of any contaminant protein was removed at this point but the purity was not assessed, mostly because the sample was too small. It would be
Figure 15: Model of GPIb-IX Liposome Interaction With vWF and Ristocetin Under Static Conditions. VWF multimer interacts with GPIb on liposomes using ristocetin as a bridging molecule that reduces the Zeta potential between the negative charge on GPIb and the negative charge on vWF.
beneficial in the future to purify the protein further before inserting into liposomes to provide a more concentrated source of GPIb-IX, which in turn would increase the amount of GPIb-IX on the surface of liposomes.

As a potential platelet substitute or clotting "filler", a GPIb-IX liposome would be a beneficial tool for aiding platelet adhesion and aggregation. During illnesses such as bone marrow disease and chemotherapy for bone marrow cancer, where platelet production is low, it would be expected that the platelet aggregation response could be enhanced with a GPIb-IX liposome. The GPIb-IX liposome would increase the number of interactions at a site of damage, increasing the number of adhered platelets. Platelet activation would then recruit more platelets to activate GPIIb-IIIa, which in turn can interact with more GPIb-IX liposomes or fibrinogen to aggregate and form a hemostatic plug. Kitaguchi et al, (1999) showed that platelet aggregation was enhanced with the addition of the GPIbα-liposomes.

Finally, whether the GPIb-IX liposome created in this thesis work can interact with platelets, still has to be assessed. This would be the next step to determine its role as a platelet substitute. The prototype can then be tested in an animal model for function in vivo.

The studies by Rybak et al, (1993) and Kitaguchi et al, (1999) have created optimism towards realizing a liposome-based platelet substitute. The prospects of a liposome-based platelet substitute are attractive and promising. The product would be able to be stored for a long time until needed. In addition, it would be donor independent and safe from potential blood transmitted diseases. It would be useful for treatment of hemorrhage due to thrombocytopenia.
Bibliography


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