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The Effect of Material Preactivation of Platelets on Deposition

by

Beverly Kristine MacDonald

A thesis submitted in conformity with the requirements
for the degree of Master of Applied Science
Graduate Departments of Chemical Engineering and Applied Chemistry and
Institute for Biomaterials and Biomedical Engineering
University of Toronto

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Abstract

The Effect of Material Preactivation of Platelets on Deposition

Master of Applied Science, 1998

Beverly Kristine MacDonald

Departments of Chemical Engineering and Applied Chemistry and
Institute for Biomaterials and Biomedical Engineering

University of Toronto

The effect of shear on platelet activation was investigated and characterized. It was found that the residence time had a more significant effect on microparticle, MP, generation in the sample than the applied shear rate.

Platelets were also activated by alternative methods, such as incubation with glass beads, to induce MP generation. Samples which produced large MP concentrations during activation were found to deposit fewer platelet on the polyethylene, PE, tubing than the rest sample.

Further characterization of the material activated platelets revealed that material contact did not result in significant loss or activation of GPIIbIIIa on the platelet surface. Nor did material contact result in the upregulation of P-Selectin to the platelet surface.

Samples which produced the greatest number of MP during activation experienced impaired sensitivity to platelet agonists such as thrombin peptide, collagen and ADP. Therefore, platelet microparticles may be markers of reduced platelet function as well as platelet activation.
Acknowledgments

Professor Michael Sefton, for taking me on and supporting my alliance with Vas-Cath

Cynthia Gemmell for answering my questions and her constant reassurance

Maud Gorbet and Elaine Cheng for their friendship, support and help with platelet analysis

Chris Sauer for over two years of listening to me, supporting my decisions and of course, proof reading

My Family for their faith in me and their support throughout my studies

All my friends for making the University of Toronto both interesting and memorable

Vas-Cath and the NSERC Industrial Post Graduate Scholarship for funding my research
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The Effect of Material Preactivation of Platelets on Deposition

1.0 Introduction
Elevated concentrations of activated platelets and microparticles have been found in blood drawn from patients having disease states such as fibrinolysis, idiopathic thrombocytopenic purpura, hemolytic uremia and those undergoing cardiopulmonary bypass operations (1,2). Blood activation and platelet microparticle, MP, formation are also consequences of blood interaction with a biomaterial (3). Just as platelets are activated when they come into contact with subendothelial layers of the vessel wall, they are likewise activated by contact with other foreign, non endothelial surfaces.

Are the activation of platelets and the formation of MP simply artifacts of interaction with biomaterials or do they pose problems as they travel further downstream? In particular, concerns have been raised as to the implications that material activated blood and its associated microparticles have on platelet deposition on biomaterials placed downstream in the circulation. This thesis was focused on the effect that biomaterial preactivation of blood has on biomaterial platelet deposition at a physiologically representative flow rate and the effect that the material contact has on the activated platelet population. Research into this area is very important to ensure that device failure due to blood preactivation and the resultant platelet deposition can be minimized.

1.1 Biocompatibility and Biomaterials
Biocompatibility is defined as “the ability of a material to perform with an appropriate host response in a specific application”(4). In the human body, if the blood comes into
contact with an incompatible surface, either biologic or foreign, the complicated complement and coagulation pathways are triggered. Without an endothelial lining, the blood constantly initiates these pathways, creating thrombi that compromise the patency of the blood vessels. The main purpose of endothelial cells is to provide a blood compatible surface which does not activate the body's protective pathways. The ultimate goal of biomaterial engineering is to design a material which the body can not distinguish as foreign.

1.2 Whole Blood vs. Platelet Rich Plasma
A significant amount of the research has been conducted using platelet rich plasma, PRP, or washed platelets suspended in buffer rather than whole blood, WB (5). In experiments where the blood is static while in contact with the biomaterial, it is possible that the PRP or washed platelets was the preferred choice since the presence of other blood components, such as the erythrocytes and leukocytes, may impede progress of the platelets towards the biomaterial surface (6). PRP is also chosen over whole blood when erythrocyte lysis during incubation with the biomaterial is a possibility, since the release of platelet activators such as ADP resulting from such lysis interferes with the evaluation of the biomaterial effect on platelet activation.

However, under non static conditions, erythrocytes are important in platelet analysis because their shape and rotating motion facilitates the delivery of platelets to the biomaterial surface. Platelet deposition is dependent on the concentration of RBC as the platelet diffusivity toward the surface increases with hematocrit (7). When testing platelet activation due to biomaterials it is beneficial to maintain a milieu which closely
resembles the true physiological situation by testing with WB, rather than PRP, at physiologically representative shear rates.

1.3 Platelets
Platelets range in size from 1.5-2 \( \mu \text{m} \) and are oval in shape. They are created in the bone marrow where they differentiate from hematopoietic cells called megakaryocytes. The platelet surface, called the glycocalyx, exhibits many glycoprotein receptors which are involved in adhesion and aggregation reactions (8,9). The main concern with blood contacting biomaterials is that they may activate platelets thereby releasing coagulation factors, increasing platelet adhesion and ultimately resulting in thrombosis (6).

With the use of blood contacting biomaterials constantly increasing, there is concern about the effect of biomaterial pre-activated blood and its microparticles in the circulation. When two biomedical devices are implanted in the blood vessels the one placed further downstream in the blood flow will be exposed to material activated blood. The question posed here is whether biomaterial preactivated platelets have enhanced propensity for adherence to the biomaterial surface.

1.3.1 Platelet Adhesion
Platelets, through their surface receptors, adhere to biomaterial surfaces via plasma proteins which have been previously adsorbed onto the surface. Within seconds of its introduction to the blood, plasma proteins (10), have already begun to be adsorbed onto the biomaterial surface. At shear stresses below 12 dynes/cm\(^2\) fibrinogen and fibronectin
are believed to be the primary platelet binding proteins but at higher shear stresses von Willebrand Factor, vWF, takes over as the most important adhesion protein (11, 12,13,14).

1.3.2  **Platelet Receptors and Granule Constituents**
There are many receptors on the platelet surface, each found at different densities varying from platelet to platelet. Monoclonal antibodies are used to indicate the expression of particular antigens on the platelet surface in conjunction with the use of a flow cytometer. This method is particularly important as it allows for the identification of the platelet population and the activated platelet subpopulation. Those receptors of greatest interest to platelet-biomaterial adhesion and determination of platelet activation and/or refractoriness, defined in section 1.5, are discussed below.

**GPIIbIIIa**
GPIIbIIIa, which can be identified by CD41(GPIIb) or CD61(GPIIIa), is a heterodimeric transmembrane molecule held together by Ca$^{2+}$. This receptor belongs to the β₃ integrin family of platelet glycoprotein receptors. The integrin family of receptors is characterized by the two nonidentical subunits, α and β, of which they are comprised. GPIIbIIIa exhibits an extremely high concentration of 40-50 thousand receptors/platelet (15) with the mean distance between the receptors on the surface being about 20 nm (16).

Although GPIIbIIIa is present on the surface of all resting platelets and thus can be used to identify resting platelet populations, it undergoes a conformational change following activation. This conformational change reveals the binding site for fibrinogen, von Willebrand factor, fibronectin and vitronectin. A monoclonal antibody called
PACl (activated GPIIbIIIa) is directed at the fibrinogen binding site and can therefore be used to identify activated platelet populations (17).

Thrombin peptide activated samples exhibit increased numbers of GPIIbIIIa receptors over the rest sample. This increase in GPIIbIIIa is believed to come as a direct result of the disruption of the platelet α granule membrane of which GPIIbIIIa is a component and from the membrane of the open cannicular system within the platelet (16).

**GPIb-IX**
The GPIb-IX complex, also called CD42, is a nonintegrin receptor which interacts with von Willebrand Factor, vWF (18). There are approximately 25000 receptors / platelet (13). There are three different polypeptide chains of which this receptor is comprised: GPIbα, GPIbβ and GPIX. It is the C-terminal fragment of GPIbα which presents the vWF binding site (14). GPIb also has a binding site for thrombin although its significance is not known (16). This receptor can be used to identify the platelet population as it is present on the surfaces of both resting and activated platelets.

**P-Selectin**
Also called GMP-140, CD62, CD62P and PADGEM protein, P-Selectin is stored in the α granule membrane within the cytoplasm of the resting platelet. Upon activation the platelet granules release their contents through the cannicular system thus allowing the P-Selectin receptor to express itself on the surface of the platelet. The P-Selectin antigen is the receptor by which activated platelets bind to neutrophils and monocytes (17). Thrombin peptide is used as a positive control agonist to elicit P-selectin expression in the platelet population for flow analysis.
Collagen Receptor: GPIaIIa
GPIaIIa, also called CD49b and CD29, comes from the VLA-family of receptors and is the main collagen receptor on platelets. The GPIaIIa receptor belongs to the VLA-2 subset which share a common β= IIa subunit (14). Platelet adhesion to collagen through this receptor requires magnesium or manganese and is inhibited by calcium (16).

Collagen binding induces platelet shape change and the release of dense granule contents. The adhesion resulting from this activation is dependent on the ADP and prostaglandin which is released from the dense granules. Collagen also binds directly to GPIV and has been shown to bind indirectly to GPIIbIIIa through association with vWF and fibronectin (13).

ADP Receptor
The ADP receptor is not very well characterized. This receptor belongs to the P₂ subset of the family of purinergic receptors which recognize ADP and ATP (13). Reports on the number of receptors found on the surface range from 1000 to 160 000 per platelet (18). ADP affects platelets by initiating a signal pathway which causes platelet shape change, the revealing of binding sites on the GPIIbIIIa receptor, platelet granule release and platelet aggregation.

Thrombin Receptor
Thrombin, having between 1700-1800 receptors/cell, is a very potent agonist for platelets which causes platelet shape change, aggregation and granule and lysosome secretion(13, 16). The thrombin receptor belongs to the superfamily of receptors which are coupled to G proteins and phospholipase C.
Granule secretion
Although platelets are anucleated and therefore can not reproduce themselves, they do have subcellular organization. All platelets have organelles including peroxisomes, mitochondria, lysosomes, dense granules and alpha granules. The latter two mentioned are important in the process of platelet activation as the release of their contents aids platelet activation as part of the positive feedback control system (16). Figure 1.1 is a partial listing of the platelet granule contents.

Figure 1.1 Dense Granule and α Granule Contents (8)

<table>
<thead>
<tr>
<th>Dense Granules</th>
<th>α Granules</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>Platelet Factor 4</td>
</tr>
<tr>
<td>ATP</td>
<td>Fibrinogen</td>
</tr>
<tr>
<td>Calcium</td>
<td>von Willebrand Factor</td>
</tr>
<tr>
<td>Serotonin</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>Pyrophosphate</td>
<td>Thrombospondin</td>
</tr>
<tr>
<td></td>
<td>Vitronectin</td>
</tr>
<tr>
<td></td>
<td>Factor V</td>
</tr>
<tr>
<td></td>
<td>Protein S</td>
</tr>
<tr>
<td></td>
<td>Factor XI</td>
</tr>
</tbody>
</table>

Dense Granule
There are fewer than fifty dense granules per platelet (16) and each contains adenine nucleotides, calcium, pyrophosphate and serotonin (8). Dense granule secretion releases ADP into the plasma, which is a very strong platelet agonist and therefore it plays an important role in positive feedback for platelet activation. Serotonin is also released during dense granule secretion and acts as a weak agonist for platelet activation.

Alpha Granule
There are between 50 and 80 alpha granules per platelet (16). The α granules contain varying concentrations of all plasma proteins, coagulation proteins and various polypeptides. Upon α granule secretion, all its contents are released into the blood.
plasma including platelet factor 4 (PF4) which binds to heparin in the circulation and neutralizes its anticoagulant activity. (16).

1.3.3 MicroParticles (MP)
Platelet microparticles are routinely used as markers for platelet activation in a clinical setting(19). The first publication dedicated to the subject of platelet microparticles, MP, appeared in the British Journal of Haematology in 1967. Peter Wolf noted the presence of minute particles originating from platelets which he termed “platelet dust” (20). These particles ranged from 200 - 500 nm in diameter and were found to possess procoagulant properties as they are rich in platelet factor 3, PF3. Wolf remarked that the production of thousands upon thousands of microparticles during platelet activation provided for a very large surface area for procoagulant reactions.

Platelet microparticles have also been shown to possess many receptors for coagulation factor Va and to provide a large surface area for prothrombinase reactions. (21, 19). In vitro studies of MP have shown these particles to possess anticoagulant properties as well, yet their significance in the circulation is yet known (22, 23).

Platelet microparticles, MP, are formed through an exocytotic budding process from the platelet membrane(1) as observed through electronmicrographs of activated platelets(24). Platelet microparticles result from many conditions such as the introduction of a strong agonist, the increase in intracellular calcium concentration, (23) platelet-biomaterial contact (3) or exposure of platelets to high shear rates (25).
Platelet microparticles are easily detected, despite their small size, through the use of flow cytometry since MP possess the same surface glycoproteins as platelets. AntiGPIIbIIIa or antiGPIIb is used preferentially over antiGPIb as the former has been shown to be more sensitive for MP detection (21), approximately 73% of detected MP were positive for GPIIbIIIa and only 43% were positive for GPIb (24). As there are fewer GPIb receptors than GPIIbIIIa receptors on the platelet surface it stands to reason that platelet microparticles are much more likely to present GPIIbIIIa receptors than GPIb, thus making antiGPIIbIIIa a more effective antibody for MP detection.

1.4 Wall Shear rate, $\gamma_w$

The first stage of research completed for this thesis involved the determination of the effect of flow, or more specifically, shear rate on platelet activation. Investigations were undertaken to determine whether increased shear rate initiated greater platelet activation and therefore greater platelet microparticle, MP, formation in the bulk. The wall shear rate is directly related to the flow rate of the blood by the equation in Figure 1.2.

$$\gamma_w = 4Q/ \pi R^3$$

$\gamma_w$ = wall shear rate
$Q$ = volumetric flow rate
$R$ = inner radius of the tubing

Figure 1.2 Wall Shear Rate Equation:

This is the standard equation that is used to evaluate wall shear rate in a tubular chamber (i.e. blood vessel or medical tubing) (6, 11, 12, 26).

Wall shear rate is a relevant calculation to perform when analyzing platelet activation since it is known that the platelets in WB are pushed out to the walls by the rotational
motion of the red blood cells. The time averaged wall shear rate of whole blood in the circulation is between 500-750 s$^{-1}$. Typical wall shear rates in the blood vessels are presented in Figure 1.3 (11).

Figure 1.3 Typical Ranges of Wall Shear Rates in the Circulation

<table>
<thead>
<tr>
<th>Blood Vessel</th>
<th>Wall Shear Rate (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large Arteries</td>
<td>300 - 800</td>
</tr>
<tr>
<td>Arterioles</td>
<td>500 - 1600</td>
</tr>
<tr>
<td>Veins</td>
<td>20 - 200</td>
</tr>
<tr>
<td>Stenotic Vessels</td>
<td>80 - 10 000</td>
</tr>
</tbody>
</table>

The analysis of blood flow deviates from that of the ideal fluid since it is a suspension of cells and proteins and consequently experiences non-Newtonian flow in areas where shear rates are less than 50 s$^{-1}$(26). All experimentation in this thesis was carried out at shear rates in excess of 50 s$^{-1}$ in order to assume newtonian fluid mechanics for all flow analysis.

1.4.1 Stenotic Flow Analysis

For patients who have developed a stenosis due to intimal hyperplasia resulting from a prosthetic implant, the areas of recirculation just distal to the stenosis lead to an increase in platelet deposition. A recirculation can be crudely modeled by an abrupt expansion in tube diameter as both cause areas of low shear distal to the change in diameter. This thesis investigated this flow situation for both resting and material activated samples.

Following an abrupt expansion in lumen diameter, fluid flow is rapidly slowed as a result of the increase in cross sectional area. During the deceleration of flow a positive pressure gradient is formed, in accordance with the Bernoulli equation, which attempts to force the
fluid in the reverse direction (26). Flow separation occurs such that the blood closest to
the wall is subject to reverse flow, creating an area of low shear recirculation immediately
distal to the increase in lumen diameter. This recirculation zone where platelets and
microparticles can gather is very similar to the flow pattern created by a stenosis as seen
in Figure 1.4. The recirculation zone is dependent on the increased diameter, as the
decrease in flow is inversely proportional to the increase in cross sectional area. The
diameter expansion ratio of the tubing used to mimic the stenosis was in section 2.5 was
1.38.

1.5 Platelet Activation and Refractoriness
Platelet activation is a general term which reflects one of a number of complex
biochemical reactions upon stimulation by various agonists(27). Platelets can be strongly
or weakly activated by agonists or materials. Weak activation can be characterized by the
formation of platelet microparticles, conformational shape change of the GPIIbIIIa
receptor revealing the fibrinogen binding site and some shape changes of the platelet
itself. Strong platelet activation results in degranulation, during which the P-Selectin
receptor which resides on the surface of the α granule when the platelet is at rest
expresses itself on the platelet surface. Degranulation results in further platelet activation
in the bulk since the α and dense granules contain strong (and weak) platelet agonists.

Platelet refractoriness is defined by the reduced ability to respond to an agonist challenge,
induced either by small concentrations of platelet agonists or from processes occurring
during material contact (i.e. during extracorporeal circulation) (28). This thesis
Figure 1.4 Flow Patterns for a Stenosis and Abrupt Increase in Tube Cross Sectional Area

a) Depiction of the flow patterns and recirculation zones associated with a stenosis (12)

b) Depiction of flow patterns and low shear recirculation zones for an abrupt expansion in lumen cross sectional area (26)
characterized the activated platelet population to determine the degree of platelet refractoriness following material activation.

Platelet refractoriness has been observed in blood subjected to extracorporeal circulation. This refractoriness was characterized by the reduced ability to respond to aggregation inducing platelet agonists and reduced affinity for glass beads, as well as prolonged post operative bleeding times in patients (8, 28). Some studies have also reported loss of receptors due to biomaterial contact during extracorporeal circulation(10,29,30).

1.6 Analysis of Platelet Activation via Flow Cytometry
Flow cytometry uses fluorescently conjugated antibodies to identify selected cell populations. A two colour-two antibody approach is often used to simultaneously identify subpopulations within the main cell population(31). The first monoclonal antibody is directed toward a receptor common to all platelets, commonly FITC (fluorescein isothiocyanate) conjugated- GPIb, GPIIb or GPIIIa while the second antibody, usually biotinylated or conjugated with PE (phycoerythrin-streptavidin), is directed toward the subpopulation of interest, in this case those platelets exhibiting P-Selectin expression.

AntiCD62P was the antibody used to identify platelets with P-Selectin expression and two different antibodies, antiCD41 and antiCD61 were used to identify the GPIIbIIIa receptor. AntiCD61 identified an epitope of GPIIIa, whether on its own or in the GPIIbIIIa formation, and antiCD41 identified an epitope on GPIIb most effectively when present in the GPIIbIIIa configuration. AntiCD61 was an important antibody in
experiments where EDTA was added to fix samples at 37°C since the addition of EDTA at this temperature is known to denature the surface glycoproteins (21).

Flow cytometry has some distinct advantages for the study of platelet activation, as outlined in Figure 1.5, taken from a review article written by Alan Michelson (31).

Figure 1.5 Advantages of Flow Cytometry for the Study of Platelet Activation

**Advantages of Flow Cytometry for the Study of Platelet Activation**

- 1) Platelet activation can be studied in the more physiological milieu of whole blood.
- 2) Minimal manipulation of samples prevents artifactual in vitro activation and potential loss of platelet subpopulations.
- 3) Both the activation state of circulating platelets and the reactivity of circulating platelets can be detected.
- 4) Activation-dependent changes in multiple surface receptors can be detected.
- 5) New MoAbs directed against novel functional epitopes can easily be incorporated into the assay.
- 6) Only 5µl of blood is required.
- 7) High degree of sensitivity for the detection of platelet subpopulations.
- 8) No radioactivity.
- 9) Platelet microparticles (MP) can be analyzed, as defined by their low forward angle light scatter and ability to bind platelet specific MoAb (monoclonal antibody).

1.7 **Anticoagulation**

To prevent the development of thrombi on biomaterials both in vivo and in vitro, anticoagulants such as heparin are used. Heparin enhances the action of antithrombin III, which is an enzyme that naturally occurs in the body through the formation of a ternary complex, thereby amplifying its effectiveness by 1000 times. Antithrombin III is present in the body to prevent blood clots from spreading too far or coagulating out of control.

The conformational change resulting from the Antithrombin III-heparin complex enables the inhibition of thrombin to proceed at a faster rate (27). Thrombin is the enzyme that causes the production of fibrin, thus the addition of heparin essentially prevents the
cleavage of fibrinogen into fibrin, consequently preventing the stabilization of activated platelets in a clot.

Working in a thrombin free environment allows for less complicated analysis of platelet-biomaterial interaction. It is common practice to administer anticoagulants to patients in a clinical setting which inhibit clot formation but do not interfere with normal platelet function (32, 33). All research conducted used 5 units heparin/mL WB which is similar to the concentration used in a clinical setting.

1.8 Hypothesis and Scope of Thesis
When blood comes into contact with a biomaterial, platelet activation occurs. In a system where blood comes into contact with two biomaterials placed in series as pictured below, it is hypothesized that there will be increased platelet deposition on the downstream biomaterial resulting from blood which has been preactivated by the biomaterial upstream (Figure 1.6).

Platelet microparticle formation is expected to play a large role in increased platelet deposition as they present a large surface area for coagulant activities. Platelet microparticles themselves do not adhere to synthetic surfaces but if their production results in enhanced deposition of platelets, biomaterial preactivated blood in the circulation could cause further problems especially for patients who are already predisposed to high MP levels in their blood, and have biomaterials in the blood stream.
As the platelets are preactivated by the biomaterial upstream in the blood flow, they consequently deposit in increased concentrations on the biomaterial downstream.
2.0 Materials and Methods

2.1 Blood Preparation

2.1.1 Whole Blood (WB) Collection
Whole blood was collected from healthy volunteers, who had not taken any medication in the previous seven days, into a syringe containing 5 units heparin/ml WB. Hepalean, the anticoagulant used, was obtained from Organon Teknika, Inc., Toronto, Ontario. Following collection, the blood was gently mixed to ensure even distribution of the heparin throughout the sample.

2.1.2 Platelet Rich Plasma (PRP) Preparation
PRP was obtained using heparinized whole blood spun at 800 rpm for 6-7 minutes in the Silencer™ centrifuge, model# 4103 FRS, from Western Scientific Services, Japan. The supernatant PRP was pipetted out of the tube into a clean polypropylene test tube, paying careful attention to not disturb the settled RBC and other whole blood constituents.

2.1.3 Washed Platelets and Gel Filtered Platelets
The protocols which were used to wash and gel filter platelets were standard laboratory procedures which can be found in the appendix. The whole blood collected for washed platelets was collected into 3.8% sodium citrate, 1:9, rather than heparin.

2.2 Flow Cytometric Analysis
Flow cytometry was used to evaluate the platelet population using platelet MP and P-Selectin as markers for material activation.
2.2.1 Control Samples
There were three control samples used for flow cytometry; two negative control rest samples and one positive control agonist activated sample.

Positive control: A volume of 5 µl of SFLLRN was added to 25 µl fresh WB. The sample was gently mixed then allowed to incubate at room temperature for 20 minutes. Following incubation, the sample was incubated with the antibodies outlined in section 2.2.2.

Negative Control: 5µl of fresh WB was taken immediately following venipuncture and allowed to incubate with the antibodies outlined in section 2.2.2. The rest sample was allowed to sit at room temperature for the duration of the experiment, at which time it was then incubated with the antibodies as described below.

2.2.2 Antibody Incubation
The flow cytometry test tubes were prepared with 50 µl of HTB (Hepes Tyrodes Buffer: NaCl 8g/L, KCl 0.2g/L, NaHCO3 1.4g/L, Mg2Cl 1.0g/L, Hapes 0.90g/L, Glucose 1.0g/L and BSA 2g/L) and the following antibodies: 5µl antiCD61, GPIIIa antibody, (Serotec, where) or 5µl antiCD41, GPIIb antibody, in a 1:10 dilution (Southern Biotech, where) and 3µl antiCD62P, P-Selectin antibody, in a 1:10 dilution (Monosan Cedarlane, where). To the antibody mixture 5 µl test WB or PRP was added and allowed to incubate at room temperature for 20 minutes. When the incubation was complete, the sample was diluted by 150 µl of HTB and fixed with 150 µl of fixative (paraformaldehyde 2g/100ml, 10 drops NaOH/100ml, 10X PBS 10ml/100ml).
2.2.3 Recording of Flow Cytometric Data

Once the samples were fixed, they were analyzed with the FACScan flow cytometer (model # 0261059-01, Becton Dickinson Immunocytometry Systems, San Jose, California) using the CellQuest software analysis program (Becton Dickinson Immunocytometry Systems, San Jose, California). With the light scatter and fluorescence channels set at logarithmic gain, 10,000 GPIIbIIIa positive events (platelets + microparticles) were counted for each sample and analyzed for forward and side light scatter, PE fluorescence and FITC fluorescence. Since antibody positive platelets may have very few receptors present on their surface, antibody binding was reported as a mean fluorescence intensity, which more closely approximates the total amount of platelet surface antigen (17).

On the basis of forward and side scatter characteristics, two platelet subpopulations were gated. The first gate, single intact platelets, had higher forward and side scatter characteristics, while platelet microparticles with the lower scatter characteristics were in the second gate. The concentration of platelet microparticles was recorded as a percentage of the total GPIIbIIIa positive events.

2.3 MP Generation via Application of Shear

In order to study the effects of shear and residence time on platelet activation and deposition, two methods (Figure 2.1) were used to apply shear to WB samples in polyethylene (PE) tubing. The first method was a single pass through a simple tube and the second was a recirculation method. Both involved the use of the dual syringe pump from Harvard Apparatus (model 55-3333, Saint-Laurent, Quebec).
### 2.3.1 Straight Through Single Pass

This mode of shear application pushed WB from a syringe straight through the Intramedic PE tubing, measuring 0.28mm x 0.60mm x 60 or 240 cm long (Becton Dickinson, Parsippany, New Jersey). Silicone tubing measuring 0.50mm x 0.94mm x 20cm long (Silastic, Dow Corning Corporation, Midland, Michigan) was used to join the biomaterial being tested to the syringe. A Falcon® polyethylene test tube (Becton Dickinson, Lincoln Park, New Jersey) was used to collect the blood as it passed through the open end. The apparatus was setup as pictured in Figure 2.1.

Samples taken from the blood collected were analyzed via flow cytometry for MP formation and platelet activation. Rest samples and agonist activated samples were retained from each run to use as a baseline for activation comparison via flow cytometry.

### 2.3.2 Recirculation Method

This mode of applying shear pushed WB back and forth through the Intramedic PE tubing, 1.57mm x 2.08mm x 80cm long (Becton Dickinson, Parsippany, New Jersey), at a temperature of 37°C and a flow rate of 5.0 ml/min, unless otherwise stated in the results. Each end of the PE tubing was attached via silicone tubing, 1.57mm x 3.18mm x 30 cm (Silastic, Dow Corning, Midland, Michigan) to a syringe on the pump. The method used was similar to that of Rhodes et al. and was attractive because it allowed for increased contact time with minimal blood-air interface.

The 0.65ml blood, which was recirculated, did not enter the syringes on the pump at any time during the experiment. Samples of the blood collected at specified time intervals (5-40 min) were analyzed via flow cytometry. Rest samples and agonist activated samples
were retained from each run to use as a baseline for activation comparison. In some trials, 1μl apyrase/ml WB was added to the sample prior to activation for the evaluation of ADP release on platelet refractoriness.

![Diagram](image.png)

Figure 2.1 Syringe Pump Set-up

2.3.3 **Variation on the Recirculation Method: Larger Volumes**

A variation on the recirculation method was used to activate larger volumes of blood through a slight change to the syringe setup as pictured in Figure 2.2. This setup allowed for the accommodation of 5ml WB in two inverted syringes, which were connected to the syringe pump via Silastic tubing and 2-19G needles (Becton Dickinson, Franklin Lakes, New Jersey). The Silastic tubing, flow rate and temperature were all the same as used in section 2.3.2. The PE tubing used had the same ID and OD as in section 2.3.2 but was 150cm long. The blood was collected after 40 minutes and retained for use in platelet deposition studies and analysis via flow cytometry for evidence of material activation. Rest and agonist activated samples were retained from each run to use as a baseline for flow cytometry platelet activation comparison.
2.4 Alternative Methods of MP Generation

When the volume of blood required for testing was increased, the recirculation method was no longer capable of activating the whole blood enough to produce the numbers of MP which were generated in section 2.3.2. The following methods for activating larger volumes of blood were investigated.

2.4.1 Polystyrene Beads

The 10μm polystyrene beads from Polysciences, Warrington, Pennsylvania were washed and resuspended in 2X BBS(2X Borate Buffer Solution: Boric Acid 12.36g/L, Sodium Tetraborate 19.08g/L) and NaHCO₃ 8.76g/L) prior to use. They were incubated for 40 minutes at 37°C with PRP at ratios of 1:20, 1:6, 1:4, 2:7 and 1:3 for a final volume of 1ml. Samples collected following the incubation period were analyzed via flow cytometry to observe increases in MP concentration and P-Selectin expression. Rest samples and agonist activated samples were retained from each run to serve as a baseline for activation comparison.

2.4.2 Freeze Thaw Method

Freeze-thaw induced MP were prepared in order to provide a consistent source of MP. The method used to create and isolate the freeze thaw MP was adapted from Bode et al.
Platelets were washed and a count was taken. The washed platelets were then frozen at -70°C overnight and thawed in a 37°C bath. This process was repeated twice, before the samples were centrifuged at 15 000 rpm to remove unlysed platelets and other large debris. The MP concentrate supernatant was then removed, counted for platelets, and stored in 200μl aliquots in the -70°C freezer for future use.

Thawed MP concentrate was added to WB to evaluate the increase in MP concentration. In each trial, the indicated volume of MP concentrate, as per Figure 2.3, was added to 1.5ml of resting WB, then gently mixed. Resting WB samples with and without the MP concentrate were subjected to flow cytometric analysis to determine the corresponding MP concentrations.

Figure 2.3: Addition of Freeze Thaw MP to Resting WB

<table>
<thead>
<tr>
<th>Trial</th>
<th>Volume WB</th>
<th>Volume of MP Concentrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1ml WB</td>
<td>30μl</td>
</tr>
<tr>
<td>2</td>
<td>1ml WB</td>
<td>100μl</td>
</tr>
<tr>
<td>3</td>
<td>1ml WB</td>
<td>200μl</td>
</tr>
<tr>
<td>4</td>
<td>100μl WB</td>
<td>100μl</td>
</tr>
<tr>
<td>5</td>
<td>1ml WB</td>
<td>1ml</td>
</tr>
</tbody>
</table>

2.4.3 Glass Beads

Glass beads were investigated for platelet activation because glass is known to be a strong platelet activating material. The density of these beads allowed easy removal from the PRP through settling, unlike the 10μm polystyrene beads which were studied first.

The WB obtained for testing was separated into two equal halves; one designated as the rest sample and the other for PRP activation. The whole blood constituents left behind
after PRP separation were saved for reconstitution with the activated PRP for the platelet deposition studies only. Five test tubes were prepared with 0.73g of glass beads (350-400 μm, Sigma, St. Louis, Missouri) and 200 μl of 2X BBS and then the PRP obtained from 1 ml WB (~200-250 μl of PRP). The PRP-bead samples were incubated (Fisher Scientific Isotemp® incubator, model # 2530, Ivyland, PA) at 37°C for 30 minutes on a hematology mixer (model 14 060 1, Fisher Scientific, Ivyland, PA). Following incubation the beads were allowed to settle to the bottom of the sample and the activated PRP was pipetted out of the centrifuge tube.

A platelet count was conducted for the activated and resting samples on the Sysmex E-2500 Cell Counter, Japan. The platelet concentration for the rest sample was diluted, through the addition of HTB, until equal to the activated sample platelet count. The rest, agonist activated and the glass bead activated samples were analyzed for MP generation and platelet activation via flow cytometry. The activated PRP was used in platelet deposition and aggregometry studies.

2.4.3.1 MP Generation with Lower Volume of Glass Beads
PRP activation using a lower concentration of glass beads was used in FITC fibrinogen binding studies and aggregometry studies to evaluate the effect of a lower reactive surface area on platelet refractoriness. The method used in section 2.7.3 was repeated except in this case using a 500μl Eppendorf centrifuge tube filled with 0.06g glass beads, 20μl 2XBBS and 600μl PRP. The tube was filled such that no air bubbles were trapped inside
when closed. In some trials, 1 μl apyrase/ml PRP was added to the tubes to evaluate the effect of ADP release on platelet refractoriness.

2.5 Platelet Deposition

2.5.1 Single Pass Platelet Deposition
The same method used in section 2.3.1 for platelet activation was used again with a larger diameter tube, 1.57mm x 2.08mm x 50cm, and flow rate of 5.0ml/min for the platelet deposition studies of resting and activated WB. Platelet deposition on the biomaterial was characterized by SEM. Silastic, silicone tubing, 1.57mm x 3.18mm x 30 cm, was used to connect the PE tubing to the syringes.

2.5.2 Recirculation Zone
Two sections of polyethylene tubing, having two different inner diameters, were joined in series using Silastic tubing to create a recirculation zone, crudely mimicking the flow conditions in an anastomosis. The PE tubing dimensions used were 1.14mm x 1.57mm x 25cm and 1.57mm x 2.08mm x 25cm. Silastic tubing measuring 1.02mm x 2.16mm x 3cm, was used to join the two pieces of PE. Figure 2.4 illustrates the step created when the tubing pieces are overlapped 1cm.

The 5ml WB sample in the syringe was run from the smaller to the larger tube at a flow rate of 5.0ml/min. This setup was run twice in each trial, first with resting WB and second with activated WB (recirculation method 2.3.3 or glass bead activated 2.4.3). Platelet deposition on the biomaterial at the recirculation zone was characterized by SEM.
2.6 Preparation of PE Tubing Samples for SEM

This method was used to prepare all tubing samples viewed using SEM. First, the tubing was drained of WB then rinsed using a syringe containing 10 ml HTB to remove anything which did not adhere to the biomaterial surface. The sample was then infused with 2.5% glutaraldehyde-PBS solution using a syringe and allowed to fix between 24 and 48 hours at room temperature. Once fixed the samples were drained of the 2.5% glutaraldehyde solution and placed in a series of graded ethanol solutions (10%, 30%, 50%, 70%, 90% and 100%), for 20 minutes each to dehydrate the cells.

The tubing was air dried, then cut to 1-1.5 cm lengths and sliced in half, lengthwise. These pieces were mounted onto SEM sample holders then gold sputter coated and silver painted on the ends to ensure conductivity between the sample and the holder. All samples were viewed by an Hitachi S-520 Scanning Electron Microscope, Japan. All film used was Polaroid Professional P53 film.
2.7 Methods of Characterizing the Activated Platelet Population

2.7.1 SFLLRN Activation
Post material contact activation studies were completed on samples which had been material activated (PE recirculation method, section 2.3.2 or incubation with glass beads, section 2.4.3) to observe whether a reduction in sensitivity to SFLLRN (ser-phenyl-leu-leu-arg-asn), thrombin peptide, had occurred as a result of material contact. Five microlitres of SFLLRN from the University of Toronto Peptide Synthesis Centre, Toronto, Ontario was added to 25μl of material activated or resting blood and allowed to incubate for twenty minutes at room temperature. Following this incubation period, flow cytometric analysis was carried out to determine whether P-Selectin levels for the two samples were comparable.

2.7.2 Aggregometry Studies
Glass bead activated PRP and PRP samples prepared from PE activated or resting WB were tested at 37°C for platelet aggregation response to the soluble agonists; SFLLRN(15μl /450μl PRP), ADP (50μl /450μl PRP) and collagen (50μl /450μl PRP). The aggregometer (Payton Associates, Scarborough, Ontario) was calibrated prior to each test, to determine the minimum turbidity (value of 9), using 450μl of PPP an aggregometry test tube(Ion Trace, Stouffville, Ontario). The PPP was obtained by spinning WB or PRP at 35 000 rpm for 5min.

For each run, 450μl of test PRP was added to an aggregometry test tube with a magnetic stir bar (Ion Trace, Stouffville, Ontario). Once the sample had normalized to a maximum
turbidity of 1, an agonist was added. The aggregometry tracing was continued until the line achieved the minimum turbidity or reached a plateau.

2.7.3 FITC Labeled Fibrinogen Binding
The presence of activated GPIIbIIIa receptors can be detected using FITC labeled fibrinogen rather than antibodies such as PAC-1 (32). PRP, both resting and activated (0.06g glass beads, 600μl PRP), was gel filtered prior to incubation with the FITC labeled fibrinogen.

Fifty microlitres of gel filtered platelets (resting or activated) and 5μl FITC fibrinogen was added to three polystyrene test tubes. To the second and third test tube 3μl EDTA and 5μl of SFLLRN was added, respectively. All samples were incubated for 20 minutes at 37°C, then diluted and fixed with 250μl HTB and 250μl fixative. Flow cytometry was conducted to determine the amount of fibrinogen bound to the platelets through the average fluorescence of the sample.
3.0 Results

3.1 Effect of Shear on Platelet Activation

3.1.1 Simple Tube
Blood was pumped single pass through PE tubing (0.28mm ID) at a wall shear rate of approximately 500 s⁻¹ to assess the effect of material contact on platelet activation.

![Graph showing MP generation](image)

Figure 3.1 Analysis of MP Generated in Simple Tube, Single Pass:

For a tube inner diameter of 0.28mm at a flow rate of 0.25mL/min, there was no increase in MP concentration between the resting and material contact samples for tubing lengths of 60cm and 240cm. There was also no change observed in the P-selectin expression for the material contact vs. the resting samples.

The results from flow cytometry of the effluent (Figure 3.1) showed no significant MP generation as a result of the blood flowing single pass through the PE tubing. Residence times for this experiment were under 1 minute for both the short and longer length tubes and were calculated by dividing the volume of the tubing by the volumetric flow rate.

Since the numbers of MP observed was not significant, it was assumed that MP formed during material contact were outnumbered by the bulk of the blood which did not contact the material.
3.1.2 Recirculation Method

In contrast to the single pass through the simple tube, the recirculation method was capable of generating a significant increase in MP concentration using a tubing ID of 1.57mm. This method allowed complete control over the residence time of the blood and the applied shear rate.

To determine which factors were most critical in the activation of blood, two distinct protocols were investigated. The first, which explored a range of shear rates while maintaining a constant residence time of 15 min, showed that there were no significant differences in the degree of platelet activation observed with shear rates between 170s\(^{-1}\) and 1000s\(^{-1}\) (Figure 3.2). There was however a significant difference in MP concentration noted between the static resting sample and the flowing samples. The degree of platelet activation was determined by the MP concentration in the activated samples.

The second protocol, which maintained a constant shear rate of 500 s\(^{-1}\) while varying residence time from 5 to 40 minutes showed residence time as the most critical of the two factors in MP generation. This protocol established a clear trend relating the residence time and increase in MP fraction present in the sample; as residence time increased, so did the number of generated MP (Figure 3.3). The percentage of platelets lost during a forty minute incubation period was found to be 16%.
Figure 3.2 MP Generated in Over Fifteen Minutes at Varying Flow Rates

For a residence time of fifteen minutes at flow rates of 1.5 ml/min, 5.0 ml/min and 8.8 ml/min, there was no apparent change in MP generation between samples. The error bars represent the average value ± standard deviation for n=3.

Figure 3.3 Effect of Increased Residence Times on MP Generation

This experiment was run at a constant flow rate of 5.0 ml/min, corresponding to a shear rate of approximately 500s⁻¹, for residence times ranging from 5 to 40 minutes. As the residence time of the blood in the PE tubing increased, so did the MP generated. The error bars shown represent the average ± standard error of the mean for n=7.
3.2 Alternative Methods of Creating Microparticles

3.2.1 Polystyrene Beads
Through cytometric analysis, small increases in the activated sample MP concentration were observed after incubation with the 10µm polystyrene beads (Figure 3.4). These increases appeared to be independent of the volume of beads added to the sample. As observed in previous material activated samples (PE), P-Selectin expression did not occur following PRP incubation with the beads indicating that no platelet granule secretion resulted from material contact.

Complete removal of the beads was difficult even following centrifugation because they were too small and light to settle to the bottom of the sample. The polystyrene bead activation method was abandoned because complete bead removal was necessary for further applications of the activated blood, including platelet deposition trials and aggregometry studies.

3.2.2 Freeze-Thaw Method
The freeze thaw method fractures the platelet, resulting in smaller pieces which no longer register as platelets when counted using the cell counter. Prior to the first freeze treatment, the platelet count of the washed platelets was found to be $\approx 600 \times 10^9$ platelets/L. After completing the freeze thaw process the platelet count was taken again and was found to have dropped significantly to $\approx 164 \times 10^9$ platelets/L, suggesting platelet rupture resulting in MP formation.
Figure 3.4: MP Generation Following Incubation with Polystyrene Beads

After 40 min incubation at 37°C there was a very modest increase in MP concentration in the activated samples. It does not appear that MP generation was dependent upon the bead concentration. All trials were run only once due to difficulty with complete bead removal following activation. All values were reported in relation to the resting sample, having a normalized value of 1.

Since the platelet count had dropped so drastically, it was expected that the MP concentration in each aliquot would be high. The first trial addition of the MP concentrate was very conservative at 30μl / ml WB and resulted in very little change to the observed MP concentration: 12.46%, compared to the resting sample: 9.67%. Even following the more liberal addition of the MP concentrate at 1ml / 1ml WB, the MP fraction still only reached 22%.

While the numbers of MP did not increase dramatically with the addition of the freeze thaw MP, the P-Selectin expression in the samples did climb. It was observed that following a 1:1 ratio of PRP:MP concentrate the P-Selectin expression was 7 times the
resting P-Selectin level. The freeze thaw generated MP and remaining platelets in the suspension appeared to have upregulated P-Selectin, consequently the P-Selectin increase was found to be directly dependent upon the amount of MP concentrate added (Figure 3.5). This increase was interesting since most modes of blood-biomaterial activation investigated so far have not induced P-Selectin expression in the resultant MP. The freeze thaw method was abandoned however, since the MP concentration was not greatly increased and the P-Selectin expression did not mimic the activation process of biomaterials.

Figure 3.5 Addition of Freeze Thaw MP Concentrate to WB:
Volumes of 30μl, 100μl, 200μl and 1000μl freeze thaw MP concentrate were added to 1ml resting WB to determine the volume required to reach MP concentrations of 20%. There was a direct relationship found between the amount of MP concentrate added and P-Selectin expression in the sample.
3.2.3 Glass Beads

Glass beads, measuring 350-400 μm in diameter with a surface to volume ratio of 687 cm⁻¹, were investigated and found to produce 30-40% MP after 30 minutes of incubation at 37°C (Figure 3.6). The observed MP concentration in the activated sample was approximately 6 times that of the resting sample. On average 50% of platelets were lost during glass bead PRP activation using 0.73g of beads.

PRP activation via this method did not induce P-Selectin expression which was consistent with other modes of blood-biomaterial activation. As seen in Figure 3.6, the SFLLRN activated sample expressed a significant amount of P-Selectin as compared to the material activated sample. This method was accepted for use as the alternative method of creating MP for platelet deposition studies, SFLLRN activation, aggregometry studies and FITC labeled fibrinogen binding.

Figure 3.6 PRP Activation via Glass Beads

<table>
<thead>
<tr>
<th>n=7</th>
<th>Rest MP (% of GPIIbIIIa positive events)</th>
<th>Glass Bead MP (% of GPIIbIIIa positive events)</th>
<th>Glass Bead P-Selectin (Signal Intensity)</th>
<th>SFLLRN P-Selectin (Signal Intensity)</th>
</tr>
</thead>
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<tr>
<td>Average</td>
<td>5.6</td>
<td>34.7</td>
<td>5.5</td>
<td>129.7</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>1.3</td>
<td>10.5</td>
<td>1.8</td>
<td>41.6</td>
</tr>
</tbody>
</table>

3.3 Platelet Deposition Studies

3.3.1 Simple Tube

In this experiment, blood (resting or material preactivated) was run through a simple PE tube (1.57mm ID), at a shear rate of approximately 500s⁻¹, then the tube was flushed, fixed and mounted for viewing via SEM to determine platelet deposition.
When the blood was preactivated by the recirculation method there was no significant difference in the platelet deposition for resting and preactivated samples (Figures 3.7 a, b, c and d). The MP concentration generated for the recirculation method in section 2.3.2.1, which used a larger volume of WB, was roughly 12-14%, slightly higher than resting MP concentrations but less than the 20% MP fraction reported in section 3.1.2.

In contrast, the glass bead preactivated samples (0.73g glass beads) experienced decreased platelet deposition as compared to resting blood (Figure 3.8a and b) despite having a much greater MP concentration and a larger surface area than the PE recirculation method. In a 50μm × 50μm grid, counted on the SEM in Figure 3.8, the glass bead preactivated sample deposited 40 platelets and the resting sample deposited 64 platelets. The decrease in platelet deposition for the glass bead preactivated sample can not be attributed to platelets lost during material activation as the platelet concentration of the resting blood samples were normalized to that of the preactivated sample prior to running the blood through the tubing.

### 3.3.2 Expansion Zone

The abrupt expansion of the PE tubing mimicked the recirculation flow patterns of the anastomosis shown in Figure 1. At the recirculation zone, distal to the expansion in diameter, there was a marked increase in platelet deposition for both preactivated and resting blood samples as compared to the single ID tube. Furthermore, the platelet deposition of the resting blood: 114, was greater than that of the glass bead preactivated blood: 71, in the recirculation zone (Figure 3.9a and b). The resting blood sample was diluted to the platelet concentration of the preactivated samples prior to material contact.
a) At 500x magnification, this is a picture of resting platelet deposition on PE tubing following 5ml WB run at a flow rate of 5.0 ml/min. Platelet count 4.7e6 platelets/cm².

b) At 300x magnification, this picture shows the platelet deposition on PE tubing following contact with 5ml PE recirculation preactivated WB run at a flow rate of 5.0 ml/min. Platelet count 3.72e6 platelets/cm².
c) At 300x magnification, this is a picture of resting platelet deposition on PE tubing following contact with 5ml WB run at a flow rate of 5.0 ml/min. Platelet count 1.88e6 platelets/cm².

d) At 300x magnification, this is a picture of the platelet deposition on PE tubing following contact with 5ml PE recirculation preactivated WB run at a flow rate of 5.0 ml/min. Platelet count 1.72e6 platelets/cm².
a) Resting WB, volume 5 ml was run through PE tubing at a flow rate of 5.0 ml/min, magnification 300x. Platelet count 2.56e6 platelets/cm².

b) Five ml of glass bead preactivated blood (0.73g glass beads, 200µl PRP) was run at a flow rate of 5.0 ml/min through a PE tube, magnification 300x. Platelet count 1.6e6 platelets/cm².
Figure 3.9: SEM taken 98/02/19

a) Resting platelet deposition on PE at the recirculation zone after 5ml WB was run at 5.0 ml/min, magnification 500x. Platelet count 4.56e6 platelets/cm²

b) Platelet deposition on PE at the recirculation zone after 5ml glass bead preactivated WB (0.73g, 200μl PRP) was run at 5.0 ml/min. Platelet count 2.84e6 platelets/cm².
therefore the differences in platelet deposition could not be attributed to platelet concentration.

3.4 Characterization of Activated Platelets
The results presented so far have indicated that platelets preactivated by a material do not adhere to a biomaterial surface more readily than resting platelets, as shown through SEM. In fact, the material preactivated platelets appear to be less adherent. An investigation was conducted to further characterize the platelet population, after biomaterial activation, for any indications which may explain this behaviour.

3.4.1 Flow Cytometric Quantification of GPIIbIIIa Signal Intensity
Platelet adherence to biomaterials is mediated through the GPIIbIIIa receptor on the platelet surface. This investigation showed no loss in ability of the GPIIbIIIa receptors to bind antiCD41 following material activation (Figure 3.10). The ANOVA analysis (p=0.69) of the three groups; T=0, Rest and Glass Bead Activated, indicated that the three groups were statistically similar in CD41 Signal Intensity.

3.4.2 SFLLRN Activation
Post material contact activation studies were also undertaken. A comparison between the thrombin peptide activated samples (resting and material activated) indicated that granule release resulting from thrombin peptide stimulation is reduced for the material activated samples when compared with the agonist activated rest sample (Figure 3.11). This loss of sensitivity to the platelet agonist indicates some degree of platelet refractoriness resulting from material contact. The material activated samples which were not SFLLRN stimulated samples did not express P-Selectin on the platelet surface.
Figure 3.10: Quantification of GPIIbIIIa Signal Intensity for Rest and Glass Bead Activated Samples
This figure was constructed from 12 sets of signals intensities for T=0, resting and glass bead preactivated samples. The analysis of variance for the groups revealed that all three were statistically similar (p = 0.69).

Figure 3.11: Post Material Contact P-Selectin Expression showed the effect thrombin peptide, SFLLRN, had on inducing P-Selectin expression in the resting blood samples but not in the material activated samples. These results indicated some degree of platelet refractoriness was resulting from material contact. All values were normalized to the resting sample. Error bars are the average value ± standard deviation for n=3.
3.4.3 Platelet Aggregation Studies
Platelet aggregation studies measured the ability of platelet agonists to induce aggregation in resting and activated PRP samples. Through this analysis it was determined that the material activated platelets had experienced a reduction in sensitivity to the platelet aggregating agents. All agonists used were capable of initiating complete aggregation in the resting PRP samples, while exhibiting difficulty in producing the same effect in glass bead preactivated samples (0.73g glass beads, 200 µl PRP), as seen in Figure 3.12.

When the lower glass bead concentration (0.06g glass beads) was compared to the higher glass bead concentration (0.73g) tracing, the lower surface area sample was found to exhibit more aggregation, as seen in Figure 3.13a. The average percentage of platelets lost during the thirty minute glass beads incubation (0.06g) was 33%. The comparison of the PE tubing (recirculation method) to the lower concentration of glass beads (0.06g) showed that both had comparable reactive surface areas and MP generation capabilities; 23% (PE recirculation) and 26% (glass beads). Further analysis of the aggregometry tracings revealed that both experienced a similar loss in sensitivity to the platelet aggregating agents (Figure 3.13b).

The extent of refractoriness seen in Figure 3.12, was thought to be affected by the air-PRP interface and the surface area of the glass beads used in the preparation of the preactivated blood. However, in the absence of an air interface, lowering the surface area of the glass beads (0.06g glass beads, 600 µl) continued to reduce platelet agonist effectiveness although resulting in the generation of fewer MP; 26%.
The addition of apyrase, to reduce all ADP to AMP, did not diminish the platelet refractoriness in the material preactivated samples (Figure 3.14). The tracing clearly showed that the sample without apyrase acted just as the sample with apyrase, confirming that ADP release during material activation was not the cause of the observed platelet refractoriness.

Figure 3.12 Aggregometry Tracings for Glass Bead and PE Recirculation Activated Samples:
This tracing illustrated the reduction in effectiveness for the platelet agonists on the material preactivated samples. From left to right the samples and agonist used were: PE preactivated with SFLLRN, Rest with SFLLRN, glass bead preactivated with collagen, glass bead preactivated with ADP, Rest with ADP, glass bead preactivated with SFLLRN and Rest with SFLLRN.

Figure 3.13a Aggregometry Tracings: Both volumes of glass beads (0.73g and 0.06g) were investigated and the higher volume was shown to result in a greater reduction in sensitivity to platelet aggregating agents.

Figure 3.13b Aggregometry Tracings:
Both the PE recirculation and glass bead (0.06g) samples have been subjected to similar reactive surface areas and consequently have experienced similar reductions in sensitivity to the platelet aggregating agents.

Figure 3.14 Aggregometry Tracing:
An aggregometry tracing for glass bead preactivated PRP with and without apyrase. The addition of apyrase did not eliminate platelet refractoriness following activation via glass beads (0.06g glass beads, 600μl PRP).

3.4.4 *FITC labeled Fibrinogen Binding*
Material preactivated platelets were also characterized for the presence of activated GPIIbIIIa receptors. Neither rest nor glass bead activated samples were found to express activated forms of GPIIbIIIa as witnessed by the relative lack of fibrinogen binding. This suggested that although material activated platelets created MP, they were not activated in such a way that platelet adhesion events should be expected to increase following
material contact. This was an interesting finding as it was in complete contradiction with the hypothesis of this thesis.

SFLLRN was used to activate platelets and correspondingly reveal the fibrinogen binding site on GPIIbIIIa. While the glass bead activated + SFLLRN samples expressed more activated GPIIbIIIa than the glass bead activated sample without SFLLRN stimulation, this amount was still less than the resting platelet sample + SFLLRN which bound the most fibrinogen (Figure 3.15). It appeared that the material activated sample was not as strongly activated by the SFLLRN as the rest sample, possibly indicating material induced platelet refractoriness.

![Graph showing FITC Labeled Fibrinogen Binding Intensities](image)

Figure 3.15 FITC Labeled Fibrinogen Binding Intensities
This figure shows that neither the resting or material preactivated samples without SFLLRN stimulation exhibited activated GPIIbIIIa on the platelet surface while both samples with SFLLRN stimulation did exhibit GPIIbIIIa receptor activation to a certain degree.
4.0 Discussion

4.1 Effect of Shear Rate on Platelet Activation

The effect of shear rate on platelet deposition has been well characterized; high flow rates enhance platelet adhesion through increased platelet transport to the surface (8,10,13).

Platelet activation via shear application however has not been as well characterized and contradictory results have been reported on this subject. Miyazaki et al. (25) claim to have observed shear rate dependent platelet activation at a shear rate of 3000s\(^{-1}\) whereas Holme et al. (2) found no differences in MP generation and platelet activation for shear rates of 420s\(^{-1}\) and 2600s\(^{-1}\). In both the recirculation method and single pass experiments, platelet activation and MP formation were found to be independent of shear rate, supporting the assumption that shear rate was not a critical factor in the creation of platelet microparticles.

High shear rates were not found to cause platelet degranulation in any of the samples. These results were supported by the work of Rhodes et al., who found that whole blood subjected to shear rates as high as 3000s\(^{-1}\) for a contact time of 30s did not have increased P-Selectin expression (6). Likewise, increased material contact times did not result in platelet degranulation, although these increases in residence time did result in more MP formation. For material contact times of up to 40 minutes, no P-Selectin upregulation was observed for any material activation trials. This suggested that MP formation resulting from platelet material contact (PE and glass beads) was independent of granule secretion hence classifying material activation as very weak, rather than strong, like thrombin induced platelet activation.
Erythrocytes are very important in platelet material interaction as the rotation of the RBC facilitates the delivery of platelets to the biomaterial surface. The effective diffusivity of platelets is directly influenced by the concentration of the erythrocytes since increased RBC concentration results in greater numbers of platelet-erythrocyte interactions. The resultant radial drift from these interactions pushes the platelets toward the biomaterial surface, causing platelet material interaction (7). Platelet activation reactions are reported to be very time dependent (6) and this dependence on residence time was well illustrated by the increased MP concentration seen in the bulk during the 40 minute incubation period of the PE recirculation method.

Unfortunately, for short contact times the bulk of the platelet population did not contact the biomaterial so the resting platelets overwhelmed any MP which had been formed during platelet-material contact (Figure 3.1). For this reason the single pass method was abandoned in favour of the recirculation method. With its arbitrary residence time and shear rate, the recirculation method was able to generate significant increases in MP concentration. As shear rate had not been found to be a critical factor in MP formation, it was determined that greater residence times accounted for the higher MP concentrations through increased numbers of platelet-biomaterial interactions. From the results discussed, it was concluded that contact time rather than shear rate was the most important factor to consider when fostering an environment for platelet biomaterial activation.
4.2 Alternative Methods of Creating MP

Polystyrene beads were investigated as an alternative method of creating material induced MP. Despite creating MP without the upregulation of P-Selectin to the activated platelet surface and the suggested benefit of high surface area to volume ratio, the polystyrene beads were troublesome to work with because of the difficulty associated with removal from the sample following incubation. Extra handling, including centrifugation, was required for bead removal which raised concerns with residual activation not associated with the material contact, consequently this method was abandoned.

The freeze thaw method of creating microparticles had been used for deposition experiments on subendothelium (35) to evaluate whether the presence of MP corresponded to increased platelet deposition. This thesis research intended to use the freeze thaw MP to observe whether the increased MP population would result in greater platelet deposition on a biomaterial, however the freeze thaw concentrate did not supply a greatly increased concentration of MP following its addition to WB.

This method was deemed unfeasible since the platelet microparticles, MP, created by the freeze thaw method, expressed elevated levels of P-Selectin as compared to the MP created through the PE recirculation method. As seen in Figure 3.5, the P-Selectin signal intensity of the 1:1 ratio of MP concentrate to WB, was a significant increase over the P-Selectin expressed in the resting sample and this change in P-Selectin expression was found to increase linearly with the addition of the MP concentrate to the WB. As the control rest samples exhibited the requisite low levels of P-Selectin expression associated
with platelet quiescence, it was concluded that the increase in P-Selectin expression came solely as a result of the freeze thaw MP concentrate.

The freeze thaw method created MP by fracturing the platelets into smaller pieces through the introduction of -70°C temperatures. The water in the suspension and inside of the platelet expanded as it froze, literally shattering the platelets into millions of pieces. It was possible that through this fracturing process the P-Selectin, stored within the α granules of resting platelets, became exposed and consequently presented on the outer surface of the MP. Therefore when this freeze thaw MP concentrate was added to the resting WB sample, the P-Selectin expression of the sample was increased. Platelet microparticles produced via this method were not believed to be fair representations of MP created through biomaterial activation and were rejected for use in further studies.

Glass is known to be a strong platelet activating material so as expected, glass beads (0.73g, 200 μl PRP) were successful in producing large increases in MP concentration following incubation with PRP. Even the lower concentration of glass beads (0.06g, 600μl PRP) was capable of creating similar numbers of MP as the PE recirculation method. This congruence in MP generation was attributed to the similarity in surface area for the two methods; 6.8 cm⁻¹ for 0.06g glass beads and 9.9 cm⁻¹ for the PE tubing. This method was able to generate large concentrations of MP with small bead volumes after 30 min incubation, thus it was chosen as the alternative method for increasing the MP concentration.
4.3 Platelet Deposition on Biomaterial

The shear rate of $500s^{-1}$ was used predominantly in this thesis as it was the time average mean shear rate of WB in the circulation (11). It was interesting to note that platelet interaction with the biomaterial at the flow rate of $500s^{-1}$ was not dominated by either diffusion or kinetic factors (7).

The hypothesis of this thesis expected that preactivated platelets would preferentially adhere to the biomaterial surface in greater numbers than their resting counterparts. This was believed to be a sound hypothesis as platelet microparticles are known to possess procoagulant properties which were hypothesized to aid in increasing the platelet deposition for preactivated samples. Glass bead and PE recirculation method preactivated platelets have not been found to have enhanced propensity to adhere to biomaterial surfaces. The deposition of the biomaterial preactivated platelets onto the biomaterial surface has been shown qualitatively through SEM to be equivalent to, or less than, the deposition of the resting platelets. The observation of decreased deposition for long material contact times was consistent with Gluszko et al. who reported that platelets lose their ability to adhere to synthetic surfaces following extended contact with synthetic surfaces (36).

Possible reasons for the decreased deposition of preactivated platelets were suggested to be (30):

1. Platelet concentration of the preactivated sample was decreased to such a degree that fewer platelet biomaterial surface interactions were occurring therefore resulting in fewer platelets deposited on the surface.
This possibility was addressed early in the research and even following the adjustment of the resting platelet concentration to that of the preactivated sample, the rest sample continued to deposit platelets at concentrations greater than or equal to that of the preactivated sample.

2. Plasma proteins in the preactivated sample were adsorbed onto the surface of the glass bead and were not present in great enough concentrations in the PRP, thereby inhibiting their adherence to the biomaterial surface.

The issue of plasma protein dilution was investigated briefly in February of 1998. The PE tubing used for the platelet deposition studies was incubated in pooled plasma prior to its contact with the preactivated blood. This incubation allowed plasma proteins to adsorb onto the biomaterial surface prior to blood contact, thereby eliminating any problems associated with plasma protein dilution. Plasma incubation was found to have no effect on platelet adhesion as the resting sample continued to deposit more platelets on the biomaterial surface than the glass bead preactivated sample.

3. The receptor by which platelets adhere to the plasma protein adsorbed on the biomaterial was unable to perform its function either due to rearrangement or internalization of the GP on the platelet surface thus rendering the platelets refractory.

The process by which platelets were preactivated by biomaterials appeared to be through damage to the cell receptors rather than through the biochemical pathways as with agonists such as thrombin peptide, which result in platelet degranulation as well
as MP formation. Cell damage by material contact could have internalized of rearranged the adhesion receptors ultimately resulting in fewer functional receptors on the platelet surface to become involved in adhesion to the biomaterial. Evidence of this platelet receptor damage has not been observed.

This third explanation for decreased platelet adherence for material preactivated samples was investigated and discussed further in section 4.4, Characterization of the Activated Platelet Population.

The platelet deposition in a simple tube was a consequence of fully developed flow which evenly deposited platelets throughout the tubing length whereas the recirculation zone created by the abrupt step in the tubing ID allowed analysis of the platelet deposition resulting from the flow patterns associated with a stenosis. It was important to study such flow patterns as most implants, once placed in the blood flow, disrupt the circulatory flow patterns by creating recirculation zones in which MP and preactivated platelets could spend extended periods of time, thus increasing the probability of adhesion. Both the preactivated and resting blood samples experienced increased platelet deposition at the recirculation zone. This increase in localized adhesion of platelets could be explained by the effect of the flow patterns associated with the stenosis (26). Platelets were swept very close to the vessel wall thereby increasing the flux of cells toward the vessel wall and the possibility of cell-biomaterial interaction and platelet deposition. This effect was amplified by the erythrocytes around the reattachment point, which served to increase the diffusive and convective transport of the platelets. Both preactivated and
resting samples experienced their greatest deposition in the recirculation zone, distal to the step, however the resting sample continued to exhibit greater platelet adhesion at this ID step than the preactivated sample. Further characterization of the material preactivated platelet population was undertaken in an attempt to explain the differences in deposition observed for the preactivated and resting samples.

4.4 Characterization of the Platelet Population
Faced with results which were in direct contradiction with the hypothesis of this work, it was imperative that the preactivated platelet population be more thoroughly characterized to understand why biomaterial preactivated platelets were less adherent than their resting counterparts. As the GPIIbIIIa receptor was an important mediator by which platelets adhered to the plasma proteins adsorbed onto the biomaterial surface, the decrease in platelet adherence following material preactivation raised questions concerning GPIIbIIIa receptors on the material preactivated platelet population. An investigation was carried out to determine whether a decreasing trend was found in the GPIIbIIIa signal intensities for the preactivated samples, thus indicating a loss in the ability to bind antiCD41 following material activation.

Papers dealing with extracorporeal circulation (29,30) reported that GPIIbIIIa receptors were lost from the platelet surface during simulated extracorporeal circulation, an explanation which would account for the decrease in platelet deposition. Four different mechanisms were postulated to result in the loss of GPIIbIIIa receptors from the material preactivated platelet population found in the bulk (29,30).
1. The platelets in the circulation having a higher number of GPIIbIIIa receptors adhered more readily to the material surface thus leaving behind the population having fewer GPIIbIIIa receptors in the bulk of the sample,

2. Fragmentation and MP formation caused by activation, surface contact and shear stress lead to lower GP concentrations,

3. Possible rearrangement of the platelet membrane following initial damage caused a decrease in binding sites, and

4. Receptor occupancy by fibrinogen prior to incubation with the antibody, did not allow the attachment of antiCD41 or cleavage of the receptor from the platelet surface. 

Despite the reports of lost GPIIbIIIa receptors, this thesis found that there were no statistical differences between the means or variances of the antiCD41 signal intensities for all three groups; T=0, Resting and Bead Activated (Figure 3.10), at a 95% confidence level (Calculations were made using Statgraphics V4.0 and are attached in the appendix.). As a result, the decrease in adhesion for the material preactivated samples could not be explained by the loss of GPIIbIIIa receptors from the platelet surface or the decreased ability of these receptors to bind antiCD41.

Platelet-material contact did not upregulate P-Selectin to the platelet surface. This finding was consistent with the paper by Musial et al. (30) which discussed the lack of platelet degranulation for samples having undergone extracorporeal circulation. Platelet activation using SFLLRN following material contact likewise failed to elicit extensive P-selectin expression. This diminished response was defended by other studies which have also reported reduced response to platelet aggregating agents following material contact
(8,28, 29, 30,36). All SFLLRN activated positive control samples had elevated P-Selectin expression as compared to the rest sample without agonist stimulation. The P-Selectin expression in the positive control sample confirmed that the inability to induce P-Selectin expression in the material preactivated sample was not a direct result of the donor blood nor the quality of the SFLLRN sample used. This decreased sensitivity to SFLLRN in the material preactivated sample reinforced that the effect appeared to be material contact related.

Reduction in the platelet response to a known platelet agonist has been termed refractoriness, as discussed in the introduction. A significant reduction in the ability of agonists, SF, collagen and ADP, to induce platelet aggregation was detected during the aggregometry studies following material contact. It has been reported that the circulating platelets of patients, who had undergone cardiopulmonary bypass surgery, exhibited a reduced ability to aggregate when platelet agonists such as ADP or epinephrine were introduced (8, 28). The same phenomena was repeated during simulated extracorporeal circulation experiments and other material contact studies (29,30,36).

Although relatively few platelets were strongly activated following material contact, it appeared that most had reduced function in the circulation (8). This statement accurately described the platelets which had been subjected to material activation, as the ability to aggregate was compromised but not completely lost. The hypotheses offered to explain the reduced platelet function were:
1. Platelets may have suffered membrane damage resulting in alteration to the agonist receptors.

2. Platelets may have partially released their granule contents, resulting in reduced function from depleted granule stores.

The first hypothesis offered seemed to be the most plausible, as the second would have been accompanied by the upregulation of P-Selectin to the platelet surface, which was not observed. Alteration to platelet receptors by which activation occurs could impair the ability of the platelet to become activated by agonists, thus become refractory. Platelet contact with a synthetic surface may be capable of causing this sort of receptor damage.

The high surface area of the glass beads (0.73g, 200µl PRP) resulted in the greatest decline in sensitivity to the platelet agonists as well as the highest concentration of platelet microparticles following activation. Although the PE recirculation method also experienced a decline in the sensitivity to the platelet agonists, it was not to the same degree as the glass bead preactivated samples. The PE tubing, with a surface to volume ratio of ~9.9 cm⁻¹, resulted in the production of fewer MP due to a smaller number of platelet-material interactions during the activation period. Ultimately this lower MP concentration lead to a less drastic decline in agonist sensitivity than observed in samples with higher MP generation. It appeared that the loss in agonist sensitivity was directly dependent on the MP concentration generated during material contact.

The glass bead concentration was reduced (0.06g, 600µl, surface to volume ratio of 6.83 cm⁻¹) to investigate whether the platelet refractoriness observed with the higher glass bead
concentration was due solely to the high surface to volume ratio of 688 cm\(^{-1}\) and its associated high MP concentration. PRP incubation with the lower concentration continued to affect the ability of the agonists to induce platelet aggregation, however the reduction in aggregation was not as drastic as was witnessed with the higher concentration (0.73 g, 200 \(\mu\)l PRP). A correlation between MP generation and agonist sensitivity was noted (Figure 4.1); as the MP concentration increased, the ability of the agonist to aggregate the platelets diminished.

![Corellation Plot](image)

**Figure 4.1 Corellation Plot Percentage MP Generated vs. Percent Refractoriness**

This figure shows the correlation between the MP generated during material activation as it relates to the percentage of refractoriness observed in the sample. The best fit line generated through regression analysis was found to be \(y=65.3+3.87x\).

The PE recirculation method and the lower concentration of glass beads experienced comparable MP generation and loss of platelet agonist sensitivity, although these were both less pronounced than observed using the high concentration of glass beads (0.76 g). In these lower surface area experiments, the platelet responsiveness was not compromised.
completely since aggregation still occurred although to a lesser degree than the resting PRP samples. The lower surface area, in combination with the lower concentration of MP, may explain why the platelets preactivated through the rocking platform-low shear experiment (used by Cynthia Gemmell) continued to be responsive to agonists. As the degree of platelet refractoriness could be correlated to the concentration of MP generated, it was suggested that the platelets preactivated by the rocking platform method were in fact slightly refractory. This meant that these samples could still be made to elicit some P-Selectin expression and be aggregated by platelet agonists, because the MP concentration generated was not high, but to a lesser degree than the resting samples.

It was important to show, via the bead experiment, that the air interface was not the root cause of the platelet unresponsiveness since the plasma-air interface would tend to conformationally alter the plasma proteins (6). The results of the air interface elimination protocol showed that the air interface was not a contributing factor since platelet refractoriness continued to be present in the glass bead PRP samples activated in the absence of air.

Both RBC and platelets store ADP, which could leak out if the membranes of the cells were compromised. Small concentrations of ADP in the plasma have been attributed to reductions in platelet agonist response (28). Apyrase is a low molecular weight enzyme which acts on ADP by removing a phosphate and reducing it to AMP (adenosine monophosphate). This enzyme was added to the WB or PRP sample prior to activation to assess whether ADP leakage was a factor in the observed platelet refractoriness.
The addition of apyrase to the samples made no changes to the observed refractoriness as was seen in the aggregometry tracings (Figure 3.14) comparing material activated samples with and without the addition of apyrase. Since the tracings with and without the apyrase were the same, it was confirmed that ADP leakage from the compromise of either RBC or platelets was not the cause of the observed platelet refractoriness in the material activated samples.

A FITC labeled fibrinogen binding study concluded the characterization of the material activated platelet population. Although no conclusions could be directly drawn from this experiment as \( n=1 \) was not statistically significant these results supported observations made in previous characterization studies. First, this test showed that no activated GPIIbIIIa was present either on material activated or resting platelets when compared to the SFLLRN stimulated samples. This observation demonstrated that although platelet-material interaction did result in platelet microparticle formation, it did not result in the activation of the GPIIbIIIa receptor nor did it contribute to platelet degranulation. The absence of activated GPIIbIIIa receptors able to bind to fibrinogen provided an explanation for why the material activated platelets did not adhere more readily to the biomaterial surface than the resting platelets.

Second, this experiment supported the idea of the material induced platelet refractoriness as seen in the SFLLRN Activation results of section 3.4.2, and the aggregometry studies, section 3.6.3. The introduction and incubation of SFLLRN with both the rest and activated samples during the FITC fibrinogen binding showed that the rest sample bound
more fibrinogen than the material activated sample, suggesting that a greater number of GPIIbIIIa receptors were activated by the SFLLRN. The fibrinogen binding results obtained for the material activated samples following SFLLRN stimulation were consistent with studies which investigated WB following biomaterial contact (30). These studies reported lower fibrinogen binding capabilities for the preactivated samples when compared to the SFLLRN activated rest samples. This test should be repeated with different materials to provide more concrete evidence of this material induced refractoriness and shall be recommended for future work.

Platelet- material interaction accompanied by substantial MP generation has been shown to result in diminished platelet adhesion to a synthetic surface. This fact may aid in understanding why platelet activating materials, such as PVA tubing, have experienced negligible platelet deposition although capable of generating MP. Perhaps in generating MP through material contact platelets have lost, rather than enhanced, their adhesion capabilities. Consequently, it would follow that all strongly activating platelet materials should display lower numbers of adherent platelets following extended blood contact with a fixed volume of blood, such as in the dog shunt experiments.

Platelet activation resulting from biomaterial contact has not been well characterized and similarly no mechanisms for this mode of activation have been identified. The thesis has shown GPIIbIIIa activation and P-Selectin upregulation was not a consequence of biomaterial contact. Biomaterial activation does not appear to be similar to agonist activation, which utilizes biochemical signalling pathways to activate receptors, initiate
platelet shape change and cause platelet degranulation. Rather material activation appears to be most likely due to physical damage to the cell resulting in MP formation and rearrangement or internalization of the surface receptors, thus accounting for the observed platelet refractoriness.
5.0 Conclusions
Platelet activation by a biomaterial was suggested to be affected by material contact time and the applied shear rate. The PE recirculation method investigated both factors and it was concluded that platelet activation was dependent upon increased contact time and independent of increases in shear rate.

Platelet contact with PE and glass did not significantly activate the GPIIbIIIa receptor or upregulate P-Selectin and therefore it was concluded that preactivation of platelets through material contact should not be expected to result in increased platelet adhesion.

Material preactivation of platelets accompanied by substantial microparticle formation appeared to inhibit rather than enhance platelet deposition on biomaterial surfaces. As GPIIbIIIa receptors were not lost during activation, it was concluded that greater MP formation during material contact was an indication of impaired platelet adhesion capability.

The MP concentration following material contact was found to correlate with the reduced sensitivity to aggregation inducing agonists: the greater the MP concentration generated through material contact, the less sensitive became the activated platelet population to agonist stimulus. The degree of platelet refractoriness and reduction in platelet adherence were both found to be related to the MP concentration generated during material contact. Aside from being an indicator of platelet activation, MP may also be indicators of lost platelet functionality.
6.0 Future Work

To compare deposition of PVA and PE activated platelets on a biomaterial surface via SEM or LDH analysis. In this way it could be determined whether a more strongly platelet activating material (i.e. one capable of generating greater MP concentrations) would result in a greater decrease in platelet adhesion when compared to the rest sample as suggested in section 4.4.

To continue the fibrinogen binding studies with blood activated by biomaterials, other than glass, to determine whether biomaterial contact does indeed result in lost agonist sensitivity and reduced ability to activate the GPIIbIIIa receptor.
References


33. Hepalean Product Information Sheet, Organon Technika, Toronto, Ontario


Appendix

A1. Definitions

ADP- Adenosine Diphosphate is a strong platelet agonist present in platelet dense granules and red blood cells. ADP is used as a platelet agonist in the aggregometry studies.

Biomaterial- A non-viable material used in the fabrication of a biomedical device which interacts with a biological system(4).

EDTA- Ethylenediaminetetraacetic acid is a strong calcium chelating agent used to inhibit platelet activation requiring calcium to proceed. (16)

HTB- Hepes Tyrode Buffer is used as a buffer in flow cytometry to dilute the 5λ sample for analysis.

MP- An abbreviation for the term microparticle. MP result from platelet vesiculation following classical activation or biomaterial contact and are reputed to demonstrate procoagulant activity.

PRP- Platelet Rich Plasma is platelets and plasma without all other blood constituents such as WBC and RBC. PRP is generated by centrifuging anticoagulated blood at 800 rev/s for 6-7 minutes.

PPP- Platelet Poor Plasma remains when all WB particle constituents have been removed. PPP is generated by spinning either PRP or WB at 35 000 rev/s for 5 minutes.

Refractory- Platelets are deemed refractory if they exhibit a weaker response than normal resting platelets to activating stimulus.
WB- An abbreviation for whole blood. Heparin was added to all WB at 5 units/ml WB.

GP- An abbreviation for glycoprotein receptors (i.e. GPIIbIIIa).

A2. Washed Platelets

1. Solution A

<table>
<thead>
<tr>
<th>Reagent</th>
<th>mass</th>
<th>molecular mass</th>
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</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>4 g</td>
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<tr>
<td>KCl</td>
<td>0.1 g</td>
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</tr>
<tr>
<td>NaHCO₃</td>
<td>0.5 g</td>
<td>84.01</td>
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<tr>
<td>NaH₂PO₄·H₂O</td>
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</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>0.1 g</td>
<td>203.31</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.5 g</td>
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</tr>
</tbody>
</table>

Make up to 500 ml with double distilled water. Store at 4°C.

2. Solution B

<table>
<thead>
<tr>
<th>Reagent</th>
<th>concentration</th>
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</thead>
<tbody>
<tr>
<td>Solution A</td>
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<td>-</td>
</tr>
<tr>
<td>CaCl₂·H₂O</td>
<td>0.03 g/100ml</td>
<td>147</td>
</tr>
</tbody>
</table>

Store at 4°C.

3. Wash #1

Solution A 45 ml
3.8% sodium citrate 5 ml
Bovine albumin 0.175 g

Use a very pure grade of albumin. Float it on the solution, then mix gently after dissolved. Adjust the pH of the solution to 6.4. It is very important to have the pH be exact.

4. Wash #2

Solution A 50 ml
Bovine albumin 0.175 g
Adjust the pH level to 7.4

5. Wash #3

Solution B 50 ml
Bovine albumin 0.175 g
Adjust the pH level to 7.4
6. Draw WB as in 2.1.1 except use 3.8% sodium citrate at a 1:10 dilution as the anticoagulant. Spin the blood as in 2.1.2 to achieve PRP. Transfer the PRP to a polypropylene tube taking care to avoid red cells.

7. Spin the PRP at 1000g (2200 rpm) for 15 min. Discard the supernatant. Resuspend in 5 -10 ml of wash #1 and transfer to a clean tube. Avoid the introduction of bubbles into the solution. Cover and allow to sit for 10 min.

8. Spin at 900g (1800 rpm) for 10 min. Discard the supernatant. Resuspend in 5 -10 ml of wash #2 and transfer to a clean tube. Cover and allow to sit for 10 min.

9. Spin at 900g (1800 rpm) for 10 min. Discard the supernatant. Resuspend in 5 -10 ml of wash #3. Perform a platelet count using the Sysmex E-2500, Japan. Adjust the count to between 250 - 350 x10^9 platelets/L, using wash #3.

A3. Gel Filtered Platelets
This method was taken from Platelets A Practical Approach (17)

Equipment and Reagents
Siliconized Glass Column
0.9% NaCl
Acetone
HTB
Sepharose 2B, Sigma

A. Column Preparation
1. Prepare a siliconized glass column containing an nylon microfilament disc of 52 µm mesh.
2. Wash the Sepharose 2B in acetone (3-4 vol.) followed by 0.9% NaCl (5-6 vol.).
3. Pour the packing material into the column. It is important to avoid air bubbles in the poured column. In this instance the column should be repacked.
4. Connect the Butterfly needle to the bottom of the column.
5. The column should be used at room temperature.

B. Isolation of Platelets
1. Apply HTB (2-3 vol.) to column and allow to pass through. When the HTB has entered the gel apply the PRP directly onto and allow to enter, the gel.
2. Add more HTB.
3. Collect the eluate which is clear in the absence of platelets.
4. Platelets are eluted as an opaque fraction prior to the plasma proteins.
SEM

SEM was indisputably, an excellent tool for the qualitative evaluation of density of platelet adhesion and the discussion of platelet morphology following biomaterial deposition. Quantitative assessment of platelet deposition using pictures from SEM and image analysis software, however, involved problems with picture quality, intensity thresholding and edge detection which lead to an artificially low platelet count.

Intensity thresholding and an edge detection algorithm were the means by which the NIH Particle Analysis program determined where particles started and ended. Any platelets which were clumped together or touching were counted as a single particle. In most instances where platelet deposition was high, the platelets were not deposited singly, but rather adhered to the biomaterial in very close proximity to one another. This contributed to the artificially low platelet counts since the touching platelets were counted as a single particle. Picture quality also contributed to artificially low platelet counts. Overexposure made thresholding difficult to adjust such that not too many single platelets were removed from the analysis.

This software program would benefit from a more sophisticated algorithm which could perform a correlation between the enclosed area and the average surface area covered by a user defined particle size. This addition would correct for platelets which are clumped together or touching and allow for a better estimate of the platelet deposition.
A5. Lactate Dehydrogenase (LDH) Assay

Lactate dehydrogenase is present in the cytoplasm of platelets and therefore is commonly used as a marker for the platelet cytosol (Holm Holmsen. Platelet Responses and Metabolism vol. II: Receptors and Metabolism, CRC Press Inc, 1987). The LDH assay was based on the activity of LDH in the bulk after lysis of adhered platelets which allowed for a direct correlation to the number of platelets adhered to the biomaterial surface (6). The LDH assay can be a very powerful tool since it is easy to use and is not time consuming.

The LDH assay works on the basis of the following test principle. In the body, LDH is the enzyme which catalyzes the conversion of pyruvate to lactate. This reaction recycles the NADH produced in the glyceraldehyde-3-phosphate dehydrogenase reaction (Holm Holmsen. Platelet Responses and Metabolism vol. II: Receptors and Metabolism, CRC Press Inc, 1987).

\[
\text{pyruvate} + NADH + H^+ \xrightarrow{LDH} \text{lactate} + NAD^+
\]

The paper by Tamada et al. states that LDH assays are sensitive enough to detect as few as 500̅ platelets/cm². The instruction leaflet supplied with the assay warned that highly active sera may experience false low absorbance readings resulting from consumption of most of the NADH prior to the absorbance measurements. Very low absorbance readings were recorded for the material activated and resting deposition samples, however no false low readings were obtained during the creation of the calibration curve.

The paper describing the LDH assay method used 2ml of Triton X100 lysing solution for a surface area of 1.8 cm² (5) whereas the method which was employed for the PE tubing lysis used a volume of 1ml Triton X100 lysing solution for a surface area of 24.5 cm².
Consequently the PE tubing resulted in a much higher than expected LDH concentration following platelet lysis by the Triton X100 solution. The bulk of the NADH in the solution was reacted very quickly with the excess LDH prior to the absorbance reading made in the spectrophotometer, therefore low, almost zero absorbance readings were made.

The LDH kit used was purchased from Boehringer Mannheim, Mannheim, Germany. Two solutions were required for the LDH analysis; NADH solution and pyruvate solution. The compositions of each solution are listed below. Tris buffer solution (composition unknown) was supplied with the kit.

**NADH Solution**
- Tris buffer: 81.3 mmol/L
- sodium chloride: 203 mmol/L
- NADH: 0.244 mmol/L

**Pyruvate Solution**
- Tris buffer: 81.3 mmol/L
- sodium chloride: 200 mmol/L
- pyruvate: 9.76 mmol/L

**Preparation of PE Tubing Samples for LDH Analysis**
The PE tubes were drained of the WB and rinsed using a syringe containing 10 ml HTB to remove any platelets and RBC not adhered to the biomaterial surface. Approximately 500µl of the Triton X100 detergent solution was injected into the tubing via syringe and allowed to incubate for one hour at room temperature. Following the incubation, the lysing solution was drained into a 0.5 ml polypropylene centrifuge tube.
LDH Analysis: Quantification of Platelet Adhesion/Lysis
To a silica glass cuvette, Sigma Chemical Company, St.Louis, Montana, USA, having a 1cm light path, 2.5 ml of NADH solution and 300µl of the lysing solution was added.
To this, 0.5 ml of the pyruvate solution was added, followed by gentle mixing of the cuvette. A Beckman DU-640 spectrophotometer, Fullerton, California, USA, at a wavelength of 340 nm and a temperature of 37°C was used to make the absorbance readings every fifteen seconds for a duration of two minutes. The dA, change in absorbance measured against air, was then correlated to the number of platelets lysed by the solution.

Preparation of LDH Calibration Curve Samples
The calibration curve was created by lysing known concentrations of washed platelets using a Triton X100 detergent solution for 1 hour at room temperature. Concentrations ranging from 72 million platelets/ml down to 1.1 million platelets/ml were used to create the curve. A 1:1 volume ratio of the washed platelet suspension to the lysing solution was used to achieve a final volume of 0.5ml.
Figure A1 LDH Calibration Curve: The LDH calibration curve was created using known concentrations of washed platelets. The arrow indicates the average number of resting platelets deposited on the PE tubing as reported in 3.5.1.
<table>
<thead>
<tr>
<th></th>
<th>Immediate</th>
<th>Rest</th>
<th>Glass Bead Activ</th>
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<tbody>
<tr>
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<td>43.88</td>
<td>42.78</td>
<td>79.87</td>
</tr>
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<td>227.09</td>
<td>300.69</td>
<td>173.07</td>
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<td>226.86</td>
<td>169.21</td>
<td>156.63</td>
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<td>4</td>
<td>131.7</td>
<td>225</td>
<td>157.97</td>
</tr>
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<td>5</td>
<td>257</td>
<td>279</td>
<td>236</td>
</tr>
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<td>6</td>
<td>105.7</td>
<td>145.54</td>
<td>251.49</td>
</tr>
<tr>
<td>7</td>
<td>12.63</td>
<td>57.5</td>
<td>75.55</td>
</tr>
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<td>8</td>
<td>23.68</td>
<td>96.78</td>
<td>87.67</td>
</tr>
<tr>
<td>9</td>
<td>62.27</td>
<td>79.54</td>
<td>20.26</td>
</tr>
<tr>
<td>10</td>
<td>26.8</td>
<td>46.48</td>
<td>51.56</td>
</tr>
<tr>
<td>11</td>
<td>62.68</td>
<td>77.79</td>
<td>27.49</td>
</tr>
<tr>
<td>12</td>
<td>64.94</td>
<td>75.87</td>
<td>43.32</td>
</tr>
</tbody>
</table>
Analysis Summary

Sample 1: Glass Bead Activated
Sample 2: immediate
Sample 3: Rest

Sample 1: 12 values ranging from 20.26 to 251.49
Sample 2: 12 values ranging from 12.63 to 257.0
Sample 3: 12 values ranging from 42.78 to 300.69

The StatAdvisor

This procedure compares the data in 3 columns of the current data file. It constructs various statistical tests and graphs to compare the samples. The F-test in the ANOVA table will test whether there are any significant differences amongst the means. If there are, the Multiple Range Tests will tell you which means are significantly different from which others. If you are worried about the presence of outliers, choose the Kruskal-Wallis Test which compares medians instead of means. The various plots will help you judge the practical significance of the results, as well as allow you to look for possible violations of the assumptions underlying the analysis of variance.
Summary Statistics

<table>
<thead>
<tr>
<th></th>
<th>Count</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass Bead Activated</td>
<td>113.407</td>
<td></td>
</tr>
<tr>
<td>immediate</td>
<td>12</td>
<td>102.936</td>
</tr>
<tr>
<td>Rest</td>
<td>12</td>
<td>133.015</td>
</tr>
<tr>
<td>Total</td>
<td>36</td>
<td>116.453</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Variance</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass Bead Activated</td>
<td>6320.48</td>
<td>79.5015</td>
</tr>
<tr>
<td>immediate</td>
<td>7399.82</td>
<td>86.0222</td>
</tr>
<tr>
<td>Rest</td>
<td>8288.71</td>
<td>91.0423</td>
</tr>
<tr>
<td>Total</td>
<td>7076.99</td>
<td>84.1248</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass Bead Activated</td>
<td>20.26</td>
<td>251.49</td>
</tr>
<tr>
<td>immediate</td>
<td>12.63</td>
<td>257.0</td>
</tr>
<tr>
<td>Rest</td>
<td>42.78</td>
<td>300.69</td>
</tr>
<tr>
<td>Total</td>
<td>12.63</td>
<td>300.69</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Stnd. skewness</th>
<th>Stnd. kurtosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass Bead Activated</td>
<td>0.836561</td>
<td>-0.671387</td>
</tr>
<tr>
<td>immediate</td>
<td>1.21241</td>
<td>-0.554855</td>
</tr>
<tr>
<td>Rest</td>
<td>1.28475</td>
<td>-0.414861</td>
</tr>
<tr>
<td>Total</td>
<td>1.82099</td>
<td>-0.884016</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Sun</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass Bead Activated</td>
<td>1360.88</td>
</tr>
<tr>
<td>immediate</td>
<td>1235.23</td>
</tr>
<tr>
<td>Rest</td>
<td>1596.18</td>
</tr>
<tr>
<td>Total</td>
<td>4192.29</td>
</tr>
</tbody>
</table>

The StatAdvisor

This table shows various statistics for each of the 3 columns of data. To test for significant differences amongst the column means, select Analysis of Variance from the list of Tabular Options. Select Means Plot from the list of Graphical Options to display the means graphically.
**ANOVA Table**

<table>
<thead>
<tr>
<th>Source</th>
<th>Sum of Squares</th>
<th>Df</th>
<th>Mean Square</th>
<th>F-Ratio</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between groups</td>
<td>5595.53</td>
<td>2</td>
<td>2797.76</td>
<td>0.38</td>
<td>0.6859</td>
</tr>
<tr>
<td>Within groups</td>
<td>242099.0</td>
<td>33</td>
<td>7336.34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total (Corr.)</td>
<td>247695.0</td>
<td>35</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**The StatAdvisor**

The ANOVA table decomposes the variance of the data into two components: a between-group component and a within-group component. The F-ratio, which in this case equals 0.381357, is a ratio of the between-group estimate to the within-group estimate. Since the P-value of the F-test is greater than or equal to 0.05, there is not a statistically significant difference between the means of the 3 variables at the 95.0% confidence level.

**Variance Check**

Cochran's C test: 0.376605  P-Value = 1.0
Bartlett's test: 1.00614  P-Value = 0.907438
Hartley's test: 1.5114

**The StatAdvisor**

The three statistics displayed in this table test the null hypothesis that the standard deviations within each of the 3 columns are the same. Of particular interest are the two P-values. Since the smaller of the P-values is greater than or equal to 0.05, there is not a statistically significant difference amongst the standard deviations at the 95.0% confidence level.

**Kruskal-Wallis Test**

<table>
<thead>
<tr>
<th>Sample Size</th>
<th>Average Rank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass Bead Activated</td>
<td>18.5</td>
</tr>
<tr>
<td>immediate</td>
<td>16.1667</td>
</tr>
<tr>
<td>Rest</td>
<td>20.8333</td>
</tr>
</tbody>
</table>

Test statistic = 1.17718  P-Value = 0.55511

**The StatAdvisor**

The Kruskal-Wallis test tests the null hypothesis that the medians within each of the 3 columns is the same. The data from all the columns is first combined and ranked from smallest to largest. The average rank is then computed for the data in each column. Since the P-value is greater than or equal to 0.05, there is not a statistically significant difference amongst the medians at the 95.0% confidence level.
Means and 95.0 Percent LSD Intervals

response

Glass Bead ActivatedImmediate  Rest

sample