
# Selective Activation of Platelets by Surfaces and Soluble Agonists

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# Summary

Platelets are anuclear cell fragments circulating in blood whose primary function is to form blood clot at the site of injury and stop bleeding (hemostasis). They perform these functions by undergoing activation—a process that involves changes in platelet shape and size, exposure of phospholipid phosphatidyl serine (PS) in the outer leaflet of plasma membrane, activation of GPIIb/IIIa receptor, membrane expression and secretion of cargo molecules stored in different intracellular granules (alpha, dense and lysosome).

Platelets' key role in hemostasis is well established. Recent findings have furthermore implicated platelets in a variety of other processes, such as wound healing, angiogenesis, implant integration and rejection, tumor metastasis, as well as adaptive and innate immune responses. Such functional diversity implies that platelets exhibit a spectrum of different functional states that are induced in a stimulus-dependent manner. In other words, there are different kinds of activated platelets tailored to performing different functions. In this context, understanding how platelet activation is regulated, promises to revolutionize therapeutic approaches to cardiovascular disorders, cancers, treatment of recalcitrant wounds, and implant integration.

In this work we focused on differences in the way platelets respond to different stimuli. By examining platelet activation at surfaces, we found that platelets adhering on glass and titania in the absence of calcium express different sets of activation markers. Glass-adhering platelets expressed CD62P (alpha granule marker), CD63 (dense granule

marker), PS, and the active form of GPIIb/IIIa, while $TiO_2$-adhering platelets expressed only CD63. In the presence of extracellular calcium, this difference between the two surfaces vanished. To investigate the mechanism underlying this phenomenon, we measured the dynamics of intracellular calcium in surface-adhering platelets. It was found to be different in platelets adhering on glass and on $TiO_2$ in the absence of extracellular $Ca^{2+}$. The differences correlated with the different platelets responses observed on these surfaces. These findings suggest that platelets can selectively undergo different sets of activation responses depending on the stimuli ($TiO_2$ surface with or without surface bound calcium, glass or $TiO_2$ surface in the absence of extracellular calcium), confirming the recent notion that platelets can tune their microenvironment in a stimuli-specific fashion.

Studying platelet activation selectivity is challenging due to the lack of proper markers for distinguishing differently activated platelets. To circumvent this problem, we capitalized on the diversity and ubiquity of cell surface carbohydrates and the specificity of the lectin-carbohydrate interactions. Like all other cells, the surfaces of platelets are covered with various glycosylated proteins and lipids. We used fluorescently labeled lectins—proteins that selectively bind certain mono- or oligosaccharides—to investigate changes in platelet surface glycosylation upon agonist treatment. We tested a range of physiological and non-physiological agonists differing in terms of strength, receptors they act upon, and the corresponding intracellular signaling pathways. Each agonist caused a unique change in the platelet surface glycosylation pattern, eliciting a unique functional state, because cell surface carbohydrates serve specific functions in cell-cell and cell-matrix communications.

Thus, for the first time, we were able to show that different agonists lead to different platelet functional states.

The work presented in this Thesis provides insights into the platelet activation selectivity. It provides clear evidence that various platelet activation responses—such as alpha and dense granule exocytosis, exposure of PS, activation of GPIIb/IIIa receptor and surface carbohydrate expression—are stimuli-dependent. These findings have significant ramifications for understanding platelet signaling from the point of view of systems biology and considerable practical implications for the development of diagnostic approaches as well as personalized, therapeutic strategies.

## Resumen

Las plaquetas son fragmentos celulares no nucleares del torrente sanguíneo cuya función primaria es formar el coágulo sanguíneo en el lugar donde se produzca la herida, de manera que se detenga el sangrado (hemostasis). Las plaquetas llevan a cabo esta función sometidas a activación - un proceso que implica cambios en la forma y tamaño de la plaqueta, exposición del fosfolípido fosfatidilserina (PS) en la cara externa de la membrana plasmática, activación del receptor GPIIb/IIIa, expresión de la membrana y secreción de la carga almacenada en diferentes gránulos intracelulares (alfa, densos y lisosomas).

El papel clave de las plaquetas en la hemostasis está bien establecido. Descubrimientos recientes han implicado además a las plaquetas en otros procesos diferentes, tales como la cicatrización de las heridas, angiogénesis, integración y rechazo del implante, metástasis del tumor así como respuestas inmunológicas adaptadas e innatas. Tal diversidad funcional implica que las plaquetas exhiben un espectro de diferentes estados funcionales que son inducidos de una manera estímulo-dependiente. En otras palabras, hay diferentes tipos de plaquetas activadas adaptadas para llevar a cabo diferentes funciones. En este contexto, la comprensión de cómo se regula la activación de las plaquetas promete revolucionar las estrategias terapéuticas para trastornos cardiovasculares, cánceres, tratamiento de heridas recalcitrantes e integración de implantes.

En este trabajo, hemos centrado nuestra atención en las diferencias en la forma en que las plaquetas responden a diferentes estímulos. Examinando la activación de las plaquetas en las superficies, descubrimos que las plaquetas adheridas a vidrio y a óxido

de titanio en ausencia de calcio expresan diferentes grupos de marcadores de activación. Las plaquetas adheridas a vidrio expresaron CD62P (marcador de gránulos alfa), CD63 (marcador de gránulos densos), PS y la forma activa de GPIIb/IIIa, mientras que las plaquetas adheridas a $TiO_2$ expresaron solamente CD63. En presencia de calcio extracelular, esta diferencia entre las dos superficies desapareció. Para investigar el mecanismo subyacente a este fenómeno, medimos la dinámica del calcio intracelular en las plaquetas adheridas a la superficie. Se encontró que era diferente en plaquetas adheridas a vidrio y a $TiO_2$ en ausencia de $Ca^{2+}$ extracelular. Las diferencias correlacionaron con las diferentes respuestas de plaquetas observadas en estas superficies. Estos descubrimientos sugieren que las plaquetas pueden selectivamente experimentar diferentes conjuntos de respuestas de activación dependiendo del estímulo (superficie de $TiO_2$ con o sin calcio unido a la superficie, superficie de vidrio o $TiO_2$ en ausencia de calcio extracelular), confirmando la idea reciente de que las plaquetas pueden modificar su microambiente de manera estímulo-específica.

El estudio de la selectividad de la activación de las plaquetas es un reto debido a la ausencia de marcadores apropiados para distinguir plaquetas activadas de modo diferente. Para esquivar este problema, nosotros aprovechamos la diversidad y ubicuidad de los carbohidratos de la superficie celular y la especificidad de las interacciones lectina-carbohidrato. Como todas las otras células, las superficies de las plaquetas están cubiertas con varias proteínas glicosiladas y lípidos. Utilizamos lectinas-proteínas marcadas de forma fluorescente que se unen selectivamente a ciertos mono- u oligosacáridos para investigar cambios en la glicosilación de la superficie de las plaquetas bajo tratamiento agonista. Probamos un rango de agonistas fisiológicos y no fisiológicos que difieren en términos de resistencia, receptores sobre los que actúan y las correspondientes vías de señalización intracelular. Cada agonista provocó un

único cambio en el patrón de glicosilación de la superficie de la plaqueta, provocando un único estado funcional, porque los carbohidratos de la superficie celular ejercen funciones específicas en las comunicaciones célula-célula y célula-matriz. Así, por primera vez, hemos conseguido demostrar que diferentes agonistas conducen a diferentes estados funcionales de las plaquetas.

El trabajo presentado en esta Tesis proporciona un nuevo conocimiento en la selectividad de la activación de las plaquetas. Proporciona clara evidencia de que varias respuestas de activación de las plaquetas -tales como exocitosis de los gránulos alfa y densos, exposición de PS, activación del receptor GPIIb/IIIa y expresión de los carbohidratos de la superficie- son estímulo-dependientes. Estos descubrimientos tienen repercusiones significativas para la comprensión de la señalización de las plaquetas desde el punto de vista de la biología de sistemas y considerables implicaciones prácticas para el desarrollo de estrategias de diagnóstico y terapeúticas personalizadas.

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# List of Publications

I. **The sweeter aspects of platelet activation: a lectin-based assay reveals agonist specific glycosylation patterns**, Swati Gupta and Ilya Reviakine, *Submitted*, 2013. (This paper is based on the work presented in Chapter 4 of the Thesis)

II. **Hemocompatibility study of a bacterial cellulose/polyvinyl alcohol nanocomposite**, Alexandre F. Leitao, Swati Gupta, Joao Pedro Silva, Ilya Reviakine, Miguel Gama, *Colloids and Surfaces B: Biointerfaces*, 2013; 111: 493-502. (Work of this publication is not presented in the Thesis. We studied interaction of platelets with biomaterials-bacterial cellulose and bacterial cellulose/polyvinyl alcohol nanocomposites (developed by Alexandre) using flow cytometry and confocal microscopy)

III. **Platelet activation profiles on $TiO_2$: Effect of $Ca^{2+}$ binding to the surface**, Swati Gupta and Ilya Reviakine, *Biointerphases*, 2012; 7: 28-40. (This paper is based on the work presented in Chapter 3 of the Thesis)

# Abbreviations

| | |
|---|---|
| 5-HT | 5-Hydroxytryptamine |
| AAL | *Aleuria Aurantia* |
| ACA | *Amaranthus Caudatus* |
| ADP | Adenosine 5′-Diphosphate |
| ATP | Adenosine 5′-Triphosphate |
| BMPs | Bone Morphogenetic Proteins |
| BPL | *Bauhinia Purpurea Alba* |
| $Ca^{2+}$ | Calcium |
| CaIoP | Calcium Ionophore |
| CaM | Calmodulin |
| Con A | *Canavalia Ensiformis* |
| DAG | Di Acyl Glycerol |
| DBL | *Dolicos Biflorus* |
| DSL | *Datura Stramonium* |
| DTS | Dense Tubular System |
| EGF | Epidermal Growth Factor |
| EMT | Epithelial-Mesenchymal Transition |
| ENA-78 | Epithelial-Derived Neutrophil-Activating Peptide 78 |
| FACS | Flow Activated Cell Sorting (Flow Cytometry) |
| FGF | Fibroblast Growth Factor |
| Fuc | Fucose |
| Gal | Galactose |
| Glc | Glucose |
| GM-CSF | Granulocyte/Macrophage Colony-Stimulating Factor |
| GPCRs | G-Protein Coupled Receptors |
| GRO-α | Growth Related Oncogene-α |
| HGF | Hepatocyte Growth Factor |
| HMWK | High Molecular Weight Kininogen |
| IBD | Inflammatory Bowel Disease |
| ICAM | Intercellular Adhesion Molecule |
| IGF-1 | Insulin-Like Growth Factor |
| IgG | Immunoglobin G |
| IL-2 | Interleukin-1 |
| IL4 | Interleukin-4 |
| IL-8 | Interleukin-8 |
| IP3 | Inositol 1,4,5 Tris Phosphate |
| LCA | *Lens Culinaris* |
| LPA | Lysophosphatidic Acid |
| LPS | Lipopolysaccharide |
| LSECs | Liver Sinusoidal Endothelial Cells |
| MAA | *Maackia Amurensis* |
| Man | Mannose |
| MCP-3 | Monocyte Chemoattractant Protein-3 |
| MFI | Mean Fluorescence Intensity |

| | |
|---|---|
| MIP-1α | Macrophage Inhibitory Protein-1α |
| MMP2 | Matrix Metalloproteinase 2 |
| MMP9 | Matrix Metalloproteinase 9 |
| GlcNAc | N-Acetyl-Glucosamine |
| GlcNAc | N-Acetyl-Galactosamine |
| NK cells | Natural Killer Cells |
| NO | Nitric Oxide |
| OCS | Open Canalicular System |
| PAR1 | Protease-Activated Receptor 1 |
| PAR1-agonist | Protease-Activated Receptor 1-Specific Agonist Peptide |
| PAR4 | Protease-Activated Receptor 4 |
| PAR4-agonist | Protease-Activated Receptor 4-Specific Agonist Peptide |
| PBP | Platelet Basic Protein |
| PDGF | Platelet-Derived Growth Factor |
| PF4 | Platelet Factor-4 |
| PGI2 | Prostaglandin I2 |
| PKC | Protein Kinase C |
| PLC | Phospholipase C |
| PMA | Phorbyl Methyl Acetate |
| PMCA | Plasma Exchange $Ca^{2+}$-Atpase |
| PRP | Platelet-Rich Plasma |
| PS | Phosphatidylserine |
| PSA | *Pisum Sativum* |
| PTII | *Psophocarpus Tetragonolobus* |
| RANTES | Regulated And Normal T Cell Expressed |
| SDF1α | Stromal Derived Growth Factor 1α |
| SDS | Sodium Dodecyl Sulfate |
| SERCA | Sarco/Endoplasmic Reticulum $Ca^{2+}$-ATPase |
| Sial | Sialic Acid |
| SIP6 | Sphingosine-1-Phosphate 6 |
| SNA | *Sambucus Nigra* |
| TARC | Thymus And Activation Regulated Chemokine |
| TGF-β | Transforming Growth Factor-β1 |
| $TiO_2$ | Titania |
| TLR | Toll Like Receptors |
| TNF-α | Tumor Necrosis Factor-α |
| TRAP | Thrombin Receptor Activating Peptide |
| TSCs | Tendon Stem Cells |
| TXA2 | Thromboxane A2 |
| U46619 | Thromboxane A2-Receptor Agonist |
| VAMP | Vesicle Associated Membrane Protein |
| VCAM | Vascular Cell Adhesion Protein |
| VEGF | Vascular Endothelial Growth Factor |
| VFA | *Vicia Faba* |
| vWF | von Willebrand Factor |
| XPS | X-Ray Photoelectron Spectroscopy |
| α-granule | Alpha-Granule |

# Table of Contents

# List of Figures

## Chapter 1



## Chapter 2



## Chapter 3

## Chapter 4

# List of Tables

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# *Chapter1*

# *Introduction*

## 1.1 Platelets: an overview

Platelets are anuclear 3–4 μm cell fragments that circulate in the blood, scouring the vascular bed for sites of injury, where they become activated. Activated platelets adhere to each other and to the tissue at the site of injury, forming a platelet plug and catalyzing coagulation cascade reactions that culminate in fibrin production and clot formation (Figure 1.1), and coordinate the subsequent inflammatory response and wound healing.[1-5] Platelets achieve these functions by releasing and expressing on their surface a variety of soluble and membrane factors—lipids, proteins, and small molecules—that are stored internally in the quiescent platelets.[1, 3, 6-8] The process of blood clot formation to stop bleeding is known as hemostasis.[3]

Platelet activation under pathological conditions, such as atherosclerosis, leads to grave thrombotic complications such as heart attacks and strokes.[9-12] Thrombosis and the consequent occlusions and emboli are also caused by blood-contacting implants (heart valves, stents, ventricular assist devices, etc).[13, 14] Antiplatelet therapy is used with some success to alleviate these complications, underscoring the critical role of platelets in hemostasis and thrombosis.[12, 15, 16]

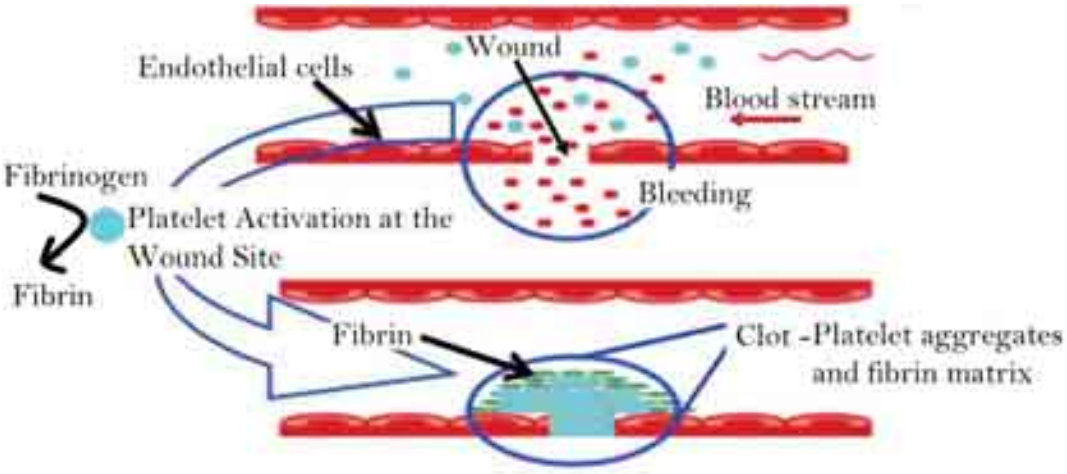



**Figure 1.1. Blood clot formation by platelets at the site of injury.**

Until recently, platelet activation was thought to be associated just with its pro-coagulant function. However, accumulating evidences suggests that platelets play numerous other physiological roles.[4, 17-21] They are involved in wound healing,[5, 20] angiogenesis and *de novo* blood vessel synthesis,[20] cancer metastasis,[19, 21] as well as adaptive and innate immune responses.[17, 18] Their role in atherosclerosis appears to be intimately related to their role in inflammation.[10, 22] These pleiotropic functions are mediated by platelets by release of hundreds of different molecules stored inside platelet granules (alpha and dense granules). The functional diversity of platelets suggests a possibility that platelets can respond to various external stimuli selectively; that there is a spectrum of platelet activation states for performing different functions. There is some evidence in literature in support of this hypothesis. Studies have shown that α-granules (alpha-granules) contain substances with contradictory functions (pro- and anti-angiogenic growth factors, pro- and anti-inflammatory cytokines, etc.),[2, 20, 23] and reports of differential packaging of these α-granule contents into distinct subpopulations and their selective secretion in response to different agonists have appeared in the literature. [2, 23, 24] These results have been recently challenged however.[25-27] At the moment, it remains unclear how, or whether, the secretion of the varied cargo stored inside the platelet granules is regulated.

Selectivity in platelet activation is the central idea of the Thesis. Until now selectivity in platelet activation has been explored only from the point of view of granule secretion. In this Thesis we have identified various other platelet activation responses that are selectively regulated in response to different stimuli. Although molecular mechanisms underlying selectivity remain unclear, our measurements allowed us to formulate hypotheses in this regard. Understanding these processes is crucial as it could revolutionize approaches to targeted, personalized treatment strategies for a variety of

disorders ranging from cardiovascular to cancer, by offering the possibility to tailor platelet response to particular goals in individual patients.

## 1.2 Platelet structure

Platelets are small cell fragments that do not contain a nucleus. Otherwise their structure is similar to that of other cells.[3] They contain microtubules, actin cytoskeleton, mitochondria and Golgi complex (Figure 1.2), and are surrounded by the plasma membrane. On the plasma membrane there are the numerous receptors.[28] Platelet structure consists of certain specific organelles that distinguishes them from other cells. Platelets contain a calcium rich network called the dense tubular system (DTS)-an internal smooth endoplasmic reticulum membrane system, invaginations of surface plasma membrane referred to as open canalicular system (OCS) and three sets of granules-dense granules, alpha granules and lysosomes—storing different substances (proteins, small molecules).[3, 29] These various structural entities play an important role in platelet activation which are discussed in the following section.

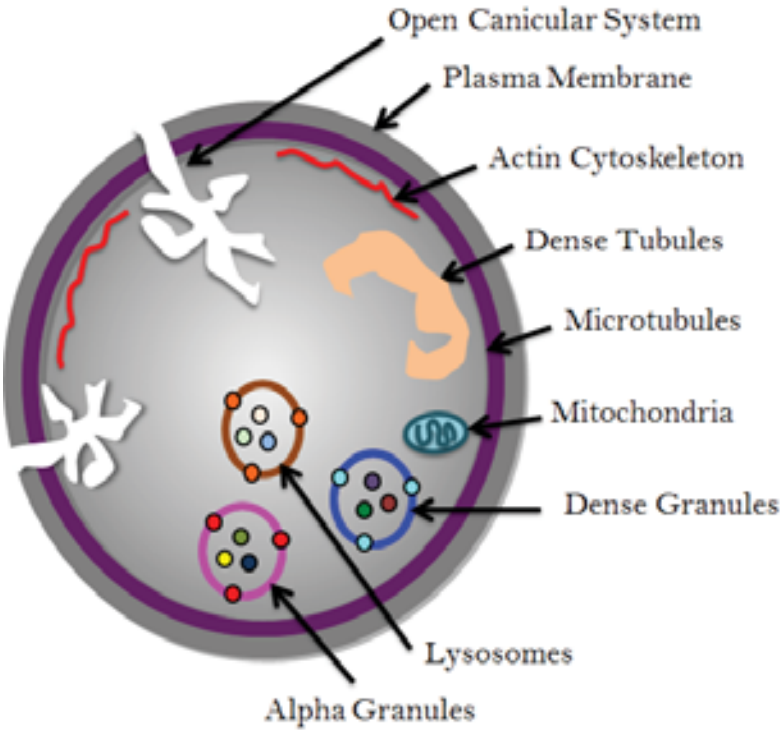



**Figure 1.2. Structure of platelets.**

## 1.3 Platelet activation

Platelets circulate in blood in a resting/quiescent state.[1, 3] They perform their pro-coagulant function by undergoing activation. This process entails changes in platelet shape and size, exposure of phosphatidylserine (PS), activation of GPIIb/IIIa receptor, secretion of TXA2 (thromboxane A2) and exocytosis of intracellular granules (Figure 1.3).[1-5] Platelet activation may be triggered by the soluble agonists such as thrombin, ADP (Adenosine 5′-Diphosphate), thromboxane A2, fibrinogen that are secreted by the platelets themselves or produced in the coagulation cascade,[3, 28] or by the interfacial agonists such as tissue factor, collagen, or collagen-bound von Willebrand factor (vWF) that become exposed to the blood when vascular endothelium is damaged.[30, 31] Below, we discuss each of the responses in more detail.

The circumferential coil of microtubules and the actin cytoskeletal structures, help in maintaining the discoid shape of quiescent platelets.[3] Stimulation of platelets results in disassembly of microtubules and depolymerisation of actin filaments that causes a change in shape of platelets from a resting state discoid form to an intermediate spheroid form.[32, 33] This is followed by polymerization of actin,[32] which results in spreading of platelets together with extension of filopodia.[33, 34] The OCS invaginations are the major source of membrane for expansion of surface area during platelet spreading.[3, 35, 36]

GPIIb/IIIa receptor is present in an inactive conformation on the surface of resting platelets. Platelets upon activation undergo a change in conformation of GPIIb/IIIa receptor that results in its activation.[30, 31, 37]

Agonist induced stimulation of platelets results in release of arachidonic acid from platelet membranes (granules and plasma membrane) by the action of phospholipase A2 and its conversion into TXA2 that is then released by platelets. The released TXA2 acts as platelet agonist.[28, 38]

Phospholipids are usually asymmetrically distributed in the plasma membrane of cells[39, 40] including platelets, with of the PS present in the inner leaflet of the plasma membrane. Stimulation of platelets with agonists results in transfer of PS into the outer leaflet of plasma membrane (Figure 1.3).[7, 41]

Platelets have three distinct sets of granules–alpha granules, dense granules, and lysosomes.[3, 29] Various granules present in the platelets store hundreds of different cargos and contain on their membranes specific markers associated with their expression. Alpha granules are the most abundant[29] and contain more than 300 distinct proteins and peptides.[42] They include hemostatic factors (e.g. fibrinogen, Factor V, vWF, GPIIb/IIIa), growth factors (e.g. Stromal Derived Growth Factor 1α /SDF1α, Platelet-Derived Growth Factor/PDGF), proteases (e.g. Matrix Metalloproteinase 9/MMP9, Matrix Metalloproteinase 2/MMP2), pro-angiogenic factors (e.g. angiogenin, Vascular Endothelial Growth Factor/VEGF), anti-angiogenic factors (e.g. Platelet Factor-4/PF4, angiostatin), pro-inflammatory factors (e.g. P-selectin, RANTES, IL-8, IL-2), and anti-inflammatory factors (e.g. Interleukin-4/IL4, Hepatocyte Growth Factor/HGF, Tumor Necrosis Factor-α/TNF-α).[29, 43, 44] Dense granules contain small bioactive molecules such as ADP, ATP (Adenosine 5′-Triphosphate), calcium, magnesium, 5-hydroxytryptamine (5-HT, serotonin) and

membrane receptors such as CD63. Lysosomes contain hydrolytic enzymes such as β-hexosaminidase, cathepsin D, and acid phosphatases.[29]

Secretion of these granule markers takes place upon platelet activation at the wound site and is critical for hemostasis.[3, 8, 44] Patients with defective granule secretion are shown to exhibit moderate to progressive bleeding disorders.[3]

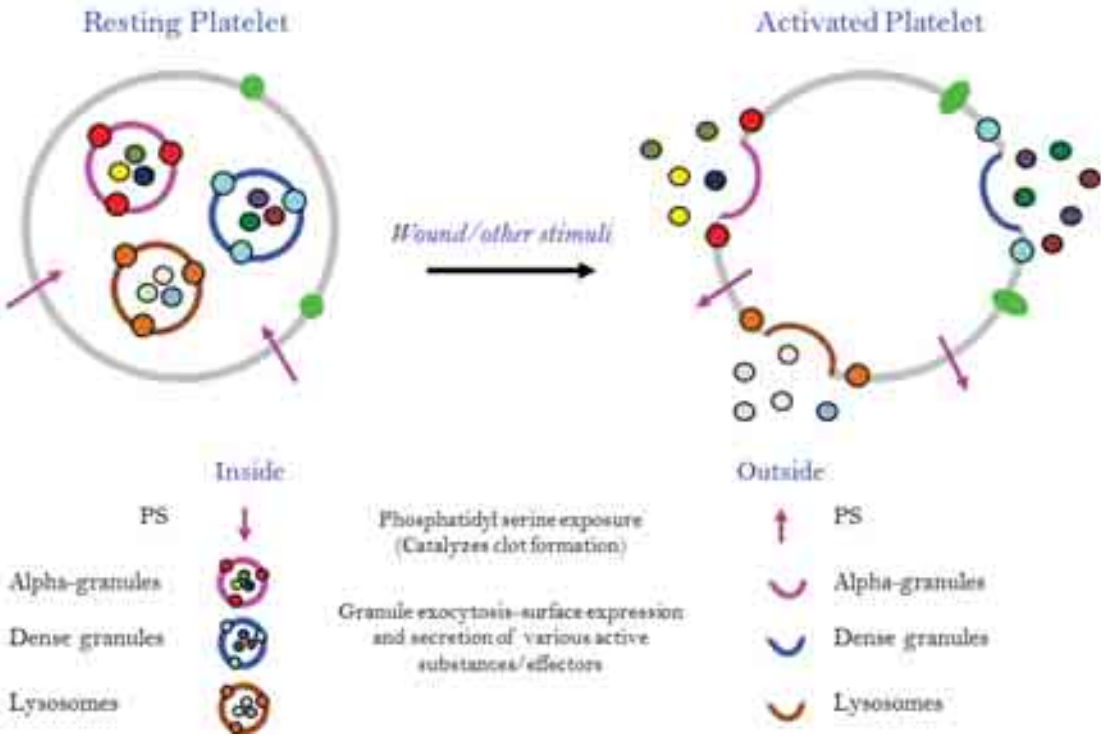



**Figure 1.3. Platelet activation. Resting platelets upon activation undergo exocytosis of intracellular granules (alpha, dense and lysosomes) resulting in secretion and membrane expression of granule cargos, transfer of PS from the inner (purple arrow facing inward) to the outer leaflet (purple arrow facing outward) of plasma membrane, change in conformation of GPIIb/IIIa from inactive (green circles in membrane) to an active state (green ellipses in membrane).**

## 1.4 Platelet agonists and their receptors

Platelet activation is induced upon binding of agonists to various receptors expressed on the platelet surface.[45] Some of these agonists such as thrombin, ADP and TXA2 act via G-protein coupled receptors (GPCRs) and other agonists-collagen, fibrinogen and vWF act via non-GPCRs (Figure 1.4).[28, 30, 31] GPCRs are seven transmembrane helix receptor proteins that are coupled to different heterotrimeric G-proteins (Gq, Gi1, Gi2, Gi3, Gz, G12, G13, Gs or G16) that induce downstream signaling upon ligand engagement of the receptors.[30]

Thrombin is a serine protease that acts via PAR (Protease Activated Receptors) family of GPCR receptors, PAR1-PAR4, and GPIb.[46] The mechanism of action of thrombin in PARs is quite unique. It acts by cleaving off N-terminal peptides at specific sites resulting in the release of short peptides SFFLRN for PAR1 receptor and AYPGKF for PAR4 receptor-that act as the receptor agonists.[47] This action results in a very high local concentration of ligand to bind to its receptor. Thrombin is one of the most potent platelet agonists.[45]

ADP acts on P2Y1 and P2Y12 receptors [48] and TXA2 acts via TP receptors.[49] Collagen stimulates GPVI and α2β1 receptors. vWF and fibrinogen exert their effects by binding to GPIb/IX/V and activated GPIIb/IIIa receptors, respectively.[3, 31, 37]

Based on the potency of the agonists to activate platelets, these agonists are classified into two sets-(1) strong agonists such as thrombin and collagen and (2) weak agonists such as ADP and TXA2.[45, 50] Weak agonists such as ADP require a much higher concentration (μM) to stimulate platelets as opposed to strong agonist that can stimulate platelets even in nanomolar ranges.[50] Irrespective of the agonist type, activation of platelets is marked by an increase in intracellular cytosolic calcium, $[Ca^{2+}]i$, achieved

through its release from internal calcium DTS stores, which in turn lead to opening of plasma membrane calcium channels (Orail), allowing influx of extracellular calcium into the platelet cytoplasm.[28, 30, 31, 51] The resulting increase in cytoplasmic calcium activates several calcium-dependent kinases. This leads to the various platelet activation responses such as shape change, granule exocytosis, PS exposure and activation of GPIIb/IIIa (Figure 4).[28-31, 41, 52, 53]

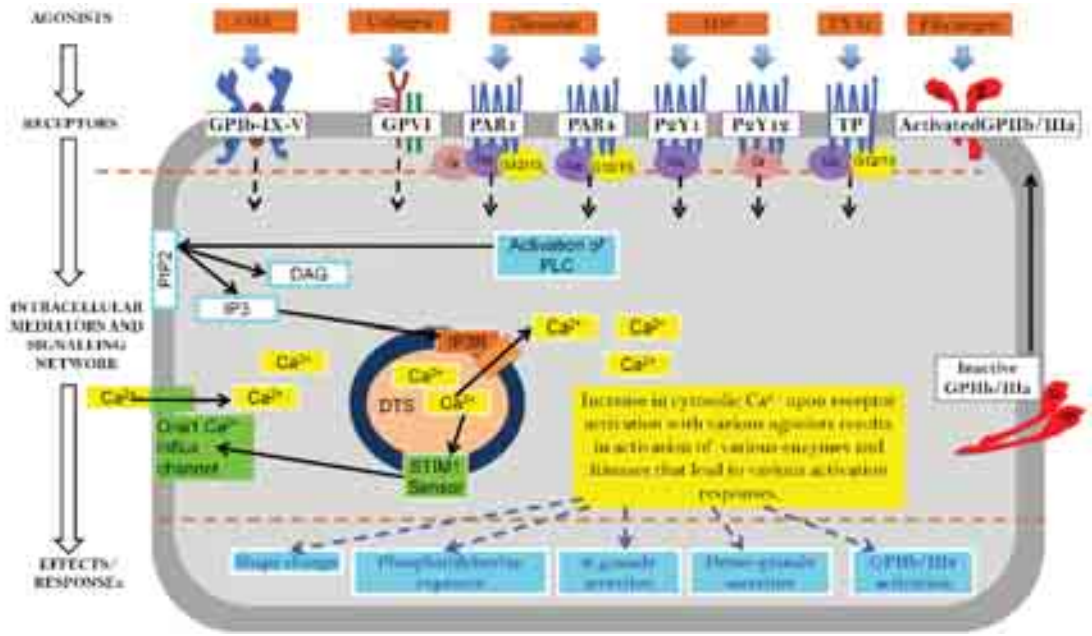



**Figure 1.4. Platelet agonists and their corresponding receptors. Various platelet agonists act on their respective receptors and initiate different signaling pathways which results in activation of intracellular phospholipase C (PLC) enzyme. PLC results in generation of ionositol triphosphate (IP3) and diacylglycerol (DAG). IP3 acts on its receptor present on dense tubular system (DTS) and results in release of calcium from the DTS into the cytoplasm. The STIM sensor present in the DTS membrane sense the decrease in calcium levels in internal DTS stores and results in activation of Orai1 calcium channels present in plasma membrane. This causes influx of calcium from exterior into the cytoplasm of platelets via Orai1 calcium channels. These process result in an increase in cytoplasmic calcium. Calcium activated various enzymes and kinases and leads to platelet activation responses such as shape change, exposure of PS, activation of GPIIb/IIIa receptor, exocytosis of alpha and dense granules.**

## 1.5 Platelets in hemostasis

Hemostasis is a physiological process of blood clot formation to stop bleeding from an injured vessel. Clot formation is a highly regulated process and is divided into three stages: initiation, extension and propagation (Figure 1.5).[3, 10, 54] During the initiation phase, circulating platelets are captured and activated at the wound site. Activated platelets aggregate with each other and form a platelet plug covering the wound site. During the extension phase, additional platelets from the circulation are activated and recruited to the wound site. These activated platelets stick to each other and to the existing platelet plug resulting in extension of platelet plug. Propagation phase involves events that help in stabilization of platelet plug and prevent immature disaggregation. During these stages thrombin is generated on the surface of activated platelets that helps in fibrin formation. Fibrin forms a meshwork around the platelet-plug forming a clot that seals the wound site.[3]

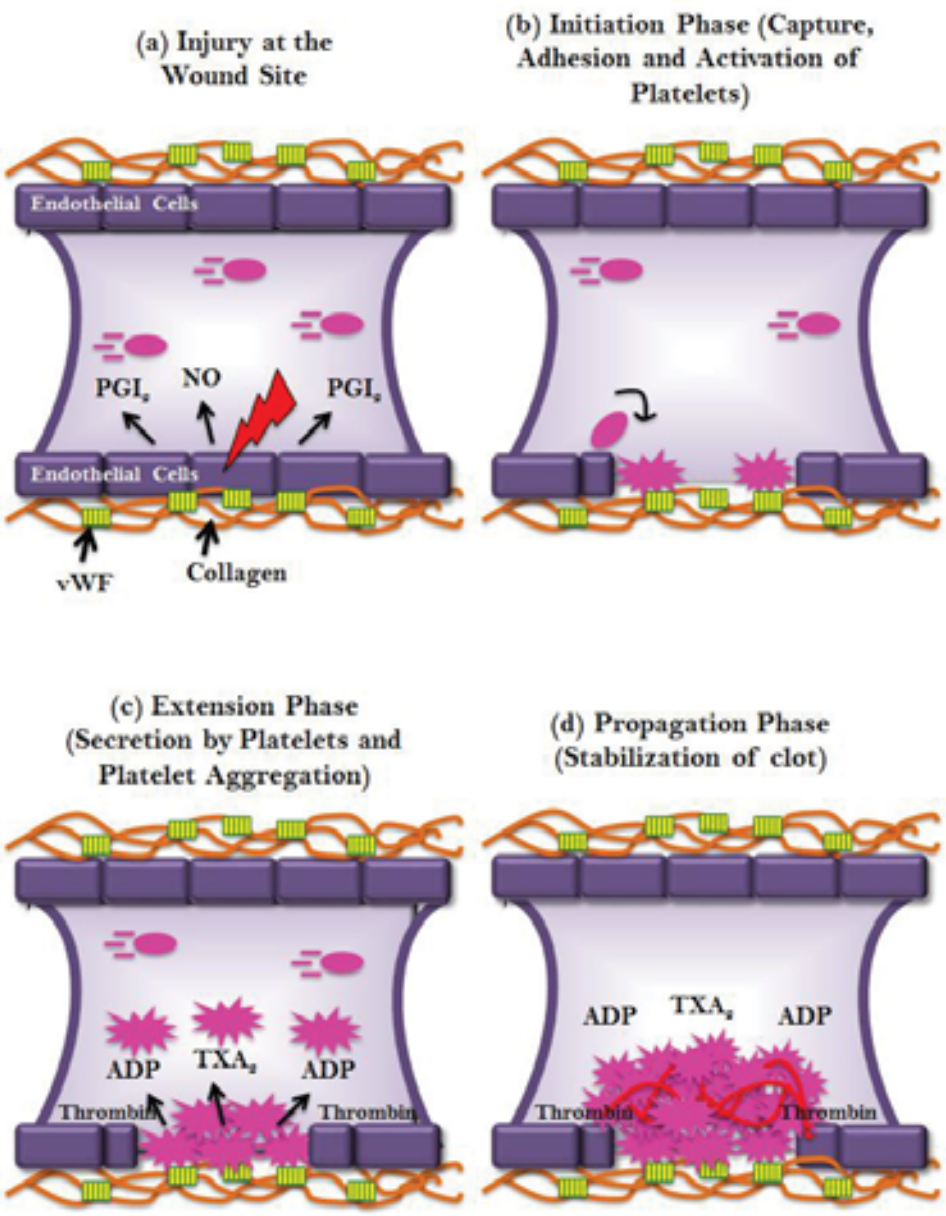



**Figure 1.5. Different Stages of Blood clot Formation at the site of injury in a blood vessel.**

## Initiation phase

Initiation phase starts with platelet adhesion and activation of platelets at the wound site. Platelets circulate in blood in a quiescent state. This quiescent state of platelets is maintained by inhibitors like nitric oxide (NO) and prostaglandin I2 (PGI2), that are released by the topmost endothelial cell layer of blood vessels.[3] A disruption of the vascular barrier (e.g. during an injury) results in the exposure of sub-endothelial

collagen and collagen bound vWF.[31] vWF is also circulating in blood but it is present in a conformation that prevents its binding to platelets.[3, 55] Exposure of these ligands is sensed by the platelet receptors GPIb/IX/V and GPVI, respectively, leading to the adhesion and activation of platelets at the wound site.[13, 31, 45] Activated platelets undergo morphological changes such as spreading and extension of filopodia. These shape changes help in strong adhesion of platelets to the vessel wall. Activated platelets undergo secretion of alpha and dense granules cargos as well as TXA2. The secreted TXA2 and granule contents (such as ADP and serotonin from dense granules and fibrinogen from alpha granules) act as platelet agonists and activate platelets, thereby amplifying platelet activation responses at the wound site. The PS exposed on surface of activated platelets acts as a catalyst for formation of thrombin by the coagulation cascade reactions that culminate in fibrin production.[56] Factor-V secreted from platelet alpha granules also participates in the coagulation cascade reactions for formation of fibrin.[3, 57] The activated GPIIb/IIIa receptor on the platelet surface binds to fibrinogen (secreted from platelet alpha granules) and results in platelet aggregation.[3, 56] All these platelet activation events culminate in the formation of platelet plug covering the exposed wound site.

## Extension phase

The next step of blood clot formation includes the extension of the platelet plug, which involves recruitment of more circulating platelets to the existing platelet plug at the wound site.[3, 54] The recruitment of additional platelets is made possible by the local accumulation of agonists that were released during the initiation phase, such as ADP and TXA2 and by thrombin generated on the surface of activated platelets.[10, 45] These agonists activate circulating platelets and recruit them to the wound site. These

activated platelets stick to each other and to existing platelet plug via crosslinking of their activated GPIIb/IIIa receptors by fibrinogen, fibrin or VWF, thereby resulting in extension of platelet plug.[3]

## Propagation phase

During this phase clot retraction occurs in which clot pulls in upon itself, thereby shrinking to a smaller volume.[3] The purpose of this process is to narrow the gaps between platelet-platelet aggregates and increase the local concentration of platelet activators. These events are facilitated by fibrinogen bound to the activated GPIIb/IIIa integrin via outside-in signaling though this integrin.[3, 37, 58] This clot remains stable, long enough until wound healing starts. The close contacts between the activated platelets (via fibrinogen bridges) within the growing hemostatic plug, together with the fibrin meshwork help in stabilization of the platelet plug and prevent premature disaggregation.

## 1.6 Platelets beyond hemostasis

Until recently platelet function was thought to be associated only with hemostasis. Recently it has been shown that platelets play an important role in various other physiological and pathophysiological processes such as wound healing and angiogenesis,[5, 20] inflammation and atherosclerosis,[44] tumor growth and metastasis,[19, 21] implant rejection/integration,[59, 60] liver regeneration,[61] adaptive and innate immune responses.[17, 18] Platelet alpha granules contain more than 300 distinct proteins and secretion of some of these granule proteins help platelets to perform such diverse functions. In addition to hemostasis-promoting proteins, platelet granules also contain mitogens (present in alpha granules) such as Insulin-Like Growth Factor (IGF-1), Vascular Endothelial Growth Factor (VEGF), and Fibroblast Growth Factor (FGF), which have wound healing and vessel re-growth promoting properties.[29, 62] Alpha-granules also contain proinflammatory chemokines and cytokines such as Regulated And Normal T Cell Expressed (RANTES) and Interleukin-8 (IL-8).[63] Around 30 angiogenesis influencing factors such as VEGF, endostatin and PF4 have also been reported to be present in alpha granules.[29, 62] The role of platelet activation and secretion in health and disease are discussed in details in the following sections.

### 1.6.1 Platelets in wound healing and angiogenesis

Wound-healing is a complex process that involves a series of events-coagulation, inflammation, ground substance and matrix synthesis, angiogenesis, fibroplasia, epithelialization, wound contraction, and remodeling.[60] Platelet granules contain both activators and inhibitors of these processes (e.g. Granulocyte/Macrophage Colony-Stimulating Factor/GM-CSF, Transforming Growth Factor-β/TGF-β, PDGF,

IGF-1, TNF-α, VEGF, FGF, IL-1β, HGF, PF4, endostatin, protease inhibitors) upon platelet activation at the wound site, in order to achieve successful healing of wound.[5, 60]

Platelet-derived granulocyte/macrophage colony-stimulating factor (GM-CSF) acts as a strong chemoattractant for the arrival of neutrophils at the wound site.[60] Neutrophils get activated by the platelets secreted TGF-β, PDGF and PF4.[64] Upon activation they kill bacteria and decontaminate the wound from foreign debris. Protease inhibitors secreted from platelet granules, protects the surrounding tissue that is not a part of wound, from the action of neutrophils.[64]

PDGF secreted from platelets acts a chemotactic stimulus for the arrival of macrophages at the wound site.[60, 64, 65] Macrophages phagocytize, digest, and kill pathogenic organisms; scavenge tissue debris; and destroy any remaining neutrophil.

Release of epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin-like growth factor (IGF-1), and TGF-β by platelets results in recruitment of fibroblasts to the wound site.[5, 60, 64] These fibroblasts proliferate and synthesize collagen, glycosaminoglycans and proteoglycans, the building blocks of the new extracellular matrix of granulation tissue to fill the wound space.

Platelets contain both pro-angiogenic (vascular endothelial growth factor-A, -C, fibroblast growth factor-2, hepatocyte growth factor, platelet-derived growth factor, IL8 and many others) and anti-angiogenic factors (thrombospondin-1, transforming growth factor-β1, platelet factor-4, plasminogen activator inhibitor-1, IL-1β and others) in their alpha granules.[64, 65] Platelets tightly regulate the process of angiogenesis (formation of new capillaries from existing blood vessels) by releasing both pro and anti- angiogenic factors in a controlled and balanced fashion so that angiogenesis stops

once the wound is healed. For example, pro-angiogenic factors such as VEGF is secreted in high concentration at the hemostatic plugs that causes proliferation and migration of proliferating endothelial cells from the clot margin into the fibrin mesh to reinstate the vessel wall.[64-66] However the released anti-angiogenic factors such as PF4 and transforming growth factor-β1 inhibit the proliferation and migration of endothelial cells.[20, 56]

Once the new tissue within the wound is formed, the remodeling phase begins to restore tissue structural integrity and functional competence. The substances released from the alpha granules (fibrinogen) and lysosomes of platelets, are proposed to participate in clot remodeling. [3, 58]

Therefore, platelets and platelets derived proteins play an important role in the natural process of wound healing and angiogenesis. Various intrinsic and extrinsic patient factors affect each step of the complex process of wound healing. By understanding the underlying biology, we can significantly influence patients' ability to heal.

### 1.6.2 Platelets in implant integration and rejection

A variety of implants are introduced in body to treat clinical conditions such as periodontal diseases, arteriosclerosis and other cardiovascular diseases. Both, during implantation of biomaterial in systemic circulation (vascular stents, catheters, artificial heart valves) or during bone implantation (dental implants), the biomaterial come into direct contact with blood, and results in activation of platelets and blood coagulation on the surface.[13, 14]

In a normal physiological condition, platelet activation and its finely tuned degranulation at the exposed sub-endothelium would result in controlled clot formation followed by wound healing and tissue regeneration.[64] However, platelet activation

and degranulation of hemostasis promoting factors (ADP, serotonin, fibrinogen, factor V, factor XIII, vWF) on the surface of titanium implants placed in blood vessels (stents), results in thrombus formation (excessive clot formation) and embolization, which is turn causes, blockage of arteries, strokes, infarcts and other complications.[14, 59, 67]

On the other hand, blood coagulation at surfaces of osseoimplants, such as dental implants, results in osseointegration of the implant (bone regeneration and integration into implant).[60] Osseointegration is basically a type of wound healing process that involves angiogenesis and tissue regeneration. It is reported that platelets at the surface of osseoimplants secrete factors such as β-thromboglobulin (β-TG) and PDGF that promote osseointegration.[60, 68] Platelets also contain factors such as bone morphogenetic proteins (BMPs)-2, -4, -6, IGF-1, which are essential for bone formation, bone matrix formation and replication of osteoblasts and their precursors.[68]

It is intriguing that platelet activation at the surface of biomaterials results in rejection of vascular implant but integration of osseoimplant. A better understanding of platelets reactions operating at the implant surfaces would aid in development of biomaterials and anti-platelet therapeutics for preventing platelet activation at the surface of vascular implants and allow better integration of implants.

### 1.6.3 Platelets in tumor growth and metastasis

The interaction between tumor cells and platelets works in a bi-directional way.[69] The tumor cells secrete ADP, thrombin, metalloproteinase-2 and cathepsin B and all these molecules can activate platelets.[69, 70] Platelets upon activation by tumor cells undergo GPIIb/IIIa activation, release lysophosphatidic acid (LPA) and they secrete

and express various alpha granule markers (e.g. CD62P, TGF-β, VEGF, pro-metastatic matrix metalloproteinase-1, -2 and -9) that promote tumor cell proliferation, tumor angiogenesis and metastasis.[20, 70, 71]

VEGF is one of the factors released from platelet alpha granules that promote tumor neoangiogensis.[71, 72] Platelets contain both pro- and anti-angiogenic factors stored in alpha granules, however upon stimulation of platelets with tumor cells, platelets exhibit an extreme pro-angiogenic effect.[72-74] Tumor angiogenesis helps in tumor growth and survival.[75]

Activated platelets release a bioactive molecule, lysophosphatidic acid (LPA) which binds to LPA receptor on tumor cells and contributes to tumor cell proliferation, survival, migration and invasion.[69, 76-78]

TGFβ released by platelets has also been reported to promote activation of epithelial-mesenchymal transition (EMT) of tumor cells that is important for tumor metastasis.[79]

Activated platelets express the active form of the GPIIb/IIIa receptor that interacts with αvβ3 receptor on tumor cells, via ligands such as fibronectin, vWF and fibrinogen. CD62P (alpha granule cargo) expressed on the surface of activated platelets is also shown to bind to mucins and heparan-sulfated glycancs expressed on the surface of tumor cells.[69, 80-82] These processes result in formation of tumor-platelet aggregates. Platelets bound to tumor cells serve as a physical guard for tumor cells by protecting them against immune elimination by natural killer cells (NK cells) circulating in the blood.[69, 71, 83]

Platelets contain both pro-metastatic (matrix metalloproteinase -1, -2 and -9) [56, 84] as well as metastasis-preventing factors (vWF)[56, 71] and these factors are released upon activation, for example during tumor cell induced platelet aggregation. It remains unclear, how platelets promote metastasis upon interaction with tumor cells, despite possessing an anti-metastasis activity.

### 1.6.4 Platelets in inflammation

Platelets play an important role in the inflammation by expressing and releasing various intracellular granule proteins (CD62P, CD40 ligand/CD40L, PF4, Macrophage Inhibitory Protein-1α/MIP-1α, Monocyte Chemoattractant Protein-3/MCP-3, Thymus And Activation Regulated Chemokine/TARC, Growth Related Oncogene-α/GRO-α, Epithelial-Derived Neutrophil-Activating Peptide 78/ENA-78, IL-1β, PDGF, TGFβ) that promote inflammation.[43]

The role of platelets in inflammation can be best exemplified by a chronic inflammatory disease-atherosclerosis.[22, 85, 86] Atherosclerosis is a pathophysiological inflammatory condition, which arises due to accumulation of minimally oxidized low density lipoprotein (LDL) at the sub-endothelial matrix.[69] It triggers the activation of the overlying endothelial cells that expresses CD62P.[87]

Platelets expressing P-selectin glycoprotein ligand-1 (PSGL-1) interact with endothelial CD62P[88-90] and undergo activation-express alpha granule markers such as CD62P[69, 82, 89, 90] and CD40L.[88, 90, 91] CD40L expressed on platelets binds to CD40 receptor on endothelial cells and induces the expression of leukocyte adhesive molecules (e.g. Vascular Cell Adhesion Protein/VCAM, Intercellular Adhesion Molecule/ICAM, E-selectin) by endothelial cells.[91, 92]

CD40L and P-selectin expressed on platelets mediates their binding to inflammation promoting cells (neutrophils, lymphocytes and monocytes).[93] The interaction between monocytes and platelets results in microparticles release that deliver platelet alpha granule-derived chemokines to endothelial cells such as RANTES.[90, 93] RANTES binds to its serpentine receptor (CCR1/CCR5) on endothelial cells that leads to an upregulation of expression of monocyte-binding adhesive receptors on endothelial cells.[94]

These processes facilitate the direct binding and rolling of inflammatory cells like monocytes to the endothelial cells. Monocytes finally migrate through the endothelial layer where under the influences of cytokines, differentiation of monocytes and macrophages, thickening of artery wall by accumulation of calcium and fatty materials such as cholesterol and triglycerides. All these processes result in inflammation of artery.[69]

Platelets are also known to contribute to etiology of other inflammation associated disorders such as inflammatory bowel disease (IBD), rheumatoid arthritis, systemic lupus erythematosus and psoriasis, however its role is not well delineated.[69]

### 1.6.5 Platelets in immunity

As platelets rapidly accumulate at the site of injury/infection; it has been proposed that platelets might play “surveillance” roles, similar to that of traditional immune cells such as mast cells, macrophages, and dendritic cells. Platelets are reported to interact with bacteria and undergo activation.[69] Platelets upon activation by bacterial antigens have been shown to secrete and express various alpha granule contents (e.g. CD40, RANTES, platelet basic protein (PBP), PF-4, thymosin-β4 and anti-bacterial proteins-TCP1 and TCP2) that help in mediating immune responses against bacterial infections.

Platelets express a class of receptors-Toll like receptors (TLR-1, TLR-2, TLR-3, TLR-6, TLR-8 and TLR-9),[95, 96] which recognize the antigens on the bacterial surface such as LPS, lipoteichoic acid and cell wall peptidoglycans. Studies in mice have demonstrated that LPS binds to platelet expressed TLR-4 receptors induces surface expression and secretion of alpha granule marker CD40L.[56, 97-101]

Expression of CD40L on activated platelet surface has been reported to affect adaptive immunity responses- acceleration of dendritic cell maturation, stimulation of IgG production by B cells and enhancement of T cell activity.[69, 102]

Bacteria-induced platelet activation also results in the secretion of a set of granular stored chemokines (such as RANTES), which serve to attract immune cells (NK cells, monocytes and macrophages) to the site of infection.[69]

Furthermore, in vitro studies have shown that RANTES, PBP, PF-4 and thymosin-β4 released from activated platelets mediate bacteriocidal effects against *S. aureus* and *E. coli*.[103] Platelets have also been proposed to mediate bacterial clearance by internalizing them, thereby removing them from the circulation.[69]

Platelets have also been reported to contain thrombocidins (anti-bacterial proteins)-TCP1 and TCP2 in their granules. [69, 90] Studies in rabbits have shown that thrombocidins contribute to host defense against *Streptococci*-induced endocarditis. [104]

A better understanding of role played by platelets in prevention of infection, could help in development of platelet based therapeutics for prevention of pathogen associated infections and diseases.

### 1.6.6 Platelets in liver regeneration

Very recently, it has been shown that platelets upon interaction with hepatocytes and other cells in liver, undergo activation and secrete alpha granule factors (e.g. IGF-1, HGF and Sphingosine-1-Phosphate 6/SIP6) that help in the process of liver regeneration.[61, 105, 106] In addition, platelets also contain serotonin, cytokines, and lipid mediators, such as, S1P, ADP, and ATP, which also contribute to the process of liver regeneration.[107]

It has been reported that the Kupffer cells present in liver mediate accumulation of platelets in the liver after hepatectomy or other types of liver injury, such as, ischemic reperfusion or lipopolysaccharide administration. Upon entering liver, platelets make a direct contact with hepatocytes and get activated.[61]

Upon activation, platelets secrete growth factors such as IGF-1 and HGF. These growth factors act on hepatocytes and activate the Akt and ERK1/2 pathways that result in hepatocyte proliferation.[61]

In addition to hepatocytes, platelets also make direct contacts with liver sinusoidal endothelial cells (LSECs) in the liver. This triggers platelets activation and release of SIP6.[61] The released SIP6 in turn acts on LSECs and induces IL-6 secretion by them. The released IL-6 causes the activation of the STAT3 pathway in hepatocytes, Akt and ERK1/2 activation that promotes hepatocyte proliferation. Furthermore, platelets also cause LSESs proliferation.

The presence of liver regenerating cytokines and growth factors in platelets, point towards the potential capability of platelets to help in tissue regeneration and stem cell research applications.

## 1.7 Hypothesis: Platelet activation is selective in response to different stimuli

Accumulating evidence shows that platelets perform a wide repertoire of functions ranging from hemostasis to tumor metastasis. Platelet secretion of granule cargo plays an important role in mediating these diverse processes. Platelet granules contain a myriad of molecules with pleiotropic functions: angiogenesis regulating proteins, metastasis regulating proteins, coagulation-regulating factors. But despite this platelets are able to perform one particular function in response to specific stimulus. For example, they catalyze clot formation and wound healing upon an injury however upon interaction with bacteria they mediate immune responses. Moreover, platelets store both pro- and anti-angiogenic factors in their alpha granules, however they have a stimulating effect on angiogenesis upon interaction with tumor cells. These observations led to the central hypothesis of the thesis i.e. platelet activation is selective, i.e., platelets selectively undergo specific sets of activation responses (for example selective secretion and membrane expression of specific sets of intracellular granule stored cargos) in response to a specific stimulus, and these responses differ for different stimuli (Figure 1.6). By undergoing different activation responses in a stimuli specific fashion, platelets tune their vascular microenvironment (result in angiogenesis, tumor metastasis or athereosclerosis or tissue regeneration as shown in Figure 1.6). There is growing body of evidence in literature, in support of this hypothesis.[108-112]

Recent findings of Italiano et al, shown that pro- and anti-angiogenic factors are present in distinct alpha granules.[23, 24] Consistently, other studies have shown that stimulation of platelets with PAR1-agonist (Protease-Activated Receptor 1-Specific Agonist Peptide) or ADP and PAR4-agonist (Protease-Activated Receptor 4-Specific

Agonist Peptide) or TXA2 results in selective secretion of VEGF (pro-angiogenic factor) and endostatin (anti-angiogenic factor), respectively (Figure 1.6).[23, 24, 74] Furthermore, Batinelli et al reported that MCF-7 breast cancer line stimulated platelets to secrete pro-angiogenic factors.[74] Sehgal et al have shown even hemostatic factors such as fibrinogen and vWF are packaged in distinct granules and there is selective secretion of fibrinogen over vWF in activated platelets.[113] Based on all these findings it is proposed that factors with opposing functions are stored in distinct alpha granules populations and that platelet secretion is contextually thematic: release of specific sets of granule proteins (e.g. pro- or anti-angiogenic factors) in response to specific agonists. While there is support for this general idea in the literature, it has also led to some controversy.

Studies by Kamykowski et al, propose that as opposed to multiple alpha-granule population storing different factors, there is only one major population of large granules in which proteins are differentially segregated in a zonal manner and there is little co-localization of factors with similar functions.[26] Furthermore, they also suggest that the different factors co-packaged within the same granule, can be differentially released in response to different stimuli, by a kiss-and-run exocytosis mechanism, as observed in chromaffin granules in neuroendocrine cells.[114]

A very recent study measured the secretion of 30 different granule markers from platelets stimulated with 4 different agonists (PAR1-agonist, PAR4-agonist, thrombin and convulxin).[25] They report that all these agonist result in release of mostly all of the tested markers, however there are differences in their release kinetics-speed and extent of release, which differed depending on the potency of the agonist. There were indeed differences in the number and composition of markers that were released, although there was no functional pattern related to these differences. There were 8

proteins that were released specifically in response to PAR1-agonist and 2 substances were specifically released in response to PAR4-agonist.

All of the studies reported above differ in the terms of agonist type, agonist concentration, duration of stimulation and the markers tested. Irrespective of that, all of them indeed support the hypothesis of selective secretion of different granule markers from platelets in response to different agonists.

Given the central role played by platelet activation in physiological and pathophysiological conditions, understanding the mechanisms of the platelet selective activation and secretion, might be useful in controlling and/or regulating platelet functions at sites of vascular lesions and pathologies. Such knowledge would help in identification of targets for development of anti-platelet therapeutics in order to treat platelet-mediated thrombosis, cancer, athereosclerosis, implant rejection. Understanding more about how the selective secretion process in response to different stimuli works, may yield a unique tool to tune the release of growth factors by platelets, which would aid in implant/graft integration, stem cell differentiation and tissue regeneration applications.

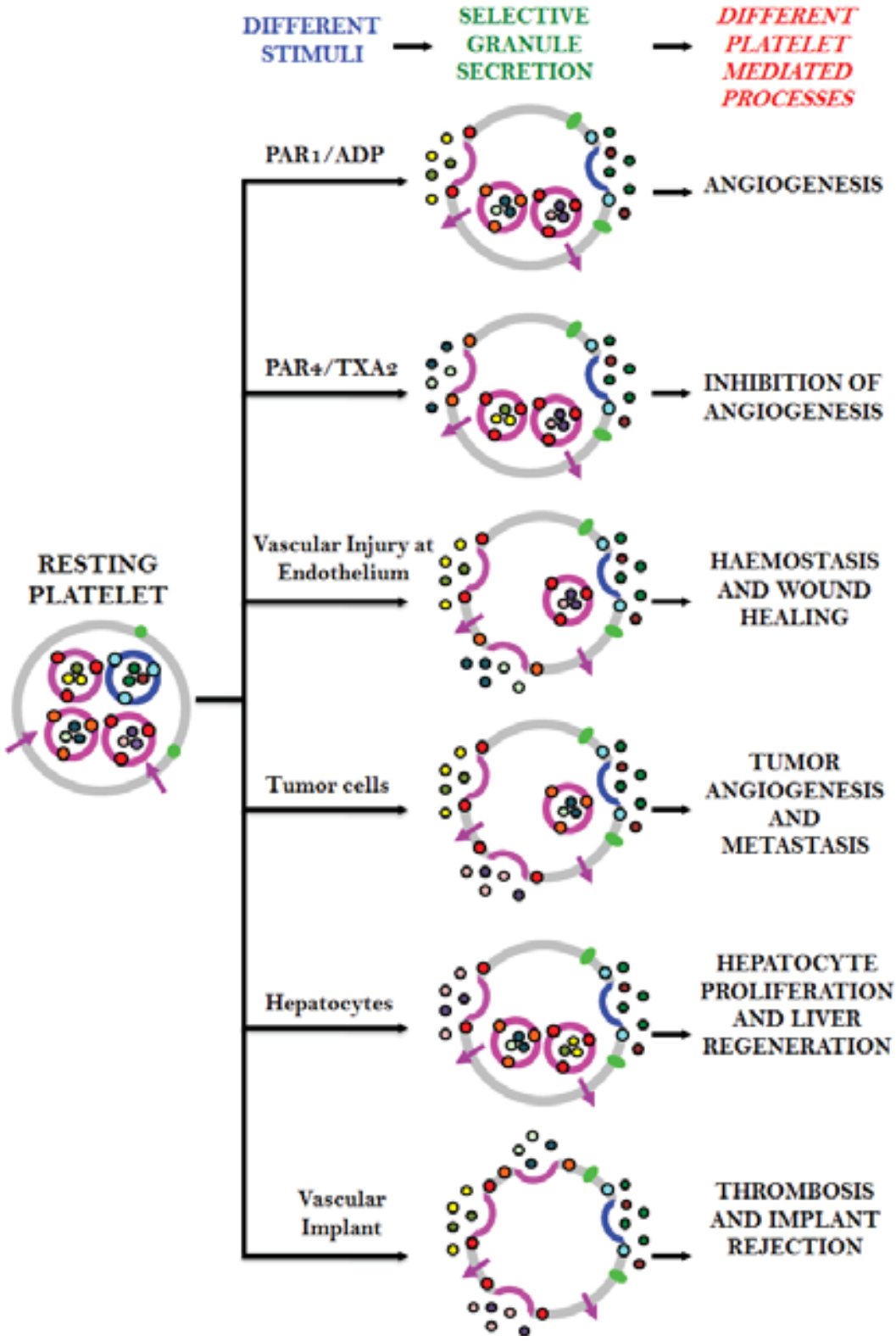



**Figure 1.6. Selective platelet activation in response to different stimuli.**

## 1.8 Exploiting selectivity in platelet activation for developing platelet based therapies

As described in previous section, platelets can undergo selective activation- depending on the stimuli they selectively secrete different activation markers, which delineates their function-inflammation, tumor angiogenesis, wound healing, liver regeneration, implant integration/rejection and tumor growth. Therefore identification of specific platelet markers (surface expressed or secreted) involved in these processes and understanding the mechanisms (intracellular pathways) behind selective expression of these markers by platelets, would allow us tailor platelet responses towards a desired goal at sites of wanted platelet activation (e.g wound healing, implant integration, tissue regeneration such as liver, immune response against bacterial infections) and sites of unwanted platelet activation (tumor growth, imflammation, implant rejection).

Potential identified markers include-platelet derived LPA and VEGF involved in tumor proliferation and metastasis; sCD40L and RANTES which are associated with atheriosclerosis; and TLR2 and TLR9 which are involved in bacterial infections. Therefore, development of approaches that can block the secretion or surface expression of specific markers by platelets while leaving the other activation responses unaffected would be beneficial.

As opposed to inhibition, induction of selective secretion of thromspondins (anti-bacterial proteins); endostatin (anti-angiogenic factor); and growth factors such as PDGF, TGFβ, FGF, VEGF, IGF-1 and EGF from platelets would help in reducing bacterial infections; tumor angiogenesis; and promote implant integration and tissue regeneration, respectively. For example, previous studies using tenocyte cultures showed that thrombin activated PRP-clot releasate (PRCK) stimulated cell

proliferation, collagen production and differentiation of TSCs (tendon stem/progenitor cells) into tenocytes.[115] Li, et al have recently reported that activated platelet rich plasma gels promoted bone marrow mesenchymal stem cell proliferation and their differentiation into two kinds cell populations- osteoblast-like cells and neural cells in a dose-dependent manner.[116] Activated platelet rich plasma consists of a myriad of platelet derived growth factors. If we could achieve selective secretion of growth factors then it would be possible to obtain desired population of cells from bone marrow mesenchymal stem cells -osteoblast-like cells or neural cells.

One has to keep in mind that some of these markers are also involved in platelet-mediated wound healing process and inhibition of these markers might affect wound-healing function of platelets. This indicates that there is an urging need to identify new platelet activation markers that contribute to platelet-mediated pathologies and the mechanism underlying their selective expression or secretion by platelets, in order to develop disease specific anti-platelet drugs.

## 1.9 Thesis overview

Evidence in literature supports that idea that platelets undergo a specific set of activation responses upon stimulation with a particular set of agonists, and responses differ for different agonists. At present, there is no clear mechanistic understanding of the molecular basis of selectivity in platelet activation.

Until now, selectivity in platelet activation has been explored only from granule secretion point of view. Physiological platelet agonists (eg. thrombin, PAR1-peptide agonist, PAR4-peptide agonists, ADP, TXA2) have been able to display selectivity only in secretion of few soluble alpha granule markers (eg VEGF and endostatin),[23, 74] while other platelet activation markers (such as CD62P, CD63, activated GPIIb/IIIa, PS) are all expressed upon stimulation with these agonists.[3, 12, 13, 45] The question remains if other platelet activation responses can also be selectively regulated by different stimuli? For example can platelets undergo selective secretion of alpha and dense granules? Can platelet undergo selective degranulation without GPIIb/IIIa activation? Are there other platelet activation responses that are selectively induced by different stimuli? The focus of this Thesis is on addressing these questions. Biomaterials are known to activate platelets and physio-chemical properties of biomaterials influences the biological response they elicit. In Chapter 3, we examine the role played by interfacial ion equilibrium in platelet activation by foreign surfaces. Unexpectedly, we found that platelets could be activated in a selective fashion, that they expressed different activation markers on the two different surfaces.

The work reported in Chapter 3 is based on standard markers of platelet activation and antibodies against them. However, to study the subtle nuances of platelet activation, new markers are sorely needed. Therefore, in Chapter 4, instead of relying on the traditional markers of platelet activation markers, we examined differences in platelet

surface carbohydrates expression in response to different agonists. For the first time, we were able to show that different agonists lead to different changes in the platelet surface carbohydrates. We have therefore provided a new assay for evaluating platelet activation, which will find applications in the clinical, diagnostic setting.

This Thesis demonstrates that platelet activation is selective and that selectivity is not just restricted to soluble alpha granule markers but also to other markers such as PS, activated GPIIb/IIIa, carbohydrate expression.

# *Chapter 2*
# *Materials and Methods*

## 2.1 Materials

### Antibodies

All antibodies used in this study-PerCPCy5.5-conjugated anti-CD41a (HIP8 clone, Ms $IgG_1$, K), APC or PE-conjugated anti-CD62P (AK-4 clone, Ms $IgG_1$, K), PE-conjugated anti-CD63 (H5C6 clone, Ms $IgG_1$, K), FITC-conjugated PAC1 (PAC1 clone, Ms IgM, K) and their isotype matched controls were purchased from Becton-Dickinson (Madrid, Spain).

### Fluorescently labelled lectins

Lectins from *Amaranthus caudatus* (ACA), *Bauhinia purpurea alba* (BPL), *Dolicos biflorus* (DBL), *Aleuria aurantia* (AAL), *Datura stramonium* (DSL), *Sambucus nigra* (SNA), *Canavalia ensiformis* (Con A), *Lens culinaris* (LCA), *Maclura pomifera* (MPL), *Pisum sativum* (PSA) were purchased from vector laboratories (Palex Medicals SA, Barcelons, Spain). Lectins from *Psophocarpus tetragonolobus* (PTII) and *Vicia faba* (VFA) and *Maackia amurensis* (MAA) were purchased from EY laboratories (Hycultec GmbH, Germany). All lectins were purchased in the FITC conjugated form.

### Other materials

Acid-citrate-dextrose (ACD), phorbyl methyl acetate (PMA), calcium ionophore A23187 (CaIoP), thrombin receptor activating peptide (TRAP) and adenosine 5′-diphosphate sodium salt (ADP) were purchased from Sigma (Madrid, Spain). PAR1/protease-activated receptor 1-specific agonist peptide (TFLLR-NH2), PAR4/protease-activated receptor 4-specific agonist peptide (AY-NH2) and TXA2/thromboxane A2-receptor agonist (U46619) were purchased from Tocris

Biosciences (Oxford, United Kingdom). BAPTA-AM, calcein-AM stain, fluo-3-AM stain and red-orange calcein-AM stain were purchased from Invitrogen (Madrid, Spain). All other chemicals and plasticware (safe lock eppendorf tubes, 15 ml and 50 ml falcon tubes) were purchased from Sigma (Madrid, Spain). PE or APC-Annexin V (A5) was purchased from Becton–Dickinson (Madrid, Spain). Glass vacutainer® tubes (4.5 ml) with 3.8% sodium citrate anticoagulant (0.129 M) were purchased from Becton–Dickinson (Madrid, Spain). NHS-activated hydrogel glass slides were purchased from Nexterion H (Schott AG, Mainz, Germany).

Surfaces used in this study were either bare 25 mm thickness #1 (0.13-0.16 mm) microscope coverslip glass slides (Menzel-Gläser, Braunschweig, Germany), or the same glass slides coated with ~ 20 nm layer of $TiO_2$ by magnetron reactive sputtering. The coating was done in a Leybold dc-magnetron Z600 sputtering unit at the Paul Scherrer Institut (Villigen, Switzerland) as well as at CIC biomaGUNE (protocol described in section 2.9.1), according to the previously published protocols.[117]

## 2.2 Equipment

Sterile laminar flow cabinet (Faster Two 30, Faster, Italy), ABX Micros 60 hematology analyzer (Horiba ABX Diagnostics, Madrid, Spain), 5417R Eppendorf centrifuge (Hamburg, Germany), Sigma 3K30 centrifuge with fixed angle rotor (Fisher Scientific), FACScalibur flow cytometer (Becton-Dickinson, Madrid, Spain), ND-1000 Nanodrop spectrophotometer system (Wilmington, DE, USA), nano-pure water dispenser (Nanopure Diamond™, Barnstead International, USA), stream of filtered nitrogen gas (99.999, AirLiquide Spain, Derio, Spain), UV-ozone cleaner (BioForce Nanosciences, USA), XPS (SAGE HR100, Specs, Berlin, Germany), LSM 510 confocal laser scanning microscope (Carl Zeiss, Jena, Germany) equipped with plan-apochromat

63x/1.40, Agilent G265BA microarray scanner (Agilent Technologies) and robotic non-contact piezoelectric spotter (S11, Scienion, Berlin).

## 2.3 Software used for processing of experimental data

Zen (Carl Zeiss, Jena, Germany) and ImageJ version 1.42 softwares were used for analyzing transmission and fluorescence channel images, obtained using confocal microscope. Flow Jo software (Tree Star Inc, Oregon, USA) was used for processing flow cytometer data and for constructing flow cytometer histograms presented in this thesis. Casa XPS 2.3.15dev87 software was used for analysis of chemical composition of $TiO_2$ coated surfaces. ProScanArray Express software (Perkin-Elmer) was used to process lectin array data and for constructing lectin array images and intensity histograms presented in Chapter 4.

## 2.4 Buffers

Table 2.1 lists the buffers used throughout the Thesis, together with the particular chapters where they were used. All buffers were prepared in nano-pure water produced with a Diamond UV water purification system. All the buffers were filtered through a 0.22 μm syringe filter, and degassed in water bath for 30 minutes immediately prior to use.

**Table 2.1. List of buffers**

| Buffers | Composition and pH | Chapter |
|---|---|---|
| Citrate buffer | 100 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM $MgCl_2$, 15 mM citrate, pH 6.5 | 3, 4 |
| Calcium free-HEPES buffer | 145 mM NaCl, 5 mM glucose, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM KCl, pH 7.4 | 3, 4 |
| 2 mM Calcium-HEPES buffer | 145 mM NaCl, 5 mM glucose, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM KCl, 2 mM $Ca^{2+}$, pH 7.4 | 3, 4 |

## 2.5 Principles of techniques used

There are two major techniques used throughout the thesis. Those are flow cytometry and confocal microscopy. The principles of those techniques are described below.

### 2.5.1 Principles of flow cytometry

Flow cytometry (FACS) is a technique in which cells or particles suspended in fluid pass one by one across a set of optical and/or fluorescence detectors. The optical parameters recorded in a typical flow cytometry experiment include the intensity of directly scattered light (forward scattering), the intensity of light scattered at 90° (side scattering), and fluorescence signals.

**Components of a flow cytometer**

A flow cytometer is made up of three main systems: fluidics, optics, and electronics. These are depicted schematically in Figure 2.1.

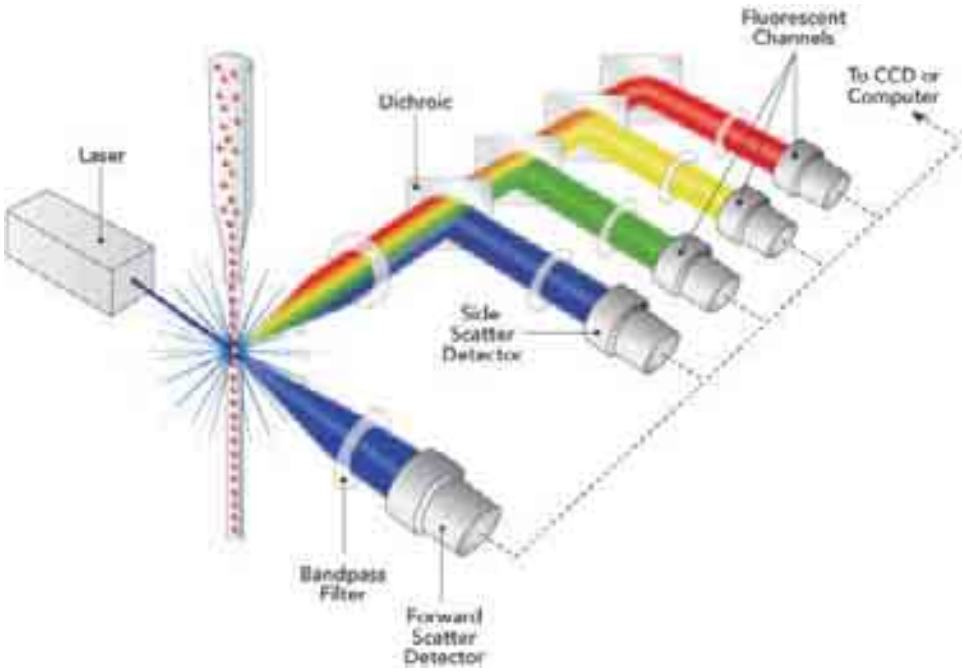



**Figure 2.1: Schematic overview of a flow cytometer. A flow cytometer consists of three main components. The optical system (set of lasers, lenses, filters, and detectors surrounding the sample cell), the fluidics system which carries the sample to and from the cell, and electronics for signal correlation, data recording and analysis.**

**Fluidic system**

When a solution containing a particulate sample is injected into the flow cytometer, the particles are randomly distributed the volume. The fluidic system of a flow cytometer consists of a central core channel through which the sample is injected into the center of a flowing stream of a sheath fluid (Figure 2.1). The combined fluid stream forces through a narrowing, forcing the cells into a single file of particles. This process is called hydrodynamic focusing.

**Optics and detection**

After hydrodynamic focusing, each particle passes through the beam of light. In modern flow cytometers, lasers and arc lamps are the most commonly used light sources. Lasers are chosen because they produce a high intensity beam of monochromatic light.

Light scattering occurs when a particle deflects the incident laser beam. The physical properties of a particle, such as size and internal complexity, determine the intensity of the scattered light. In particular, the intensity of the directly scattered light is related to the particle size. This signal is called forward-scattered light (FSC) is used to distinguish between cells of different size, cell debris vs. living cells, and so on. FSC is a measurement of mostly diffracted light and is detected just off the axis of the incident laser beam in the forward direction (Figure 2.2-a).

The intensity of the side-scattered light is a more complex function of particle shape, state of aggregation, and internal complexity. It is called side-scattered light (SSC; Figure 2.2-b). SSC signal is sensitive to changes in cell shape, cell aggregation, and fission. It is also useful for distinguishing granular from agranular cells (Figure 2.3).

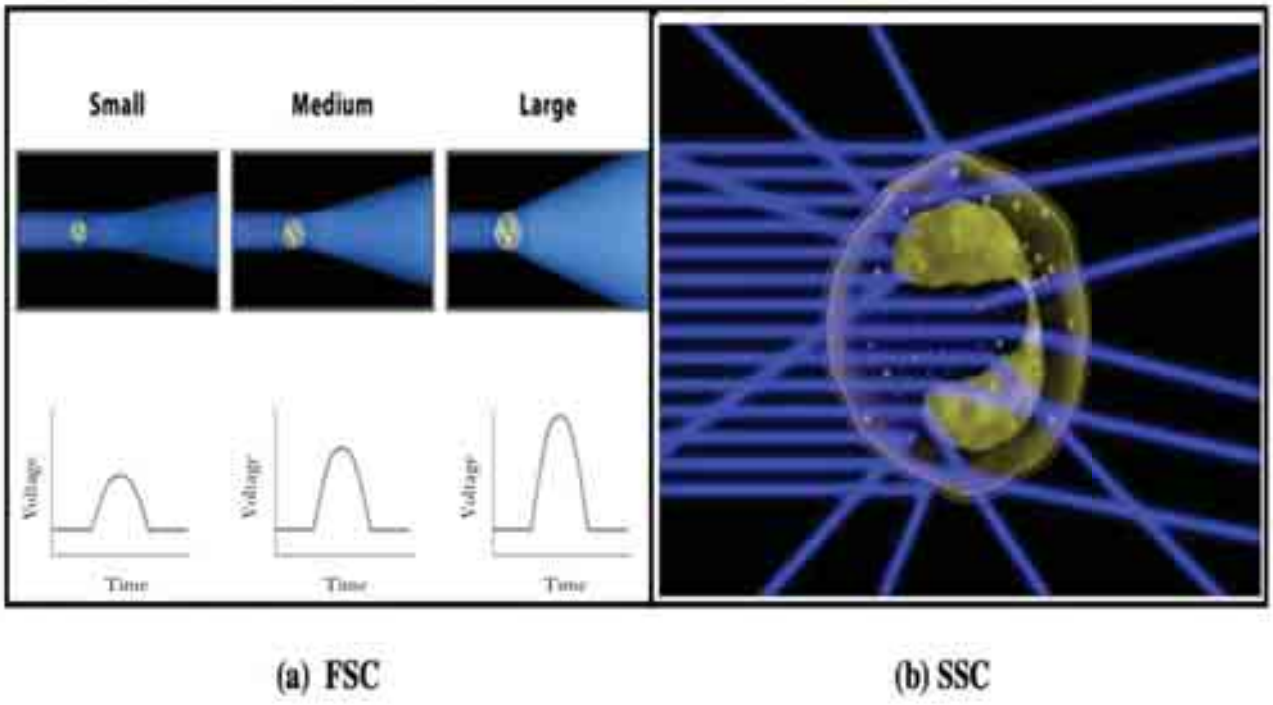



**Figure 2.2. Light scattering profiles of cells by flow cytometry.**

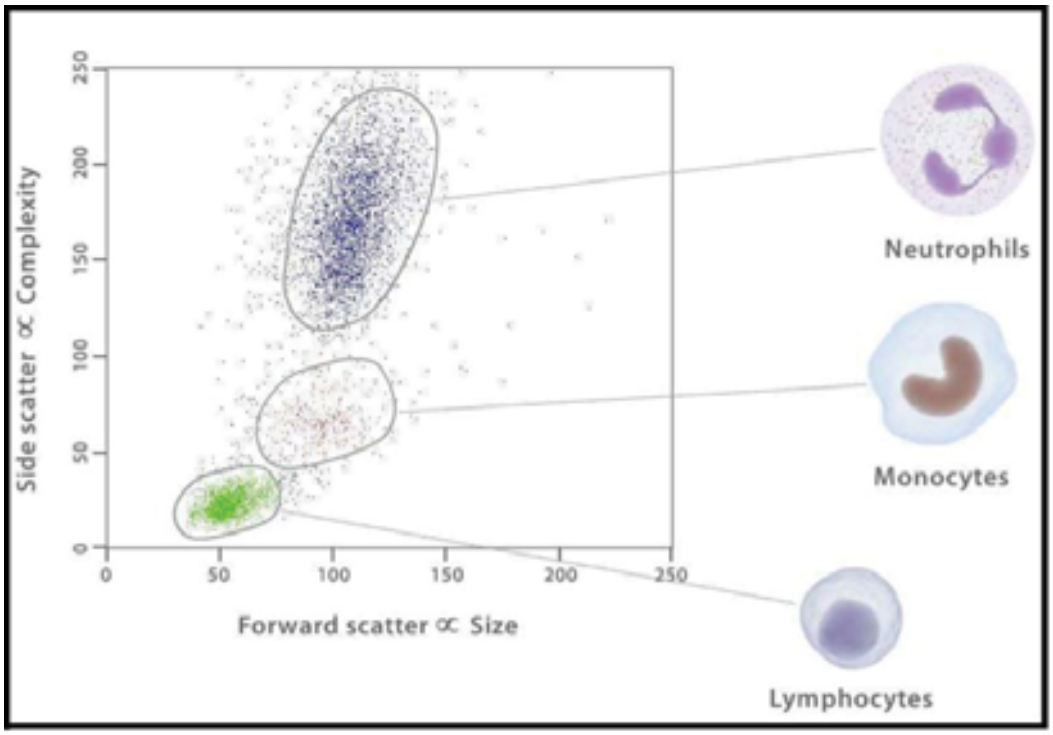



**Figure 2.3: Forward (x, FSC) and side (y, SSC) scattering analysis of a leukocyte population. All leukocytes have approximately same size as visible looking at x-axis (FSC). However, due to differences in the internal complexity of the cells, they can be distinguished by their SSC signals. In the 2D plots shown above, it is clear that this cell populations consist of several sub-populations, each cloud of points corresponding to one leukocyte sub-type. Each dot in the plot represents an individual cell that has passed through the instrument.**

### Fluorescence measurements correlated to the light scattering detection

Each cell displays a set of glycoproteins on its surface that uniquely identifies a particular cell type. Moreover, this set changes depending on the state of the cell. Availability of fluorescently labeled monoclonal antibodies and other reagents (such as lectins-carbohydrate binding proteins) against these surface glycoproteins enables cells to be distinguished from each other and enables identification of activated vs. quiescent cells, etc. The key to the ability of the flow cytometer to distinguish different cell populations is the correlated detection of light scattering and fluorescence signals.

Specifically, the fluorescence channels are activated by cells registered ("gated") by the optical detection system—and only by those cells (objects smaller than a certain size will not be registered by the optical detection system). Each time a cell passes through the light beam, it activates the fluorescence detectors. If the cell happens to be labeled by one or more of the fluorescently-tagged antibodies, one or more fluorescence signals will be associated with that scattering event (with that cell). This, in turn, allows different fluorescence signals to be cross-correlated. Figure 2.4 illustrates this principle.

Once the data is collected, this correlation can be visualized in the computer by selecting groups of points (events) and analyzing the FSC, SSC, and fluorescence signals associated with these events. This process is also called "gating".

A single parameter such as FSC or fluorescence (FL1) can be displayed as a single parameter histogram, where the horizontal axis represents the parameter's signal value in channel numbers and the vertical axis represents the number of events per channel number. Each event is placed in the channel that corresponds to its signal.

Two parameters can be displayed simultaneously in a dot plot. One parameter is displayed along the x-axis and the other parameter is displayed along the y-axis.

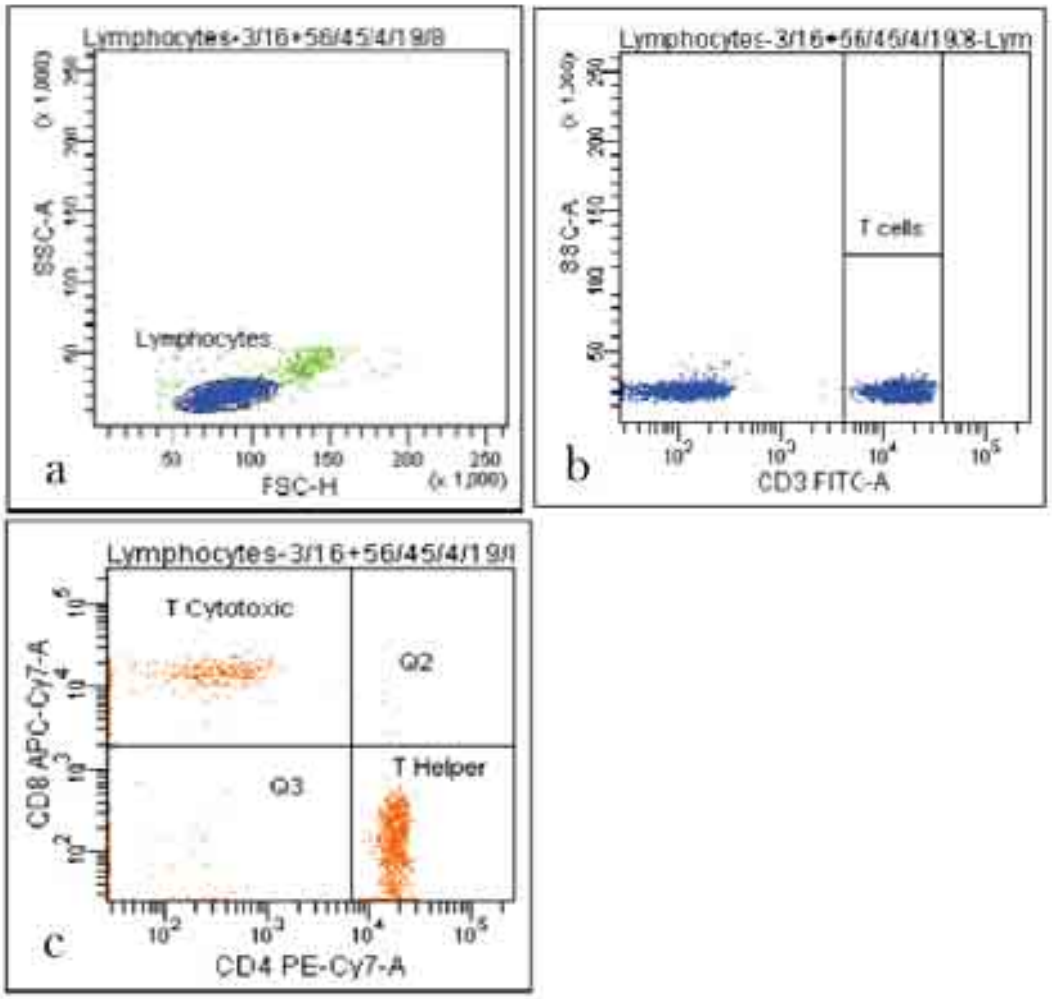



**Figure 2.4. Illustrates how the correlation between the light scattering signals (FSC or SSC) and fluorescence signals is used to identify different types of T cells in a population of lymphocytes (Follow plots from a to c). Lymphocytes in whole blood were labeled with fluorescently tagged antibodies specific for the particular surface receptors. In plot (a), the side scattering intensity (SSC) is plotted vs. forward scattering intensity (FSC). Lymphocytes population is selected (blue). Analysis of this population according to fluorescence from the anti-CD3 FITC labeled antibody shown (b) reveals the presence of two populations: CD3-positive (lymphocyte B) and CD3-negative (lymphocyte T). Finally, the T-cell (CD3+) population is analyzed according to the presence of CD4 or CD8 surface antigen in plot (c).**

### 2.5.2 Principles of laser scanning confocal microscopy

Confocal laser scanning microscopy (CLSM or LSCM) is a technique for obtaining high-resolution in-focus-optical images with depth selectivity. This process is known as optical sectioning. Images are acquired point-by-point and reconstructed with a computer, allowing three-dimensional reconstructions of topologically complex objects. Surface profiling as well as interior structures of biological objects labeled with fluorescent markers can be obtained with this technique.

In a confocal laser scanning microscope, a laser beam passes through an aperture, is focused by an objective lens into a small volume within or on the surface of a specimen. Specimens can be fluorescent (e.g. labeled with fluorescent antibodies). Scattered and reflected laser light together with fluorescent light that is coming from the illuminated spot is then re-collected by the objective lens, where it passes through the same aperture (Figure 2.5). The purpose of the aperture is to restrict the light to a certain volume (confocal volume). Optical sectioning is performed by moving the aperture parallel to the beam.

Wavelength-selective beam splitters and filters illuminate the overly intense incident light allowing only the fluorescence signals to reach the detectors.

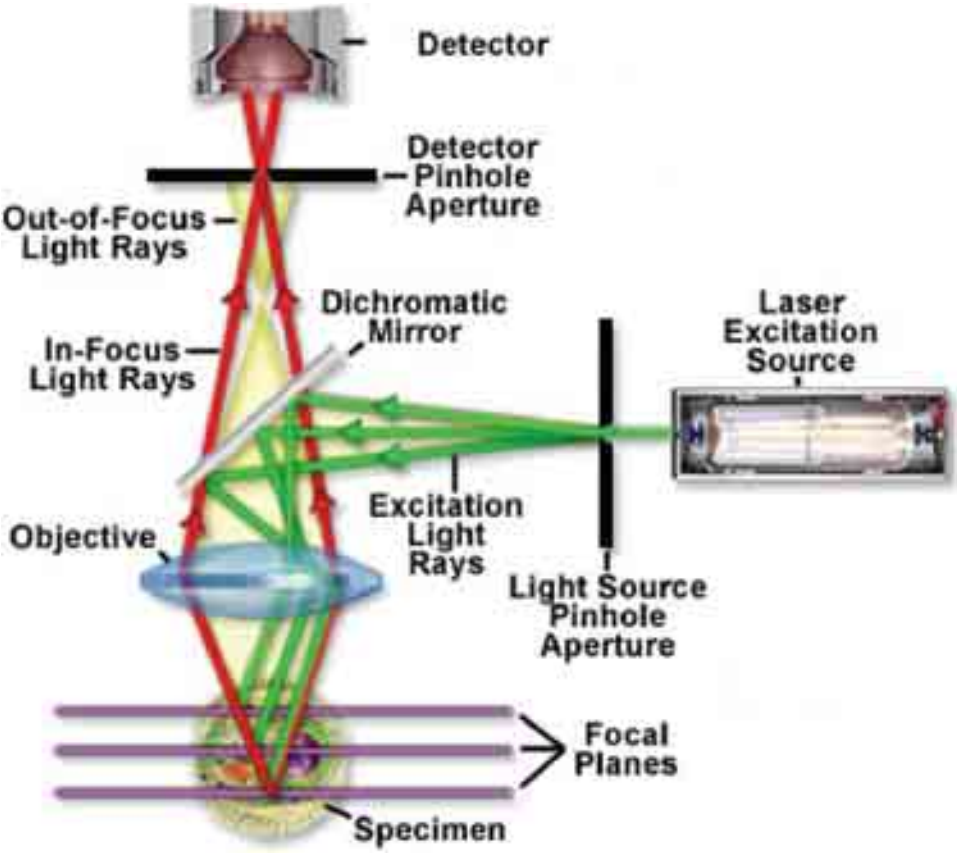



**Figure 2.5. Schematic representation of confocal microscope.**

## 2.6 Blood collection protocol

Blood collection was organized by the Biobanco Vasco para la Investigación (Basque Biobank for Research, Galdakao, Spain) and performed with informed consent according to the appropriate legal and ethical guidelines. Donors were healthy volunteers without the history of exposure to medication (such as aspirin) or exposure to alcohol in the two weeks prior to collection. For every experiment, 10 ml of blood was collected by venipuncture with a 21-gauge needle into two 4.5 ml glass Vacutainer® tubes with 3.8% sodium citrate anticoagulant and stored at 37 °C. First 2 ml of blood was discarded during the collection to avoid platelet activation by residual thrombin. Both tubes were from the same donor.

## 2.7 Preparation of washed platelets from whole blood

All procedures involving dispensing, pipetting, and transferring blood were carried out in a sterile laminar flow cabinet to avoid contamination. Glass- and plasticware was autoclaved before use unless it was already sterile when purchased. Citrate-anticoagulated whole blood was transferred from the vacutainer tubes to 1.5 ml safe-lock microcentrifuge eppendorf tubes. Platelet count in blood was determined with a ABX Micros 60 hematology analyzer and was typically in the range 1.5 – 2.5 × $10^8$ platelets per ml. Platelet-rich plasma (PRP) was prepared from whole blood by centrifugation of blood at 600 rpm (37×g) for 20 min at 37 °C in a 5417R Eppendorf centrifuge equipped with a fixed angle rotor.

PRP layers were collected carefully (without disturbing the underlying white blood cells/WBC and red blood cell/RBC layers) into a 15 ml falcon tube and acid-citrate-

dextrose was added to the PRP in a ratio 1:6 by volume. Platelets were incubated with ACD for 10 mins at 37 °C and then centrifuged at 700×g at 22 °C for 20 mins. The pellet was re-suspended in citrate buffer (100 mM NaCl, 5 mM KCl, 5 mM glucose, 1 mM $MgCl_2$, 15 mM citrate, pH 6.5) and centrifuged again at 700×g at 22 °C for 10 mins. The pellet was finally suspended in the calcium free-HEPES buffer (145 mM NaCl, 5 mM glucose, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM KCl, pH 7.4). For experiments to be carried out in the presence of extracellular calcium, the pellet was re-suspended in the 2 mM calcium-HEPES buffer (145 mM NaCl, 5 mM glucose, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM KCl, pH 7.4). Red blood cell (RBC) and white blood cell (WBC) counts were below $0.01 \times 10^6$ and below $0.1 \times 10^6$ cells per ml, respectively. Platelet concentration varied from experiment to experiment and was adjusted to ~ 1 x $10^8$ cells per ml prior to experiments.

Platelet purity in the washed platelet suspension was ascertained by flow cytometry (described below in section 2.8) and was found to be above 99%.

Protein concentration in the purified washed platelet suspension was measured with a ND-1000 Nanodrop spectrophotometer system using an extinction coefficient of ~ 1 [$ml \cdot g^{-1} \cdot cm^{-1}$]. Protein concentration in our samples was ≈ 0.05 ± 0.01 mg/ml, compared to ≈ 130 mg/ml in the blood plasma.

Isolation of platelets was performed within ~ 12 hrs of blood collection because, due to the stringent safety regulations, testing for infectious agents had to be performed off-cite before the blood could be used for experiments. All experiments were completed within 24 hours of blood collection.

## 2.8 Characterization of isolated platelets for their purity and functional state by flow cytometry

Platelets are extremely sensitive to environmental conditions. Before every experiment, it was necessary to ensure that the isolated platelets were pure, minimally activated, and properly responded to agonists. For this reason, prior to each experiment, the freshly isolated platelets in the calcium free-HEPES buffer were analysed by flow cytometry (principle described in section 2.5.1), untreated and treated with agonists such as TRAP and PMA following the protocol as described below.

Flow cytometry was performed on freshly isolated platelets to analyze their purity, basal activity, and response to agonists, with a FACScalibur flow cytometer (Becton-Dickinson, Madrid, Spain). 50 µl of the washed platelets were mixed with 50 µl of calcium free-HEPES buffer or with 2 mM calcium-HEPES buffer, as required. For stimulating platelets, either TRAP (thrombin receptor activating peptide) at a final concentration of 70 µM, or PMA (phorbyl methyl acetate) at a final concentration of 10 µM, were added to the samples. Platelets were identified by staining samples with PerCPCy5.5-conjugated anti-CD41a antibody (antibody against platelet specific marker CD41a). Activation was evaluated by staining them with PE-conjugated anti-CD62P (P-selectin), PE-conjugated anti-CD63, FITC-conjugated PAC1 (antibody against the active conformation of GPIIb/IIIa) and APC-conjugated Annexin A5 (staining agent for phosphatidyl serine, PS), in separate tubes. Untreated and agonist treated platelets samples were incubated with antibodies (mentioned above) and A5 for 30 min at 37 °C without agitation before being diluted with 2 ml of the respective buffer and analyzed by flow cytometery. Unstained platelet sample and isotype-matched controls were run in parallel to all monoclonal antibodies. Light scatter and

fluorescence data from 10,000 events were collected with all the detectors in the logarithmic mode. Antibody binding was expressed as percentage of platelets positive for an antibody staining, using Flow Jo software. The data (in Chapter 3) is presented as light scatter plots (side scatter, SSC vs forward scatter, FSC) and fluorescence histograms and histogram overlays (fluorescence intensity on the X-axis and % of cells positive for antibody binding on Y-axis). Only if platelets were in their quiescent state, they were used for subsequent experiments. If isolated platelets were activated they were discarded.

## 2.9 Protocols for Chapter 3

### 2.9.1 Preparation of $TiO_2$-coated glass slides [117]

$TiO_2$-coated glass slides were prepared by Danijela Gregurec and Luis Yate, at CIC biomaGUNE, Spain. 25 mm $TiO_2$-coated glass coverslips were prepared by direct current (d.c.) and radio frequency (r.f) magnetron reactive sputtering, respectively in an ATC 1800 UHV sputtering system (AJA International Inc., MA, USA) equipped with a load-lock transfer chamber. The base pressure in the chamber was kept at ~ $1.2\times10^{-8}$ Pa. Prior to the deposition process, glass slides were cleaned for 10 minutes at 50 °C in a mixture of $H_2O$ : $NH_4OH$ : $H_2O_2$ at a volume ratio of 1.5 : 1 : 1, followed by a rinse with nano-pure water (3x). This was followed by cleaning in a mixture of $H_2O$ : HCl : $H_2O_2$, also at a volume ratio of 1.5 : 1 : 1 for 10 minutes at 50 °C and then rinsing with water. Cleaned slides were rinsed multiple times in nano-pure water and dried under a stream of nitrogen. They were then installed in the sputtering plant where they were further cleaned in Ar plasma generated with a negative radio frequency (r.f.) bias of 162 V in a 4 Pa Ar atmosphere for 3 min.

Briefly, a 2-inch diameter Ti target (99.99% purity, AJA International Inc., MA, USA) was used, with a power of 228 W, in the $Ar/O_2$ atmosphere generated by combining 10 sccm of Ar flow with 20 sccm of $O_2$ flow at 0.4 Pa working pressure. Substrate-to-target distance was kept at 4 cm and the holder with the glass slides was rotated at 80 rpm. Sputtering was performed at room temperature for 120 mins. This resulted in generation of transparent $TiO_2$ films of ~ 30 nm thickness. The thickness of films was calculated based on the sputtering rate of 0.42 nm/sec measured with a built-in QCM sensor under identical conditions and verified by optical ellipsometry. Surface chemical composition of the $TiO_2$ films was analyzed by XPS after SDS-UV ozone cleaning procedure as described below.

### 2.9.2 Surface cleaning

All the surfaces (uncoated glass slides, $TiO_2$-coated glass slides) used for all the experiments (Chapter 3) throughout the thesis were cleaned in the same way. Immediately prior to each experiment, surfaces were cleaned in 2% SDS (Sodium Dodecyl Sulfate) solution that was filtered through 0.2 µm pore diameter syringe filter for 30 minutes, washed under a stream of nano-pure water, dried with a stream of filtered nitrogen gas, and further cleaned for 30 minutes in a UV-ozone cleaner (BioForce Nanosciences, USA) that was pre-heated for 30 minutes immediately prior to this step. The UV-ozone treatment cleans the surface of organic contaminants, and makes the surface hydrophilic. This procedure results in minimal carbon contamination of the surface (<10%) as judged by X-ray photoelectron spectroscopy/XPS (described below in section 2.9.3). No other impurities were detected on the surfaces cleaned in this way. Several groups for a wide variety of biophysical studies have used this cleaning procedure.[118-121]

### 2.9.3 Analysis of chemical composition of $TiO_2$ coated surfaces by X-ray photoelectron spectroscopy (XPS)

X-ray photoelectron spectroscopy (XPS) is a technique for characterizing the chemical composition of surfaces with a depth resolution of a few nm. The technique is based on photoelectric effect. The surface of a material is irradiated with x-rays that causes an emission of the photoelectrons. The elements in the sample are identified from the kinetic energies of the emitted photoelectrons, while peak intensities, that were suitably normalized, are used to determine their relative contents. The kinetic energy of a photoelectron can be determined from the Einstein equation:

$$KE = h\nu - BE - e\Phi$$

Where KE is the kinetic energy of the emitted photoelectron, $h\nu$ is the characteristic energy of x-ray photon, $BE$ is the binding energy of the atomic orbital from which the photoelectron originates and $e\Phi$ is the spectrometer work function.

Surface chemical composition of the $TiO_2$-coated glass slides was analyzed by XPS in a SPECS SAGE HR 100 spectrometer equipped with a Mg Kα (1253.6 eV) non-monochromatic source operated at 12.5 kV and 250 W. The take-off angle was fixed at 90° and the analysis was conducted at a pressure of ~$10^{-8}$ Torr. Surfaces were brought into the XPS chamber within 5 min after SDS-UV cleaning (protocol described above). Survey spectra were obtained with pass energy of 30 eV. For $TiO_2$-coated surfaces, detailed spectra were acquired for Ti 2p3/2, Ti 2p1/2, O1s, and C1s regions with the pass energy of 15 eV. Flood gun was used for charge compensation, set at 0.7 mA and 1.0 eV. Spectra were analyzed with the Casa XPS 2.3.15dev87 software. The analysis for determination of the atomic percentages of various elements present on the surface consisted of- satellite removal, Shirley background subtraction, calibration to the C1s peak at 285 eV, and peak fitting with Gaussian-Lorentizan line shapes. Freshly cleaned

$TiO_2$-coated surfaces typically contained < ≈ 10 atomic% of carbon and ≈ 95% of the Ti2p peak could be assigned to TiIV, with ≈ 5% corresponding to the lower oxidation states.[122-124] Samples that contained extraneous elements or higher amounts of carbon (contamination) were discarded.

### 2.9.4 Platelet adhesion and activation studies on glass and $TiO_2$ surfaces.

Platelet adhesion experiments were started no later than 2 hours after preparation of washed platelets. The entire experiment was performed at 37 °C.

Freshly SDS-UV cleaned surfaces were mounted in the center of circular home-made, 500 µl hollow teflon cells (hollow in the centre) using dental glue. It was made sure that the dental glue did not spread to the portion of surface that came in contact with platelets. 250 µl of the buffer-calcium free- or 2 mM calcium-HEPES buffer (depending on the experiment) was added to the teflon cell immediately after the glue had set (~ 2 minutes) to reduce as much as possible the chances of contaminating the surface, followed by 250 µl of the washed platelets in the same buffer, and incubated either for 10 mins or for 3 hrs.

After the incubation, the samples were washed by adding and withdrawing 250 µl of the buffer 20×, to remove any non-adhered platelets. Care was taken not to touch the surface with the pipette tip, not to introduce bubbles, and not to dry the sample out.

Washed samples were co-stained with 5 µl of PerCPCy5.5 anti-CD41a antibody and with either of these reagents specific for platelet activation markers (CD62P/CD63/activated GPIIb/IIIa or PS)-PE anti-CD62P or PE anti-CD63 antibody

or FITC-PAC1 antibody or PE-A5 for 30 mins. The samples were then analyzed for platelet adhesion and antibody staining of surface adhered platelets, using confocal microscopy (described below in section 2.9.5). Settings of microscope were adjusted to make sure there was no mixing of fluorescence signals between channels. The sample mounting stage of microscope was set at 37 °C.

For experiments involving stimulation of surface adhered platelets with an agonist, the above-described steps were followed by the addition of TRAP or PMA to the sample cell. In other experiments calcium ionophore along with 2 mM calcium was added to the surface adhered platelets. Samples were incubated with agonists for 30 mins, followed by analysis with confocal microscopy. For experiments involving intracellular calcium chelation with BAPTA-AM, platelets were isolated in calcium free-HEPES buffer and incubated with desired concentration of BAPTA-AM (1, 2, 5, 8, 10, 100 μM) for 30 min before they were treated with agonist (TRAP) in solution or allowed to interact with the surfaces (glass and $TiO_2$) following the same protocol as described above.

### 2.9.5 Confocal microscopy and image analysis

Fluorescence and transmission images of the above-described samples were obtained using an LSM 510 confocal laser scanning microscope equipped with a plan-apochromat 63x/1.40 NA oil immersion objective. All experiments were carried out in a temperature controlled stage at 37 ºC. PerCP Cy5.5, FITC and PE fluorescence was excited with the 488 nm line of the Ar laser and emission was measured at 695 nm, 519 nm and 578 nm respectively. 143 × 143 $\mu m^2$ 512 × 512, 1024 × 1024 or 2048 × 2048 pixel images were recorded and analyzed using the Zen or ImageJ version 1.42 software

to extract the numbers and sizes of the adhering platelets as well as fluorescence intensity of antibody stained platelets, to highlight the different types of platelets observed on the surface. The image analysis procedure was as follows.

First, the multi-channel images were split into separate channels—one corresponding to the anti-CD41a fluorescence and one corresponding to the CD62P or CD63 or PAC1 or A5 fluorescence. Second, a Gaussian blur filter was applied to both sets of images to highlight platelet edges.

The numbers of adhering platelets were quantified using the ImageJ cell counter plugin. Platelets adhering on the surfaces for 3 hr were counted manually because cells were overlapping and it was difficult to separate them.

Platelet areas were measured manually by drawing regions of interest around the platelets using the free hand drawing tool. Based on the area analysis of 180 cells, radii were calculated to determine the size distribution of the platelets, adhered on the surface for different experiment conditions. Due to the aggregation of platelets after 3 hrs, the exact number and size of adhered platelets were difficult to quantify.

Following method was used to determine which of the adhering platelets were activated i.e. if they stained with antibody specific for platelet activation markers- CD62P, CD63, PAC1 and PS.

First, blurred images for the CD41a and CD62P or CD63 or PAC1 or A5 channel were false colored green and red respectively and then merged using Image J software. Co-localization of fluorescence between green and red channel was visualized in the merged images in terms of the appearance of the yellow color. These images are shown in Chapter 3.

To calculate the background subtracted average fluorescence intensities for red channel (for CD62P/CD63/PAC1/A5), individual cells were selected in ImageJ and median intensity within the selected region in the red channel was calculated. Median intensities for regions where there were no cells were measured as well. The intensity for each cell in a given image was than normalized by the background intensity I = [(I(cell)-I(background)]/I(background). These normalized intensities were averaged over the cells observed in different experiments performed under nominally the same experimental conditions but with blood taken from different donors. If the normalized value was greater than 1, it was taken as positive staining, however if less than equal to 1, it was designated as negative staining.

### 2.9.6 Evaluation of platelet $[Ca^{2+}]i$ changes during the platelet adhesion and activation processes on $TiO_2$ and glass surfaces

Isolated PRP was co-incubated with fluo-3 AM (10 µM) and red-orange calcein AM (5 µg/ml) for 40 mins at 37 °C and platelets were isolated from PRP following same protocol as described in section 2.7.

Platelet purity and state in the washed platelet suspension was ascertained by flow cytometry (as described in section 2.8) and was found to be above 99%.

### 2.9.7 Measurement of intracellular calcium by confocal microscopy

Freshly SDS-UV cleaned surfaces (glass or $TiO_2$) were mounted in the center of circular home-made, 500 µl hollow teflon cells (hollow in the center) using dental glue as described in section 2.9.4. 400 µl of the calcium free-HEPES buffer was added to the teflon cell. This teflon cell set up was placed on the stage of LSM 510 confocal laser scanning inverted microscope equipped with a plan-apochromat 40x oil immersion objective. The temperature of the stage was maintained at 37 ºC throughout the experiment. Before addition of labeled platelets to the teflon cell, the surface of the glass or $TiO_2$ coated glass coverslip was focused with a 40x objective lens in the transmission channel. This was done to make sure that calcium changes in the platelets are recorded the moment they arrive at the surface. This was followed by addition of 20 µl of the labeled platelets in the same buffer, to the teflon cell chamber. Only platelets interacting with the surfaces were analyzed in this study. Fluorescence from fluo-3 (AM) and red-orange calcein (AM) dyes was measured simultaneously. For fluorescence measurement, fluo-3 (AM) fluorescence was measured at 505 nm excitation, 530 nm emission and red-orange calcein (AM) fluorescence was measured at 570 nm excitation, 590 nm emission, respectively. With these optical conditions, there was no interference of the fluo-3 (AM) and red-orange Calcium (AM) fluorescence signals. The intracellular $[Ca^{2+}]i$ changes, entire adhesion and spreading processes were recorded with a video rate of 1 frame per 1.964 seconds (143 × 143 $\mu m^2$ 512 × 512 or 1024 × 1024 pixel images). The frames were analyzed using the Zen or ImageJ version 1.42 software to extract sizes of the adhering platelets as well as changes in the fluorescence intensity of fluo-3 (AM) and red-orange calcien (AM)

stained platelets, to highlight the different types of platelets observed on the surface. The image analysis procedure was as follows.

The analysis for area measurements and calcium measurements was done for all frames recorded, starting with the frame where platelets hit the surface, followed by rolling on the surface, arrest at the surface and spreading.

For each frame, platelet areas were measured manually by drawing regions of interest around the platelets using the free hand drawing tool. Based on the area analysis of platelets, radii were calculated to determine the size of the platelets, adhered on the different surfaces (glass and $TiO_2$).

To calculate the background subtracted average fluorescence intensities for fluo-3 (AM) and red-orange calcien (AM) channels, individual cells were selected in ImageJ in every frame and median intensity within the selected region was calculated in the both channels. Median intensities for regions where there were no cells were measured as well for both channels, which were then subtracted from the cell intensities of respective channels to obtain background subtracted intensities for both channels for each platelet. The obtained fluo-3 (AM) intensities were then divided by red-orange calcein (AM) intensity for every frame, which gave us the normalized increase in fluo-3 (AM) intensities for every frame. These normalized values are plotted against time in Figure 3.19 in Chapter 3 which gives us the increase in calcium in platelets with time upon interaction with surfaces.

### 2.9.8 Cell viability analysis

Membrane integrity in platelets adhering on the bare glass and $TiO_2$-coated glass surfaces was measured by staining surface adhered platelets with calcein-AM stain. This marker is retained in the cytoplasm and degraded by esterases, unless the cell membrane is damaged. After the adhesion of cells on the surface for 10 mins and 3 hrs, they were washed, and then 200 µl of 2 mM calcein-AM solution was added to the surface. After staining for 30 mins at 37 ºC, labeled cells were visualized using confocal microscope. This analysis showed that adhesion to the surface did not affect the integrity of the platelet membranes.

**Table 2.2. Summary of samples prepared for Chapter 3:**

| Platelets and buffer type for sample preparation | Surface used and incubation time for platelet adhesion and activation experiments | Parameters analyzed |
|---|---|---|
| Resting platelets in calcium free-hepes buffer | $TiO_2$ and glass<br><br>(10 mins and 3 hrs) | Expression of CD62P, CD63, PS, activated GPIIb/IIIa, number and size distribution of adhered platelets, changes in intracellular calcium upon adhesion, effect of TRAP or PMA or calcium ionophore on surface adhered platelets |
| Resting platelets in 2 mM calcium-hepes buffer | $TiO_2$ and glass<br><br>(10 mins and 3 hrs) | Expression of CD62P, CD63, PS, activated GPIIb/IIIa, number and size distribution of adhered platelets |
| BAPTA-treated platelets in calcium free-HEPES buffer | $TiO_2$ and glass<br><br>(10 mins and 3 hrs) | Expression of CD62P and CD63 effect of TRAP on surface adhered platelets |

## 2.10 Protocols for Chapter 4

### 2.10.1 Lectin binding studies of platelets

Lectins are carbohydrate binding proteins often derived from plant seeds, which bind weakly but highly selective glycan epitopes found on glycoproteins. Lectins have long been used as a tool to characterize cell surface glycans because of their ability to distinguish glycans with different structures- branching, linkage, and terminal modifications.

In this thesis, we characterized the binding of a range of lectins to resting platelets and agonist treated platelets using two techniques- lectin arrays and flow cytometry. Flow cytometry is solution-based technique where platelets are stained individually with different fluorescently labeled lectins in solution and the fluorescence of the platelet bound lectins is detected by flow cytometry. The principle of Flow cytometry has been described in section 2.5.1. Lectins array on the other hand is a surface based technique where multiple lectins (upto 70-100) are printed as spots on the same glass slide and then platelets are allowed to interact with these spots simultaneously (Figure 2.6). The lectin bound platelets are stained with platelet specific antibodies and the fluorescence arriving from lectin-spot bound platelets is measured by a microarray scanner. All the lectins used in this study along with their sources of extraction and carbohydrate specificity are mentioned in Chapter 4.

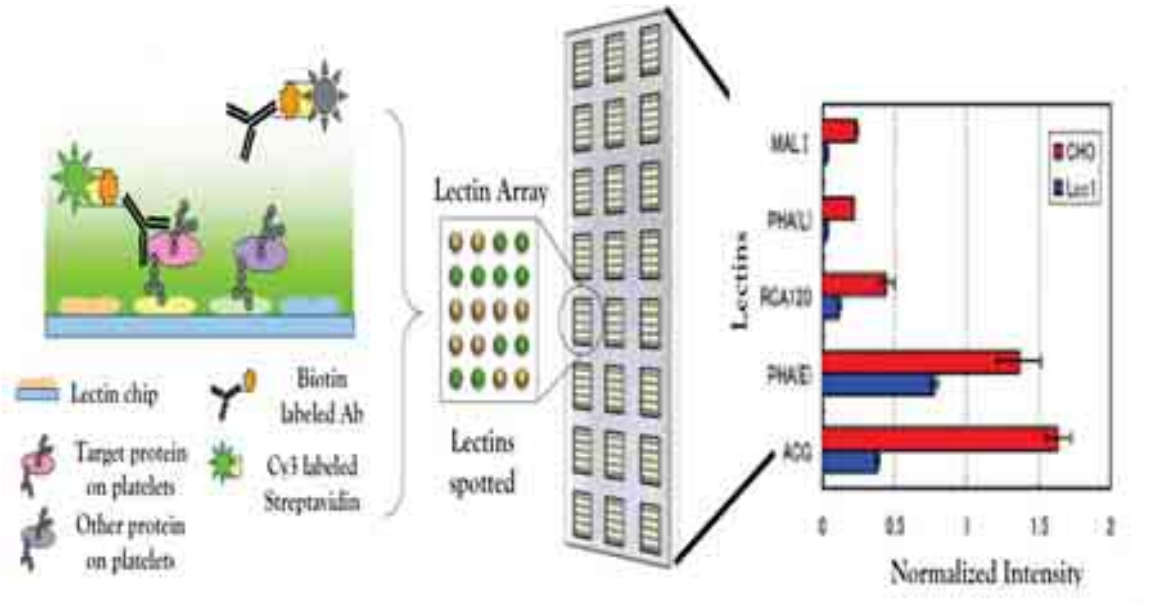



**Figure 2.6. Lectin arrays for detecting membrane bound glycoproteins on platelets.**

## 2.10.2 Lectin binding studies of platelets using flow cytometry

Resting platelets, platelets treated with BAPTA-AM, and platelets treated individually with different agonists-PAR1 and PAR4 receptor agonist peptides (SFLLRN and AY-$NH_2$), ADP, thromboxane A2, calcium ionophore (CaIoP), and phorbol 12-myristate 13-acetate (PMA), were incubated with 20 µg/ml of FITC-labelled lectins (ACA, BPL, AAL, DSL, SNA, ConA, LCA, MPL, PSA, PTII, VFA or MAA) for 30 min at 37 °C without agitation. The concentration of platelets was kept constant at ≈ 50 × $10^3$ platelets per µl in all samples (final concentration after the addition of the lectin solution). Following the incubation, samples were diluted in 2 ml of buffer and immediately analyzed by flow cytometry. 10,000 events per sample were counted. Lectin binding was expressed as mean fluorescence intensity (MFI) of platelets showing positive staining for a particular lectin using Flow Jo software (Tree Star Inc,

Oregon, USA). For each individual experiment, the MFI of the agonist-treated platelets was divided by that of the resting platelets, giving the fold-change of the MFI between the agonist-treated and resting platelets. Averages of these for various lectins, taken from at least four individual experiments, are plotted in Chapter 4.

The effect of lectins on CD62P expression by platelets was determined by staining the FITC-lectin treated platelets with APC-anti-CD62P antibody for 30 mins at 37 °C, followed by analysis with flow cytometry.

For certain experiments platelets were stained with both FITC-lectin and APC-CD62P but in different order of addition to determine the effect of one labeling agent on the binding of the other labeling agent to the platelets.

### 2.10.3 Lectin binding studies of platelets using lectin arrays

Juan Echevarria, who works in the Niels-Christian Reichardt Group, at the CIC biomaGUNE, prepared lectins arrays for us according to the below described protocol.[125, 126] These lectin arrays were then used by me for studying interaction of platelets with various lectins printed in these arrays.

**Lectin array preparation:** Lectin solutions in print buffer (0.4 mg/mL; PBS that contained 1 % glycerol and 0.001 % of Tween-20) were freshly prepared for every print run to avoid activity loss due to protein denaturation in a source plate (384-well plate) loaded with aliquots (20 µL) of every lectin, which could be stored overnight at 4 °C without loss of function. These dilutions were spotted onto NHS-activated hydrogel glass slides by employing a dispensing volume of 0.8 nL and at 300 µm pitch between spot centers and a robotic non-contact piezoelectric spotter.

**Lectin array design:** Lectin arrays were printed on a glass slide. Each lectin array consisted of 62 lectins spots printed in the following order-ConA (lectin 1), WGA, RCA, ECA, SNA, MAL-I, AAL, PSA, LCA, GNA, NPL, BSII, PHA, JAC, WFL, PNA, VVL, LTL, BS-I, PT-I, LEL, ACA, BS-I(EY), SJA, GML-SBA, STL, AIA (JAC), BPL, PT-II, EEA, MPA, HHL, PAL, ABL, DSL, HAL, AHP, LPL, CFL, PWA, BSI-B4, CAL, DBL, HPL, VFA, PHAL, PHAE, PMA, ASA, SSA, HMA, UEA-I (EY), LFA, Calsepa, Moa, LAL, AAA, LBA, MAL-II and AOL (lectin 62). In each array, these lectins were printed in 6 replicates as 6 × 3 matrixes in well format. Printing buffer was also spotted as a negative control. Humidity for the slide printing was maintained around 50% before and during printing. The printed slides were incubated at 75 % humidity in a custom-made humidity chamber filled with saturated NaCl solution at 18 °C over-night and stored at －20 °C without quenching unless the slides were used immediately. Before use of the lectin array slides for studying interaction with platelets, unreacted NHS-activated carboxylate groups were quenched by placing the slides in a 50 mM ethanolamine solution in 50 mM borate buffer for 1 h and washed with PBS for 5 mins. Slides were dried by centrifugation without any additional washing step and used immediately for platelet studies.

**Interaction of the platelets with the lectin arrays:** A gasket (cleaned with cobas and water) was used to separate the 14 lectin arrays on each slide and this set was inserted into a slide holder. This resulted in division of slide into 14 lectin array wells with a volume of 100 µl. 100 µl of platelet suspensions in calcium free-HEPES buffer (BAPTA-AM treated platelets, TRAP treated platelets, PMA treated platelets and calcium ionophore treated platelets) with a concentration of $5 \times 10^4$ platelets/µl were

loaded into these lectin array wells and incubated for 2 hrs at room temperature inside a sterile laminar flow hood. It was made sure that there was no leaking of solutions between the wells. This was followed by washing of slides with the same buffer (1×) and addition of fluorescent antibodies to these wells. Antibodies used for staining array bound platelets included 0.5 μg of Cy5-anti-CD41a antibody (antibody against platelet specific marker). The lectin arrays were incubated with antibodies for 1 hr at room temperature with gentle shaking. This was followed by washing of slides with PBS buffer and 1x with nano-pure water. The slides were then dried with a slide spinner and immediately analyzed with a microarray scanner for measuring fluorescence emission from the antibody-stained array bound platelets. All the samples of a particular experiment were done on the same slide and each sample (platelet condition) was done in duplicates on the same slide for that experiment. The concentration of the platelets and antibodies used in this experiment were optimized to get a good saturating signal from platelets bound to lectin spots. Negative controls included-unstained platelets for checking autofluorescence and antibody added to wells without platelets to check for non-specific binding of antibodies to the lectin spots.

**Image analysis:** Fluorescence measurements were performed on an Agilent G265BA microarray scanner, in green (for PE-anti-CD62P and PE-anti-CD63) and red channels (for Cy5-anti-CD41a) with a laser intensity of 10%, 20%, 50% and 100%. Scanning provides us with fluorescent images of the spots (bound to platelets stained with antibody) on the lectin arrays. Image quantification was performed with ProScanArray Express software, and interaction profiles of platelets with the lectin array were displayed as fluorescence histograms using Microsoft Excel. The adaptive circle method with a diameter range of 65−70 μm was employed for spot quantification on the arrays. The median value (after background subtraction) was used for the

fluorescence of each spot, and for every lectin the average of 6 replicate spots was used to construct histograms showing the binding profile of lectins to the platelets for every experiment. Error bars are included showing the standard deviation for each lectin/platelet interaction for every individual experiment.

# *Chapter 3*
# *Development of a model system for the in vitro modulation of platelet activity: Selective activation of platelets by surfaces.*

## 3.1 Introduction

Contact with biomaterial surfaces activates platelets, leading to thrombosis.[13, 14] This is a well-known problem for the development of blood-compatible biomaterials and considerable research efforts are focused on the discovery and development of materials with minimal procoagulatory properties.[13, 14] On the other hand, blood coagulation at surfaces of osseoimplants is beneficial for implant integration. Because of their importance, interactions between blood, its components, and various biomaterials, have been extensively studied.[13, 14, 108, 127-130] There is the overall understanding of the sequence of events, viz., protein adsorption – platelet activation – thrombosis, and it is established that surface protein adsorption and the dynamics of the adsorbed protein films play a major role in the process of platelet activation by foreign materials,[13, 59, 131] although direct surface effects have also been described.[132, 133] Adverse effects of biomaterials can be successfully managed with anticoagulants, but complications continue to arise despite the anticoagulant therapy.[13] The nuances of processes operating at the surface of biomaterials remain poorly understood; in particular detailed, multifactorial studies of platelet activation by surfaces are hard to come by. There is a distinct lack of suitable biophysical models that would allow the principles underlying these interactions to be established. For this reason we chose to investigate direct interaction between purified platelets and a widely used biomaterial, $TiO_2$.[134-137] We specifically focused on the role played by surface ion equilibrium in influencing interaction between platelets and $TiO_2$ surface.

The interplay between surface physico-chemical properties of materials and the biological response they elicit is an active area of research.[138] The role of interfacial ion equilibrium in this process is an aspect that has not received sufficient attention despite the recognition of its importance.[138] In this context, titania-$Ca^{2+}$ interactions

represent an interesting example. Titania ($TiO_2$) is a relatively inert and stable material responsible for the favorable, if poorly understood, biocompatibility properties of titanium (Ti), which is used in the production of various implants—stents, heart valve housings, dental prostheses and other osseoimplants.[135-137] Titania interactions with $Ca^{2+}$ are manifested in terms of changes of several surface properties, such as the isoelectric point (IEP, the pH at which proton dissociation from the surface is balanced by adsorption and the surface charge is neutralized) and ζ-potential: the IEP of $TiO_2$ is shifted in the presence of calcium to higher pH,[118, 139-141] and its ζ-potential is inverted at a near-physiological $Ca^{2+}$ concentration of ~ 3 mM at physiological pH.[118] These changes can be interpreted in terms of adsorption of $Ca^{2+}$ ions at the oxide surface; this is the so-called "chemical" interpretation.[142, 143]

In simplest terms, $Ca^{2+}$ adsorption to the surface changes the balance of interactions between the surface and the material (proteins, lipids, cells) adsorbing or adhering to it. Indeed, there is some evidence suggesting that $Ca^{2+}$ adsorption to titania affects $TiO_2$-protein interactions.[144] Furthermore, on $TiO_2$, $Ca^{2+}$ elicits a clear response in terms of lipid behavior in phosphatidyl serine (PS)-containing liposomes and supported lipid bilayers—response that is absent on substrates such as silica or glass.[119, 120, 145, 146] Interactions of $Ca^{2+}$ with silica are not manifested by the ζ-potential reversal,[147] and at neutral pH the fraction of adsorbed $Ca^{2+}$ ions is quite small.[148] For more details, see Figure 3.1.

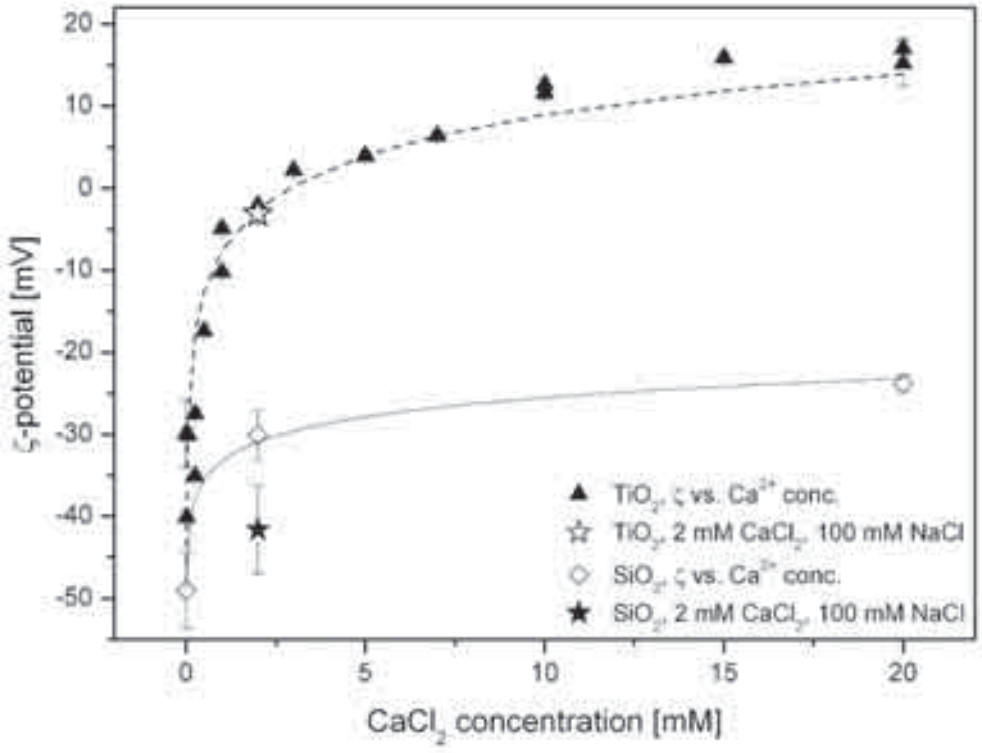



**Figure 3.1. ζ-potentials of various silica-based materials and $TiO_2$. The data for Pyrex, Vycor, Quartz and vitreous silica are taken from table III in Hednačak and Pravdić [149]. $TiO_2$ data were measured by Rossetti et al. using washed 270 nm $TiO_2$ particles from Kronos International, Inc., (Leverkusen, Germany) and are presented in Ref [118]. In the same study, amorphous silica colloids with a mean diameter of ~ 300 nm from Kisker GbR were used to measure the ζ-potential of silica. This figure is taken from Rossetti et al.[118] Note the inversion of the ζ-potential in the case of titania colloid and its saturation in the case of silica colloid. Saturation of the electrophotetic mobility of silica colloids at high calcium concentrations has been noted before.[147]**

In view of these observations, we hypothesized that the behavior of more complex biological systems (such as platelets) on $TiO_2$ would also depend on the presence or absence of surface bound calcium. Therefore, we compared $TiO_2$ and glass with respect to their ability to activate adhering platelets. Because we were looking for clear cause-and-effect relationships between surface properties and platelet activation, we focused on purified platelets. We studied the expression of a set of common platelet activation markers (alpha granule marker CD62P, dense granule marker CD63, activated GPIIb/IIIa and the expression of phosphatidylserine) to elucidate the effect of surfaces such as $TiO_2$ and glass on the platelets under different ionic conditions. We show that the pattern of activation marker expression on platelets interacting with $TiO_2$ differs depends on whether the calcium is present or not. In the absence of $Ca^{2+}$, platelets adhering on $TiO_2$ selectively express dense granule maker CD63 and not the other tested markers, however they express all the markers in the presence of extracellular calcium. Similarly, the express all four markers on glass surface irrespective of presence or absence of extracellular calcium. Differences in the expression profiles of these four markers depending on the surface and the ionic conditions prevalent at the surface, point towards the selective regulation of platelet activation responses upon different kinds of stimulation and support the recent notion that platelets can tune their microenvironment in a trigger-specific fashion.

We have furthermore taken steps towards elucidating the molecular mechanisms underlying this selectivity. To this end, we looked at the intracellular calcium dynamics in the platelets adhering on different surfaces. Calcium is a well-known second messenger that mediates inside-out signaling in platelets and many other excitable cells.[28, 30, 31, 41, 52, 150, 151] Intracellular calcium is maintained at ~ nM level in the resting platelets through the action of ATP-requiring pumps that extrude it into the

extracellular space and into the calcium storage organelles inside the platelets via plasma exchange $Ca^{2+}$-ATPase (PMCA) and sarco/endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA).[28, 30, 151, 152] Binding of agonists to the receptors on the platelet surface leads to a rise in the intracellular calcium level to μM range, which in turn activates signaling cascades that lead to various platelet responses.[28, 30, 152-155] How the intracellular calcium dynamics is manifested in the context of the selectivity of platelet activation is unclear. It is for this reason our model system exhibiting selective activation is so interesting for studying intra-platelet signaling processes.

Their understanding is essential for the development of new, targeted and personalized treatment strategies for a variety of platelet-mediated pathophysiological processes ranging from thrombosis on implants to cancer metastasis.

## 3.2 Results

### 3.2.1 Platelet isolation and purification

Platelets were isolated from human whole blood collected by venipuncture into sodium citrate anticoagulant. In order to establish a clear cause-and-effect relationship between $Ca^{2+}$-$TiO_2$ interactions and platelet activation at the $TiO_2$ surface, we purified platelets from the plasma proteins. Purification was done by a two-step centrifugation procedure as described in Materials and Methods, section 2.7. Protein concentration in our samples was ~ 0.05 ± 0.01 mg/ml, compared to ~ 130 mg/ml in the blood plasma.

Prior to the surface experiments, platelet behavior in solution was analyzed by flow cytometry to ensure their purity, minimal activation, and appropriate response to agonists.[156-160] The results of this analysis are shown in Figure 3.2. Freshly isolated platelets in the calcium free-HEPES buffer were analysed by flow cytometry, untreated and treated with agonists such as TRAP and PMA. Isolated platelet suspension consisted of only one population of cells, and 95% of it stained positive for a platelet marker, CD41a, (Figure 3.2A) which confirmed that the suspension was pure and not contaminated by other cells.

Isolated platelets do not express activation markers (Figure 3.2B-3.2E) such as CD62P, CD63, active form of GPIIb/IIIa, or phosphatidyl serine (PS). CD62P and CD63 are indicative of the exocytosis of α- and dense granules, respectively, that occurs in response to activation. GPIIb/IIIa is a constitutively expressed platelet surface integrin that changes conformation upon activation, and the procoagulant phospholipid PS is usually present in the inner leaflet of plasma membrane and gets exposed in the outer leaflet upon platelet activation.[3, 8, 156, 157, 160] Stimulation with soluble agonists (PMA or TRAP) leads to the expression of these markers (Figure 3.2).

Consistent with previous findings, CD62P and CD63 expression did not depend on the presence of the extracellular $Ca^{2+}$. GPIIb/IIIa activation and the detection of exposed PS with annexin A5 require extracellular $Ca^{2+}$ (Figure 3.2D and 3.2E).[157, 161, 162] Only platelets that were not activated upon isolation but responded to the agonists as shown in Figure 3.2 were used in subsequent experiments. The average of results from three different experiments (3 donors) is shown in Figure 3.3A.

Isolation of platelets was performed within ~ 12 hrs of blood collection because, due to the stringent safety regulations, testing for infectious agents had to be performed off-cite before the blood could be used for experiments. We have checked that platelets isolated from fresh blood behaved in the same way as platelets isolated after 12 hrs of blood extraction, in regards to activation with soluble agonists (Figure 3.3B). In both cases, the % of isolated platelets expressing CD62P was in the range of ~ 3-8% and CD63 – 2-6 %. For PS it was 0-5% and active form of GPIIb/IIIa, 0-8% (Figure 3.3B). Upon stimulation with TRAP or PMA (activated platelets), the % of platelets expressing these markers increased to 80 – 90% for CD62P, CD63 and GPIIb/IIIa. PS expression in response to TRAP fluctuated between 5-12% (Figure 3.3B). Each experiment was carried out at least three times with blood from three different donors. All experiments were completed within 24 hours of blood collection.

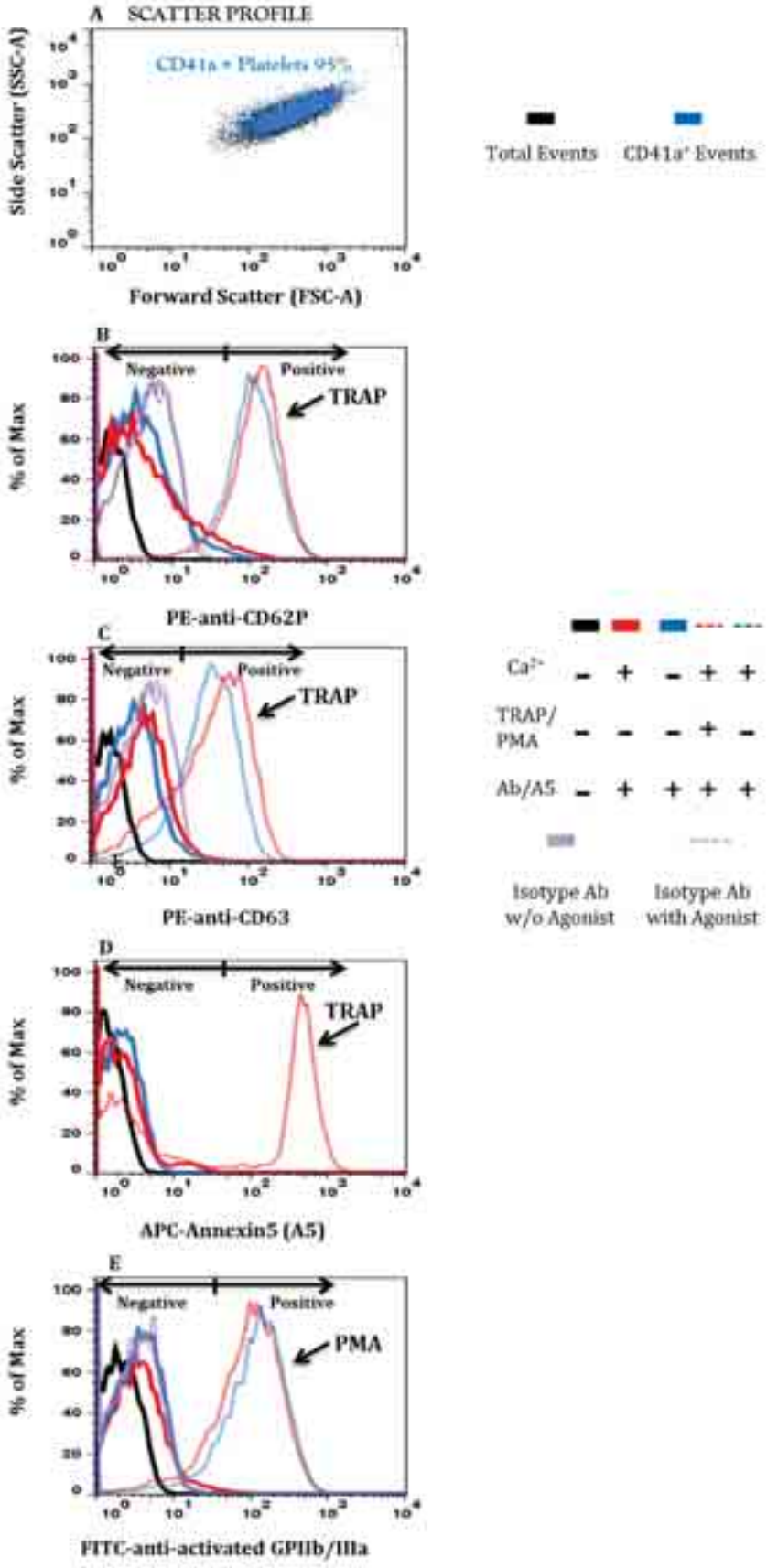

A SCATTER PROFILE
CD41a+ Platelets 95%
Side Scatter (SSC-A)
Forward Scatter (FSC-A)
Total Events
CD41a+ Events
B
Negative
Positive
TRAP
% of Max
PE-anti-CD62P
C
Negative
Positive
TRAP
% of Max
PE-anti-CD63
Ca2+
TRAP/ PMA
Ab/A5
Isotype Ab w/o Agonist
Isotype Ab with Agonist
D
Negative
Positive
TRAP
% of Max
APC-Annexin5 (A5)
E
Negative
Positive
PMA
% of Max
FITC-anti-activated GPIIb/IIIa

**Figure 3.2. Flow cytometry analysis of purified platelets.**
**Platelets isolated from sodium citrate-anticoagulated blood were re-suspended in a nominally $Ca^{2+}$-free HEPES buffer and analyzed by flow cytometry. Results of one representative experiment are shown as percentages of positive events out of a total of 10000 events are shown on the plots. Average values from several experiments are shown in Figure 3.3. (A) The scatter profile shows one population of cells, 95% of which stained positive for the platelet-specific marker CD41a (platelet transmembrane glycoprotein GPIIb). This is consistent with what is expected of isolated quiescent platelets that are free of contaminants such as erythrocytes and white blood cells. (B and C) Platelets stain positive for CD62P (B) and CD63 (C) upon addition of 70 μM TRAP (dotted blue and dotted red histograms) but negative in its absence (solid blue and solid red histograms). Expression of CD62P and CD63 in TRAP-activated platelets is independent of the extracellular $Ca^{2+}$: the dotted blue (absence of $Ca^{2+}$) and the dotted red (presence of $Ca^{2+}$) histograms nearly overlap; this is consistent with previous findings. Isotype controls are negative in the presence and in the absence of TRAP (dotted and solid light purple histograms). Sample auto-fluorescence recorded in the absence of antibodies is shown in black. (D) Platelets expose PS upon activation with 70 μM TRAP as judged by the binding of APC-labeled annexin A5 in the presence of extracellular calcium (dotted red histogram). Platelets that were not activated with TRAP do not expose PS (solid blue histograms) even when $Ca^{2+}$ is present (solid red histograms). Annexin A5 binding is $Ca^{2+}$-dependent; therefore, it is not possible to check whether PS is expressed on the surface of TRAP-activated platelets in the absence of $Ca^{2+}$ with this reagent. Black: sample auto-fluorescence in the absence of APC-annexin A5. (E) Platelets activated with 10 μM PMA express the active form of GPIIb/IIIa, as judged by the binding of PAC1 antibody in the presence of $Ca^{2+}$ (dotted red histogram). No binding is detected in platelets that were not activated by PMA (solid blue and solid red histograms). The inactive-to-active conformation change in GPIIb/IIIa is $Ca^{2+}$-dependent. The $Ca^{2+}$ requirement for PAC1 antibody binding is well-established in the literature and was not tested in our experiments. Isotype controls are negative in the presence and in the absence of PMA (dotted and solid light purple histograms).**

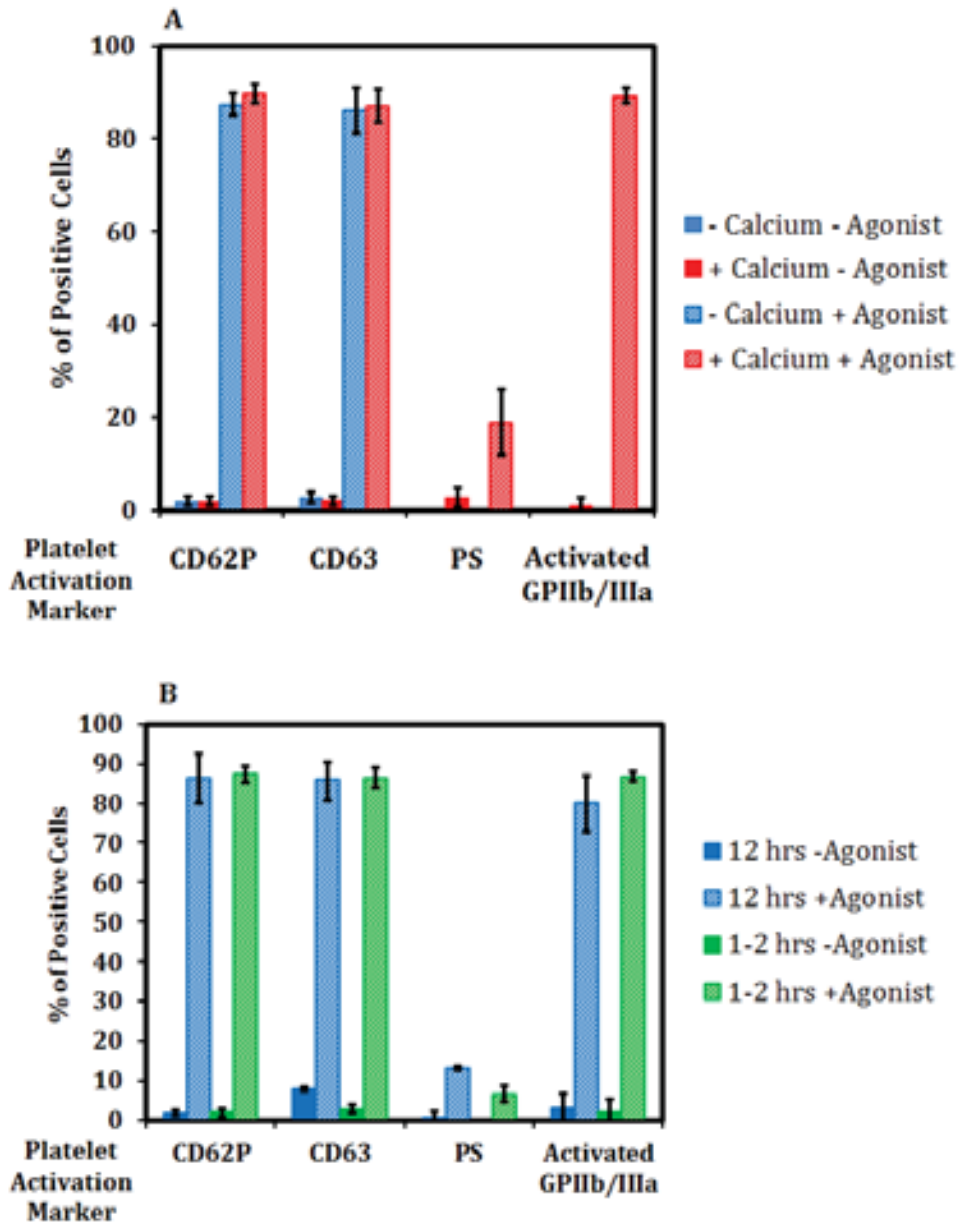



**Figure 3.3. Flow Cytometry characterization of platelets for different experiments. (A) Assaying for activation marker expression on purified platelets in the absence and in the presence of agonists was used to ascertain their state of activation and viability before using them in surface experiments. Results of one such experiment are shown in Figure 3.2 in the main text. Average results from three different experiments (different donors) are shown here. The markers used are the same as those discussed in the legend of Figure 3.2 and in the main text. (B) Effect of Blood storage period on state of isolated platelets. Platelets isolated from blood stored for 1-2 hrs or 12 hrs after extraction, were characterized by flow cytometry for their state of activation and response to agonists by assaying for same set of markers discussed in legend of Figure 3.2. Platelets isolated from fresh blood behaved in the same way as platelets isolated after 12 hrs of blood extraction, in terms the state of activation and response to agonists as analyzed by flow cytometry.**

### 3.2.2 Platelet adhesion and activation studies on glass and $TiO_2$ surfaces

Previous studies in our lab showed that $Ca^{2+}$-titania interactions affect the distribution of phosphatidyl serine (PS) in model lipid bilayer systems, such as surface adsorbed liposomes and planar lipid bilayers- a response that that is absent on silica or glass.[119, 120, 145, 146] Following these findings, we propose to investigate the role played by $Ca^{2+}$-titania interactions on affecting platelet activation responses. We studied the interaction of purified platelets with $TiO_2$ and glass surfaces for 10 mins and 3 hrs, in the presence and absence of extracellular calcium and analyzed their adhesion, spreading and expression of four activation markers-CD62P, CD63, activated GPIIb/IIIa and PS.

### 3.2.3 Platelet adhesion and spreading on glass and $TiO_2$ surfaces

Freshly isolated, purified platelets were allowed to interact with freshly cleaned, bare glass surfaces or with $TiO_2$-coated glass surfaces in a $Ca^{2+}$-free or 2 mM calcium buffer. Platelets that remained on the surface were stained with anti-CD41a antibody and observed in the confocal fluorescence microscope. The number of adhered platelets and their spreading area were determined from the confocal images using Image J software as described in the materials and methods section.

Platelets adhered and spread on both surfaces (Figure 3.4) both in the presence and absence of extracellular calcium. In the absence of extracellular calcium, the numbers of adhering platelets (Figure 3.4A) were similar on glass and on $TiO_2$ surfaces. In both

cases, more platelets adhered after three hours than after 10 minutes (Figure 3.4A). The number of platelets adhering on glass and TiO2 are similar both in the presence and absence of extracellular calcium.

In the absence of extracellular calcium, the degree of platelet spreading (Figure 3.4B-3.4E) is similar on glass and on $TiO_2$ surfaces. Some of the platelets adhering on $TiO_2$, after three hours in the absence of extracellular calcium were not spread (Figure 3.4E, indicated with arrows).

After 10 minutes (Figure 3.4B and 3.4C) for both glass and $TiO_2$ surfaces, platelets are significantly more spread in the presence of calcium (red) than in the absence (blue), but after three hours, the degree of spreading is similar in both cases, probably because at this point it is controlled by the amount of space available on the surface (Figure 3.4D and 3.4E).

### 3.2.4 Viability of platelet adhered on glass and $TiO_2$ surfaces

Platelets adhered to glass and $TiO_2$ surfaces in the absence of extracellular calcium were analyzed for their viability and membrane damage by staining them with intracellular cytoplasm staining dye (calcein-AM).[163] The results for these experiments are shown in Figure 3.5. Platelets adhered to glass and $TiO_2$ were viable and had an intact membrane as they show staining with calcein-AM dye. If the membranes were damaged then this dye would have leaked out of platelets and they won't show staining with calcein AM.

A

| Platelets in Buffer | $Tio_2$, 10 mins | Glass, 10 mins | $Tio_2$, 3 hrs | Glass, 3 hrs |
|---|---|---|---|---|
| Platelets in $Ca^{2+}$ free HEPES | 8000 ± 1200 | 7000 ± 3000 | 18000 ± 2500 | 18000 ± 2500 |
| Platelets in 2 mM $Ca^{2+}$ HEPES | 8000 ± 1176 | 9000 ± 4910 | 16000 ± 450 | 17000 ± 2248 |
| BAPTA-AM Platelets in $Ca^{2+}$ free HEPES | 6000 ± 800 | 7000 ± 3000 | 27000 ± 6000 | 17000 ± 1500 |

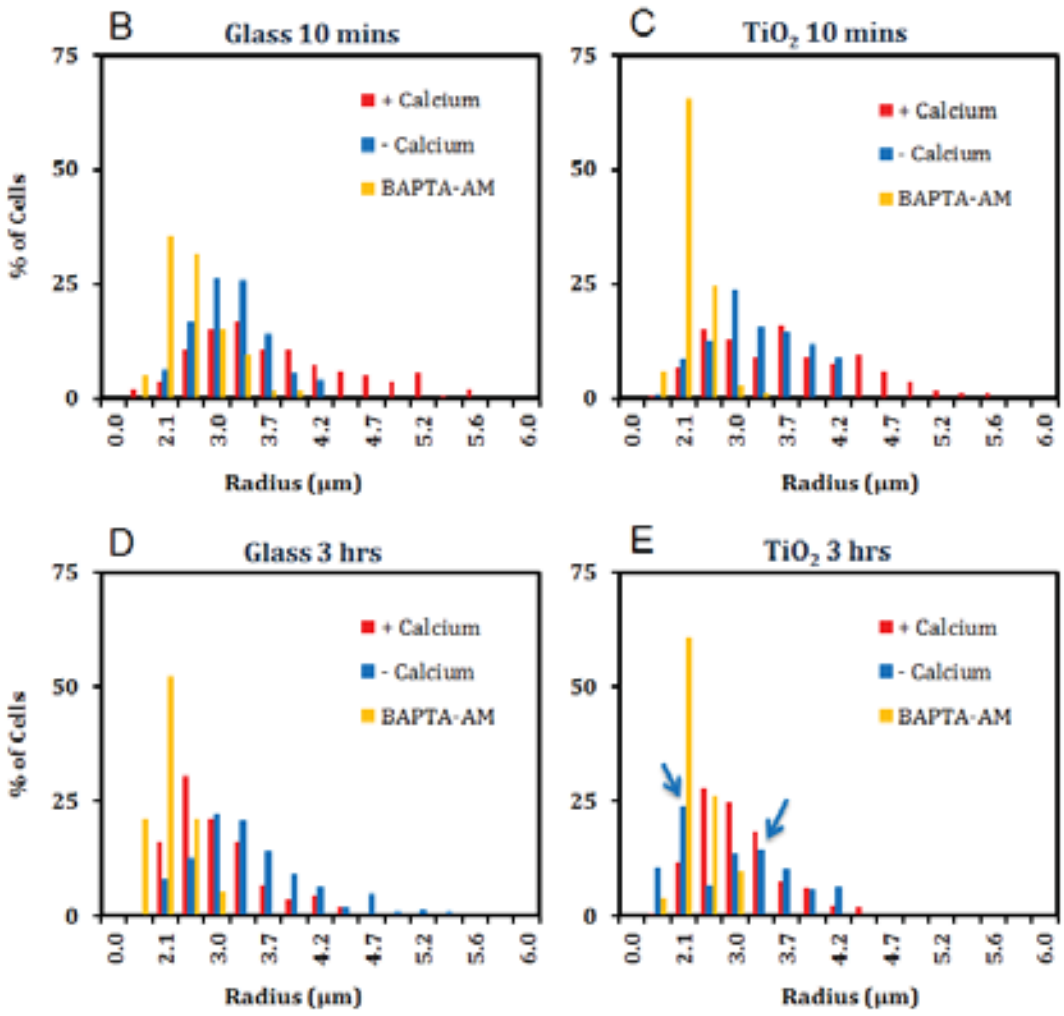



**Figure 3.4. Platelet adhesion and spreading on surfaces. (A) The numbers of adhering platelets on glass and $TiO_2$ in the absence and presence of calcium, per $mm^{-2}$. (B-E) Platelet spreading in the presence of extracellular calcium (red), in the absence of extracellular calcium (blue), and in the presence of intracellular calcium chelator BAPTA-AM (yellow).**

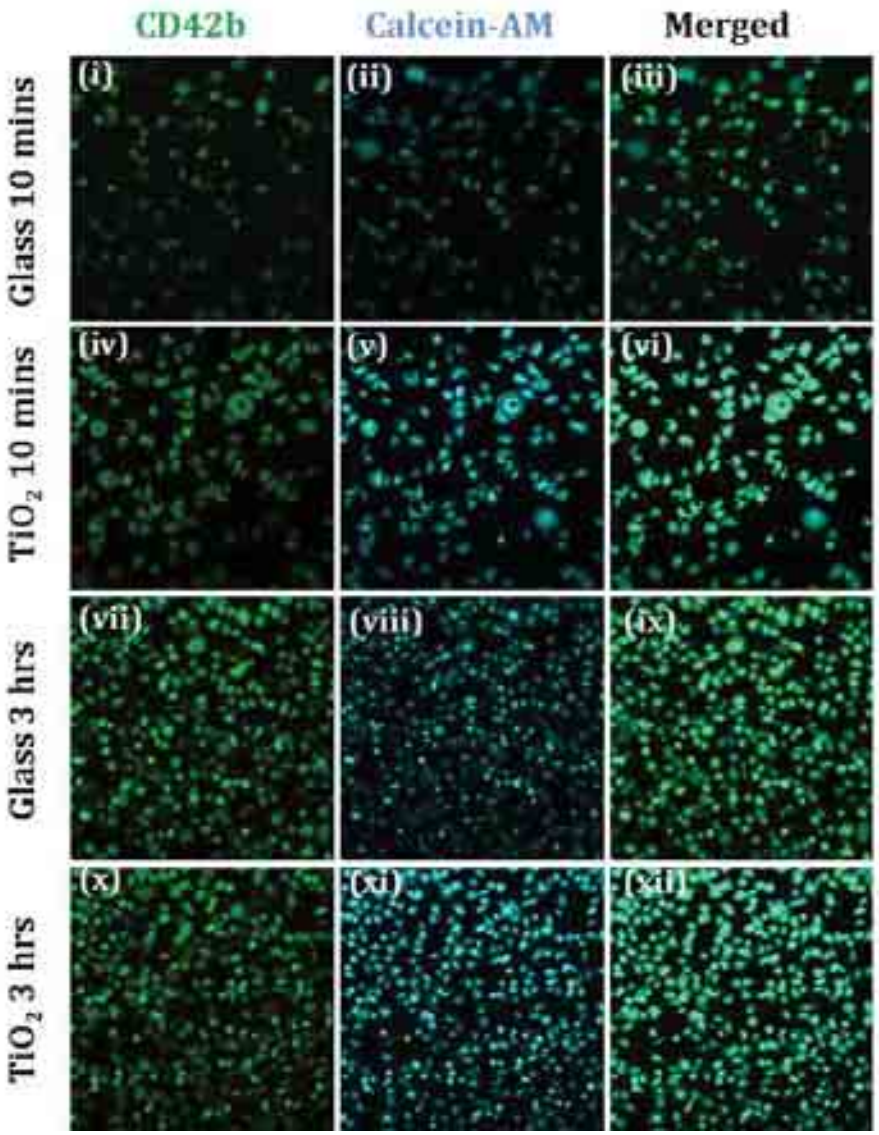



**Figure 3.5. Cell viability assay of platelets adhering on glass and $TiO_2$ surfaces.**
**Platelets were incubated with $TiO_2$-coated (iv-vi and x-xii) or bare glass cover slips (i-iii and vii-ix) for 10 minutes or for 3 hours in a nominally $Ca^{2+}$-free buffer and extensively washed with the same buffer. Platelets that remained on the surface were incubated with the following anti-CD42b antibody (green) and an intracellular cytoplasmic dye calcein AM (blue) and observed in the confocal microscope. Cells adhered on both glass and $TiO_2$ surfaces were viable as they stained with calcein AM dye (blue and merged images), which would have leaked out if platelets were not viable or if membrane was damaged.**

### 3.2.5 Platelets are selectively activated by $TiO_2$ depending on the presence or absence of extracellular calcium

Platelets adhered on the surfaces (glass and $TiO_2$) in the presence or absence of extracellular calcium, were stained with anti-CD41a antibody along with either of these reagents specific for different platelet activation markers- anti-CD62P antibody, anti-CD63 antibody, A5 (detects PS) or anti-PAC1 antibody (detects activated GPIIb/IIIa) and observed in the confocal fluorescence microscope for the expression of different activation markers upon interaction with these surfaces.

In the absence of extracellular calcium, on glass, adhering platelets expressed CD62P (Figure 3.6-iii and x), CD63 (Figure 3.7-iii and ix), PS (Figure 3.8-iii and x) and activated GPIIb/IIIa (Figure 3.9-iii and x) markers, signaling their activation. Activation was already noted in many of the platelets incubated with glass for 10 minutes (Figure 3.6-iii, 3.7-iii, 3.8-iii and 3.9-iii), when platelets were well-separated. Incubation for 3 hr led to a near-confluent layer of activated platelets on the surface (Figure 3.6-x, 3.7-ix, 3.8-x and 3.9-x). These observations are consistent with previous reports of platelet activation on glass in the absence of anticoagulants, both in the presence and in the absence of plasma proteins.[164-167]

Crucially, platelets incubated for 10 minutes on $TiO_2$ in a $Ca^{2+}$-free buffer did not express the alpha granule markers CD62P (Figure 3.6- vi) or respond to TRAP (Figure 3.6-vi). In $Ca^{2+}$-free buffer after 10 mins of incubation on $TiO_2$, adhered platelets also did not express activated GPIIb/IIIa (Figure 3.9-vi) or PS (Figure 3.8-vi). The expression of these markers could not be elicited in these platelets by TRAP (Figure 3.9 and 3.8-vii). After 3 hr of incubation in a $Ca^{2+}$-free buffer, there were platelets on $TiO_2$ that did express CD62P (Figure 3.6-xiii) and PS (Figure 3.8-xiii) and responded

to TRAP, but these were the non-spread platelets (Figure 3.6 and 3.8-xiv). Non-spread, activated platelets have been observed before. On collagen, they appeared in the very early stages of the adhesion process and contribute to thrombus formation through platelet-platelet interactions.[168] While platelet spreading and activation are usually coupled,[3] this is not always the case. For example, Broberg et al. shows several examples where platelet spreading and activation (as judged by the expression of CD62P) are not correlated.[169] Similarly Pandey et al. show that low concentrations of lysophosphatidic acid induce platelet shape changes but not secretion.[170] After 3 hrs, there was no expression of GPIIb/IIIa on both spread and non-spread platelets (Figure 3.9-vi, vii, xiii, xiv).

Platelets adhering on $TiO_2$ in the absence of $Ca^{2+}$ did express the dense granule marker CD63 (Figure 3.7-vi and xii), suggesting that the exocytosis of the α-granules and dense granules is regulated selectively.

In the presence of 2 mM $Ca^{2+}$, platelets adhered, spread, and expressed all fours activation markers-CD62P (Figure 3.10A), CD63 (Figure 3.10B), PS (Figure 3.11A) and activated GPIIb/IIIa (Figure 3.11B), both on $TiO_2$ (Figure 3.10A,B- vi and xii and 3.11A,B- vi and xii) and on glass (Figure 3.10A,B-iii and ix and 3.11A,B-iii and ix), after 10 minutes and after three hours. However, expression of activated GPIIb/IIIa on platelet adhered to TiO2 after 3 hrs was less as compared to 10 mins (Figure 3.11B-vi and xii).

Furthermore, there was a difference in the distribution of the integrin GPIIb as observed by CD41a marker staining. It appeared to be concentrated at the periphery of the spread platelets in presence of $Ca^{2+}$ (Figure 3.10-i, iv, vii and x), while it was uniformly

distributed in its absence (Figure 3.6-i, iv, viii and xi). The significance of this difference is not clear. Such non-uniform staining has been previously observed with this protein in activated platelets,[171] though platelets adhering on glass are reported to have a centrally located pool of GPIIb/IIIa surrounded by concentric rings.[3]

The results suggest that in the presence of the extracellular calcium, $TiO_2$ acts as any physiological agonist, activating adhering platelets, as judged by the expression of the alpha- and dense granule markers CD62P and CD63, activated GPIIb/IIIa and PS expression. Its effect is similar to that of glass or of soluble agonists such as TRAP. In the absence of the extracellular calcium, the effect of TiO2 on the adhering platelets is markedly different from that of the other agonists: platelets adhering on $TiO_2$ selectively express CD63 but not CD62P, activated GPIIb/IIIa or PS, while platelets adhering on glass, or challenged with agonists in solution, express both markers. These trends are visible in the images of individual experiments shown in Figure 3.6-3.11, and are also apparent in the average data from different experiments shown in Figure 3.12. These results suggest that platelets can undergo selective activation responses upon stimulation with different stimuli. They can undergo selective exocytosis and degranulation of alpha or dense granules. Moreover they can undergo selective degranulation without causing PS exposure and GPIIb/IIIa activation. This selectivity in platelet activation responses has not been achieved before using soluble physiological platelet agonists or surface agonists. These results have provided us with a $TiO_2$ based model system that results in selective platelet activation. We have further used this system to understand the mechanism underlying selectivity in platelet activation responses.

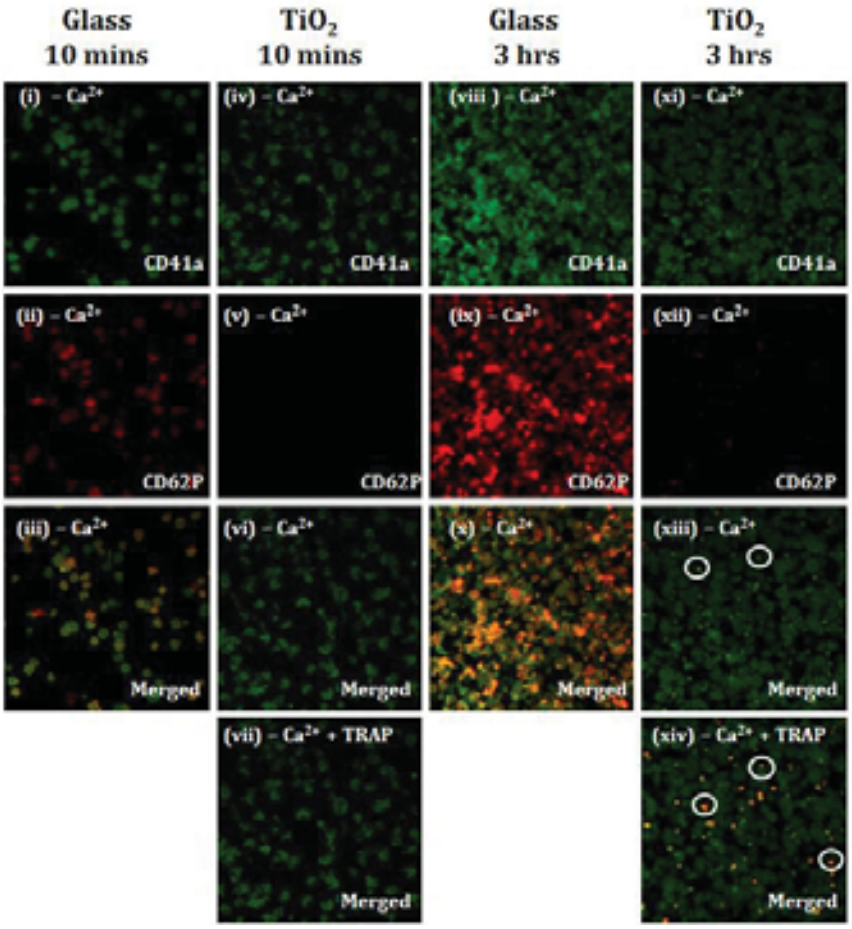



**Figure 3.6. CD62P expression profiles of platelets adhered on titania and glass in the calcium-free buffer.**

**Platelets were incubated with $TiO_2$-coated or bare glass cover slips for 10 minutes or for three hours in a nominally $Ca^{2+}$-free buffer and extensively washed with the same buffer. Platelets that remained on the surface were incubated with the following antibodies and observed in the confocal microscope: anti-CD41a (green) and anti-CD62P (α-granule marker; red); Platelets incubated on glass stain positive with both CD41a and CD62P, showing clear evidence of activation after 10 minutes (i-iii) and after three hours (viii-x). Platelets incubated on $TiO_2$ show no evidence of activation (no staining with CD62P) after 10 minutes even in the presence of TRAP (iv-vii). Platelets incubated on $TiO_2$ for three hours do show evidence of activation (xi-xiv), however, the co-localization analysis (xii, white circles) shows that it is the second (non-spread) population of platelets that expresses CD62P. The spread platelets from the first layer do not express CD62P even after 3 hr of incubation on $TiO_2$ and in the presence of TRAP (xiv). Only non-spread platelets expressed CD62P after addition of TRAP (xiv, white circles).**

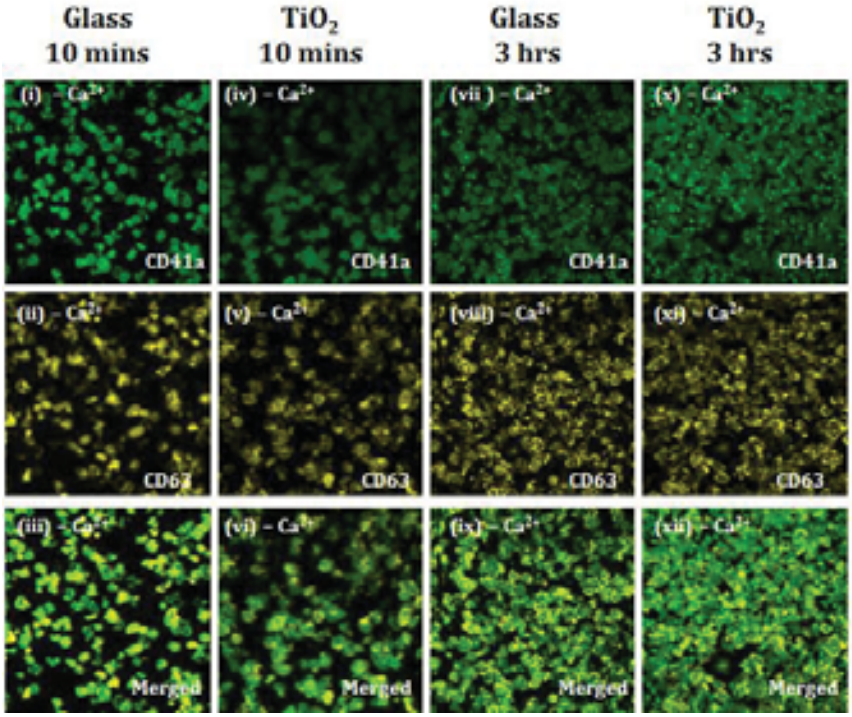



**Figure 3.7. CD63 expression profiles of platelets adhered on titania and glass in the calcium-free buffer.**

**Platelets were incubated with $TiO_2$-coated or bare glass cover slips for 10 minutes or for three hours in a nominally $Ca^{2+}$-free buffer and extensively washed with the same buffer. Platelets that remained on the surface were incubated with the following antibodies and observed in the confocal microscope: anti-CD41a (green) and anti-CD63 (dense-granule marker; yellow); Platelets incubated on glass (i-iii and vii-ix) and $TiO_2$ (ii-iv and x-xii) stain positive with both CD41a and CD63 (merged images), after 10 mins and 3 hrs.**

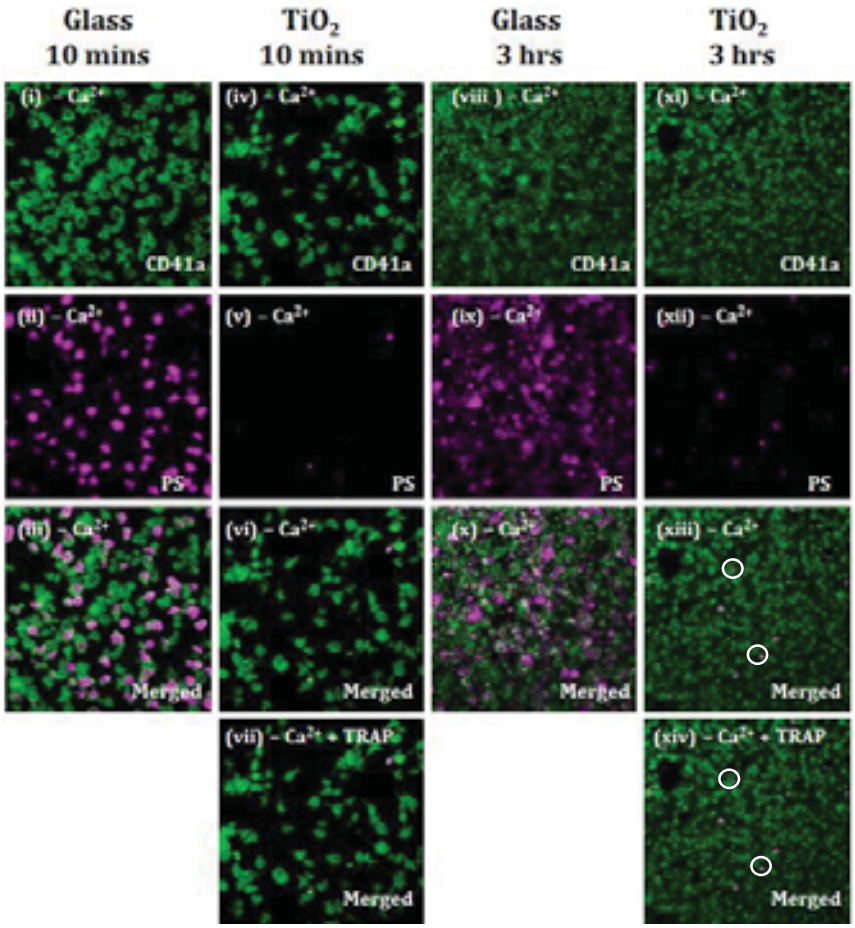



**Figure 3.8. Phosphatidylserine (PS) expression profiles of platelets adhered on titania and glass in the calcium-free buffer.**
**Platelets were incubated with $TiO_2$-coated or bare glass cover slips for 10 minutes or for three hours in a nominally $Ca^{2+}$-free buffer and extensively washed with the same buffer. Platelets that remained on the surface were incubated with the following reagents and observed in the confocal microscope: anti-CD41a antibody (green) and A5 (pink); Platelets incubated on glass stain positive with both CD41a and A5, showing clear evidence of activation after 10 minutes (i-iii) and after three hours (viii-x). Platelets incubated on $TiO_2$ show no evidence of activation (no staining with A5) after 10 minutes even in the presence of TRAP (i-vii). Platelets incubated on $TiO_2$ for three hours do show evidence of activation (xi-xiv), however, the co-localization analysis (xiii) shows that it is the second (non-spread) population of platelets that expresses PS. The spread platelets from the first layer do not express PS even after 3 hr of incubation on $TiO_2$ and in the presence of TRAP (xiv). Only non-spread platelets expressed PS after addition of TRAP (xiv).**

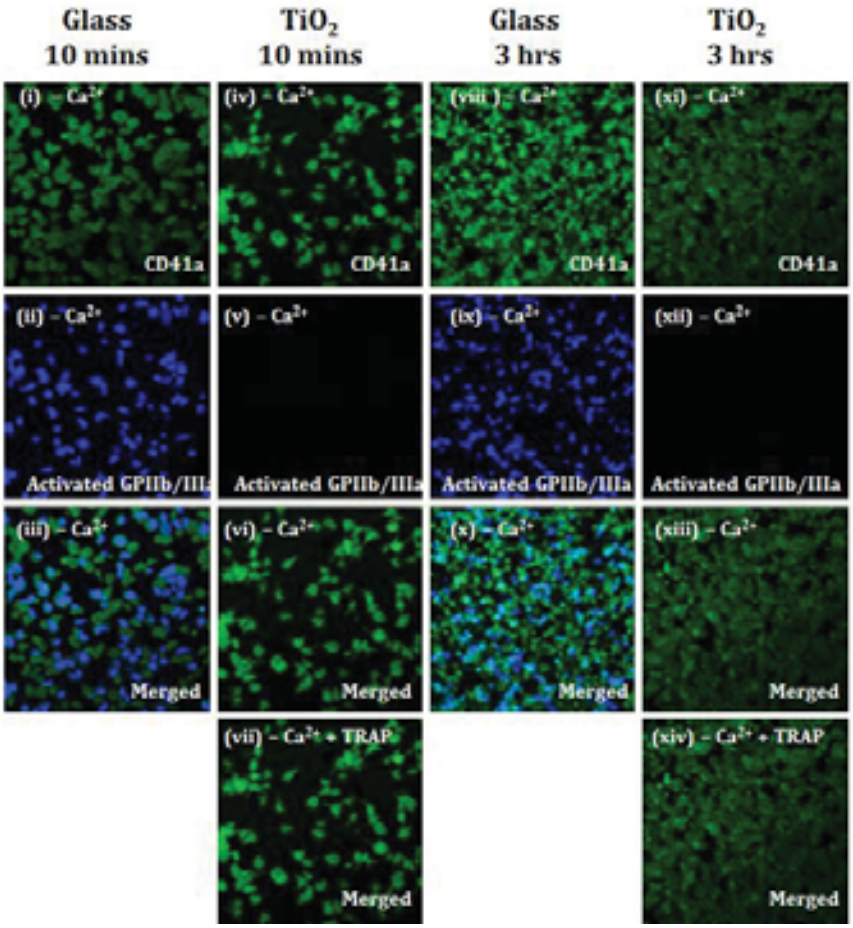



**Figure 3.9. Activated GPIIb/IIIa expression profiles of platelets adhered on titania and glass in the calcium-free buffer.**
**Platelets were incubated with $TiO_2$-coated or bare glass cover slips for 10 minutes or for three hours in a nominally $Ca^{2+}$-free buffer and extensively washed with the same buffer. Platelets that remained on the surface were incubated with the following antibodies and observed in the confocal microscope: anti-CD41a (green) and anti-PAC1 (activated GPIIb/IIIa binding antibody; blue); Platelets incubated on glass stain positive with both CD41a and PAC1, showing clear evidence of activation after 10 minutes (i-iii) and after three hours (viii-x). Platelets incubated on $TiO_2$ show no evidence of activation (no staining with PAC1) after 10 minutes (iv-vii) and 3 hrs (xi-xiv) even in the presence of TRAP (vii and xiv).**

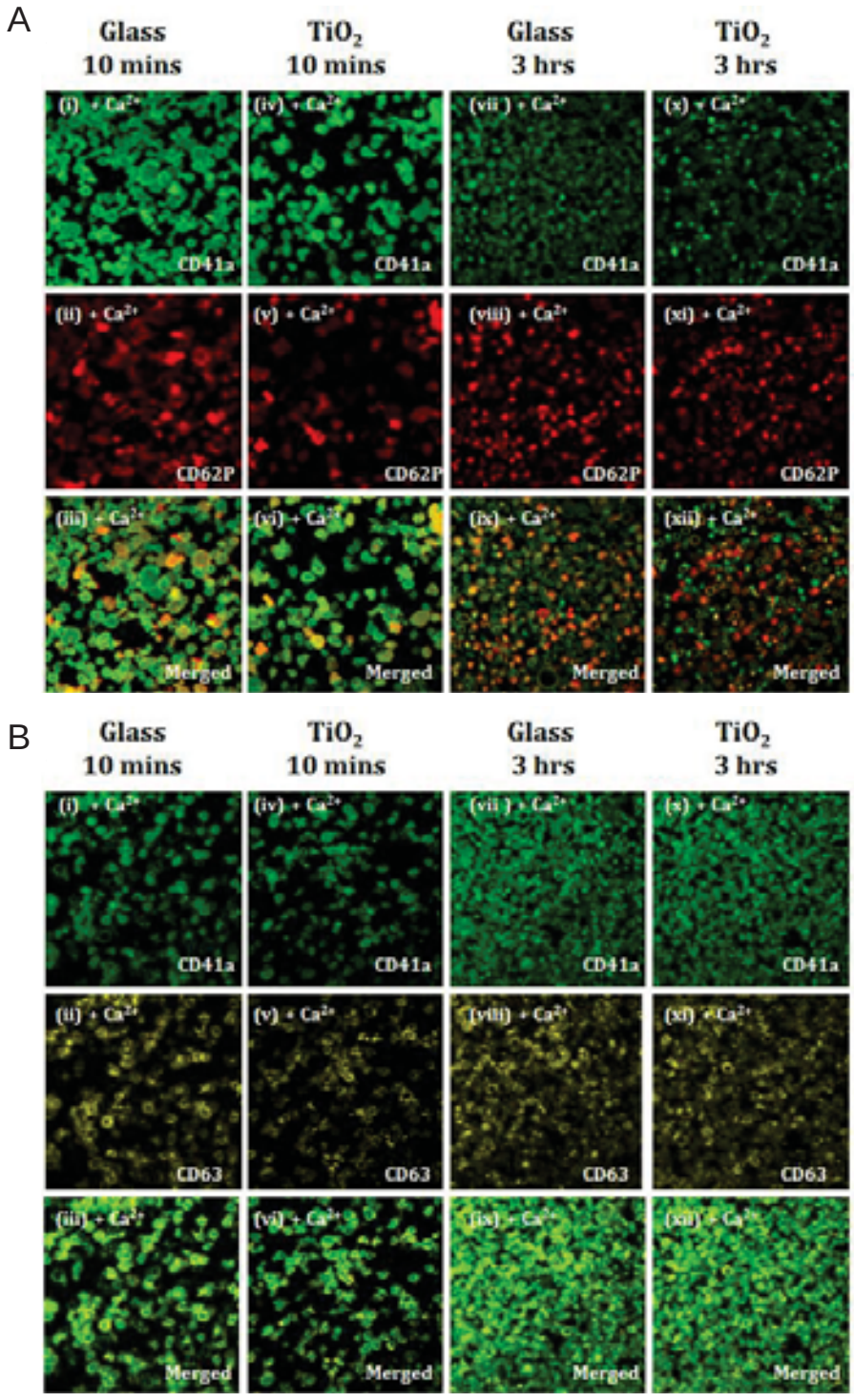



**Figure 3.10. CD62P (A) and CD63 (B) expression profiles of platelets adhered on titania and glass in the 2 mM calcium buffer.**

**Platelets were incubated with $TiO_2$-coated or bare glass cover slips for 10 minutes or for three hours in a 2 mM Calcium buffer and extensively washed with the same buffer. Platelets that remained on the surface were incubated with the following antibodies and observed in the confocal microscope: anti-CD41a (green) and anti-CD62P (A, red) or anti-CD63 (B, yellow); the expression of CD62P (A) and CD63 (B) was observed on the majority of platelets both after 10 mins and 3 hr of contact with either surface.**

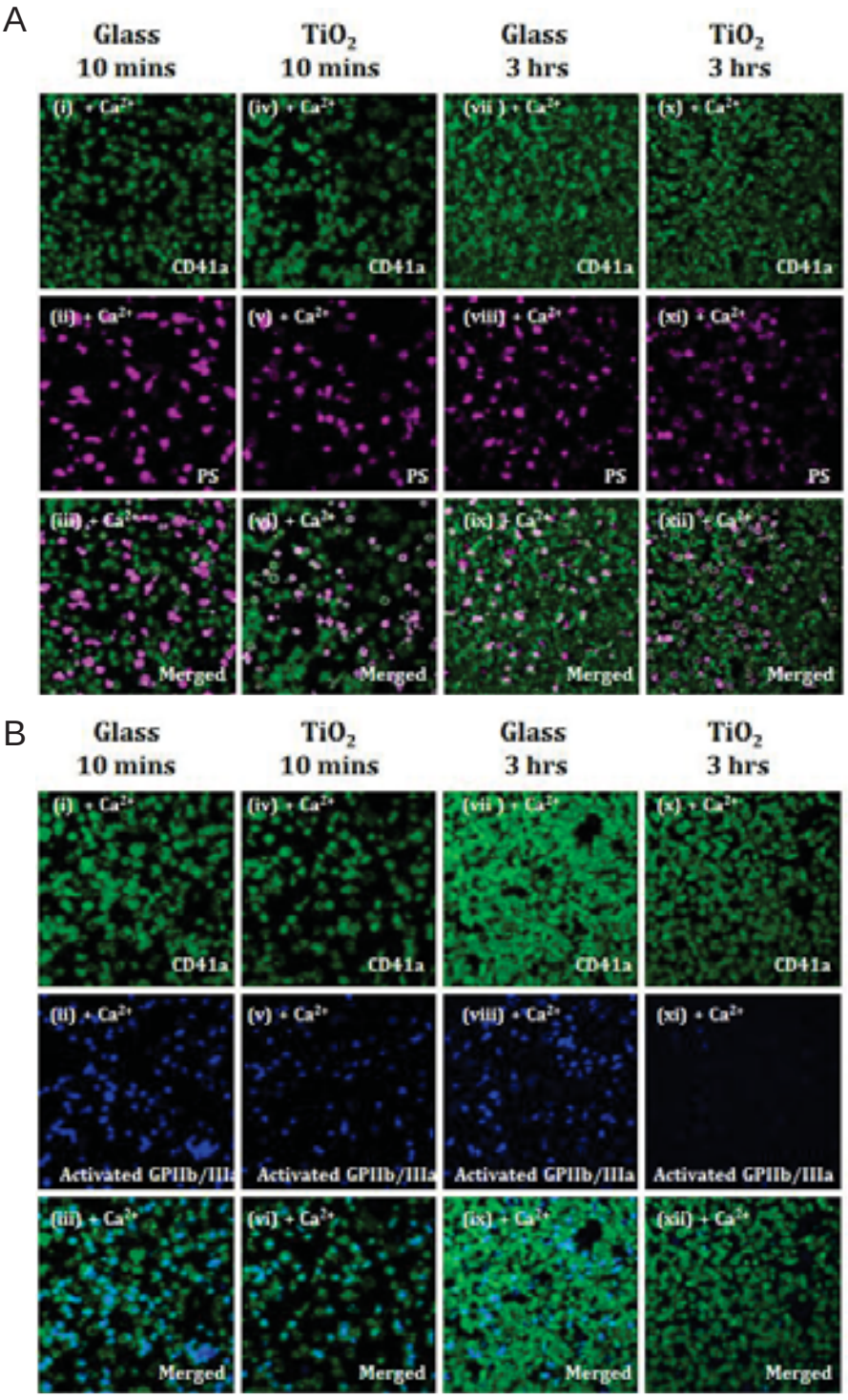



**Figure 3.11. PS (A) and Activated GPIIb/IIIa (B) expression profiles of platelets adhered on titania and glass in the 2 mM calcium buffer.**

**Platelets were incubated with $TiO_2$-coated or bare glass cover slips for 10 minutes or for three hours in a 2 mM calcium buffer and extensively washed with the same buffer. Platelets that remained on the surface were incubated with the following reagents and observed in the confocal microscope: anti-CD41a (green) and A5 (A, pink) or anti-PAC1 (B, blue); the expression of PS (A) and activated GPIIb/IIIa (B) was observed on the platelets both after 10 mins and 3 hr of contact with either surface.**

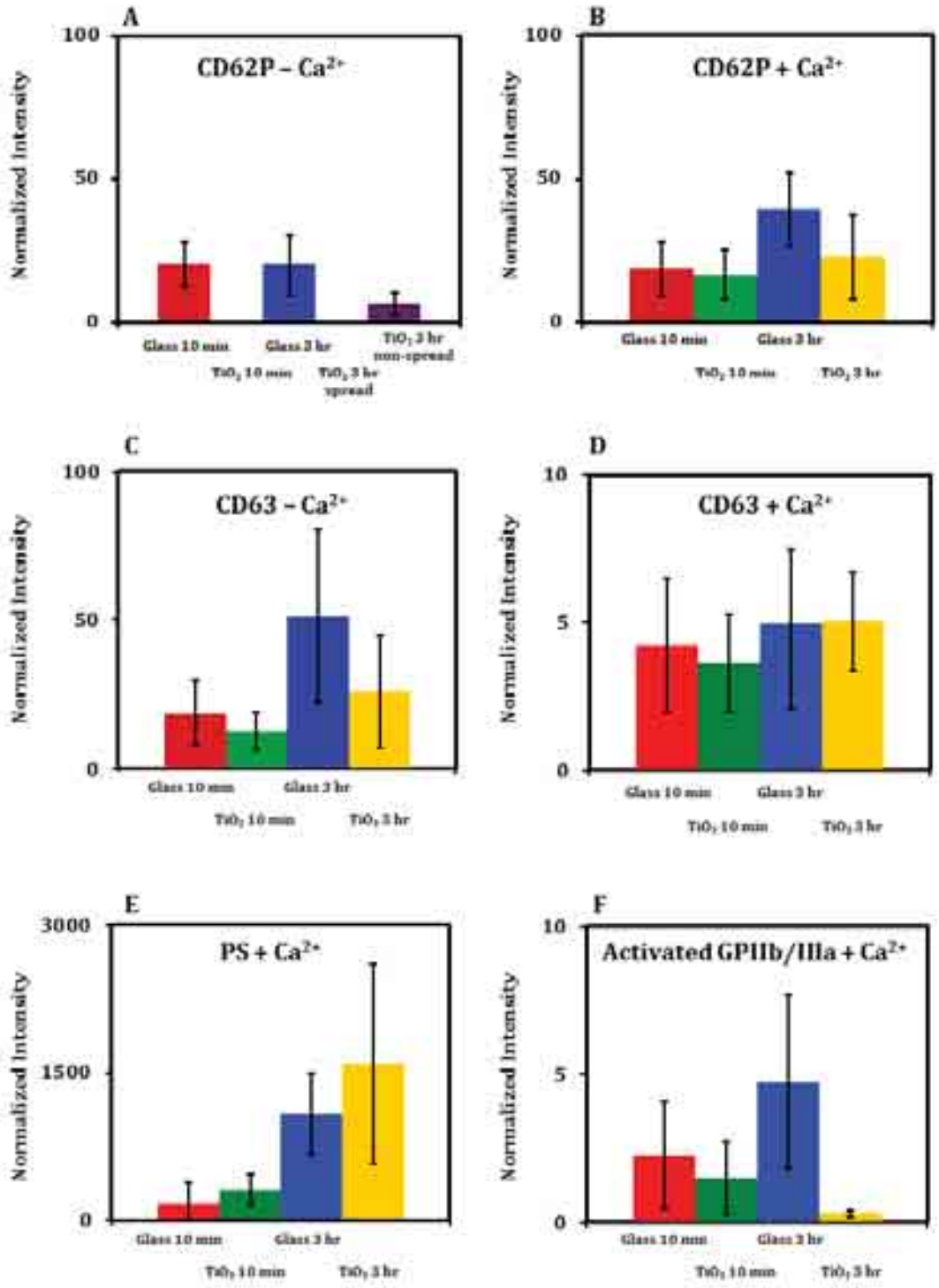

A
CD62P – Ca2+
Normalized Intensity
100
50
0
Glass 10 min
Glass 3 hr
TiO2 3 hr non-spread
TiO2 10 min
TiO2 3 hr spread
B
CD62P + Ca2+
Normalized Intensity
100
50
0
Glass 10 min
Glass 3 hr
TiO2 10 min
TiO2 3 hr
C
CD63 – Ca2+
Normalized Intensity
100
50
0
Glass 10 min
Glass 3 hr
TiO2 10 min
TiO2 3 hr
D
CD63 + Ca2+
Normalized Intensity
10
5
0
Glass 10 min
Glass 3 hr
TiO2 10 min
TiO2 3 hr
E
PS + Ca2+
Normalized Intensity
3000
1500
0
Glass 10 min
Glass 3 hr
TiO2 10 min
TiO2 3 hr
F
Activated GPIIb/IIIa + Ca2+
Normalized Intensity
10
5
0
Glass 10 min
Glass 3 hr
TiO2 10 min
TiO2 3 hr

**Figure 3.12. Summary of the platelet activation profiles under different conditions employed in this study.**

**Shown in this figure is the background-subtracted fluorescence intensity, [(S-B)/B], where S is signal and B is background, in the red channel, corresponding to the expression of the activation markers CD62P, CD63, PS or activated GPIIb/IIIa under different conditions used in this study. For each condition, the data was collected from at least two or three experiments using the blood of three different donors. The trends confirm those apparent in the individual images shown in Figures 3.6, 3.7, .310 and 3.11. Note the change in scale: while in (A)–(C), the fluorescence intensity is plotted on a scale from 0 to 100, in (D) and (F) it is plotted on the scale from 0 to 1, and on plot (E) it is plotted 0-3000, because the average intensity is different for various conditions and activation markers. (A) Expression of CD62P in platelets adhering on $TiO_2$ and on glass in the absence of $Ca^{2+}$. Significant levels of CD62P expression are seen on glass. On the other hand the expression is not detectable on $TiO_2$ after 10 minutes. After 3 hrs, the spread platelets do not express CD62P on $TiO_2$, while the non-spread platelets do express some. (B) Expression of CD62P in platelets adhering on $TiO_2$ and on glass in the presence of extracellular $Ca^{2+}$. CD62P is expressed on platelets adhering on both surfaces after 10 minutes and after 3 hrs. (C and D) Platelets express CD63 in the absence and presence of $Ca^{2+}$ on both surfaces. (E and F) Platelets express PS and activated GPIIb/IIIa on both glass and $TiO_2$ in the presence of $Ca^{2+}$.**

### 3.2.6 Role of intracellular calcium dynamics in regulation of selectivity in platelet activation responses by $TiO_2$ in the absence of extracellular calcium

A major signaling event that occurs in platelets upon stimulation with agonists is a rise in the intracellular calcium.[28, 30, 31, 52, 151] In the resting state, internal calcium concentration is kept at nM level by the action of transmembrane protein pumps that extrude it into the extracellular space and into the intracellular calcium stores (DTS/ER) (Figure 3.13).[3, 28, 153-155] Receptor activation leads to mobilization of this ion from intracellular stores, which in turn leads to the opening of the store operated calcium channels (Orai) in the cell membrane, allowing the extracellular calcium to flood into the cell. This mechanism is called store-operated calcium entry, or SOCE (Figure 3.13).[51, 172, 173]

The resulting rise in cytosolic calcium leads to the activation of various calcium-dependent kinases involved in signal transduction pathways.[28, 30] These lead to the platelet activation responses such as shape change, adhesion, granule secretion, inside-out GPIIb/IIIa activation and PS translocation to the outside of the membrane (Figure 3.13).[28-31, 41, 52, 53]

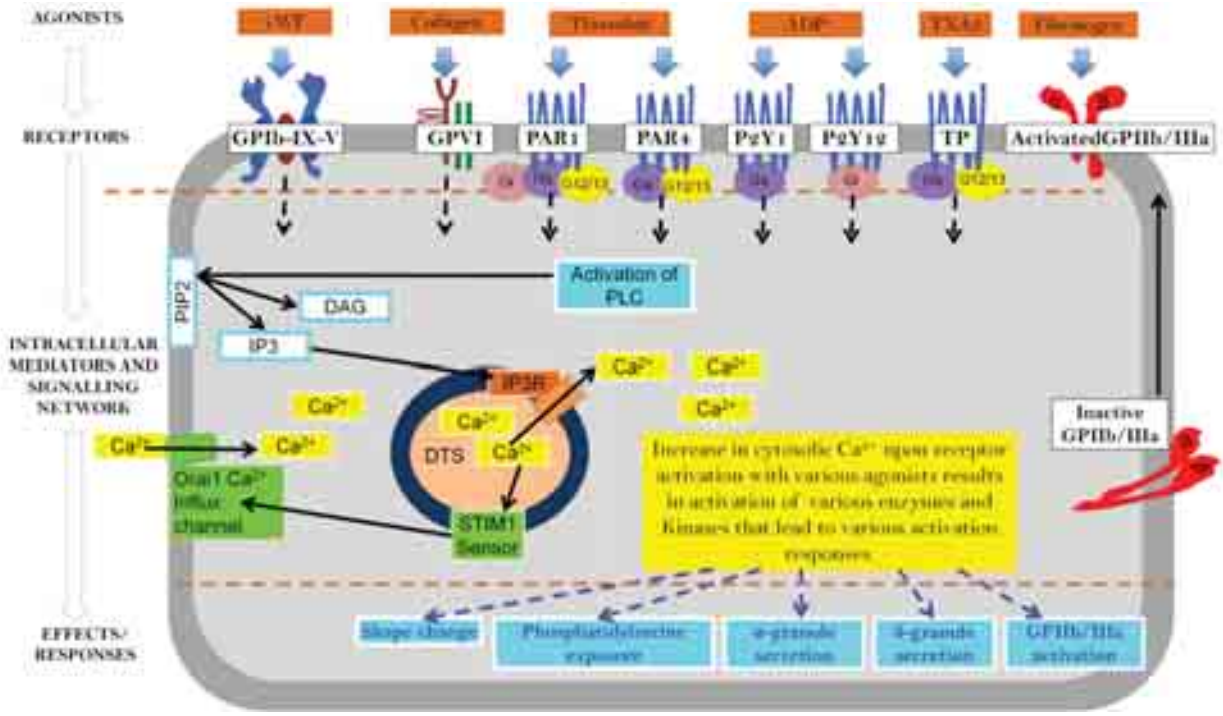



**Figure 3.13. Rise in cytosolic calcium in response to different agonists.**

**Different agonists act via different receptors. vWF acts on GPIb-IX-V receptor, collagen acts on GPVI receptor, thrombin acts on PAR1 and PAR4 receptors, TXA2 acts on TP receptor, ADP acts on P2Y1 and P2Y12 receptors and fibrinogen acts on activated GPIIb/IIIa receptor. These agonists upon binding to their receptors activate different intracellular mediators and signaling pathways, all of which result in activation of phospholipase C (PLC) activation (different isoforms for different agonists). PLC acts on membrane PIP2 and converts it into IP3 and DAC. The IP3 acts on IP3 receptor on calcium channels present on dense tubule system (calcium stores). This results in release of calcium from internal stores into the cytoplasm. This release from stores activates a calcium sensor (STIM1) which then sends signal to Orai calcium channels in the plasma membrane causing influx of calcium from exterior into the platelets. This phenomenon is called store operated calcium entry. These processes result in an increase of cytosolic calcium levels which leads to different platelet activation responses- shape change, PS exposure, secretion of alpha and dense granules and activation of GPIIb/IIIa receptor.**

Selectivity of platelet responses presents a conundrum in this context: if all agonists lead to cytosolic calcium rise, how can platelets respond differently to different agonists? A recent study suggests that internal platelet calcium dynamics is different for different agonists,[174] while our observations of a selective response from platelets adhering on surfaces under different conditions present us with a unique opportunity to examine intracellular calcium dynamics under conditions, where platelets responses were different.

To elucidate the role of intracellular calcium in selective platelet activation at surfaces, we first chelated all intracellular calcium by treating platelets with BAPTA-AM (an intracellular calcium chelator) and studied the surface expression of CD62P and CD63 in the BAPTA-AM treated platelets in solution and upon adhesion to glass and $TiO_2$ surfaces. We then measured the dynamics of intracellular calcium in platelets adhering to the surfaces and showed that it is surface-dependent. Finally, we manipulated the intracellular calcium levels in adhering platelets artificially with a calcium ionophore. The results of these studies allowed us to arrive at hypotheses with regards to the potential molecular mechanism underlying selectivity of platelet responses to the two different surfaces.

### 3.2.7 Chelating intracellular $Ca^{2+}$ affects the expression of both alpha and dense granule markers (CD62P and CD63)

To elucidate the role of calcium in the selective activation of platelets we observed at surfaces, the platelet cytoplasmic calcium was chelated by incubating them with BAPTA-AM. BAPTA-AM is an ester that can freely cross the plasma membrane but is hydrolyzed once inside the platelet. The resulting membrane-impermeable acid acts as a chelator of cytoplasmic $Ca^{2+}$ and thereby is known to block all calcium dependent pathways and processes (Figure 3.14).[175, 176] Figure 3.15 shows that platelets in solution lose their ability to express CD62P (Figure 3.15A) and CD63 (Figure 3.15B) in response to stimulation with TRAP if their cytoplasmic calcium is chelated by BAPTA-AM. This is expected, because both processes require the rise in cytosolic $Ca^{2+}$.[3] Similarly, chelation of intracellular $Ca^{2+}$ significantly reduced the ability of platelets adhering on glass in the absence of extracellular calcium, to express these two markers both after 10 mins and 3 hrs (Figure 3.16A–iii, ix and Figure 3.17A for CD62P, and 3.16B–iii, ix and Figure 3.17B for CD63). BAPTA-AM treatment also significantly reduced the extent of expression of CD63 in platelets interacting with $TiO_2$ (Figure 3.16B-vi, xii and Figure 3.17B). Since CD62P is not expressed in platelets interacting with $TiO_2$ in the absence of extracellular $Ca^{2+}$, BAPTA-AM treatment had no further effect on the expression of this marker (Figure 3.16A-vi, xii and Figure 3.17A). Chelation of intracellular $Ca^{2+}$ did reduce the extent of platelet spreading on $TiO_2$ and to a lesser extent on glass (Figure 3.4B-E and 3.16). A similar effect has previously been observed on polystyrene.[177]

Since chelation of intracellular calcium abolished the expression of both CD62P and CD63 on glass and of CD63 on $TiO_2$, it suggests that rise in intracellular calcium does

take place on both $TiO_2$ and glass and it is required for expression for expression of both CD62P and CD63 markers by platelets. If that's the case then how these two surfaces result in differential expression of activation markers by platelets? It led us to hypothesize that probably the kinetics of calcium mobilization from internal stores is different between platelets adhered to two surfaces which may account for the differences in platelet activation patterns on two surfaces. We therefore measured the kinetics of calcium release in platelets upon adhesion to glass and $TiO_2$ surfaces in the absence of extracellular calcium.

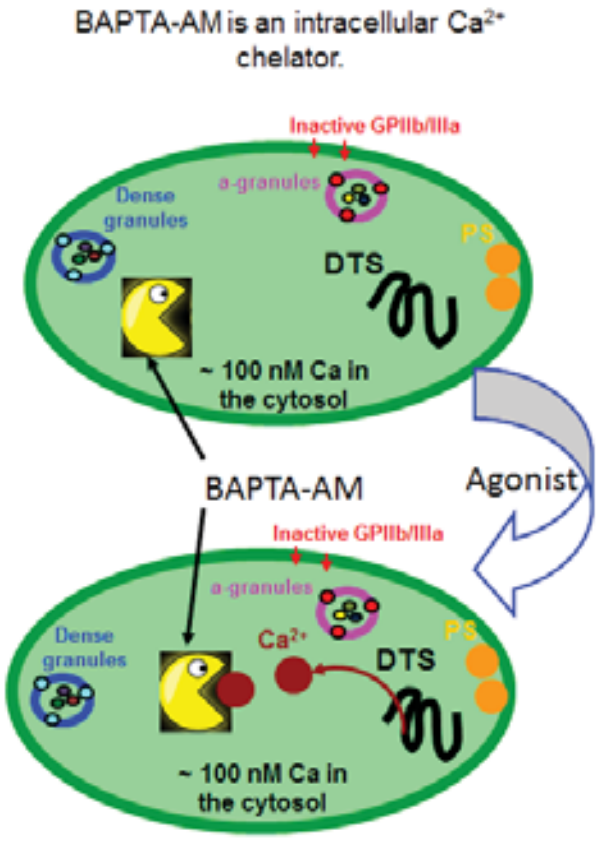



**Figure 3.14. Principle of intracellular calcium chelation by BAPTA-AM. BAPTA-AM is membrane permeable intracellular calcium chelator that prevents the rise of cytosolic calcium upon stimulation of platelets with an agonist, thereby inhibiting platelet activation responses such as exocytosis of alpha and dense granules (green and pink circles), PS exposure (orange circles) and activation of GPIIb/IIIa receptor (red arrows in membrane).**

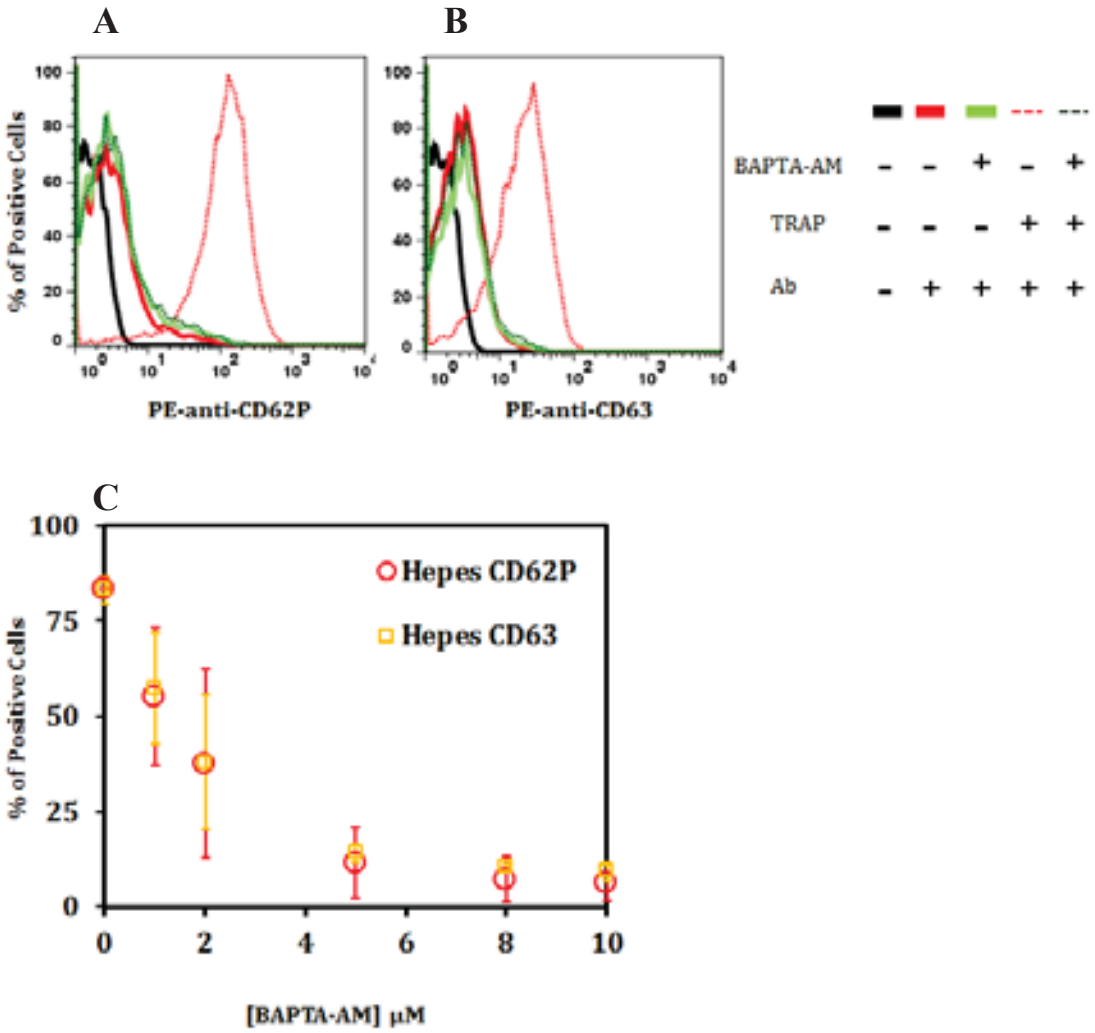



**Figure 3.15. Intracellular chelation of calcium with BAPTA-AM inhibits expression of CD62P and CD63 upon stimulation of platelets with TRAP in solution.**
**(A and B) Histograms: Platelets isolated in the nominally calcium-free buffer were treated with BAPTA–AM (100 µM) for 30 mins to chelate the intracellular $Ca^{2+}$, and analyzed by flow cytometry. They do not express CD62P (left, dotted green) or CD63 (right, dotted green) upon stimulation with an agonist (TRAP). Untreated stimulated platelets do (dotted red). Non-stimulated platelets do not express CD62P or CD63 independently of the BAPTA-AM treatment (solid red and green). Sample auto-fluorescence recorded in the absence of antibodies is shown in black. (C) Plot: % of CD62P (red)- and % of CD63 (yellow)- positive platelets as determined with flow cytometry by analyzing the histograms such as those shown on the left upon stimulation with 70 µM TRAP as a function of the BAPTA-AM concentration. Each point represents an average of six experiments, three of which were performed in the $Ca^{2+}$-free HEPES buffer and three–in the citrate buffer; no differences were detected between the two sets. This plot demonstrates that the chelation of intracellular $Ca^{2+}$ affects the expression of both of these markers in the same way.**

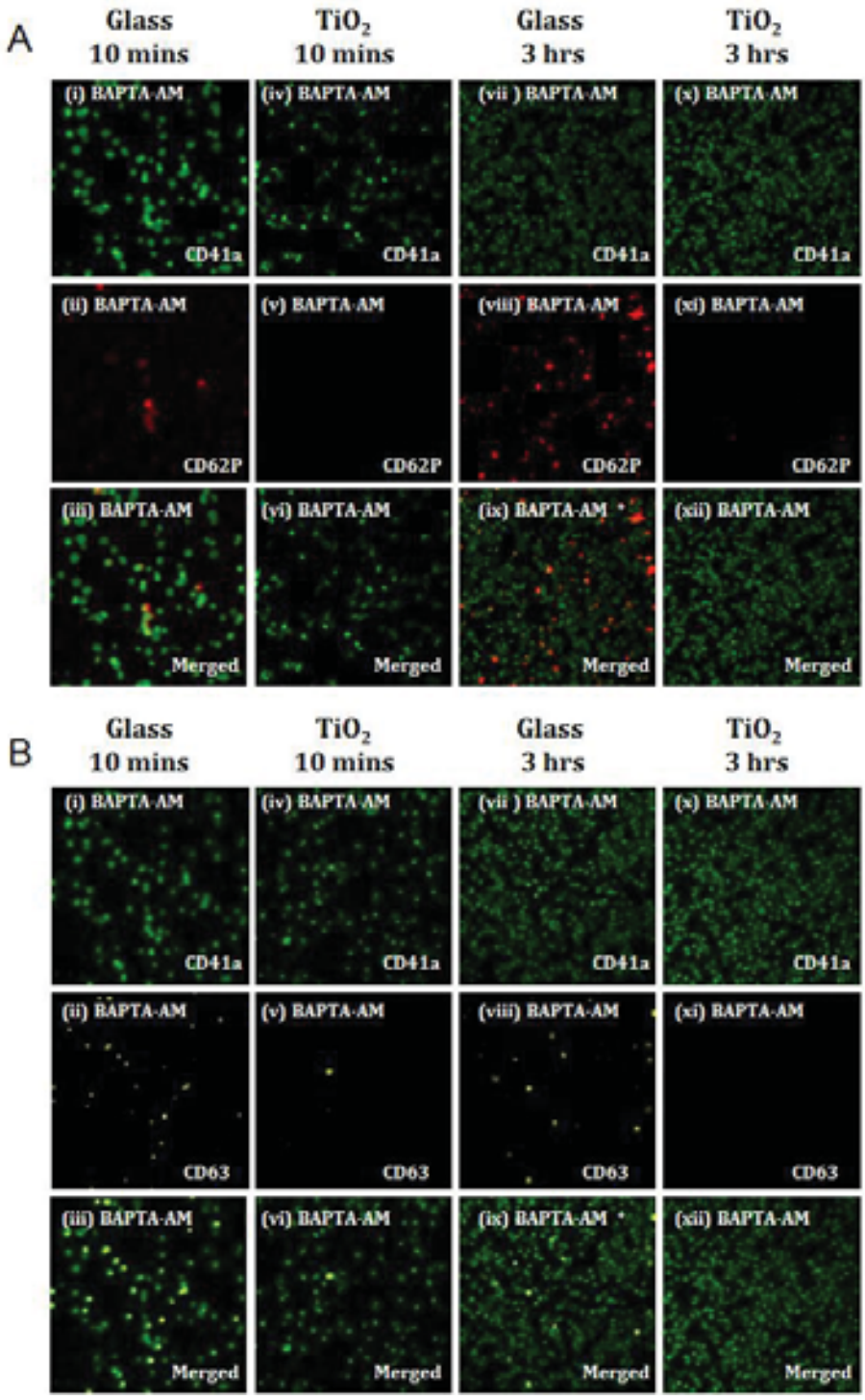



**Figure 3.16. CD62P (A) and CD63 (B) expression profile of BAPTA-AM treated platelets on glass and $TiO_2$ in the absence of extracellular calcium.**
**BAPTA-AM treated platelets were incubated with the glass and $TiO_2$ surfaces and analyzed for CD41a (green) and CD62P (A, red) or CD63 (B, yellow) expression by confocal microscopy. No spreading was observed on either of the two surfaces after 10 min or after 3 hr of incubation (see Figure 3.4B-3.4E). Some CD62P (A) expression is evident in platelets incubated on glass (iii and ix). On the contrary, no CD62P expression was observed in platelets incubated on $TiO_2$ (vi and xii). Some CD63 (B) expression is evident on both glass (iii and ix) and $TiO_2$ (vi) surfaces.**

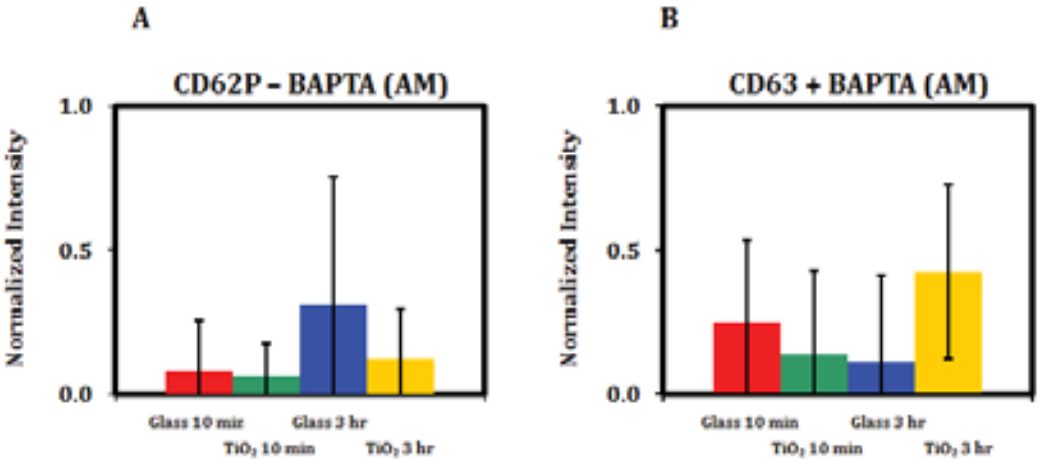



**Figure 3.17. Summary of the activation profiles of BAPTA-AM treated platelets in the absence of intracellular calcium.**
**Shown in this figure is the background-subtracted fluorescence intensity, [(S-B)/B], where S is signal and B is background, in the red channel, corresponding to the expression of the activation markers CD62P (A) and CD63 (B) of BAPTA-AM treated platelets adhered to glass and $TiO_2$ in the absence of extracellular calcium. BAPTA-AM affects the expression of both CD62P (A) and CD63 (B) activation markers.**

### 3.2.8 Kinetics of intracellular calcium mobilization in platelets is different on the two surfaces

The changes in intracellular calcium of platelets upon interaction with $TiO_2$ and glass surfaces were followed with fluo-3 AM dye. Fluo-3 is a fluorescent $Ca^{2+}$ chelator probe that is coupled to an acetoxymethyl ester (AM) which masks the $Ca^{2+}$-binding region and makes the fluo3-molecule lipid soluble, allowing it to cross the cell membrane (Figure 3.18). Following de-esterification by the cytoplasmic esterases, the fluo-3 is trapped inside the cell.[153, 178, 179] Upon binding the cytoplasmic $Ca^{2+}$-ions, the fluo-3–$Ca^{2+}$-complex emits a substantially increased fluorescence following excitation, with an intensity that is proportional to $[Ca^{2+}]i$ (Figure 3.18).[179] This fluorescence signal can be detected by fluorimetry or visualized with a confocal microscope.[178, 179] Another cell membrane permeable dye (red orange calcein AM) was used to co-label platelets together with fluo-3 (AM). This dye stains the cytoplasm and therefore would help in visualizing platelets at the surface.[180] Also as opposed to fluo-3 (AM) dye whose intensity increases with an increase in intracellular calcium, the intensity of the second cytoplasm staining dye is independent of the $Ca^{2+}$ concentration. Therefore the red orange calcein dye serves as a control and can be used to normalize the fluo-3 (AM) intensity signals.

Platelets co-stained with these dyes were allowed to adhere to glass and $TiO_2$ surfaces in the absence of extracellular calcium and changes in intracellular calcium dynamics were followed as a function of time using confocal microscopy. The process of platelet adhesion consisted of touching, rolling, and then finally spreading (images in Figure 3.19A and 3.19B-i, ii and iii). Changes in the intracellular calcium level were followed throughout these stages by taking advantage on the normalization procedure: for every

individual cell, in every image frame, fluo-3 (AM) intensities were divided by red orange calcein (AM) intensities. This gave a signal that was proportional to the changes in the internal $Ca^{2+}$ concentration and independent of the cell motion. This is the signal that is plotted in Figure 3.19 for the two surfaces. We observed that both the surfaces result in an increase in the level of intracellular calcium. That increase is very similar on the two surfaces (glass and $TiO_2$). However, in platelets adhered on glass, the increase in calcium level is sustained (Figure 3.19A), while in platelets adhered on $TiO_2$, it is transient and is followed by decay to a negligible level (Figure 3.19B). These patterns of calcium changes inside platelets are also represented as merged images of transmission channel (gray) and fluo-3 AM channel (red) show spreading of a platelet and the changes in fluo-3 AM intensity on glass (Figure 3.19A-i, ii and iii) and $TiO_2$ (Figure 3.19B-i, ii and iii).

We suspect that these differences in intracellular calcium dynamics on glass (sustained rise) and $TiO_2$ (transient rise with decay) might be responsible for the differential regulation of platelet activation observed on these two surfaces. We confirmed this hypothesis by stimulating $TiO_2$ adhered platelets with calcium ionophore in the presence of 2 mM calcium and thereby inducing an increase in intracellular calcium inside platelets.

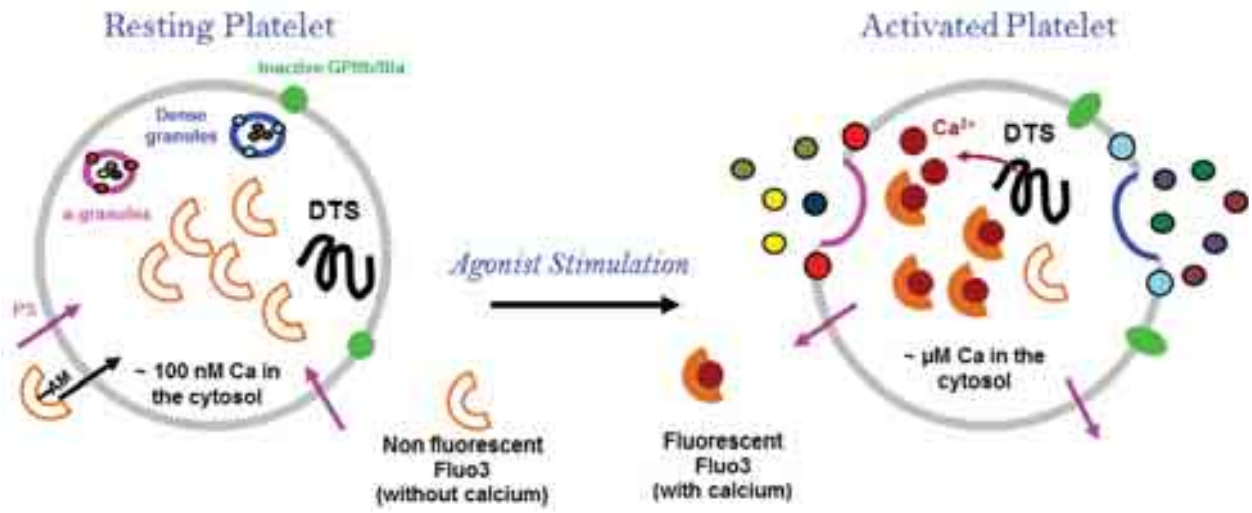



**Figure 3.18. Fluo3-AM is a membrane permeable intracellular $Ca^{2+}$ indicator dye. The AM esters of the indicators themselves do not bind $Ca^{2+}$. However, once they have entered cells, they are readily hydrolyzed by intracellular esterases into the parent $Ca^{2+}$ indicators, thus becoming responsive to $Ca^{2+}$. Fluo-3 is non-fluorescent without calcium present (like in resting platelets) but its fluorescence increases at least 40 times upon calcium binding (in activated platelet).**

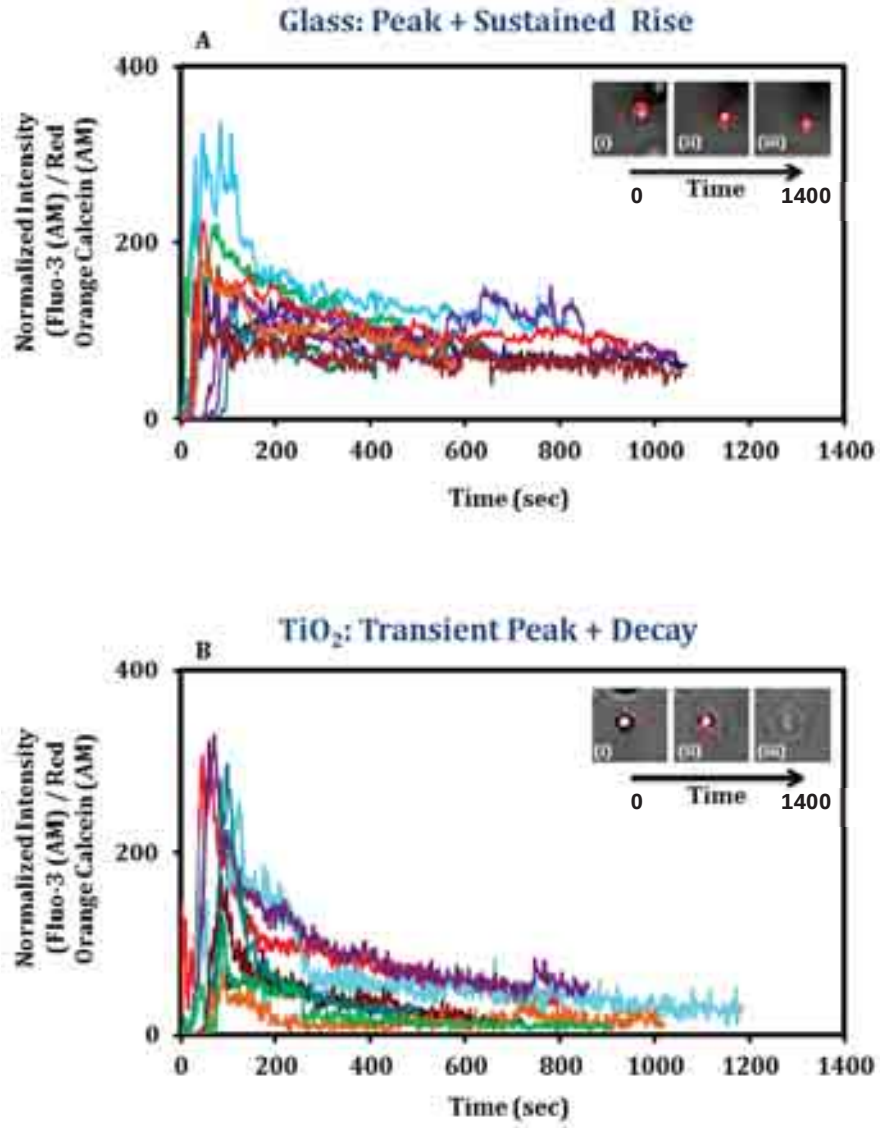



**Figure 3.19. Intracellular calcium changes inside platelet adhered in glass and $TiO_2$ in the absence of extracellular calcium.**

**Shown in the plots is the change in normalized fluo-3 AM (intracellular calcium binding dye) intensity with time for platelets adhered on glass (A) and $TiO_2$ (B) for different cells from 2 experiments. The intensity were measured starting from the frame where platelets adhered to the surface (0 time point on x-axis). Inside each plot merged images of transmission channel (gray) and Fluo-3 AM channel (red) show spreading of platelets and the changes in fluo-3 AM intensity on glass (A) and $TiO_2$ (B) with time (i-iii) where iii corresponds to the last frame/time point. Glass shows a sustained increase in calcium and $TiO_2$ shows a transient increase followed by decay to a negligible level.**

### 3.2.9 Artificial manipulation of intracellular calcium in $TiO_2$ adhered platelets induces selective expression of CD62P and PS but not GPIIb/IIIa

In order to determine the role in calcium kinetics in selective regulation of platelet activation responses, we treated the $TiO_2$ adhered platelets with calcium ionophore alone or in combination with 2 mM calcium. Calcium ionophore is a molecule that transports calcium across membranes down the concentration gradient.[154, 181] Therefore, it will transfer calcium from outside to the cytoplasm as well as from the intracellular stores into the cytoplasm.

We observed that calcium ionohphore alone did not induce expression of CD62P on platelets adhered on $TiO_2$ (spread platelets) after 10 mins and 3 hrs (Figure 3.20A-iii and ix). In case of platelets adhered to $TiO_2$ for 3 hrs, calcium ionophore alone could induce expression of CD62P on the non-spread platelets (Figure 3.20A-ix). This suggests that the calcium pools inside the spread platelets are empty. These observations explain why stimulation of $TiO_2$-adhered platelets with agonists did not result in CD62P expression (Figure 3.6-vii and xiv). Stimulation of platelets with calcium ionophore and 2 mM calcium induced expression of CD62P on platelets adhered to $TiO_2$ (Figure 3.20A-vi and xii) for 10 mins and 3 hrs (both spread and non-spread). Similarly, platelets adhered to $TiO_2$ (both 10 mins and 3 hrs) expressed PS (Figure 3.20B-iii and vi) after stimulation with calcium ionophore and 2 mM calcium. However no expression of GPIIb/IIIa could be observed (Figure 3.20B-ix and xii). We could not check the effect of calcium ionphore alone on the PS and GPIIb/IIIa expression on $TiO_2$ adhered platelets, as the addition of calcium to the sample was

necessary for binding of annexin A5 and of the PAC1 antibody to the PS and activated GPIIb/IIIa markers, respectively.[3, 8, 156, 157, 160]

We observed that the kinetics of cytosolic calcium increase in platelets adhered to glass and $TiO_2$ surfaces are different. This is correlated with the differential regulation of platelet activation at these surfaces. A transient increase in calcium in platelets is sufficient to induce CD63 expression, as was observed on $TiO_2$ surface. However, a sustained and high level of calcium is required for expression of CD62P and PS, as observed on glass surface. That is why platelets adhered to TiO2 upon stimulation with calcium ionophore and 2 mM $Ca^{2+}$, started expressing CD62P and PS. This indicates that calcium dependent signaling pathways involved in platelet activation are intact in platelets adhered to $TiO_2$. These pathways could not be activated in $TiO_2$ adhered platelets stimulated with TRAP, PMA or just ionophore, probably because the internal stores were empty in these platelets.

For reasons that remain unclear, stimulation of $TiO_2$ adhered platelets with calcium ionophore and 2 mM $Ca^{2+}$ could not restore expression of activated GPIIb/IIIa.

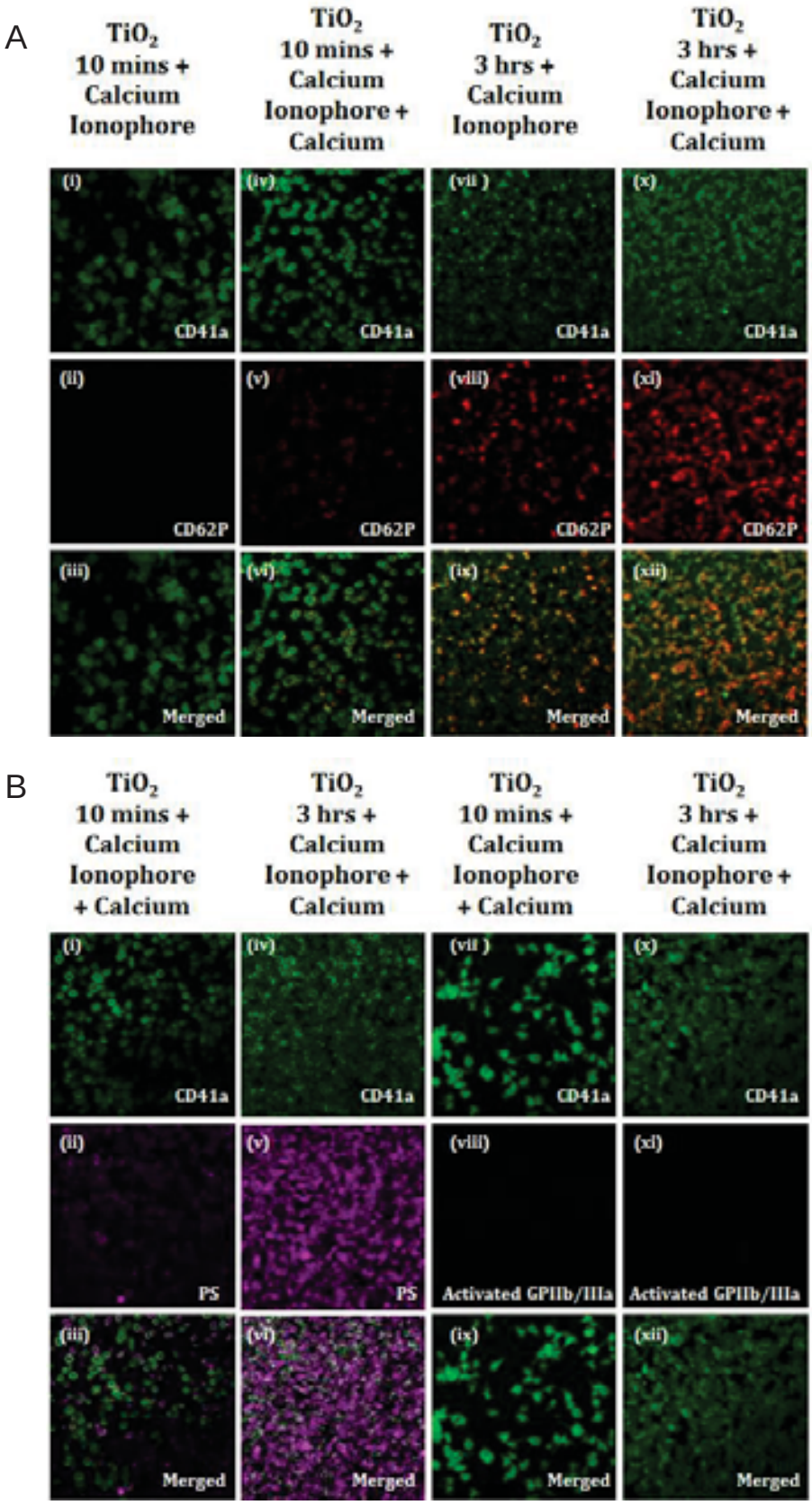

A
TiO2 10 mins + Calcium Ionophore
TiO2 10 mins + Calcium Ionophore + Calcium
TiO2 3 hrs + Calcium Ionophore
TiO2 3 hrs + Calcium Ionophore + Calcium
(i)
CD41a
(iv)
CD41a
(vii)
CD41a
(x)
CD41a
(ii)
CD62P
(v)
CD62P
(viii)
CD62P
(xi)
CD62P
(iii)
Merged
(vi)
Merged
(ix)
Merged
(xii)
Merged
B
TiO2 10 mins + Calcium Ionophore + Calcium
TiO2 3 hrs + Calcium Ionophore + Calcium
TiO2 10 mins + Calcium Ionophore + Calcium
TiO2 3 hrs + Calcium Ionophore + Calcium
(i)
CD41a
(iv)
CD41a
(vii)
CD41a
(x)
CD41a
(ii)
PS
(v)
PS
(viii)
Activated GPIIb/IIIa
(xi)
Activated GPIIb/IIIa
(iii)
Merged
(vi)
Merged
(ix)
Merged
(xii)
Merged

**Figure 3.20. Artificial manipulation of intracellular calcium of $TiO_2$ adhered platelets induces selective expression of CD62P and PS but not GPIIb/IIIa.**
**Platelets were incubated with the glass and $TiO_2$ surfaces in the absence of extracellular calcium for 10 mins and 3 hrs, washed and then treated with calcium ionophore alone or along with 2 mM calcium and analyzed for CD41a (green) and CD62P (A, red) or PS (B i-vi, pink) or activated GPIIb/IIIa (B vii-xii, blue) expression by confocal microscopy. (A) Addition of calcium ionophore induced CD62P expression only on non-spread platelets (viii and ix). (A and B) Addition of calcium ionophore along with 2 mM calcium induced expression of CD62P (A) and PS (B, i-vii) on platelets adhered to $TiO_2$ for 10 mins and 3 hrs (both spread and non-spread). Expression of activated GPIIb/IIIa was not detected after addition of calcium ionophore and 2 mM calcium (B, vii-xii).**

## 3.3 Discussion

Surface ion equilibrium is hypothesized to play an important role in defining the interactions between foreign materials and biological systems.[138] There is some evidence suggesting that $Ca^{2+}$ adsorption to titania affects $TiO_2$-protein interactions.[144] Furthermore, previous studies in our lab have shown that, on $TiO_2$, $Ca^{2+}$ elicits a clear response in terms of lipid behavior in phosphatidyl serine (PS)-containing liposomes and supported lipid bilayers—response that is absent on substrates such as silica or glass.[119, 120, 145, 146] In this study, we compared platelet activation profiles on glass (which does not bind calcium) and $TiO_2$ (which binds calcium) in the presence and absence of extracellular calcium. One of the main findings of this study is that $TiO_2$ results in selective platelet activation depending on the presence or absence of surface bound calcium.

In the presence of $Ca^{2+}$, $TiO_2$ acts as an agonist, activating adhering platelets and causing the expression on their surface of four well-known activation markers, alpha granule marker CD62P, dense granule marker CD63, phosphatidylserine and activated GPIIb/IIIa. On the contrary, in the absence of $Ca^{2+}$, platelets adhering on $TiO_2$ express only one of the four markers, CD63 and no other three markers. Platelets adhering on glass, as well as platelets challenged with soluble agonists in solution, express all four markers independently of whether $Ca^{2+}$ is present or not. These findings suggest that platelets can selectively undergo different sets of activation responses depending on the stimuli ($TiO_2$ surface with or without surface bound calcium, glass or $TiO_2$ surface in the absence of extracellular calcium), confirming the recent notion that platelets can tune their microenvironment in a trigger-specific fashion.[4, 17-21]

The simplest mechanism behind the observed selective pattern of platelet activation on $TiO_2$ and not on glass in the absence of extracellular calcium would be that $TiO_2$, because of its affinity for $Ca^{2+}$,[118, 139-141] acts as a $Ca^{2+}$-chelator: it could deplete intracellular $Ca^{2+}$, the way BAPTA-AM does, and/or remove structurally important ions from the outer surface of the platelet membranes, the way EDTA does.[3] This mechanism does not account for our observations, however: firstly, we see a clear difference between the effect of the intracellular $Ca^{2+}$ chelator BAPTA-AM and the effect of $TiO_2$. The former affects the expression of both CD62P and CD63 markers, while the latter concerns only CD62P. Secondly, chelation of the extracellular $Ca^{2+}$ by EDTA or citrate does not prevent the expression of either of the two markers in platelets challenged with various agonists.[182, 183] In other words, platelets must directly sense differences in the surface properties between glass and $TiO_2$ in the presence and in the absence of $Ca^{2+}$ and undergo selective activation.

### 3.3.1 Selective platelet activation by surface properties (surface composition, protein adsorption, ion adsorption)

In our study we used nearly purified platelets to study the direct effect of ion equilibrium (calcium ion) in mediating interaction between purified platelets and biomaterial surface and have shown that depending on its presence or absence on $TiO_2$, platelets undergo selective activation. However isolated platelets suspension still had minimal amount of proteins (~ 0.15 mg/ml) which might also play a role in influencing platelets adhesion and selective activation on the surfaces. Moreover, platelets secrete their own proteins. We suspect that this is what happens during the “sniffing” stage of platelet adhesion, before platelets settle into place. The steps involved in the adhesion and subsequent activation of platelets on foreign surfaces such as glass and $TiO_2$ are

complex and incompletely understood. The initial observable event is the instantaneous adsorption of plasma proteins (such as fibrinogen, Immunoglobin G/IgG, albumin, vWF, complement factor C1q and C3, factor XII) at the exposed surfaces.[59, 133, 134, 169, 184] The composition and properties of surface govern the composition of protein adsorbed and the conformational changes in the adsorbed proteins. For example, gold (Au) surfaces have been reported to result in adsorption of albumin, IgG, fibrinogen, High Molecular Weight Kininogen/HMWK, pre-kallikrein, factor XII and C1q complement factor after plasma exposure for 1hr while adsorbed lower amounts of fibrinogen, C1q and IgG as compared to Au surface.[133] The conformation of the adsorbed protein was also shown to be influenced by the surface. For example, atomic force microscopy (AFM) studies have revealed the trinodular shape of fibrinogen (Fg) molecules adsorbed on hydrophilic surfaces, whereas all of the molecules appeared globular on hydrophobic surfaces.[185] Studies by Latour et al have shown that the plasma proteins such as Fg and albumin undergoes a change in conformation (unfolding) upon adsorption to surfaces exposing sites in Fg and albumin that that induces platelet adhesion.[186, 187] These plasma proteins are unable to bind platelets in solution however once adsorbed onto a surface, they changes their conformation, and mediates binding to their respective receptors on platelet surface.[184, 186, 187] Furthermore, protein adsorption on surfaces has been reported to be influenced by the presence or absence of calcium ions. Studies have reported that calcium bound to $TiO_2$ surface resulted in selective adsorption of high amounts of albumin, pre-albumin and IgG from the human serum proteins.[144] On the other hand $TiO_2$ in the absence of calcium, adsorbed very little albumin.[188] We speculate that different proteins adsorbed on different surfaces (glass, $TiO_2$, $Ca^{2+}$-$TiO_2$) might result in differential platelet activation responses on these surfaces.

The type and conformation of protein adsorbed on the surfaces have been reported in literature to influence the platelet response on the surface. It has shown that platelet adhesion and spreading on artificial surfaces is correlated with conformation of fibrinogen adsorbed on the biomaterial surface.[186, 189] On the other hand albumin adsorption on surface minimizes platelet adhesion and spreading.[184, 190] Factor XIII is a plasma protein that upon adsorption to negatively charged surfaces (such as glass) undergoes conformational change that make it 500 fold more susceptible for proteolytic cleavage and generation of activated Factor XIIa.[191, 192] Activated Factor XIIa is associated with initiation of blood coagulation reactions and blood clot formation at the surface.[191, 192] Because proteins are readily adsorbed to the surface, it is speculated that they play a role in platelet activation at the surfaces, however direct surface effects have also been observed.[132] Several studies have reported that platelets responses to materials remain dependent upon the material regardless of presence of absence of plasma proteins.[193-195] Goodman et al have shown that GPIIb/IIIa receptor is involved in spreading of purified platelets on synthetic surfaces.[132]

In view of above observations, we propose that selective platelet activation observed on $TiO_2$ surface depending on the presence or absence of surface bound calcium, could either be attributed to the direct interaction of platelets receptors with surfaces of different chemical composition ($TiO_2$ or $Ca^{2+}$-$TiO_2$) or by the different proteins adsorbed onto $TiO_2$ surface in the presence and absence of surface bound calcium (Figure 3.21). Similarly selective activation of platelets by $TiO_2$ (only expresses CD63) and not by glass surface (expresses both CD62P and Cd63) could be due to direct surface effects or different proteins adsorbed onto these surfaces (Figure 3.21). It has

been reported that of the different proteins present in plasma, adsorption of vWF on surface is required for induction of CD62P expression on platelets.[169]

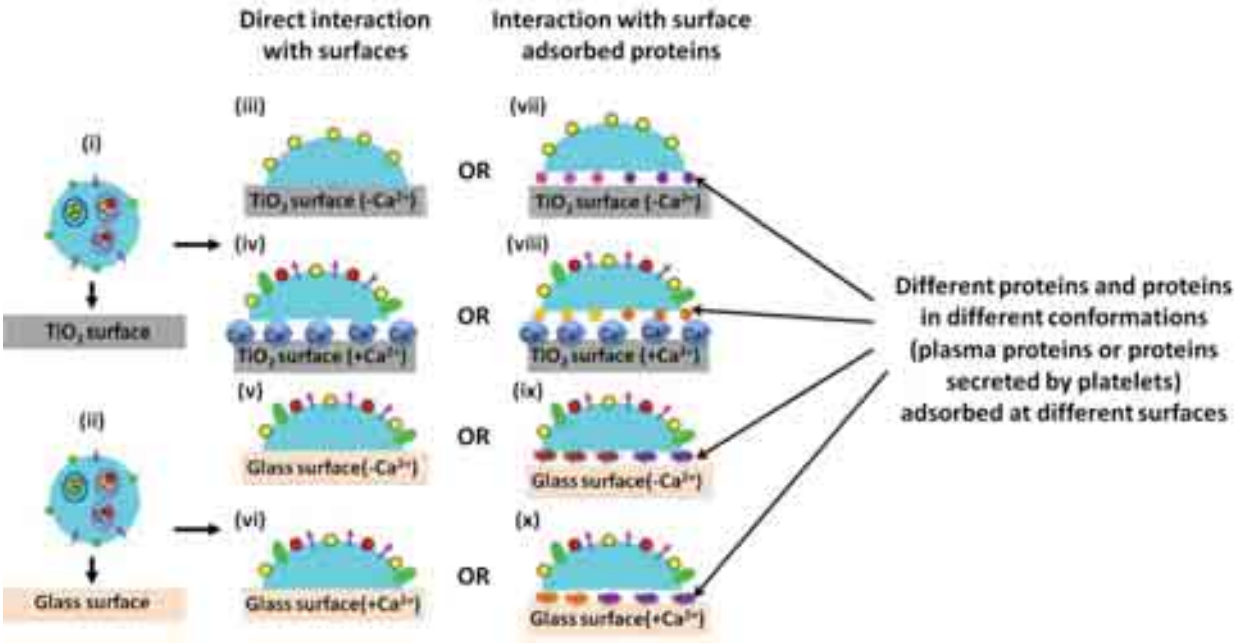



**Figure 3.21. Selective platelet activation by surface properties (surface composition, protein adsorption, ion adsorption).**
**Platelet adhesion and differential activation on $TiO_2$ and glass surfaces in the presence and absence of extracellular calcium via direct interaction with the surfaces ($TiO_2$, $Ca^{2+}$-$TiO_2$ or glass) or via different set of proteins and proteins in different conformations adsorbed onto the surface.**

There is a possibility that the platelets upon interaction with glass and $TiO_2$ surface-direct interaction or via different surface adsorbed proteins undergo different intracellular signaling pathways that results in differential pattern of platelet activation responses on these surfaces. It is well established that different agonists (soluble or surface) activate platelets via different intracellular signaling pathways (Figure 3.22).[28, 30] As shown in Figure 3.22, collagen, thrombin, TXA2 and ADP activate different isoforms of protein Kinase C (PKC) via different intracellular signaling pathways.[28, 30] Dense granule secretion upon stimulation of platelets with thrombin (PAR1 and PAR4 receptor) takes place by activation of intracellular PKC α, PKC β, PKC θ and PKC δ.[196, 197] However dense granule secretion by platelets in response to collagen is dependent on activation of PKC α, PKC θ and PKC β.[53, 197, 198] In fact PKC δ, activated upon collagen stimulation of platelets, results in inhibition of dense granule secretion.[196] Dense granule secretion in response to ADP takes place by activation of PKC δ and PKC η via generation of TXA2 while PKC β inhibits dense granules secretion in response to ADP.[199-201] These studies as well as our results suggest that different intracellular signaling reactions are initiated in response to different stimuli.

Indeed, in our studies we observed that kinetics of rise in cytosolic calcium in platelets adhered to $TiO_2$ (transient increase followed by a decay) and glass surfaces (sustained increase) were different. Furthermore, artificial manipulation of intracellular calcium (increase) of $TiO_2$ adhered platelets by calcium ionophore along with 2 mM calcium resulted in expression of CD62P and PS, which were not expressed before. These results imply that differences in platelets intracellular calcium kinetics on $TiO_2$ and glass surfaces correlate with the differential pattern of platelet activation markers expressed on platelets adhered to $TiO_2$ (only CD63 expressed) and glass surfaces

(CD62P, CD63, PS and activated GPIIb/IIIa expressed). Studies by Tadokoro et al have reported that platelets stimulated with PAR1 and PAR4 result in different calcium release kinetics, and that these differences correlated with the differences in the activation of GPIIb/IIIa receptor and CD62P expression levels.[174] PAR1 stimulation resulted in a transient calcium increase and GPIIb/IIIa activation was transient upon stimulation of platelets with PAR1 agonist peptide while PAR4 peptide resulted in a sustained calcium increase and GPIIb/IIIa activation. Similarly studies in other cells have shown how differences in calcium kinetics in response to different stimulation agents affect cell differentiation. For example, stimulation of Chinese hamster ovary cell proliferation by gastrin correlated with its ability to maintain an oscillatory $Ca^{2+}$ signal for at least two hours. Conversely, carbachol, acting through muscarinic M3 receptors, gave a short-lived $Ca^{2+}$ response and failed to stimulate proliferation.[202, 203]

In this study we have shown that differential intracellular calcium dynamics plays a role in selective platelet activation in response to different stimuli ($TiO_2$ and glass surfaces). Calcium was known to be crucial for different platelet activation responses but it was not known how differences in calcium kinetics can selectively regulate these processes in platelets

### 3.3.2 Calcium transients regulate selective platelet activation via different calcium sensors

Our studies suggest that extent and duration of calcium increase inside cells play an important role in regulating cell processes including selective activation of platelets. But how do these different types of calcium signals produce changes in cell functions? The information encoded in the kinetics of the $Ca^{2+}$ rise is sensed by various intracellular $Ca^{2+}$-binding proteins that convert these signals into a wide range of biochemical responses.[203, 204] Platelets contain such calcium sensors such as PKC isoforms, calmodulin and α-actinin that have been reported to play various roles in platelet activation processes including secretion and GPIIb/IIIa activation.[53] [174, 205, 206]

We propose here an essentially new way to look at the interplay between intracellular calcium dynamics and the way the cell responds to it. We propose that there is a *set* of calcium sensors whose state and function is differentially regulated depending on the level of calcium rise as well as its duration. Different events are then evoked by these sensors in response to different levels of intracellular calcium and by different types of calcium transients (sustained, short-lived, and oscillating). Calcium dynamics in platelets is not studied sufficiently well for this hypothesis to be tested, but some results present in the literature offer support for this hypothesis.

First possibility is that depending on the level and type of calcium transient, a particular sensor performing a particular function is either activated or not. Protein kinase C is a calcium regulated protein whose activation has been reported to be regulated by this mechanism. It has been reported that frequency of calcium spikes and temporal co-ordination of calcium spikes with diacyl glycerol (DAG) controls the extent and timing

of PKC activation. A low frequency and shorter calcium spikes induce small and reversible protein kinase activation in the presence of diacyl glycerol (DAG).[207] However, high frequency calcium spikes mediate maximal activation of kinases. It is known that PKCs isoforms perform different functions upon platelet stimulation. For example, PKC α and β promotes alpha granule secretion upon stimulation of platelets with collagen.[53] There is a possibility that depending on the calcium level and duration of calcium, the activity of PKC α and β is differentially regulated. It is activated in response to one type of calcium transient (or at one calcium concentration) but not by other type of calcium transient. Different calcium transients in response to different stimuli-glass and $TiO_2$ surfaces, might result in differential regulation of PKC, resulting in selective platelet activation responses by these surfaces.

Another possibility is that a calcium sensor exerts its action by binding to various effectors and the binding of calcium sensors is affected by the type of calcium level or transient. Calmodulin (CaM) is a member of a class of calcium binding proteins that exerts its action by binding to various effectors and the binding of these effectors is regulated by calcium.[203, 204] For example, certain classes of effectors bind calmodulin in the presence of low concentration of calcium but are activated by CaM only at high concentration of calcium upon formation of high affinity complexes with CaM.[204] Smooth-muscle myosin-light-chain kinase (MLCK), protein phosphatase, and calcineurin belong to such class of effectors which bind to CaM at low concentration but require high concentration to get activated and exert their action.[204, 208, 209] Activation of calmodulin in platelets mediates various platelet activation processes such as platelet aggregation and granule exocytosis via various effectors (e.g. myosin light chain, Vesicle Associated Membrane Protein/VAMP).[205, 206] There is a possibility that binding of these effectors and various other effectors (known and

unknown) to CaM and activation by CaM require low and high concentration respectively, which might result in differential pattern of platelet activation in response to different pattern of calcium transients.

These examples suggest that activity of calcium sensors can be regulated by the level of calcium and duration of calcium rise. This phenomenon might account for our observations-regulation of selective platelet activation on $TiO_2$ and glass surface by different calcium transients in platelets upon interaction with these surfaces. We can only make speculations at this point as studies are needed to determine the regulation of various calcium sensors by different calcium transients to support this hypothesis. Until now platelets have been studied only from the perspective of determining effect of individual intracellular signaling mediators on different platelet activation responses. There is a need to solve the mystery of how these different signaling mediators and pathways communicate with each other and result in a particular activation response.

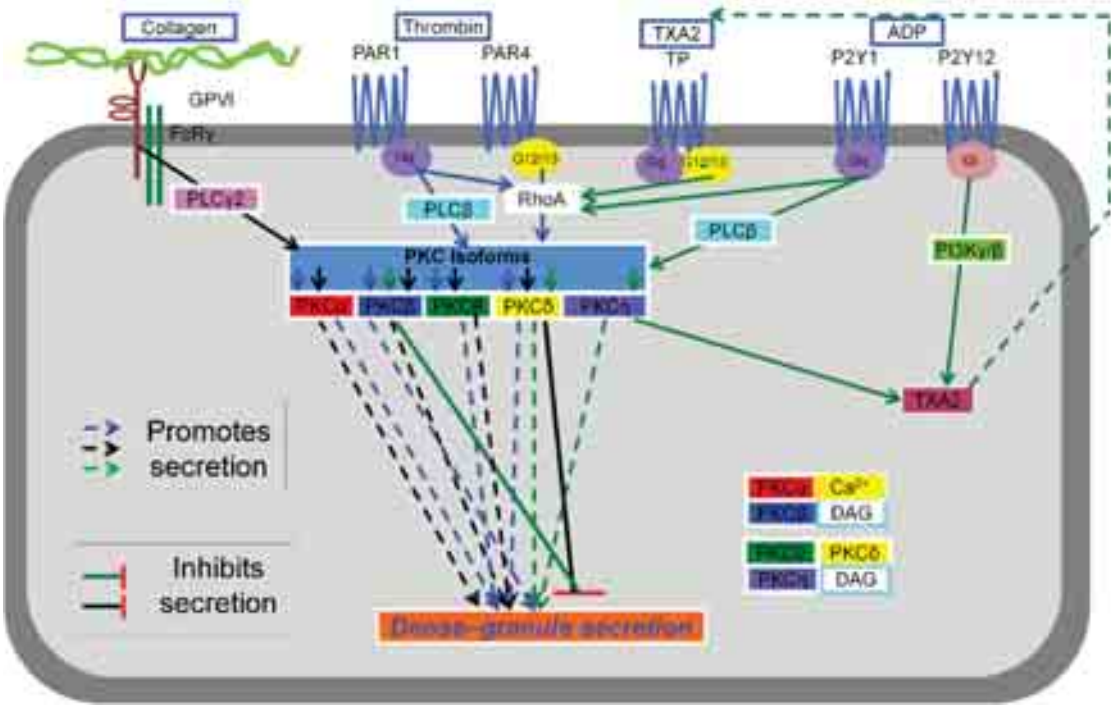


**Figure 3.22. Different intracellular signaling pathways initiated by different agonists.**

**Collagen acts on GPVI receptor, thrombin acts on PAR1 and PAR4 receptors, TXA2 acts on TP receptor and ADP acts on P2Y1 and P2Y12 receptors. The signaling pathways initiated by different agonists are color coded- black arrows for collagen, blue arrows for thrombin and green arrows for ADP and TXA2. These agonists activate different intracellular Protein Kinase C isoforms (PKCs) via different intracellular mediators. Collagen via PLCγ2, thrombin via PLCβ and RhoA, TXA2 via Rho and ADP via PLCβ and RhoA.**

**PKC isoforms activated by different agonists are indicted by colored arrows specific to that agonist (above each PKC isoform). Black for collagen, blue for thrombin and green for ADP.**

**Dense granule secretion in response to different agonists takes place by activation of different isoforms isoforms-PKC α, β and θ for collagen (dashed black arrows), PKC α, β, θ and δ in case of thrombin (blue dashed arrows), PKC δ and η (via TXA2 generation) in case of ADP (green dashed arrows).**

**PKCβ and δ activated by ADP (solid green line ending with red bar) and collagen (solid black line ending with red bar) respectively, inhibit granule secretion upon stimulation of platelets by these agonists. Depending on the PKC isoform, they require both calcium and diacylglycerol (DAG) or just DAG for their activation.**

## 3.4 Conclusions and Outlook

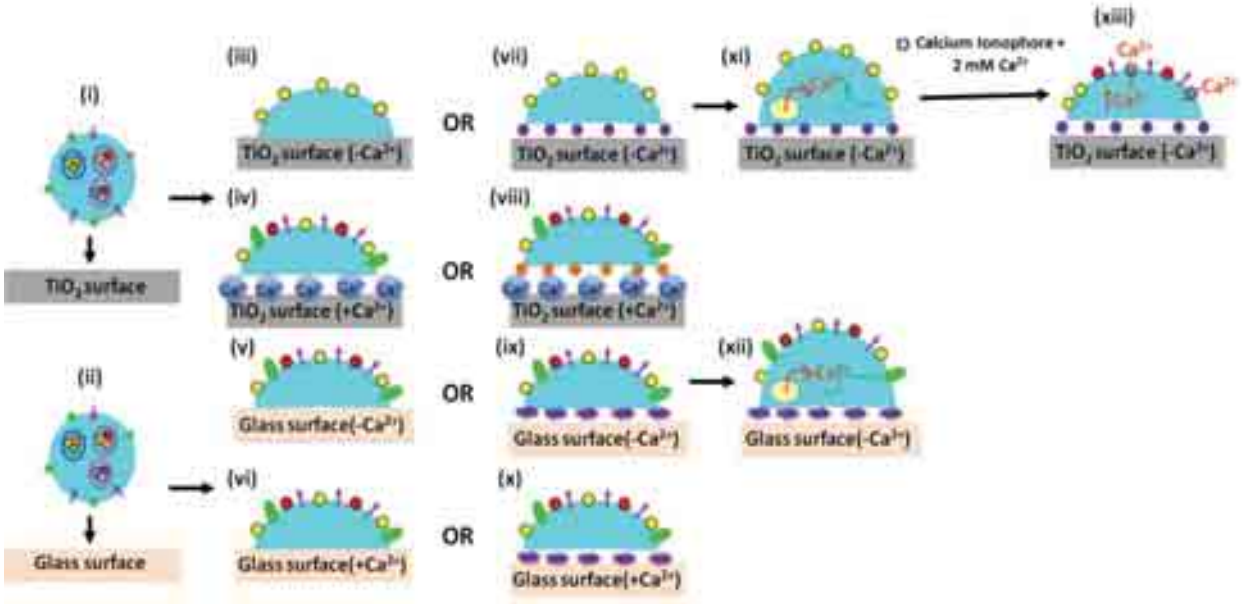



**Figure 3.23. Selective activation of platelets by different stimuli–(iii and iv) $TiO_2$ in the presence and absence of calcium and (iii and v) glass and $TiO_2$ in the absence of calcium (summarized pictorially).**

**(i and ii) Resting Platelets: Pink arrowhead refers to phosphatidyl serine (PS), the phospholipid that is asymmetrically distributed (facing inwards) in the membranes of resting platelets but is facing outwards in the membranes of activated platelets. PS catalyses clot formation. Dark blue spheres refer to GPIIb-IIIa integrin in the inactive conformation on the resting platelets. Dark blue ellipses designate the active conformation of this protein. Granules (α, pink hollow ellipses inside resting platelets, and dense, blue hollow ellipses inside resting platelets), with their respective markers (CD62P, red spheres, and CD63, yellow spheres) are shown inside the resting platelet. They undergo exocytosis upon activation, and the two markers appear on the cell membrane.**

**(vii-xiii) Purple circles and stars on the surface are same protein in different conformations adsorbed on $TiO_2$ (in the absence of calcium) and glass surface (in the absence and presence of extracellular calcium). Orange circles are the proteins adsorbed on $TiO_2$ surface in the presence of surface bound calcium. For explanation of figure, see text below.**

In this work we studied the role played by surface ion equilibrium (calcium ion) in defining the interactions between foreign materials and platelets. The findings of this study are summarized pictorially in Figure 3.23. One of the main findings of this study is that platelets undergo selective activation on $TiO_2$ surface (express different activation markers) depending on the presence or absence of surface bound calcium. Similarly, in the absence of calcium, platelets express different sets of activation markers on glass and $TiO_2$. Selective platelet activation can either take place by direct

interaction of platelets with surfaces of different compositions or via different proteins adsorbed on the surfaces.

When investigating the mechanism underlying this phenomenon, we focused on the intracellular calcium dynamics in the adhering platelets. We found that intracellular calcium transients are different in platelets adhered to glass and $TiO_2$ and they correlate with the different platelets responses observed on these surfaces. We propose that different calcium transients cause selective platelet activation on these surfaces via action of different calcium regulated sensors or differential regulation of these sensors.

These studies have provided us with a biomaterial ($TiO_2$) based model system that allows selective manipulation of platelet activation responses. Using this system, we have been able to show role of one of the player (calcium) of intracellular signaling pathways in regulation of selective platelet activation in response to different stimuli ($TiO_2$ and glass). Future studies would aim at understanding other pathways and intracellular mediators (e.g PKCs) involved in the selective expression of different activation markers on platelets. We would also expand the repertoire of activation markers that are selectively regulated by $TiO_2$ surface. An understanding of mechanism underlying selective platelet activation would allow us to manipulate microenvironment at sites of platelet activation. It would also aid in identification of targets for developing therapies against platelet mediated diseases ranging from thrombosis on implants to cancer metastasis.

# *Chapter 4*
# *The sweeter aspects of platelet activation: a lectin-based assay reveals agonist-specific glycosylation patterns.*

## 4.1 Introduction

Platelets play a key role in minimizing traumatic blood loss. When activated at the wound site, they aggregate to form a platelet plug and provide a catalytic surface for the formation of the fibrin clot that seals the wound, stopping the bleeding.[3, 7, 13, 210] Until recently, platelet activation was thought of as a transition from the resting to the procoagulant state. This process entails changes in platelet shape and size, expression of phospholipid phosphatidyl serine, of surface glycoproteins such as P-selectin (CD62P), conformational changes in the constitutively expressed glycoproteins such as the integrin GPIIb-IIIa, membrane expression and secretion intracellular granule cargos (growth factors, cytokines, coagulation pathway intermediates)—a repertoire of responses underlying platelets' hemostatic role.[3, 12, 13, 45]

Recent findings call for a more nuanced view of platelet activation. Platelets have recently been shown to play numerous physiological roles.[4, 17-21] They are involved in wound healing,[5, 20] angiogenesis and *de novo* blood vessel synthesis,[20] inflammation,[10] cancer metastasis,[19, 21] as well as adaptive and innate immune responses. [17, 18] [77, 78] [73, 74] [3, 4] [14, 15] This functional diversity suggests a possibility that platelets can respond to various external stimuli selectively; that there is a spectrum of platelet activation states for performing different functions. If true, this could revolutionize approaches to targeted, personalized treatment strategies for a variety of disorders ranging from cardiovascular to cancer, by offering the possibility to tailor platelet response to particular goals in individual patients.

Selectivity of platelet responses has been explored from the point of view of regulation of granule secretion. There are several types of granules in the platelets. α- granules contain high molecular weight substances (growth factors, cytokines, etc), while dense

granules contain small molecular weight substances like ADP and serotonin.[2, 3, 20] Recent studies reported that, α-granules contain substances with contradictory functions (pro- and anti-angiogenic growth factors, pro- and anti-inflammatory cytokines, etc.),[2, 20, 23] and reports of selective secretion and differential packaging of the α-granule contents into distinct subpopulations appeared in the literature.[2, 23, 24] Other studies, including our own, suggest that exocytosis of dense- and α-granules is differentially regulated.[108, 110, 111, 211, 212] In the previous chapter, we have demonstrated that this selectivity in platelet activation is not just restricted to granule markers but also other platelet activation markers such as expression of PS and activation of GPIIb/IIIa. We showed that in the absence of extracellular calcium, platelets adhered to $TiO_2$ selectively underdo exocytosis of dense granules and did not express other activation markers-CD62P, PS and activated GPIIb/IIIa. Moreover, artificial manipulation of intracellular calcium in $TiO_2$ adhered platelets induced expression of CD62P and PS but not activated GPIIb/IIIa. These findings led us to speculate that there might be other platelet activation responses (other than granule exocytosis, PS and activated GPIIb/IIIa expression) that are selectively regulated by different stimuli.

In this chapter, we approach the concept of platelet activation selectivity from a different perspective. If different ways of stimulating platelets result in platelets with distinct properties, it must be possible to distinguish between them. To do so, we chose lectins, a class of carbohydrate-binding proteins other than enzymes or antibodies.[213] Platelets, like all cells, are covered with a coat of carbohydrates, originating mostly from cell surface glycoproteins.[214] While it is relatively poorly studied, it is known that its chemical nature of carbohydrate coat changes as platelets age, and that these changes signal platelet clearance by macrophages and hepatocytes.[215-217] Changes

upon stimulation with thrombin have also been reported, as have differences between platelets from healthy individuals and those from uraemic or diabetic patients.[218, 219] Several members of a class of endogenous lectins, e.g. galectins, have recently come to prominence as platelet agonists,[220] and platelet activation by some plant lectins has also been reported.[221] In other cells, changes in surface glycosylation have clear functional implications, such as cells turning malignant.[222] In other words, changes in platelet surface glycosylation are functionally significant. We therefore hypothesized that treating platelets with different agonists will result in changes in their glycosylation, detectable with lectin binding assays, and that these changes will be agonist-specific.

We studied platelet surface glycosylation and its changes upon agonist stimulation by characterizing binding of a range of fluorescently labeled lectins to resting platelets and different agonist treated platelets by flow cytometry. Using fluorescently labeled lectins, we indeed demonstrate agonist-specific lectin binding "fingerprints" and comment on their potential functional significance. In view of previous findings that exogenous and endogenous lectins can serve as platelet agonists,[220, 221] we furthermore examined platelet activation by some of the lectins we used. While doing so, we observed that some of them competed for binding with the antibodies against P-selectin (CD62P, a glycosylated lectin). We consider our results to be highly significant for future research into platelet activation as well as for the development of new diagnostic tests.

Flow cytometry served to be a useful technique for determining platelet surface glycosylation patterns and agonist specific changes in these patterns using fluorescently labeled lectins, but this approach is very time consuming. It requires staining of

platelets separately with each of the fluorescently labeled lectins to determine binding level of a particular lectin by FACS. Therefore, only one lectin at a time could be analyzed. This has restricted us in terms of the number of lectins (9 in this study) that could be used for analyzing platelet surface glycosylation. Clearly, a multiplexing, high-throughput approach is needed. Such an approach is provided by the lectin arrays.[223] We therefore tried to apply them for the analysis of platelet surface glycosylation and its changes upon agonist stimulation. Lectin arrays is a surface-based technique where multiple lectins (up to 100) are printed as spots on a glass slide[125, 223] and platelets are allowed to interact with these spots. Adhering platelets are stained with specific fluorescent antibodies and the fluorescence is measured by a microarray scanner. Its intensity is proportional to the binding level. Lectins arrays have been used to study bacteria, stem cells, breast cancer cells, and lymphocytes.[224-226] However, in our case, we found that lectin arrays were not a suitable approach for studying platelet surface glycosylation because we could not obtain reproducible patterns of platelet binding to the various lectins printed in the array. The reasons for these variations are discussed.

## 4.2 Results and Discussion

### 4.2.1 Analyzing platelet surface glycome changes using lectins by flow cytometry

Lectins are proteins that bind with high affinity to sugar moieties containing specific oligosaccharide structures.[213] They are ubiquitously expressed in plants, animals, and microorganisms and have been commonly used as tools for studying protein glycosylation and for profiling cell surface carbohydrates.[213] In this study, we used nine fluorescently labeled plant lectins to study platelet surface glycosylation patterns and investigate how these patterns change when platelets are stimulated with various agonists. The lectins we used, together with their haptens, are listed in the Table 4.1. The agonists we used include thrombin, two of its analogues—PAR1 and PAR4 receptor agonist peptides (TFLLRN and AY-$NH_2$), ADP, thromboxane A2, calcium ionophore (CaIoP), and phorbol 12-myristate 13-acetate (PMA). The schematic of the experiment is shown in Figure 4.1A and 4.1B using the ACA lectin, thrombin, PAR1 agonist, and CaIoP as examples. In all our experiments, we used freshly isolated platelets purified by centrifugation and washing as described in the Materials and Methods, section 2.7. Prior to lectin binding experiments, platelets were tested by flow cytometry to insure that they were in the resting state and that they correctly responded to agonists (see Materials and Methods, section 2.8).

As shown in Figure 4.1, resting and agonist-treated platelets were incubated with the fluorescently labeled lectins. Their mean fluorescence intensity (MFI) was then determined by flow cytometry (Figure 4.1B). MFI signifies the level of lectin binding, which, depending on the agonist, was found to remain unchanged, increase, or decrease,

relative to that of the resting platelets (states ii, iii, or iv, respectively, in Figure 4.1B). Crucially, responses to different agonists could be distinguished: treatment of platelets with thrombin did not lead to a change in the ACA binding, while treatment of platelets with its analogue PAR1 agonist led to an increase in the level of ACA binding, and treatment with calcium ionophore—to a decrease.

For each individual experiment, the MFI of the agonist-treated platelets was divided by that of the resting platelets, giving the fold-change of the MFI between the agonist-treated and resting platelets. Averages of these for ACA and DBL, taken from at least four individual experiments, are plotted in Figure 4.1C. Statistically significant differences in the amount of ACA binding (relative to the resting platelets) were observed upon treating platelets with CaIoP, PAR1, and PAR4 agonists. On the other hand, in the case of DBL, statistically significant differences were observed for PAR1 agonist, PAR4 agonist, thrombin, and thromboxane. Therefore, not only the changes in the lectin binding to platelets upon agonist treatment are agonist-specific (Figure 4.1C), but they are also different for different lectins: thrombin and thromboxane treatment resulted in no change in the ACA binding but in an increase in the DBL binding (c.f. Figure 4.1C-i and ii). The flow cytometry data and the quantification results for the other lectins are shown in Figures 4.2 and 4.3, which we are discussed in the next section.

### 4.2.2 Lectin fingerprinting: changes in lectin binding to platelets upon agonist treatment are agonist-specific

Shown in Figure 4.4A is a three-color heat map of the changes in the lectin binding levels to platelets treated with various agonists, for all nine lectins and seven agonists. Importantly, each agonist is seen to affect lectin binding in a unique fashion, resulting in a "fingerprint".

The agonists we used in this study evoke the same set of responses in platelets: aggregation, secretion of active substances contained in platelet granules, expression of activation markers such as CD62P, CD63, and PS, and the change in the conformation of GPIIb/IIIa.[3, 12, 13, 45] However, different agonists act via somewhat different signaling pathways.[3, 45, 111, 227] Consequently, there are differences in the time-courses and levels of responses different agonists evoke. For example, CD63 and CD62P expression levels are different for different agonists (Figure 4.4B). Similarly, different levels of expression of the activated form of GPIIb/IIIa in thrombin vs. ADP-treated platelets were observed.[228] Lectin binding fingerprints appear to be sensitive to the differences in the signaling pathways through which different agonists evoke their responses.

To illustrate the sensitivity of lectin fingerprinting to the differences between agonists, we consider the case of thrombin and two of its analogues: PAR1 activating peptide TFLLRN and PAR4 activating peptide AY-NH2. Thrombin is a protease that exerts its action on the so-called protease-activated receptors (PARs, recently reviewed in ref.[47]) by cleaving their N-terminal exodomain sequences at specific sites, releasing a unique peptide for each receptor. These peptides then act as agonists, binding to the

receptors, leading to various intracellular effects through the coupled G-proteins. Synthetic analogues of these peptides have been designed to target individual receptors: (T/S)FLLRN targets PAR1 while AY-NH2 targets PAR4. While all three agonists induce intracellular calcium rise leading to aggregation, secretion, expression of CD62P and CD63 (Figure 4.4B), and GPIIb/IIIa conformational change, there are subtle differences in responses to these three agonists, because the signaling pathways of the PAR1 and PAR4 receptors are different.[227] For example, the intracellular calcium rises arising from PAR1 and PAR4 stimulation are different.[174] This correlates with different levels of CD62P expression (see Figure 4.4B and ref.[174]). Other differences have also been reported.[174, 227, 229-233] As shown in Figure 4.4A, these three agonists result in rather different changes in lectin binding.

Furthermore, a rather striking difference is observed between physiological agonist thrombin and the non-physiological agonists PMA and calcium ionophore. Calcium ionophore bypasses the signaling pathways and causes the rise in the intracellular calcium levels by directly channeling this ion into the cytoplasm from both the intracellular and extracellular stores. It may also have a direct effect on platelet membrane. PMA bypasses G-protein coupled receptors and exerts its action on one of their downstream effectors, protein kinase C (PKC). Although both sets of agonists (physiological and non-physiological) are classified as strong and evoke the same responses, lectin binding reveals differences between them (Figure 4.4A). Indeed, differences in lectin binding to thrombin-, PMA-, and CaIoP-treated platelets are quite striking. This result suggests that care must be taken when using non-physiological agonists to activate platelets in vitro, as the results might not be the same as with physiological ones.

Last but not least, we consider weak physiological agonists ADP and thromboxane A2. They are secreted during platelet activation and potentiate the effects of strong agonists. ADP acts via so-called purigenic receptors and the TXA2 has its dedicated receptors.[3, 45, 111] One of the ADP receptors is the target of the anti-platelet drug clopidogrel used in the treatment of patients receiving implants such as artificial heart valves and stents,[12] while the well-known drug aspirin inhibits thromboxane synthesis. Once again, platelets treated with ADP and thromboxane can be clearly distinguished from platelets treated with the other agonists, as well as from each other, by the changes in the lectin binding profiles (Figure 4.4A).

These results illustrate the capability of lectins for distinguishing between platelets activated in different ways. Lectins appear to be far more sensitive to these differences than the traditionally used antibodies (c.f. Figure 4.4A and 4.4B). Therefore, we believe that lectin fingerprinting of platelets will find uses in research and clinical practices.

**Table 4.1. List of plant lectins used in this study and their corresponding haptens.**

| Lectin (source) | Abbr. | Hapten specificity | Ref. |
|---|---|---|---|
| *Amaranthus caudatus* | ACA | Galβ1-3GalNAcα-Ser/Thr (T-antigen) Siaα2-3Galβ1-3GalNAcα-Ser/Thr (cryptic T-antigen) | [234, 235] |
| *Bauhinia purpurea alba* | BPL | Galβ1-3GalNAc, Galβ1-3/4GlcNAc, not to terminal α-GalNAc | [236, 237] |
| *Dolicos biflorus* | DBL | GalNAcα-Ser/Thr (Tn) and GalNAcα1-3GalNAc | [238-240] |
| *Aleuria aurantia* | AAL | Fucose (Fucα1-6,2,3,4GlcNAc)-both core and peripheral fucose | [241-243] |
| *Maackia amurensis* | MAA | Siaα2-3Galβ1 | [244-246] |
| *Sambucus nigra* | SNA | Siaα 2-6Galβ1-4Glc(NAc) | [241, 245-247] |
| *Pisum sativum* | PSA | Fucα1-6GlcNAc (core fucose), αMan, αGlc, GlcNAcα | [243, 248] |
| *Psophocarpus tetragonolobus* | PTII | D-GalNAcα-(1-3)DGal(β1-3/4)Glc (α-GalNAc, D-GalNAcα-(1-3)DGal) preference for α over β | [249, 250] |
| *Vicia faba* | VFA | Manα | [248] |

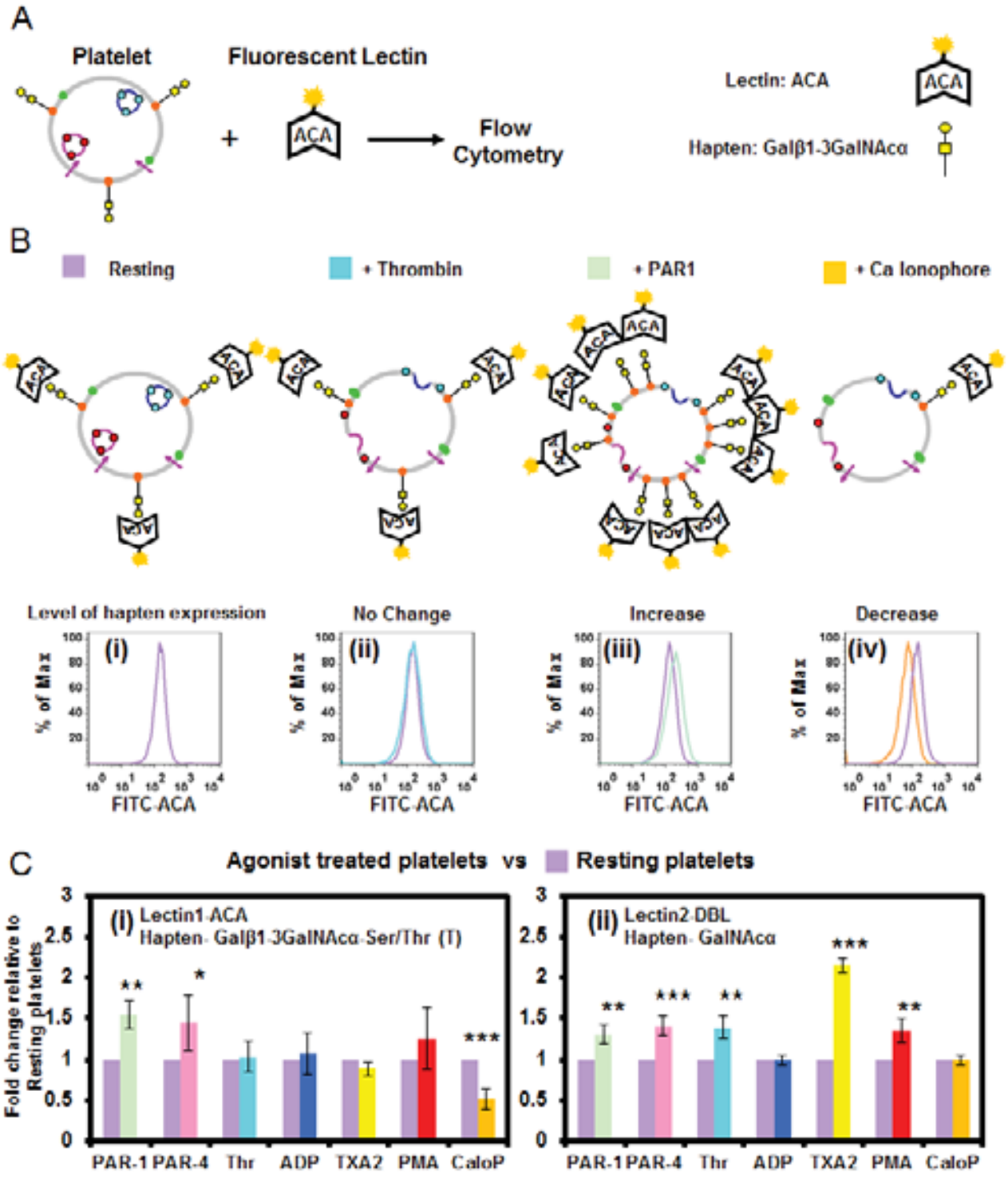
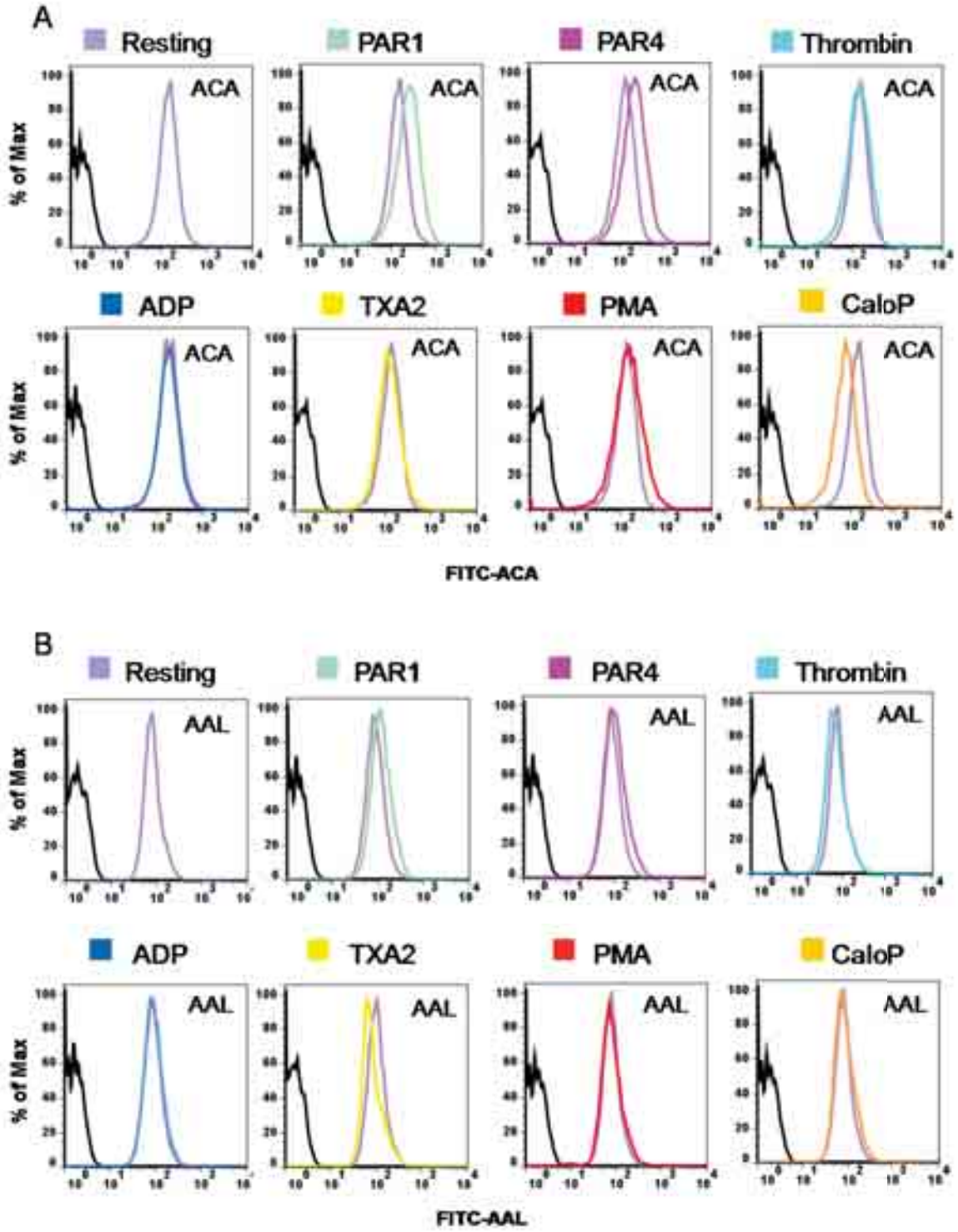
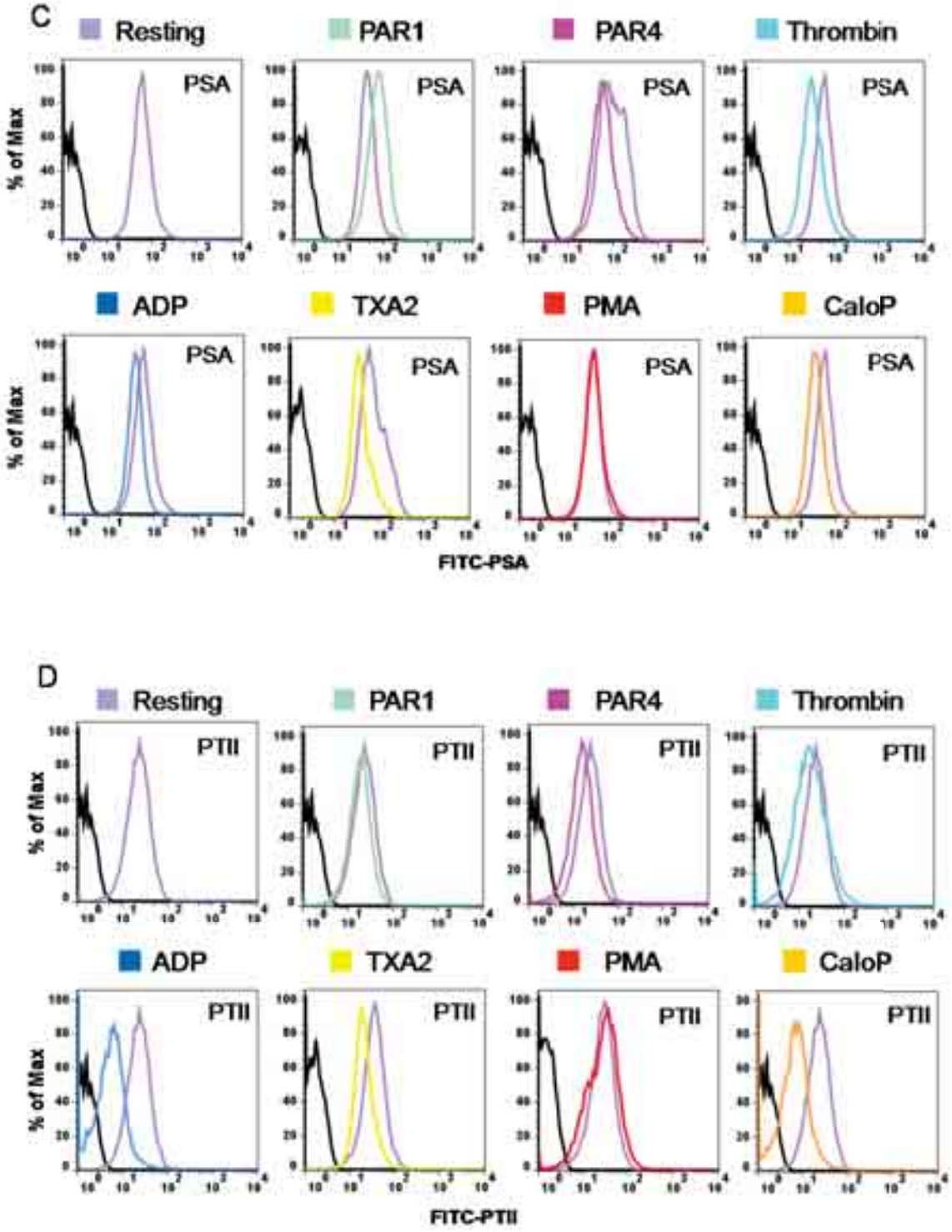
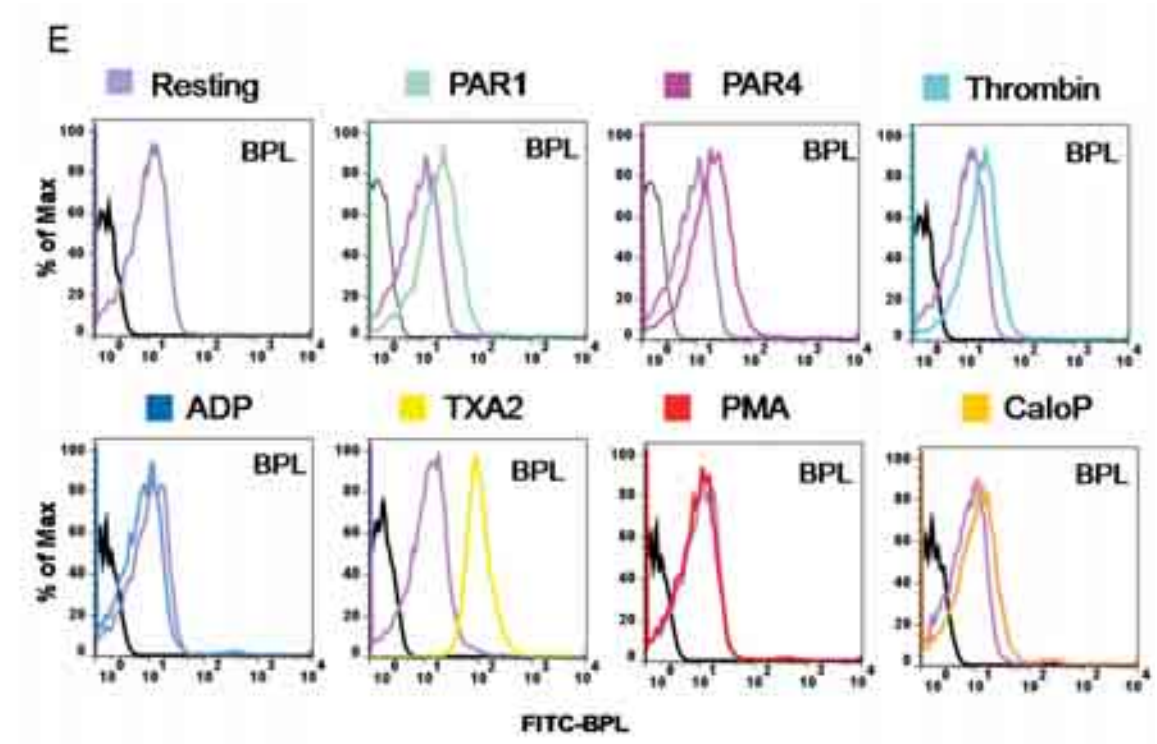
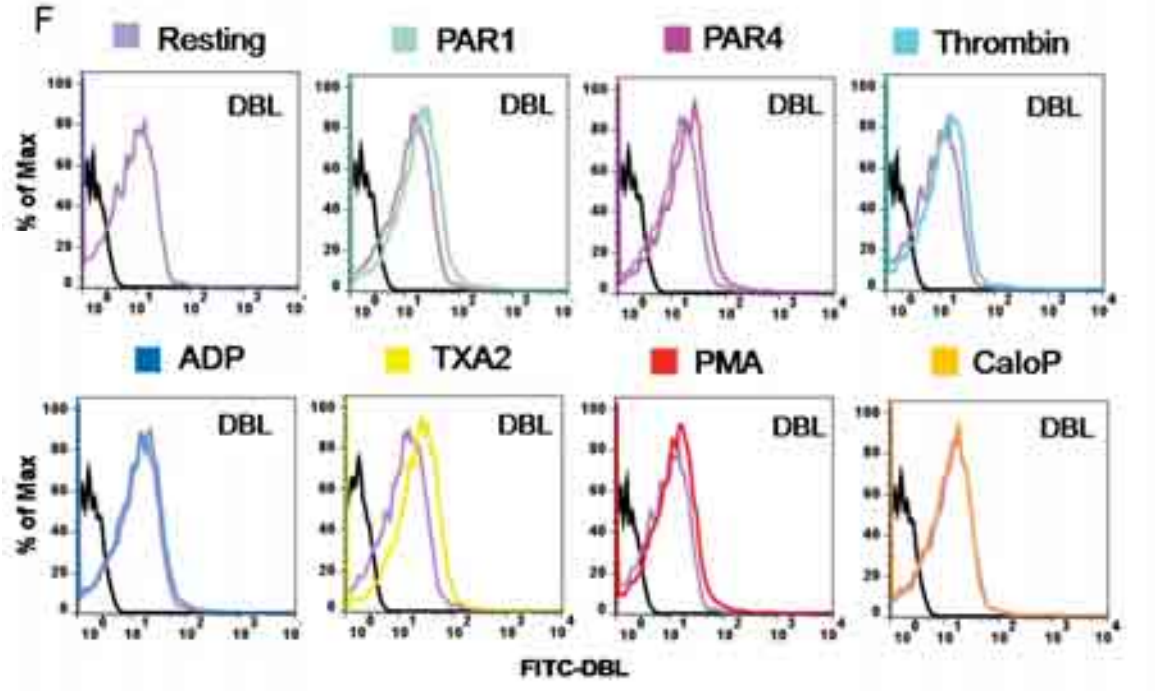

A
Platelet
Fluorescent Lectin
ACA
Flow Cytometry
Lectin: ACA
Hapten: Galβ1-3GalNAcα
B
Resting
+ Thrombin
+ PAR1
+ Ca Ionophore
Level of hapten expression
No Change
Increase
Decrease
% of Max
(i)
(ii)
(iii)
(iv)
FITC-ACA
C
Agonist treated platelets vs Resting platelets
Fold change relative to Resting platelets
(I) Lectin1-ACA
Hapten- Galβ1-3GalNAcα-Ser/Thr (T)
(II) Lectin2-DBL
Hapten- GalNAcα
PAR-1 PAR-4 Thr ADP TXA2 PMA CaloP

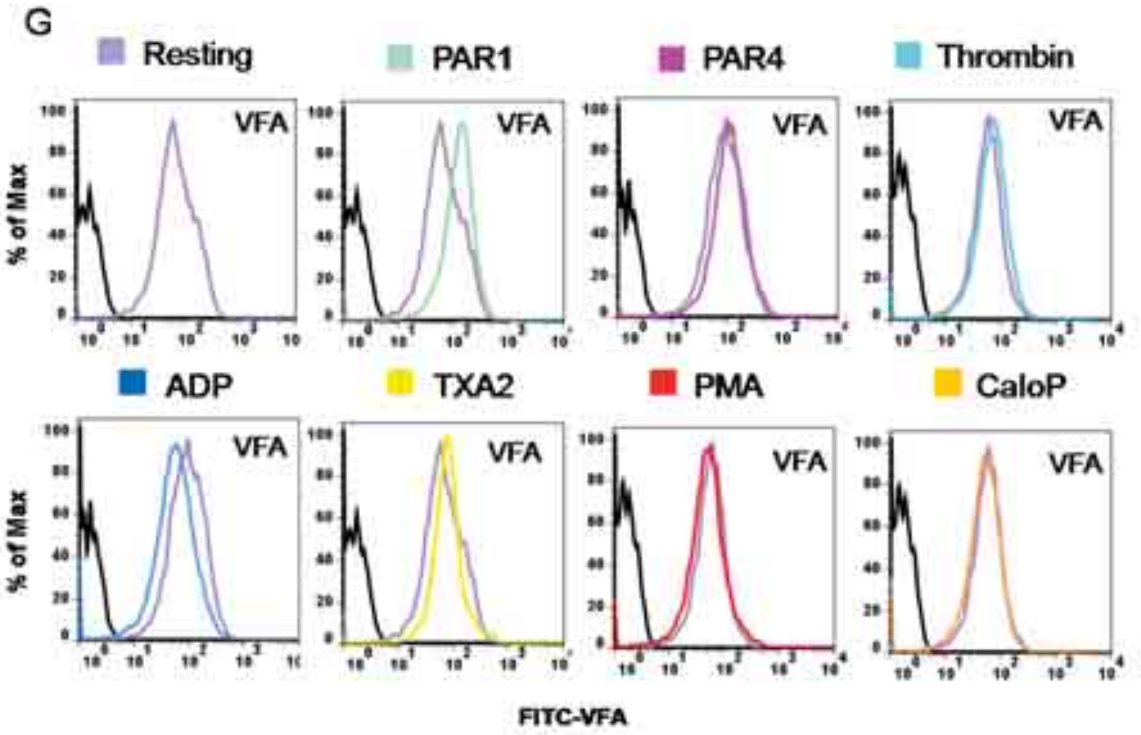
**Figure 4.1. Profiling platelet glycome with lectins. (A) Platelets were mixed with fluorescently tagged lectins and analyzed by flow cytometry. In this case, the ACA lectin is shown as an example. It binds to the Galβ1-3GalNAcα-Ser/Thr (T) structure. (B) Top: A schematic representation of platelet responses to treatment with different agonists (thrombin, PAR1 and calcium ionophore): no change in the level of the hapten expression (ii) as compared to the resting platelets (i), increase (iii), or decrease (iv). Bottom: the corresponding flow cytometry results depicting the relevant changes in the mean fluorescence intensities, MFIs. The histograms showing binding of ACA and other lectins to platelets treated with other agonists (PMA, PAR4, ADP and TXA2) are presented in Figure 4.2. (C) Quantification of the flow cytometry results, such as the ones shown in (B), for two different lectins: ACA (hapten: Galβ1-3GalNAcα-Ser/Thr) and DBL (hapten: GalNAcα-Ser/Thr). The results are represented as fold changes of MFI of the agonist treated platelets over the resting platelets; means of 4 independent experiment ± SD are shown. Statistical significance was evaluated using a 1-way ANOVA test with *p<0.05, **p<0.01 and ***p<0.001 vs resting platelets. These results show that the pattern of lectin binding to platelets depends on the agonist used to activate them. The quantified flow cytomtery results for binding pattern of other lectins to resting and agonist treated platelets are presented in Figure 4.3.**

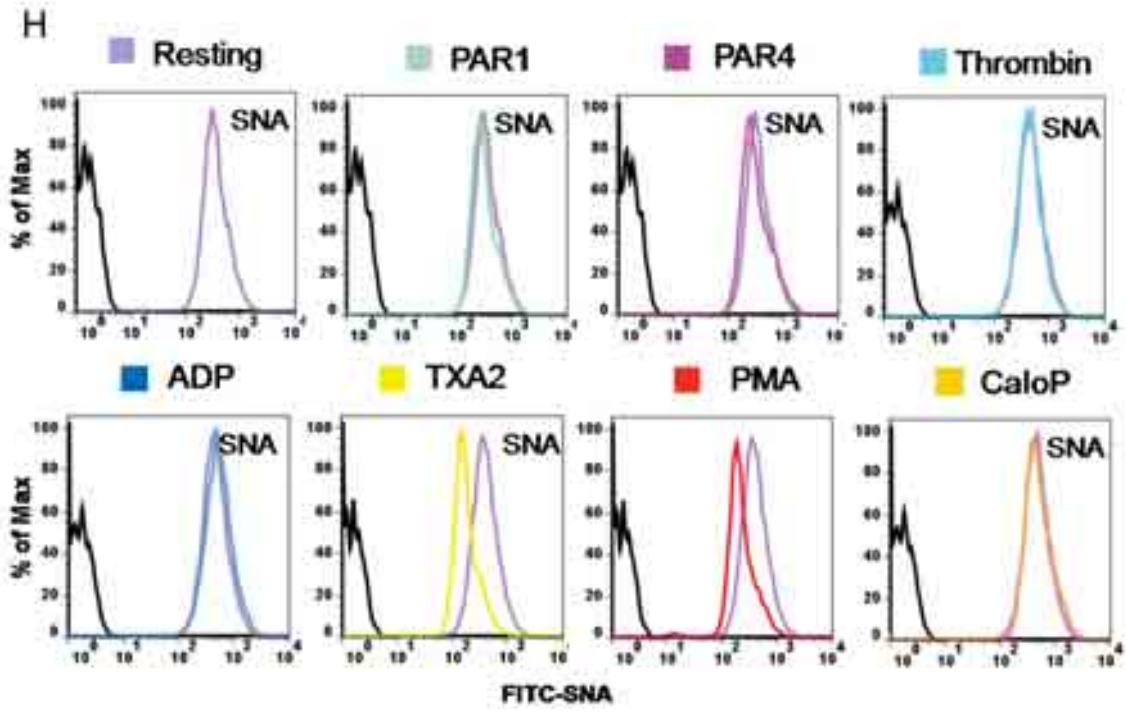

A
Resting
PAR1
PAR4
Thrombin
ACA
% of Max
ADP
TXA2
PMA
CaloP
FITC-ACA
B
AAL
FITC-AAL


C
Resting
PAR1
PAR4
Thrombin
ADP
TXA2
PMA
CaloP
PSA
% of Max
FITC-PSA
D
PTII
% of Max
FITC-PTII


E
Resting
PAR1
PAR4
Thrombin
ADP
TXA2
PMA
CaloP
BPL
% of Max
FITC-BPL


F
Resting
PAR1
PAR4
Thrombin
ADP
TXA2
PMA
CaloP
DBL
% of Max
FITC-DBL


G
Resting
PAR1
PAR4
Thrombin
ADP
TXA2
PMA
CaloP
VFA
% of Max
FITC-VFA


H
Resting
PAR1
PAR4
Thrombin
ADP
TXA2
PMA
CaloP
SNA
% of Max
FITC-SNA

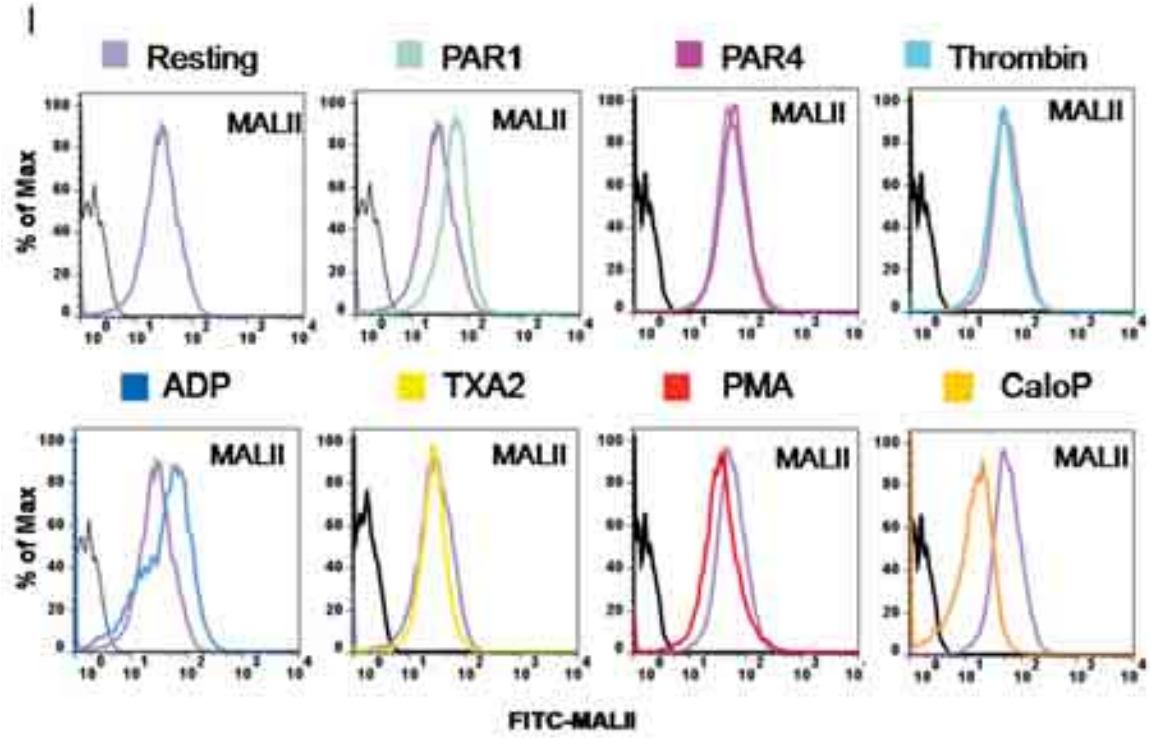



**Figure 4.2. Flow cytometry characterization of lectin binding to resting and agonist-treated platelets.**
**(A-I) Resting and agonist-treated platelets were incubated with various lectins and their mean fluorescence intensity (MFI) was determined using flow cytometry. The MFI represents the level of lectin binding. See Figure 4.1 for additional information. Lectin abbreviation is indicated on each histogram (A-I). Further information about the lectins can be found in Table 4.1. Agonist abbreviation is indicated above each histogram overlay. The histograms are also color-coded by agonist: purple for resting platelets, green for PAR1 treated platelets, pink for PAR4 treated platelets, fluorescent blue for thrombin treated platelets, royal blue for ADP treated platelets, yellow for thromboxane treated platelets, red for PMA-treated platelets and mustard for calcium ionophore treated platelets. The results show that binding levels of lectins to platelets change upon treatment of platelets to the agonists as compared to resting platelets (purple curves in histogram overlays).**

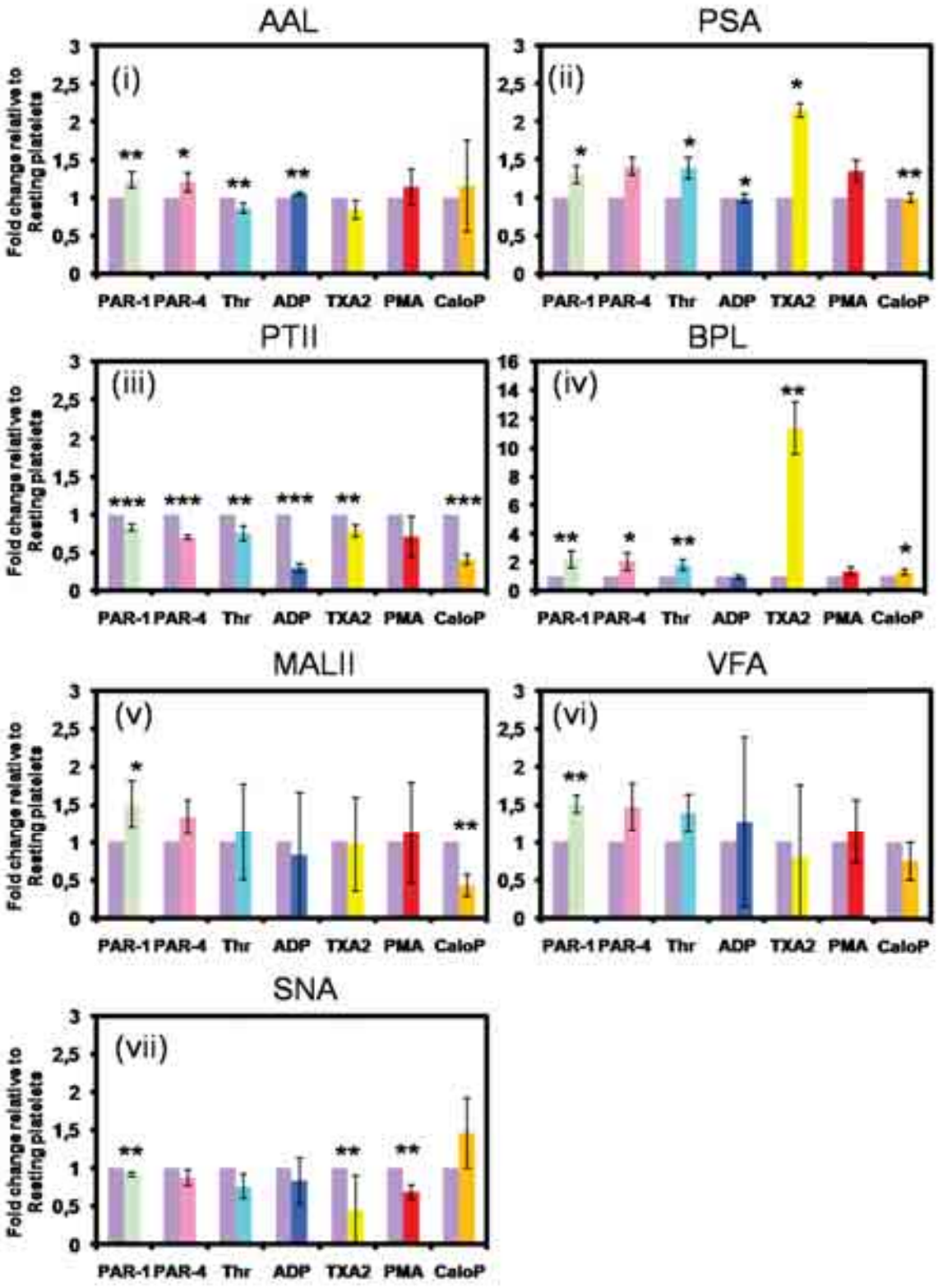

AAL
(i)
PSA
(ii)
PTII
(iii)
BPL
(iv)
MALII
(v)
VFA
(vi)
SNA
(vii)
Fold change relative to Resting platelets
PAR-1 PAR-4 Thr ADP TXA2 PMA CaloP

**Figure 4.3. Quantification of the flow cytometry results.**
**For each measurement, the MFI for the agonist-treated platelets was divided by the MFI for the resting platelets. The ratios (fold-changes) were averaged over at least four separate experiments and plotted in this figure (± standard deviation). Lectin abbreviations are indicated on each plot and agonists are listed along the X-axes. Statistical analysis was done for these fold changes using a 1-way Anova test to determine if the fold changes (upon agonist treatment) are significant relative to 1 (resting platelets) with *p<0.05, **p<0.01 and ***p<0.001 vs resting platelets. This figure shows the same data as shown in Figure 4.1 C, but for all the agonists and all the lectins.**

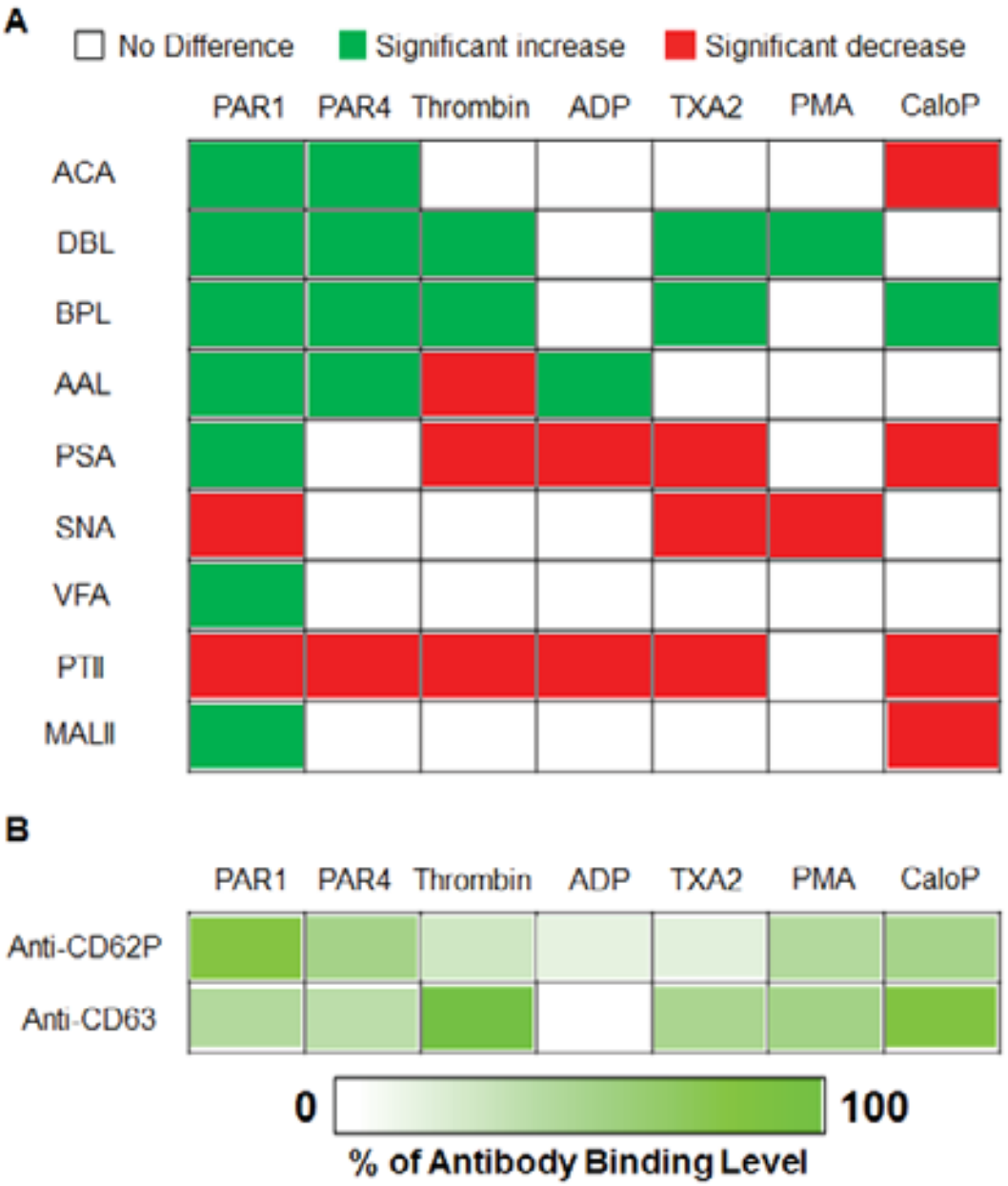



**Figure 4.4. Agonist-specific changes in the platelet surface lectin and antibody binding profiles. (A) A three-color heat map depicting a significant increase (green), a significant decrease (red), or no change (white), in the binding of the respective lectin to platelets treated with the respective agonist as compared with that to the resting platelets. Statistical significance ($p < 0.05$) was evaluated using a 1-way ANOVA test run on the ratios [MFI(agonist-treated platelets)/MFI(resting platelets)] relative to 1 [MFI(resting platelets)/MFI(resting platelets)], obtained from at least four separate experiments for each agonist/lectin pair. (B) The expression of two common platelet activation markers, CD62P and CD63, in response to agonist treatment, evaluated by measuring the binding of respective antibodies. Maximum antibody binding for each marker (PAR1 for CD62P and thrombin for CD63) was taken as 100%. Both markers are expressed in response to all the agonists. The level of expression depends on the agonist.**

### 4.2.3 Functional significance of changes in platelet glycosylation

Lectins specifically and selectively bind to particular carbohydrate sequences (haptens, Table 4.1. Therefore, changes in their binding to platelets that we observe upon agonist treatment reflect changes in platelet surface glycosylation. These may involve changes in the number of certain haptens expressed on the platelet surface or changes in their organization (clustering[251]) that cause changes in affinity for the lectin. Changes in the number of expressed haptens may arise from the transfer of glycoproteins between the interior of the cell and cell surface as well as from enzymatic modification of surface carbohydrates by the glycosyltransferases, which are known to be present in platelets.[252] These possibilities are schematically illustrates in Figure 4.5.

Surface carbohydrates mediate cell-cell and cell-matrix interactions via specific binding events between the carbohydrate haptens and endogenous lectins. Therefore, changes in the platelet surface glycosylation will affect their interactions with other cells and extracellular matrixes. Some of the effects are known (Table 4.2). Best-studied among them is the effect of surface glycosylation changes on platelet clearance (reviewed recently in ref.[253]), which includes the exposure and/or clustering of the β-GlcNAc-terminated glycans on the GPIbα receptor leading to the clearance by macrophages through the $\alpha_M\beta_2$ integrin binding, and the exposure of the terminal β-galactose or β-GalNac residues responsible for the clearance mediated by the hepatocyte Ashwell-Morrell receptors.[254] Activated, as well as damaged, platelets are rapidly cleared from the circulation via these pathways,[251] although other pathways exist, as well.[216] Of the lectins used in our study, *Bauhinia purpurea alba lectin* (BPL) and *Dolicos biflorus lectin* (DBL) are sensitive to haptens containing

terminal β-galactose and GalNac residues, respectively (Table 4.1). Significantly, the binding of both lectins to platelets increases upon treatment with PAR1, PAR4, thrombin, and thromboxane—all of the physiological agonists or analogues used in this study, except ADP. Treatment with ADP does not lead to a change in the level of binding of either of the two lectins, while treatment with the non-physiological agonists PMA and calcium ionophore cause discordant changes in their binding (Figure 4.4A).

An attempt to correlate agonist-induced glycosylation changes to one particular aspect of platelet physiology (clearance) would of course be overly simplistic, as numerous other processes with different time constants are expected to be at play. The hapten recognized by BPL, Galβ1–3GlcNAc, also serves as a binding site for galectin-1 (Gal1). Gal1 is an endogenous lectin with hemostatic and immunoregulatory functions[220, 255] that is expressed on the surface of activated endothelial cells and exposed subendothelium,[256] on tumors, atherosclerotic plaques, where it affects plaque growth and stability,[257] as well as on platelets themselves, where it appears to mediate ADP-induced aggregation.[255] It can trigger platelet activation by itself, but appears to act synergistically with thrombin and ADP, amplifying their effects and mediating platelet interactions with other cells (e.g., leukocytes).[255, 258]

Further complexity is revealed by considering that the exposure of terminal galactose and GalNac residues is thought to result at least partially from desialylation, because terminal sialic acid residues on mature glycans protect platelets, red blood cells, and blood glycoproteins, from clearance.[216, 254] *Sambucus nigra* lectin (SNA) is sensitive to the Siaα2-6Galβ1-4Glc(NAc) hapten, while *Maackia amurensis* (MAA) lectin is specific for the Siaα2-3Galβ1-4Glc(NAc) hapten (Table 4.1). We do find a

decrease in the SNA binding to platelets treated with PAR1 agonist, thromboxane, and PMA, and in these instances it is correlated with an increase in BPL binding, DBL binding, or both (Figure 4.4A). However, we also find situations where there is no change in the binding of SNA while there are changes in the binding of DBL, BPL, or both (Figure 4.4A). This suggests that the increase in the galactose and/or GalNac residues may occurs not only as a result of desialylation, but as a result of the appearance of immature glycans. The possibility of this mechanism is mentioned in ref.[253]

Sialic acid can occur in a 2-3 or a 2-6 linkage. While SNA is sensitive to the 2-6 linked sialic acid, MAA is specific for the 2-3 linked residue (Table 4.1). In the case of platelets treated with PAR1 agonist, the binding of SNA decreases while the binding of MAA increases, suggesting a change in the balance of differently linked sialic acid residues on the platelet surface. Wandall et al. did report an increase in platelet surface sialylation upon treatment with TRAP, another PAR1 agonist, but in that study 2-3 vs 2-6 linkages were not distinguished.[252]

Further illustrative examples of functions associated with various carbohydrates or correlated with lectin binding are compiled in the Table 4.2. A wider range of lectins, kinetic studies, and complementary measurements of platelet interactions with other cells and extracellular matrixes are needed to understand the changes we observe in functional terms. It is clear, however, that our results reveal agonist-specific differences in platelet functionality.

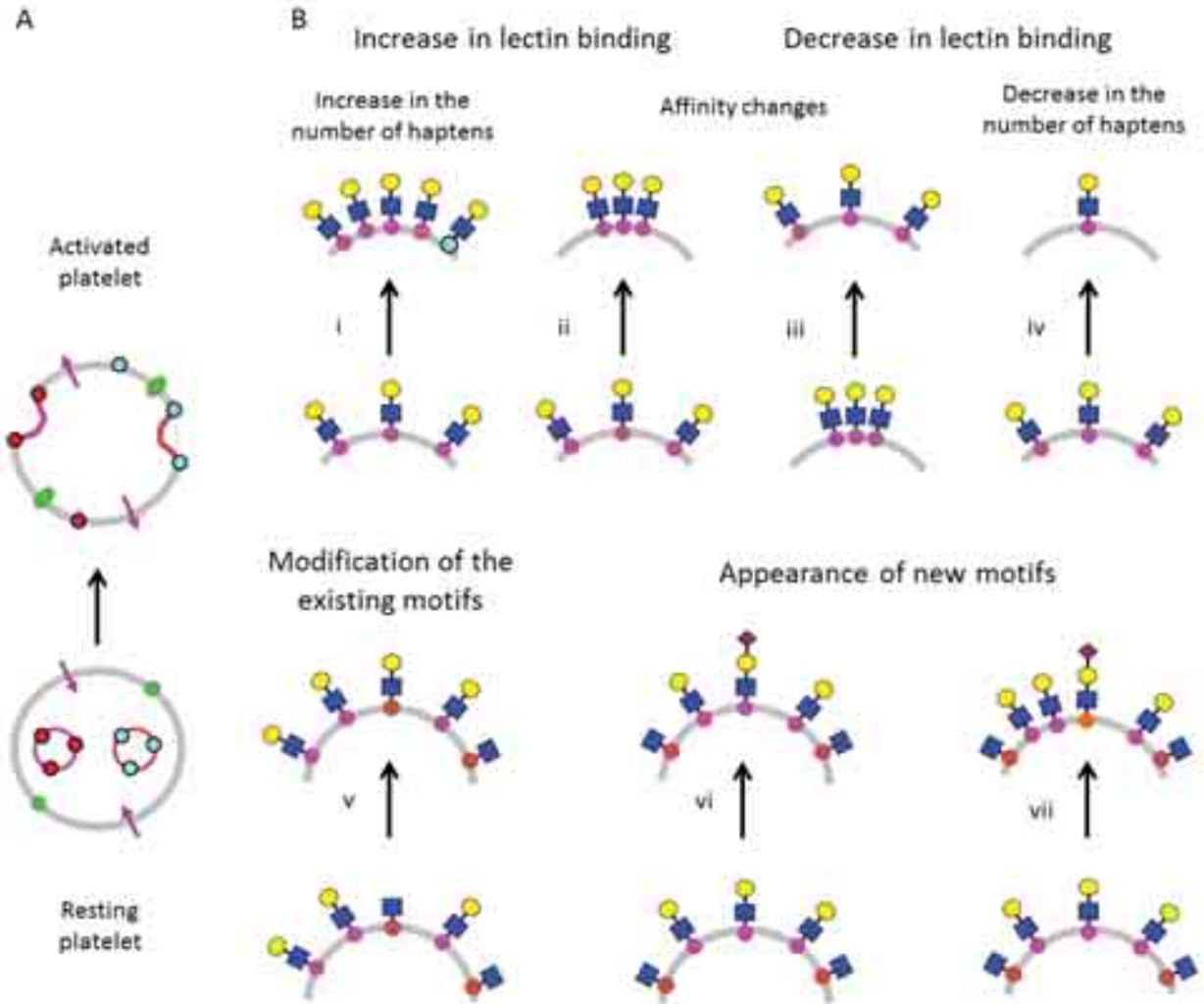

A
B
Increase in lectin binding
Decrease in lectin binding
Increase in the number of haptens
Affinity changes
Decrease in the number of haptens
Activated platelet
i
ii
iii
iv
Modification of the existing motifs
Appearance of new motifs
v
vi
vii
Resting platelet

**Figure 4.5. Mechanistic links between the changes in the lectin binding levels and glycosylation patterns.**

**(A) Resting (bottom) and activated (top) platelets. Platelet membrane is shown in grey. Purple arrowhead refers to phosphatidyl serine (PS), the phospholipid that is asymmetrically distributed (facing inwards) in the membranes of resting platelets but is facing outwards in the membranes of activated platelets. PS catalyses clot formation. Green spheres refer to GPIIb-IIIa integrin in the inactive conformation on the resting platelets. Green ellipses designate the active conformation of this protein. Granules (α, purple, and dense, brown), with their respective markers (CD62P, red, and CD63, turquoise) are shown inside the resting platelet. They undergo exocytosis upon activation, and the two markers appear on the cell membrane. As we discuss in the main text (see Figure 4.4B), these activation-related events are similar for all agonists and are therefore omitted from (B) below for clarity. It should be understood, however, that on all agonist-treated platelets these changes occur *in addition* to the glycosylation pattern changes shown in (B).**

**(B) Eight possible changes in the platelet surface glycosylation patterns upon treatment with agonists are shown in this figure. In each diagram, the resting platelet is below and the agonist-treated platelet is above. Variously coloured circles in the platelet membrane represent glycoproteins. Structures projecting outside the membrane represent the carbohydrate moieties of these glycoproteins. Yellow coloured circles: Galactose (Gal); blue squares: N-acetylglucosamine (GlcNAc); brown rhombi: N-acetylneuraminic acid (sialic acid).**

- **Increase in lectin binding can be due to an actual increase in the number of haptens (i) as new glycoproteins are brought to the platelet surface or due to clustering of existing haptens (ii) that leads to an increase in the avidity for the corresponding lectin. The most prominent example of the latter mechanism is clustering of the GPIbα receptor carrying the βGlcNAc hapten. The clustering leads to the rapid clearance of chilled platelets through the lectin domain-containing $\alpha_M\beta_2$ integrin receptors on the macrophages of the reticuloendothelial system. [251]**
- **Decrease in lectin binding can similarly be due to dissociation of clusters (iii) or actual loss of haptens (iv) through endocytosis or shedding of microparticles. For example, platelets internalize GPIb upon stimulation with most of the agonists (thrombin, PAR1 and PAR4 peptide agonists, ADP, PMA and TXA2).[259] This process is reversible for some of the agonists (thrombin, PAR1 and PAR4 receptor agonists, and ADP) but not for others (PMA and TXA2).[260] Moreover, the kinetics (extent and rate) of return of the GPIb to the platelet surface varies depending on the concentration and type of agonist.[260] On the other hand, platelets shed CD62P-expression microparticles, leading, over time, to a decrease in the expression of this glycoprotein on the platelet surface.[261]**
- **Modifications of the existing carbohydrates by glycosyltransferases[252] may lead to an increase in the number of some haptens at the expense of other haptens (v). As shown in panel (v), the removal of galactose residues from the Gal-GlcNAc haptens results in a decrease in the number of of Gal-GlcNAc haptens expressed and a corresponding increase in the number of GlcNAc haptens expressed on the cell surface. An example of this mechanism is the exposure of galactose residues on platelet surfaces due to desialylation, leading to platelet clearance through the hepatocyte Ashwell-Morell receptors (asialoglycoprotein receptors, ASGPR),[217] and vice-a-versa.[262] Note that this process leads to an increase in the amount of existing haptens on the platelet surface, not to the appearance of new haptens.**

- **Essentially new haptens may also appear on the platelet surface, either through the action of glycosyltransferases (vi) or on the newly-expressed glycoproteins (vii). While we do not observe any evidence of this mechanism in our study, others have reported such events in T and B cells upon activation. [225]**

**Table 4.2. Functional significance of the expression of particular carbohydrates on cell surfaces inferred from the literature.**

In this table, we highlight the functional relevance of certain cell surface carbohydrates that also serve as haptens for various lectins and/or indicate correlations between lectin binding and functionality.

| Hapten and receptor | Lectins used for characterization | Effect and comments | Ref. |
|---|---|---|---|
| Terminal sialic acid residues on cell surface glycans | | Protect red blood cells and platelets from clearance by the macrophages of the reticuloendothelial system (RES); protect platelets from clearance by through the lectin asialoglycoprotein receptors on hepatocytes (ASGPR, Ashwell-Morell receptors) and resident liver macrophages (Kupffer cells). | [217, 254, 263] |
| | MALII<br>SNA | Loss of terminal sialic acid residues serves as an "eat me" signal on apoptotic lymphocytes. | [264] |
| Terminal β-GlcNAc on the platelet GPIbα receptor N-glycan. | | Platelet clearance by hepatic macrophages of the RES through binding to the $\alpha_{M\beta2}$ integrin upon clustering of the GPIbα. | [215, 216] |
| Terminal βGalactose exposed due to desialylation:<br>• On the platelet GPIbα receptor N-glycan.<br>• On plasma VWF. | RCA and ECA | Platelet and vWF clearance through the hepatocyte Ashwell-Morell receptors (ASGPR).<br>Increased binding of VWF to GPIb causes platelet activation and also accelerates their clearance. | [216, 217, 254, 265] |
| | | Sialic acid is also involved in the VWF–GPIb-IX-V interactions. | [217] |

| | | | |
|---|---|---|---|
| Terminal sialic acid (e.g., on sLeX, sLacNAc of the GPIbα) | | Binding site for the SSL5 toxin from *Staphylococcus aureus.* Cause of thromboembolitic complications of *S. Aureus* infection such as disseminated intravascular coagulation, or DIC. | [266] |
| Increased sialylation of platelets surface glycans | | Diminishes platelet responsiveness to activation (for example in response to TRAP)-reduced CD62P expression. | [252] |
| | LcH, ConA, WGA, RCA | Identification of platelet surface glycoproteins on polyacrylamide gels.<br>LcH and ConA: bind GPIb, GPIIb, GPIIIa and GPIV (IIIb)<br>WGA: GPIb<br>RCA: no targets found. | [267] |
| Defucosylation (removal of the terminal α-L-Fucose) | | Reduces tumor cell adhesiveness to a wide variety of ECM components, endothelial cells, and selectin matrixes. Impaired interactions with external milieu. | [268] |
| | An array of 94 lectins | Lectin array was used to profile and distinguish between cell surface glycosylation of 24 human cell lines.<br>The same lectin array was used to distinguish between B cells (resting and activated), thymocytes, and T cells (resting, PMA/Ionomycin- and allogeneically activated).<br>Lectins with more complex haptens provide better selectivity. | [225] |
| | ConA, LCA, SNA, PHA-L | Lectin binding was used to differentiate between endothelial cells from different vascular beds based on their glycosylation patterns. This correlated with monocyte adhesiveness to these cells. | [269] |
| | ConA, PHA-L, HPA, SBA, MAA, SNA, WGA | Differences in carbohydrate expression patterns were observed on different human breast cancer cell lines. These correlated with metastatic potential and target-tissue preference. | [222] |
| α(2,6)-linked sialic acid<br>N-β1-6GlcNAc<br>Galβ1,3GalNAc | SNA<br>ECA, PHA<br>PNA | Carbohydrate expression profiles on osteolytic and non-osteolytic breast cancer cell lines are different.<br>Increased expression of α(2,6)-linked sialic acid, N-β1-6GlcNAc, sialylated Lewis-A antigen (sLe A) and decreased expression of Galβ1,3GalNAc (T antigen), on osteolytic cell line. | [270] |

| | | | |
|---|---|---|---|
| | | These differences affect binding to bone marrow endothelial cells and ECM, ultimately leading to differences in the invasive potential (ability to metastasise to bone). | |
| Unsialylated (Galβ1,3GalNAcα-) disaccharide (oncofetal Thomsen-Friedenreich antigen, or TF). | Galectin-3 | Galectin-3, elevated in the sera of patients with certain types of cancers, mediates adhesion between cancer cells and endothelial cells promoting metastasis, by binding to TF on cancer cells. | [271] |
| Galβ1,4-GlcNAc | Galectin-1 | Binding of galectin-1 to platelets via the $\alpha_{IIb}\beta_3$ integrin (GPIIb/IIIa) triggers platelet activation. | [272] |
| HIV-1 infection of T cells results in altered glycosylation patterns of CD43 and CD45: loss of sialic acid, increased expression of core 2 *O*-glycans and polylactosamine sequences. | SNA, PNA, MAA, ECA, STA, LEA, GNA and GS-I-B4 | These oligosaccharides are recognized by endogenous galectin-1. The normal function of galectin-1 is to induce activated lymphocyte cell death. | [273] |

### 4.2.4 Platelet activation by lectins and competition between lectin and antibody binding.

We felt it prudent to examine how our fingerprinting results might be affected by the ability of some lectins to activate platelets,[220, 221] which for plant lectins has been known at least since the 70s of the last century. To do so, we looked at the expression of a common platelet activation marker, CD62P, upon treatment of platelets with the lectins. As a positive control, we used platelets treated with the PAR1 agonist (c.f. Figure 4.4B), while platelets treated with BAPTA-AM, a membrane-permeable calcium chelator that prevents platelet activation upon agonist stimulation, were used as a negative control (Figure 3.15 in Chapter 3). The results of this study are shown in Figure 4.6, where it can be seen that treatment of platelets with lectins resulted in three types of responses.

Treating platelets with DBL (panel i in Figure 4.6), BPL, ACA, or PSA (panels iv – vi in Figure 4.6) did not lead to positive staining of the platelets with the anti-CD62P antibody. These lectins also did not interfere with the ability of the antibody to bind to CD62P in platelets treated with the PAR1 agonist. These lectins do not activate platelets.

AAL (panel ii in Figure 4.6) and SNA (panel vii in the Figure 4.6), interfered with the detection of CD62P expression in the PAR1 agonist-treated platelets. Because no binding of anti-CD62P antibody could be detected when platelets were treated with lectins from this group, it is not possible to ascertain whether these lectins activated

platelets or not, but we view this possibility as unlikely because of the results obtains with the lectins from the third group.

Lectins from the third group included VFA (panel iii in Figure 4.6), MAA, and PTII, (panels viii and ix in Figure 4.6). They both induced CD62P expression in platelets and interfered with the binding of anti-CD62P antibody to the PAR1-agonist treated platelets. These lectins activate platelets, and in fact, they are not very efficient in distinguishing between agonists (Figure 4.4A).

To better understand the nature of interference between some of the lectins and the anti-CD62P antibody, we checked whether the reverse was true: whether antibody binding would interfere with the lectin binding, as well. This was done by comparing lectin binding to platelets previously incubated with the anti-CD62P antibody with that in platelets that were not incubated with it. The results are shown in Figure 4.7. VFA (Figure 4.7B) and PTII (Figure 4.7D) interfered with the anti-CD62P antibody binding, while AAL (Figure 4.7A) and SNA (Figure 4.7C) did not. We conclude that CD62P carries glycans containing the haptens for VFA and PTII (Table 4.1), but not AAL or SNA. Therefore the interference between the binding of AAL, SNA, and the anti-CD62P antibody to the platelets must have a different origin. For example, these lectins may cause glycoproteins to reorganize in the membrane, hindering the ability of the antibody to bind to CD62P.

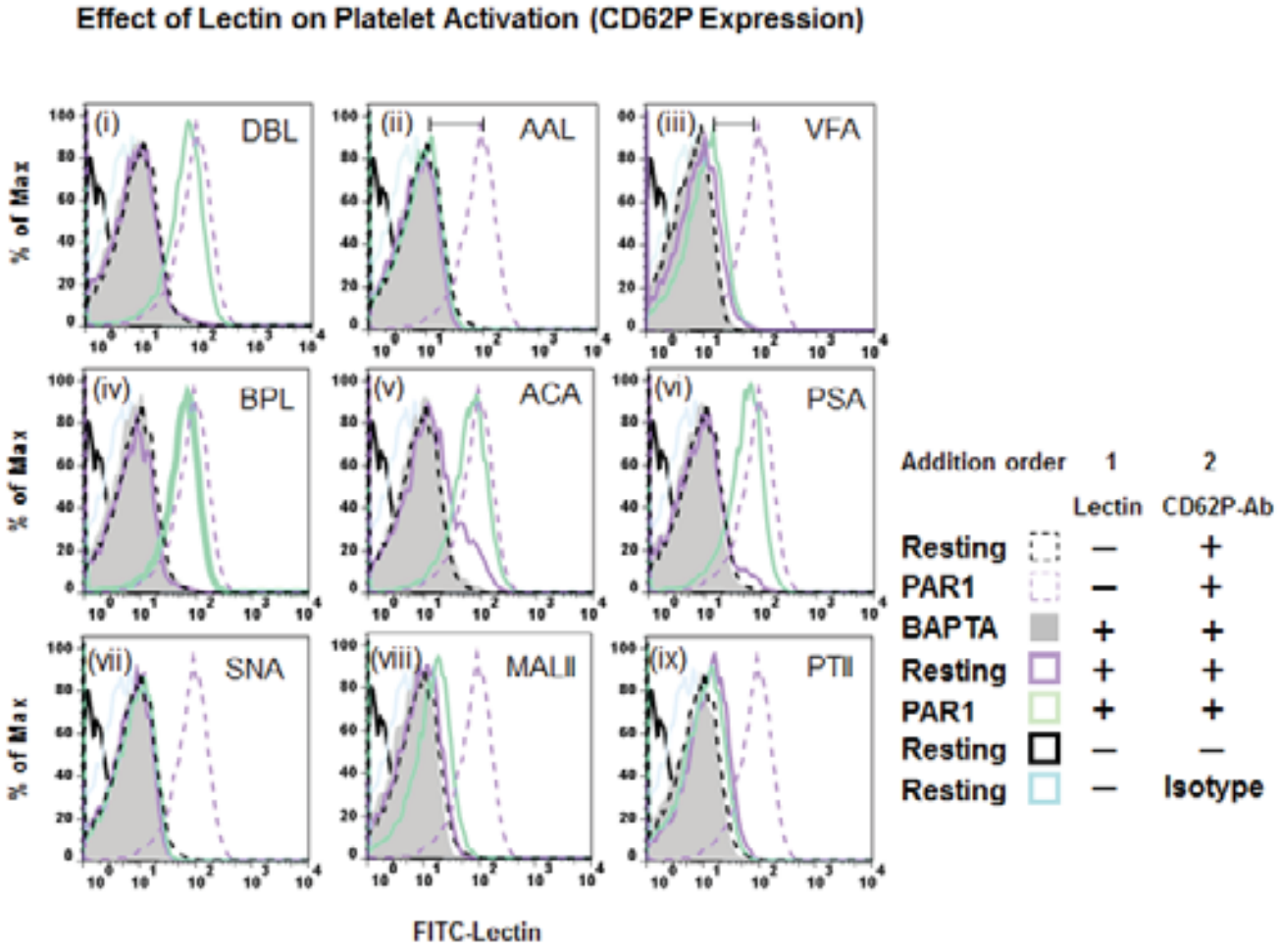



**Figure 4.6. Platelet Activation by lectins.**
**Effect of lectin binding on the expression of platelet activation marker CD62P. Platelets (resting, treated with BAPTA, or with PAR1 agonist) were further treated with one of the nine lectins followed by staining with the APC-anti-CD62P antibody as shown in the legend, and analyzed by flow cytometry to compare surface expression of CD62P marker. Three types of responses were differentiated based on the overlap (i, iv-vi) or lack thereof (ii, iii, vii-ix) between the dashed purple and green histograms, and the overlap (i, ii, iv-vii) or lack thereof (iii, viii and ix) between the purple, grey, and dashed black histograms, and the overlap (ii and vii) or lack thereof (i, iv-vi, iii, viii and ix) between green and purple histograms.**

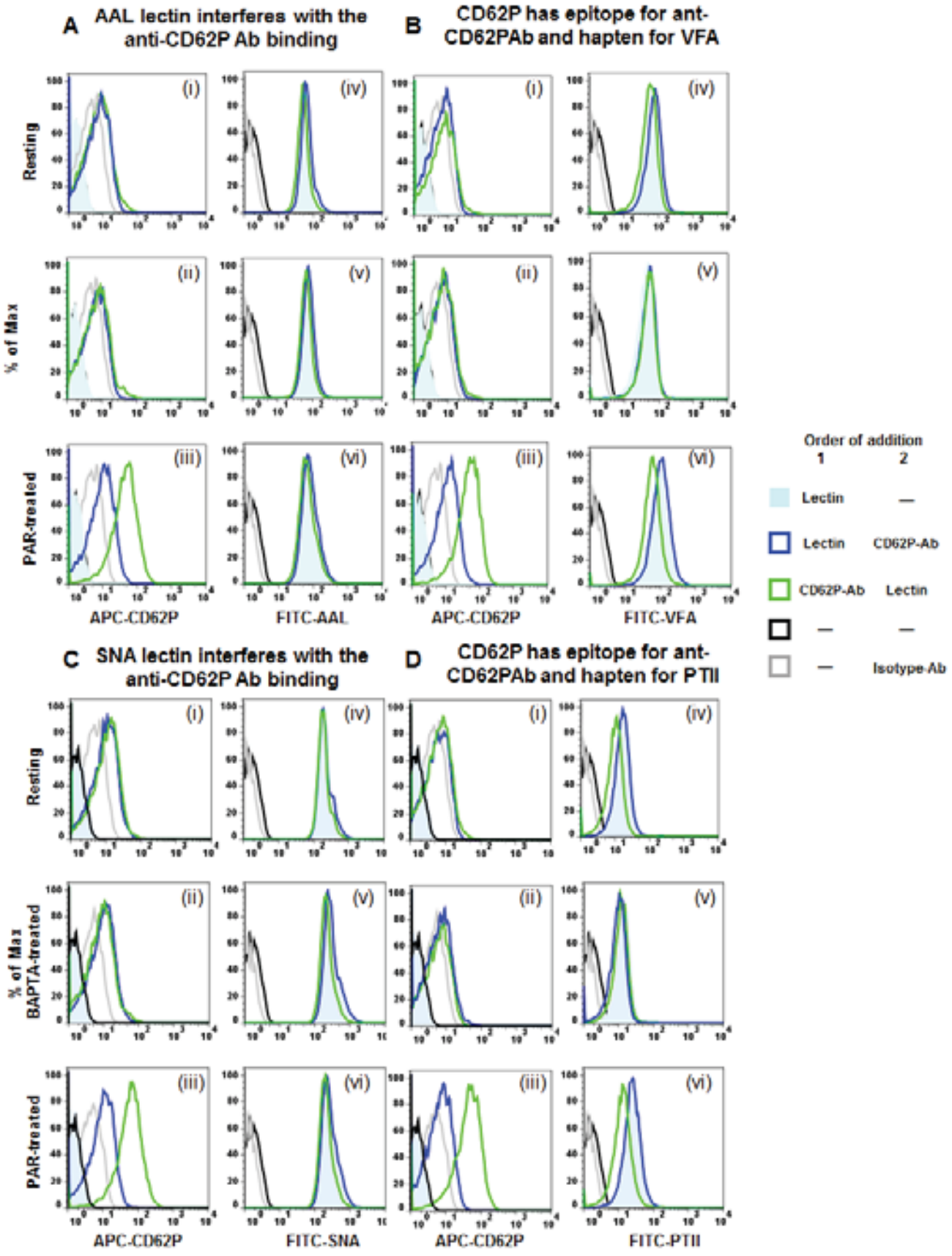

A
AAL lectin interferes with the anti-CD62P Ab binding
B
CD62P has epitope for ant-CD62PAb and hapten for VFA
Resting
% of Max
PAR-treated
APC-CD62P
FITC-AAL
APC-CD62P
FITC-VFA
Order of addition
1
2
Lectin
—
Lectin
CD62P-Ab
CD62P-Ab
Lectin
—
—
—
Isotype-Ab
C
SNA lectin interferes with the anti-CD62P Ab binding
D
CD62P has epitope for ant-CD62PAb and hapten for PTII
Resting
% of Max
BAPTA-treated
PAR-treated
APC-CD62P
FITC-SNA
APC-CD62P
FITC-PTII

**Figure 4.7. The mechanism of interference between lectin binding and antibody staining of CD62P. Resting, BAPTA-AM- or PAR1 agonist-treated platelets were treated with FITC-labeled AAL (A) or VFA (B) or SNA (C) or PTII (D) and the APC-anti-CD62P antibody. Antibody treatment either followed (dark blue) or preceded (green) lectin treatment. For both lectins, lectin binding interfered with the antibody binding to the PAR1-treated platelets (panel iii in A, B, C and D), but only in the case of VFA (B) and PTII (D) did the antibody binding interfere with the lectin binding (panel vi).**

### 4.2.5 Analyzing platelet surface glycosylation and its changes upon agonist stimulation using lectin arrays

Lectin array is a high throughput assay that allows characterization of a binding pattern of multiple lectins [223] to platelets simultaneously as opposed to flow cytometry technique which allows only single lectin analysis at a time. Lectin arrays used in this study were prepared by Juan Echevarria (the group of Niels-Christian Reichardt, CIC biomaGUNE), using the protocol described in materials and methods chapter (section and page to be added).[125, 126] The design of the lectin array is presented in Figure 4.8. Each lectin array consists of 62 lectins spots printed in the following order- ConA (lectin 1), WGA, RCA, ECA, SNA, MAL-I, AAL, PSA, LCA, GNA, NPL, BSII, PHA, JAC, WFL, PNA, VVL, LTL, BS-I, PT-I, LEL, ACA, BS-I(EY), SJA, GML-SBA, STL, AIA (JAC), BPL, PT-II, EEA, MPA, HHL, PAL, ABL, DSL, HAL, AHP, LPL, CFL, PWA, BSI-B4, CAL, DBL, HPL, VFA, PHAL, PHAE, PMA, ASA, SSA, HMA, UEA-I (EY), LFA, Calsepa, Moa, LAL, AAA, LBA, MAL-II and AOL (lectin 62). In each array, these lectins were printed in 6 replicates as 6 × 3 matrixes in a well format (Figure 4.8).

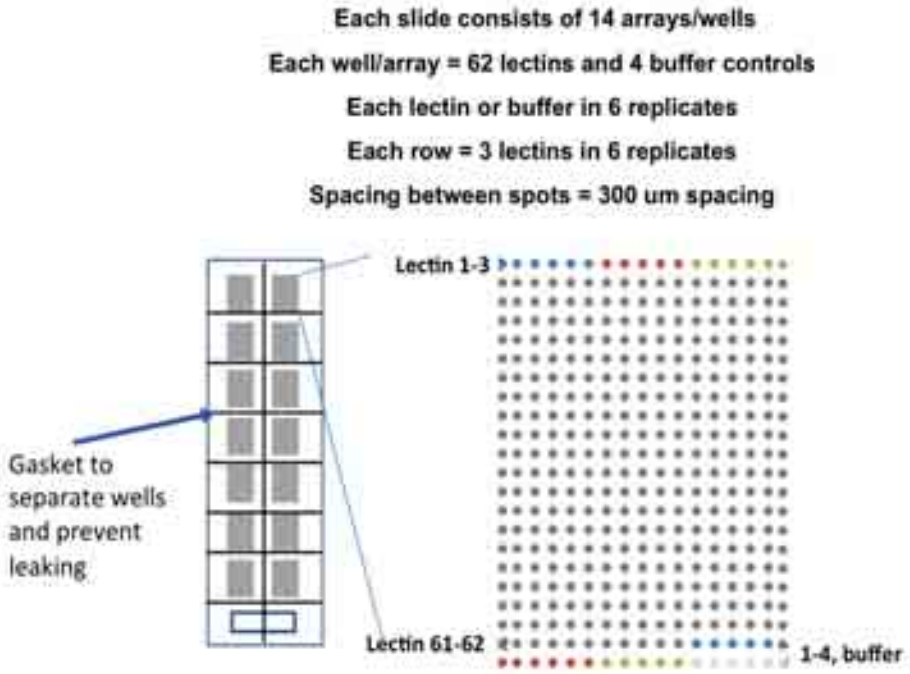



**Figure 4.8. Lectin array design.**

**Table 4.3. List of plant lectins used for fabrication of lectin arrays and their corresponding haptens.**

| Lectin number | Lectin | Hapten specificity | Lectin number | Lectin | Hapten specificity |
|---|---|---|---|---|---|
| 1 | ConA | Man | 35 | VVL | T antigen |
| 2 | GNA | Man | 36 | JAC | T antigen |
| 3 | NPL | Man | 37 | AIA (JAC) | T antigen |
| 4 | HHL | Man | 38 | ABL | T antigen |
| 5 | VFA | Man | 39 | ACA | T antigen |
| 6 | PMA | Man | 40 | AHP | T antigen |
| 7 | ASA | Man | 41 | MPA | T antigen |
| 8 | Calsepa | Man | 42 | PT-II | a-Gal |
| 9 | WGA | GlcNAc | 43 | BS-I | a-Gal |
| 10 | BSII | GlcNAc | 44 | BS-I (EY) | a-Gal |
| 11 | LEL | GlcNAc | 45 | BPL | a-Gal |
| 12 | STL | GlcNAc | 46 | PAL | a-Gal |
| 13 | PWA | GlcNAc | 47 | EEA | a-Gal |
| 14 | RCA | Gal, LacNAc | 48 | Moa | a-Gal |
| 15 | ECA | Gal, LacNAc | 49 | BSI-B4 | a-Gal |
| 16 | PHA | Gal, LacNAc | 50 | CFL | GalNac |
| 17 | PHA E | Gal, LacNAc | 51 | HPL | GalNac |
| 18 | PHA L | Gal, LacNAc | 52 | SJA | GalNac |
| 19 | CAL | Gal, LacNAc | 53 | GML-SBA | GalNac |
| 20 | MAL-II | Gal, LacNAc | 54 | DBL | GalNac |
| 21 | SNA | Sial | 55 | DSL | GalNac |
| 22 | MAL-I | Sial | 56 | SBA | GalNac |
| 23 | LFA | Sial | 57 | PNA | GalNac |
| 24 | LPL | Sial | 58 | WFL | GalNac |
| 25 | HMA | Sial | 59 | SSA | GalNac |
| 26 | AAL | Fuc | 60 | HAL | GalNac |
| 27 | UEA-I | Fuc | 61 | LBA | GalNac |
| 28 | UEA-I (EY) | Fuc | 62 | PT-I | GalNac |
| 29 | PSA | Fuc | | | |
| 30 | LCA | Fuc | | | |
| 31 | LTL | Fuc | | | |
| 32 | LAL | Fuc | | | |
| 33 | AAA | Fuc | | | |
| 34 | AOL | Fuc | | | |

The lectins used for fabrication of these lectin arrays, together with their haptens, are listed in the Table 4.3.[125] The schematic of the experiment is shown in Figure 4.9 using the resting platelets as an example.

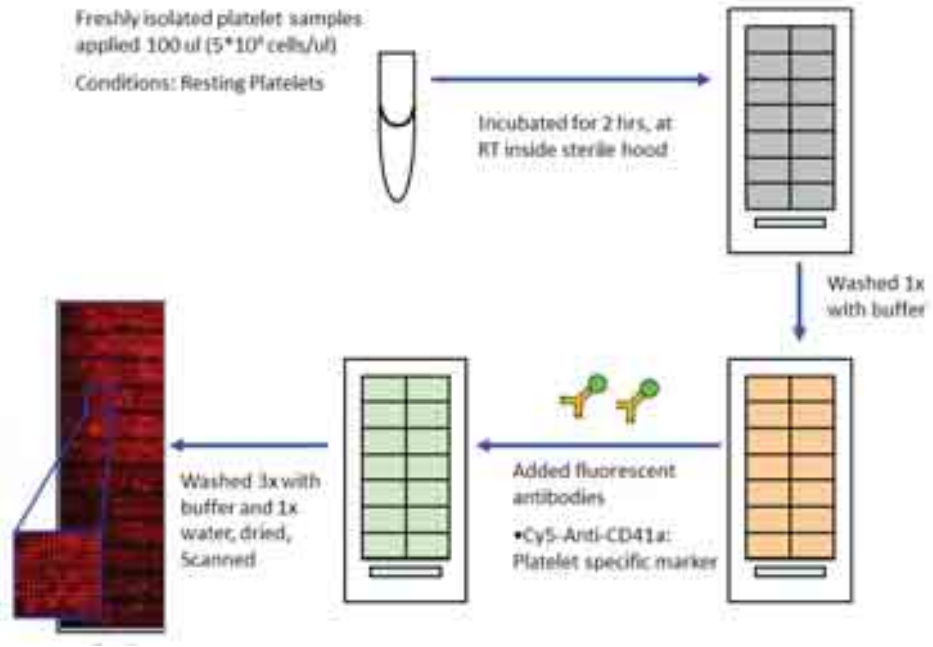



**Figure 4.9. Experimental set up for lectin array studies of platelets.**

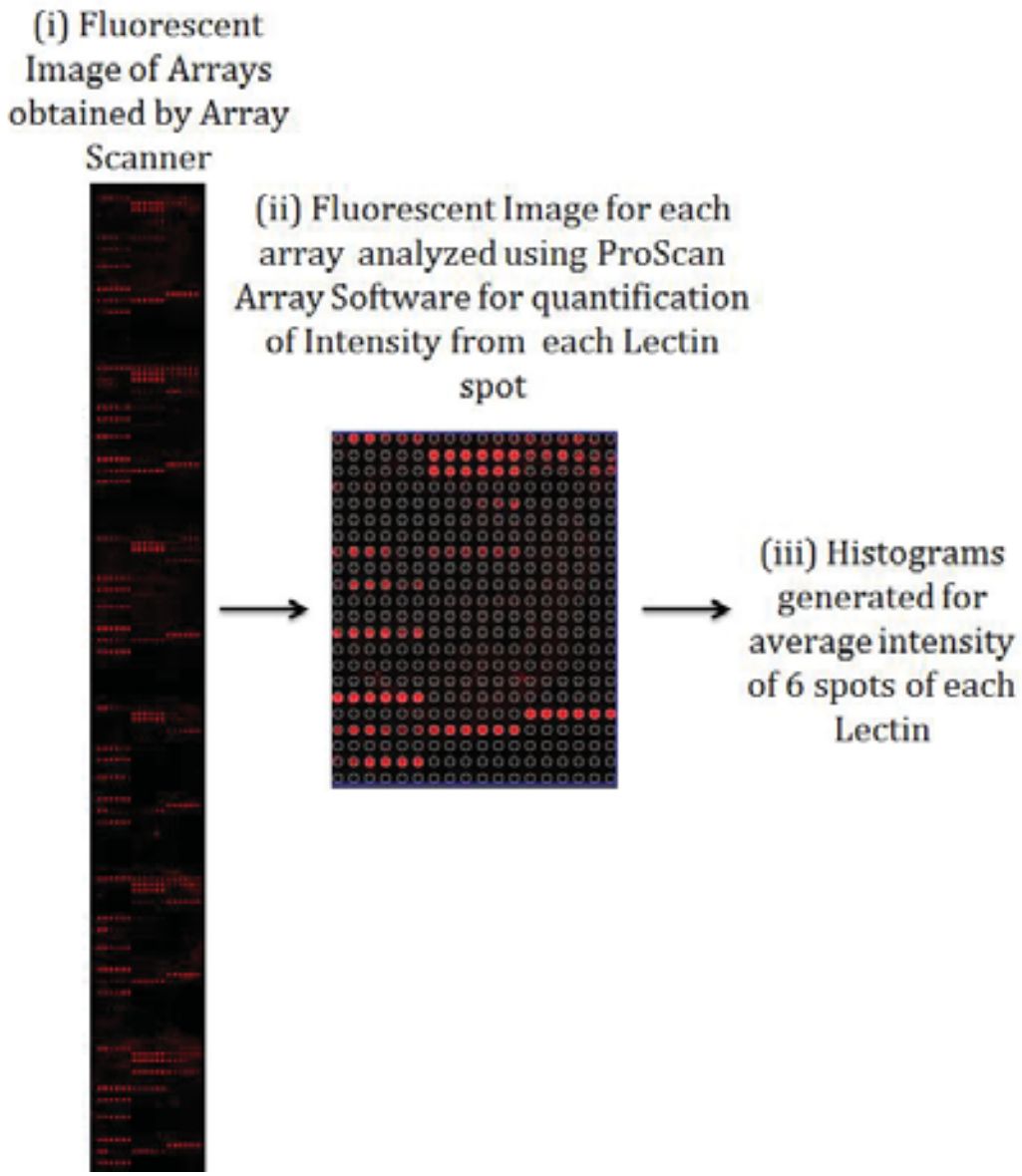


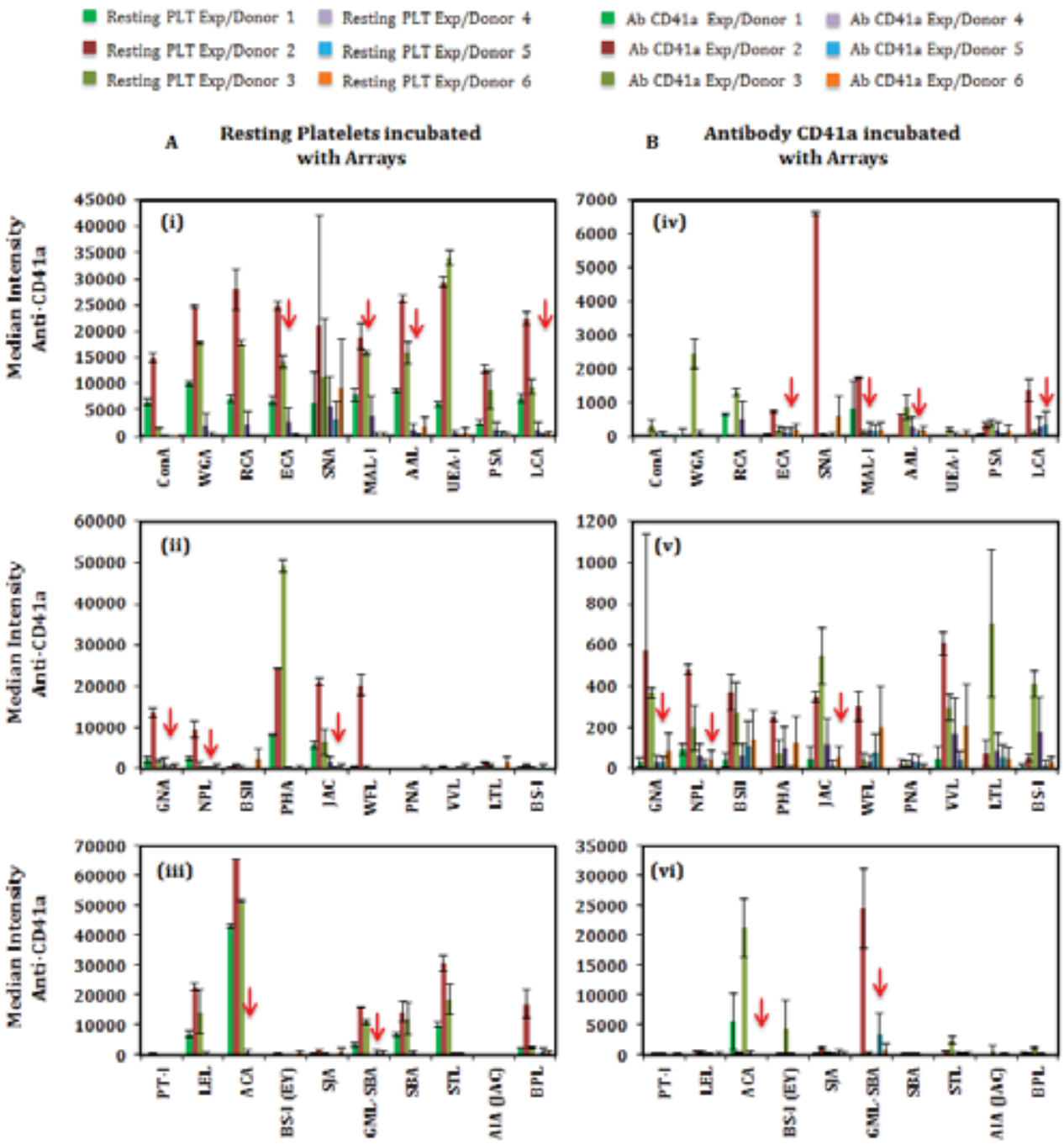

**Figure 4.10. Schematic representation of the fluorescent images of arrays obtained with array scanner and processing of images to obtain lectin binding profile histograms as shown in Figure 11.**

As shown in Figure 4.9, lectin arrays were incubated with resting platelets, washed and then incubated with fluorescent platelet specific antibody (Cy5-anti-CD41a) for staining lectin bound platelets. The arrays were then analyzed with a microarray scanner which provided us with fluorescent images of the spots (spots bound to platelets stained with antibody) on the lectin arrays (Figure 4.9 and 4.10). For every array, the median fluorescence intensity, MFI (after background subtraction) was determined for each lectin spot. For every lectin, an average of MFI of 6 replicate spots within an array was used to construct histograms giving the binding profile of 62 lectins to the platelets. The results for 6 such array experiments (6 Donors) are presented in Figure 4.11. The donors 1-6 are color coded. The median fluorescence intensity (MFI) signifies the level of lectin binding to platelets. As shown in Figure 4.11A (i-iii, vii-ix) for Donor 1 (dark green bars), resting platelets bind a certain set of lectins: ConA, WGA, RCA, ECA, SNA, MAL-I, AAL, UEA-I, PSA, LCA (Figure 4.11A-i); GNA, NPL, PHA, JAC (Figure 4.11A-ii); LEL, ACA, GML-SBA, SBA, STL, BPL (Figure 4.11A-iii); PTII, EEA, MPA, HHL, PAL, ABL, DSL (Figure 4.11A-vii); CFL, CAL, VFA, PHAE, PMA, ASA (Figure 4.11A-viii); UEA-I(EY), LFA, Calsepa, MAL-II and AOL (Figure 4.11A- ix). For the same donor (green bars in Figure 4.11A), a certain set of lectins showed higher binding (e.g. ConA, WGA, Calsepa, ACA, PMA, LFA, DSL) than others (e.g. PTII, PAL, PSA). There was some non-specific binding of antibody to the some of the lectins in the arrays as some signal was detected at the lectin spots for the sample where lectin arrays were incubated just with antibody Cy5-anti-CD41a without any platelets (Donor 1 in Figure 4.11B-iv, v, vi, x, xi, xii, dark green bars). Crucially, the results obtained for binding of platelets to lectins were different for different experiments (Donors) as shown in Figure 4.11A (i-iii and vii-ix) for Donors 1-6. As shown in Figure 4.11A-i, resting platelets showed fluctuating but good binding with

ConA, WGA, RCA, ECA, MAL-I, AAL, UEA-I, PSA, LCA for Donors 1 (dark green bars), 2 (maroon bars) and 3 (light green bars) but this binding completely disappeared (shown by red arrows) in case of donor 4 (purple bars), 5 (light blue bars) and 6 (orange bars). Similar kind of variations were observed for platelet binding to other lectins (Figure 4.11A-i, ii, iii, vii, viii and ix) between different array experiments for different donors (indicated by red arrows). These variations in binding of platelets to various lectins in the array between different experiments could not be attributed to donor differences as the non-specific binding of antibodies to these lectins for the corresponding experiments showed similar pattern of variations (red arrows, c.f 4.11B-iv, v, vi, x, xi and xii with Figure 4.11A-i, ii, iii, vii, viii and ix). The non-specific binding of antibodies to lectins was higher for experiments 1 (dark green bars), 2 (maroon bars) and 3 (light green bars) as compared to donors 4 (purple bars), 5 (light blue bars) and 6 (orange bars) as shown in Figure 4.11B-iv, v, vi, x, xi and xii (indicated by red arrows).

We also analyzed changes in lectin binding pattern of platelets upon agonist stimulation for different experiments described above. The results for 3 such experiments/donors are presented in Figure 4.12 (donor 1, 3 and 6 out of the 6 donors presented in Figure 4.11). There were variations in the agonist induced changes in lectin binding pattern of platelets between donors. As shown in Figure 4.12, TRAP stimulated platelets showed an increase in binding to WGA (relative to resting platelets) for donor1 (Figure 4.12-i) but not for donor3 (Figure 4.12-ii) where TRAP stimulated platelets showed a decrease in binding and in case of donor 6, both resting and TRAP stimulated platelets showed no binding to WGA (Figure 4.12-iii). Similarly, PMA treated platelets showed a decrease in binding to AAL (relative to resting platelets) in case of donor 1 (Figure

4.12-i), an increase in binding in case of donor 2 (Figure 4.12-ii) and no change in binding (relative to resting platelets) in case of donor 3 (Figure 4.12-iii). Similar kind of variations in lectin binding patterns of agonist treated platelets (relative to resting platelets) between different experiments were observed for other lectins (Figure 4.12-i-ix). Based on these observations we conclude that these lectin arrays could not be used as a suitable tool for determining the surface glycosylation patterns of platelets and their changes upon agonist stimulation.


Resting PLT Exp/Donor 1
Resting PLT Exp/Donor 2
Resting PLT Exp/Donor 3
Resting PLT Exp/Donor 4
Resting PLT Exp/Donor 5
Resting PLT Exp/Donor 6
Ab CD41a Exp/Donor 1
Ab CD41a Exp/Donor 2
Ab CD41a Exp/Donor 3
Ab CD41a Exp/Donor 4
Ab CD41a Exp/Donor 5
Ab CD41a Exp/Donor 6
A
Resting Platelets incubated with Arrays
B
Antibody CD41a incubated with Arrays
Median Intensity Anti-CD41a
(i)
(ii)
(iii)
(iv)
(v)
(vi)
ConA
WGA
RCA
ECA
SNA
MAL-I
AAL
UEA-I
PSA
LCA
GNA
NPL
BSII
PHA
JAC
WFL
PNA
VVL
LTL
BS-I
PT-I
LEL
ACA
BS-I (EY)
SJA
GML-SBA
SBA
STL
AIA (JAC)
BPL

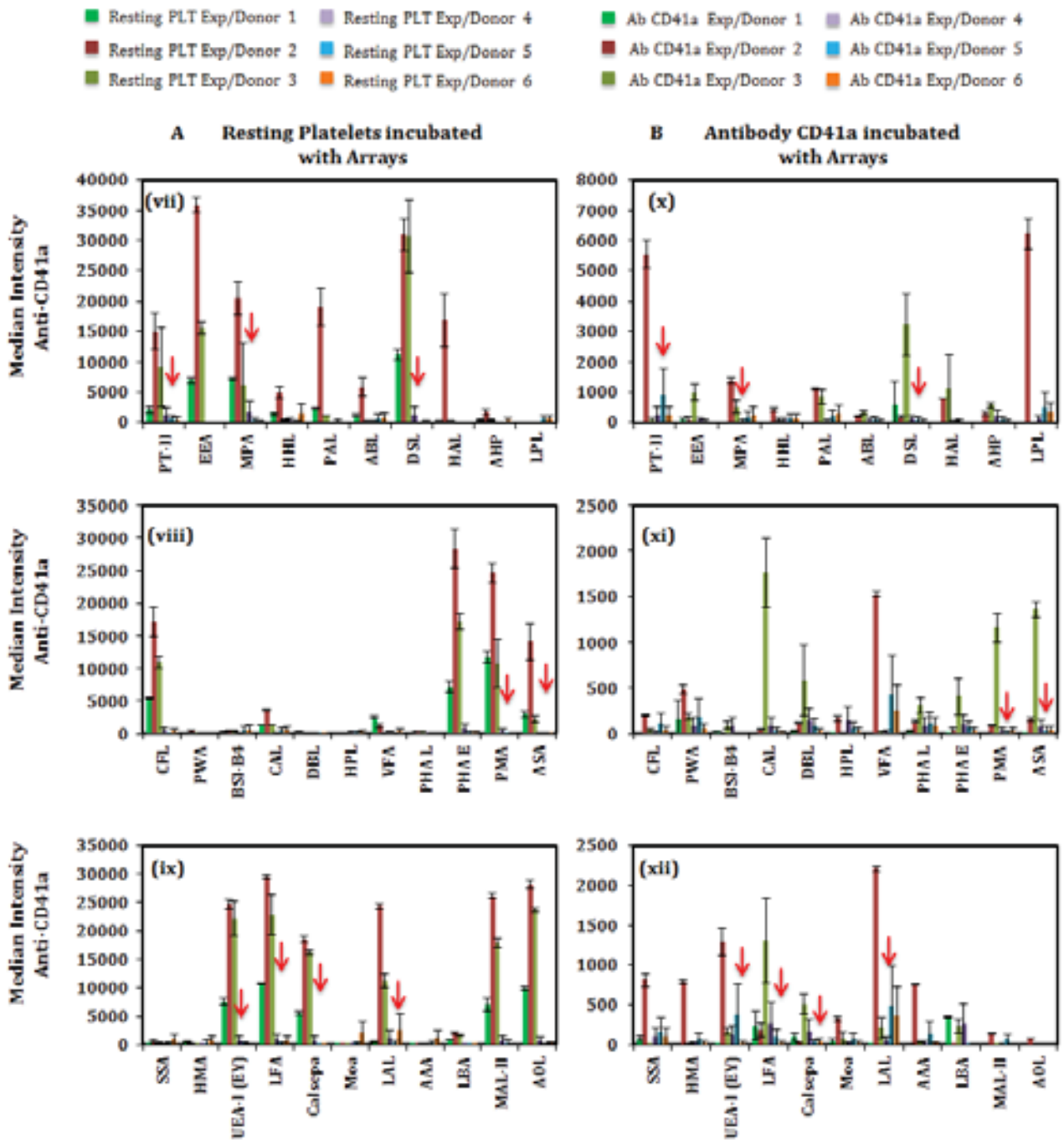
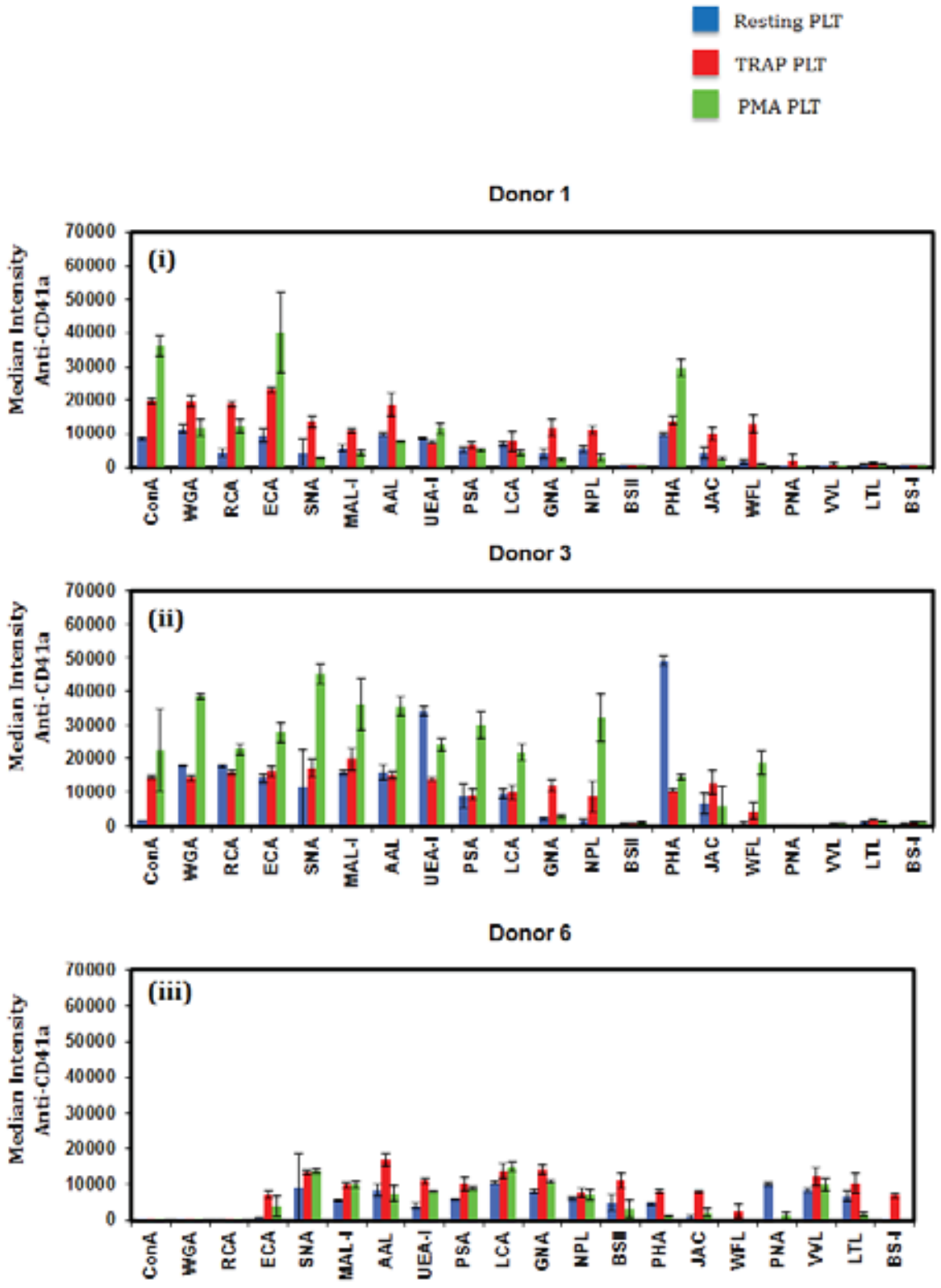
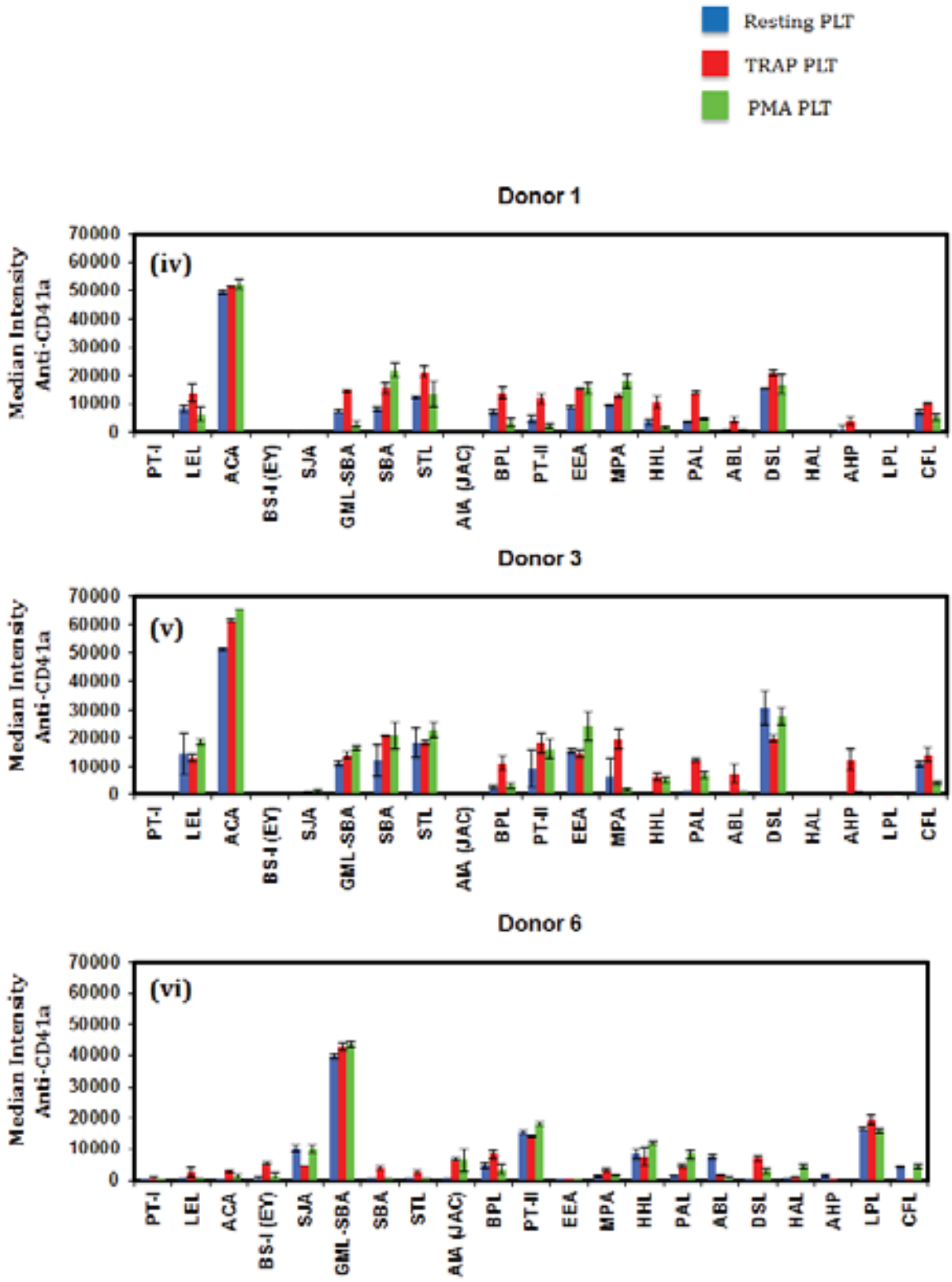

Resting PLT Exp/Donor 1
Resting PLT Exp/Donor 2
Resting PLT Exp/Donor 3
Resting PLT Exp/Donor 4
Resting PLT Exp/Donor 5
Resting PLT Exp/Donor 6
Ab CD41a Exp/Donor 1
Ab CD41a Exp/Donor 2
Ab CD41a Exp/Donor 3
Ab CD41a Exp/Donor 4
Ab CD41a Exp/Donor 5
Ab CD41a Exp/Donor 6
A Resting Platelets incubated with Arrays
B Antibody CD41a incubated with Arrays
Median Intensity Anti-CD41a
(vii)
(x)
(viii)
(xi)
(ix)
(xii)
PT-II
EEA
MPA
HHL
PAL
ABL
DSL
HAL
AHP
LPL
CFL
PWA
BSI-B4
CAL
DBL
HPL
VFA
PHAL
PHAE
PMA
ASA
SSA
HMA
UEA-I (EV)
LFA
Calsepa
Mea
LAL
AAA
LEA
MAL-II
AOL

**Figure 4.11. Variations in lectin binding profiles of resting platelets between different experiments (exp)/donors.**
**Lectin arrays were incubated with resting platelets followed by addition of Cy5-anti-CD41a (A) or just Cy5-anti-CD41a (B) for each of the 6 different experiments/donors and analysed by array scanner to generate fluorescent images of lectin spots (bound to platelets stained with fluorescent antibodies or to fluorescent antibodies). For each lectin spot in an array, the median fluorescence intensity was determined using ProScan array software. The median fluorescence intensity (MFI) signifies the level of lectin binding to platelets (A) or to antibody (B). The MFI of the 6 replicate spots of each lectin were averaged individually for each array experiment and are plotted (Y-axis) in the figure for all 62 lectins (X-axis) for resting platelets (A) and for antibody (B) for different experiments. The different experiments/donors are color coded-Exp1 (dark green), Exp2 (maroon), Exp3 (light green), Exp4 (purple), Exp5 (light blue) and Exp6 (orange). The results show that there are inconsistencies in binding pattern of platelets to lectins between different experiments (A) which correlate with the non-specific binding pattern of antibody to lectins (B) as indicated by red arrows in A and B.**


Resting PLT
TRAP PLT
PMA PLT
Donor 1
(i)
Donor 3
(ii)
Donor 6
(iii)
Median Intensity Anti-CD41a
70000
60000
50000
40000
30000
20000
10000
0
ConA
WGA
RCA
ECA
SNA
MAL-I
AAL
UEA-I
PSA
LCA
GNA
NPL
BSII
PHA
JAC
WFL
PNA
VVL
LTL
BS-I


Resting PLT
TRAP PLT
PMA PLT
Donor 1
(iv)
Donor 3
(v)
Donor 6
(vi)
Median Intensity
Anti-CD41a
70000
60000
50000
40000
30000
20000
10000
0
PT-I
LEL
ACA
BS-I (EY)
SJA
GML-SBA
SBA
STL
AIA (JAC)
BPL
PT-II
EEA
MPA
HHL
PAL
ABL
DSL
HAL
AHP
LPL
CFL

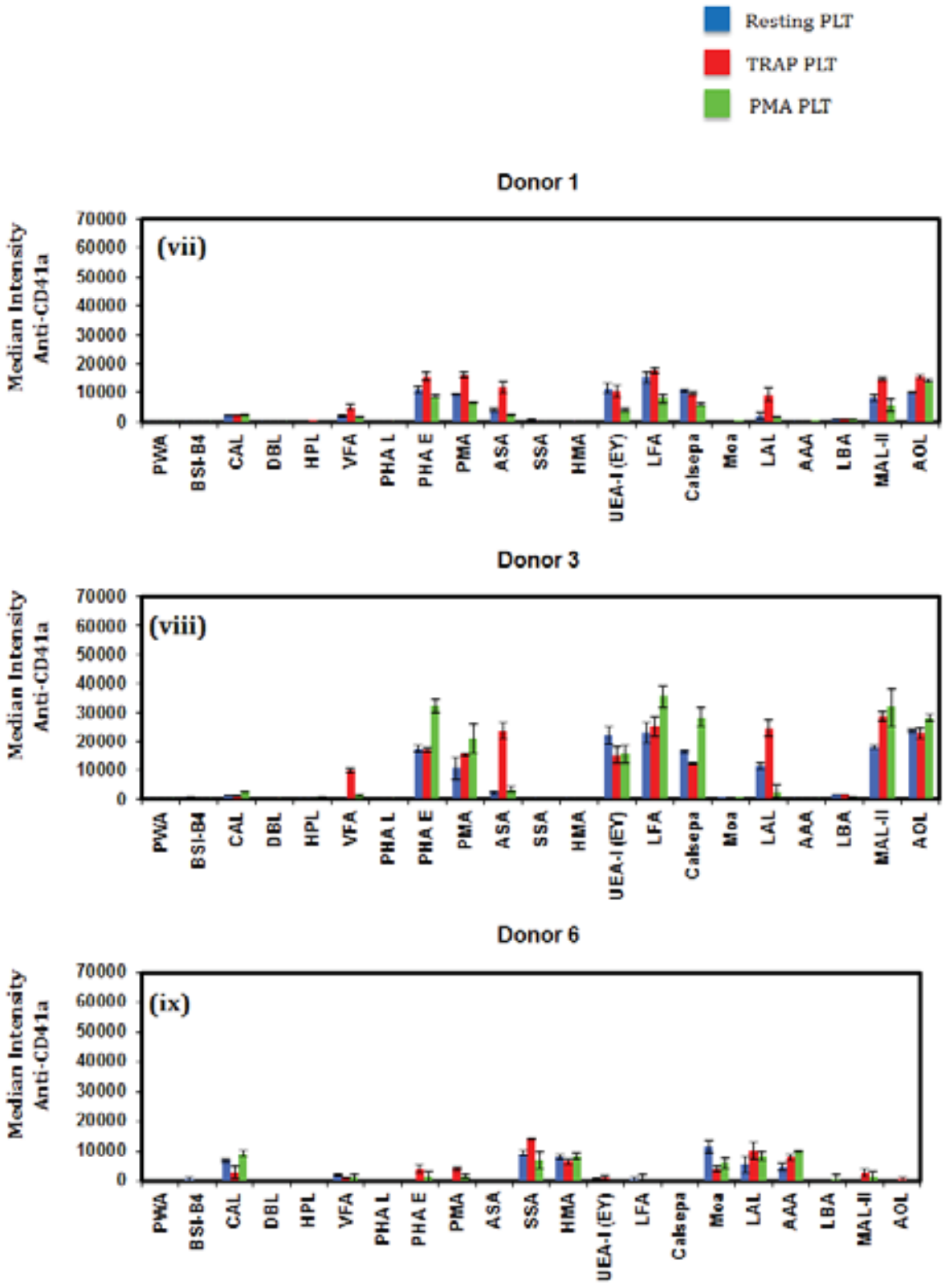

Resting PLT
TRAP PLT
PMA PLT
Donor 1
(vii)
Median Intensity Anti-CD41a
70000
60000
50000
40000
30000
20000
10000
0
PWA
BSI-B4
CAL
DBL
HPL
VFA
PHA L
PHA E
PMA
ASA
SSA
HMA
UEA-I (EY)
LFA
Calsepa
Moa
LAL
AAA
LBA
MAL-II
AOL
Donor 3
(viii)
Median Intensity Anti-CD41a
70000
60000
50000
40000
30000
20000
10000
0
PWA
BSI-B4
CAL
DBL
HPL
VFA
PHA L
PHA E
PMA
ASA
SSA
HMA
UEA-I (EY)
LFA
Calsepa
Moa
LAL
AAA
LBA
MAL-II
AOL
Donor 6
(ix)
Median Intensity Anti-CD41a
70000
60000
50000
40000
30000
20000
10000
0
PWA
BSI-B4
CAL
DBL
HPL
VFA
PHA L
PHA E
PMA
ASA
SSA
HMA
UEA-I (EY)
LFA
Calsepa
Moa
LAL
AAA
LBA
MAL-II
AOL

**Figure 4.12. Variations in lectin binding profiles of TRAP and PMA activated platelets (relative to resting platelets) between different experiments/donors.**
**Lectin arrays were incubated with resting platelets, TRAP treated or PMA treated platelets for each of the 3 donors (out of 6 donors presented in Figure 4.11) and analysed by Array scanner to generate fluorescent images of lectin spots (bound to platelets stained with fluorescent antibodies). For each lectin spot in an array, the median fluorescence intensity was determined using ProScan array Software. The median fluorescence intensity (MFI) signifies the level of lectin binding to platelets. The MFI of the 6 replicate spots of each lectin were individually for each array experiment and are plotted (Y-axis) in the figure for all 62 lectins (X-axis) for resting platelets (blue bars), TRAP activated platelets (red bars) and PMA treated platelets (green bars) for different donors-Donor 1 (i, iv and vii), Donor 3 (ii, v and viii) and Donor 6 (iii, vi and ix). The results show that there are inconsistencies in agonist induced changes in lectin binding patterns of platelets (relative to resting platelets in red bard) between different experiments/donors.**

### 4.2.6 Lectin arrays failed to duplicate flow cytometry results

The lectin arrays failed to duplicate results obtained using flow cytometry. For example, flow cytometry results showed that DBL binds to platelets and its binding increases upon stimulation of platelets with PAR1 agonist peptide (thrombin analogue for PAR1 receptor) and PMA (Figure 4.4A). However both resting platelets and TRAP (thrombin analogue for PAR1 receptor) or PMA treated platelets showed no binding to DBL lectin in lectin arrays (Figure 4.12-vii, viii and ix). Similarly PMA treated platelets showed no changes in binding to ACA lectin according to agonist specific fingerprints determined by FACS (Figure 4.4A) however lectin array results show an increase in binding of PMA treated platelets to ACA lectin (Figure 4.12-iv, v and vi). PAR-1 agonist peptide treated platelets showed a decrease in binding to PTII lectin according to flow cytometry results (Figure 4.4A), however PAR-1 agonist peptide treated platelets showed either no change (Figure 4.12-vi) or increase (Figure 4.12-iv and v) in binding to PTII lectin according to lectin arrays results.

### 4.2.7 Possible sources of error for inconsistencies between lectin array measurements

We suspect that variations we observe in the lectin array studies are due to the degradation of the lectins spots. The degradation could take place while printing the arrays or while storing them. Lectin arrays are printed with a tip that is dipped in the lectin solution and which then prints it on the glass slide. This process is repeated multiple times and there is a possibility that the lectin gets degraded during this printing process, due to exposure to air or contamination of tip, resulting in each lectin spot to be different in terms of its binding activity. That would explain why we observed

variations between the experiments for a particular sample. For example, as shown in Figure 4.13, for resting platelet sample, on array 2 (for experiment 2), resting platelets showed binding to all 6 spots of ConA (green box) and RCA lectin (dark blue box) whereas on array 1 (experiment 1), only 1 or 2 out of 6 ConA and RCA spots showed binding with platelets. Similarly for TRAP platelets, on array 3 (experiment 1), all 6 spots of both PAL (yellow box) and LEL (fluorescent blue box) lectins show good binding with TRAP platelets, however on array 4 (experiment 2), there is hardly any binding detected.

Another source of variation between experiments could be due to degradation of lectins during storage of arrays. Experiments conducted using freshly printed arrays showed much higher binding of lectins to resting platelets than arrays that were 2-3 weeks old (such as donor 4, 5 and 6 presented in Figure 4.11A-i-ix). In case of arrays that were 2-3 weeks old, the binding of majority of lectins to platelets was found to be abolished (Figure 4.11A-i-ix, indicated by red arrows).

Another thing we noticed was that the mostly all lectins printed in corners (Figure 4.13) and borders of the arrays show variation in binding on 6 replicates of a lectin within a array and between different array experiments as shown in Figure 4.13 for lectins (ConA, RCA, LEL and MALII). This could be due to masking of corners of the arrays with the gasket for separation of array while setting up the experiment as described in materials and methods.

Based on these observations we conclude that these lectin arrays are not fabricated properly and therefore cannot be used as a suitable tool for determining the surface glycosylation patterns of platelets and their changes upon agonist stimulation. There is a need for redesigning these arrays with fewer lectins instead of 62 lectins and with

more number of replicates of each spot to prevent experiment to experiment variations. Also the lectins need to be concentrated more towards the array centre to prevent the corner lectins being masked by the gasket. One the major factor that needs to be worked upon is prevention of degradation of lectins while printing or storage. Conditions need to be optimized to keep lectins stable for a longer period of time.

Staining of platelets with an antibody may also present a source of error, and alternative staining strategies may be attempted, as well. However, in this particular case, it was the control of incubating the arrays with the antibody alone that revealed significant array-to-array variations that we believe caused the reproducibility problems.

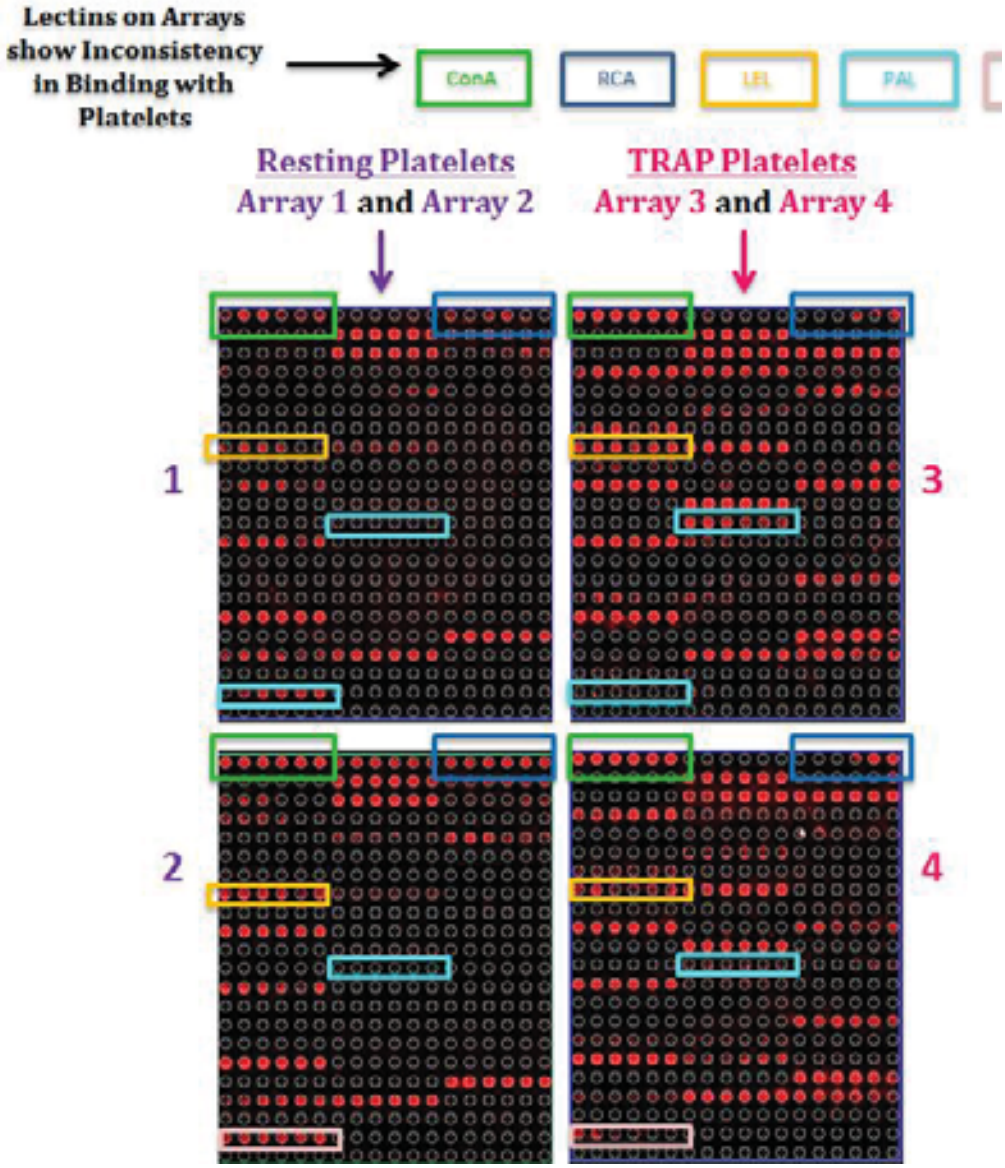



**Figure 4.13. Sources of error in lectin array results for variations in the lectin binding profiles of platelets between different experiments. Fluorescent Images of lectins arrays incubated with resting platelets (left image, array 1 and array 2) and TRAP activated platelets (right image, array 3 and array 4). Some of the lectins showing variation in their binding to platelets between arrays (resting or TRAP activated platelets) are illustrated on the images in color coded boxes. Refer to main text for explanation.**

## 4.3 Conclusions and Outlook

We present essentially new results on lectin-based fingerprinting of platelet activation by various agonists. The main finding of our work is that agonists can be distinguished by examining platelet surface glycosylation changes. Each agonist or agonist analogue exhibits a unique pattern of lectin binding changes relative to the resting platelets (Figure 4.14). Lectin binding appears to be sensitive to the differences in the signaling pathways utilized by the agonists.

We summarize our findings pictorially in Figure 4.14. A platelet can be thought of as a piano with numerous keys that are linked to responses by partially overlapping signaling pathways, where each agonist strikes a particular chord i.e. a particular set of platelet activation responses (platelet surface glycosylation pattern changes in this case). Within the scope of our findings, a possibility worth considering is that non-physiological agonists may be slightly "out of tune".

The piano analogy brings into focus the need for an integrated—systems—approach to studying platelet activation in order to gain insight into the kinds of "chords" different agonists and their combinations "strike". Until now, systems approaches have not been widely used to investigate platelet behavior, although some attempts are being made.

On the more pragmatic side, our findings have potential applications towards developing new clinical and research assays of platelet activation.

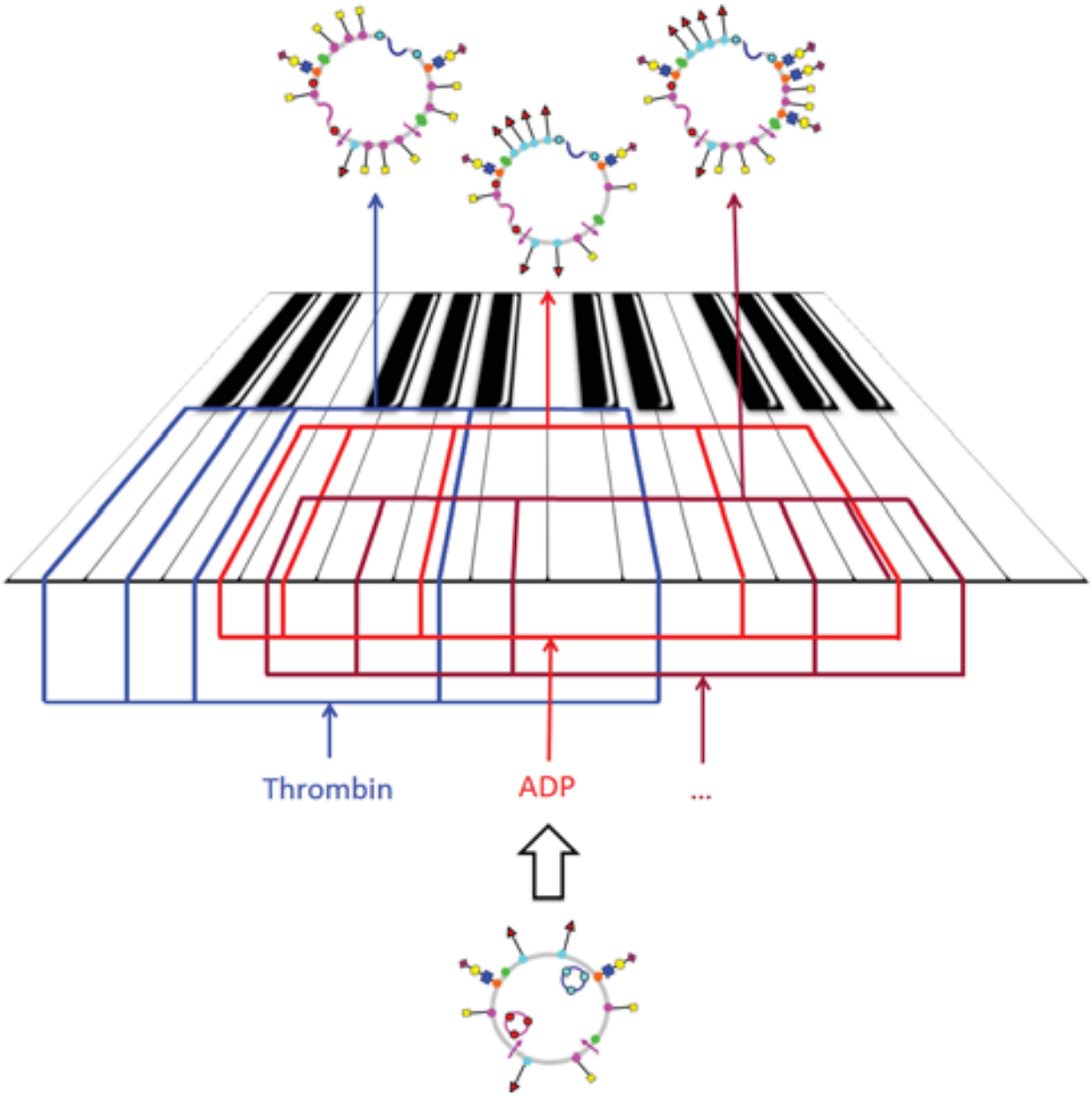



**Figure 4.14. Spectra of platelet activation profiles.**
**In this figure, we schematically depict platelet activation (changes in platelet surface glycosylation) by agonists. A platelet is likened to a piano, with each agonist eliciting a unique "chord" of a response (platelet surface glycosylation changes in this case). Some of the keys overlap between agonists, but some are different. The inspiration for this figure came from an analogy used by J. Tepperman and H. M. Tepperman in their endocrinology text to describe the role of sympathetic nervous system and adrenal medulla in the fight-or-flight response (Tepperman J, Tepperman HM. *Metabolic and endocrine physiology: an introductory text.* Year Book Medical Publishers, Chicago, 1987 (fifth edition). Russian translation, Mir, 1989).**

## Concluding Remarks

The work presented in this Thesis demonstrates that different soluble and surface agonists selectively regulate various platelet activation responses such as exocytosis of alpha and dense granules, expression of PS in outer leaflet of plasma membrane, activation of GPIIb/IIIa receptor and changes in surface glycosylation pattern, as shown in Chapter 3 and 4 of this Thesis.

Soluble agonists such as thrombin, PAR1 agonist, PAR4 agonists, ADP, TXA2, calcium ionophore and PMA all result in expression of CD62P and CD63 (alpha and dense granule marker, respectively), exposure of PS in outer leaflet of plasma membrane and activation of GPIIb/IIIa receptor. However, the surface expression of different carbohydrates (increase, decrease or no change) upon platelet stimulation are differentially regulated by these soluble agonists as determined by lectin binding patterns in Chapter 4. Each agonist upon stimulating platelets resulted in a unique lectin binding fingerprint.

Similarly, as opposed to soluble agonists, which result in expression of CD62P, CD63, PS and activated GPIIb/IIIa, surface agonists such $TiO_2$ display selectivity in regulation of these activation responses as illustrated in Chapter 3. Platelets upon interaction with $TiO_2$ (in the absence of extracellular calcium) selectively express only CD63 and no other three markers mentioned above. In contrast to $TiO_2$, another surface agonists-glass, results in expression of all these 4 markers on platelets.

Selective regulation of different platelet activation responses by different stimuli could be attributed to the differences in intracellular signaling pathways that are initiated by these stimuli. We have shown that intracellular calcium kinetics in platelets upon stimulation with $TiO_2$ and glass are different and this differential calcium kinetics correlates with the different pattern of platelet responses observed on these surfaces.

Taken together, our results suggest that platelet activation is a stimuli-driven selective process. The notion about selective platelet activation has been out only recently and the mechanisms underlying this selectivity are not understood. The partial reason for this is that the knowledge about the responses that are selectively regulated is very limited. Our results provide significant insight on the repertoire of responses that are selectively regulated by different stimuli and has paved a way to understand phenomenon of selective platelet activation.

The selective nature of platelet activation allows platelets to perform different functions (hemostasis, wound healing, angiogenesis, tumor metastasis, adaptive and immune responses) in response to different stimuli. An understanding of these processes is crucial as it could revolutionize therapeutic approaches to cardiovascular disorders, cancers, treatment of recalcitrant wounds, and implant integration.

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# Curriculum Vitae

Name: **Swati Gupta**
E-mail: swati.gupta@outlook.in
Date of Birth: 03/02/1988
Nationality: Indian

**Education and Qualifications**

Dec 2009 – current: PhD student in Biosurfaces, CIC biomaGUNE, San Sebastian, Spain
Research topic: *Selective activation of platelets by surfaces and soluble agonists.*

2008 – 2009: M.Sc. in Biological Sciences (Specialization in Infection and Immunity), University of Leeds, Faculty of Biological Sciences, UK.
Dissertation: *Investigation of pathway of nuclear import of KSHV ORF57 protein by studying Importin-α / Transportin-NLS interactions.* Defended, August 2009. Mark: First Division with Distinction.

2005 – 2008: B.Sc. (Hons.) in Biochemistry, University of Delhi, India. Mark: 82% (First Division with Distinction).

2006 – 2007: Add on Bioinformatics and Computational Course, University of Delhi, India.

**Previous Research experience**

October 2011 – Visiting Student in the lab of Prof. J. Voros, Institut f. Biomedizinische Technik, Gloriastrasse 35, Zürich.
Project title: *Use of Fluid FM technology to study interaction between blood components and implant surfaces, cell staining procedures, vesicle injections on surfaces.*

September 2008 – December 2008: Research project, under supervision of Dr. Aysha Divan, Faculty of Biological Sciences, University of Leeds, UK.
Project title: *Cloning of mutant GFPuv created by site directed mutagenesis and expression, purification and analysis of this protein.*

September 2007 – December 2007: Research project, under supervision of Dr. P. Hemalatha Reddy, Faculty of Biochemistry, University of Delhi, India.
Project title: *Cloning of genes of two component system of Mycobacterium.*

**Publications**

Swati Gupta Ilya Reviakine. The sweeter aspects of platelet activation: a lectin-based assay reveals agonist specific glycosylation patterns. *Submitted*, (2013)

Alexandre F. Leitão, Swati Gupta, João Pedro Silva, Ilya Reviakine, and Miguel Gama. *Hemocompatibility study of a bacterial cellulose/polyvinyl alcohol nanocomposite*. Colloids and Surfaces B: Biointerfaces 111, 493-502 (2013)

Swati Gupta, Ilya Reviakine. *Platelet Activation Profiles on $TiO_2$: Effect of $Ca^{2+}$ Binding to the Surface.* Biointerphases 7, 28 - 40 (2012)

**Distinctions**

Awarded merit based scholarship for M.Sc. in Biological Sciences, 2008-2009, University of Leeds, UK.

Awarded merit based funding for FEBS workshop (2013) on Biological Surfaces and Interfaces, Sant Feliu de Guixols, Spain

**Science events organization and teaching experience**

July 2010: FEBS: Physical Chemistry of Biointerfaces – PhysChemBio II, San Sebastian, Spain
As a **Tutor**, I organized the tutorial on the *Flow Cytometry and vesical preparation and characterization by Dynamic Light Scattering* and participated in other aspects of the general organization of the workshop. More information about this workshop can be found at http://www.physchembio.net/

July 2012: FEBS: Physical Chemistry of Biointerfaces – PhysChemBio II, San Sebastian, Spain
As a **Tutor**, I organized the tutorial on the *characterization of platelets by Flow Cytometry* and participated in other aspects of the general organization of the workshop. More information about this workshop can be found at http://www.physchembio.net/

Feb 2008: CATALYSIS- Biochemistry Society workshop. University of Delhi, India. I organized the debates, quizzes, presentations, biochemistry magazine editing and release.

**Science events participation**

July 2010: FEBS: Physical Chemistry of Biointerfaces – PhysChemBio II, San Sebastian, Spain
Poster presentation: *Platelet activation profiles on Titanium oxide: Effect of calcium binding to surface*.

June 2010: Zeiss on your campus workshop-Curso de microscopia avanzada, Universidad Autonoma de Barcelona, Spain
Seminars and hands on session-latest updates on applications of Confocal Microscopy.

July 2013: FEBS Workshop on Biological Surfaces and Interfaces, Sant Feliu de Guixols, Spain
Poster presentation: Selectivity in Platelet Activation by the Titania Surface: A model system for in vitro modulation of platelet activity.

**Practical skills**

Flow cytometry (FACS), Scanning Laser Confocal Fluorescence microscopy, ELISA, Chromatography (gel filteration, ion exchange, thin layer, and paper, affinity chromatography), Spectrophotometry, Polymerase chain reactions (PCR), Gene cloning techniques, Expression and purification of recombinant proteins, SDS-PAGE and western blotting, Immuno-precipitation techniques, GST pull down assays, Lipid handling and preparation of liposomes

**Other**

Languages: Hindi (native), English (native)