Isolation and characterization of platelet microvesicles

Project Report submitted to Central University of Punjab

For the Award of

Master of Science

In

Life Sciences (Specialization in Animal Sciences)

By
KAPIL KUMAR

Supervisor
Dr. Sunil Kumar Singh

Department of Animal Sciences
School of Basic and Applied Sciences
Central University of Punjab, Bathinda
May, 2018

DECLARATION

I,Mr.Kapil Kumar hereby declare that this project entitled "**Isolation and characterization of platelet microvesicles**" submitted by me in partial fulfillment for the requirements of the M.Sc. Degree in Life Sciences with specialization in Animal Sciences under the guidance of Dr. Sunil Kumar Singh. Further, this work is an original and independent review which has not been submitted previously in part or full to this university or any other university or institution for the award of any degree or diploma or fellowship.

Name: Kapil Kumar
Registration No.: 16mslsas08
Department of Animal Sciences,
School of Basic and Applied Sciences,
Central University of Punjab,
Bathinda-151001

Date:
Signature:

CERTIFICATE

This is to certify that this project entitled "**Isolation and characterization of platelet microvesicles"** is a record of original and independent review done by Mr. Kapil Kumar, a student admitted for M.Sc. program in Centre for Animal Sciences, School of Basic and Applied Sciences, Central University of Punjab under my guidance and supervision. This project has not been submitted previously in part or full to this university or any other university or institution for the award of any degree or diploma or fellowship.

Dr. Sunil Kumar Singh
(Supervisor)
Department of Animal Sciences,
School of Basic and Applied Sciences,
Central University of Punjab,
Bathinda-151001

ACKNOWLEDGEMENT

Completion of this Project is an outcome of continual work over and intellectual support from various sources, instrumental in imparting this work a successful status. It is a matter of pleasure to express my gratitude and appreciation to them.

First and foremost, I take this opportunity to express a deep sense of gratitude and thank my mentor Dr. Sunil Kumar Singh, for supervising and lending me stimulating suggestions, innovative quality guidance, creative thinking. I am grateful to him for being patient with my mistakes and boasting my confidence.

I am also obliged to Dr. Anil Mantha, Head of Department, Animal Sciences, Central University of Punjab, for providing me an opportunity to pursue my field of my interest and I would also like to acknowledge with much appreciation the crucial role of the staff of the Central University of Punjab, who gave me the permission to use all required equipment and all the necessary material to complete the task.

I'm particularly grateful to Dr. R.K. Kohli, honorable Vice Chancellor Central University of Punjab for providing the research facilities to carry out my project.

I'm also thanks to Mr. Sumit Srivastava to help while image processing in the technique FESEM and Mr. Gaurav Joshi (Ph.D. Scholar, Molecular Medicine) in Flow Cytometer.

Special thanks to all my friends and colleagues who provided me with the great enthusiasm to work upon the project. I am thankful to my friend Sneha Santra for her help and support.

Lastly, I thank almighty and a heart full of thanks to my parents who supported me at every stage of my life and encouraged me throughout my work.

(Kapil Kumar)

ABSTRACT

Isolation and characterization of platelet microvesicles.

Name of the student : Kapil Kumar

Registration No. : 16mslsas08

Degree for which submitted : M.Sc. Life Sciences (Sp. Animal Sciences)

Name of Supervisor : Dr. Sunil Kumar Singh

Name of the Department : Department of Animal Sciences

Keywords: Platelet microvesicles, cardiovascular diseases, prognostic biomarker, differential centrifugation, Flow cytometer, hyaloplasmic extensions

In the recent years, Platelet microvesicles (PMVs) are projected as potential diagnostic and prognostic biomarkers of many diseases like cardiovascular diseases, diabetes and infection. Due to the small size (100-1000 nm), diverse structure and functions, PMVs is associated with different challenges during isolation, quantification and characterization. Hence, it is pertinent to optimize methods to isolate, characterize and quantify MVs in blood plasma. Therefore, in this study it is aimed to isolate and characterize the PMVs release upon platelet activation. The released PMVs were isolated by differential centrifugation and characterized through scanning electron microscopy (SEM) and flow cytometer (FACS). SEM images showed resting platelets as smooth spherical cells of 2-3 µm in diameter separated from each other having presence of small hyaloplasmic extensions. In contrast, platelet activation elicits significant morphological change exhibited well-developed pseudopods (hyaloplasmic processes), which connected the cells forming large aggregates (clumps). Electron microscopy and flow cytometry data showed platelet microvesicles release of size in the range of 200-500 nm from the activated platelets. Thus, in this study an isolation and characterization technique for platelet microvesicles was optimized that can be further validated with clinical samples and exploit to study microvesicles structure, function and clinical importance.

(Kapil Kumar) (Dr. Sunil Kumar Singh)

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Sr. No.	Full Form	Abbreviation
1.	Platelet Microvesicles/Microparticles	PMVs/PMPs
2.	Field Emission Electron Microscope	FESEM
3.	Von Willebrand factor	VWF
4.	Lysosomal membrane proteins	LAMP
5.	Diacylglycerol	DAG
6.	Inositol-1,4,5-trisphosphate	IP3
7.	Multivesicular bodies	MVBs
8.	Intraluminal vesicles	ILVs
9.	Transmission electron microscopy	TEM
10.	Atomic force microscopy	AFM
11.	Cyclic voltammetry	CV
12.	Indium Tin oxide	ITO
13.	Horseradish peroxidase	HRP
14.	Citrate-phosphate-dextrose adenine	CPDA
15.	Ethylene diamine tetra acetic acid	EDTA
16.	Hydroxyethyl piperazine ethane sulfonic acid	HEPES
17.	Platelet rich plasma	PRP
18.	Platelet poor plasma	PPP
19.	Activated platelets	APs
20.	Resting platelets	RPs
21.	Forward scatter	FSC
22.	Side scatter	SSC

CHAPTER- I

INTRODUCTION

Platelet-derived microvesicles (PMVs) are small membrane-bound vesicles (0.1 to 1.0 μm in size) formed by budding of plasma membrane during platelet activation or apoptosis. PMVs represents about 70–90% of all circulating microvesicles (MVs). PMVs elevation in blood shows strong correlation with the pathogenesis of diseases including atherosclerosis, arterial thrombosis, acute coronary syndrome, sepsis, cancer, hypertension and diabetes. Platelets (or Thrombocytes) are the component of blood derived from megakaryocytes of the bone marrow that are required for human survival by virtue of their ability to prevent and arrest bleeding. Owing to their small size of PMVs, diverse structure, functions and polydispersed nature; special techniques and skills need to be applied for their accurate estimation and characterization. PMVs estimation is affected by many factors that includes sample collection, processing, methods and techniques used for estimation.

1.1 Blood Overview:

Blood is a specialized fluid connective tissue that circulates throughout the body performing various essential functions. It is made of Red Blood Cells or Erythrocytes, White Blood Cells or Leukocytes, Platelets and Plasma. Plasma consists of approximately 92 % of water and the rest is a combination of proteins, salts, vitamins, fats, gases. Plasma makes up to 55 % of blood. Rest of the 45% is made up by the other cellular components. Blood performs several essential functions like, supplying oxygen to the cells and tissues, removing waste material, protecting the body from foreign bodies and infections, regulating body temperature, pH levels, providing essential nutrients to cells.

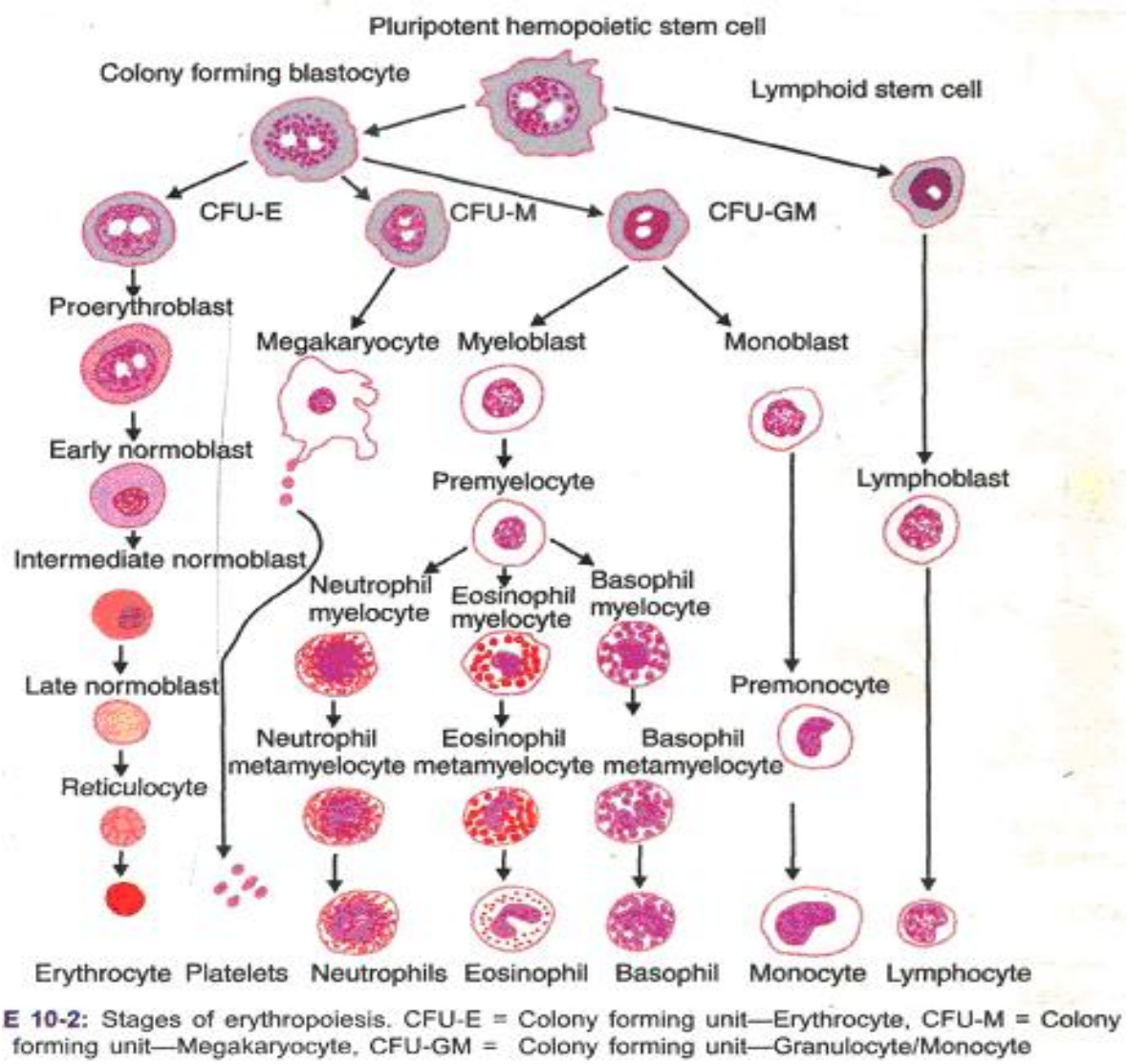
Red Blood Cells was first observed by Jan Swammerdam under the microscope for the first time in 1658. Later in 1695, Antoni Van Leeuwenhoek, acquaintance of Swammerdam described the precise size and shape of the Red Blood Cells. Red Blood Cells are round with an indented center. They appear to be red in color, due to the presence of Hemoglobin protein, which helps the RBCs to carry Oxygen (O_2).

Further in 1843, Gabriel Andral and William Addison reported the first description about Leukocytes, simultaneously. White Blood Cells are the cells that form the vital line of

defense against disease and infections. These landmark discoveries led to the establishment of a new field in Medicine i.e., Hematology.

Alfred Donne, a French Biologist, in 1842 discovered the third element in blood, the platelets. Accurate description of the same was reported later in 1865 by Max Schultze and by G. Bizzozero in 1882. Platelets (or Thrombocytes) are the component of blood. They are remarkable mammalian adaptation that is required for human survival by virtue of their ability to prevent and arrest bleeding. They are fragments of cytoplasm that are derived from Megakaryocytes of the Bone Marrow.

Blood cell formation**,** also called Hematopoiesis**,** or Hemopoiesis is a continuous process by which the cellular constituents of blood are replenished as needed. Blood cells do not originate in the bloodstream itself but in specific blood-forming organs, notably the marrow of certain bones. In the human adult, the bone marrow produces all of the red blood cells, 60–70 percent of the white cells (*i.e.,* the granulocytes), and all of the platelets. The platelets, which are small cellular fragments rather than complete cells, are formed from bits of the cytoplasm of megakaryocytes of the bone marrow. The formation of various blood cells is shown in the figure 1 below.



E 10-2: Stages of erythropoiesis. CFU-E = Colony forming unit—Erythrocyte, CFU-M = Colony forming unit—Megakaryocyte, CFU-GM = Colony forming unit—Granulocyte/Monocyte

Figure 1: Formation of different blood cells

Source: http://nhealthcare.blogspot.in/2013/04/red-blood-cell-formation-erythropoiesis.html

1.2 Platelets:

Platelets are megakaryocyte produced cellular fragments with the diameter of 2-5 micrometer and thickness of about 0.5 micrometer. Despite their lack of genomic DNA, platelets contain megakaryocyte-derived messenger RNA (mRNA) and the translational machinery required for protein synthesis (Weyrich AS *et al*., 2009). Once individual platelets are fragmented from megakaryocytes, they are released into circulation under

thrombopoietin hormonal regulation where they circulate for approximately 7-10 days. . Aged platelets are removed from blood in the spleen. A normal platelet count ranges from 150,000 to 450,000 platelets per µl of blood. The primary physiological function of platelets is to arrest hemorrhage at sites of vascular injury where they have the ability to rapidly form platelet-rich thrombi.

Unfortunately, platelets are unable to distinguish between physiological wounds and pathological lesions, such as those that occur in diseased atherosclerotic vessels, which can result in the formation of blood clot in the blood vessels which hinder the flow of blood through them. (Sachs UJ *et al*., 2007).

1.2.1 Structure of Platelets:

Platelets surface show an open canalicular system, connected "tunnels" of platelet membrane folds. Specific glycoproteins (GP) elements of platelet outer surface contribute to various platelet functions. Principle GPs involved in the main platelet function, haemostasis are GP Ib-V-IX and GP IIb-IIIa. While former is involved in platelet shear-stress based activation and in the binding of von Willebrand factor (VWF) and collagen, the later is responsible for fibrinogen binding of activated platelets and in the formation of platelet aggregates. These receptors are able to move horizontally in the membrane even inside the canalicular system. This movement enables to increase local receptor density in case of activation. Platelets harbor different types of inner vesicles of various physiological functions. They are known as platelet granules.

1.2.2 Platelet Granules:

Platelets contain two different secretory organelles, α-granules and dense core granules, as well as the OCS system that allows them rapidly release a variety of factors and increase their expression of surface proteins (Reed G *et al*., 2007, May AE *et al*., 2008). Some other membrane enclosed organelles include a few simple mitochondria for energy metabolism and glycosomes for glycogen storage (White JG *et al*., 1999). α-Granules, the most abundant granules, with an average of 40-80 per platelet, contain procoagulant molecules, fibrinolytic regulators, growth factors, chemokines, immunologic modulators, adhesion molecules, and other proteins (Reed G *et al*., 2007) (Table 1). α- Granule

proteins can be either *platelet-specific* such that they are synthesized solely by megakaryocytes or *platelet-selective* whereby they are present in megakaryocytes along with a few other cells. Dense granules are smaller, less abundant, and contain fewer proteins compared to α- granules. They store mainly small molecules like ADP, serotonin and Ca^{2+}, as well as some lysosomal membrane proteins like CD63 (LAMP-3) and LAMP-2 (Table 1). A few lysosomes can also be found in platelets containing acid hydrolases, cathepsins, and lysosomal membrane proteins. The release of these factors particularly from α- and dense granules results in an organized and precisely regulated series of events influencing a variety of biological functions such as cell adhesion, cell aggregation, chemotaxis, cell survival, proliferation, coagulation, proteolysis, and cell recruitment**.** Platelet degranulation occurs following platelet activation by specific ligands including thrombin, collagen, and thromboxane A2. Typically through a Gq protein-coupled mechanism, plasma memberane phosphatidylinositol 4,5 bisphosphate (PIP2) is cleaved into diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP3), which activate protein kinase C (PKC) and increase intracellular Ca2+ (from 40 – 100 nM to 2 -10 μM), respectively (Reed G *et al*., 2007). Direct increases in cytoplasmic Ca2+ along with the synergistic effects of PKC result in platelet degranulation and the initiation of a hemostatic response. The structure of platelet and its major receptors are shown in figure 2a and 2b.

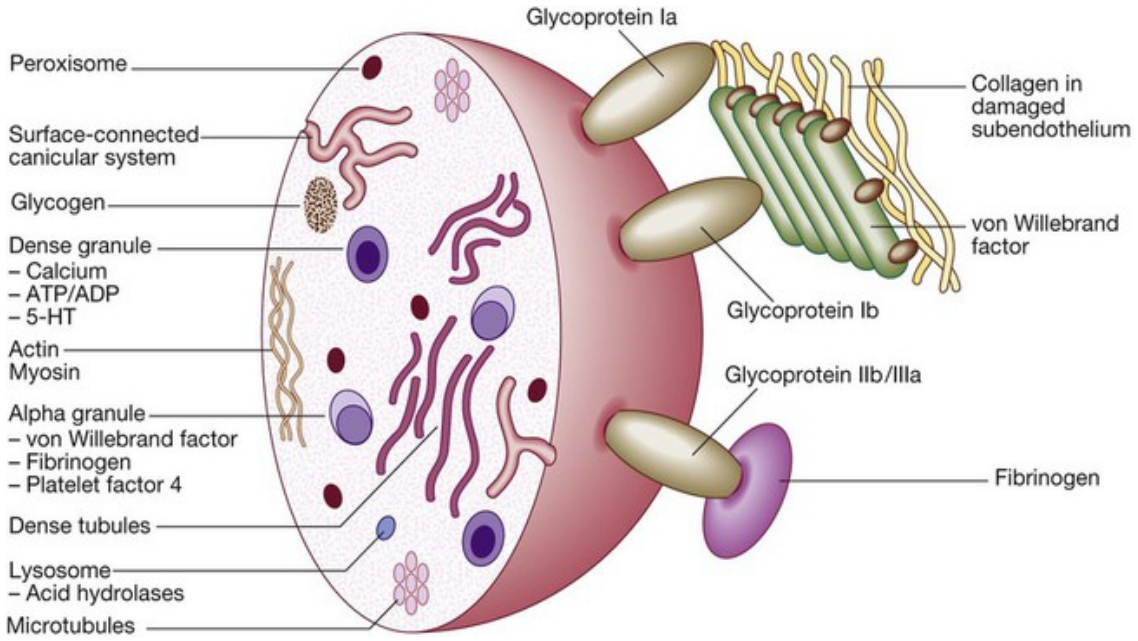


Figure 2 (a): Normal platelet structure. (5-HT = 5-hydroxytryptamine, serotonin; ADP = adenosine diphosphate; ATP = adenosine triphosphate

Source: https://basicmedicalkey.com/blood-disease/

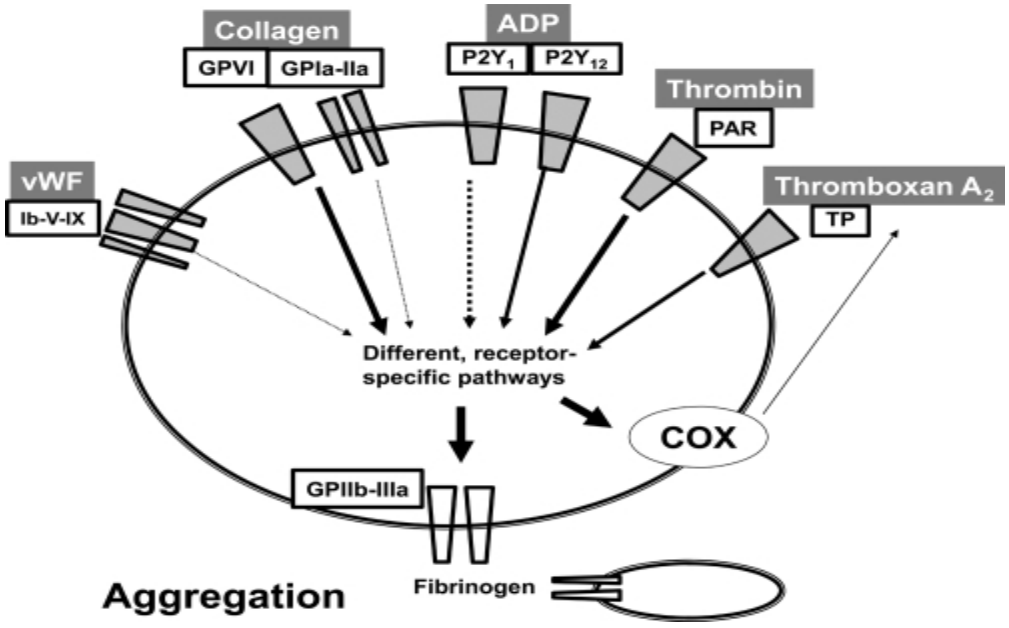


Figure 2 (b): Different receptors present on platelets

Source: https://openi.nlm.nih.gov/detailedresult.php?img=PMC2515423_vhrm0403-637-01&req=4

1.3 Extracellular vesicles (EVs):

The existence of extracellular particles and vesicles was observed in platelet-free serum by Chargaff and West (1946). EVs are classified into three major classes according to their biogenesis as exosomes, microvesicles and apoptotic bodies (see figure 3 & table-1).

1.3.1 Exosomes:

Vesicles that are heterogeneous in size, with a diameter ranges from 20nm to 100nm, and they are shed from the plasma membrane of dying cells undergoing programmed cell death. These are generated under multivesicular bodies (MVBs) which are endosomal compartments in which “intraluminal vesicles” (ILVs) develop; these ILVs later become secreted into the extracellular space as “exosomes” (*Pitt et al., 2016b*). Exosomes are as durable as viruses, with the ability to deform elastically while maintaining vesicle integrity (Calo *et al*., 2014; Riazifar *et al*., 2017). Exosomes are highly enriched in certain tetraspanins, such as CD63, CD81, and CD9. Endosome related protein TSG101 is a recognized marker (Raposo and Stoorvogel, 2013; van Niel *et al*., 2006).

1.3.2 Microvesicles (MVs):

Microvesicles (MVs), which directly originate from the membrane surface, are characterized by phosphatidylserine (PS) exposure and are generally referred to be between 0.1 and 1.0 μm of diameter (Wiedmer *et al*., 1990). MVs with densities between 1.04 and 1.07 g/mL, are of irregular shape and very heterogeneous in size. MVs are formed by direct shedding from the plasma membrane *via* outward invaginations. Microvesicles contain plasma membrane proteins, cytosolic proteins, nucleic acids, and other small molecules (Riazifar *et al*., 2017). Their sizes average approximately 200 nm, but range from 50 to 1500 nm (Pitt *et al*., 2016b; Raposo and Stoorvogel, 2013; Xu *et al*., 2016).

1.3.3 Apoptotic Bodies:

Apoptotic bodies (ABs), are released during cell apoptosis, resulting in vesicles composed of organelle and plasma membranes with nuclear and cytoplasmic contents (Riazifar *et al*., 2017). ABs are larger than exosomes and microvesicles, typically ranging from 1,000 to 5,000 nm. Phagocytes usually take up ABs after being shed and eliminated rapidly from the human circulation, but can interact with many recipient cells before phagocytosis (Pitt *et al*., 2016b; Raposo and Stoorvogel, 2013; Xu *et al*., 2016).

ABs are more likely to contain genomic DNA and histones and can play a role in apoptosis-induced proliferation and regulation of immune responses (Ryoo and Bergmann, 2012). They carry large amounts of phosphatidylserine and annexin to recruit and activate phagocytosis (Poon *et al.,* 2014).

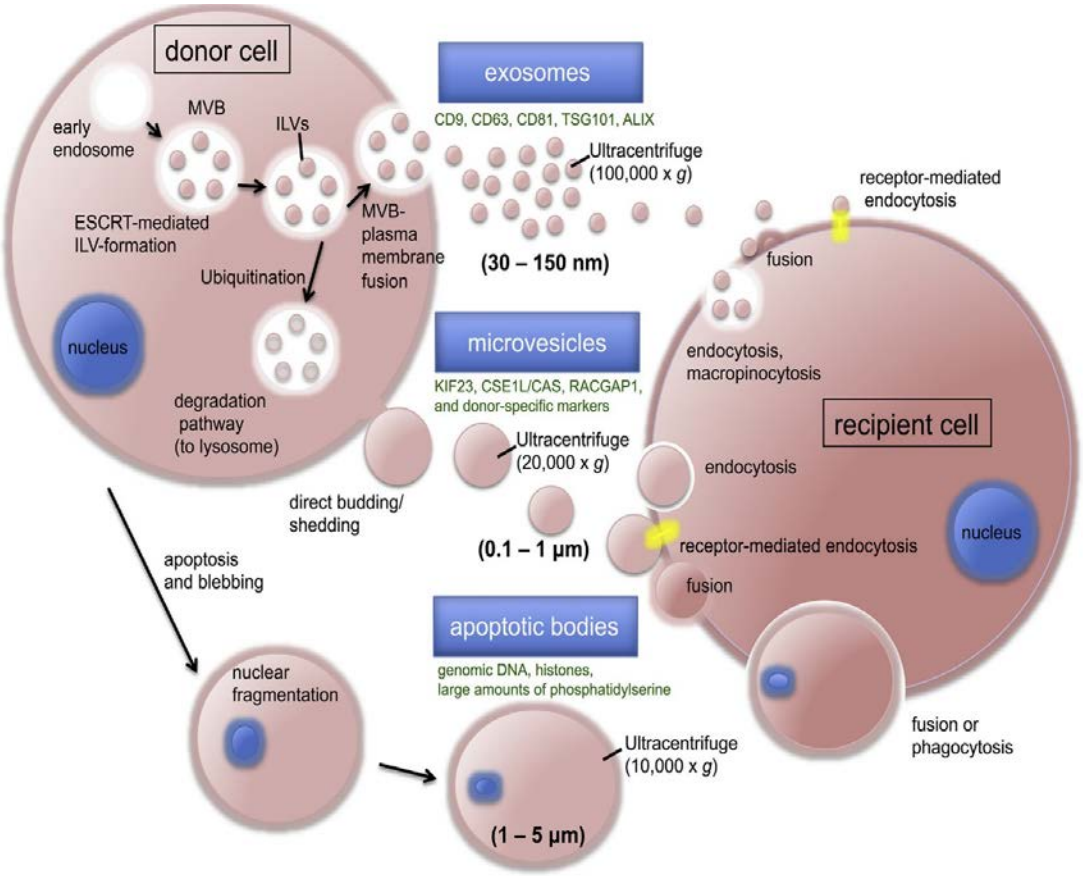


Figure 3: Typical structure of extracellular vesicles (EVs)

Source: (McBride *et al*., 2017)

	Exosomes	Microvesicles	Apoptotic Bodies
Size	≈ 20-100 nm	≈ 0.1-1 µm	> 1 µm
Origin	Multivesicular bodies, internal compartments	Plasma membrane	Cellular fragments
Markers	– Tetraspanins (CD63, CD9, and CD81) – ALG-2-interacting protein X – Tumor susceptibility gene 101 protein – Heat shock 70-kDa proteins – Major histocompatibility complex class and class II	– Phosphatidylserine (PS) – Integrins – Selectins – CD40 ligands – Other antigens of parental cell	– Histones –Fragmented DNA – PS

Table: 1. Characteristics of extracellular vesicles (EVs)

Source: Zaldivia *et al*., 2017

1.4 Blood Microvesicles:

In 1967, Peter Wolf described the presence of elements, “originating from platelets, but distinguishable from intact platelets” in the plasma and serum of healthy individuals that were capable of supporting coagulation. The tiny elements, smaller than platelets, were extracellular vesicles produced by platelets in response to activation or at the time of apoptosis. Further investigations revealed that there are two types of vesicles released by activated platelets: exosomes (approximately 40–100 nm in diameter), released by exocytosis from the multivesicular body and alpha-granules, and microvesicles (MVs)

(approximately 100–1000 nm in diameter), also called microparticles (MPs) or ectosomes that are released by budding of the cytoplasmic membrane. Usually, the term microparticles is the most commonly used to describe platelet-derived microvesicles PMVs. MVs level in healthy individuals is 1,560±143/µl (Meng H *et al.,* 2017).

Although many cell release subcellular particles or extracellular microvesicles, platelet derived microparticles (PMPs) are most abundant MPs in human blood and may originate from platelets in circulation, as well as from megakaryocytes. 90% of vesicles released from platelets are below 500 nm in size, majority being in range of 100–250 nm. PMP play significant role in cell to cell communication, homeostasis, angiogenesis and other functions owing to their procoagulant surface, rich in phosphatidylserine, tissue factor and many other receptors and their ability to interact with leukocytes and endothelial cells.

They have a potent pro-inflammatory effect, promote coagulation and affect vascular function. The levels of circulatory PMVs are altered during several disease manifestations such as coagulation disorders, rheumatoid arthritis, systemic lupus erythematosus, cancers, cardiovascular diseases e.g. myocardial infarction, acute coronary syndrome, stroke, venous thrombo-embolism, hereditary thrombophilia, thalassemia, antiphospholipid antibody syndrome, etc. pointing to their potential contribution to disease and their development as a biomarker (J. Kailashiya *et al.,*2018).

CHAPTER- II

REVIEW OF LITERATURE

2.1 Platelet Microvesicles (PMVs):

Microvesicles (MPs) can originate from platelets, endothelial cells, leukocytes, erythrocytes, and smooth muscle cells, and are found in circulating blood at relative concentrations determined by the pathophysiological context. Platelet-derived microvesicles (PMVs) are the most abundant in the bloodstream, representing about 70–90% of all circulating MVs and contribute many of the biological processes (Suades, Rosa, *et al*., 2012; Badimon *et al*., 2016). Platelet-derived microvesicles are much higher in peripheral blood as a result of chronic platelet activation in various disease states (Tan *et al*., 2005). In terms of protein composition, PMVs from activated platelets are highly enriched in a-granule-derived factor Va and Xa (Monkovic and Tracy, 1990) and thus possess procoagulant properties (Connor *et al*., 2009).

A very high percentage of PMVs express surface activation markers such as glycoprotein (GP)-Iba (CD42b), P-selectin(CD62P), b3-integrin(GPIIIa,CD61), aIIb-integrin (GPIIb,CD41), lysosomal-associated membrane protein-3 (LAMP3,CD63), and thrombospondin-1(TSP-1). However, healthy individuals may also have CD41+/CD61+/PS+ -MVs derived from megakaryocytes instead of activated platelets (APs). Megakaryocyte-derived MVs differ from PMVs in that they do not express granule fusion markers ($CD62P^{-}$, $LAMP\text{-}1^{-}$) and only contain full-length filamin A (Flaumenhaft *et al*., 2009). PMVs carry as well other platelet proteins such as platelet activating factor (PAF), b-amyloid precursor, anticoagulant protein C/S, complement C56b-9, and the chemokines CXCR4, CXCL4, CXCL7, and CCL5, which may exert potent biological effects.

It has been demonstrated that the generation of PMVs can be triggered by various mechanisms: *via* (a) platelet activation by soluble agonists or (b) shear stress or (c) glycoprotein (GP) IIb/IIIa outside-in signaling (Connor DE *et al*., 2010; Diehl P *et al*., 2008). Once generated, PMVs have generally been observed to have a relatively short lifespan with studies demon-strating PMVs to have half-lives of 30 min in mice (44), 10 min in rabbits (45), and approximately 5.5 h in apheresis-derived PMVs (Rank A *et al*., 2011). PMVs have recently been described to carry mitochondria, which can influence

inflammatory responses (Boudreau LH *et al*., 2014). Thus, the formation of the platelet microvesicles PMVs and its characteristics is shown in the figure 4 below.

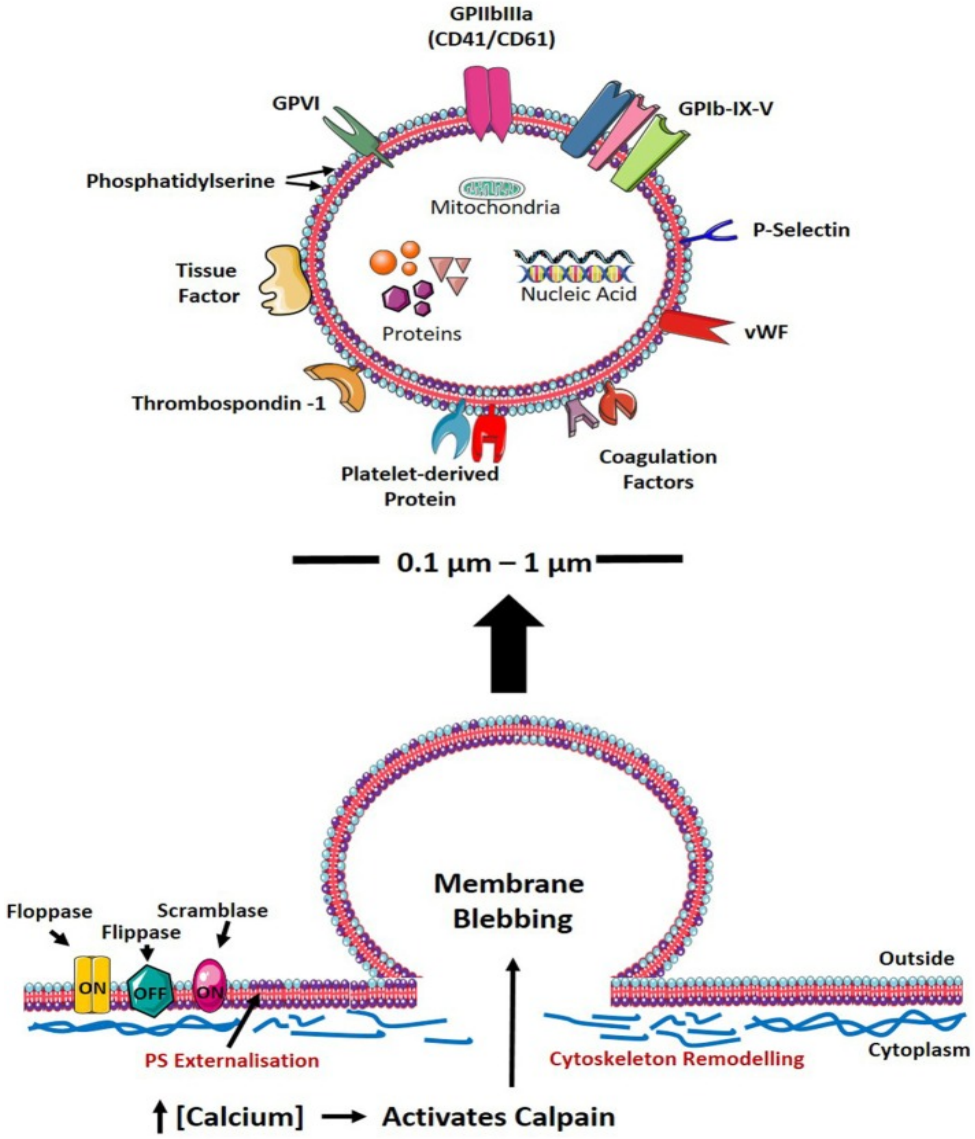


Figure 4: PMV formation and characteristics

Source: Zaldivia, Maria TK, *et al*., 2017

2.2 Isolation of platelet microvesicles:

PMVs are naturally present in human blood, but are mixed with microparticles (MP) originated from other cells. To isolate platelet-derived microvesicles, there are many existing techniques are there:

2.2.1 Gel Filtration:

Gel filtration is used to separate the molecules differing in their hydrodynamic radius and is widely exploit for separation of biopolymers i.e. proteins, polysaccharides, proteoglycans, etc. (Konoshenko, Maria Yu, *et al*., 2018). Gel filtration is also called as Molecular Sieving or Size Exclusion Chromatography. Cross linked hydrophilic polymer (gel) of different porosity (average pore size) are employed for this purpose. The porosity is determined by the degree of cross linking (Böing, Anita N., *et al*., 2014). Sepharose 2B beads of 60-200 µm diameters are used to separate platelets suspended in plasma into the medium of buffer. In view of the size, the platelets are excluded from Sepharose 2B beads and eluted in the void volume, whereas plasma components are included inside the beads (Aatonen, Maria T., *et al*., 2014). Platelet isolation by gel filtration substitutes the last two centrifugation steps of differential centrifugation method of platelet isolation (Gámez-Valero, Ana, et al., 2016; Lozano-Ramos, Inés, *et al*., 2015)

2.2.2 Centrifugation:

The classical method for EVs isolation utilizes the separation of particles according to their buoyant density by centrifugation. At the first stage, the particles with a high buoyant density are sedimented, such as cells, cell debris, apoptotic bodies, and aggregates of biopolymers (Clotilde, *et al*., 2006; Mikhail A., *et al*., 2015; Kenneth W., *et al*., 2012). In order to reduce losses caused by co-sedimentation and to decrease contamination of the preparations with the products of cell lysis, this step also includes several sub steps, namely, centrifugation at 300–400 ×g for 10 min to sediment a main portion of the cells, at 2000 ×g to remove cell debris, and at 10,000 ×g to remove the aggregates of biopolymers, apoptotic bodies, and the other structures (Jan, *et al*., 2014). EVs contained

in the resulting supernatant are sedimented by ultracentrifugation at >100,000 ×g (100,000–200,000 ×g) for 2 h (Maria Yu, *et al*., 2018). The non-EV proteins in the EV pellet are removed by suspending followed by repeated ultracentrifugation (Natasa, *et al*., 2015; Kenneth W., *et al*., 2013).

2.3 Characterization of isolated platelet microvesicles:

Characterization of platelet microvesicles (PMVs) can be carried out through the following existing techniques:

2.3.1 ELISA:

The enzyme-linked immunosorbent assay (ELISA) is a test that uses antibodies and color change to identify a substance. It is a popular format of "wet-lab" type analytic biochemistry assay that uses a solid-phase enzyme immunoassay (EIA) to detect the presence of a substance, usually an antigen, in a liquid sample or wet sample. Antigens from the sample are attached to a surface. Then, a further specific antibody is applied over the surface so it can bind to the antigen. This antibody is linked to an enzyme, and, in the final step, a substance containing the enzyme's substrate is added. The subsequent reaction produces a detectable signal, most commonly a color change in the substrate. Coating ELISA plate wells with PMVs specific antibody (e.g. CD41, CD42, CD9, CD62P, and KMP-9) and capture assays facilitates simple spectrophotometric or fluorimetric detection of PMVs. It observe that KMP-9 captured antibody based method correlated best with FCM analysis of PMVs (Kazuoki, et al., 2001). ELISA can perform other forms of ligand binding assays instead of strictly "immuno" assays, though the name carried the original "immuno" because of the common use and history of development of this method. The technique essentially requires any ligating reagent that can be immobilized on the solid phase along with a detection reagent that will bind specifically and use an enzyme to generate a signal that can be properly quantified. Advantage of this ELISA technique is that multiple samples of small volume can be analyzed at the same time making it suitable for clinical applications.

2.3.2 Microscopy:

Microscopy is a valuable tool for detecting small size particles and is considered gold standard for imaging.

2.3.2.1 Field Emission Scanning Electron Microscope (FESEM):

FESEM is a microscope that works with electrons (particles with a negative charge) instead of light. These electrons are liberated by a field emission source. The object is scanned by electrons according to a zig-zag pattern. It is used to visualize very small topographic details on the surface or entire or fractioned objects. Researchers in biology, chemistry and physics apply this technique to observe structures that may be as small as 1 nm. Sharma, Shivani, *et al*., 2012). The FESEM may be employed for example to study organelles and DNA material in cells, synthetic polymers, and coatings on microchips. Electrons are liberated from a field emission source and accelerated in a high electrical field gradient. Within the high vacuum column these so-called primary electrons are focused and deflected by electronic lenses to produce a narrow scan beam that bombards the object. As a result secondary electrons are emitted from each spot on the object (Hamdan, Halimaton, *et al*., 1997). The angle and velocity of these secondary electrons relates to the surface structure of the object. A detector catches the secondary electrons and produces an electronic signal (Aksoy, Seval, *et al*., 2012). This signal is amplified and transformed to a video scan-image that can be seen on a monitor or to a digital image that can be saved and processed further (Jiao, Kun, *et al*., 2014; Pap, Andrea Edit, *et al*., 2002).

2.3.2.2 Transmission electron microscopy (TEM):

TEM uses electrons instead of photons to create an image. The best achievable imaging resolution depends largely on the spatial stability of the electron beam in combination with the chemical stability of the sample. As the wavelength of electrons is more than three orders of magnitude shorter than the wavelength of visible light, the resolution of TEM can be lower than 1 nm. Because of this high resolution, it is possible to determine the size and morphology of microvesicles (Pisitkun *et al*., 2004). As TEM is performed in a vacuum, biomaterials require fixation and dehydration, which affect size and morphology.

Furthermore, the concentration of microvesicles has to be increased by ultracentrifugation. As a consequence, the size distribution depends upon pre analytical conditions, and the concentration of microvesicles cannot be determined. With immuno-gold labeling, it is possible to provide biochemical information. The measurement time is in the order of hours (Van Der Pol, E., *et al*., 2010)

2.3.2.3 Atomic force microscopy (AFM):

AFM was developed in 1986 (Binnig *et al*., 1986) and provides sub nanometer resolution topography imaging. An atomic force microscope consists of a cantilever with a sharp tip at its end that scans a sample surface without physical contact. Movements of the tip are measured, and a three-dimensional image is created by software. AFM can be used to measure the relative size distribution of microvesicles in their physiologic state (Siedlecki *et al*., 1999 and Yuana *et al*., 2009). Because of the high resolution, platelet microvesicles must be bound to an extremely flat surface. Antibodies can be used to bind microvesicles to the surface, so that biochemical information can also be obtained. The surface binding may affect the morphology of microvesicles, and this may hamper the determination of the real diameter (Yuana, Y., *et al*. 2010; Frank AW, *et al*., 2017).

2.3.3 Electrochemical Biosensor:

Biosensors are newer tools for convenient assay. Selective binding of PMVs on specially fabricated electrode surface applies resistance in the electrical circuit, which is then detected by electro-chemical analyzer as concentration dependent increased impedance. This biosensor design is most suitable for quick, cost effective, sensitive and user friendly point of care testing. Another design of biosensor based on nanosilica-PAC1 antibody, P-selectin antibody and conjugated HRP on ITO (Indium Tin oxide) electrode was reported. Analysis can be done by cyclic voltammetry (CV) and successfull detection of PMVs numbers in samples (Kailashiya, Jyotsna, *et al*., 2015; Singh, Priti, *et al*., 2017).

2.2.4 Flow Cytometer:

Flow cytometry is the most widely used technique for estimation and characterization of PMPs. It is most suitable for phenotyping of PMPs, but not very accurate for size distribution analysis. Standard flow cytometer is based on single particle based analysis where laser is targeted at flowing particle and scattered light is measured by detectors as forward scatter (for size), side scatter (for complexity) and fluorescence intensity (for specific fluorochrome binding). There are impedance based flow cytometer also, working on Coulter principle, where particle volume and size is measured by replaced electrolyte volume for each particle passing through instrument aperture (Lacroix, Romaric, *et al*., 2010; Cointe, Sylvie, *et al*., 2017). After acquisition, software compiles data from certain numbers of particles analyzed and presents it in terms of scatter plot, histograms and many other graphical forms. It facilitates analysis of large number of MPs in very short period of time (Robert, S., *et al*., 2009) Absolute count, surface and content characterization, procoagulant activity etc. can be analyzed by flow cytometer (Helmond, Sarah E *et al*., 2013; Mobarrez, Fariborz, *et al*., 2010).

CHAPTER- III

MATERIALS AND METHODS

3.1 Materials:

Citrate-phosphate-dextrose solution with adenine (CPDA), were purchased from Sigma-Aldrich; ethylene diamine tetraacetic acid (EDTA) were from Merck; Buffer A (20 mM HEPES ($C_8H_{18}N_2O_4S$), 138 mM NaCl, 2.9 mM KCl, 1 mM MgCl2, 0.36 mM NaH2PO4, 1 mM EGTA, supplemented with 5mM glucose), buffer B (20 mM HEPES, 138 mM NaCl, 2.9 mM KCl, 1 mM MgCl2, 0.36 mM NaH2PO4, supplemented with 5mM glucose), glutaraldehyde ($C_5H_8O_2$), Glucose ($C_6H_{12}O_6$) and ethyl alcohol (C_2H_5OH) were purchased from Himedia. All other chemicals were also of analytical grade.

3.2 Platelet microvesicles preparation:

Venous blood was drawn from healthy volunteers with proper safety under proper medical guidance with fresh and new syringe in a 15 ml tube in a citrate-phosphate-dextrose adenine. Put the tube in the ice bath and centrifuge (Company: Sigma (3-18 KS) at the differential centrifugation at the room temperature (RT) with required acceleration and deceleration & isolated the PMVs. Buffer A (20 mM HEPES, 138 mM NaCl, 2.9 mM KCl, 1 mM MgCl2, 0.36 mM NaH2PO4, 1 mM EGTA, supplemented with 5mM glucose) and buffer B (20 mM HEPES, 138 mM NaCl, 2.9 mM KCl, 1 mM MgCl2, 0.36 mM NaH2PO4, supplemented with 5mM glucose) were simultaneously used for the platelet microvesicles preparation.

For further details of the experimental procedure see the flowchart below:

3.2.1 Isolation of blood plasma:

Collected Blood total volume of 10ml (1.6ml CPDA (0.109M, final Concentration) as anticogulant+8.4ml blood) with healthy donor in a tube

Centrifuged at 180×g for 15 min at RT (acceleration of 09 and deceleration of 02, see figure: 5)

We get PRP (Platelet Rich Plasma) as supernatant and discarded the remaining cells (RBC & WBC, See figure- 6)

Added EDTA (5mM) in PRP (10 µl/ml)

Again Centrifuged at 800×g for 15 min at RT (acceleration 09 and deceleration 04)

Taken out PPP (Platelet Poor Plasma) as supernatant

Figure 5: Platelet Rich Plasma (PRP)

Figure 6: Remaining blood cells (RBC, WBC, etc.)

3.2.2 Isolation of microvesicles from all cell derived:

PPP were taken in 2 tubes of volume 1.6 ml each for Ultracentrifuge (See figure: 7)

Ultracentrifuge it at 20,000×g for 30 min at the RT

Added 500µl in the pallet and resuspend it and after that fixed the pallet with Glutaraldehyde. Here, we get the **Sample-1** for the Characterization with the help of various techniques i.e., SEM, flow cytometer, etc.

Figure 7: Platelet Poor Plasma (Supernatant) & Platelets (Pellet)

3.2.3 Isolation of platelet-derived microvesicles (PMVs):

Add 5ml Buffer A in the remaining pellet and centrifuged at 800×g for 15 min at RT with acceleration 09 and deceleration 04

Discarded the supernatant and added 1ml Buffer B in the pellet and resuspend it in the tube

Separated it in the 2 tubes (2ml tube) of volume 500µl each I.e., 500ml RP (Resting Platelets) and 500µl AP (Activated Platelets)

Taken RP as a **Sample-2** and vortexed the AP

Centrifuged the vortexed tube at 800×g for 15 min at RT

We get the PMPs (Platelet Microparticles) as supernatant (**Sample-3**) and the pellet

Resuspend the pellet in Buffer B

Separated it into 2 tubes with volume of 200µl and 200µl each and this is **Sample-4** for Characterization process

3.3 Characterization of isolated platelet microvesicles (PMVs):

For the characterization of platelet-derived microvesicles (PMVs) with the SEM (Company: Carl Zeiss, Germany, Model No: Merlin Compact) the prepared samples were adhered on coverslips for 45 to 60 minutes and dehydrated the adhered sample with 10, 50, 70 and 100 (absolute) percentage of ethyl alcohol on the coverslip and also the sample were gold coated for the comprehensive study of size and surface morphology.

For absolute counting of PMPs through flow cytometry (Company: BD Diagnostic, USA Model No: Accuri C6), the sample were taken into the 2 ml eppendorf tubes and for the reference purpose working solution of buffer B was used. At the time of analysis, an appropriate gate on PMVs population was drawn to differentiate it from platelets and also to exclude background noise. Forward scatter (FSC) and side scatter (SSC) was plotted on logarithmic scale. Events were acquired using collection criteria of the software to stop acquisition after 30 seconds has elapsed (time limit).

CHAPTER- IV

RESULT AND DISCUSSION

4.1 Characterization of resting, activated platelets and platelet-derived microvesicles (PMVs) through FESEM:

Surface morphology of isolated platelets was characterized through FESEM. SEM images showed resting platelets as smooth spherical cells of 2-3 μm in diameter separated from each other having presence of small hyaloplasmic extensions (Figure 8). Small filopodial extensions is might be due to weak activation of these platelets. In contrast, platelet activation elicits significant morphological change exhibited well-developed pseudopods (hyaloplasmic processes), which connected the cells forming large aggregates (clumps). Activated platelets exhibited fully spread activation morphology (Figure 9a). Figure 9b SEM image at a scale bar of 200 nm clearly showed platelet microvesicles release of size in the range of 200-500 nm from the activated platelets.

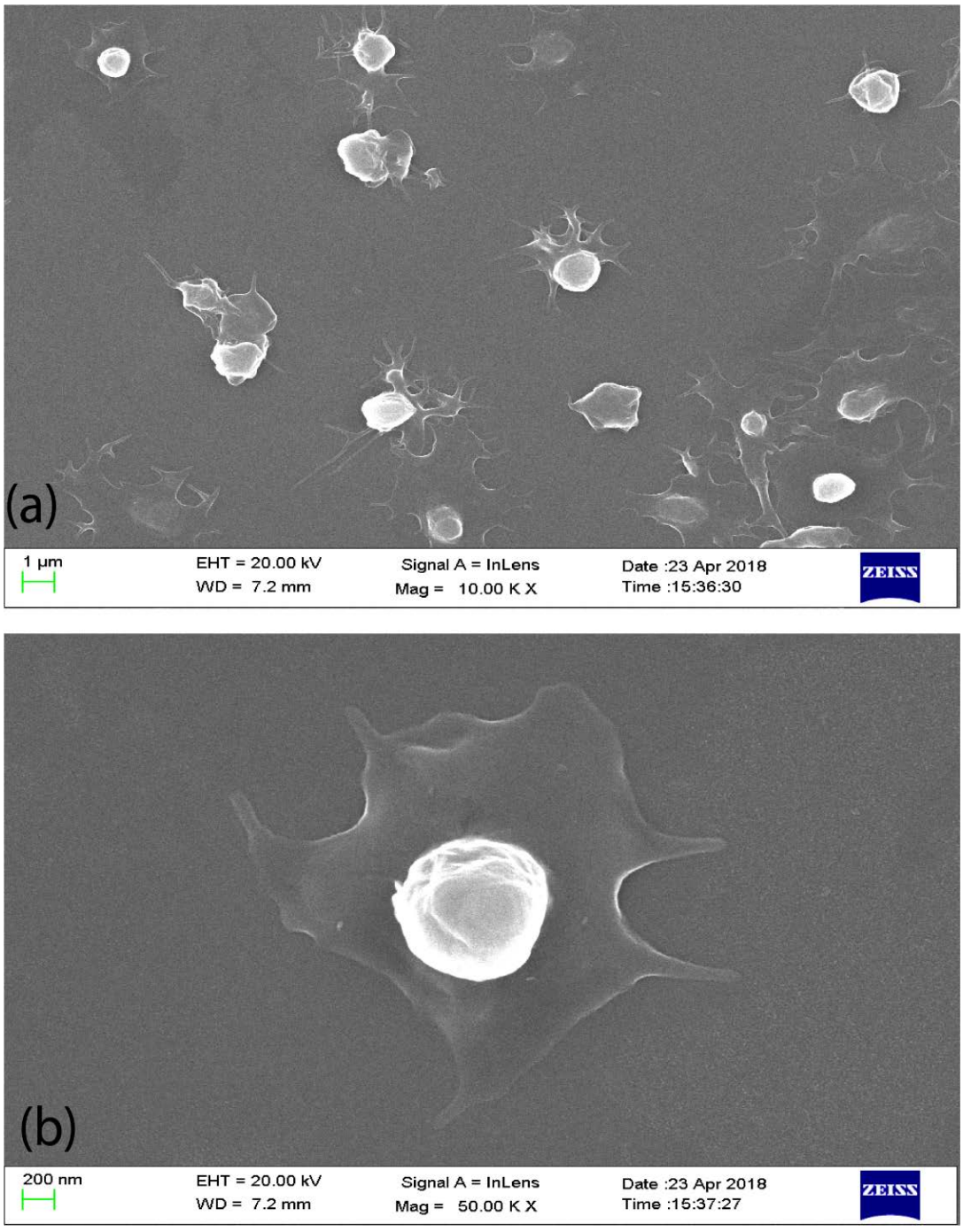


Figure 8: SEM characterization of resting platelets (RPs) **(a)** at a scale bar 1 µm **(b)** at a higher magnification (i.e. 50 KX) with scale bar of 200 nm.

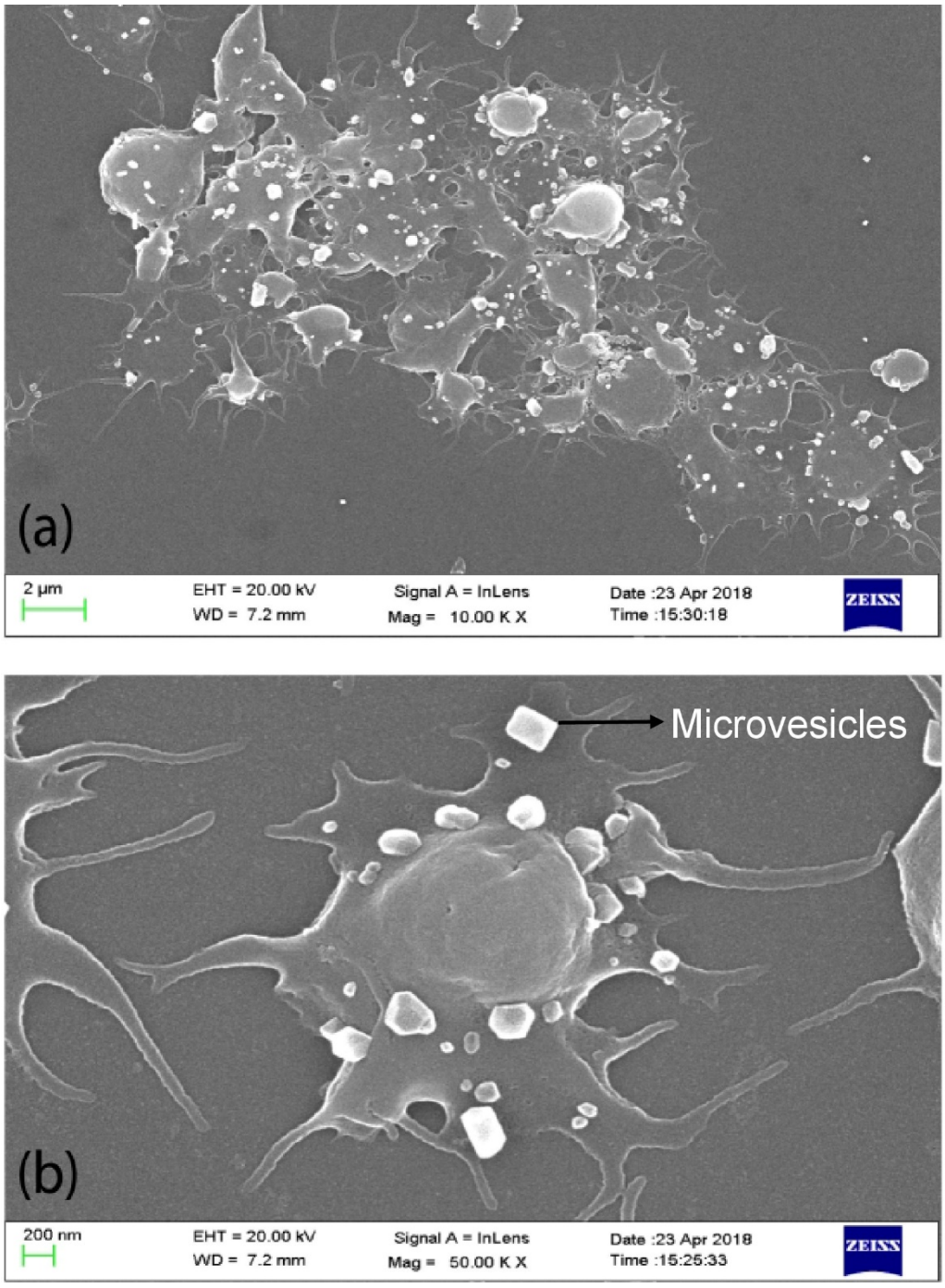


Figure 9: SEM characterization of activated platelets (APs) at different magnifications **(a)** 10.00 KX (at a scale bar 1 µm) and **(b)** 50.00 KX (at a scale bar 200 nm)

4.2 Characterization of resting, activated platelets and platelet-derived microvesicles (PMVs) through Flow Cytometry:

Isolated platelets was also characterized through flow cytometry analysis based on their FSC and SSC parameters. Isolated platelets and platelet microvesicles population were acquired and displayed in the dot plot acquisition quadrants (Figure 10). Buffer fluid was acquired to determine background signal/noise. Majority of the Microvesicles and platelet population was found to be distributed in the upper and right regions/quadrants (Figure 1b-d) reasonably well distanced from background noise, while the latter was confined to the left lower quadrant close to the origin (Figure 10a). However, some microvesicles signals existed in the lower regions/quadrant along with noise. Gate was imposed on the microvesicles and platelet population present in both the upper or right quadrants for exclusive analysis.

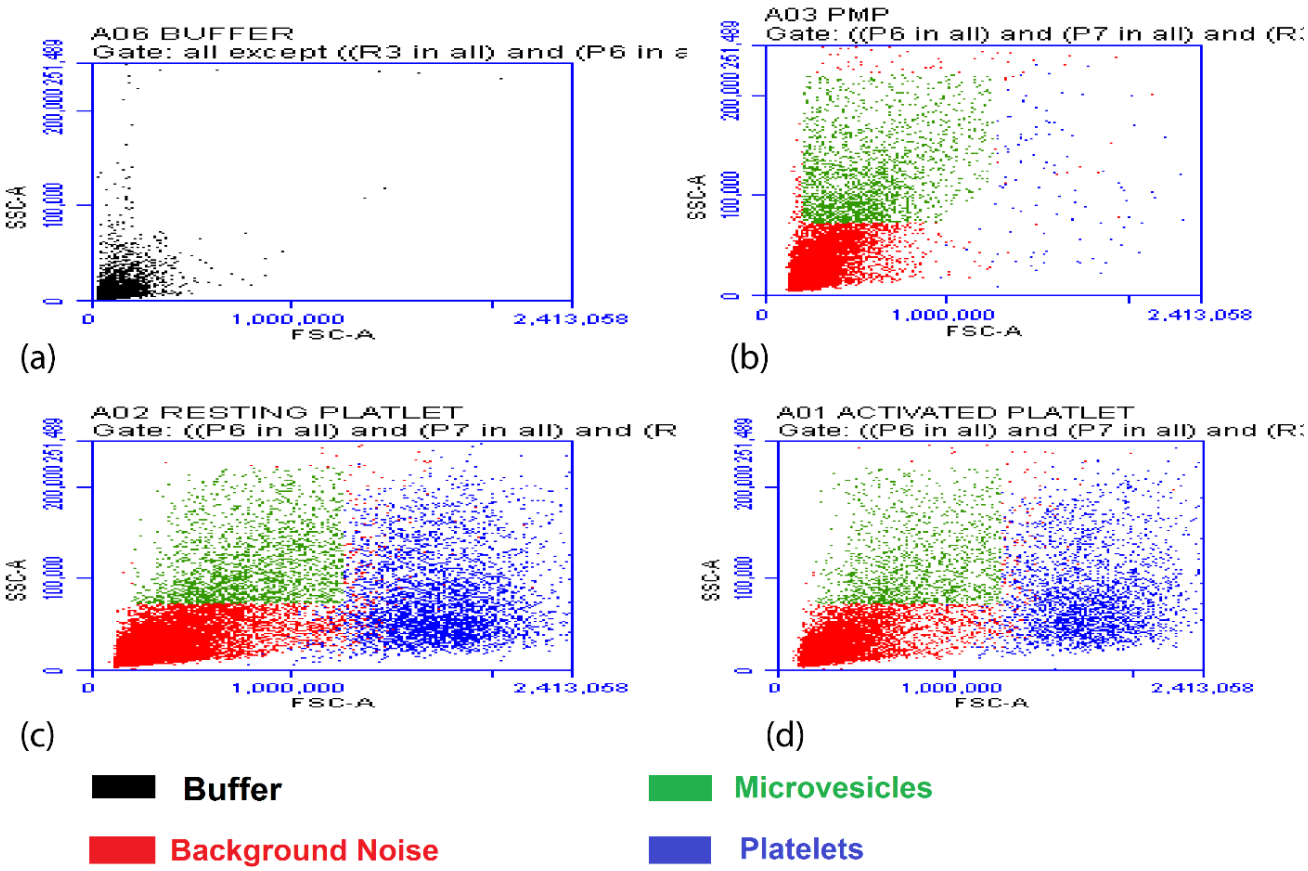


Figure 10: FSC–SSC dot plot displaying size distribution of platelets and PMVs population were acquired for 30 s at constant flow rate. a) Buffer fluid (b) PMVs (c) Resting platelets (d) Activated platelets

Thus, in this study an isolation technique for obtaining platelet microvesicles was optimized by first isolating platelets from whole blood and then separating all PMVs populations by differential centrifugation. MVs release upon platelet activation were characterized by SEM, and flow cytometry to further strengthen the earlier studies carried out for the determination of PMVs size and its release during platelet activation.

CHAPTER- V

CONCLUSION AND FUTURE ASPECTS

As per the available literatures, it is well understood that PMPs act as markers of various diseases like diabetes, thrombosis, coronary artery diseases and have prognostic potential. In this study, MVs released from activated platelets was isolated and characterized. The released PMVs were isolated by differential centrifugation and characterized through scanning electron microscopy (SEM) and flow cytometer (FACS). SEM images showed remarkable differences in surface morphology between resting platelets and activated platelets. It was also clearly evident that PMVs are released during platelet activation while it is absent in resting platelets. Electron microscopy and flow cytometry data showed PMVs release upon platelet activation and have size in the range of 200-500 nm. Thus, in this study an isolation and characterization technique for platelet microvesicles was optimized that can be further validated with clinical samples to throw more light on PMVs as a prognostic biomarker for various diseases.

REFERENCES

Singh, P., Srivastava, S., Chakrabarti, P., & Singh, S. K. (2017). Nanosilica based electrochemical biosensor: A novel approach for the detection of platelet-derived microparticles. *Sensors and Actuators B: Chemical*, *240*, 322-329.

Kailashiya, J. (2018). Platelet-derived microparticles analysis: Techniques, challenges and recommendations. *Analytical biochemistry*.

Van Der Pol, E., Hoekstra, A. G., Sturk, A., Otto, C., Van Leeuwen, T. G., & Nieuwland, R. (2010). Optical and non-optical methods for detection and characterization of microparticles and exosomes. *Journal of Thrombosis and Haemostasis*, *8*(12), 2596-2607.

Suades, R., Padro, T., Vilahur, G., & Badimon, L. (2012). Circulating and platelet-derived microparticles in human blood enhance thrombosis on atherosclerotic plaques. *Thrombosis and haemostasis*, *108*(6), 1208-1219.

Kadota, T., Fujita, Y., Yoshioka, Y., Araya, J., Kuwano, K., & Ochiya, T. (2017). Emerging role of extracellular vesicles as a senescence-associated secretory phenotype: Insights into the pathophysiology of lung diseases. *Molecular aspects of medicine*.

Lässer, C., Jang, S. C., & Lötvall, J. (2018). Subpopulations of extracellular vesicles and their therapeutic potential. *Molecular aspects of medicine*.

Suades, R., Padro, T., Alonso, R., Lopez-Miranda, J., Mata, P., & Badimon, L. (2014). Circulating CD45+/CD3+ lymphocyte-derived microparticles map lipid-rich atherosclerotic plaques in familial hypercholesterolaemia patients. *Thrombosis and haemostasis*, *112*(01), 111-121.

Laberge, A., Arif, S., & Moulin, V. J. (2018). MICROVESICLES: INTERCELLULAR MESSENGERS IN CUTANEOUS WOUND HEALING. *Journal of cellular physiology*.

Badimon, L., Suades, R., Arderiu, G., Chiva-Blanch, G., Peña, E., & Padró, T. (2017). Microvesicles in Atherosclerosis and Angiogenesis: From Bench to Bedside and Reverse. *Frontiers in cardiovascular medicine*, *4*, 77.

Van Niel, G., D'Angelo, G., & Raposo, G. (2018). Shedding light on the cell biology of extracellular vesicles. *Nature Reviews Molecular Cell Biology*.

Zaldivia, M. T., McFadyen, J. D., Lim, B., Wang, X., & Peter, K. (2017). Platelet-Derived Microvesicles in Cardiovascular Diseases. *Frontiers in cardiovascular medicine*, *4*, 74.

Badimon, L., Suades, R., Fuentes, E., Palomo, I., & Padró, T. (2016). Role of platelet-derived microvesicles as crosstalk mediators in atherothrombosis and future pharmacology targets: a link between inflammation, atherosclerosis, and thrombosis. *Frontiers in pharmacology*, *7*, 293.

Aatonen, M. T., Öhman, T., Nyman, T. A., Laitinen, S., Grönholm, M., & Siljander, P. R. M. (2014). Isolation and characterization of platelet-derived extracellular vesicles. *Journal of extracellular vesicles*, *3*(1), 24692.

McBride, J. D., Rodriguez-Menocal, L., & Badiavas, E. V. (2017). Extracellular vesicles as biomarkers and therapeutics in dermatology: a focus on exosomes. *Journal of Investigative Dermatology*, *137*(8), 1622-1629.

Arraud, N., Linares, R., Tan, S., Gounou, C., Pasquet, J. M., Mornet, S., & Brisson, A. R. (2014). Extracellular vesicles from blood plasma: determination of their morphology, size, phenotype and concentration. *Journal of Thrombosis and Haemostasis*, *12*(5), 614-627.

Yuana, Y., Böing, A. N., Grootemaat, A. E., van der Pol, E., Hau, C. M., Cizmar, P., ... & Nieuwland, R. (2015). Handling and storage of human body fluids for analysis of extracellular vesicles. *Journal of extracellular vesicles*, *4*(1), 29260.

Shai, E., Rosa, I., Parguiña, A. F., Motahedeh, S., Varon, D., & García, Á. (2012). Comparative analysis of platelet-derived microparticles reveals differences in their amount and proteome depending on the platelet stimulus. *Journal of proteomics*, *76*, 287-296.

Schmidmaier, G., Herrmann, S., Green, J., Weber, T., Scharfenberger, A., Haas, N. P., & Wildemann, B. (2006). Quantitative assessment of growth factors in reaming aspirate, iliac crest, and platelet preparation. *Bone*, *39*(5), 1156-1163.

Lamparski, H. G., Metha-Damani, A., Yao, J. Y., Patel, S., Hsu, D. H., Ruegg, C., & Le Pecq, J. B. (2002). Production and characterization of clinical grade exosomes derived from dendritic cells. *Journal of immunological methods*, *270*(2), 211-226.

Lamparski, H. G., Metha-Damani, A., Yao, J. Y., Patel, S., Hsu, D. H., Ruegg, C., & Le Pecq, J. B. (2002). Production and characterization of clinical grade exosomes derived from dendritic cells. *Journal of immunological methods*, *270*(2), 211-226.

Isola, A. L., Eddy, K., & Chen, S. (2016). Biology, therapy and implications of tumor exosomes in the progression of melanoma. *Cancers*, *8*(12), 110.

Mobarrez, F., Antovic, J., Egberg, N., Hansson, M., Jörneskog, G., Hultenby, K., & Wallén, H. (2010). A multicolor flow cytometric assay for measurement of platelet-derived microparticles. *Thrombosis research*, *125*(3), e110-e116.

Helmond, S. E., Catalfamo, J. L., & Brooks, M. B. (2013). Flow cytometric detection and procoagulant activity of circulating canine platelet-derived microparticles. *American journal of veterinary research*, *74*(2), 207-215.

Lacroix, R., Robert, S., Poncelet, P., & Dignat-George, F. (2010, November). Overcoming limitations of microparticle measurement by flow cytometry. In *Seminars in thrombosis and hemostasis* (Vol. 36, No. 08, pp. 807-818). © Thieme Medical Publishers.

Robert, S., Poncelet, P., Lacroix, R., Arnaud, L., Giraudo, L., Hauchard, A., ... & Dignat-George, F. (2009). Standardization of platelet-derived microparticle counting using calibrated beads and a Cytomics FC500 routine flow cytometer: a first step towards multicenter studies?. *Journal of Thrombosis and Haemostasis*, *7*(1), 190-197.

Cointe, S., Judicone, C., Robert, S., Mooberry, M. J., Poncelet, P., Wauben, M., ... & Lacroix, R. (2017). Standardization of microparticle enumeration across different flow cytometry platforms: results of a multicenter collaborative workshop. *Journal of Thrombosis and Haemostasis*, *15*(1), 187-193.

Brisson, A. R., Tan, S., Linares, R., Gounou, C., & Arraud, N. (2017). Extracellular vesicles from activated platelets: a semiquantitative cryo-electron microscopy and immuno-gold labeling study. *Platelets*, *28*(3), 263-271.

Poncelet, P., Robert, S., Bouriche, T., Bez, J., Lacroix, R., & Dignat-George, F. (2016). Standardized counting of circulating platelet microparticles using currently available flow cytometers and scatter-based triggering: Forward or side scatter?. *Cytometry Part A*, *89*(2), 148-158.

Central University of Punjab, Bathinda

Declaration

I declare that all the changes suggested by the external examiner in the dissertation/thesis entitled **“isolation and characterization of platelet microvesicles"** submitted by me for the award of degree of Master in the Department of Animal Sciences has been incorporated in the dissertation/thesis.

(Name and signature of the student)

Department of Animal Sciences

School of Basic and Applied Sciences

Date:

(Name and signature of the supervisor)

Date:

Department of Animal Sciences

School of Basic and Applied Sciences

URKUND

Urkund Analysis Result

Analysed Document:	kapil thesis.docx (D39266131)
Submitted:	5/26/2018 1:50:00 PM
Submitted By:	Sunil.singh@cup.edu.in
Significance:	11 %

Sources included in the report:

https://docksci.com/extracellular-vesicles-as-biomarkers-andtherapeutics-in-dermatology- afocuson-exo_59eefad8d64ab25585d5e5bf.html
https://basicmedicalkey.com/blood-disease/

Instances where selected sources appear: 6