Development of lactadherin based electrochemical biosensor for the detection of platelet microvesicles

Project report submitted to Central university of Punjab

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In

Life Sciences (Specialization in Animal Sciences)

By

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May, 2018

DECLARATION

I, Ms Sneha Santra hereby declare that this project entitled "**Development of lactadherin based electrochemical biosensor for the detection 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.

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CERTIFICATE

This is to certify that this project entitled "**Development of lactadherin based electrochemical biosensor for the detection of platelet microvesicles" is a** record of original and independent review done by Ms Sneha Santra, a student admitted for M.Sc. program in Department of 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.

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ABSTRACT

Development of lactadherin based electrochemical biosensor for the detection of platelet microvesicles.

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Keywords: Platelets, Microvesicles, Lactadherin, Cardiovascular disease, Electrochemical biosensor

Platelet derived microvesicles (PMVs) are the most abundant membrane vesicles in the blood having a potent pro-inflammatory effect, promote coagulation and affect vascular function which are involved in the pathogenesis of cardiovascular disease including diabetes, thrombosis, and coronary artery diseases. Therefore, it is pertinent to detect PMVs level in blood of an individual which have prognostic potential for cardiovascular diseases. As per literature available, lactadherin is a small (53-66 kDa) multifunctional glycoprotein which plays an important role in the clearance of microvesicles. In the present study, lactadherin based electrochemical biosensor for the detection of PMVs was explored. Polythionine film (as a good electron mediator) was electrochemically deposited on ITO-coated glass through electrochemical process involving cyclic voltammetry (CV) and chronoamperometry. Electrochemically deposited electrode provides ideal adsorbing platform for immobilization of RGDS peptide sequences (Lactadherine binding motif) having binding affinity against active conformation of integrin on PMVs surface. CV and Diffusion Pulse Voltammetry (DPV) measurements showed gradual decrease of current with the subsequent adsorption of microvesicles poor plasma (MPP), microvesicles rich plasma (MRP) and activated platelets. Decrease of current clearly

depicts the presence of microvesicles in blood plasma. Our developed fabricated electrode can have a promising potential for its efficient application in clinical testing of various pathological conditions.

(Sneha Santra) (Dr. Sunil K Singh)

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

S. No	Full form	Abbreviation
1	Activated Platelets	AP
2	Cyclic voltametry	CV
3	Differential Pulse Voltammetry	DPV
4	Electrochemical Impedance Spectroscopy	EIS
5	Energy-Dispersed Spectrum	EDS
6	Fourier-transform infrared spectroscopy	FTIR
7	Indium Tin Oxide	ITO
8	Messenger RNA	m RNA
9	Microparticles	MPs
10	Microvesicles	MVs
11	Microvesicle Poor Plasma	MPP
12	Microvesicle Rich Plasma	MRP
13	Phosphatidylserine	PS
14	Phosphate Buffer Saline	PBS
15	Phospholipids	PL
16	Platelet derived microparticles	PMPs
17	Platelet derived microvesicles	PMVs
18	Platelet Poor Plasma	PPP
19	Polythionine	PTh
20	Scanning Electron Microscopy	SEM

Chapter- I

INTRODUCTION

Thrombotic diseases include myocardial infarction (heart attack), stroke which affects the brain tissues and deep vein thrombosis are one of the major causes of death. Thrombosis occurs due to the formation of a clot (platelets and fibrin) within a blood vessel that subsequently leads to blockage of blood vessels. As per recent reports, platelets are not only the factor responsible for thrombosis but also platelet derived microvesicles (PMVs) actively participate in thrombosis. In addition, the externalization of phosphatidylserine (PS) on the outer PMVs membrane has been shown to cause increased blood coagulation. To understand the clinical significance of PMVs, one should have the basic knowledge of blood and its various components which will further enhance our understanding of platelets and PMVs.

1.1 Blood Overview

Blood is a specialized bodily fluid 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 vital functions like, supplying oxygen to cells and tissues, removing waste material, protecting the body from foreign bodies and infections, regulating body temperature, pH levels, providing essential nutrients to cells.

Jan Swammerdam was the first person to observe Red Blood Cells 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 RBC carry Oxygen.

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 following figure.

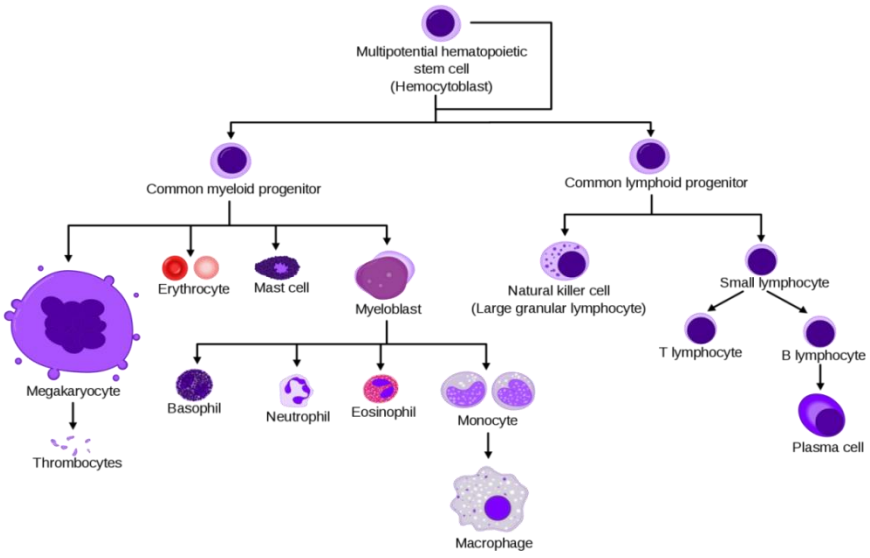


Figure 1: Formation of different blood cells

(Adapted from: https://en.wikipedia.org/wiki/Haematopoiesis)

1.2 Blood coagulation system

Hemostasis is a phenomenon, in order to arrest blood loss at the time of injury and protect us from an injured blood vessel wall. Blood clot is the main end product of hemostasis, that can be lifesaving but also life threatening. Imbalance of coagulation factors or platelets disorders can lead to excessive bleeding or thrombotic disorders. In order to form a clot, two factors are playing a crucial role, formation of fibrin and activated platelets. This process is divided into following phases:

• First, that resulted in platelets aggregation

• Second, is the one that lead to formation of fibrin

• Third, is the formation of solid thrombus by platelets and fibrin.

The following schematic diagram shows pathway of platelets activation and aggregation:

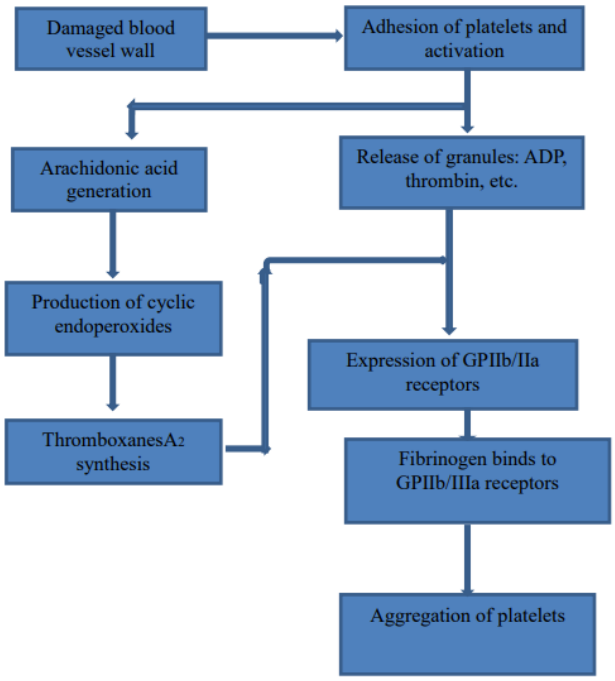


Figure 2: Pathway of platelets in blood coagulation system

(Adapted from: Literature review in field of blood clotting influencing drugs (Diploma Thesis) by Hradec Králové, 2015Konstantinos Kolotsios)

Activation and aggregation of platelets is a very complicated pathway that involves many protein, factors and receptors. Platelets usually are in resting phase but after the wall of blood vessels gets damaged they are activated. To have a better understanding of this topic lets venture in detail about platelets.

1.3 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).

1.3.1 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.

1.3.2 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., 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., 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 Ca2+, 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., 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.

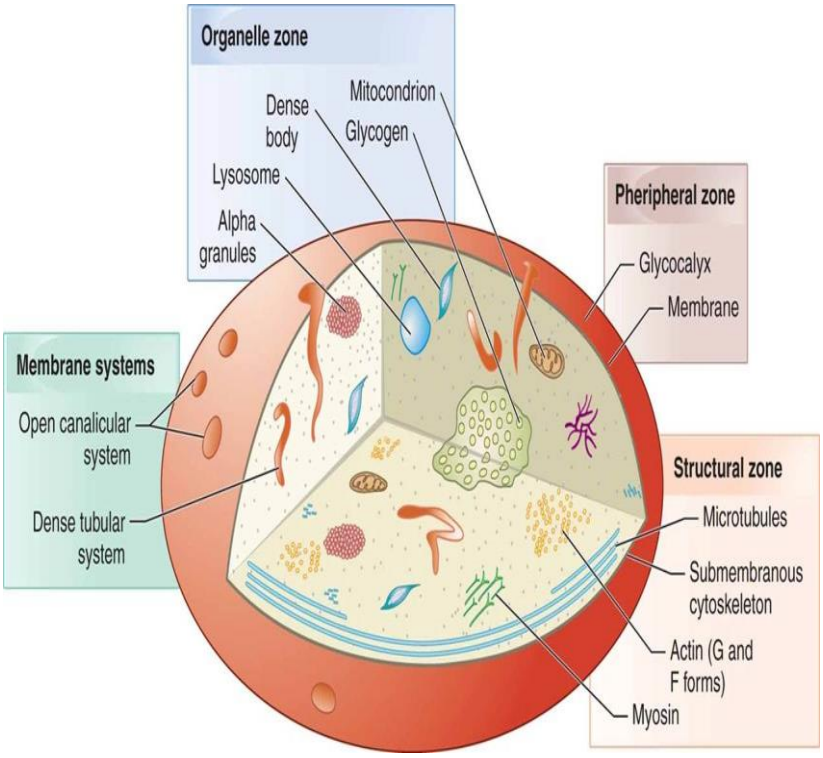


Figure 3a: Structure of platelet

(Adapted from: http://passtheclassandnotfail.blogspot.in)

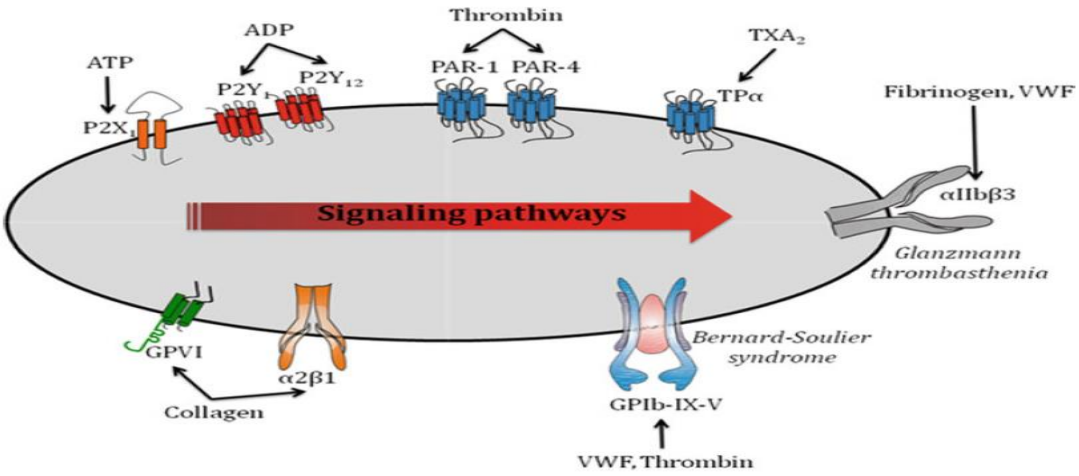


Figure 3b: Major receptors of platelets

(Adapted from: Kauskot, A., & Hoylaerts, M. F. (2012). Platelet receptors. In *Antiplatelet Agents* (pp. 23-57). Springer Berlin Heidelberg.)

α-Granules	
Adhesion molecules	[a]P-selectin (CD62P), [a]Von Willebrand Factor (VWF), [a]thrombospondin, fibrinogen, [a]integrin [b]$\alpha_{IIb}\beta_3$, integrin $\alpha_v\beta_3$, fibronectin
Chemokines	[b*]Platelet basic protein [[b]platelet factor 4 (PF4, CXCL4) and [b]β-thromboglobulin], MIP-1α (CCL3), RANTES (CCL5), MCP-3 (CCL7), growth-regulated oncogene-α (CXCL1), ENA-78 (CXCL5), IL-8 (CXCL8)
Coagulation pathway	[a]Factor V, [a]multimerin, factor VIII
Fibrinolytic pathway	α_2-Macroglobulin, plasminogen, plasminogen activator inhibitor-1 (PAI-1)
Growth and angiogenesis	Basic fibroblast growth factor, epidermal growth factor, hepatocyte growth factor, insulin-like growth factor 1, transforming growth factor β (TGF-β), vascular endothelial growth factor-A, vascular endothelial growth factor-C, platelet-derived growth factor (PDGF)
Immunologic molecules	β1H Globulin, factor D, c1 inhibitor, IgG
Other proteins	Albumin, α_1-antitrypsin (α_1-AT), Gas6, histidine-rich glycoprotein, high molecular weight kininogen, osteonectin protease nexin-II (amyloid beta-protein precursor)
Dense Granules	
Ions	Ca, Mg, P, pyrophosphate
Nucleotides	ATP, GTP, ADP, GDP
Membrane proteins	CD63 (granulophysin), LAMP-2
Transmitters	Serotonin

Table 1: Platelet α and dense granule contents.

(Adapted from: Reed G. Platelet Secretion. In: Michelson A, ed. *Platelets.* Vol 2nd Edition. San Diego: Elsevier; 2007)

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. **MV 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 (PMP) are most abundant MP 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)

Surprisingly, little is known regarding the half-time of PMVs in the circulatory system, with reports ranging from 10 minutes to several hours depending on the study. Among the mechanisms proposed to explain rapid clearance, is lactadherin-promoted phagocytosis mediated by macrophages. The endothelium has also been implicated in the elimination of PS+ PMVs from the circulation, notably by endocytosis through developmental endothelial locus-1 (Del-1), a protein mainly expressed by the endothelium that serves as an anchor between endothelial cells and PS-exposing MVs.

1.5 Lactadherin

Lactadherin is a secreted glycoprotein of milk-fat globule that shares structural domain homology with Del-1. It is also known as milk-fat globule-EGF factor 8 (MFG-E8). **Lactadherin level in healthy individuals is 790.2 ± 38.8 pg/**mL (Chikako Kishi *et al.,* 2017). Lactadherin is produced in and around blood vessels. In arteries, lactadherin is mainly expressed by adventitial microvessels, medial smooth muscle cells and some luminal endothelial cells (Silvestre et al., 2005). Lactadherin is composed of four functional domains starting from the N terminus: EGF1-EGF2-C1-C2 (Oshima,K *et al*., 2014). The EGF2-like domain (homology with epidermal growth factor) contains an arginine-glycine-aspartic acid (RGD) motif which enables the binding to the integrins and the C2 discoidin domain has homology to coagulation factors VIII and V. C2 domain contains a phosphatidylserine (PS) binding domain. Hence, lactadherin act as a bridge between macrophage containing integrins and PS rich microvessicles and help in MV phagocytosis by macrophages.

1.6 Electrochemical biosensor

An electrochemical biosensor is a small device that can be used for direct measurement of the analyte in the sample matrix. They are normally based on reaction that produces or consumes electrons (such reactions are called redox reaction).As compared to other biosensor electrochemical sensors have more advantages because; in these, the electrodes can sense the materials which are present within the host without doing any damage to the host system Comparative to other technologies, electrochemical biosensor is rapid, cheap, low- tech and easy to operate at ambient temperatures without external heating.

The ongoing research on new sensing concepts, combined with numerous technological advancements, has expanded horizons for extensive clinical applications of electrochemical biosensor (Wang J., 1999). The enhanced sensitivity, specificity, simplicity, and inherent miniaturization of modern electrochemical bioassays allow them to compete with the most advanced optical protocols (Wang J., 2006). In the recent few years electrochemical biosensor is widely used for the detection of various biomarkers. Feng et al reported an electrochemical biosensor for label free neoplastic cell detection using aptamer and functionalized graphene. The

high binding affinity of the aptamer to the overexpressed nucleolin on the neoplastic cell surface enabled the electrochemical aptasensor to detect as low as thousand cells (Feng L *et al*., 2011). Chen et al developed a simple, label free electrochemical biosensor for oral cancer detection based on nuclease-assisted target recycling and DNAzyme for the detection of DNA species related to oral cancer in saliva (Chen J *et al*., 2011). Also a few number of different nanoparticle-based biosensors all with its own specific properties have been recently published emphasizing the principal advantages of such nanoparticle usages for the electrochemical detection of MVs (Singh, P *et al*., 2017, Kailashiya, J *et al*., 2015). From this it can be concluded that electrochemical biosensor is the immerging process in the field of biosensors.
Electrochemical biosensor includes the following functional parameters:

a) Cyclic Voltammetry or CV

Cyclic voltammetry is a type of potentiodynamic electrochemical measurement in which the working electrode potential is changed linearly versus time. Cyclic voltammetry experiment ends when it reaches a set potential value. When cyclic voltammetry reaches the set potential, potential ramp of the working electrode is inverted back. This inversion can happen multiple times during a single experiment until a set cycle number is obtained. The plot of the current at the working electrode vs. the applied voltage gives the cyclic voltammogram of the reaction. Cyclic voltammetry is a general way to study the electrochemical properties of an analyte in a solution. (Bard, Allen J *et al*., 2000, R.S Nicholson *et al*., 1964)

b) Differential Pulse Voltammetry or DPV

DPV is a derivative of linear sweep voltammetry and staircase voltammetry, which is extremely useful to detect trace levels of organic and inorganic analytes. In this technique, there are a series of regular voltage pulses superimposed on the potential linear sweep or stair steps. Just before each potential change and late in the pulse life, the currents are recorded. The current difference is then

plotted against the applied potential. In the differential pulse voltammogram, the height of the current peak can be directly proportional to the concentration of corresponding analytes. The peak potential varies with different analytes, which can also be used to distinguish the detected species. DPV can not only help improve the sensitivity of the detection and the resolution of the voltammogram, but also provide information about the chemical form of the analytes, such as oxidation and complexation status, which is very important for an analysis. Therefore, this technology has also been widely used for the electrochemical analysis of proteins and cells.

c) Chronoamperometry

A potential is applied to the working electrode and steady state current is measured as a function of time for chronoamperometric measurement. There is a diffusion layer between electrode surface and solution media. The concept of a diffusion layer was introduced by Nernst. Diffusion controls the transfer of analyte from the bulk solution of higher concentration to the electrode. Thus there is a concentration gradient from solution media to the electrode surface.

Polythionine (PTh) as matrix

Electrochemical detection techniques use enzymes or peptide sequences because they have specific binding capabilities and biocatalytic activity. Some of the other biorecognition elements are antibodies, nucleic acids, cells and micro-organisms. Biorecognition elements should be immobilized on the electrode surface. Adsorption, covalent attachment and cross linking methods are the most well known immobilization methods. Mostly polythionine (PTh) is electrochemically deposited on the working electrode to form a matrix for immobilization on peptide sequences. It is widely used for matrix because it is electrochemically standardized dye, thionine molecule can oxidize and reduced through CV and it contains $–NH_2$ terminal that can interact with peptide sequence through covalent bond. Also PTh contains a nano-network structure composed of relatively uniform fibers and particles which helps in adsorption of the biorecognition elements.

Hypothesis

In case of various diseases such as diabetes and certain cardiovascular diseases it is found that the levels of MVs have increased. It is hypothesized that in such cases either there is reduction in the level of lactadherin or there is excessive production of MVs. It is yet to strengthen the correlation between lactadherin and microvesicles level in blood plasma. It is also expected that lactadherin based detection of microvesicle in blood plasma can be used for early diagnosis of thrombotic disorders.

Knowledge gap

After reviewing the recent progress in the field of microvesicles and lactadherin and their clinical correlation, it is found that there is not enough data establishing the link between the level of microvesicles and the level of lactadherin in the blood plasma. At the same time, there is no work done in the field of lactadherin based PMV detection through electrochemical biosensor.

Objective

- **To develop lactadherin based electrochemical biosensor for the detection of PMVs.**

To address the knowledge gap, the present experiment was designed to establish the link between the level of microvesicles and the level of lactadherin in the blood plasma with the help of electrochemical biosensor.

Chapter- II

REVIEW OF LITERATURE

2.1 Platelets hyperactivity (Activation/ Aggregation)

Platelets are the cells that circulate within our blood and bind together when they recognize damaged blood vessels. Platelets, the smallest of our blood cells are literally shaped like small plates in their non-active form. A blood vessel will send out a signal when it becomes damaged. When platelets receive that signal, they'll respond by traveling to the area and transforming into their "active" formation.

Platelet function is highly regulated by the surrounding endothelial lining and various factors. Classically, platelet aggregation is discussed in 3 consecutive steps: initiation, extension and perpetuation. The initiative activation may rise from exposure of collagen and VWF following vascular wall injury, gathering a monolayer of activated platelets lining the injury site. Collagen acts through α2β1 and GPVI, increasing platelet Ca2+ through a phospholipase $C_{\gamma}2$ mediated way, while VWF activates through GPIbα and α2bβ3 receptors. The VWF is crucial in platelet adhesion and rolling in high-shear situations, where VWF have a linear structure compared to low-shear where VWF has a globule like form (Schneider SW *et al*., 2007). The initial binding cause a smaller Ca2+ peak in platelets, resulting in the release of ADP. ADP can act on the P2Y1 receptors of resting platelets, activating platelets locally in a positive loop in a Gi-protein coupled manner, decreasing intraplatelet cyclic- AMP levels. Following firm adhesion to VWF through α2bβ3, an increased Ca2+ surge precedes platelet aggregation. From various collagen receptors, GPIV plays a pivotal role in platelet activation and aggregation. Other possible form of activation is through PAR receptors in the presence of thrombin, usually reported in thrombotic diseases. Patients receiving heparin can have platelet activation through FcR-IIA, while pathogens (mainly bacteria and fungi) may act both on FcRs and TLRs. The extension phase is mainly a positive loop of platelet activation, initiated by the monolayer of activated platelets. Among many other molecules, these fragments secrete thromboxane A2, ADP and help local thrombin formation, activating resting platelets of the circulation. These activating substances induce phospholipase C activity in platelets, increasing internal Ca2+ levels and the presence of surface α2bβ3. The α2bβ3 complex changes its conformation following the binding of phosphorylated talin (Ye F *et al*., 2012). Binding of fibrinogen fibers and platelet plug formation is mainly developed through these receptors. In the perpetuation phase, G-protein coupled receptor signals are already faded, integrins

and receptor tyrosine kinase signalling is responsible for platelet plug stability (Brass LF *et al*., 2007). Besides the classical role of platelets in haemostasis, their contribution to innate immune response is of high interest. The review of Beaulieu et al. summarises platelet reactions to pathogens and pathogen associated patterns. The activation of platelets through TLR-2 may induce PI3K-Akt-Erk pathway activation, causing platelet adhesion, aggregation, α-granule secretion and reactive oxygen species production, while TLR-4 activation and signal transduction through MyD88 cause cytoskeletal rearrangement, α-granule secretion and aggregation (Beaulieu LM *et al* ., 2010). The following figure shows the main things that happen when a platelet is activated.

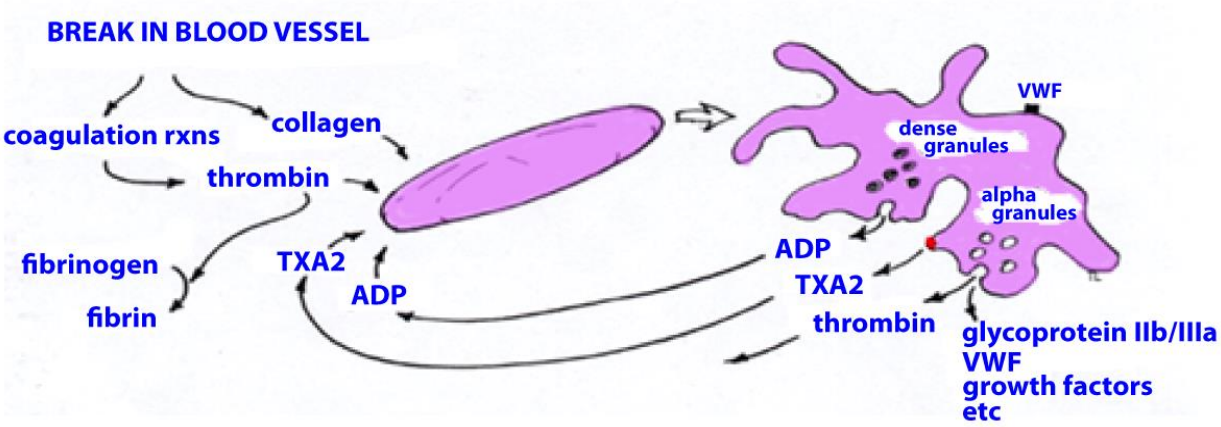


Figure 4: Major step involved in platelet activation

(Adapted from: https://courses.washington.edu/conj/bloodcells/platelets.htm)

2.2 Platelets Microvesicles

The first description of microvesicles (MVs) came from Wolf, in 1967, who first noted them as platelet-dust in platelet free plasma. He proved their procoagulant activity and that this feature is removable by ultracentrifugation (Wolf P *et al*.,1967). Although platelet-dust was known for more than four decades, the technical difficulty of MP measurement withheld their extensive analysis until the last decade. Nowadays we are aware that hypothetically all eukaryotic (and some prokaryotes) cells are capable of forming MPs, even though in the circulation of healthy individuals small amounts of MPs are present, most of them harboring platelet-specific proteins (>80%) (Flaumenhaft R *et al*., 2009). There was and still is

confusion in the naming of different bodily fluid vesicles. Although, the phrase microvesicle (MVs) is synonym for microparticles (MPs), MPs is the widely accepted term for 100-1000nm vesicular fragments produced by cell membrane shedding. Exosomes are <100nm doughnut shaped vesicles, generally produced during exocytosis from multivesicular bodies, or also by membrane blebbing (Cocucci E *et al.*, 2009)

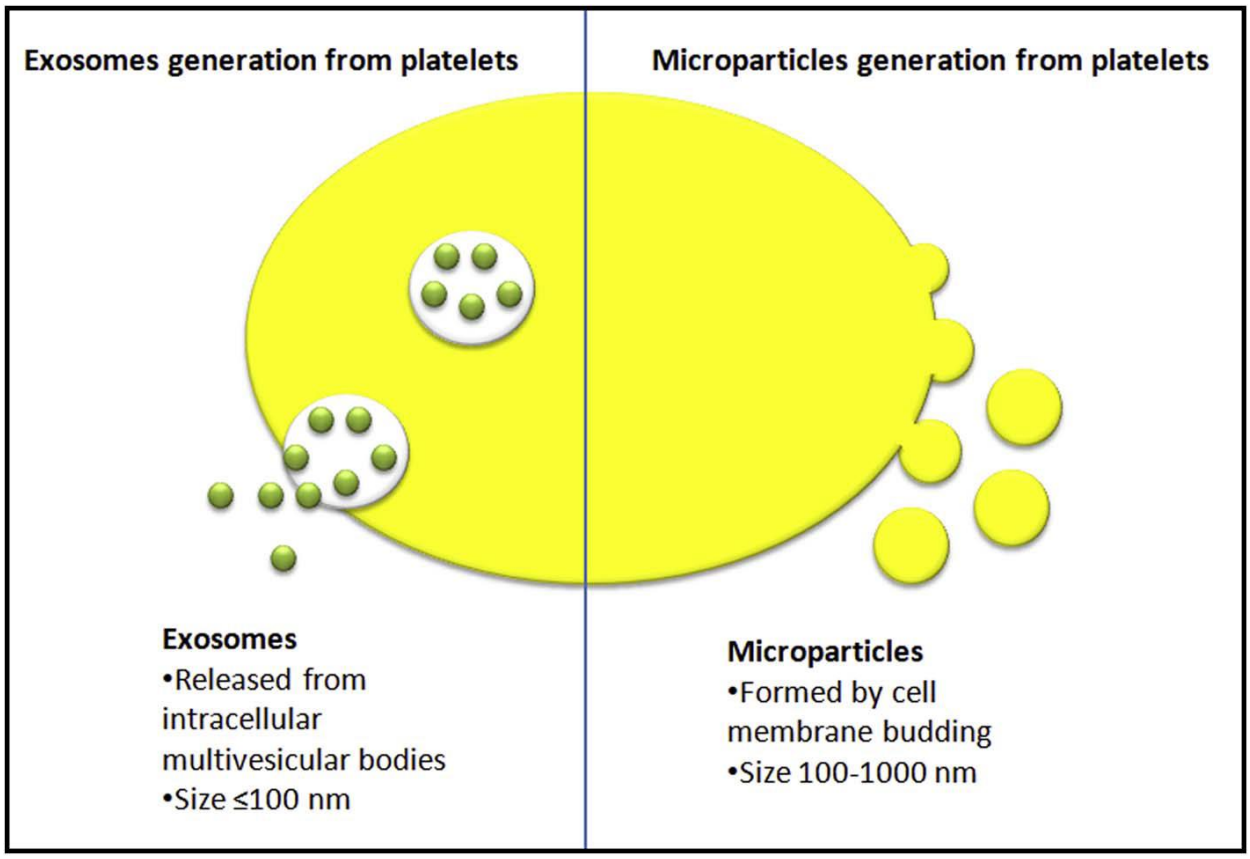


Figure 5: Schematic diagram showing formation of Platelet-derived microparticles and Exosomes from platelets.

(Adapted from: Platelet-derived microparticles analysis: Techniques, challenges and recommendations. Jyotsna Kailashiya, Department of Biochemistry, Institute of Medical Sciences, Banaras Hindu University, Varanasi, UP, 221005, India)

2.2.1 Clinical relevance of microvesicles

Many cells, including platelets, endothelial cells, leukocytes, and erythrocytes, shed MVs. There is quite a lot of evidence that these microparticles, have important physiological roles. It is also well proved that PMPs are the most abundant MVs in blood. From several sources it is evident that platelet microvesicles participate in

thrombus formation. Increased platelet microparticle levels are associated with many disease states including thrombotic thrombocytopenia (Galli M *et al*., 1996), heparin-induced thrombocytopenia (Hughes M *et al*., 2000), arterial thrombosis (Mallat Z *et al*., 2000, Lee YJ *et al*., 1993), sickle cell disease (Tomer A *et al*., 2001), rheumatoid arthritis (Boilard E *et al*., 2010), malignancy (Varon D and Shai E., 2009), and uremia (Ando M *et al*., 2002). Platelet microparticles have also been implicated in the pathogenesis of atherosclerosis as well as the regulation of angiogenesis (Tan KT *et al*., 2005). Because of all these reasons it becomes very important to monitor the level of PMPs so that we can modify the diseases with early precautionary steps.

2.2.2 Production and elimination of microparticles

Generally thought MPs are formed during the loss of membrane phospholipid asymmetry. Under normal resting conditions phosphatidylserine (PS) and phosphatidylethanolamine is located mainly in the inner leaet of the membrane, while phosphatidylcholine and sphingomyelin are abundant in the outer leaflet (Manno S *et al*., 2002,. Seigneuret M *et al*., 1984). This asymmetry is sustained by the ippase enzyme transferring aminophospholipids (PS and phosphatidylethanolamine) into the cytoplasmic membrane layer. During cell activation, apoptosis or increased shear stress the presence of PS in the outer leaet is one of the first signs of this processes (Daleke DL ., 2003). Besides random phospholipid movement, the activation of oppase(s) (an enzyme capable to direct aminophospholipids towards the outer leaet) and scramblase (enzyme catalysing random movement of phospholipids) accelerate the appearance of PS in the outer membrane. According to the "classical" view of MPs production, intracellular Ca2+ increase is the main determinant of MP formation. Calcium-induced degradation of cytoskeleton by calpains and the transient mass difference between membrane leaets support the formation of MPs and (Ca2+) inux following cell activation may inhibit ippase and activate oppase enzymatic activity (Bevers EM *et al*., 1999). Several signal transduction pathways are known to be involved in MP formation. Phosphorylation state of intracellular compartments as well as myosin light chain kinase, calmodulin and other calmodulin-related proteins are known to be responsible for platelet MP formation. For platelets, stable Ca2+ levels are necessary to prevent platelet activation and particle formation. Ca2+-dependent ATPases pump calcium ions back into the sarcoplasmatic and endoplasmatic

reticuli, or out from platelets. Calcium concentration may increase by agonist-dependent pathways (store-operated calcium entry) or through the purinerg P2X1 receptors (Varga-Szabo D *et al*., 2009). The store-operated entry pathway causes a rapid PS exposure without receptor-ligand interactions. In human erythroleukaemic cell line this pathway is regulated by the RhoA GTPase, instead of ROCK (Kunzelmann C *et al.,* 2004). The importance of the storage-operated pathway is hypothesised by the protection of collagen-induced arterial thrombus formation by the inhibition of Orai1 and STIM1 key proteins of this pathway. In platelets, STIM1 translocates from the dense tubuli into the membrane and co-localise with Orai1 upon store-depletion, to and STIM1 key proteins of this pathway. In platelets, STIM1 translocates from the dense tubuli into the membrane and co-localise with Orai1 upon store-depletion, to induce PS externalisation and thrombus formation (Munnix ICA *et al*., 2003). Also, cyclic adenosine monophosphate-dependent protein kinase activation reduced activation-dependent MP formation of platelets, while inhibition of this protein increased shedding. Caspases are known to take part in cell apoptosis (Cohen GM., 1997). These proteins are able to cleave filamin-1, gelsolin, talin and also myosin (Fox JE *et al*., 1990). The induced activation of caspases in platelets resulted in proportional PS exposure and membrane blebbing in the study of Schoenwaelder et al. Other vascular cell lineages are also producing MPs following caspase activation. Research on Jurkat T-cells provided information regarding caspases and cytoskeletal reorganization leading to MP formation. The cleavage of ROCK I kinase by caspase-3, or ROCK II activation by caspase-2 can both induce particle formation. ROCK I and II Rho kinases are responsible for myosin light chain phosphorylation which induces cell contraction and MP release during apoptosis. Regardless of the cause of PS+ MP release, the presence of these particles may start a positive loop of further PS-MP release from previously resting endothelial cells and platelets (Sebbagh M *et al*., 2001). Although the above described mechanism is the currently accepted model for MP formation, certain data question the single role of Ca2+ concentration in MP shedding. Most of our knowledge based on the use of Ca-ionophores (Connor DE *et al*., 2010). These compounds are able to increase intracellular Ca2+, triggering membrane changes even without storage-operated pathway activation (Galitzine M *et al*., 2005).

This effect of ionophore treatment is widely used in microparticle shedding assays, while the normalisation of cytosolic-cytoplasmatic Ca2+ levels may reduce particle

numbers. Other research data implicates not only Ca2+, but other electrolyte uses may cause the release of particles. Ca2+-sensitive K+ channels (Gardos channels) may cause the efflux of potassium, causing cell dehydration and PS exposure in platelets.

Protein composition of particles generally follows their host cells, which can be used in particle origin characterisation. Platelet-derived MPs (PMPs) harbour glycoproteins (GP) and different proteins characteristic for resting platelets, as well as ones expressed in activated platelets (see Table 2). Besides PS bearing MPs another group of MPs are PS negative. There is still a debate on the actual number and role of these MPs, as labeling of PS by annexin V is highly dependent on pH and the presence of Ca2+. Characteristics of these vesicles are not completely clear. Some hypothesise that the loss of PS is due to interaction with other membrane vesicles, cell debris and lipophilic proteins (Morel O *et al*., 2011).

Epitope	Antibody clones
Constitutional markers:	
glycoprotein Ib	6D268, N-19, C-20
glycoprotein IX	GR-P, Beb1, ALMA.16
glycoprotein IIb	5B12, VIPL3, HIP8
glycoprotein IIIa	F11, VIPL2
Platelet endothelial cell adhesion molecule-1 (also present on endothelial MPs)	CD31
Activation markers:	
glycoprotein IIb-IIIa fibrinogen binding conformation	PAC1
P-selectin exposure from α-granules	S12, AC1.2, AK-4
CD40 ligand	TRAP1
Factor Va binding	V237
Factor VIII binding	1B3

Table 2: Constitutional and activation-dependent markers and representative antibody clones of platelets and platelet-derived microparticles, generally used in flow cytometry measurements (Michelson AD *et al*., 2000).

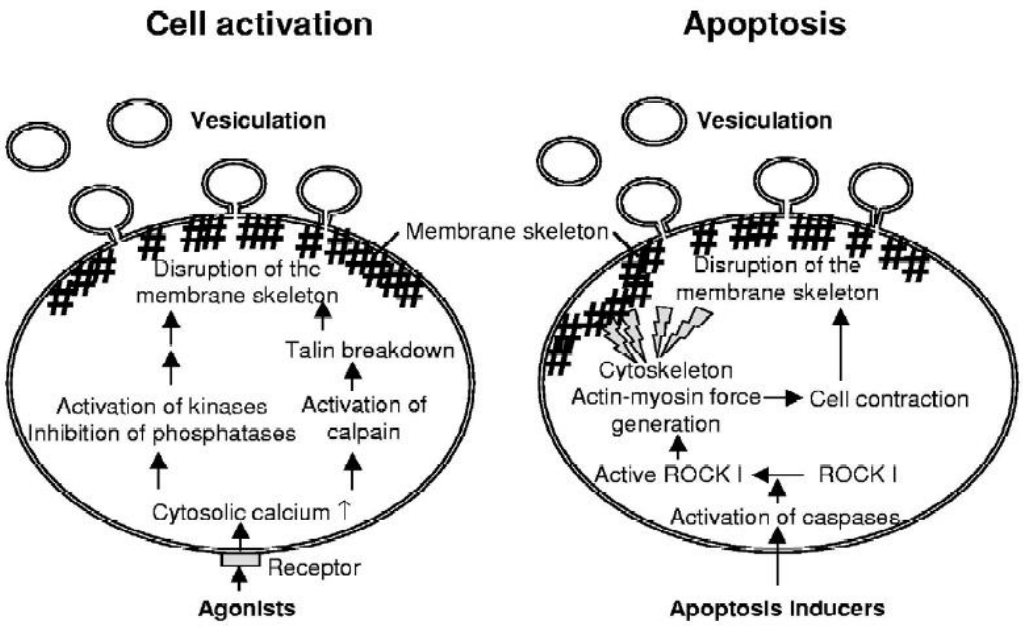


Figure 6: Schematic representation of general mechanisms involved in microparticle formation during cell activation (left panel) and apoptosis (right panel).

(Adapted from: VanWijk, M. J., VanBavel, E., Sturk, A., & Nieuwland, R. (2003). Microparticles in cardiovascular diseases. *Cardiovascular research*, *59*(2), 277-287).

Our knowledge regarding the elimination of MPs is considerably limited. Rank et al. followed MP numbers after platelet transfusion in patients suffering from aplastic phase of haematological diseases. According to their data, transfused MP half-life was 5-6 hours. Phagocytosis by macrophages, as well as the role of lactadherin (binding and phagocytosis by platelets and leukocytes) is hypothesised in the removal of MPs from circulation (Rank *et al*., 2011).

2.3 Lactadherin structure

Lactadherin also known as MFG-E8 (Milk Fat Globule-Epidermal Growth Factor 8) belongs to the family of secreted ECM glycoproteins. In most species, lactadherin occurs as two splice variants: ~53 kDa and ~66 kDa that include an O-glycosylated proline/threonine rich sequence (Oshima K *et al*., 1999). The smaller variant is present in the mammary glands of adolescent female animals as well as other tissues and organs (sweat glands, bile ducts) and body fluids (serum, urine,

cerebrospinal fluid). It can be expressed and released by activated macrophages, epithelial cells, immature dendritic cells, pancreatocytes, and keratinocytes (Aziz M *et al*., 2011, Watanabe T *et al*., 2005). The larger variant of the lactadherin globule is secreted into milk by mammary epithelial cells of humans, cows, or mice and it is most abundant in the fraction of milk-fat-globule membranes (Oshima K *et al*., 1999, Butler JE *et al*., 1980). Bovine *lactadherin* is composed of four functional domains starting from the N terminus: EGF1-EGF2-C1-C2 where the EGF2-like domain (homology with epidermal growth factor) contains an arginine-glycine-aspartic acid (RGD) motif and the C2 discoidin domain has homology to coagulation factors VIII and V (Oshima K *et al*., 2014). The *EGF2* domain of lactadherin which contains a very conservative RGD cell-adhesion motif is recognized by integrin heterodimers: αVβ3 and αVβ5 (Hvarreggard J *et al*., 1996). The following figure shows the structure of lactadherin with its major domains:

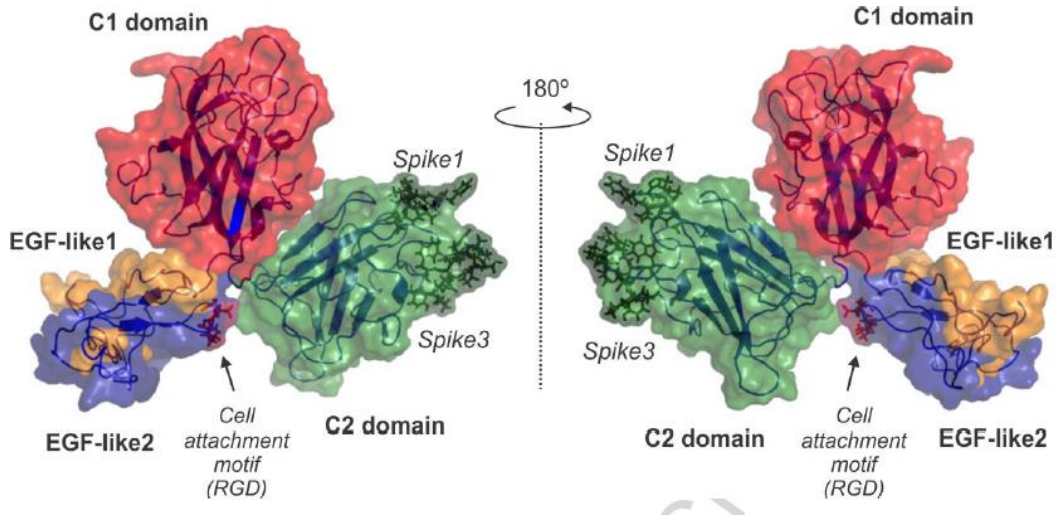


Figure 7: Structural model of bovine *lactadherin* showing its domains and principal functional features

(Adapted from: Kamińska, A., Enguita, F. J., & Stępień, E. Ł. (2017). Lactadherin: An unappreciated haemostasis regulator and potential therapeutic agent. *Vascular pharmacology*)

2.4 Interaction between Microvesicles and Lactadherin

The role of *lactadherin* in the initialization of phagocytosis has been shown in a number of studies. *Lactadherin* acts as an opsonin that targets apoptotic cells. After their secretion by macrophages, *lactadherin* molecules form a kind of a bridge between PS exposed on apoptotic cells and αVβ3 integrin on phagocytes for uptake by macrophages (Neutzner M *et al*., 2007, Andersen MH *et al*., 2000, Hanayama R *eta l*., 2002).

The platelet plasma membrane is composed of a phospholipid bilayer, with an outer leaflet enriched in phosphatidylcholine and sphingomyelin, and an inner leaflet that comprises anionic phospholipids such as phosphatidylserine (PS) (Zwaal RF *et al*., 1989). In response to activation by agonists like thrombin, collagen or ADP, or by the calcium carrier ionophore, an increase in intracellular calcium induces the activation of scramblase, a bidirectional phospholipid transporter, thereby leading to rapid exposure of the negatively charged phospholipid PS at the platelet surface (Bevers EM *et al*., 1983). Of importance is that the disruption of the platelet plasma membrane results in PMV release (Comfurius P *et al*., 1990). Therefore there is more PS exposure on the outer surface of PMVs.

In another study, it has been proven that positively-charged residues in the C2 domain are engaged in the binding to PS-exposed cells and that hydrophobic aromatic residues (Trp26, Phe81) stabilize the docking of PS with the C2 domain (Ye H *et al*., 2013). From the above mentioned points it can be inferred that the properties of lactadherin to bind small PS-positive vesicles through C2 domain and to bind with integrin of macrophages through RGD can be considered as possible regulators of microvesicle clearing processes. Following figure shows the interaction between PMPs and *lactadherin:*

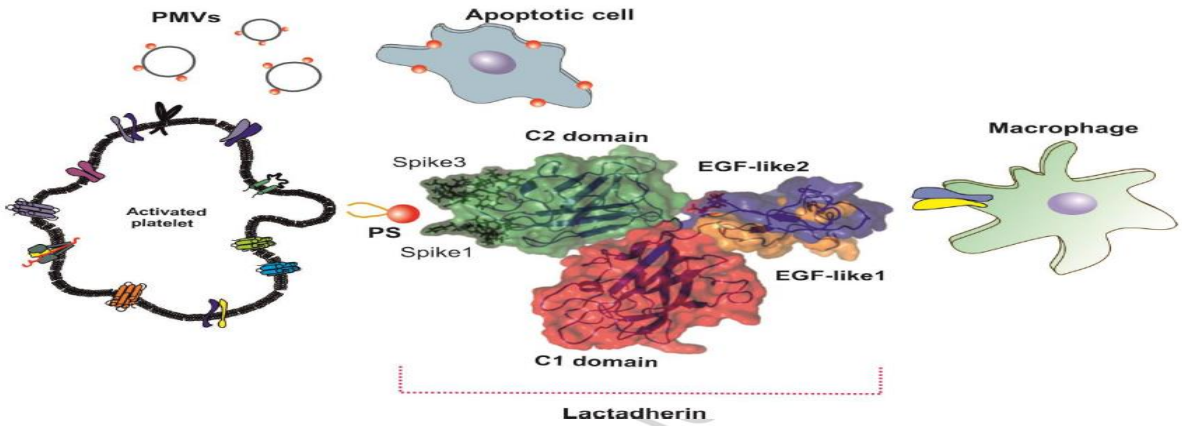


Figure 8: Interaction between PMPs and lactadherin

(Accepted from: Kamińska, A., Enguita, F. J., & Stępień, E. Ł. (2017). Lactadherin: An unappreciated haemostasis regulator and potential therapeutic agent. *Vascular pharmacology*)

Chapter III

MATERIALS AND METHODS

3.1 Materials

Thionin acetate salt (287.34g/mol), the tetrapeptide Arg-Gly-Asp-Ser (RGDS) and Citrate-phosphate-dextrose solution with adenine (CPDA) were purchased from Sigma-Aldrich; sodium chloride (NaCl), potassium chloride (KCl), potassium dihydrogen phosphate (KH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4) from Thomas Baker. EDTA was from Merck Potassium ferricyanide ($K_3[Fe(CN)_6]$) and potassium ferrocyanide ($K_4[Fe(CN)_6]$)•$3H_2O$ were from Himedia. ITO glass was a product of Techinstro. Ag/AgCl Electrode was used as reference electrode and counter electrode was made up of platinum. Phosphate Buffer Saline (PBS) was prepared with 0.05 g KH_2PO_4, 0.75 g Na_2HPO_4, 0.05 g KCl and 2 g NaCl in 250mL distilled water and the pH was adjusted to be 7.0 with HCl or NaOH.Electrolytic solution of Potassium Ferri/Ferro-Cyanidewas prepared by dissolving 24mg/ 30mL both potassium ferricyanide and potassium ferrocyanide in PBS containing 1.11gm KCl. All other reagents/chemicals were also of analytical grade.

3.2 Electrochemical measurement

The Cyclic voltammetry (CV), differential pulse voltammetry (DPV) and electrochemical impedance spectroscopy (EIS) measurements were carried out on a Potentiostat Galvanostat Autolab (Nova 1.11) in a solution containing 24mg/ 30mL $[Fe(CN)_6]^{3/4}$ and 1.11gm/ 30mL KCl in PBS (pH 7.0). A conventional three electrode system containing a modified ITO electrode as the working electrode, a platinum electrode as the counter electrode, and an Ag/AgCl electrode as the reference electrode was employed for the electrochemical detection. The detailed parameters of all DPVs are as follows: initial potential was -0.5 V; final potential was 0.5 V; increment was 0.004 V; amplitude was 0.05 V; pulse width was 0.05 s; pulse period was 0.2 s; and quiet time was 2 s.

3.3 Deposition of poly-thionine layer on ITO plate

Electropolymerization of thionine was done through Electrochemical analyzer (PotentiostatGalvanostatAutolab) in two steps: (a) Chronoamperometry: Fixed the ITO plate under a constant potential of -1.5 V for 10 min. in PBS containing thionine

(0.02 M) (Figure 9a) (b) Cyclic voltammetry: Potential cycling at scan rate of 100 mV/sec for 35 cycles between -0.4 to 0.8 V in pH 7PBS (0.1 M) (Figure 9b).

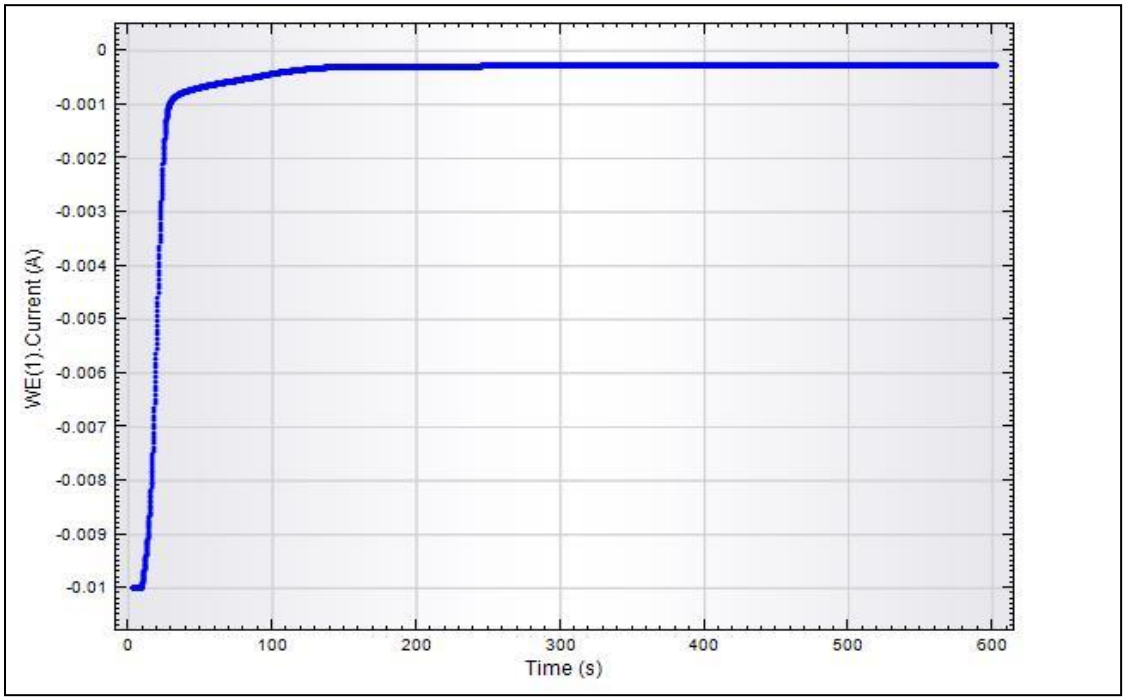


Figure 9a: Deposition of poly-thionine layer through chronoamperometry

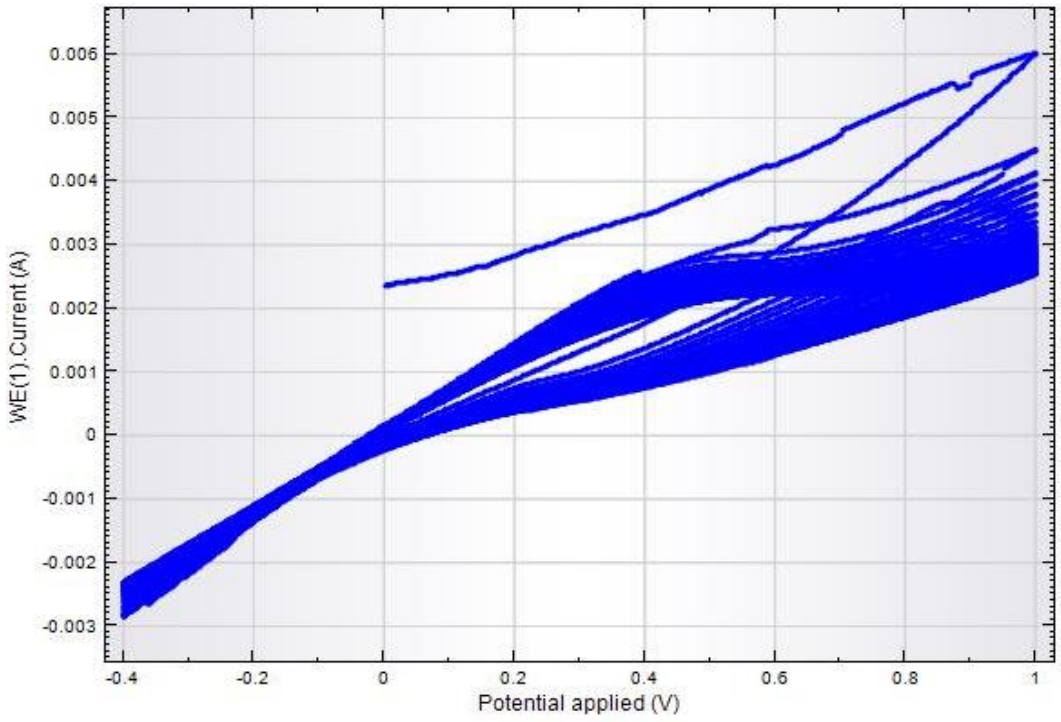


Figure 9b: Deposition of poly-thionine layer through cyclic voltammetry.

3.4 Characterization of thin film of polythionine

Surface morphology of the thin film was characterized through scanning electron microscopy (SEM) [Carl Zeiss, Germany Model: Merlin Compact]. Additionally, the chemical composition of polythionine (PTh) was determined using the energy-dispersed spectrum (EDS) in the SEM system. FTIR [Bruker Model: Tensor 27] and UV-vis spectra [Shimadzu, Japan Model: UV-2450] were further used to discuss the molecular structure of PTh.

3.5 Microvesicles Preparation

Collected total amount of 10ml blood (1.6ml CPDA as anticogulant+8.4ml blood) from healthy donor in a tube

Centrifuged it at 180×g for 15 min at RT along with acceleration of 09 and deceleration of 02

Got PRP (Platelet Rich Plasma) as supernatant and discarded the remaining cells (RBC & WBC)

Kept PRP at 37ºc for 10 min and added EDTA in it according to the amount 10µl/ml

Again Centrifuged at 800×g for 15 min at RT along with acceleration 09 and deceleration 04

Taken out PPP (Platelet Poor Plasma) as supernatant

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) with 500µl each i.e., 500ml RP (Resting Platelets) and 500µl for AP (Activated Platelets) preparation

Taken RP as a Sample-1 and vortexed the other to form AP

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

We got the PMPs (Platelet Microparticles) as supernatant (Sample-2) and the pellet

Resuspend the pellet in Buffer B

Separated it in the 2 tubes with 200µl and 200µl each and this is Sample-3 for Electrochemical Biosensing

The PPP which we got in the earlier steps, taken 1ml for the Electrochemical Biosensing use and the remaining were taken in 2 tubes 1.6ml each for Ultracentrifuge

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

Then, collected the supernatant for Electrochemical Biosensing and added 500µl buffer B and glutaraldehyde in the pellet. Here, we got the Sample-4for characterization with the help of various techniques such as SEM, Flow Cytometer, etc.

3.6 Modification of electrochemically deposited polythionine for microvesicles detection

The thin film was modified by immobilizing an aliquot of RGDS(100µg/ 100µL) solution on fabricated thionine modified electrode and incubated it for 2 hrs. After that 2.5% glutaraldehyde solution for cross linking was dropped over immobilized RDGS layer and allowed to dry. Finally the electrodes were washed thoroughly and dipped in electrolytic solution to check its electrochemical behavior.

3.7 Electrochemical analysis of Microvesicles

Microvesicles poor plasma (representing supernatant collected via ultracentrifuge of PPP) were dropped on the RGDS/Glutaraldehyde/PTh/ITO and kept at 37ºC for 2hrs. Then CV, DPV and EIS measurements were carried out on a Potentiostat Galvanostat Autolab (Nova 1.11) in a solution containing 24mg/ 30mL $[Fe(CN)_6]^{3/4}$ and 1.11gm/ 30mL KCl in PBS (pH 7.0). After this PMVs (representing pellet collected via ultracentrifuge of PPP) were incubated on it for 2hrs and CV, DPV and EIS measurements were carried out. Thereafter activated platelets were incubated on fabricated electrode for 2hrs and readings were taken by keeping the parameters same. Finally measured parameters were compared to conclude the results.

Chapter IV

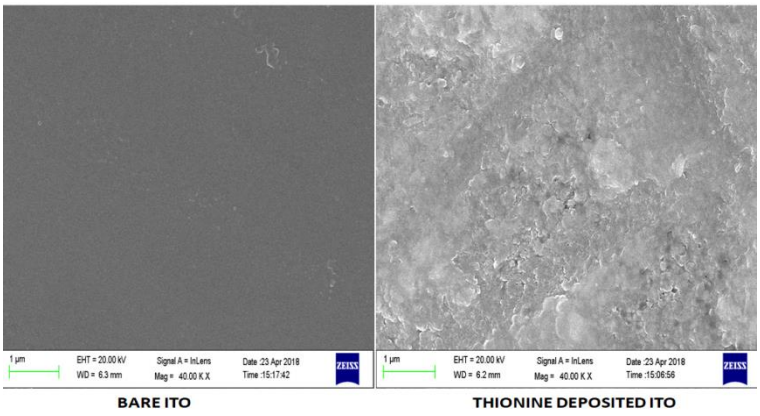
RESULTS and DISCUSSION

4.1 Characterization of electrochemically deposited Polythionine

As shown in Figure 10A, the SEM image of the PTh indicated that the morphology of PTh contains a nano-network structure composed of relatively uniform fibers and particles. The fibers with tens of nanometers widths and particles form an ordered nanostructure. Further, the high magnification of SEM image revealed that the fibrous and granular PTh can form a uniform and even partially tight film on the solid surface.

Besides, the chemical composition of PTh was determined using the energy-dispersed spectrum (EDS) in the SEM system, as shown in Figure 10B. The peaks of C, N, O, Sn and In are observed, indicating that PTh is successfully deposited on ITO electrode. The peak of S is not observed because of its very minute presence. The peak of silicon is observed due to glass electrode nature. ITO electrode is made by coating a thin layer of indium tin oxide on a glass electrode.

A



B

Element	Weight%	Atomic%
C K	12.05	22.39
N K	5.11	8.15
O K	31.61	44.12
Si K	25.66	20.40
In L	19.63	3.82
Sn L	5.95	1.12
Totals	100.00	

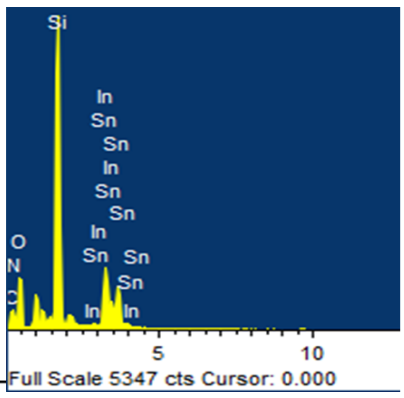


Figure 10. SEM images(A) and EDS (B)

FTIR was further used to explore the molecular structure of PTh, as shown in Figure 11. The two strong bands of PTh at 2931 cm^{-1} and 2851 cm^{-1} indicate thearomatic C–H stretching vibration of PTh during thepolymerization of thioninethat is concurrent with the earlier findings (C. F. Zhao *et al*., 2015, Y. Kong *et al*., 2001).

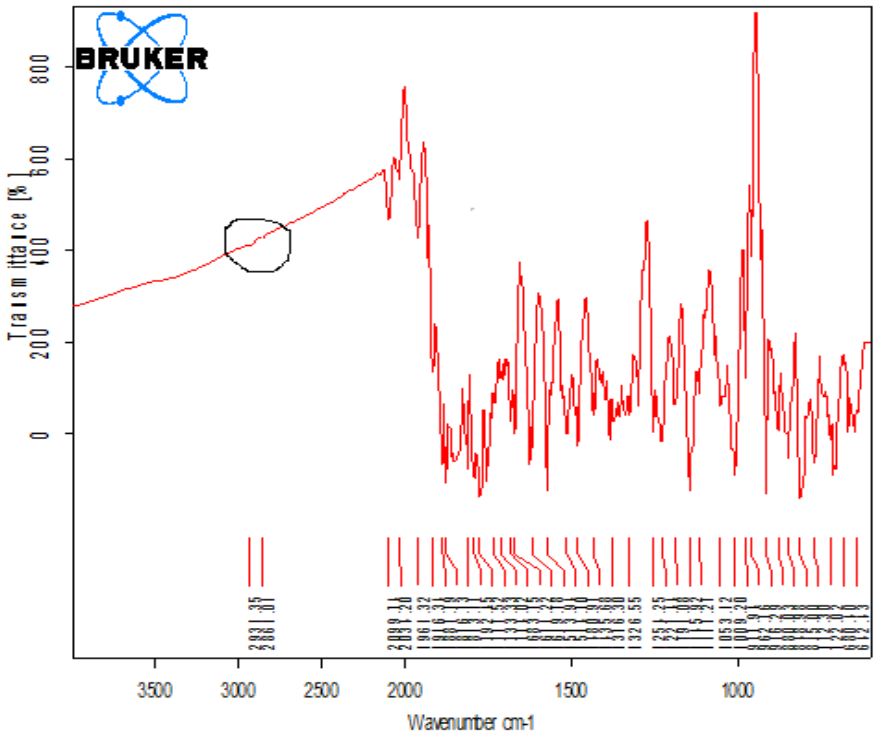
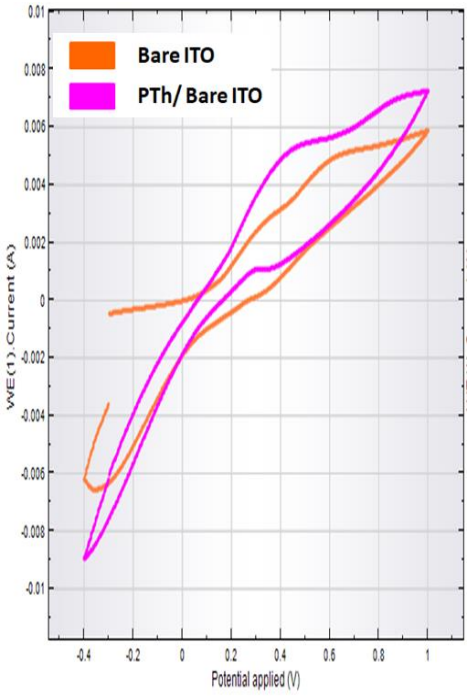


Figure 11: The FTIR spectra of Polythionine

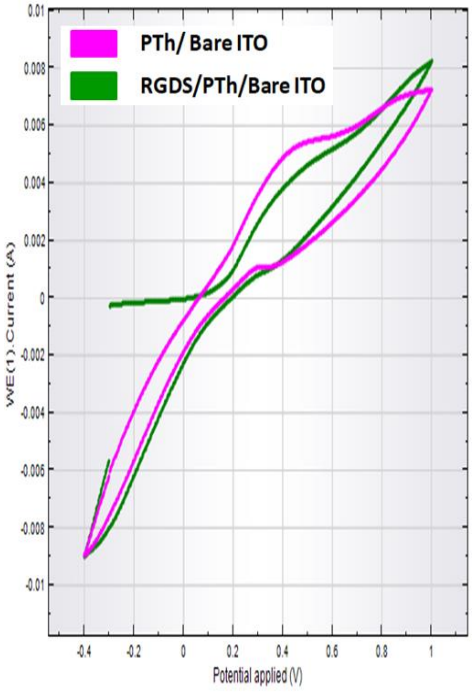
4.2 Electrochemical monitoring of different modified electrodes

CV was used to characterize the modification process of the bare ITO electrode. Polythionine deposited ITO showed increase in oxidation peak of the CV as compared to bare ITO electrode due to enhanced electron transfer kinetics properties of polythionine resulting in the increased current flow (Figure 12A). RGDS immobilized electrode showed decrease in electrochemical redox response as compared to characteristic thionine redox peak observed in Figure 12B at 100 mV/ sec confirms the successful immobilisation of RGDS on poly-thionine film. Observed decrease in conductance (redox response) was due to the insulating nature of RGDS biomolecule. After incubating microvesicle poor plasma (MPP) on RGDS/PTh/ITO electrode, further reduction in the peak is observed implying that the

microvesicles are interacting with the RGDS sequences which hinders the transferof $[Fe(CN)6]^{4-/3-}$ as shown in Figure 12C. MPP/RGDS/PTh/ITOelectrode showed further decrease of the redox peak on incubation with microvesicles rich plasma (MRP) due to increased insulating properties that hinders the transferof $[Fe(CN)6]^{4-/3-}$. In the last, activated platelets was deposited on the MRP/MPP/RGDS/PTh/ITO electrode that results in further peak decrease as shown in Figure 12E, indicating that platelets are having larger surface area as compared to microvesicles and hence have more insulating effect.



A



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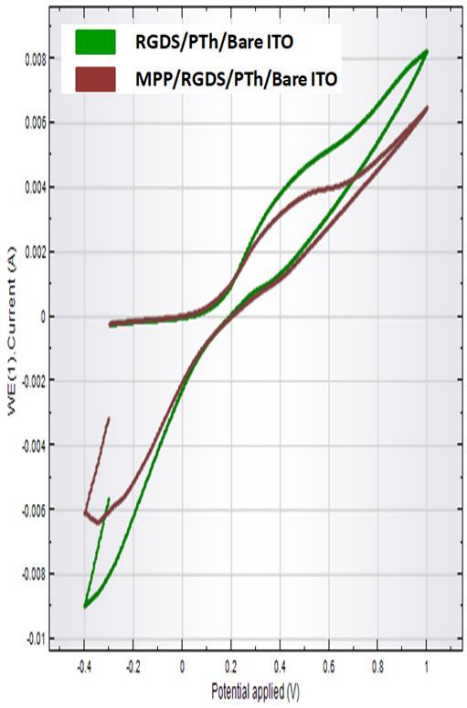

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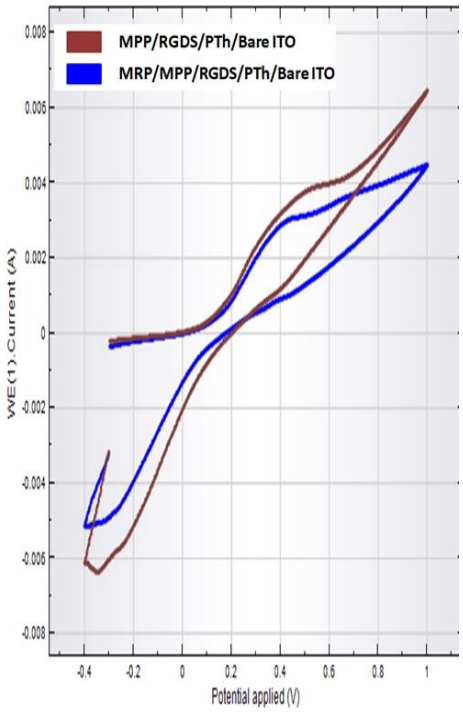

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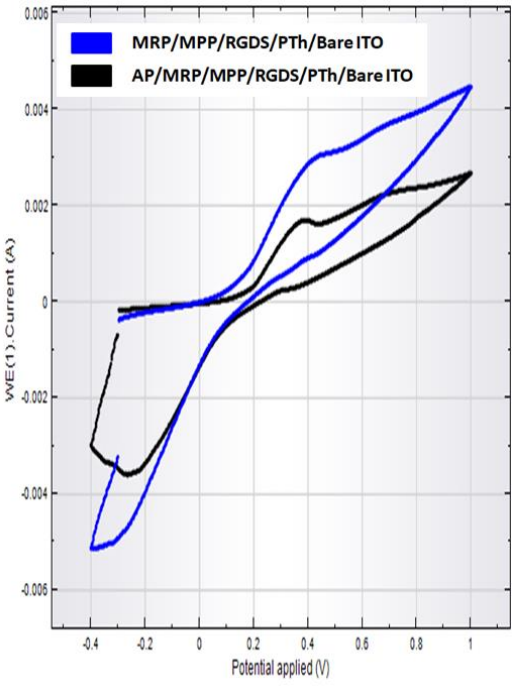

E

Figure 12: CV measurements of (A) Bare ITO vs. Polythionine deposited ITO (B) Polythionine deposited ITO vs.RGDS/PTh/Bare ITO (C) RGDS/PTh/Bare ITO vs MPP/RGDS/PTh/Bare ITO (D) MPP/RGDS/PTh/Bare ITO vs MRP/MPP/RGDS/PTh/Bare ITO (E) CV ofMRP/MPP/RGDS/PTh/Bare ITOvs AP/MRP/MPP/RGDS/PTh/Bare ITO

In the meantime, we investigated DPV of different steps involved in electrochemical detection of microvesicles, as shown in Figure 5.The DPV of PTh/ITO electrode showed an obvious well-defined anodic peak that appears at about 0.03 V, implying that PTh can easily adhere to the electrode surface and produce an obvious response. When RGDS covered the PTh/ITO, there is slight decrease in the DPV anodic peak due to the increase resistance and charge hindrance of RGDS, suggesting that the adjustment of the surface of the PTh/ITO could change the electrochemical response sensitively. The anodic response of PTh gradually decreases with the subsequent process of immobilization/incubation of MPP, MRP and activated platelets.

Thus, the results indicate that developed RGDS based electrochemical method could provide suitable platform for the detection of platelet microvesicles in blood plasma. Further specificity, stability and reproducibility can be checked before their clinical validation.

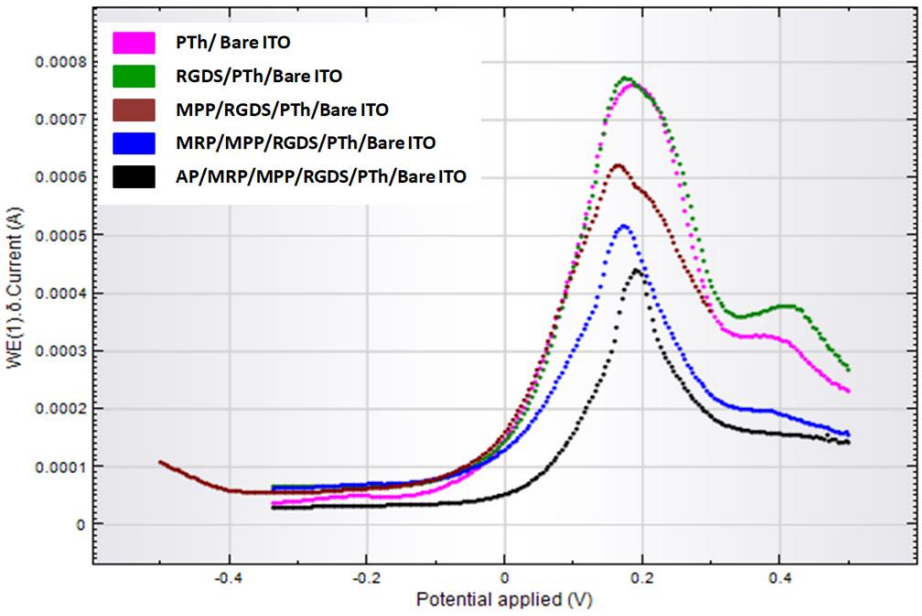

Figure 13: DPV of various modified electrode

Chapter V

CONCLUSION and FUTURE PROSPECTS

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. Therefore, it is pertinent to detect PMPs level in blood of an individual. In this study, we try to develop a novel approach for the electrochemical detection of PMPs in blood plasma using lactadherin binding motif (RGD domain). Polythionine film (as a good electron mediator) was electrochemically depositedon ITO-coated glass through electrochemical process involving CV and Chronoamperometry. Electrochemically deposited electrode provide ideal adsorbing platform for immobilization of RGDS peptide sequences. CV and DPV measurements showed gradual decrease of current with the subsequent adsorption of microvesicle poor plasma (MPP), microvesicle rich plasma (MRP) and activated platelets. Decrease of current clearly depicts the presence of microvesicles in blood plasma. Our developed fabricated electrode can have a promising potential for its efficient application in clinical testing of various pathological conditions. Despite achieving expected outcomes, sensitivities, specificity, stability and reproducibility of the developed electrochemical biosensor may need careful examinations. Also, batch-to-batch variations in results need more attention and may be minimized by performing the experiment for more number of times. Further validation of this microvesicle detection method can be done with existing conventional methods like flow cytometry, ELISA etc and with various clinical samples. Besides, the electrode modification can be carried out to monitor simultaneously the level of lactadherin and MVs so that it becomes possible to correlate the level of both. If successful, this lactadherin based electrochemical biosensor can be a valuable cost effective label free tool for microvesicles detection and this methodology for microvesicles detection can be subsequently patented.

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