THE DEVELOPMENT OF BIOHYBRID PLATELET-BASED DRUG DELIVERY VEHICLES FOR POTENTIAL TREATMENT OF ISCHAEMIC STROKE

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A Dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master of Pharmacy

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DECLARATION

I, Sarah Mahmood Kola, student number: 719180, declare that this Dissertation is my own, unaided work and that I contributed adequately towards research findings published in the articles included in my Dissertation. This Dissertation is being submitted for the Degree of Master of Pharmacy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

The front page of the originality report, showing the similarity index of this Dissertation, obtained from the Turnitin software can be found in Appendix A.

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(Signature of candidate)

Signed this 15th day of January 2021 in Johannesburg.

ETHICS DECLARATION

I, Sarah Mahmood Kola, hereby confirm that the study entitled “The Development of Biohybrid Platelet-Based Drug Delivery Vehicles for Potential Treatment of Ischaemic Stroke” received approval from the Human Research Ethics Committee (Medical) of the University of the Witwatersrand, Johannesburg. Ethics Clearance Number M180978 (Appendix B).

I, Sarah Mahmood Kola, hereby confirm that the study entitled “The Development of Biohybrid Platelet-Based Drug Delivery Vehicles for Potential Treatment of Ischaemic Stroke” also received approval from the South African National Blood Service (SANBS) Human Research Ethics Committee. Ethics Clearance Number 2018/-0451 (Appendix C).

This dissertation is dedicated to my parents, Mahmood Suliman Kola and Fatima Mahomed Surtee, who always help me in all matters large and small. I will treasure you forever.

PUBLICATIONS ARISING FROM THIS STUDY

The following manuscripts have been published:

1. Review Paper

Kola SM, Choonara YE, Kumar P, Kondiah PP, Pillay V. Platelet-inspired therapeutics: current status, limitations, clinical implications, and future potential. Drug Deliv and Transl Res. 2020:1-25.

(Abstract in Appendix D)

2. Research Paper

Kola SM, Kumar P, Choonara YE, du Toit LC, Pillay V. Hypothesis: Can drug-loaded platelets be used as delivery vehicles for blood-brain barrier penetration? Med Hypotheses. 2019; 125:75-78.

(Abstract in Appendix E)

The following manuscripts are to be submitted to international peer-reviewed journals:

1. Research Paper

An investigation into the suitability of commonly used nanomaterials in the design of a biohybrid platelet-based tissue plasminogen activator delivery system.

(Abstract in Appendix F)

2. Research Paper

Tissue plasminogen activator (tPA)-loaded platelets for clot lysis in the potential treatment of ischaemic stroke.

(Abstract in Appendix G)

ABSTRACT

Ischaemic strokes are caused when areas of the brain are deprived of oxygen, due to an interruption of blood flow that may be caused by a blood clot. The current ischaemic stroke treatment methods have many associated limitations that impede their use. Nonspecificity of the treatment and damage to the blood-brain barrier (BBB) are two examples of major limitations. In an attempt to overcome these limitations and develop a system that may improve the likelihood of positive treatment outcomes, scientists have suggested that a targeted thrombolytic approach be adopted. Therefore, this study investigated the possibility of using natural platelets as drug delivery vehicles for the thrombolytic agent tissue plasminogen activator (tPA). Platelets may be modified in a number of ways to give rise to different therapeutic modalities that are aimed at targeted treatment. Due to the structure and multiple functions of platelets within the body, these different platelet-inspired therapeutic modalities have been investigated for their use in an array of conditions. However, no known research has been undertaken on the use of platelets to cross the BBB for the targeted treatment of neurological conditions that illicit a platelet response. In this study platelets were explored as carriers for tPA, for the potential penetration of the BBB. This approach potentially has two ways in which platelets may be loaded with tPA. The first way involves the direct loading of tPA into the platelets. The second way involves the loading of a tPA-encapsulated nanoparticle into the platelets, and the system that is formed is known as a biohybrid delivery system as it is composed of a natural element (platelets) as well as the synthetic nanoparticle (biopolymer-based). An investigation was conducted on the suitability of five nanomaterials that may potentially be used to encapsulate tPA, in the design of a biohybrid platelet-based system. The nanomaterials that were investigated included silica nanoparticles, carbon nanotubes, cerium (IV) oxide nanoparticles, nanoliposomes, and poly methyl methacrylate (PMMA) particles. The investigation analysed the effects of the nanomaterials on erythrocyte morphology and aggregation behaviour, in an attempt to investigate the erythrocyte compatibility of the particles. From the nanomaterials tested, the nanoliposomes exhibited the greatest erythrocyte compatibility, therefore, it was recommended that further research be conducted on their use in the design of a biohybrid platelet-based system. Experiments were then conducted on the ability of natural platelets having undergone direct tPA loading, to induce clot lysis. The

experiments conducted showed that the platelets were able to encapsulate tPA with an average encapsulation efficiency of 63%, and that the platelets were effective in releasing 15.2% of the encapsulated tPA upon activation by thrombin. It was also observed that loading the platelets with tPA did not result in any morphological changes to the platelets, nor did it alter the integrity and ability of the platelets to respond to vascular injury. The tPA-loaded platelets on average were able to cause 72% clot lysis when tested using an *in vitro* whole blood clot model. Additionally, the tPA-loaded platelets were not seen to alter the colloidal suspension stability of blood as well as the degree of erythrocyte aggregation, thereby promoting their erythrocyte compatibility profile. The results from this research provided *in vitro* evidence to support the use of tPA-loaded platelets in the potential treatment of ischaemic stroke.

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LIST OF ABBREVIATIONS

ACAN: Aggrecan

Anti-PD-L1: Antibodies against programmed-death ligand 1

Arg-Gly-Asp: Arginylglycylaspartic acid

ASCs: Adipose-derived stromal cells

BBB: Blood-brain barrier

BMSCs: Bone marrow mesenchymal stem cells

CBP: Collagen-binding peptide

CNS: Central nervous system

COL1A1: Collagen type I

COL2A1: Collagen type II

COL3A1: Collagen type III

cRGD: Cyclic-RGD peptide

CSIR: Council for Scientific and Industrial Research

CTCs: Circulating tumour cells

DEA: Degree of erythrocyte aggregation

DOX: Doxorubicin

EDTA: Ethylenediaminetetraacetic acid anticoagulant

EE: Encapsulation efficiency

EGF: Epidermal growth factor

ESR: Erythrocyte sedimentation rate

FBS: Foetal bovine serum

FDA: United States Food and Drug Administration

FGF: Fibroblast growth factor

FIX: Clotting factor IX

FMP: Fibrinogen mimetic peptide

FVIII: Factor VIII

GIT: Gastrointestinal tract

GP: Platelet glycoprotein

GP6: Glycoprotein 6

HA: Hyaluronic acid

HGF: Hepatocyte growth factor

HMDS: Hexamethyldisilizane

HSCs: Human stem cells

ICSH: The International Council for Standardisation in Haematology

IGF: Insulin-like growth factor

IL-1β: Interleukin-1β

IL-6: Interleukin-6

IV: Intravenous

K: Potassium

LPS: Lipopolysaccharide

MM: Multiple myeloma

MMP-3: Matrix metallopeptidase 3

MMP-13: Matrix metallopeptidase 13

MSCs: Mesenchymal stem cells

OA: Osteoarthritis

OCS: Open canalicular system

OD: Optical density

OI: Osteogenesis imperfecta

Pa: Pascals

PBS: Phosphate buffered saline

PCR: Polymerase chain reaction

PD-1: Programmed death 1

PD-L1: Programmed-death ligand 1

PDGF: Platelet-derived growth factor

PED: Persistent epithelial defect

PEM: Polyelectrolyte multilayer

PF4: Platelet factor 4

PL: Platelet lysate

PLNs: Platelet-like nanoparticles

PLPs: Platelet-like particles

PMMA: Poly methyl methacrylate

PMPs: Platelet-derived microparticles

PPP: Platelet-poor plasma

PRG4: Lubricin

PRP: Platelet-rich plasma

RBC: Red blood cell

RBCs: Red blood cells

rtPA: Recombinant tissue plasminogen activator

SANBS: South African National Blood Service

SD: Standard deviation

SEM: Scanning electron microscopy

SSI: Suspension stability index

TCIPA: Tumour cell-induced platelet aggregation

TGFβ: Transforming growth factor beta

TNF-α: Tumour necrosis factor alpha

tPA: Tissue plasminogen activator

TRAIL: Tumour necrosis factor-related apoptosis-inducing ligand

VBP: vWF-binding peptide

VEGF: Vascular endothelial growth factor

v/v: Volume per volume

vWF: Von Willebrand factor

WADDP: Wits advanced drug delivery platform

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CHAPTER 1

INTRODUCTION

1.1 Background to this Study

A stroke is a cerebrovascular condition that is the second leading cause of death globally [1]. The effects and symptoms of strokes vary, depending on the area of the brain that has been affected and the severity of the damage. Strokes are of two main types, namely, ischaemic and haemorrhagic [2]. Only a small percentage of strokes are haemorrhagic in nature. Ischaemic strokes are the most common type of stroke, accounting for approximately 80-87% of the known cases [1]. An ischaemic stroke occurs when brain cells are deprived of oxygen and other nutrients due to a decrease in blood flow to areas of the brain, which may lead to irreversible brain damage [1, 3]. Ischaemic strokes are most likely caused by cranial artery occlusion due to thrombus formation, or a thromboembolism originating in the heart. Alternatively, the cause of an ischaemic stroke may be cryptogenic in nature [4]. Thrombi composition differs depending on whether the thrombus is arterial or venous in nature [5]. Majority of strokes are caused by arterial thrombi [6], therefore, they are mainly composed of platelet aggregates that are joined together by fibrin strands to form a stable thrombus [5].

The therapy available for ischaemic stroke aims to achieve rapid revascularization of the affected tissue, as well as the management of cerebral oedema and blood pressure. Thereafter, the therapeutic interventions are focused on reducing the patients' risk factors with the hope of preventing the occurrence of a subsequent stroke [4]. Currently the only United States Food and Drug Administration (FDA) approved thrombolytic agent for the treatment of ischaemic stroke is intravenous (IV) administered alteplase. Alteplase is the recombinant form of tissue plasminogen activator (rtPA), which is a serine protease that achieves thrombolysis by promoting the formation of plasmin from plasminogen [3, 7]. A number of drawbacks that limit the use of tissue plasminogen activator (tPA) have been identified. For the treatment to be effective, IV administration needs to occur within three hours of the onset of the stroke, however, studies have shown that the timeframe may be extended to 4.5 hours in

select patients [3]. As these studies are recent the exact patients that may benefit from the extended timeframe is not fully known and therefore warrants further research. Delaying the administration of tPA may increase the patient's risk of haemorrhage and may prove to be fatal [8]. The use of tPA has also been linked to cerebral oedema, cell death, and damage to the blood-brain barrier (BBB). Additionally, as the treatment is given via IV administration it is entering the systemic circulation, therefore, systemic side effects may occur and non-specificity of the treatment may be inevitable [1].

The use of aspirin in the treatment of ischaemic stroke has shown to only provide a small benefit. In the event of an emergency there are medical procedures, like a mechanical thrombectomy, which doctors perform in order to treat the stroke. These procedures have shown to be successful in a number of patients, however, the full safety and efficacy profile of the newer procedures, such as those based on nanotechnology, still need to be examined [2]. Additionally, medical procedures may pose a challenge to patients as they are more invasive than alternative therapeutic interventions, and these procedures may be associated with complications as well as adverse effects.

The use of nanotechnology in the early detection and treatment of strokes has been gaining popularity as an alternative to the current treatment options. Studies have focused on investigating the use of nanocarriers to deliver medication to the stroke site in the brain, as nanocarriers may be designed to have diverse functionalities. A myriad of nanocarriers have even proven to be effective in reducing ischaemia [2]. However, nanocarriers are associated with challenges that may limit their use and translation into clinical settings. Potential toxicity to the body, biocompatibility challenges, and restricted biodegradability are some of the challenges impeding the use of nanocarriers [9]. In addition, the nanocarriers need to penetrate the BBB easily and without causing any barrier damage [2]. Due to these limitations, researchers have investigated the use of biological agents to deliver drugs to different sites of injury [10]. Some of the biological agents that have been investigated include: leukocytes [11], macrophages, erythrocytes, and platelets [10].

The BBB is a highly restrictive and regulated barrier that separates the central nervous system (CNS) and the peripheral blood [12, 13]. The function of the BBB is to protect the brain from fluctuations in plasma composition and from circulating agents, which

are capable of disturbing neural function, and to ensure that CNS homeostasis is maintained [12, 13, 14]. In addition, the presence of the BBB protects the CNS from injury and disease [13]. It is extremely important for the integrity of the BBB to be maintained as damage to the BBB can result in neuronal degeneration and CNS dysfunction [13]. The BBB comprises endothelial cells, neurons, pericytes, astrocytes, and the extracellular matrix [12]. The CNS microvasculature endothelium is responsible for stringently regulating the movement of cells, ions, and molecules between the blood and the CNS [12, 13]. This endows the BBB with selective diffusivity [12]. Due to the innate structure and functions of the BBB, its presence is probably the greatest challenge impeding the success of ischaemic stroke treatment options [1], as drug delivery to the CNS is difficult to achieve [13]. The drug delivery systems that transport medication through the BBB, and consequently to the brain, should aim to deliver and sustain effective drug solutions to the brain tissue; while ensuring neural function protection. This should be done to prevent morbidity and/or mortality associated with CNS diseases [14].

Due to the severity of the consequences of ischaemic stroke, and the drawbacks associated with the current therapeutic interventions for ischaemic stroke, there is a definite need for the development of safer and more effective therapeutic modalities that may safely penetrate the BBB and improve the likelihood of positive patient outcomes occurring.

1.2 Rationale and Motivation for this Study

Platelets are one of the biological agents that are being researched as therapeutic alternatives to the current conventional methods of treatment for a number of different disorders. Due to the innate structure and multiple functions of platelets, an array of different platelet-inspired therapeutic modalities have been successfully designed and reported. The use of natural platelets as vehicles for drug delivery has not shown to pose biocompatibility challenges [10]. Additionally, when platelets cross the BBB during times of vascular disturbances, they do not cause damage to the BBB or brain tissue [15]. Therefore, in an attempt to develop a system that can potentially cross the BBB effectively, protect neural functioning, and overcome some of the challenges posed by the current methods of treatment of ischaemic stroke, this study investigated

different elements related to the development of biohybrid platelet-based drug delivery vehicles for the potential treatment of ischaemic stroke.

This study aimed at providing a more targeted and controlled release of medication at the site of a thrombus and therefore an overall improvement in therapeutic outcomes. Platelets are naturally signalled to the site of a thrombus due to the clotting cascade process that occurs after vascular injury. This is the principle upon which the proposed drug delivery system has been designed. To date there is no such delivery system, therefore the findings of this study may contribute to knowledge generation and exploration with regards to platelets and their versatility, and using biological agents in the field of pharmaceutics. Figure 1.1 provides a schematic representation of the process of formation and mechanism of action of the proposed natural platelet drug delivery vehicles. This schematic represents only the method of drug loading that was selected to investigate the *in vitro* clot lytic ability of the platelet drug delivery vehicles (presented in chapter 5).

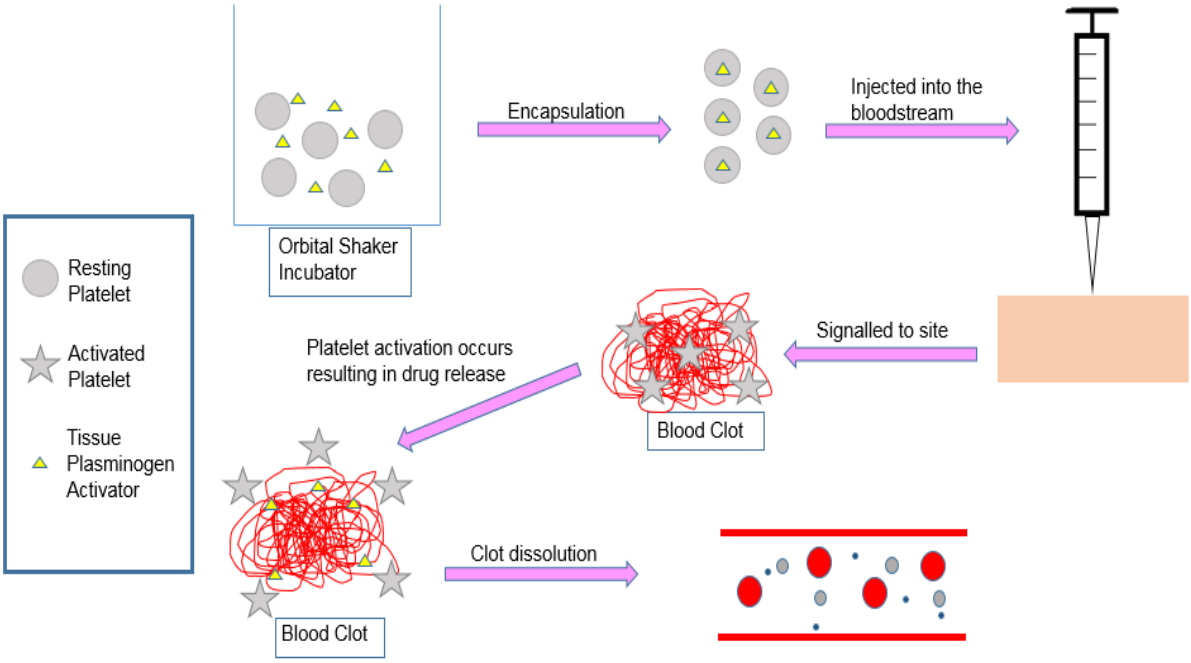

Figure 1.1: Schematic representation of the process of formation and mechanism of action of the natural platelet drug delivery vehicles

1.3 Aim and Objectives of this Study

The aim of this study was to investigate different elements related to the development of biohybrid platelet-based drug delivery vehicles that may potentially be used in the treatment of ischaemic stroke. In order to achieve this aim, the following objectives were fulfilled:

1. To undertake preliminary studies on the effects of five commonly used nanomaterials on erythrocyte morphology and aggregation behaviour in an attempt to investigate their suitability in the potential design of a biohybrid platelet-based "hitchhiker" nano system.
2. To use the results from the preliminary study to decide on a manner in which to load the platelets with tPA for clot lytic analyses.
3. To acquire isolated platelets from the South African National Blood Service (SANBS), followed by loading tPA into the platelets using the method of drug loading that was previously selected.
4. To use ELISA kits to conduct *in vitro* analyses of the platelet's encapsulation efficiency and drug release efficacy upon activation.
5. To analyse the morphology of the native (unloaded) and tPA-loaded platelets using light microscopy and scanning electron microscopy.
6. To perform ELISA assays to analyse the effect of tPA loading on two platelet surface proteins.
7. To perform a whole blood clot lysis assay in order to analyse the *in vitro* efficacy of the tPA-loaded platelets system.
8. To conduct novel *in vitro* studies to determine the effects of the tPA-loaded platelets on erythrocyte aggregation, and the colloidal suspension stability of blood.

1.4 Novelty of this Study

The points mentioned below highlight the novelty of this study.
1. Proposing the use of natural platelet drug delivery vehicles for the penetration of the BBB (Chapter 3).
2. The use of the ElastoSens^{TM}Bio2 (Rheolution, Inc., Montreal, Quebec, Canada), Turbiscan^{LAB} (Formulaction, L'Union, France) analyser, and erythrocyte sedimentation rates (ESR) for the purpose of analysing erythrocyte aggregation and sedimentation

behaviour following the introduction of silica nanoparticles, nanoliposomes, cerium (IV) oxide nanoparticles, poly methyl methacrylate (PMMA) particles, and carbon nanotubes into the blood (Chapter 4).
3. Employing natural platelets as drug delivery vehicles for tPA, in order to achieve clot lysis (Chapter 5).

1.5 Overview of this Dissertation

This dissertation is composed of six chapters, the contents of each have been detailed herein.

Chapter 1 introduces the study. It provides a brief background into ischaemic strokes, the BBB, the current conventional ischaemic stroke treatments and some limiting drawbacks associated with these treatment options that warrant the development of safer and more effective therapeutic modalities. Thereafter, this chapter presents the rationale and motivation for this study. The chapter goes on to delineate the aim and objectives, novelty of the study, and a summary of the dissertation.

Chapter 2 provides a comprehensive review of the literature detailing all of the forms of platelet-inspired therapeutics. It discusses the current available modalities of platelet-inspired therapeutic systems to provide comprehensive information that will enhance the readers understanding of how platelets function as targeted therapeutic agents in research, clinical settings, and the pharmaceutical industry. The literature review focusses on the current status, limitations, and clinical implications of six platelet modalities. These include platelet-rich plasma (PRP), platelet lysate (PL), natural platelet drug delivery vehicles, genetically engineered platelet membranes, biomimetic platelet-based systems, and hybridized platelet membrane systems. Thereafter, an analysis of the future prospects of each platelet-inspired therapeutic modality is presented.

Chapter 3 provides a clear and detailed hypothesis that explains the theoretical use of platelet drug delivery vehicles in crossing the BBB. After completing the literature review, and based on the principles of extraction and extrapolation of data, it was hypothesised that platelets may be the ideal drug carriers for the potential penetration of the BBB. The hypothesis mentions two ways in which platelets may be drug-loaded

in order to be used as drug carriers; the first being direct loading of the drug into the platelets, and the second being the loading of a drug-encapsulated nanoparticle acting as a “hitchhiker” within the platelets. The chapter discusses the use of platelet drug delivery vehicles for the potential treatment of ischaemic stroke and glioblastoma multiforme. As this study is novel, the section of the chapter based on ischaemic stroke provides the context upon which this research project was based, and the section of the chapter based on glioblastoma multiforme provides a description of an example of the future potential of platelet drug delivery vehicles for the penetration of the BBB.

Chapter 4 provides a preliminary investigation into the suitability of five nanomaterials in the potential design of a biohybrid platelet-based hitchhiker nano system. This is related to the second method of drug loading the platelets that was mentioned in chapter 3. The research presented in this chapter focused on determining the effects of silica nanoparticles, carbon nanotubes, cerium (IV) oxide nanoparticles, nanoliposomes, and PMMA particles on erythrocyte morphology and aggregation behaviour, in an attempt to analyse the erythrocyte compatibility aspect of haemocompatibility of these nanomaterials. Light microscopy was used for morphological characterisation of the erythrocytes and three novel *in vitro* methods, namely analysis of erythrocyte sedimentation rates (ESR), and use of the ElastoSens^{TM}Bio2 and a Turbiscan analyser were employed to investigate the degree of erythrocyte aggregation (DEA) and the colloidal suspension stability of blood, respectively. Based on the results obtained, it was decided that for this research the platelets should be loaded with tPA via the process of direct drug loading in order to investigate whether natural platelets may be used as potential tPA delivery vehicles.

Chapter 5 describes the use of natural platelets as delivery vehicles for tPA to achieve targeted clot lysis, for the potential treatment of ischaemic stroke. Natural platelets were loaded with tPA, through direct drug loading, and the encapsulation efficiency and drug release efficacy upon platelet activation were investigated. Thereafter, the tPA-loaded platelets were analysed in order to determine the effects of the encapsulation of tPA on platelet morphology and integrity. The clot dissolution ability of the tPA-loaded platelets was evaluated, as well as their effects on the DEA and the colloidal suspension stability of blood.

Chapter 6 concludes the dissertation. This chapter is composed of the conclusion and limitations of this research project, and recommendations for future studies based on haemocompatibility testing and studies related to platelet drug delivery vehicles have been highlighted.

1.6 Concluding Remarks

The ability of platelets to perform their normal functions within the body and above that to show promise as agents that may play an active role in drug delivery and therapeutic interventions represents the marvellous nature of the human body. Before novel platelet-inspired therapeutic modalities may be designed, it is important to gain an understanding of the current mechanisms in which platelets function as therapeutic agents.

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CHAPTER 2

PLATELET-INSPIRED THERAPEUTICS:

CURRENT STATUS, LIMITATIONS, CLINICAL IMPLICATIONS, AND FUTURE POTENTIAL

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Kola SM, Choonara YE, Kumar P, Kondiah PP, Pillay V. Platelet-inspired therapeutics: current status, limitations, clinical implications, and future potential. Drug Deliv and Transl Res. 2020:1-25.

2.1 Introduction

Platelets are only 2–4 micrometres in diameter, and are not considered to be true cells but cell fragments of megakaryocytes, which are produced in the bone marrow. They are irregular, discoid shaped and lack a nucleus but contain mitochondria, and therefore they do not contain nuclear DNA but possess mitochondrial DNA [1, 2]. On average, platelets live between 7 and 10 days, before being removed by the reticuloendothelial cells in the liver and spleen [2]. Numerous platelets (approximately 2×10^{11} to 5×10^{11} in a human adult) are produced on a daily basis with the number rising up to tenfold as the need of the body increases [3]. Not all of the platelets that are produced are found in the peripheral blood. One of the most important factors determining the number of platelets present in the peripheral blood is the hormone thrombopoietin. The rest of the produced platelets are present in a splenic pool. The most important role played by platelets in the body is that of haemostasis [2]. Additionally, platelets interact with tumour cells, and they play roles in wound repair, inflammation, and antimicrobial defence [3].

Like the other cells found in the peripheral blood, i.e. erythrocytes [4] and leukocytes [5], platelets are continuously being researched as promising strategies to achieve supreme systems of therapeutic intervention, as they are used as devices that allow

for precise and targeted therapy, therefore leading to minimal interaction with non-target cells, which reduces the likelihood of adverse effects occurring and causing the prolongation of the interaction between the drug or therapeutic modality and the diseased tissue [3, 6, 7]. There are advantages to using platelets instead of erythrocytes or leukocytes for the purpose of targeted therapy. More platelets are produced on a daily basis, with a shorter lifespan than the other two cell types, resulting in a quick replenishment time. The multiplicity of roles that platelets play in the body allows them to be used in the targeted therapy for an array of conditions [3].

Majority of the platelet-based therapeutic systems, which include the use of natural platelets acting as drug delivery vehicles, biomimetic systems based on platelet membranes, and genetically engineered, or hybrid platelet membranes, take advantage of the natural pathophysiological affinity and the ability of platelets to target sites of vascular distress [8]. The use of platelet-rich plasma (PRP) as a therapeutic modality is based on the inherent ability of platelets, suspended in plasma, to promote tissue healing and regeneration via the establishment of increased levels of platelets and their associated bioactive molecules at the local site of damage [9]. Recent research has focused on investigating the use of platelet lysate (PL) as an alternative therapeutic modality to PRP, as it also contains a high concentration of platelet-associated bioactive molecules. However, unlike PRP the process of preparation of PL involves the destruction of platelets to release their bioactive molecules, therefore, PL does not contain any cellular matter [10].

In this review the current status of the available modalities of platelet-inspired therapeutic systems will be discussed, to provide comprehensive information that will enhance the readers understanding of platelet use in research, clinical settings, and the pharmaceutical industry. The information has been compiled after analysing the evidence found within the research of this field. The six modalities that will be discussed include: PRP, PL, natural platelet delivery vehicles, genetically engineered platelet membranes, biomimetic platelet-based systems, and hybridized platelet membrane systems. The review goes on to highlight the obstacles posing limitations in the use of each system, and finally the potential future prospects of each system will be analysed based on extraction and extrapolation of data.

A discussion on the use of fully synthetic platelet analogues as a therapeutic intervention able to enhance storage life, and decrease the platelet therapeutic side effect profile is out of the scope of this review. A comprehensive discussion on this topic has been presented in the form of a review article written by Modery-Pawlowski and co-workers [7]. Interested readers are encouraged to read the article entitled "Platelet for drug delivery" [8], which provides insight into platelet drug delivery by taking advantage of platelet physiological functions. The review presented in this chapter differs from the abovementioned article by being more comprehensive with regards to platelet drug delivery in a clinical setting, discussing PRP and PL as therapeutic modalities, as well as by providing comprehensive hypothesized future prospects of each platelet therapeutic modality. It is important to note that currently the use of blood-cell-derived therapeutics is investigational, therefore, there is great scope available for researchers to develop well designed *in vitro* and *in vivo* studies based on the information presented in this review. Figure 2.1 presents a schematic illustration that provides a brief overview of the review.

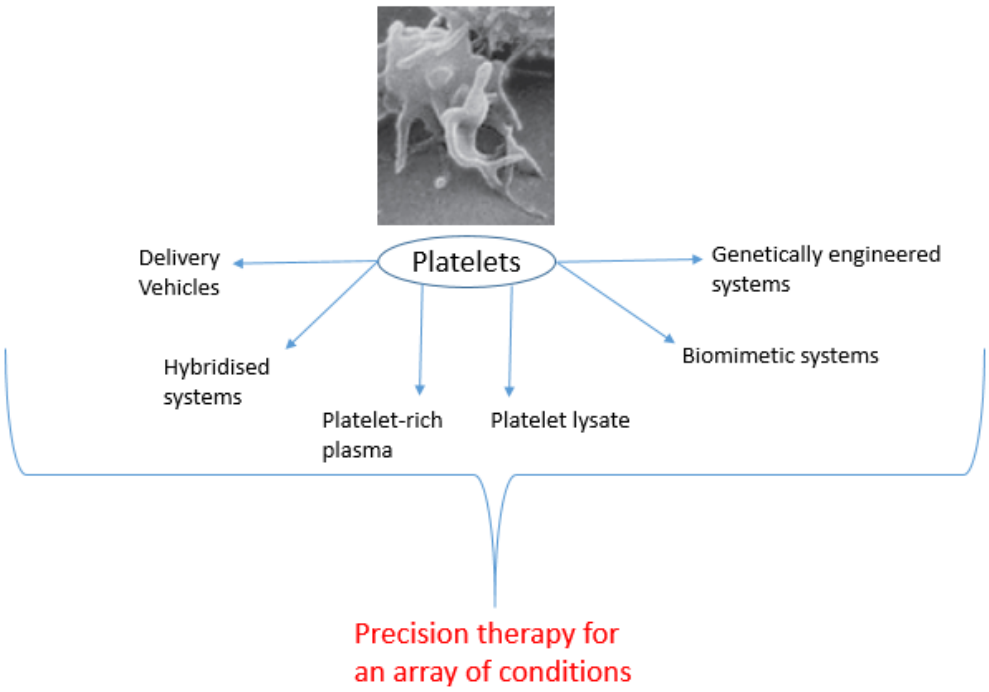


Figure 2.1: Schematic representation providing a brief overview of the literature review presented in this chapter.

2.2 The Use of Platelet Rich Plasma as a Therapeutic Modality

Platelet-rich plasma is defined as an amount of blood plasma containing a higher concentration of platelets than that which is found in the peripheral blood [11]. PRP is obtained via the process of centrifugation, which involves the separation of the blood components based on differing values of specific gravity, following the drawing of blood [12], although a number of manufacturers have developed commercial devices in an attempt to simplify the process of PRP preparation [13]. Aside from platelets, PRP contains plasma proteins, chemokines, cytokines, clotting factors, and a number of growth factors [14, 15], which will modify the surrounding environment upon activation of the platelets, as platelet activation results in their release [16].

PRP is widely being researched as a therapeutic modality that is innovative, simple, non-invasive, and a promising alternative to the conventional therapeutic interventions for a number of disorders [17]. The use of PRP therapeutics is based on the principle that the presence of growth factors released from platelets will cause the promotion of healing and attraction of stem cells to the local site [17], in addition to promoting tissue regeneration [18]. The alpha granules present within platelets contain growth factors, such as platelet-derived growth factor (PDGF), insulin-like growth factor (IGF), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and epidermal growth factor (EGF) [19]. PRP use has been reported in fields of medicine; such as dermatology, plastic surgery, oral surgery and dentistry, ophthalmology, gynaecology, urology, and orthopaedics [20].

PRP preparations differ in the preparation method, their content, and the proposed application of the sample [17]. As a result, there are differing classifications of PRP preparations. A multi-disciplinary consensus committee recommended the classification proposed by Dohan Ehrenfest and co-workers [17], which is based on the presence or absence of leukocytes and the density of fibrin present in the sample, as seen in Table 2.1 [21]. Another method of classification based on the presence or absence of white blood cells in the PRP sample, activation status, and the concentration of platelets present was introduced by Mishra and co-workers. A more complete classification method called the DEPA classification of PRP preparations was proposed by Magalon and co-workers [17]. This classification takes into account the dose of injected platelets, the efficiency of production by the device used, the purity of

the PRP obtained, and the activation process of the PRP obtained [22]. The development of classification methods provides insight into PRP preparations and how they may be used as therapeutic modalities for different pathologies.

Table 2.1: Platelet-containing preparations, as classified by Dohan Ehrenfest et al. [21]

	Rich	**Poor**
Leukocytes	Leukocyte and platelet-rich plasma	Pure platelet-rich plasma
Fibrin density	Leukocyte and platelet-rich fibrin	Pure platelet-rich fibrin

The use of PRP as a therapeutic modality has been widely researched, therefore, this review will only provide an overview of the use of PRP in gynaecology, orthopaedics, dermatology, and neuropathic and musculoskeletal disorders.

2.2.1 Platelet Rich Plasma in Gynaecological Conditions

The use of PRP within the field of gynaecology is limited due to the extreme novelty of the nature of the treatment, therefore, only a few studies have been conducted in the form of case reports and pilot studies. The field of gynaecology is broad and varied, however, PRP was researched for the treatment of a number of different gynaecological conditions due to its known mechanism of action, which is to promote healing and initiate inflammatory responses [17]. For a more detailed analysis of the use of PRP in gynaecological conditions, interested readers are encouraged to read the review article written by Dawood and Salem [17].

PRP has been studied as a potential means of treatment against certain urogenital disorders, namely, genital prolapse [23, 24], urinary incontinence [25], and genital fistulae [26-28]. The use of PRP against genital fistulae caused by Crohn disease proved to be successful in the majority of trials. Patients received a mucosal advancement flap, and thereafter PRP was injected into the patient's fistula tracts. The regenerative and healing properties of PRP was believed to be a contributing factor in the successful healing of the fistulae [29]. Both genital prolapse and urinary incontinence require reconstructive procedures and the strengthening of ligaments in order for their successful treatment to occur, therefore, PRP therapy was believed to

be a promising therapeutic modality for both of these conditions. However, due to a lack of evidence, PRP use against genital prolapse and urinary incontinence has not been sufficiently proven [17].

A niche has been created for the use of PRP in the field of reproductive medicine, as PRP is believed to be effective in causing endometrial growth and ovarian rejuvenation, which is essential for the successful treatment of all of the reproductive conditions that have been researched and mentioned below [17]. The PRP therapy proved to be successful in its aim of causing endometrial growth and ovarian rejuvenation, and therefore, it was effective in the treatment of premature ovarian failure [30, 31], ovarian torsion in a rat model [32], growth of the endometrium [33-35], and improving the outcome of a successful pregnancy in patients suffering from repeated implantation failure [36].

The study conducted by Hua and co-workers proved that with regards to the time of healing as well as adverse effects, the application of PRP is a more promising means of treatment for cervical ectopy, by causing squamous re-epithelialization, when compared to the current standard of care, which is laser therapy [37]. Behnia-Willison and co-workers [38] proved the efficacy of PRP injections in decreasing the size of lesions in lichen sclerosis that is resistant to steroid therapy. A retrospective study conducted by Morelli and co-workers [39] found the use of a platelet-based gel to be effective against locally advanced vulvar cancer - post-surgical vulvar reconstruction. In both lichen sclerosis and vulvar reconstruction, the PRP therapy worked by promoting skin regeneration, however, the precise mechanism of action is not known, thereby creating opportunities for research to be undertaken in this field [38, 39].

PRP has also proven to be an asset in aesthetic gynaecology. The study led by Runels [40] found that injecting PRP into the areas of the vagina and clitoris activated tissue regeneration, thereby increasing arousal and lubrication, and leading to an increase in the incidence of orgasm occurring. Furthermore, these intravaginal injections have been found to increase sensitivity and cosmetic appearance of the genitalia by enhancing vaginal tissue rejuvenation [41]. The combination of these findings suggests the use of PRP therapy for women suffering from sexual dysfunction. Salgarello and co-workers [42], and Gentile and co-workers [43] mixed PRP with fat grafts in order to treat patients with breast soft-tissue defects, with both studies having reported positive

results. Recent research has found PRP to be effective in promoting the viability of fat grafts by acting as a biologic scaffold [44].

In the research conducted by Chignon-Sicard and co-workers [45], the effect of PRP treatment on adipose-derived stromal cells (ASCs) was assessed in order to determine whether PRP has an effect on ASC differentiation as ASCs have been shown to promote graft survival, thereby acting as a promising means of treating soft tissue defects. The PRP, due to the released transforming growth factor beta (TGFβ) pathway activators, showed to have anti-adipogenic and pro-myofibroblastic effects, preventing ASCs from differentiating into adipocytes and causing the genesis of myofibroblast-like cells. It was concluded that PRP participates in the repairing of ASCs, and therefore combining PRP with ASCs may greatly enhance fat grafting, making PRP a promising component in the treatment of soft tissue defects [45]. Figure 2.2 represents an illustration from the research showing the effect of the presence and absence of PRP on chin- and knee- ASC primary cultures. The presence of PRP was successful in inhibiting ASC adipocyte differentiation, which can be seen in Figure 2.2 (B) obtained following polymerase chain reaction (PCR) analysis [45].

In vitro experiments conducted by Lewi and co-workers [46] reported evidence promoting the use of PRP in repairing foetal membrane defects in the premature rupture of neonatal membranes, as PRP enhances the process of membrane healing [46].

Majority of the studies mentioned yielded promising data together with minimal risks associated with the therapeutic modality, showing that PRP use in the abovementioned gynaecological conditions may be promising.

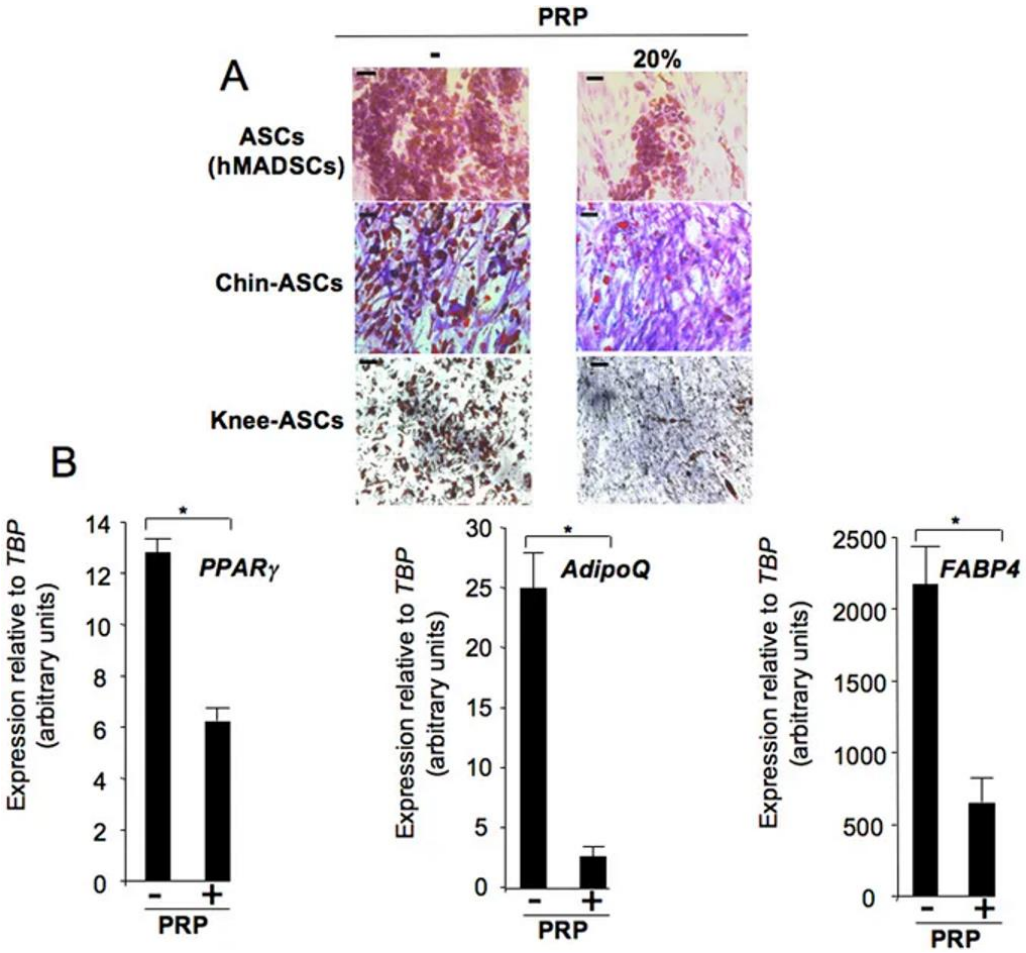


Figure 2.2: PRP inhibited ASC adipocyte differentiation. **(A)** ASCs (hMADScs), chin- and knee-ASC primary cultures were induced to undergo adipocyte differentiation in the absence (−) or presence (+) of 20% PRP. After 10 days, cells were fixed and stained with Oil Red O to visualize lipid droplets, then with nonspecific violet crystals to stain the cell layer. Bar scale: 50 µm. **(B)** ASCs were maintained as in **(A)** and RNAs were prepared to analyse expression of the indicated genes by real-time PCR. Values are means ± SEM (n = 3 individual PRPs). *p < 0.05. This figure and caption have been reprinted with permission from Chignon-Sicard et al [45], under a Creative Commons Attribution 4.0 International License.

2.2.2 Platelet Rich Plasma in Orthopaedics

A number of studies have been conducted in order to test the efficacy and feasibility of PRP use in orthopaedics, as the released growth factors from concentrated platelets aid in osteogenesis. In the research conducted by Cheng and co-workers [47], PRP-loaded scaffolds were designed by using coaxial electrospinning to integrate PRP into various nanofibers to determine whether the osteogenic properties of the nanofibers are enhanced by the presence of PRP. *In vivo* studies performed on nude mice showed an increase in the migration and proliferation of bone marrow mesenchymal stem cells (BMSCs), thereby resulting in the enhancement of the scaffold bioactivity.

Furthermore, the presence of the PRP-loaded nanofibers resulted in an increase in type II collagen expression. These results prove that PRP may be a beneficial addition to the field of bone-tissue engineering [47].

PRP is thought to be an effective and minimally invasive treatment modality for patients suffering from osteoarthritis (OA) of the knee, as well as a range of orthopaedic and sporting injuries [48]. The study conducted by Glynn and co-workers [49] involved the injecting of patients suffering from symptomatic knee OA with three PRP injections, each one being four weeks apart from the other, and clinical outcomes prior to and following the period of therapy of the patients were evaluated. Adverse events, pain and disability, patient satisfaction, health utility, and goal-orientated outcomes were the criteria used to evaluate the effectiveness of the PRP therapy. The results of the study showed improvements in all of the clinical outcomes measured leading to the conclusion that PRP may be used as an effective therapeutic modality for the treatment of symptomatic knee OA [49]. The results of the studies conducted by Dai and co-workers [50], and Shen and co-workers [51] proved the therapeutic potential of PRP to be consistent with the findings of Glynn and co-workers [49], however, heterogeneity of the clinical outcomes was reported in both of the studies.

In the review conducted by Hussain and co-workers [52], studies that assessed the effective use of PRP for a wide range of orthopaedic conditions were analysed. Following the analysis, it had been concluded that PRP may be beneficial in the treatment regimens of knee osteoarthritis and lateral epicondylitis, while conditions such as anterior cruciate ligament repair, hamstring injuries, medial epicondylitis, rotator cuff repair, and patellar and Achilles tendinopathies showed inconsistent results in some studies, while other studies proved that patients had not experienced significant benefit from the use of PRP injections as a part of their treatment regimens [52].

2.2.3 Platelet Rich Plasma in Dermatology

The growth factors present in PRP as well as the natural healing properties of platelets have led to the increasing interest in using PRP within the field of dermatology; specifically, for alopecia, tissue regeneration, skin rejuvenation, scar repair, and wound healing [16, 53]. In the article written by Alves and Grimalt [11], PRP was shown to be a bio regenerator acting as a source of growth factors to increase the growth factor

concentration at the site of a wound, thereby stimulating the process of wound healing for recalcitrant wounds [11].

Androgenic alopecia is a genetic condition causing patterned baldness in both men and women [54]. Several different studies that have been conducted in order to assess the effects of PRP on hair regrowth in sufferers of androgenic alopecia proved positive results, whereas a smaller number of studies concluded that PRP may be beneficial in promoting hair regrowth in alopecia areata [55]. The cycle of hair growth is dependent on a number of cytokines and growth factors [56], many of which are present in PRP, therefore, the presence of PRP at the local site promotes angiogenesis of the cells [11].

PRP stimulates human dermal fibroblast proliferation, and increases the synthesis of type 1 collagen and elastic fibres resulting in the use of PRP injections within the cosmetic and aesthetic sector of dermatology. Histological evidence proved the induction of soft-tissue augmentation by PRP resulting in facial rejuvenation [57]. A number of studies have been conducted in order to determine the effects of PRP injections into the dermis and sub dermis of the facial skin on the volume, tone of the facial skin, texture, acne scars, burn scars, postsurgical scars, and appearance of wrinkles on the face. All of the studies concluded that the PRP injections had a positive effect on the tested parameters, making PRP use in cosmetics and aesthetics promising [11].

2.2.4 Platelet Rich Plasma in Neuropathic and Musculoskeletal Disorders

Recent strategies are aimed at moving towards the implementation of non-surgical interventions for the treatment of peripheral neuropathies. Although the growth factors within platelets are not neurotrophic in nature, they have gained considerable attention within the field of nerve regeneration [58]. In the study performed by Anjayani and co-workers [59], patients suffering from Hansen's disease (a condition caused by *Mycobacterium leprae*) were given PRP perineural injections in order to determine the effect of the PRP treatment on improving peripheral neuropathy, compared to the control group, which consisted of patients being treated with platelet-poor plasma (PPP) perineural injections. The study found that the PRP injections were highly promising in the treatment of nerve regeneration [59]. The work reported by Sanchez and co-workers [60], describes the use of ultrasound-guided PRP intraneural injections

in a sufferer of peroneal nerve palsy who had attempted alternative treatment for a period of 11 months, to no avail. The follow-up investigations proved a partial recovery by the patient suggesting a novel mechanism of treating nerve palsy using PRP [60].

Kuffler and co-workers [61] reported on a strategy of treatment that involved the filling of a collagen tube with platelet-rich fibrin in order to be used in the treatment of a patient who suffered ulnar nerve damage over three years ago in an attempt to improve his sensory and motor functions. The study found that the motor function of the patient was improved, he was able to feel vibration, and his neuropathic pain was significantly decreased, indicating the potential benefits of PRP use in neurological recovery [61]. The work by Hibner and co-workers [62] mentions the successful use of PRP therapy for the treatment of pudendal neuralgia failing to respond to surgical decompression interventions. Finally, the work performed by Scala and co-workers [63] demonstrated the positive effects of PRP in preventing neurological deficits of the facial nerve in patients who underwent surgery for a superficial parotidectomy.

Bhatia and Chopra [64] designed a pilot study in which they tested the efficacy of PRP in patients suffering from lumbar radiculopathy as a result of a prolapsed intervertebral disc. Patients were injected with autologous PRP via interlaminar lumbar epidural injection, and the clinical outcomes were measured using the Modified Oswestry Disability Questionnaire (MODQ), Straight Leg Raising Test (SLRT), and Visual Analogue Scale (VAS). The results of the evaluation tests showed improvements in the patients' scores without any observed complications, indicating that PRP may be considered as an alternative treatment modality for radiculopathy associated with prolapsed intervertebral disc [64].

In the article published by Aufiero and co-workers [65], the application of PRP injections within the cervical, lumbar, and thoracic spine were analysed, and based on the observed clinical results it was concluded that PRP injections may be a promising alternative treatment option for cervical, lumbar, and thoracic spinal pain associated with ligaments, capsules, and facet joints [65]. In the review report by Mohammed and Yu [66], the team investigated the effectiveness of PRP therapy in degenerative disc disease with specific reference to discogenic low back pain. They demonstrated that PRP therapy is a safe and effective modality for the treatment of discogenic low back pain, and a promising alternative treatment modality for degenerative disc disease [66].

Mautner and Kneer [67] described the use of PRP in the treatment of tendinopathies, because currently no gold standard of treatment exists in order to relieve the dysfunction and pain associated with tendinopathy. They elaborated on the finding that PRP injections may represent a new, effective, and multifaceted approach to treatment, which has a greater safety profile compared with corticosteroids and surgery, as PRP injections neither caused tendon degradation nor systemic complications [67].

The literature proves that PRP injections may be a promising therapeutic modality for the treatment of neuropathic and musculoskeletal disorders.

2.2.5 Limitations of Platelet Rich Plasma Use

Due to the rapid degradation of the growth factors that PRP releases, a limitation in their clinical use is imminent [47]. The greatest limitation however with regards to the use of PRP as a therapeutic modality is the lack of sufficient clinical data to adequately confirm the benefits of its use in clinical settings, as well as the presence of trials consisting of smaller patient numbers [17, 52, 58]. During the process of PRP preparation there is no standard operating procedure indicating the number and speed of the centrifugation process, therefore, this lack of uniformity may lead to different types of PRP being produced in different settings [11]. The lack of standard operating procedures also leads to a debate with regards to the frequency of PRP application for each condition, the dose of administration, and the site at which the injection should be administered [11]. The patient and process variability of PRP may make it an inaccurate treatment modality [68]. The use of PRP within orthopaedics has been gaining popularity based on the available data, however, majority of the studies conducted on PRP use in orthopaedics are smaller studies conducted within a single country consisting of a single population type [49]. Substantial heterogeneity exists across the various studies with regard to the patient population selected, statistical pooling, and the dose, frequency and duration of the PRP injections [52]. Another factor limiting the use of PRP as a therapeutic intervention is the cost of treatment. A PRP injection is approximately $540.00 more expensive than a corticosteroid injection for orthopaedic use [69].

It is of paramount importance to recognize that while PRP use in orthopaedics should be considered as a therapeutic modality, the current evidence suggesting the benefits

of PRP proves to be inconclusive [52]. PRP use in aesthetic dermatology yielded encouraging results, however, some studies failed to ensure standardization within their preparation techniques, they lacked objective means of analysing the quality of the skin prior to and following PRP treatment, and other studies did not provide evidence with regards to the potential benefits of the PRP therapy over time [17, 53, 57]. PRP use in neuropathic and musculoskeletal disorders has proven to be effective in the studies that have been conducted, however, further research is needed in order to provide greater, more substantial evidence for use of PRP in this field [58].

Based on the information present in all of the articles mentioned, it is evident that the mechanism of action by which PRP benefits these conditions needs to be more extensively researched, and the optimal frequency of PRP injection administration needs to be determined. The inquiry regarding whether the outcome of the treatment will be more effective with earlier administration of the injections needs to be addressed. The lack of sufficient randomized controlled trials for PRP therapy has led to an increase in the potential for bias when reporting on the effectiveness of PRP as a therapeutic modality.

2.2.6 Future Prospects of Platelet Rich Plasma Use

PRP use in radiculopathy may be further researched with specific emphasis on the use of PRP for cervical radiculopathy [64]. There is great scope for the development and undertaking of well-designed randomized controlled clinical trials in order to assess the clinical outcomes of PRP therapy in the various conditions mentioned above. Future analyses can focus on researching the different PRP preparations, and the subsequent effects of the differing formulations. The successful performance of these trials and experiments may lead to a new and improved understanding of PRP therapy in a practical setting, ensuring that clinicians are better equipped to recommend the therapy with confidence, and researchers are able to determine which gaps within PRP therapy need to be explored. Biomedical researchers can analyse the intricate functions of PRP that give rise to their healing properties, and postulate which conditions not already researched may respond favourably to PRP. I believe that an example of such a condition is osteogenesis imperfecta (OI), which is a group of bone disorders resulting in highly fragile bones that are easily susceptible to fractures [70]. OI is believed to be caused by mutations in the genes that allow for the successful coding of type 1 collagen [70]. As PRP is known to increase the synthesis of type 1 collagen

[57], the use of PRP as an adjunctive therapy for OI may be explored. The current status, limitations, and future potential of PRP therapeutics have been summarized in Table 2.2.

Table 2.2: A summary of the current status, limitations, and future potential of PRP therapeutics

Platelet System	Brief Description	Current Status	Limitations	Methods to Overcome the Limitations	Future Prospects
Platelet-rich plasma (PRP)	Blood plasma containing a higher concentration of platelets, than that which is found in the peripheral blood.	Proven to be effective in the treatment of certain gynaecological, orthopaedic, dermatological, neuropathic, and musculoskeletal disorders.	There is a lack of sufficient clinical data to adequately confirm the benefits of PRP use within clinical settings, as well as the presence of trials consisting of small patient numbers.	Well-designed, randomised, controlled clinical trials, with a sufficient patient population need to be developed and undertaken.	The use of PRP in the treatment of cervical radiculopathy, and osteogenesis imperfecta may be researched.

2.3 The Use of Platelet Lysate as a Therapeutic Modality

Due to the presence of process and patient variability that exists within PRP use, researchers have been looking into using platelet lysate as an alternative therapeutic modality [68]. As is evident from the studies mentioned below, the majority of research undertaken thus far focuses on the use of PL as a novel and interesting treatment option for conditions that are somewhat unresponsive to the current conventional treatment methods.

PL is obtained following the process of lysing, which involves either cycles of freezing and thawing, or ultrasonic treatment of apheresis PRP, followed by the removal of debris thereby resulting in a hemoderivative that is rich in growth factors [71, 72]. Briefly, the process of producing PL via freeze-thaw cycles involves freezing PRP at approximately -70°C for a minimum period of 10 minutes, followed by thawing the PRP in a water bath at 37°C. The lysate is then centrifuged in order to remove the debris,

and the supernatant is filtered to ensure the removal of any lysed platelet membranes [72, 73]. This freeze-thaw cycle is repeated based on pre-set values determined by individual research teams. This method of production has the advantages of being cost-effective and simple to apply, making it a good option for hospital settings where the PL needs of the local community may be met [72]. However, the disadvantages associated with the freeze-thaw method of production need to be taken into account. These include the time taken for the process to be completed, and the lack of consensus on the number of freeze-thaw cycles that need to be performed. The differing cycle number pre-sets have shown to yield PL preparations with great differences in their growth factor levels, making it difficult to compare data obtained from the different available research [72].

The process of producing PL via ultrasonic treatment involves collecting PRP in plastic bags (for example, ethylene vinyl acetate freezing bags) and submerging the bags in an ultrasound bath containing sterile distilled water. The exposure of the PRP to ultrasonic waves causes lysis to occur. Following sonication, the lysate is centrifuged to allow for the removal of debris. Thereafter, the supernatant is filtered to ensure the removal of any lysed platelet membranes. PL production via sonication has proven to be a fast, safe, and highly efficient method that is easy to monitor [71]. However, being a relatively new method, it is not well established, requiring further research into its use. Clinicians or research teams that are interested in investigating PL use should choose a process of preparation based on their individual needs and preferences. In this section of the review, the use of PL in stem cell research, orthopaedics, ophthalmology, and dermatology will be detailed.

2.3.1 Platelet Lysate in Stem Cell Research

PL has gained interest as a therapeutic modality following reports stating its proliferating ability of certain cells, including mesenchymal stem cells (MSCs) [74]. In the research undertaken by Bieback and co-workers [75], it was proven that PL is a positive replacement for foetal bovine serum (FBS) as it prevents the likelihood of stem cell contamination occurring, which is usually associated with FBS use. Therefore, PL is a promising addition to the field of stem cell therapeutics. Due to the significant presence of human serum within PL, the PL liquid contains cytokines, tissue inhibitor of metalloproteinase, alpha-2-macroglobulin, and interleukin receptor antagonist,

which are significant anti-inflammatory proteins [76, 77], thereby endowing PL with inherent anti-inflammatory and nerve repair properties [78].

Recent research has been focusing on the use of MSCs seeded on biomaterials as a potential alternative to bone grafts for the purpose of bone regeneration [78]. The study performed by Chevallier and co-workers [79], aimed to determine whether PL or FBS has greater osteogenic capacity, by culturing human MSCs with PL and FBS respectively. *In vitro* and *in vivo* studies performed in a mouse model showed that the MSCs cultured in PL were able to enhance osteoblastic gene activity and promote ectopic bone formation, proving that human PL is able to accelerate the proliferation and enhance osteogenic differentiation of MSCs thereby making PL a promising alternative for the promotion of MSC expansion.

2.3.2 Platelet Lysate in Orthopaedics

One of the current methods used to treat both lumbar radicular pain as well as OA involves steroid injections, containing corticosteroid anti-inflammatory agents, into the local affected area [80, 81]. A number of adverse effects have been associated with corticosteroid use. These include negative effects on the cardiovascular, endocrine, dermatologic, musculoskeletal, metabolic, gastrointestinal, and nervous systems, as well as effects on blood pressure and glucose levels. The use of corticosteroid injections has also shown to cause bone loss, and result in the suppression of the hypothalamic-pituitary-adrenal axis. As a result, researchers are looking into alternative methods that may show therapeutic promise, as well as pose lower risks to patients due to adverse effects. As has been mentioned in section 2.3.1, PL is believed to be beneficial as an anti-inflammatory and nerve repair agent, due to the anti-inflammatory proteins that it contains. This finding has led researchers to investigate the use of PL injections as an alternative to corticosteroid injections, as the successful use of PL will not result in the multitude of adverse effects associated with corticosteroid use [80].

In the study performed by Centeno and co-workers [80], the use of PL for the purpose of treating lumbar radicular pain was evaluated. Patients who were given epidural injections of PL instead of corticosteroids were assessed in terms of clinical outcomes such as functional rating index (FRI), numeric pain score (NPS), and a modified single assessment numeric evaluation (SANE) rating. The results proved that PL injections

are a sound alternative to corticosteroid use as the patients experienced significant improvements in their clinical outcomes [80]. In the study conducted by Gilbertie and co-workers [68], the joint environment of OA was mimicked *in vitro* in order to test the effects of pooled PL on chondrocytes and synoviocytes affected by inflammatory mediators. Interleukin-1β (IL-1β) and lipopolysaccharide (LPS) were used to stimulate the synoviocytes for a period of 24 hours respectively, thereafter, treatment was initiated with PL from PPP or PL from PRP, while other samples received no treatment. Following the treatment period, the growth of the synoviocytes was assessed, and the concentrations of interleukin-6 (IL-6), tumour necrosis factor alpha (TNF-α), IL-1β, and hyaluronic acid (HA) present in the synoviocyte conditioned media were analysed. Upon 48 hours challenging of the chondrocytes in synoviocytes media, they were examined for the gene expression of matrix metallopeptidase 3 (MMP-3) and 13 (MMP-13), lubricin (PRG4), aggrecan (ACAN), and collagen types I (COL1A1), II (COL2A1), and III (COL3A1). The synoviocytes treated with PL from PRP experienced an increase in growth, as well as an increased synoviocyte production of HA and IL-6. The chondrocytes that were challenged with media from synoviocytes treated with PL from PRP exhibited a decrease in MMP-13 gene expression and an increase in aggrecan and collagen type II gene expression proving that PL from PRP may be a promising modality in the treatment of OA [68].

2.3.3 Platelet Lysate in Ophthalmology

Current treatment methods for corneal damage differ depending on the type, cause, and severity of the damage. These treatment methods include oral medication, steroid eye drops, and in severe cases surgery is performed to remove the unhealthy tissue. However, ophthalmologists find damage to the cornea difficult to treat, as the treatment needs to be localized, delivered directly to the precorneal area, prolonged contact between the cornea and the treatment modality needs to be maintained, and the presence of tears results in dilution of the drug, thereby causing a smaller than intended dose to reach the site of action. Corneal damage may result in persistent epithelial defect (PED), which may severely impact the quality of life of the patient, resulting in the need for more effective therapeutic modalities. Research has shown that some of the components found in blood plasma are the same as those found in tears. This has led to investigating the use of hemoderivatives in the treatment of ophthalmic conditions. The platelet-derived growth factors and cytokines present in PL play a role in maintaining the integrity of the cornea, mediating corneal epithelium

renewal, and enhancing wound healing [10, 82]. As a result, the studies mentioned below aimed at investigating the effects of PL in the treatment of corneal lesions, by developing products that would allow the PL to have a longer contact time with the affected area.

In one study conducted by Sandri and co-workers [10], PL was loaded into a chondroitin sulphate sodium and hydroxypropylmethyl cellulose based sterile vehicle in order to develop eye drops that are thermosensitive and mucoadhesive, which will allow for the increased permanence of contact between PL and the corneal lesions. *In vitro* studies performed using corneal epithelial cells derived from rabbits, proved the success of the formulation in enhancing corneal cell growth. In addition, this study proved that PL and chondroitin sulphate have a synergistic relationship in enhancing cell proliferation [10].

An additional study conducted by Sandri and co-workers [82] investigated the effect of loading contact lenses with PL in order to create a product allowing for the permanent contact between PL growth factors and the corneal surface, thereby improving the treatment of corneal lesions. Various types of contact lenses that are available on the market were loaded with either chondroitin sulphate or chondroitin sulphate mixed with PL. Following the process of loading, *in vitro* studies proved that PureVision® contact lenses improved cornea cut closure by enhancing the proliferation of corneal cells, proving that the addition of PL was beneficial in the treatment of corneal lesions [82]. The results of both studies prove the beneficial effects of employing a strategy that allows for contact between PL and the surface of the cornea when treating corneal lesions.

2.3.4 Platelet Lysate in Dermatology

When the process of wound healing of tissue injuries is delayed for longer than 12 weeks, chronic skin lesions are formed. Wound healing may be delayed due to repeated injury to the skin or to the presence of another disease that the patient may be suffering from. Examples of these conditions include diabetes, persistent infections, and malignancies. Wound healing is a natural biological process that doesn't usually require medical intervention. However, chronic skin lesions require therapeutic interventions to facilitate healing. Essential to the process of wound healing are platelet-derived growth factors and cytokines, as these bioactive substances facilitate

the processes required for tissue regeneration to occur. The current first line treatment of chronic skin lesions is topical medication administered via gauzes or semisolid preparations. However, research has shown that the use of PL in the treatment of chronic skin lesions may be a promising alternative due to the high concentration of growth factors and cytokines that it contains. Additionally, the PL formulations that have been designed thus far have proven to be easier and less painful to administer than the conventional treatment methods [83, 84].

In an attempt to design a formulation that can successfully treat chronic skin ulcers, using the process of ionotropic gelation, Tenci and co-workers [83] prepared a powder formulation consisting of pectin, with and without chitosan particles possessing the optimal size, mechanical resistance and hydration properties. These particles were loaded with PL and Manuka honey. The Manuka honey used was either rich in methylglyoxal or rich in polyphenols. *In vivo* tests performed on a rat model proved that PL was successfully loaded into the particles without any effects on its biological activities. Moreover, the particles containing chitosan and Manuka honey rich in methylglyoxal demonstrated an enhancement of cell proliferation, proving the positive effects of the loaded particles on wound healing [83].

Mori and co-workers [84] developed a powder formulation in the form of calcium alginate particles, able to deliver a combination of PL and vancomycin hydrochloride (VCM) to treat chronic skin ulcers. Upon investigation it was proven that calcium alginate particles are able to load PL without causing any alteration to the PL bioactivity. Observations proved that the presence of PL in the calcium alginate particles enhanced the proliferation of human fibroblasts, increased viable cell count, and caused a greater cell presence in the proliferative phase. These findings indicate an alternative mechanism to wound healing [84]. In addition to these studies, Rossi and co-workers [85] were successful in the development of wound dressings with a sponge-like consistency, able to deliver PL to chronic skin wounds, without altering the biological PL activities.

2.3.5 Limitations of Platelet Lysate Use

PL is a relatively novel method of treatment, therefore, just like PRP the greatest limitation with regards to the use of PL is the lack of sufficient clinical data to adequately confirm the benefits of its use in clinical settings. There is no standardized method of

producing PL, thereby posing a challenge in determining the best manner of PL production in the laboratory, and preventing its widespread use [73]. The variations in the methods of production result in PL preparations that vary in the amount of platelet growth factors present [72]. The mechanism of action of PL is believed to be similar to that of PRP, i.e., to promote tissue growth and regeneration due to the high concentration of platelet bioactive molecules that the PL preparation contains. However, the exact effect and mechanism of action of each individual growth factor is not well known, warranting the performance of thorough *in vitro* and *in vivo* studies [86].

2.3.6 Future Prospects of Platelet Lysate Use

The study conducted by Gilbertie and co-workers [68], suggests that further research should be performed regarding the use of PL for OA. As PL has been shown to have many of the beneficial properties that PRP contains, without some of the limitations that PRP use possesses, further studies may be conducted regarding the use of PL for the conditions that have already shown to respond well to PRP, allowing patients to have a variety of treatment options to choose from, and PL may prove to be a safer, more effective tool of treatment compared to PRP. If the mechanism of action of PL can be precisely determined, researchers may use the information to test the effects of PL in a wider range of conditions, hypothesized to respond favourably to the treatment. Scaffolds that are porous in nature have the ability to promote bone regeneration, and therefore may benefit greatly by being combined with factors able to promote bone growth [87]. Therefore, the positive osteogenic effects of PL create opportunities for the use of PL in the field of bone-tissue engineering. Future research may focus on the effects of using PL, the enhancing culture supplement, on biomaterials and 3D printed scaffolds designed for bone-tissue engineering. In addition, there is ample room for the synergistic relationship between PL and chondroitin sulphate to be further investigated [82]. The current status, limitations, and future potential of PL therapeutics have been summarized in Table 2.3.

Table 2.3: A summary of the current status, limitations, and future potential of PL therapeutics

Platelet System	Brief Description	Current Status	Limitations	Methods to Overcome the Limitations	Future Prospects
Platelet lysate (PL)	A liquid obtained following the process of freezing and thawing, or ultrasonic treatment of platelets.	PL is an effective supplement for foetal bovine serum (FBS), it enhances the osteogenic differentiation of mesenchymal stem cells (MSCs), and it improved clinical outcomes in sufferers of lumbar radicular pain and osteoarthritis (OA). PL has proven to be effective in the treatment of corneal lesions when a strategy that allows for contact between PL and the surface of the cornea, is employed. PL can be incorporated into wound healing modalities with great success.	There is a lack of sufficient clinical data to adequately confirm the benefits of PL use within clinical settings.	Well-designed, randomised, controlled clinical trials, with a sufficient patient population, need to be developed and undertaken.	The use of PL in the treatment of OA, and 3D printed scaffolds designed for bone tissue engineering may be researched. The use of PL in the treatment of conditions that respond well to PRP therapy may be further researched. The use of PL in ophthalmology and dermatology should be further explored.

2.4 The Use of Natural Platelets as a Therapeutic Modality

Platelets are an ideal drug delivery system as they only target the desired affected cells, and thereby have a minimal effect on surrounding healthy tissue, causing the

incidence of systemic side effects to be greatly reduced. Drug release from platelet carriers occur in a controlled manner; and good biocompatibility, and immune evasion are ensured [88]. Platelets have been found to have the ability to encapsulate drugs through their canalicular system, and when platelet activation occurs, granular matter as well as the encapsulated drug are released from the platelet [89]. Therefore, the success of this drug delivery system is dependent on platelet aggregation and subsequent activation.

When platelets encapsulate drugs, they cloak and protect the drug in a manner where the body does not detect it as foreign matter, therefore, increased clearance of these platelets does not occur [90]. This leads to a prolonged circulation of the drug in the blood, thereby increasing the concentration of the drug in the body and decreasing the clearance rate of the drug from the body. As the platelet carrier system is not synthetic, it will not have to be removed from the body once the treatment is complete [91]. The use of natural platelets as a therapeutic modality has been investigated for cancer therapy, and the use of platelet decoys have been investigated in the treatment of thrombosis and metastatic tumours. These studies are discussed below.

2.4.1 Natural Platelet Use in Cancer Therapy

Currently, the most widely used therapy for cancer is chemotherapy. However, due to the non-specific nature of chemotherapy many adverse effects are likely to affect healthy tissue in the body [89]. As a result, the introduction of targeted therapy will greatly benefit the fight against cancer.

Thus far, the only research conducted on natural platelets as drug carriers has been performed by Sarkar and co-workers [92] and Xu and co-workers [89], wherein natural platelets were used to encapsulate the chemotherapeutic drug Doxorubicin (DOX) for the treatment of lymphoma. Scanning electron microscopy (SEM) and western blot testing revealed no changes to the morphological structure and functions of the platelets following drug loading. Tumour cell-induced platelet aggregation (TCIPA) is the phenomenon by which the successful functioning of the system was based [93]. This phenomenon is explained in further detail in Chapter 3, Section 3.3.2. *In vivo* studies demonstrated an enhanced anti-tumour efficacy of DOX-loaded platelets, thereby proving the potential success of using natural platelets as delivery vehicles in the treatment of lymphoma [89, 92]. Figure 2.3 is a schematic representation of the

natural platelet drug delivery system. The illustration shows the potential *in vitro* and *in vivo* mechanism of activity of the DOX-platelet system, following intravenous (IV) administration in a tumour-bearing mouse model. Due to the TCIPA phenomenon, the DOX-platelet system leads to an induction of tumour cell apoptosis [89].

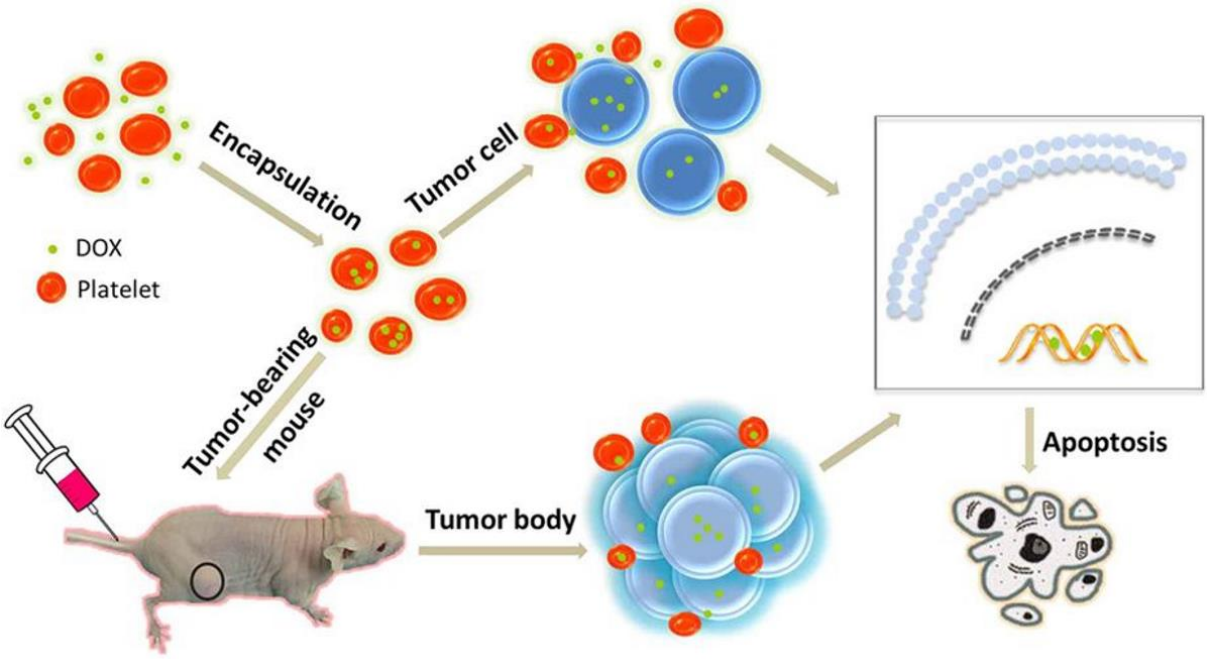


Figure 2.3: Schematic illustration of the possible mechanism of enhanced anti-tumour activity of DOX-platelet *in vitro* and *in vivo*. Tumour-bearing mice are injected with DOX-platelet by tail vein. DOX-platelet targets tumour cells passively by "tumour cell-induced platelet aggregation". DOX induces tumour cell apoptosis through regulating the expression of apoptosis-related genes. This figure and caption have been reprinted from Xu et al [89], under a Creative Commons Attribution 4.0 International License. *DOX: doxorubicin

2.4.2 The Use of Platelet Decoys in Thrombosis and Metastatic Tumour Formation

Platelet decoys are formed by removing platelet membranes and platelet cellular contents in order to obtain natural platelets possessing only their cytoskeleton and surface receptors. These platelet structures may be used as anticoagulants, as well as agents that may inhibit metastatic tumour growth [94]. Papa and co-workers [95] developed modified human platelets, with the aim of creating platelet decoys capable of normal platelet binding functions but incapable of normal platelet functional activation and aggregation behaviour. Their obtained results showed that these platelet decoys could represent a potentially effective and beneficial strategy for treating

thrombosis and metastasis. *In vitro* studies showed that the designed platelet decoys were able to inhibit platelet adhesion and aggregation on thrombogenic surfaces, which was easily reversible following the addition of unmodified platelets. *In vivo* studies conducted within a rabbit model proved the success of the platelet decoys in inhibiting thromboembolism caused by arterial injury. Additionally, the platelet decoys proved to be successful in the fight against metastatic cancer by causing a decrease in platelet cell aggregation, and tumour cell arrest as well as extravasation within a breast cancer model. *In vivo* studies performed in a metastatic mouse model proved the effectiveness of the platelet decoys in inhibiting tumour metastatic growth following injection of the decoys [95].

2.4.3 Limitations of Natural Platelet Use

As not many experiments have been conducted using natural platelets acting as drug delivery vehicles and on platelet decoys as a therapeutic modality, the true nature and long-term effects of the system is unknown. The first limitation of the natural platelet drug delivery vehicle system is the cost that the patient may have to incur due to the specifics of the procedure. Blood is necessary, so either the patient's own blood would need to be drawn, or blood from an acceptable donor would be used to ensure biocompatibility. Therefore, the price of such a treatment may be greater than if the drug was administered without being encapsulated within a delivery vehicle. As platelets are biological products, they are highly sensitive in nature and require specialist care in order to maintain their natural structure and functions. So far, only DOX has been proven to be compatible as an encapsulated drug within platelets; therefore, whether other drugs may be encapsulated successfully remains unknown, raising the need for scientific experimentation to be conducted within this field. The basis by which this system operates is that platelet aggregation and activation must occur for successful release of the encapsulated drug. While TCIPA is one situation within the body that induces platelet aggregation and activation, there may be another condition of vascular distress concurrently occurring within the body that would elicit a platelet response. Unless a specific targeting ligand is attached to the platelets intended to aggregate at the tumour site, there would be no way of controlling where the encapsulated drug release occurs, which may result in the release of the drug at the incorrect site of action, leading to an array of adverse effects and complications that the patient would experience.

2.4.4 Future Prospects of Natural Platelet Use

As it has already been proven that platelets are able to successfully encapsulate DOX without any structural and functional changes to the platelet itself, platelet use for cancers treatable by DOX, other than lymphoma, may be explored. The use of DOX-loaded platelets may also be employed in cancers where post-surgical chemotherapeutic treatment of the remainder of the tumour is necessary. Platelet aggregation and activation occurs during times of vascular distress in the body as the innate function of platelets is to target vascular disorders. These disorders include, but are not limited to, thrombosis, haemorrhage and strokes. As a result, there is an increasing interest in using platelets, as drug delivery vehicles for the purpose of treating these disorders. Therefore, there is scope for the future performance of studies to determine the effectiveness of natural platelet use in the abovementioned conditions [91]. As the use of platelet decoys has not been thoroughly investigated, there is room for knowledge generation and exploration within this field, thereby contributing to the development of cell-mediated therapeutics. Following the further research conducted on platelet decoys, there is great scope for their use within the many conditions associated with thrombosis and metastasis. The current status, limitations, and future potential of natural platelet therapeutics have been summarized in Table 2.4.

Table 2.4: A summary of the current status, limitations, and future potential of natural platelet therapeutics

Platelet System	Brief Description	Current Status	Limitations	Methods to Overcome the Limitations	Future Prospects
Natural platelet delivery vehicles	Platelets in their pure form are used as carriers for a suitable drug, to achieve targeted drug delivery.	Natural platelet carriers have been used to encapsulate doxorubicin (DOX) for the treatment of lymphoma. Platelet decoys may be used as anticoagulants, and to inhibit metastatic tumour growth.	The delivery vehicles may be activated at another site of the body that is concurrently eliciting a platelet response; therefore, the drug would be released at the incorrect target site.	A specific targeting ligand may be attached to the platelets to ensure that drug release occurs at the appropriate target site.	DOX-loaded platelet use in the treatment of other cancers may be researched. Natural platelet use in stroke, thrombosis, and haemorrhage may be investigated.

2.5 The Use of Genetically Engineered Platelet Membranes as a Therapeutic Modality

Gene therapy is an experimental approach for the treatment of diseases based on the inception of genetic material into the cells selected for genetic alteration [96]. This is performed with the aim of successfully overcoming the limitations of standard pharmaceutical interventions [97]. Recent research has found the storage and delivery functions of platelets to be essential components in making platelets a promising cell target for the application of gene therapy, in order to successfully treat diseases such as haemophilia A and cancer [98, 99], as is discussed below.

2.5.1 Genetically Engineered Platelet Membrane Systems in Haemophilia A

Haemophilia A is a rare genetic disorder resulting from a deficiency in the clotting protein - factor VIII (FVIII), causing sufferers to experience prolonged periods of bleeding [100]. The current treatment for haemophilia A is protein infusions [101, 102], however, following protein replacement approximately 30% of sufferers develop inhibitory antibodies against FVIII, which inactivate FVIII activity resulting in the rejection of standard FVIII replacement therapy, thereby leading to unsuccessful treatment outcomes [103, 104]. Von Willebrand factor (vWF) is a plasma glycoprotein that binds tightly to FVIII forming a complex that circulates to the site of injury. The studies conducted on platelet gene therapy for the treatment of haemophilia A focused on directing FVIII to platelets, as platelets are responsible for the synthesis of vWF, with the primary aim of forming an enhanced intracellular complex of vWF and FVIII that is delivered to the site of injury [98]. Different platelet specific promoters, such as platelet factor 4 (PF4) promoter, platelet glycoprotein (GP) IIB (αIIb) promoter, and GPIb promoter, in addition to lentivirus-mediated and transgenesis-mediated delivery were utilized in the various studies to direct FVIII expression to the platelets. The studies that were conducted in both animal models and human clinical trials were successful in demonstrating the storage of FVIII together with vWF in α-granules, resulting in the effective correction of haemostasis in haemophilia A sufferers even in the presence of FVIII inhibitory antibodies [105-123].

2.5.2 Genetically Engineered Platelet Membrane Systems in Cancer Therapy

Circulating tumour cells (CTCs) are cells that have separated from the primary tumour and are disseminated within the blood or lymphatic system; therefore, they are

responsible for cancer metastasis [124, 125]. It is difficult to successfully treat CTCs, even with the introduction of nanomedicines, as the CTC microenvironment is significantly different to that of the solid tumour environment [126]. The intrinsic ability of platelets is to promote metastasis by interacting with CTCs via biomolecular binding, like P-Selectin to CD44 receptors, and structure-based capture, and allowing for their immune evasion, and extravasation [99]. This function of platelets was used as the inspiration for studies that employed genetic engineering of platelets in order to establish a Trojan-horse strategy of targeting CTCs [124]. The strategy is called a Trojan-horse strategy as it employs the platelets as carriers allowing for the non-invasive delivery of therapeutics to the target site.

The research conducted by Li and co-workers [124] was successful in creating genetically modified platelets that expressed tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), a cytokine causing the initiation of cell death. *In vitro* and *in vivo* studies proved the success of the genetically modified platelets in significantly reducing metastasis to the liver [124]. Programmed-death ligand 1 (PD-L1) is a protein that binds to the programmed death 1 (PD-1) receptor and is upregulated on the surface of cancer cells in an attempt to inhibit the function of T cells, thereby resulting in immune evasion. Attempts are made to block the interaction between PD-L1 and PD-1 so that an immune response may occur against the tumour cells [127]. In order to cause a decline in the post-surgical recurrence of tumours as well as metastasis, Wang and co-workers [99] conjugated engineered monoclonal antibodies against PD-L1 (anti-PD-L1) to platelet surfaces, as illustrated by Figure 2.4. The effective release of anti-PD-L1 following platelet activation by microparticles was observed, and *in vivo* studies proved the success of the platelet-conjugated system in reducing post-surgical recurrence and metastasis in a mouse model [99].

Instead of the genetic engineering of platelet membranes, Zhang and co-workers [128] genetically engineered membranes of the megakaryocyte parent cell, in an attempt to overcome challenges associated with harvesting platelets. The result was the expression of the PD-1 protein on the megakaryocyte cell membrane, as well as production of PD-1 presenting platelets upon maturation of the megakaryocyte. This approach proved to be successful in eradicating residual tumour cells, post-surgery [128]. The findings of the above studies suggest that genetically engineered platelet membrane systems play an insurmountable role in the fight against metastatic cancer.

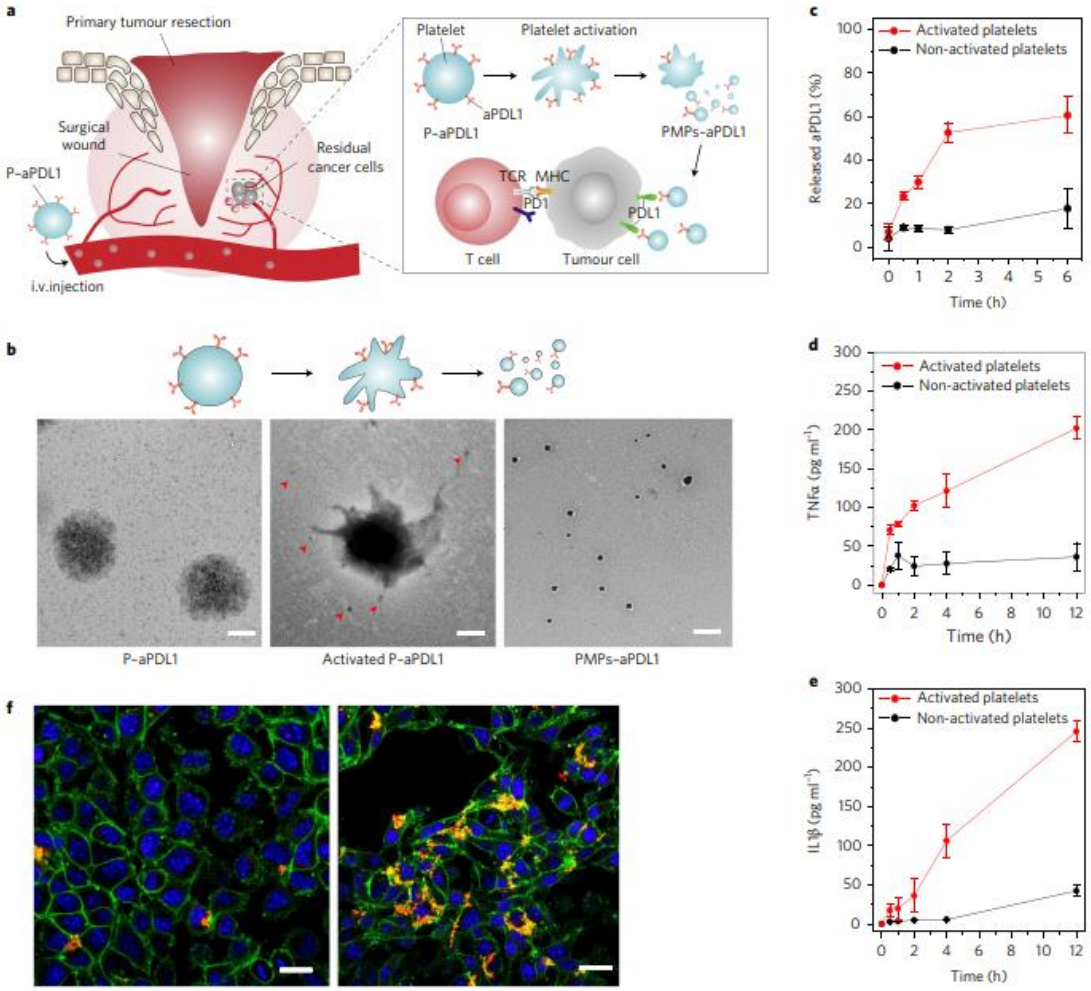


Figure 2.4: (a), Schematic illustration of the delivery of aPDL1 to the primary-tumour resection site by platelets, where TCR is T cell receptor and MHC is major histocompatibility complex. **(b)**, TEM images of P–aPDL1 before (left) and after (middle and right) activation. The red arrowheads indicate platelet-derived microparticles (PMPs) released from the platelet; a large number of these were observed via electron microscopy. Scale bars, 0.5 μm. **(c)**, Percentage of aPDL1 released from non-activated and activated platelets at different time points. **(d)**, **(e)**, Amount of TNFα and IL1β released from non-activated and activated platelets at different time points. **(f)**, Confocal immunofluorescence images of B16 cancer cells co-incubated with non-activated (left) and activated (right) P–aPDL1 in a trans well system (pore size, 1 μm). P–aPDL1 and B16 cancer cells were cultured in upper and lower compartments, respectively. Red, blue and green fluorescence indicate aPDL1, nucleus and plasma membrane, respectively. Scale bars, 20 μm. Error bars represent the standard deviation of three independent experiments (n = 3). This figure and caption have been reprinted from Wang et al [99] with permission from RightsLink: Springer Nature, Nature Biomedical Engineering. Copyright © 2017.

2.5.3 Limitations of Genetically Engineered Platelet Membrane Systems

Three limitations have been reported with regards to the use of genetically engineered platelet membrane systems for the treatment of haemophilia A; namely, the potential of an immune response, abnormal apoptosis, and genotoxicity [98].

Gene therapy involves the introduction of transfected cells into the body which may elicit an immune response in the form of elimination of the cells, or a humoral response causing inactivation of the neo-protein [129], both of which resulting in failure of the gene therapy. Two studies involving gene therapy were conducted by Chen and co-workers. The first study concluded that the transduction of 2bF8 (a vector in which the α11b promoter drives human B-domain-deleted FVIII expression) significantly increased T-regulatory cells in recipients, indicating a system of the establishment of immune tolerance following the 2bF8 platelet gene therapy [130]. The second study found that the transfusion of 2bF8 in a mouse model primed with FVIII did not elicit a primary or secondary immune response, indicating that the expression of FVIII on platelets does not activate an immune response [131]. However, the need to conduct further studies is still required in order to fully investigate this limitation.

Ectopic expression of FVIII in platelet lineage has shown to cause abnormal apoptosis of megakaryocytic cells. However, further studies into abnormal apoptosis in megakaryocytes is required, as the phenotype driving the cellular toxicity remains unclear [120, 132].

The use of a viral vector for the purpose of introducing transgene expression may result in associated genotoxicity. Current studies have shown a potential risk of genotoxicity when lentivirus-mediated gene transfer is utilized. However, more thorough studies using advanced techniques are required to determine the consequences of vector integration [133, 134]. The use of genetically engineered platelet membrane systems to treat CTCs and cancer recurrence relies completely on the clotting cascade mechanism of the body as the system is based on successful platelet activation. As a result, the use of this therapeutic modality may result in the development and occurrence of bleeding disorders [124, 99]. Further research is required in order to determine the effect of this limitation, and whether it may be wholly eliminated.

2.5.4 Future Prospects of Genetically Engineered Platelet Membrane Systems

Thus far, the majority of studies have focused primarily on the use of platelet gene therapy for the treatment of haemophilia A, with a slight interest being shown toward using the therapeutic modality for sufferers of haemophilia B, which results from a lack in the clotting factor IX (FIX). There is scope for research to be conducted on the potential use of genetically engineered platelets for the treatment of haemophilia B,

using a similar mechanism of action as that used in the studies already performed against haemophilia A. The work conducted by Shi [98] suggests a system of autologous transplantation for haemophilic as well as non-haemophilic sufferers of severe bleeding, involving the manipulation of harvested human stem cells (HSCs) derived from peripheral or cord blood, and using a platelet-specific promoter to introduce a corrected version of the gene coding for FVIII or FIX.

Approximately 90% of cancer-related mortality occurs as a result of metastasis [99]. Surgery is used as the main therapeutic intervention for solid tumours; however, the development of CTCs remains a challenge to treat [124, 99], resulting in the tremendous need for the development of effective strategies that may be used to treat metastasis and prevent post-surgical cancer recurrence. This highlights the paramount need for further research and development of genetically engineered platelets that may be used for cancers, aside from those already researched. The concept of genetically engineered platelet membranes may be investigated in respect to the other conditions which elicit a platelet response. The current status, limitations, and future potential of genetically engineered platelet membrane therapeutics have been summarized in Table 2.5.

Table 2.5: A summary of the current status, limitations, and future potential of genetically engineered platelet membrane therapeutics

Platelet System	Brief Description	Current Status	Limitations	Methods to Overcome the Limitations	Future Prospects
Genetic engineering of platelet membranes	Gene therapy involves the inception of genetic material, in order to achieve genetic alteration of the selected platelets.	Current research has focused on the use of genetic engineering of platelet membranes in order to successfully treat haemophilia A and cancer.	The use of this therapeutic modality may result in the development and occurrence of bleeding disorders.	Further, more extensive research is required to determine whether the limitation may be wholly eliminated.	There is scope for research into the use of genetically engineered platelet membranes for haemophilia B, cancers not already researched, and other conditions that elicit a platelet response.

2.6 The Use of Biomimetic Platelet-Based Systems as a Therapeutic Modality

Biomimetic systems are designed in a manner where they contain at least one biological component so that they may better resemble their source cells, thereby allowing an improvement in their interaction with other biological systems in the body, and resulting in the enhancement of their intended therapeutic efficacy [135]. Biomimetic platelet therapeutic systems are designed with the aim of mimicking and amplifying the body's natural platelet functions at the site of action, whilst ensuring the maintenance of systemic safety. Research thus far has focused on using biomimetic platelet-based systems in vascular injury, to target sites of thrombi and restenosis, and in cancer therapy.

2.6.1 Biomimetic Platelet-Based Systems in Vascular Injury

The role of platelets in primary haemostasis is well established. Therefore, the use of biomimetic platelets to target sites of vascular injury has been widely researched, with the primary aim being that the biomimetic platelets will enhance clot formation at the site of injury. Researchers have found that when creating biomimetic platelet-based systems for haemostatic purposes, it is imperative that the biomimetic system contains both the adhesion and aggregation functions of platelets. Studies that focused on designing synthetic particles only possessing either adhesion functions or aggregation functions proved to be ineffective in clinical trials. The lack of success of these studies in clinical settings has not been proven to be due to the possession of only one platelet function, however, this is believed to be the case as the importance of both adhesion and aggregation in bringing about haemostasis is well established [7].

In an attempt to develop a synthetic particle containing both platelet adhesion and platelet aggregation functions for haemostasis, Ravikumar and co-workers designed a liposome containing an active platelet clustering cyclic-RGD (cRGD) peptide, vWF-binding peptide (VBP), and collagen-binding peptide (CBP) as surface decorations. *In vitro* results showed that the liposomes were able to aggregate with activated platelets, and under flow they demonstrated the ability to adhere to vWF/collagen surfaces. This proves the success of the system in exhibiting both adhesion and aggregation functions [136]. The study conducted by Anselmo and co-workers [137] had a similar aim as that of Ravikumar mentioned above, however, Anselmo and co-workers had the additional aim of creating synthetic particles that mimic the discoid shape and

flexibility of natural platelets. In order to achieve this, the team designed platelet-like nanoparticles (PLNs) in the form of flexible capsules, by adopting a layer-by-layer approach onto spherical polystyrene nanoparticles. *In vitro* studies showed that the PLNs had a greater ability to bind to surfaces than did synthetic particles containing spherical or discoid shapes. Just like the study conducted by Ravikumar and co-workers, the results of this study proved that the PLNs exhibited platelet adhesive and aggregatory properties under flow conditions. *In vivo* analysis performed in a mouse model showed an accumulation of the PLNs at the site of injury in the mouse tail, and a reduction in the bleeding time by approximately 65%. These results exhibit great promise for using PLNs as a synthetic alternative to natural platelets [137].

The study conducted by Brown and co-workers [138] also aimed to create synthetic platelet-like particles (PLPs) able to enhance clotting. However, the research team had the additional aim of incorporating platelet behaviours into the particles, like clot contraction, which other studies did not include. The combination of microgel particles and recognition motifs was used to create the PLPs. The microgel particles endowed the PLPs with the ability to easily undergo deformation, and the recognition motifs allowed the PLPs to exhibit clot specificity. *In vitro* analysis showed that under flow conditions the PLPs were able to enhance clotting, and it was also observed that the PLPs possessed clot contraction abilities, as they demonstrated active collapsing of fibrin networks. *In vivo* analysis in a traumatic injury model showed that the PLPs were able to promote haemostasis, thereby decreasing the bleeding time [138].

Hickman and co-workers [139] aimed to solve the problem of uncontrolled, non-compressible traumatic haemorrhage within conditions where access to platelet transfusions is highly limited, by creating the lipid-based SynthoPlate; an IV synthetic platelet nano-structure able to produce haemostatic functions at the site of bleeding. Figure 2.5 is a schematic representation of the SynthoPlate structure and mechanism. The nanoparticles underwent decoration with VBP, CBP and fibrinogen mimetic peptide (FMP) motifs in order to allow for interactions with vWF, collagen, and GPIIb-IIIa, in an attempt to amplify haemostatic functions at the site of injury. The research team began by performing *in vitro* characterization tests on the post-sterilization biofunctionality and storage stability of the nanoconstructs, thereafter, *in vivo* testing within a pig model with induced femoral artery traumatic haemorrhage was performed. The *in vivo* results showed a stabilized blood pressure, and a reduction in blood loss,

leading to a significant improvement in survival, proving that SynthoPlate is a promising modality for the emergency treatment of traumatic haemorrhage if administered within 1-2 hours of injury [139].

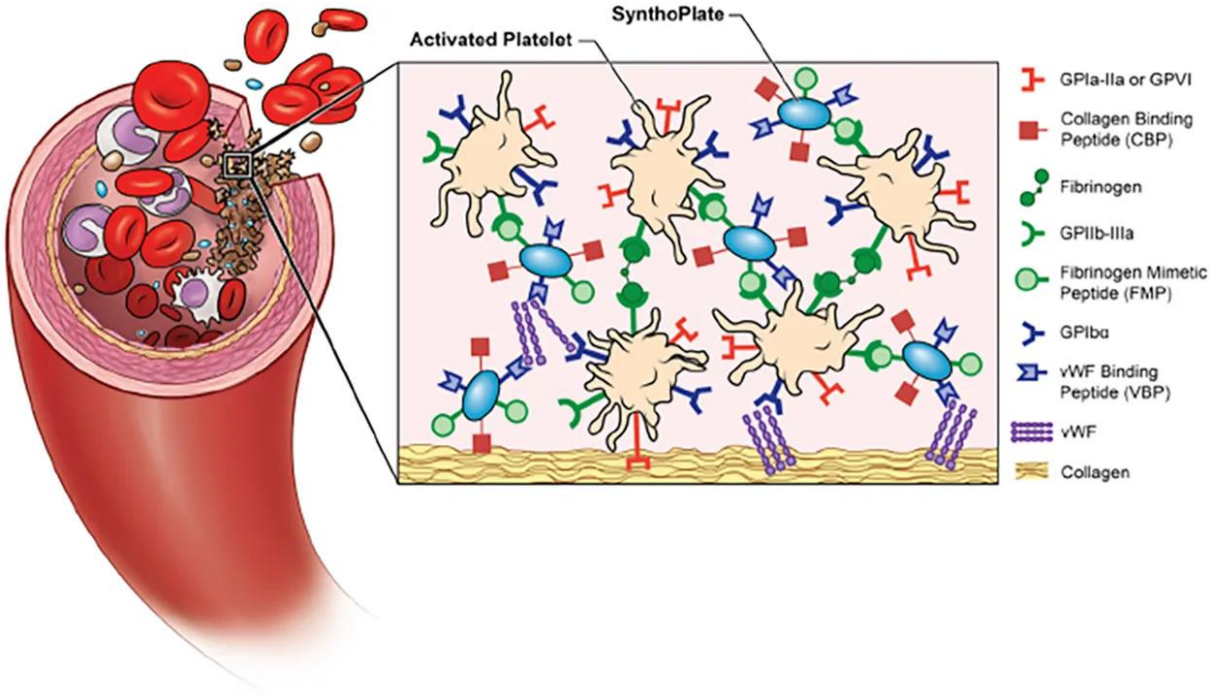


Figure 2.5: Schematic representation of SynthoPlate design and mechanism, showing nanoconstructs heteromutlivalently decorated with VBP, CBP and FMP motifs to render platelet-mimetic interactions with vWF, collagen and active platelet integrin GPIIb-IIIa respectively, and thus amplify platelet-mediated primary haemostatic mechanisms at the injury site. This figure and caption have been reprinted from Hickman et al [139], under a Creative Commons Attribution 4.0 International License.

The research conducted by Bertram's group [140] had the same aim as that of Hickman and co-workers, however they proceeded by creating arginylglycylaspartic acid (Arg-Gly-Asp) based functionalized nanoparticles that they compared with the standard clinical treatment for uncontrolled bleeding, which is recombinant factor VIIa. The Arg-Gly-Asp nanoparticles were tested in major trauma within a rat model. The study showed that the nanoparticles halved bleeding time, the system was cleared from the body within one day, and no complications were observed within one week of the IV infusion in the rats. This proves that the Arg-Gly-Asp nanoparticles may be a beneficial alternative to the treatment of traumatic haemorrhage [140].

In another study conducted by Ravikumar and co-workers [141], the team aimed to mimic the dual adhesion ability of platelets to both vWF and collagen, by creating nanoliposomes containing vWF-binding and collagen-binding ligand motifs as a

surface decoration. *In vitro* analysis showed that under flow conditions these synthetic particles demonstrated shear-dependent adherence to vWF surfaces and shear-independent adherence to collagen surfaces, as well as enhanced adherence to a mixed vWF/collagen surface. The results of this study proved that the system was successful, however, the success of this dual adhesion mimetic system is dependent on the motifs not spatially interfering with each other [141].

2.6.2 Biomimetic Platelet-Based Systems Targeting Sites of Thrombi and Restenosis

Platelets have a natural affinity to sites of thrombi and restenosis, due to their pivotal role in the occurrence of these phenomena [7]. As a result, research teams have used this function of platelets to design synthetic particles to target sites of thrombi and restenosis, in an attempt to investigate whether this approach to therapy will improve treatment outcomes. Doshi and co-workers [142] developed synthetic platelet particles that possess similar geometric features as that of natural platelets, in order to target sites of thrombi. Discoid shaped particles were created by coating polymeric microparticles with proteins using the layer-by-layer approach. Following the coating, which helped to endow the synthetic particles with an actin cytoskeleton, the polymeric core was removed. The results of the study showed that these particles were able to mimic the ability of natural platelets to target sites of thrombi in blood flow conditions [142].

In the research conducted by Pawlowski and co-workers [143], platelet-inspired nanovesicles were developed to encapsulate a thrombolytic drug in order to allow for platelet-rich thrombus site-specific drug release triggered by thrombus-related enzymes, thereby decreasing systemic side effects. *In vivo* studies performed in mice showed that the nanovesicles were successful in causing site-specific fibrinolysis of a thrombus in the carotid artery, without causing any effects to the systemic haemostatic functions. The results of this research prove that this system of drug delivery may be applied to other thrombus-related conditions [143].

Hu and co-workers [144] prepared polymeric nanoparticles coated by the plasma membrane of human platelets, resulting in functionalized nanoparticles that possess immunomodulatory and adhesion antigens. Following experimentation, it was proven that the coated nanoparticles reigned superior to the uncoated particles in terms of

immunocompatibilty, adhesion to damaged vasculature in human and rodent models, and enhanced adhesion to pathogens. The platelet-mimetic system was used with great success for delivering docetaxel and vancomycin in a coronary restenosis rat and bacterial infection mouse model, respectively. This method of nanoparticle functionalization has been translated into use within clinical settings [144].

2.6.3 Biomimetic Platelet-Based Systems in Cancer Therapy

Circulatory cell uses to deliver nanoparticles to tumour sites has gained significant attention due to the many advantages associated with this system of drug delivery, the greatest being the extended period in which the drug remains within blood circulation [145]. A greater number of studies have been performed on the use of biomimetic platelet-based systems as part of cancer therapy, in the hope of achieving a targeted delivery system of drug-loaded nanoparticles.

Li and co-workers [146] developed a Trojan-horse inspired system in order to neutralize CTCs, thereby reducing metastasis. The study involved the development and functionalization of synthetic silica particles with activated platelet membranes that had been conjugated with TRAIL. The system proved to be effective in neutralizing CTCs, and thereby decreasing lung metastases in a breast cancer model performed in a mouse [146]. In the research conducted by Pan and co-workers [147], platelet-inspired heteromultivalent interactions were used to allow for nanoliposomes, loaded with DOX, to bind effectively to metastatic breast cancer cells. Two cell lines were used in order to determine whether the system had a certain specificity, MDA-MB-231 - high-metastatic human breast cancer cells, and MCF-7 - low-metastatic human breast cancer cells. The findings demonstrated the success of this delivery system in the active and specific binding of the nanoliposomes to the high-metastatic cells, proving the potential efficacy of this strategy to treat metastasis-specific cancer [147].

The nature of the relationship between platelets and CTCs is the main principle underlying the research conducted by Hu and co-workers [148], who developed an IV system that is able to deliver both TRAIL and DOX to primary tumours as well as CTCs by coating drug-loaded nanogels with platelet membranes. *In vivo* tests performed in an animal model demonstrated a decrease in breast cancer related lung metastases, and significant primary tumour shrinkage, proving the promising effects of this system [148]. Current treatment strategies designed to treat multiple myeloma (MM) may result

in the emergence of thrombi, therefore, in another study performed by Hu and co-workers [149] the team designed a promising platelet membrane-coated nanoparticle with tissue plasminogen activator (tPA) and alendronate, able to target the bone microenvironment, and thereby treat the MM as well as any emerging thrombi. The system was tested in a mouse model, and exhibited survival prolongation of the MM-bearing mice, and a decrease in lung thrombi complications [149]. Hu and co-workers [150] also developed an enhanced delivery strategy based on signal amplification and the sequential transportation of two nanocarriers; the first being a nanogel 'signal transmission' drug carrier capable of inducing inflammation by the specific targeting of tumour blood vessels, and the second being an 'execution' biomimetic carrier capable of signal identification and subsequent accumulation at the site of the tumour, thereby enhancing drug bioavailability. This dual system proved to be effective in a mouse model, with no signs of inducing systemic toxicology [150].

2.6.4 Limitations of Biomimetic Platelet-Based Systems

Despite the advantages associated with the use of biomimetic platelet-based systems, a number of obstacles exist, limiting the translation of this therapeutic modality into clinical practice. The system relies heavily on the availability of platelet membranes, requiring large scale production of the membranes and availability of the technology and monetary capital required in the process. Due to the prevalence of disease outbreaks, as well as natural and military disasters, access to donated blood is limited. The infrastructure required to test that donated blood is safe from infections is expensive, thereby further decreasing supply to donated blood especially in lower-income countries [151].

Another obstacle attached to this system is ensuring that the synthetic structures created duplicate the complex platelet make-up with great accuracy, as the entire system is dependent on the body recognizing the vehicles as natural platelets in order to avoid the occurrence of immunogenicity. As platelets are an integral component of the clotting cascade, the greatest concern related to the biomimetic therapeutic system is its effect on coagulation, and the possibility of bleeding complications occurring. Due to the complexity and heterogeneity associated with cancer biology, there may be a variety of receptor-ligand interactions available for platelet adhesion to the cancer cells, therefore, a greater understanding of the exact interaction between platelets and cancer cells is required in order for the production of platelet-mimicking carriers

capable of precision therapy to occur [152]. The functionalization of nanoparticles affects nanoparticle effectiveness in physiological systems [146]. Current paradigms of nanoparticle functionalization appear to be inadequate in mimicking natural interfaces and preventing exposure to foreign matter [153].

2.6.5 Future Prospects of Biomimetic Platelet-Based Systems

Given the demonstrated capabilities of biomimetic platelet-based systems as a therapeutic modality, a range of opportunities are available to be explored. Future studies aimed at determining a mechanism of the systems interaction to the target site, without influencing haemostatic functions, may be conducted. With the hope of producing structures that resemble platelets more closely, Li and co-workers [146] suggests the future use of platelet-like, disc shaped particles with great deformability, as the nanocarriers coated by platelet membranes, the plateloid structure, has shown to accumulate within solid tumours with an enhanced efficacy compared to alternatively structured particles. Pan and co-workers [147] suggest future studies be conducted on the alteration of the nature of the delivery particle, as well as the effect of optimization of the ligand chosen for cell binding, in order to enhance the effects of the system. Another recommendation made by the research team is the exploration of the use of the platelet-based biomimetic system across different cell lines, to determine whether the system is applicable to a wider variety of conditions.

The positive results observed in the study performed by Hu and co-workers [149] offer prospects for the potential development of a novel, stimuli responsive paradigm of treatment for alternative conditions, based on alterations of physiological signals. The study conducted by Kona and co-workers [154] proved that the development of a biodegradable nanoparticle, able to mimic platelet adhesion functions to aid in the treatment of cardiovascular diseases, is possible. However, there hasn’t been many studies focusing on the aspect of biodegradability. This is a very important element to consider when designing a synthetic delivery system as the synthetic particles need to be broken down and eliminated from the body in a safe and effective manner in order to prevent an immune response. Therefore, I believe that there is ample room available for research to be conducted on a biodegradable platelet mimetic particle, in order to contribute to the field of platelet-inspired therapeutics. The current status, limitations, and future potential of biomimetic platelet-based therapeutics have been summarized in Table 2.6.

Table 2.6: A summary of the current status, limitations, and future potential of biomimetic platelet-based therapeutics

Platelet System	Brief Description	Current Status	Limitations	Methods to Overcome the Limitations	Future Prospects
Biomimetic platelet-based systems	Biomimetic platelet-based systems involve combining a man-made system with the body's natural platelets in order to amplify platelet functions at the site of action.	Biomimetic platelet-based systems have proven to be effective in the emergency treatment of traumatic haemorrhage, in targeting sites of thrombi and restenosis, and in the neutralisation of circulating tumour cells (CTCs).	The synthetic structures created may not duplicate the complex platelet make-up with great accuracy. This will pose challenges regarding immunogenicity.	The characterisation tests performed on the synthetic structures need to be extremely sensitive in picking up any deviations from the natural platelet make-up, so that researchers may make the necessary alterations.	The development and use of nanocarriers that have a plateloid structure and possess great deformability, should be explored.

2.7 The Use of Hybridized Platelet Membrane Systems as a Therapeutic Modality

Platelet membranes have shown great potential in the field of hybridized delivery systems, which aim to synergize therapeutic outcomes by combining two systems in order to bring together the benefits of the varying structural components of both of the systems [155].

2.7.1 Hybridized Platelet Membrane Systems in Haemophilia A

As mentioned in Section 2.5.1, the current treatment for haemophilia A can cause complications to the patient, resulting in unsuccessful treatment outcomes. In an attempt to overcome these complications, Hansen and co-workers [156] looked into using hybridized platelet membrane systems in the treatment of haemophilia A. During the formation of a blood clot platelets are the first cells in the body to elicit a response, initiating the genesis of the clotting cascade [157]. This function of platelets was used as the basis of the research conducted by Hansen and co-workers, whose aim was to develop a hybridized, cell-mediated system that achieves controlled and targeted drug delivery of a haemostatic agent for sufferers of haemophilia A that have developed

inhibitory antifactor VIII antibodies, by using the platelet contractile force of a patient in the delivery of the drug, thereby augmenting clot formation during haemorrhage, or during the occurrence of bleeding disorders. Factor VIII was encapsulated and shielded within polyelectrolyte multilayer (PEM) capsules, which were biochemically and mechanically adapted to allow for platelet adhesion and subsequent capsule rupture following platelet activation and contraction. These PEM capsules were able to hybridize with the platelets of the patient, following IV administration. The study demonstrated that the PEM loaded capsules caused an increase in fibrin formation by a factor of 3.8 and an induction of clot formation 18 minutes faster, compared to the delivery of an equal concentration of factor VIII administered via standard systemic delivery. Figure 2.6 is a schematic representation of this newly developed delivery system, making use of the patients own platelets in order to achieve targeted drug delivery [156].

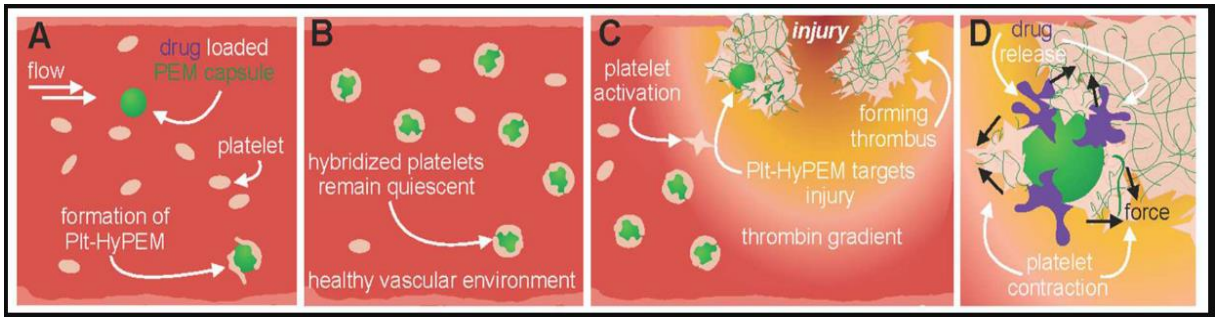


Figure 2.6: Schematic of "smart" drug delivery paradigm leveraging the patient's own platelets for targeted delivery and controlled release. **(A)** Polyelectrolyte multilayer (PEM) capsules loaded with a biotherapeutic payload are intravenously administered to the patient, whereupon the patient's own platelets adhere to the PEM capsule via surface fibrinogen (green) during circulation to form a platelet-hybridized microcapsule (plt–HyPEM). **(B)** The patient's platelets that comprise the hybrid microcapsule remain quiescent during circulation. Only when the hybrid microcapsules enter an area undergoing active clotting **(C)** do the hybridized platelets activate upon exposure to thrombin and target the platelet–HyPEM to the site of injury by adhering to exposed collagen at the site of vascular injury and/or integrating into the forming fibrin network of the nascent clot. **(D)** Finally, the physical force exerted by platelet-mediated contraction within the platelet–HyPEM and nearby platelets physically ruptures the platelet–HyPEM capsule, enabling delivery of a high dose of the encapsulated biotherapeutic (purple) to the target site. This figure and caption have been reprinted from Hansen et al [156].

2.7.2 Hybridized Platelet Membrane Systems in Cancer Therapy

As previously mentioned, using genetically engineered platelet membranes to express anti-PD-L1 or anti-PD1 antibodies has proven to be successful, as a result there is scope for research to be undertaken on how to further enhance these genetically engineered platelet systems to bring about a greater therapeutic effect. An example of

this enhancement is found in the research conducted by Hu and co-workers [158], which was successful in designing a conjugated system consisting of hematopoietic stem cells conjugated to platelets via a click reaction, followed by platelet membrane decoration with anti-PD1 antibodies, for the treatment of leukaemia. The system was systemically delivered, via IV injection, into mice infected with leukaemia. The results of the research showed that the system increased mice survival time by causing a suppression in the growth and recurrence of the leukaemia cells. This system of drug delivery took advantage of the inherent ability of both the hematopoietic stem cells and the platelets, as the success of this system was based on the ability of the hematopoietic stem cells to deliver the conjugated system to the bone marrow, which is the leukaemia site, and the ability of the platelets to undergo *in situ* activation at the leukaemia site, thereby causing the release of the anti-PD1 antibodies [158].

2.7.3 Hybridized Platelet Membrane Systems in Enhancing Nanoparticle Coating

The advent of cell-membrane-coated nanoparticles has had a significant impact on the enhancement of nanoparticle functionality in terms of imaging, treatment applications, retention of properties of the source cell, and biological compatibility. The study conducted by Dehaini and co-workers [159] aimed to create a novel, hybridized, biological, nanoparticle coating, consisting of red blood cell (RBC) membrane material fused with platelet membrane material in an attempt to further enhance the functionality of the coated nanoparticles. The group was successful in fabricating a nanocarrier, which was extensively characterized in order to prove that the hybrid-coated nanoparticles possess properties of both RBCs and platelets. These hybrid-coated nanoparticles also revealed a long circulation time, as well as suitability for *in vivo* study generation and exploration. The results of this study provide a promising natural alternative to synthetic nanoparticle coating strategies [159].

2.7.4 Limitations of Hybridized Platelet Membrane Systems

Hybridized platelet membrane systems are an extremely novel means of drug delivery, therefore, there is a lack of sufficient research and data available to make predictions regarding the efficacy of the system within a clinical setting. In the study conducted by Hansen and co-workers [156] the fabrication of the PEM capsules was extremely time-consuming, in addition to requiring a large amount of material resources. In order for this system to be successful in a commercial setting, a manufacturing process that provides the same mechanical and biochemical properties to the capsules, all-the-

while reducing material consumption, human labour, and batch-to-batch variation, needs to be developed and implemented [156, 159]. In the study conducted by Hu and co-workers, it was not determined whether the activation of the platelet carriers caused any adverse effects due to the release of pro-inflammatory molecules, which get released upon platelet activation. Additionally, the study did not investigate whether any systemic T-cell activation occurred while the conjugated system was circulating in the blood stream, and as a result it was not investigated whether any adverse effects occurred due to any potential T-cell activation. Further studies would need to be performed to investigate the occurrence of any potential adverse events before this system may be used in human clinical trials [158].

2.7.5 Future Prospects of Hybridized Platelet Membrane Systems

The development of hybridized platelet membrane systems represents a shift in the paradigm towards novel and smart drug carriers, which may be applied to other conditions that elicit a platelet response, such as cancer, thrombotic diseases, and innate immunity [156]. The success observed in the study conducted by Hu and co-workers indicates that adopting a conjugated cell-mediated drug-delivery system could be useful in the treatment of other diseases that elicit a similar biological response, in addition to leukaemia. Additionally, there is scope for research to be conducted on the use of other bioparticulates in a similar conjugated drug-delivery system [158]. The approach of using hybridized membranes may be used across a number of fields - including detoxification and immune modulation [160]. Hybridized membranes, with varying hybrid functionalities, providing a coating around nanoparticles may be used in situations where biocompatibility and imaging needs to be enhanced. Just as RBC membranes were able to fuse effectively with platelet membranes, the experimentation of other cell membrane combinations may be explored, which may result in a new method of natural multi-membrane coating of nanoparticles better able to perform within the body compared to synthetic and single membrane coatings [152]. The current status, limitations, and future potential of hybridized platelet membrane therapeutics have been summarized in Table 2.7.

Table 2.7: A summary of the current status, limitations, and future potential of hybridized platelet membrane therapeutics

Platelet System	Brief Description	Current Status	Limitations	Methods to Overcome the Limitations	Future Prospects
Hybridised platelet-membrane systems	The development of a system with the aim to synergize therapeutic outcomes by combining structural components of platelets and the additional chosen system.	Currently, this system has proven to be useful in the treatment of haemophilia A, cancer therapy, and in the further enhancement of the functionality of coated nanoparticles.	There is a lack of sufficient research and data to make adequate predictions regarding the efficacy of this system in a clinical setting.	Well-designed, comprehensive experiments on this system need to be developed and undertaken.	This approach to treatment may be further researched for its use in detoxification, immune modulation, and situations where biocompatibility and imaging needs to be enhanced.

2.8 Summary

From the six platelet-inspired therapeutic systems that have been introduced in this review, readers may be interested in knowing how to choose which system they should employ based on their needs. The use of PRP and PL as therapeutic modalities should be investigated for diseases that may respond favourably to an influx of platelet-derived growth factors, and/or other platelet bioactive molecules. This includes diseases that require tissue healing, growth, rejuvenation, or regeneration, in order for them to be successfully treated [17, 18, 68]. Researchers may prefer using PL instead of PRP as PL is acellular, decreasing the likelihood of an immune response being elicited [68]; however, PRP has been more widely researched. Using PL as an alternative to FBS for the expansion of MSCs may be investigated, as PL use prevents the problems associated with FBS use [75], as mentioned in Section 2.3.1.

The use of natural platelets, genetically engineered platelet membranes, biomimetic platelet systems, and hybridized platelet membrane systems may all be investigated as therapeutic modalities for conditions that would normally elicit a platelet response in the body [91, 149, 156]. Natural platelets acting as drug delivery vehicles would be the ideal carrier system, as immunogenicity would not be likely to occur [88]. However, the short lifespan of platelets outside the body and the special conditions in which the

platelets would need to be stored may make this delivery system difficult to successfully implement [161]. In this instance biomimetic platelet systems may be a better alternative as they would be easier to work with in a laboratory setting, however, it may be difficult to successfully mimic all of the functions of natural platelets, and immunogenicity may occur due to the synthetic material [137].

In my opinion, the best use for genetically engineered platelet membranes, based on the available research, is in cancer treatment for the purpose of immune checkpoint inhibition, where anti-PD-L1 or anti-PD-1 is engineered onto the platelet membrane and delivered to the tumour site [158]. Hybridized platelet membrane systems should be investigated as a therapeutic modality when the aim is to design a conjugated system that will bring together the positive therapeutic effects of another component together with that of the platelet. Figure 2.7 provides a summarized illustration of the different mechanisms of platelet modification in order to achieve the platelet form used in the different therapeutic modalities, as well as detailing the various conditions that have been shown to respond favourably to each of the six modalities.

Whole blood

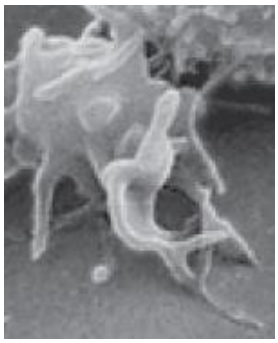
Centrifugation

PRP

- Effective in the treatment of conditions within:
  - Gynaecology
  - Orthopaedics
  - Dermatology
  - Neuropathy
  - Musculoskeletal system

Further centrifugation of PRP

Isolated platelets



Freezing and thawing, or ultrasonic treatment → PL

- Supplement for FBS
- Enhances osteogenic differentiation of MSCs
- Effective in the treatment of lumbar radicular pain
- Effective in the treatment of OA

Encapsulation of a drug → Natural platelet delivery vehicles

- Used to encapsulate DOX for the treatment of lymphoma

Inception of genetic material → Genetically engineered platelet membranes

- Effective in the treatment of:
  - Haemophilia A
  - Cancer

Addition of synthetic components → Biomimetic platelet-based systems

- Effective in the treatment of:
  - Emergency cases of traumatic haemorrhage
  - Neutralisation of CTCs

Introduction of structural components from another system → Hybridised platelet-membrane systems

- Useful in the treatment of haemophilia A
- Further enhances the functionality of coated nanoparticles

Figure 2.7: A summarized illustration regarding the different mechanisms of platelet modification in order to achieve the platelet form used in the different therapeutic modalities, as well as detailing the various conditions that have been shown to respond favourably to each of the six modalities.

2.9 Concluding Remarks

Platelet-inspired therapeutic systems are available in an array of forms, and have proven to be promising mechanisms of treatment in a wide range of conditions, due to the natural pathophysiological characteristics of platelets allowing these systems to be applied to any disorder that elicits a platelet response, all-the-while ensuring biocompatibility. However, a number of key obstacles have been observed with each therapeutic modality, limiting the use of these systems within clinical settings. Due to the fact that platelet-inspired therapeutics are still a relatively new field, the observed obstacles have not been thoroughly researched, presenting opportunities for experts to look into methods in which these obstacles may be overcome or significantly decreased. The encouraging results demonstrated has given rise to a number of hypothesized future prospects for each modality, providing the potential for knowledge generation and exploration into each of the future prospects mentioned. Thus, platelet-inspired therapeutics are a crucial addition to the field of research and therapeutic science.

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CHAPTER 3

HYPOTHESIS: CAN DRUG-LOADED PLATELETS BE USED AS DELIVERY VEHICLES FOR BLOOD-BRAIN BARRIER PENETRATION?

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Kola SM, Kumar P, Choonara YE, du Toit LC, Pillay V. Hypothesis: Can drug-loaded platelets be used as delivery vehicles for blood-brain barrier penetration? Med Hypotheses. 2019; 125:75-78.

3.1 Introduction

The literature review presented in the previous chapter revealed that platelet-inspired therapeutics may be effective in the treatment of conditions that normally elicit a platelet response in the body. However, the available literature thus far has not mentioned the investigation of platelet-inspired therapeutics for the penetration of the blood-brain barrier (BBB) to treat neurological conditions that elicit a platelet response. This had been identified as a gap in the research that warrants further investigation, as platelets are an important component of blood that are able to cross the BBB easily during times of vascular disturbance [1]. Therefore, they may prove to be an effective alternate to the current methods of treatment of neurovascular disorders.

The brain is the control centre of the body and therefore requires efficient protection in the form of the BBB. Endothelial cells lining the cerebral vasculature form the BBB [2] that serves to separate the extracellular fluid of the brain from the circulating blood. The high selectivity as well as sensitivity of this permeability barrier makes drug delivery to the brain tissue difficult to achieve [3], and as a result the clinical failure of many potentially effective central nervous system (CNS) therapeutics are often associated with shortcomings in their methodology of drug delivery, and not as a result of a lack in the drugs pharmacological efficacy [4].

Majority of the existing therapeutics, which are available on the market, are rendered ineffective in the treatment of CNS diseases, due to the fact that they cannot be

delivered and sustained within the brain effectively. This has led to an increase in the need for invasive procedures to treat these diseases, or alternatively, an increase in the mortality and morbidity from fatal and debilitating CNS conditions respectively [4]. This is evident from the following statistics: 6.5 million deaths occurred due to strokes in 2013, making strokes the second highest cause of death, globally [5]. Brain tumours are the most common cancer, and the leading cause of cancer-related deaths in children aged 0-14 years [6], while brain and CNS tumours are the third most common causes of cancer related deaths among the age group of 15-39 years [6].

Vascular disease refers to a class of disorders affecting the blood vessels of the body that results in a range of disorders, which may be severe and even fatal [7]. Gliomas and ischaemic strokes are two examples of neurovascular disorders [8]. Glioblastoma multiforme is the most aggressive and the most common type of primary brain tumours [9] that have gained attention due to their complex character which gives them the ability to evade various therapies. The majority of glioblastoma multiforme sufferers die of the disease within a year of diagnosis, and almost none of the patients experience long term survival [10]. Due to the infiltrating nature of these gliomas, the current treatment involves the surgical resection of as much of the tumour as is considered safe, followed by treating the remainder of the tumour via chemotherapy and radiotherapy [11]. However, mortality levels still remain high due to the presence of the BBB, as well as the resistance to radiotherapy of the tumour cells present in areas of hypoxia [12]. As a result, there is a great need for more effective therapeutic interventions to treat glioblastoma multiforme.

The grim statistics associated with ischaemic strokes coupled with the drawbacks limiting the use of the current conventional ischaemic stroke treatments, outlined in chapter 1, demonstrate the importance of the development of safer and more effective therapeutic interventions for ischaemic strokes.

3.2 Hypothesis

This hypothesis proposes the use of platelets as drug delivery vehicles, able to penetrate the BBB, for use in the treatment of glioblastoma multiforme, as well as ischaemic stroke**.** The primary aim of the platelet system is to achieve delivery of the necessary drugs directly to the sites of action. The proposed delivery system, which

may be administered via intravenous (IV) injection, takes advantage of the innate function of platelet activation in order to deliver drugs to the affected areas of the brain. The formulation can be prepared by encapsulating drugs into the platelet canalicular system. The success of this system may result in the achievement of targeted drug delivery, a decrease in systemic side effects, and a great reduction in the time to the pharmacological onset of action of the drug**.** The ultimate goal of the hypothesised treatment paradigm is a decline in the morbidity and mortality associated with the abovementioned neurovascular conditions.

3.3 Evaluation of the Hypothesis

3.3.1 Platelet Drug Delivery Vehicles

Although not much research is available on natural platelets as drug carriers, the available research has proven that platelets act as drug delivery vehicles by encapsulating drugs through their canalicular system, and when platelet activation occurs granular matter as well as the encapsulated drug are released from the platelet in a controlled manner. Therefore, the success of this drug delivery system is dependent on platelet aggregation and subsequent activation. These platelet vehicles may be an ideal therapeutic modality as they possess good biocompatibility, biodegradation does not pose a challenge, they are simple to produce, and they ensure immune evasion [13, 14].

When platelets encapsulate drugs, they cloak and protect the drug in a manner where the body does not detect it as foreign matter, and therefore does not cause increased clearance of these platelets. This leads to a prolonged circulation in the blood, thereby increasing the concentration of the drug in the body and decreasing the clearance rate of the drug from the body. Platelet carriers have the ability to be manipulated so that they only target the desired cells, thereby having a minimal effect on surrounding healthy tissue. This causes the incidence of systemic side effects to be greatly reduced. The use of natural platelets acting as drug delivery vehicles may also mitigate the potential problem of biocompatibility associated with biomimetic platelet-based systems [13, 14].

Drug entrapment by platelets occurs during exposure of the platelet to the drug, and this entrapment occurs at a relatively high concentration [15]. The innate function of

platelets is to target vascular disorders, such as, but not limited to, cancer, thrombosis, haemorrhage and strokes [8], leading to an increasing interest in using platelets, as a drug delivery vehicle for the purpose of treating these disorders [16].

3.3.2 The Application of Platelets as Delivery Vehicles for the Treatment of Glioblastoma Multiforme

Cancer cells are able to interact with platelets when the cells enter into blood vessels, with the aim of causing the release of platelet factors that will increase, as well as improve cancer cell vascular permeability, and promote motility and survival of the cells, thereby aiding cancer cell growth and metastasis [17]. The process whereby tumour cells and platelets interact is called tumour cell-induced platelet aggregation (TCIPA) [18], which involves the generation of receptor cells on the surfaces of the tumour cells that allow for platelet aggregation [17]. As has been mentioned before, platelet aggregation and subsequent activation is necessary for the effective functioning of the platelet drug delivery system. Following the process of TCIPA, platelet cells are activated and subsequently release factors such as factor VIIa, serine proteases, factor Xa, fibrinogen, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and thrombospondin [17]. As these factors are released from the platelets, the encapsulated drug within the platelet carrier is released as well [13].

Studies conducted by Xu and co-workers [13], and Sarkar and co-workers [14] proved that the chemotherapeutic drug, doxorubicin (DOX), was effectively encapsulated into natural platelets. Their findings proved that these drug-loaded platelets still maintained their morphology and integrity and were therefore still detected by the body as natural, native platelets. I postulate that this finding ensures that these DOX-loaded platelets would still be able to cross the BBB without being detected as foreign matter. The studies also found that the drug-loaded platelets were successful in significantly inhibiting tumour growth, all the while ensuring immune system evasion and biocompatibility [13, 14]. DOX has been proven to be an effective drug against glioblastoma multiforme [19], which indicates that DOX-loaded platelets can be used as a post-surgical treatment modality allowing for targeted drug release, directly to the tumour sites unable to be surgically resected. Consequently, decreasing the harmful systemic side effects of the chemotherapeutic drug, which is problematic for the majority of patients, and ensuring a better chance of positive treatment outcomes.

3.3.3 The Application of Platelets as Delivery Vehicles for the Treatment of Ischaemic Stroke

In the process of genesis of an ischaemic stroke, platelets aggregate to form a blood clot and send out signalling molecules which act to initiate subsequent platelet arrival to the site of the clot [20]. Following platelet arrival to the site, as part of the clotting cascade process, the platelets undergo aggregation as well as activation to enhance clot formation [21]. Figure 3.1 shows the three stages that need to occur in order for clot formation and haemostasis to be achieved [22].

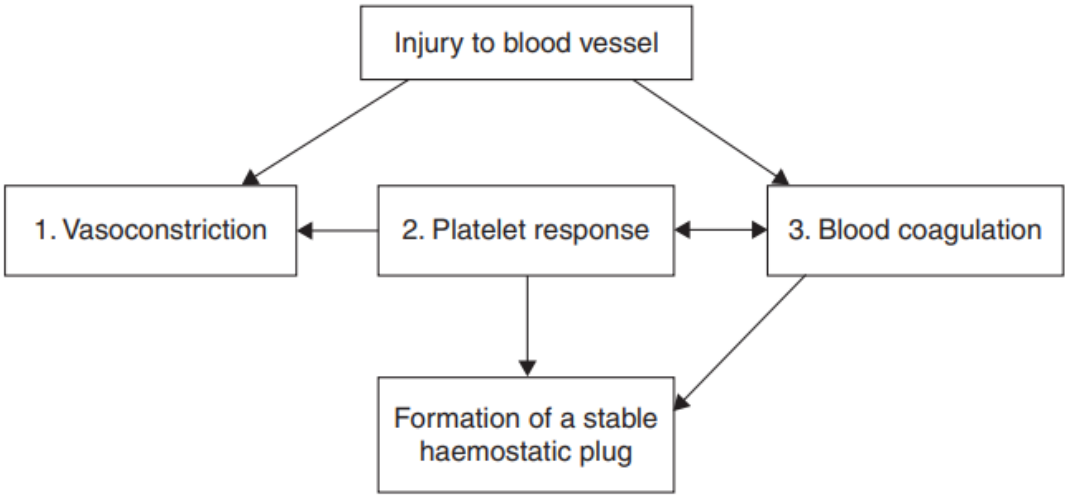

Figure 3.1: The three stages involved in bringing about clot formation and haemostasis. Reprinted from Gajjar et al [22], with permission from Elsevier. Copyright 2013.

Figure 3.2 depicts the role of platelets in haemostasis and the clotting cascade [22]. I hypothesize using platelets as drug carriers for an effective and appropriate anticoagulant drug. The platelet drug carriers will encapsulate and cloak the drug and transport it through the BBB directly to the ischaemic site. When the platelet becomes activated, by being exposed to the clot, it will release its granular contents as well as the drug, in a similar manner as has been described above. The patient will not have to undergo any medical procedure, the treatment time will be much shorter, the incidence of positive treatment outcomes will be greater, and there will be less complications due to drug therapy, thereby increasing the likelihood of successful treatment outcomes.

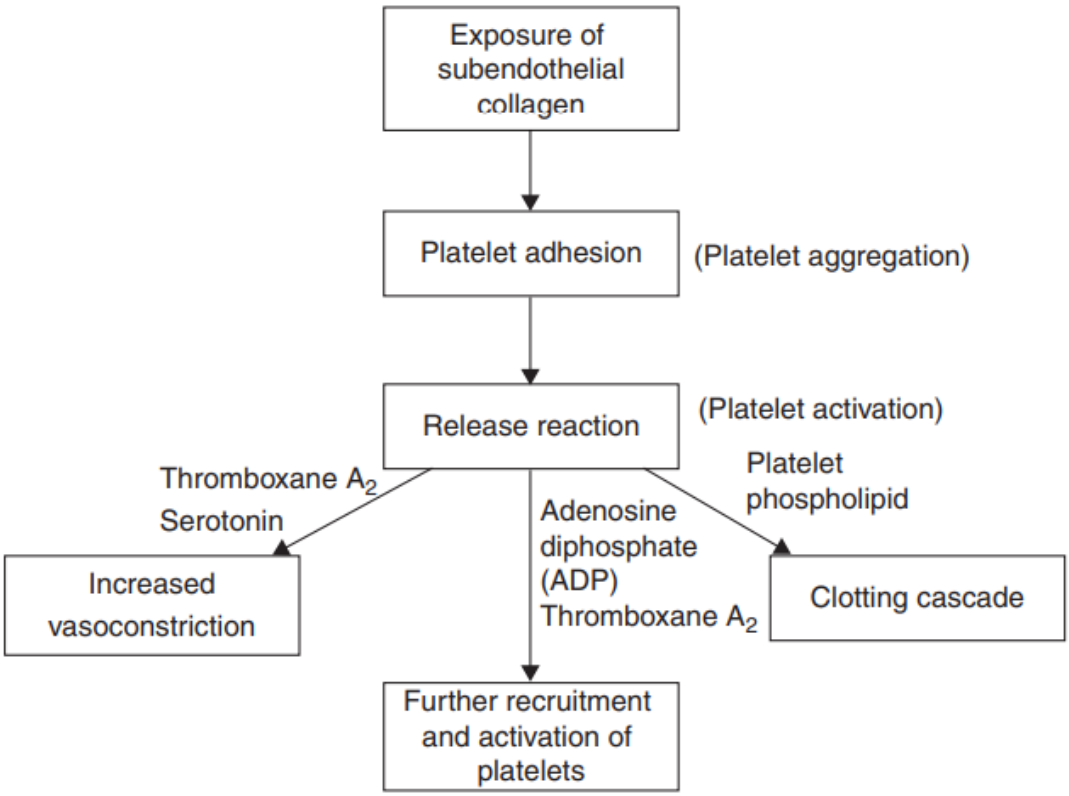

Figure 3.2: The role of platelets in haemostasis and the clotting cascade. Reprinted from Gajjar et al [22], with permission from Elsevier. Copyright 2013.

3.3.4 The Stability of Platelet Drug Delivery Vehicles

In the study conducted by Xu and co-workers [13] the stability and aggregation behaviour of the DOX-loaded platelets were compared with that of natural, native platelets, by tests performed at different time points. The results indicated that the loss of functionality of the DOX-loaded platelets and the native platelets were similar and occurred in a time-dependent manner. This finding proves the stability of the DOX-loaded platelet system. I have used these results to postulate the stability of the platelet drug delivery system within the context of crossing the BBB.

3.4 Discussion on the Significance of the Platelet Drug Delivery System

Using platelets as drug delivery vehicles that carry medication across the BBB, may emanate in a large improvement, as well as enhancement in the treatment of glioblastoma multiforme and ischaemic stroke. The hypothesis may be tested by carrying out the necessary experimentation in the laboratory to determine the effects

of the drug and platelet conjugation, as well as the formulations' ability to cross the BBB successfully. *In vitro*, *ex vivo* and *in vivo* stability testing of the developed formulation may be performed in order to determine the safety, efficacy and drug release behaviour of the different formulations [23].

Recent experimentation has shown a wide array of advantages in employing erythrocytes using hitchhiking mechanisms to deliver nanoparticles [24]; I therefore further hypothesise the use of the platelet-based drug delivery system as a carrier for drug-encapsulated nanoparticle hitchhikers that may cross the BBB with ease. The implications of the success of this overall hypothesis will be the development of a much-needed drug delivery system, able to easily penetrate the BBB during times of vascular distress. This approach to treatment may lead to the formation of a novel advancement in the treatment of glioblastoma multiforme, ischaemic strokes and all neurovascular conditions that elicit platelet activation, leading to a decrease in the mortality and morbidity associated with these conditions, and thereby enhancing and improving the quality of life of the affected patients worldwide.

The hypothesised delivery system is not applicable to synthetic platelet-like particles as there is a great obstacle in ensuring that the synthetic structures created duplicate the complex platelet make-up with great accuracy. This entire system is dependent on the body recognising the vehicles as natural platelets in order to avoid the occurrence of immunogenicity, as well as allowing the platelets to penetrate the BBB, therefore this hypothesis excludes synthetic platelet-like particles [25].

3.5 Implications of the Hypothesis

This hypothesis raises a number of key inquiries such as, (1) when would the platelet formulation need to be administered to the patient suffering from an ischaemic stroke? (2) How can it be ensured that the drug is released specifically in the brain and not at another site of the body that may simultaneously elicit a platelet response? (3) Is it possible to attain and control effective dosing, based on the amount of drug that can be encapsulated within a platelet vehicle? (4) Could the anticoagulant drug change the morphology of the platelet, and thereby cause the BBB to deem the platelet as a foreign substance? (5) Is it possible to control drug release from the platelet carrier, by investigating the exact chemical mechanism of entrapment of drugs within the platelet?

The first inquiry can be deduced by performing haematological tests to determine the time frame in which additional platelets will still be signalled to the site of the clot in the brain, thereby giving an indication of the duration in which, this system needs to be administered in order to perform the function effectively.

The second inquiry can be addressed by the process of selective brain targeting using a ligand [26]. A specific ligand may be chosen that can form a complex with a biomolecule on the surface of the tumour in glioblastoma multiforme, and a biomolecule at the site of the clot in an ischaemic stroke, ensuring that the platelet vehicle travels to the brain and subsequent release of the drug will occur specifically at the affected area of the brain only.

Previous research has not shown any negative results relating to inquiries 3 and 4, however research has never been done on the conditions mentioned in this hypothesis. Therefore, beyond extrapolation, laboratory testing will be essential to determine the outcome of inquiries 3 and 4; as specific analytical tests, such as microscopy and western blot tests, are required to make these necessary deductions.

Computational biological analyses may be used to determine the mechanism by which drug entrapment occurs within the platelet carrier [27], and the results obtained from these assessments may be used to investigate whether drug release from the platelet can be controlled effectively, in order to address inquiry number 5. Figure 3.3 is a schematic representation detailing the first 4 inquiries and consequences of the hypothesised platelet drug delivery system, intended to cross the BBB.

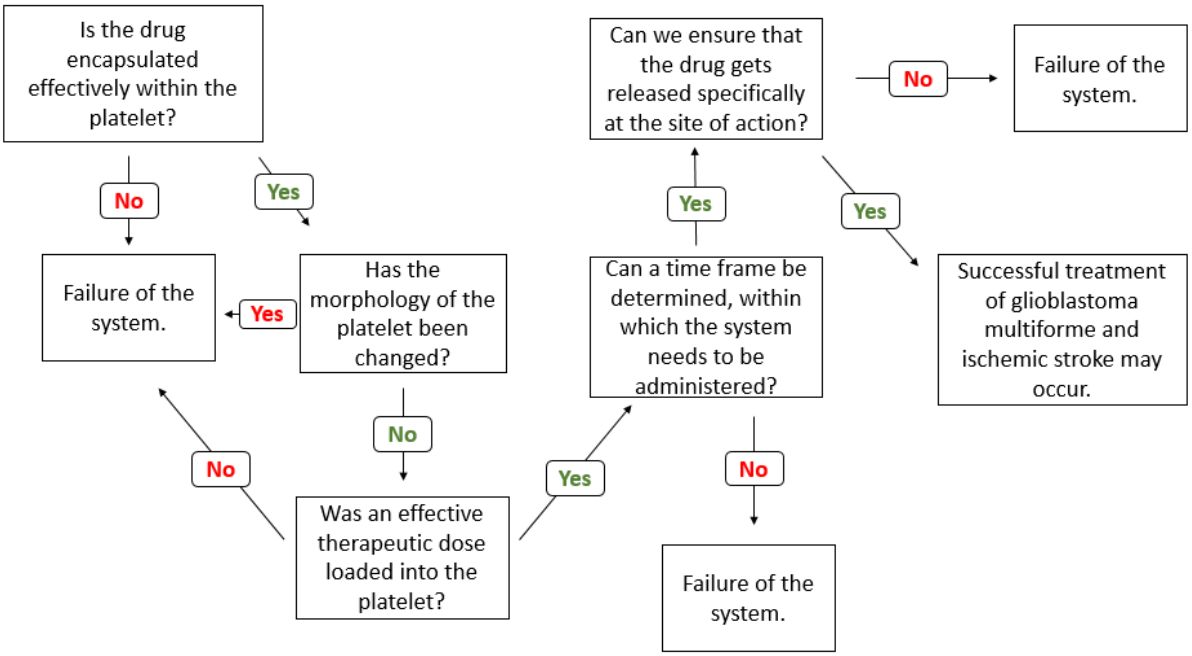

Figure 3.3: Schematic detailing the first 4 inquiries and consequences of the platelet drug delivery system, intended to cross the blood-brain barrier.

3.6 Concluding Remarks

It can be concluded that novel drug-loaded platelets may be an ideal, theoretical approach for drug delivery across the BBB in order to greatly improve the treatment outcomes of glioblastoma multiforme and ischaemic stroke. Two methods of drug loading the platelets have been mentioned in this chapter. The first, and the main method mentioned involves direct loading of the drug into the platelets, and the second method involves the loading of a drug-encapsulated nanoparticle, acting as a "hitchhiker", within the platelets. Chapter 4 focuses on the preliminary experiments that were performed to investigate certain elements related to the latter method, and chapter 5 focuses on the *in vitro* experiments that were performed to investigate certain elements related to the former method.

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CHAPTER 4

AN INVESTIGATION INTO THE SUITABILITY OF COMMONLY USED NANOMATERIALS IN THE DESIGN OF A BIOHYBRID PLATELET-BASED TISSUE PLASMINOGEN ACTIVATOR DELIVERY SYSTEM

4.1 Introduction

The published hypothesis outlined in Chapter 3 section 3.4 of this dissertation highlighted the use of platelets as carriers for drug-encapsulated nanoparticles to penetrate the blood-brain barrier (BBB). The nanoparticles “hitchhike” onto platelets for transport across the BBB. This form of transport occurs rapidly since platelets are inherently signalled to sites of vascular injury. This system of drug delivery is referred to as a biohybrid system, as the system is composed of both biological and synthetic components. The hypothesis was based on previous research undertaken by Brenner and co-workers [1], in which erythrocytes were used as carriers for nanoparticle “hitchhikers”.

The first step in the design of the biohybrid “hitchhiker” nano system to penetrate the BBB is selecting a biocompatible nanomaterial to encapsulate the drug (in this study tPA). There are a number of essential elements related to biocompatibility. One example is haemocompatibility. Haemocompatibility testing aims to evaluate the interactions between the nanomaterials and different constituents of blood [2]. Within haemocompatibility testing, there are a number of components that may be investigated and are related to the three main plasma cell types; i.e., erythrocytes, leukocytes, and platelets [3]. It is essential to analyse the haemocompatibility of the nanomaterial that will be used for potential tPA encapsulation in the biohybrid nano system, as the IV route of administration is the proposed route for the system to be introduced into the body. Therefore, the blood will be the first point of contact of the system and the body. Additionally, as the system is proposed to be used in ischaemic stroke treatment the nanomaterial will be primarily exposed to the blood.

Erythrocytes constitute approximately 40% of the total blood volume. After birth, erythrocytes are produced in the bone marrow under the influence of erythropoietin.

The main function of erythrocytes is gaseous exchange between the lungs and tissues/organs of the body, i.e., oxygen and carbon dioxide, made possible by haemoglobin that constitutes approximately 90% of the biostructure of erythrocytes [4]. The presence of erythrocytes allows blood to behave as a non-Newtonian fluid with sufficient viscoelasticity [3, 5]. The viscoelastic property of blood affects circulation and plays a critical role in the development of cardiovascular conditions such as strokes and heart disease [6]. Erythrocyte aggregation is a normal phenomenon under low shear conditions. However, nanomaterials may interact with the erythrocytes in a manner that alters normal erythrocyte aggregation, leading to changes in blood viscosity and potentially occlusion of blood vessels with altered tissue perfusion. Therefore, it is imperative to understand the effects of nanomaterials on erythrocyte aggregation [3]. Apart from erythrocyte aggregation behaviour, experiments may also be undertaken to investigate the effects of the nanomaterial presence on erythrocyte deformation, morphology, and haemolysis [3, 7]. This will provide further information on the effects of the nanomaterials on blood viscosity [3].

This phase of the research aimed at undertaking a preliminary investigation into the erythrocyte compatibility of certain commonly used nanomaterials, with the aim of determining their suitability in the potential design of a biohybrid platelet-based hitchhiker nano system. The effects on erythrocyte morphology and aggregation of silica nanoparticles, carbon nanotubes, cerium (IV) oxide nanoparticles, nanoliposomes, and poly methyl methacrylate (PMMA) particles were investigated to assess the haemocompatibility of these nanomaterials to erythrocytes. Light microscopy was used for morphological characterisation of the erythrocytes. Three novel *in vitro* methods were established to analyse the erythrocyte sedimentation rate (ESR), the degree of erythrocyte aggregation (DEA) and the blood suspension stability index (SSI). These methods provided an indication of erythrocyte aggregation behaviour following the addition of the nanomaterials to blood.

4.2 Materials and Methods

4.2.1 Materials

Silica nanoparticles, nanoliposomes, and carbon nanotubes were obtained from the laboratories of the Wits Advanced Drug Delivery Platform (WADDP) of the University of the Witwatersrand, Johannesburg. Cerium (IV) oxide nanoparticles (in the form of a

nanopowder) and anhydrous trisodium citrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Eudragit S100 particles were purchased from Röhm GmbH (Kirschenallee, Darmstadt, Germany).

Fresh whole blood was collected via clean venepuncture from a healthy male donor, over the age of 50, into potassium (K^2) ethylenediaminetetraacetic acid anticoagulant (EDTA) vacutainer tubes. The tubes were inverted 10 times to ensure that the blood and the anticoagulant were adequately mixed. The blood was immediately refrigerated and experiments were initiated within two hours of blood collection [8].

4.2.2 Ethics Clearance

Before blood collection could take place, the donor read the participant information sheet (Appendix J) and thereafter signed an informed consent form (Appendix K). The process of blood collection was performed with approval from the Human Research Ethics Committee (Medical) of the University of the Witwatersrand, Johannesburg. Ethics clearance certificate number: M180978.

4.2.3 The Incorporation of the Blood and the Nanomaterials

Prior to the incorporation of the blood and the nanomaterials the blood-filled vacutainer tubes were inverted up to five times to allow for agitation of the samples, and the blood was then placed into 10 mL sterile glass bottles. Each of the five nanomaterials selected were mixed with blood to obtain a nanomaterial concentration of 0.11 mg/mL. This concentration was chosen after reviewing the available literature on nanomaterial haemocompatibility testing, as it was believed that it would provide the most accurate results. Blood with no added nanomaterials was designated as the control sample. The samples were incubated at 37°C in an orbital shaker incubator (YIHDER, Taiwan) for one hour at 50 rpm [9]. The analyses were conducted immediately following incubation and all experiments were performed in triplicate.

4.2.4 Morphological Characterisation of the Erythrocytes and Erythrocyte Aggregates

A drop (50 µL) of each of the blood-nanomaterial samples and the control sample were carefully transferred onto sterile glass microscope slides equipped with cover slips causing an even distribution of the blood sample over the entire slide. Samples were

then viewed using an Olympus Microscope (CK2, Shibuya-ku, Tokyo, Japan), and the images were captured at a magnification of 40x.

4.2.5 The Use of ESR Values to Analyse Erythrocyte Aggregation Behaviour

The Westergren method was used to perform the ESR testing according to the recommendations outlined by the International Council for Standardisation in Haematology (ICSH) [10].

4.2.5.1 Preparation of a 3.8% Trisodium Citrate Solution

Anhydrous trisodium citrate (3.8 g) was mixed with sterile distilled water and made up to 100 mL. The solution was refrigerated at approximately 4°C, and checked prior to use to ensure stability was maintained. This solution was used as a diluent for the blood samples, as directed by the ICSH recommendations [10].

4.2.5.2 ESR Testing Procedure

The blood-nanomaterial samples and the blood control sample were placed in sterile test tubes and diluted with the 3.8% trisodium citrate solution in the ratio 4:1. Plastic sedimentation ESR pipettes (Sedi-Rate^{TM}, Globe Scientific, USA) were inserted into the tubes to fill the pipettes. Thereafter, the pipettes were placed vertically in a Westergren rack. In order to prevent any interference with the results, ESR testing was conducted at constant room temperature (25°C), and the tests were performed away from direct sunlight and from any source of heat or vibration. After one hour the levels of erythrocyte sedimentation were recorded in mm/hr.

4.2.6 Investigating the Effects of the Nanomaterials on the Degree of Erythrocyte Aggregation

In order to determine the relatively long-term effects of the nanomaterials on the DEA, analyses using an ElastoSens^{TM}Bio2 instrument (Rheolution, Inc., Montreal, Quebec, Canada) was performed to compute the shear storage modulus values of the different samples. A syringe was used to place 5 mL of each blood-nanomaterial sample and 5 mL of the control sample into individual sample holders. A small drop, the size of a pin head, of silicone oil was added to the top of each sample to prevent dehydration of samples during the analysis as this would invalidate the results. The instrument was set for the analyses of soft samples at 37°C over a period of 5.5 hours (+-20 000s),

after which the shear storage modulus values of the samples were obtained. The duration of the analyses was selected to be 5.5 hours as it provided enough time for a proper observation to be made regarding the effects of the nanomaterials on the DEA.

4.2.7 Investigating the Suspension Stability Index of Blood Post Addition of the Nanomaterials

In an attempt to determine the effects of the nanomaterials on the colloidal suspension stability of blood, a Turbiscan instrument was used to measure the intensities of transmission and backscattering of emerging light passed through 10 mL of each sample. The sample vials were sealed and placed individually in the Turbiscan^{LAB} (Formulaction, L'Union, France) platform with the parameter settings maintained at a constant temperature of 37°C throughout the analyses, over a period of one hour. One hour was selected as the time period for the analyses as it was the time period used in the ESR analyses. Therefore, the aim was to investigate the SSI during the same period of time. The process of analysis of the stability of the samples consisted of the instrument performing scans of the entire length of the sample vial, for a period of one hour. Thereafter, data illustrating the transmission and backscattering profiles of the emerging light as a function of the height of the glass sample vials were obtained.

4.3 Results and Discussion

4.3.1 Features of the Nanomaterials Used for Haemocompatibility Testing

4.3.1.1. Silica Nanoparticles

Silica nanoparticles are composed of silicon and oxygen [11]. The Stöber method is the most commonly used method to synthesize silica nanoparticles [12]. The surface of silica nanoparticles is able to be functionalized [12], thereby allowing them to be used for biological purposes such as encapsulating drugs to achieve targeted drug delivery. Silica nanoparticles are believed to possess low toxicity [13], leading to the initial belief that they may exhibit greater biocompatibility [14]. However, recent research has shown silica nanoparticles to have resulted in a number of adverse effects like cytotoxicity and genotoxicity [14], therefore, it is essential to study their biocompatibility. Mesoporous silica nanoparticles were employed in this research. These particles were synthesised according to the method described by Yang and co-workers [15] with slight modifications.

4.3.1.2. Carbon Nanotubes

Carbon nanotubes are cylindrical shaped and composed of graphene [16]. They may be classified as single-walled or multiple-walled depending on the number of rolled graphene sheets that they possess [17]. Single-walled carbon nanotubes have the advantages of possessing greater pliability and they may be characterised with ease. Multiple-walled carbon nanotubes have the advantages of possessing a high level of purity and they do not require a catalyst in their process of production [16]. Carbon nanotubes possess noteworthy mechanical strength [17] and electrical conductivity, and they are able to integrate drugs into their structure with a high efficacy [18]. Therefore, their use in biomedical applications is believed to be promising. However, they possess extremely poor solubility, which poses a challenge to their efficacy and biocompatibility. Functionalization of the surface of the carbon nanotubes is necessary to overcome this challenge [16]. Carbon nanotubes have shown signs of toxicity in some studies [16], therefore, it is necessary to conduct further experiments into their biocompatibility. Multiple-walled carbon nanotubes were employed in this research and they were synthesised according to the method described by Komane and co-workers [19].

4.3.1.3. Nanoliposomes

Nanoliposomes are bilayered in structure and they are mainly composed of phospholipids [20] with cholesterol sometimes being incorporated into their structure [21]. Nanoliposomes are used to encapsulate drugs due to their potential in improving bioavailability, enabling a more controlled drug release and resulting in targeted treatment at the site of action [21]. Additionally, they aid in decreasing any toxic effects associated with the drugs encapsulated within them and they may be formulated in a manner to target any site of the body [22]. Due to the composition of nanoliposomes, they are believed to possess greater biocompatibility and lower toxicity compared to alternative nanocarriers [22]. The nanoliposomes employed in this research were synthesised according to the method described by Ghanbari Safari and co-workers [23] with slight modifications.

4.3.1.4. Cerium (IV) Oxide Nanoparticles

Cerium oxide nanoparticles are being investigated for their potential use in an array of different applications. Their biomedical applications are largely related to their antioxidant properties, including their ability to function as scavengers of reactive

oxygen species. Additionally, they may be synthesized as drug carriers for diseases like cancer. There are a number of methods that may be used to synthesize cerium oxide nanoparticles. These methods may be classified as being either traditional or green. The green methods are believed to endow the nanoparticles with greater biocompatibility [24]. In some studies that were conducted the cerium oxide nanoparticles exhibited cytotoxic and toxic effects. These effects were believed to have occurred as a result of some of the physicochemical properties of the nanoparticles, and their reaction with the biological systems tested [25]. This substantiates the need for further tests to be conducted on the biocompatibility of cerium oxide nanoparticles. The cerium oxide nanoparticles employed in this research were purchased and not synthesised at the WADDP laboratories.

4.3.1.5. PMMA

PMMA is a polymer sold under the trade name eudragit. The copolymer eudragit S100 is derived from methacrylic acid and methyl methacrylate [26]. It is used in drug delivery applications due to its ability to promote controlled and targeted drug release, due to its pH sensitive nature [27]. Additionally, eudragit S100 is believed to be nontoxic [28]. Eudragit S100 particles are used in the synthesis of nanoparticles that are designed for oral administration, with the aim of preventing drug release in the upper gastrointestinal tract (GIT). These nanoparticles are mainly aimed at providing targeted treatment of diseases of the colon [26]. Eudragit polymers are widely believed to be biocompatible, thereby promoting their use [29]. However, due to the novelty of nanoparticle therapeutics, there is room for analyses to be performed on the *in vitro* and *in vivo* biocompatibility profiles of eudragit polymers. The eudragit S100 particles employed in this research were purchased and not synthesised at the WADDP laboratories.

4.3.2 Morphological Characterisation of the Erythrocytes and Erythrocyte Aggregates

Erythrocytes are ideally biconcave discoid shaped in order to perform their functions optimally [30]. Erythrocyte aggregation under normal conditions results in the formation of rouleaux [3]. Standard rouleaux comprise stacked erythrocytes providing a single or branched column-like appearance [31]. Figure 4.1 depicts the morphology of the erythrocytes and the erythrocyte aggregates present in (A) the blood control sample and following the addition to blood of (B) the PMMA particles, (C) carbon nanotubes,

(D) cerium (IV) oxide nanoparticles, (E) nanoliposomes, and (F) silica nanoparticles as observed under light microscopy. Erythrocyte aggregates have been encircled in red dashed lines and changes in erythrocyte morphology have been encircled in blue solid lines. The images obtained and presented in Figure 4.1 are representative of the different replicates analysed.

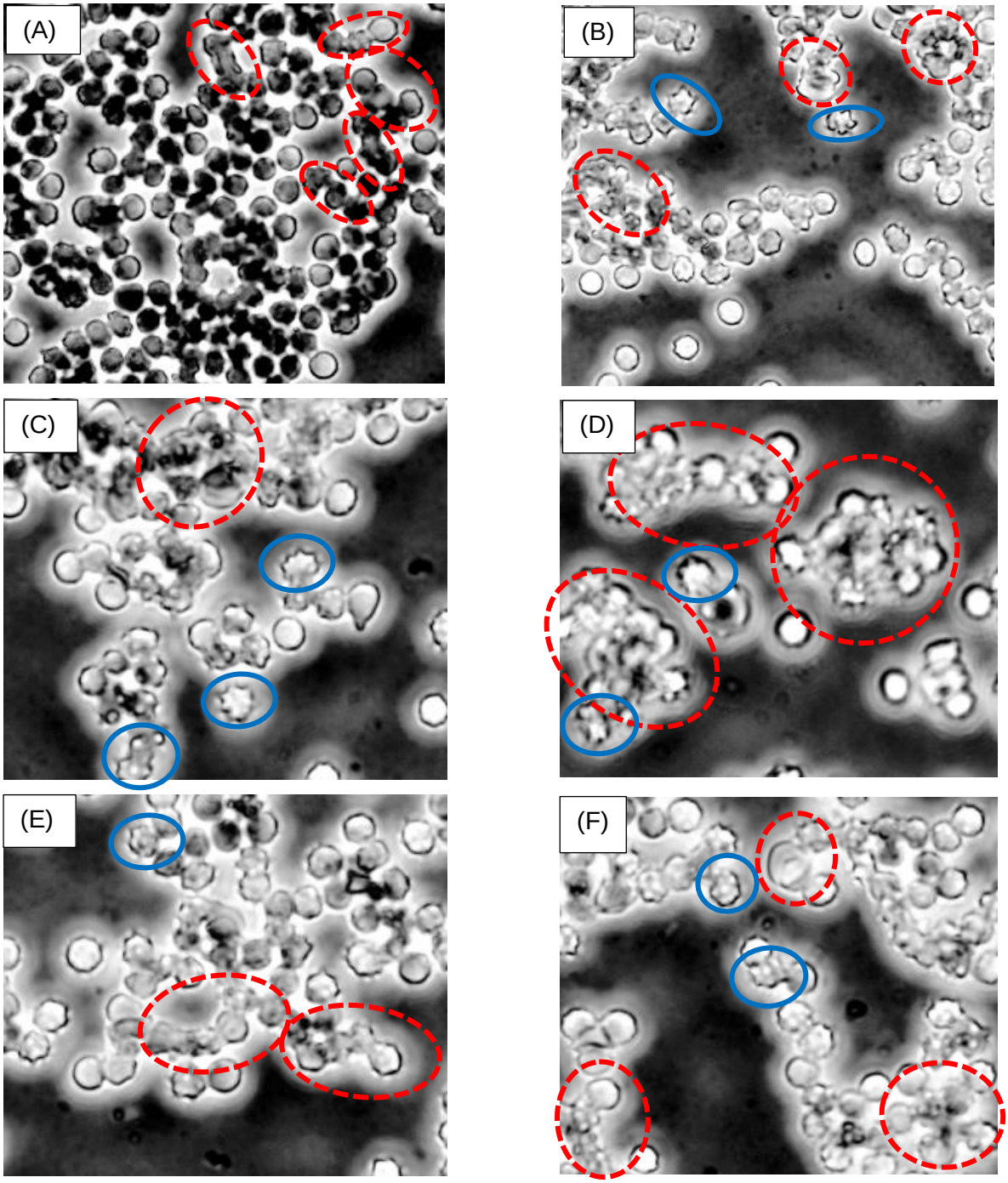


Figure 4.1: Erythrocyte morphology and aggregates present in **(A)** the blood control sample and following the addition to blood of **(B)** the PMMA particles, **(C)** carbon nanotubes, **(D)** cerium (IV) oxide nanoparticles, **(E)** nanoliposomes, and **(F)** silica nanoparticles as observed under light microscopy. Erythrocyte aggregates have been encircled in red dashed lines and changes in erythrocyte morphology have been encircled in blue solid lines.

The (A) control sample shows the erythrocytes present in their standard discoid shape, and it can be seen that the rouleaux that were formed (encircled in red) have the standard structure resembling single and branched columns. This proves that under low shear conditions erythrocyte aggregation was indeed occurring with no alteration to erythrocyte morphology or standard rouleaux characteristics.

It is evident from the images that the addition to blood of (B) the PMMA particles, (C) carbon nanotubes, (D) cerium (IV) oxide nanoparticles, and (F) silica nanoparticles resulted in erythrocyte aggregates that were larger than normal and not in the standard rouleaux shape. Instead, the aggregates resembled erythrocytes that have undergone agglutination [32]. The sample containing cerium (IV) oxide nanoparticles appeared to have the largest clumps of erythrocyte aggregates. These clumps were approximately 2.5 times larger than the erythrocyte aggregates present in the control sample. The samples with PMMA particles, carbon nanotubes, and silica nanoparticles had smaller clumps that were only slightly larger than normal erythrocyte aggregates. These clumps were approximately 1.5 times larger than the erythrocyte aggregates present in the control sample. The addition of (E) nanoliposomes to the blood resulted in erythrocyte aggregates that possessed the standard rouleaux size and branched column-like shape.

When analysing the erythrocytes morphology after the addition of the nanomaterials, it was observed (encircled in blue) that the addition of the nanomaterials had a negative effect on the discoid geometry of some of the erythrocytes. Although majority of the erythrocytes that were present on the microscope slide retained a discoid shape, a small percentage of the erythrocytes possessed a spiculated shape normally present when drugs are used as a result of disease [30]. Spiculated erythrocytes may be classified as either echinocytes or acanthocytes, depending on the number of spicules present and their distribution around the surface of the erythrocytes [33]. Due to the limitations of light microscopy, it cannot be said with certainty whether the spiculated cells that formed were echinocytes or acanthocytes.

The presence of spiculated erythrocytes could have been caused by a number of different factors, which resulted in the formation of the spiky projections. The nanomaterials may have altered the normal ionic balance of the blood, or disturbed the erythrocyte cell membrane resulting in an alteration of normal erythrocyte osmolarity.

Additionally, this malformation in erythrocyte morphology may have been due to an altered electrical state within the blood [33]. The changes observed in the shape and size of the erythrocyte aggregates indicated that the PMMA particles, carbon nanotubes, cerium (IV) oxide nanoparticles, and silica nanoparticles were either adsorbed to the erythrocyte membrane in a manner that promoted abnormal erythrocyte aggregation, or erythrocyte aggregation was altered due to electrostatic interactions between the erythrocytes and the nanomaterials, thereby altering the manner in which the erythrocytes interacted with each other [34]. The reasons behind the observed changes cannot be elucidated with absolute certainty as the exact mechanisms by which nanomaterials in general interact with erythrocytes is not clearly known.

4.3.3 ESR Values as Indicators of Erythrocyte Aggregation Behaviour

The ESR test measures the rate of formation of erythrocyte aggregates that sediment over a period of time [35]. This is performed by measuring the distance that a diluted, anticoagulated blood sample present in a vertical tube has fallen after a period of one hour [36]. ESR testing is commonly conducted in clinical medicine to detect the presence of inflammatory disease in a patient, because inflammatory diseases result in a significant increase in erythrocyte aggregation [36, 37]. When performing an ESR test it is important to note the age and gender of the donor of the blood sample because ESR values are age and gender specific. ESR testing has also been used in studies assessing nanoparticle haemocompatibility, due to the ability to measure erythrocyte aggregation. However, it has only been used in a limited number of studies for this purpose. ESR testing is not time consuming and it is relatively easy. Additionally, the ESR values may be compared to the clinically normal range. This will give an indication of the extent of the impact that the nanomaterial being analysed has on erythrocyte aggregation. For these reasons ESR testing was chosen as a novel method to investigate the impact of the nanomaterials on the rate of erythrocyte aggregation.

The ESR values that were measured are presented in Figure 4.2. The profile indicates that the addition of cerium (IV) oxide nanoparticles to blood increased the rate of erythrocyte aggregation, as the average ESR value of the sample containing cerium (IV) oxide was 12 mm/h and the average ESR value of the control sample was 8 mm/h. The addition of nanoliposomes to blood resulted in an average ESR value of 8 mm/h, equal to that of the control sample, indicating that after a period of one hour the rate of

erythrocyte aggregation was not affected by the presence of nanoliposomes. The samples containing carbon nanotubes and silica nanoparticles had the same average ESR value i.e., 7 mm/h. This indicated that both samples decreased erythrocyte aggregation to the same extent. The sample containing the PMMA particles had an average ESR value of 6 mm/h, which was the lowest ESR value from all the samples tested. This indicated that it had the greatest impact on decreasing erythrocyte aggregation.

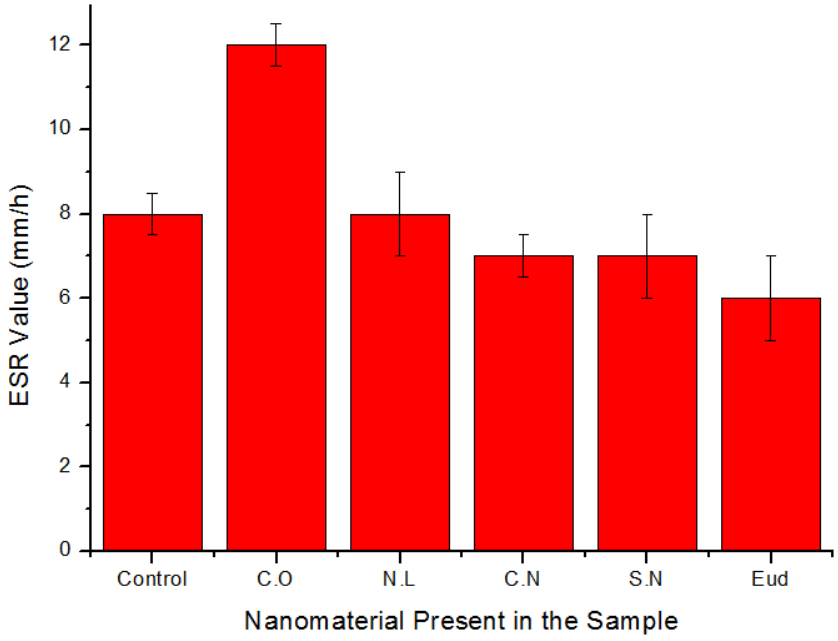


Figure 4.2: Graph showing the average ESR values of blood in mm/h following the addition of the 5 nanomaterials. The results are presented as the average ± SD (n = 3).

***C.O: cerium (IV) oxide nanoparticles; N.L: nanoliposomes; C.N: carbon nanotubes; S.N: silica nanoparticles; Eud: PMMA particles.

The blood sample obtained for this research was from a healthy male over the age of 50 years, therefore, the normal ESR range is between 0 and 20 mm/h [38]. Although the ESR values of the samples containing cerium (IV) oxide, carbon nanotubes, silica nanoparticles, and PMMA particles were different to that of the control they were all within the normal range. This indicated that even though these nanomaterials altered the rate at which erythrocyte aggregation occurred after one hour, they did not have as great an impact on erythrocyte aggregation as that of diseased states.

4.3.4 The Effects of the Nanomaterials on the Degree of Erythrocyte Aggregation

To determine whether the aggregation impact of the nanomaterials observed by the ESR tests would persist longer than one hour (3600 s), the ElastoSens^{TM}Bio2 (Rheolution, Inc., Montreal, Quebec, Canada) was used to measure the shear storage modulus of the samples by applying vertical vibration and detecting a response. The shear storage modulus (G`), measured in Pascals (Pa), was used to assess the elasticity of the samples [39]. The shear storage modulus of the blood samples indicated the erythrocyte energy storage (elasticity) intermitting their DEA in the presence of the nanomaterials [40]. For this reason, the shear storage modulus values obtained were used to analyse the rate at which erythrocyte aggregation occurred over a period of 5.5 hours (+- 20 000 s).

Figure 4.3 provides a comparison of the changes in the shear storage modulus values of the blood samples, over a period of 5.5 hours, following the addition of the five different nanomaterials. The graphs show with time passed the shear storage modulus for all of the samples increased, indicating that the elasticity of blood increased due to erythrocyte aggregation [6, 40] with simultaneous sedimentation until samples became progressively more solid [40]. The elasticity of blood following the addition of cerium (IV) oxide nanoparticles increased rapidly compared to the control sample, indicating that the presence of cerium (IV) oxide nanoparticles greatly promoted erythrocyte aggregation. This is further elucidated by the plateau phase in the profile of the shear storage modulus of cerium (IV) oxide, indicating that the sample transformed to a more solid-like structure. In contrast, the addition of the PMMA particles to the blood caused the elasticity of blood to increase at a lower rate than that of the control, indicating that the presence of the PMMA particles decreased erythrocyte aggregation.

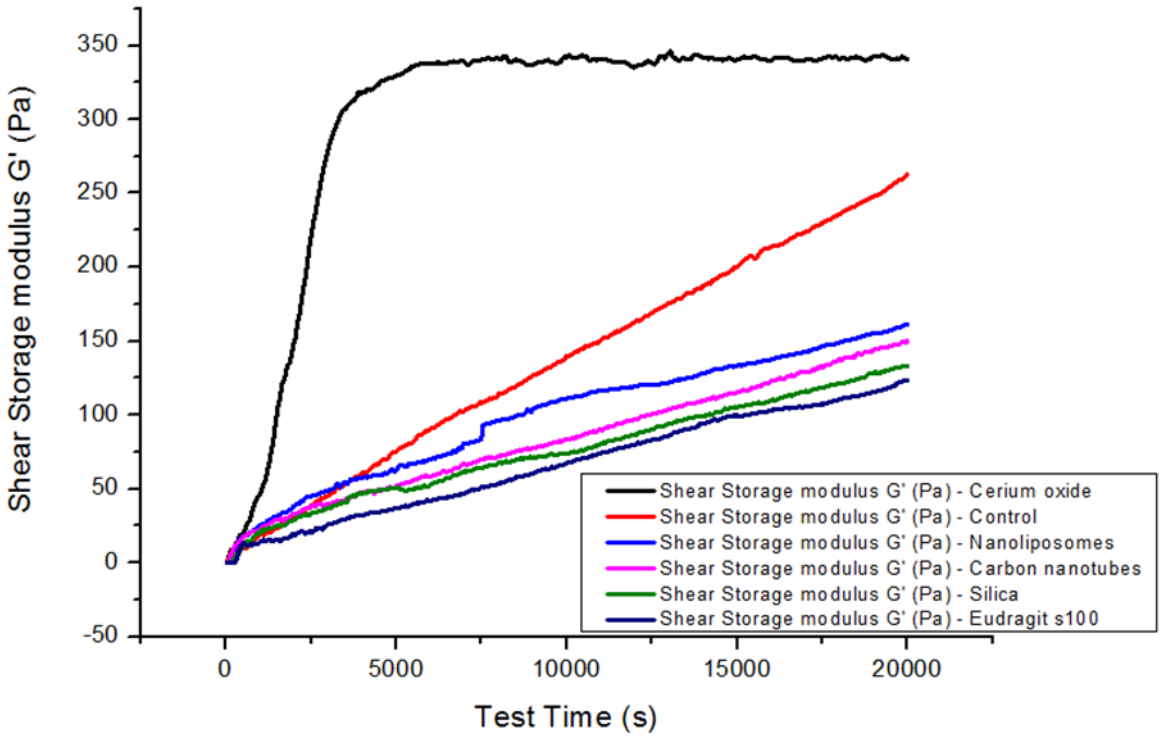


Figure 4.3: Comparative graphic representation of the changes in the shear storage modulus values of blood, over a period of 5.5 hours (+-20 000s), following the addition of the five different nanomaterials.

The initial effects on erythrocyte aggregation post addition of nanoliposomes, carbon nanotubes and silica nanoparticles could not easily be distinguished from the control sample, as there is considerable overlap of the shear storage modulus graphs. This indicated that the initial effects of these nanomaterials did not greatly alter normal erythrocyte aggregation. After approximately one hour it became apparent that the shear storage modulus values of the control sample and the sample containing nanoliposomes were the same. This value was approximately 53 Pa. Additionally, the shear storage modulus values of the silica nanoparticles sample and the sample containing carbon nanotubes were the same, approximately 48 Pa. This indicates that at this time point the erythrocyte aggregation of the respective samples was the same. These findings correlate with the results obtained from the ESR tests conducted, and presented in the preceding section. After 1.5 hours (5400 s) it was clear that the effect of the addition of nanoliposomes, carbon nanotubes and silica nanoparticles to the blood caused a decrease in the elasticity of the blood, indicating that erythrocyte aggregation occurred more slowly compared to that of the control sample.

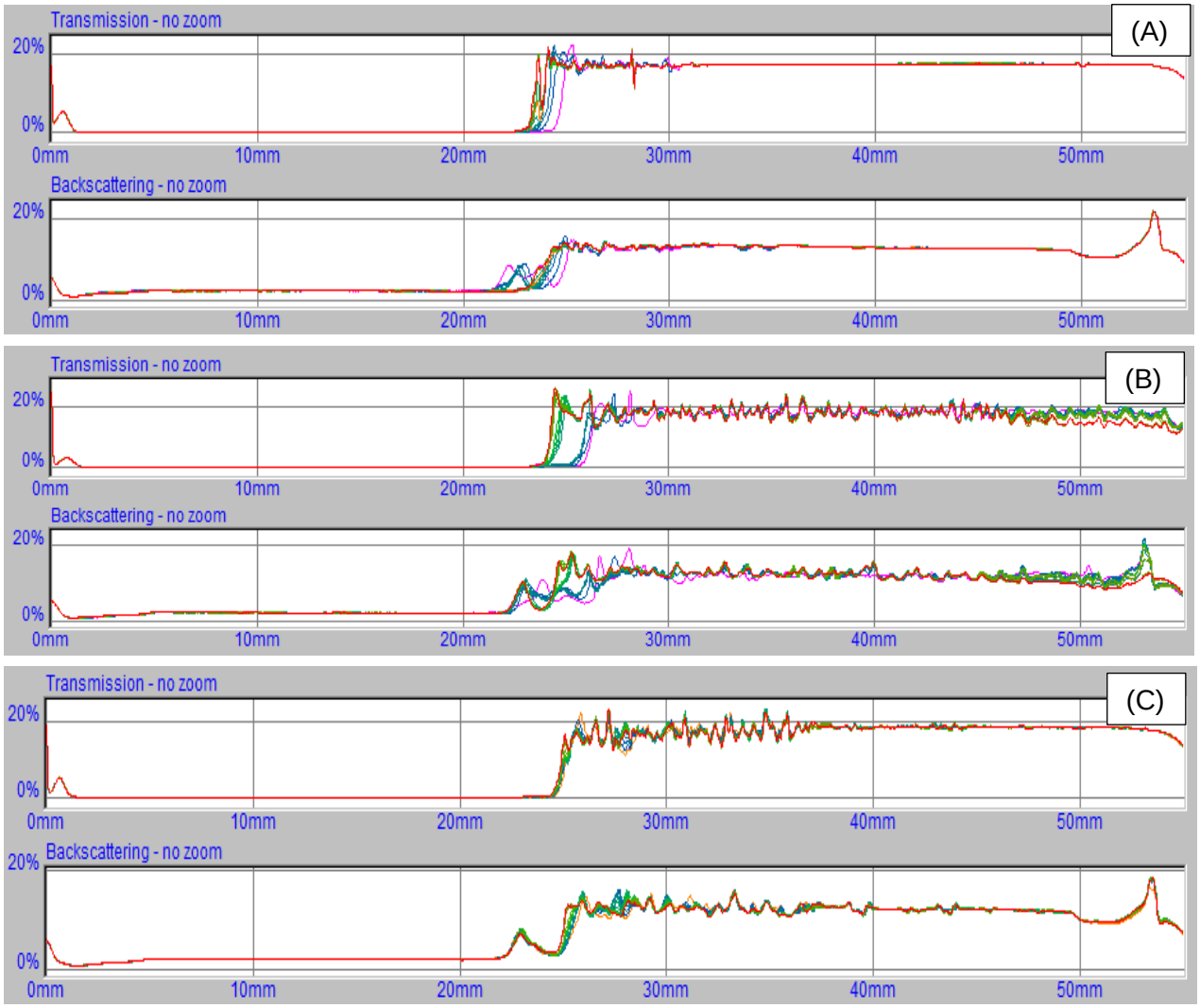
The decreased storage modulus values observed for the samples containing nanoliposomes, carbon nanotubes, silica nanoparticles and PMMA particles indicate that when analysed over a longer period of time than the one-hour testing period used in the ESR analyses, these nanomaterials interacted with the erythrocytes in a manner that delayed their normal aggregation behaviour, each by a different degree.

4.3.5 Determination of the Colloidal Suspension Stability of Blood Post Addition of the Nanomaterials

The Turbiscan^{LAB} (Formulaction, L'Union, France) analyser was used to analyse the effects of the nanomaterials on the colloidal suspension stability of blood over a period of one hour, using photo-deflection. Data was recorded in terms of the transmission and backscattering of emerging light through the turbid medium. This data was used to analyse the presence of any destabilisation phenomena occurring in the blood sample due to the addition of the various nanomaterials [41, 42].

Blood naturally exists as a colloidal suspension. Therefore, when nanomaterials are introduced into blood, they should not alter its colloidal suspension stability. When investigating the colloidal suspension stability of blood, in essence it is the rate of erythrocyte aggregation and sedimentation that is investigated [43]. Results described in sections 4.3.3 and 4.3.4 showed that the nanomaterials had an impact on erythrocyte aggregation and sedimentation. However, there was no indication of whether this impact was large enough to alter the colloidal suspension stability of the blood.

Figure 4.4 depicts the transmission and backscattering profiles of the various samples analysed. For each data set the transmission and backscattering profiles shown before approximately 2 mm (on the x-axis) and after approximately 23 mm (on the x-axis) represents the transmission and backscattering data of the emerging light passed through the sample bottle alone and not the actual blood sample, as the sample bottles were not filled in their entirety with the blood samples. Therefore, it is only the data shown between 2 mm and 23 mm that was relevant for the investigation.


(A)
Transmission - no zoom
20%
0%
0mm
10mm
20mm
30mm
40mm
50mm
Backscattering - no zoom
20%
0%
0mm
10mm
20mm
30mm
40mm
50mm

(B)
Transmission - no zoom
20%
0%
0mm
10mm
20mm
30mm
40mm
50mm
Backscattering - no zoom
20%
0%
0mm
10mm
20mm
30mm
40mm
50mm

(C)
Transmission - no zoom
20%
0%
0mm
10mm
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Backscattering - no zoom
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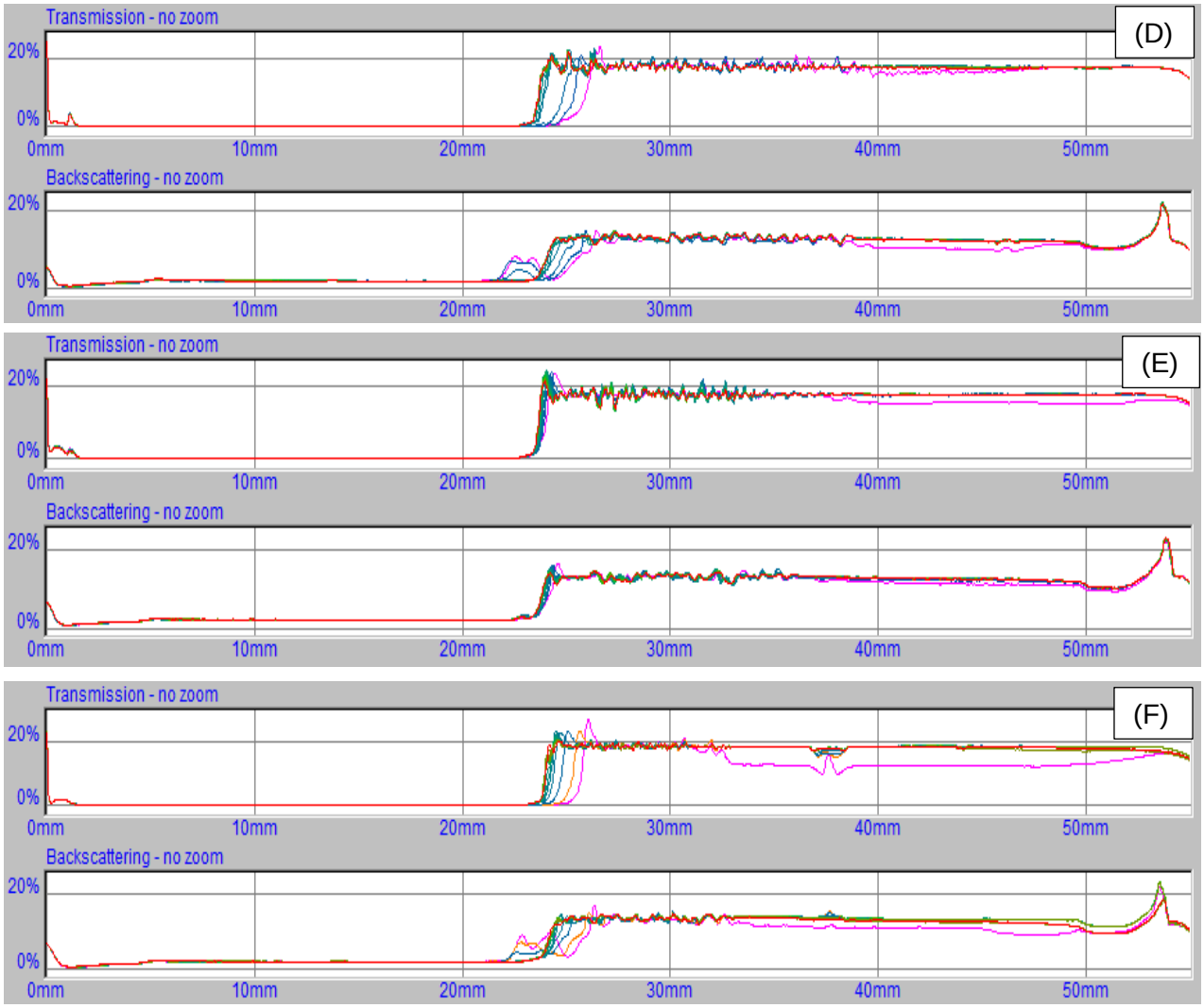

Figure 4.4: Transmission and backscattering profiles indicating colloidal suspension stability of: **(A)** the control sample, **(B)** the sample containing cerium (IV) oxide nanoparticles, **(C)** the sample containing carbon nanotubes, **(D)** the sample containing PMMA particles, **(E)** the sample containing nanoliposomes, and **(F)** the sample containing silica nanoparticles. The x-axes represent the height of the sample bottle from the bottom to the top, and the y-axes represent the % transmission and % backscattering of the emerging light obtained over a period of 1 hour. The different coloured graphs represent the data obtained at different time intervals within the 1-hour testing period.

The light intensities of transmission and backscattering are dependent on the concentration of the sample and the size of the particles within the sample. It is clear from Figure 4.4 that there were no significant changes in both the transmission and backscattering profiles, over the one-hour testing period for all of the samples. This is evident by the superimposed profiles of the data obtained. Blood is opaque in nature, therefore, no transmission of the emerging light occurred through the samples, as expected. The intensities of backscattering were low and similar for all the

nanomaterials. This indicated that no significant changes in the concentration of the sample and the size of the particles was detected.

4.3.6 Discussion

The results obtained in this research provide important information regarding the effects of the silica nanoparticles, carbon nanotubes, cerium (IV) oxide nanoparticles, nanoliposomes, and PMMA particles on erythrocyte morphology and aggregation behaviour. The following points are important to note regarding the findings of this study:

1. From previously published studies it is known that the effects of nanomaterials on erythrocytes are dependent on the concentration and contact time with the nanomaterial. Therefore, an increase in either of these two parameters from what was used in this research will result in an increase in all of the effects presented in the results obtained.

2. PMMA particles, carbon nanotubes, and silica nanoparticles resulted in erythrocyte aggregates that did not have the normal rouleaux characteristics. Instead, the aggregates resembled agglutination-like structures. However, the same nanomaterials had decreased the ESR value of erythrocyte aggregation. This indicates that the rate at which the erythrocytes were aggregating was decreased, but the erythrocyte aggregates that formed had an altered size and geometry.

3. The nanomaterials tested had effects on the rate of erythrocyte aggregation, however the photo-deflection study using a Turbiscan was unable to detect changes in the concentration of the sample and the size of the particles present in the blood samples due to diminutive sedimentation behaviour not producing significant light backscattering. This indicates that although the addition of the nanomaterials to the blood altered the rate of erythrocyte aggregation and morphology of the aggregates this change did not impact the colloidal suspension stability of the blood.

4. Although some research has been performed on the effects of different nanomaterials on erythrocyte morphology and aggregation behaviour, the exact mechanisms by which nanomaterials in general interact with erythrocytes is not clear [44, 45, 46]. Additionally, there are a sparse number of studies that have investigated

the effects of the nanomaterials on erythrocyte morphology and aggregation behaviour. Therefore, the reasons behind the observed changes in the rate of erythrocyte aggregation presented in this research cannot be elucidated with certainty. However, a number of points are presented below in an attempt to explicate the observed changes:

4.1 The cerium (IV) oxide nanoparticles employed in this research were uncoated. Cerium oxide nanoparticles that are uncoated are known to aggregate easily [25]. It is possible that the cerium oxide nanoparticles formed aggregates, and these aggregates were detected in the analyses performed. Alternatively, as the images obtained through microscopy showed the presence of large agglutination-like clumps, the cerium oxide nanoparticles may have been unintentionally adsorbed to the erythrocyte surfaces and the nanoparticles on different erythrocyte surfaces may have been attracted towards each other, thereby leading to the formation of larger aggregates more quickly.

4.2 The addition to blood of the silica nanoparticles, carbon nanotubes, nanoliposomes, and PMMA particles resulted in decreases in the rate of erythrocyte aggregation. A possible reason for the observed decreases is that these nanomaterials, as synthetic and foreign bodies, caused damages to the erythrocytes that altered erythrocyte integrity thereby resulting in decreased erythrocyte aggregation behaviour [47, 48]. It is safe to say that an erythrocyte that has experienced damages or disturbances to its normal structure may exhibit alterations in its normal functions, due to the link that exists between structure and function in biological systems. The possible mechanisms by which each of the nanomaterials caused damage to the erythrocytes are mentioned below.

4.2.1 The silica nanoparticles may have caused a degree of oxidative stress to the erythrocytes, thereby resulting in damage to the cell membrane [49]. This would also explain the formation of spiculated erythrocytes in the blood sample containing the silica nanoparticles.

4.2.2 Carbon nanotubes have the ability to enter into erythrocytes by passing through the cell membrane. The exact mechanism by which this occurs is not well known,

however, it is believed that the movement of the carbon nanotubes causes damage to the erythrocyte and may even result in cell death [44].

4.2.3 From all of the nanomaterials tested, the nanoliposomes had the least impact on erythrocyte morphology and aggregation behaviour. The nanoliposomes did not alter the normal size and shape of erythrocyte aggregates, and the decrease in the rate of erythrocyte aggregation was only observed after a longer time period. The observed effects of the nanoliposomes on the erythrocytes may possibly be attributed to the properties of the materials used in their synthesis. The length of the acyl chain of the lipids used to synthesize nanoliposomes has shown to negatively impact the erythrocyte membrane, due to its ability to cause changes in the lipids and cholesterol of the erythrocyte membrane [50, 51]. This effect may have become more pronounced with an increase in the interaction time between the nanoliposomes and the erythrocytes. This would explain why the decrease in erythrocyte aggregation behaviour was only observed in section 4.3.4 and not section 4.3.3.

4.2.4 Nanoparticles that contain PMMA are usually designed for administration via the oral route. Therefore, the observed effects of the PMMA particles may be due to the direct incubation of the particles with the erythrocytes. Damages to erythrocytes exposed to PMMA particles may occur as a result of methyl methacrylate [52].

5. In general, erythrocyte aggregates present in an agglutination-like clump and erythrocytes present as spiculated cells represent malformations which negatively impact the manner in which erythrocytes function, therefore, the presence of these malformations suggest erythrocyte incompatibility. However, it is important to analyse to what extent the malformation occurred. With regards to the impact of the nanomaterials on erythrocyte morphology, the majority of the erythrocytes present on the microscope slide retained their normal shape. Regarding the changes in shape and size of the erythrocyte aggregates, only the cerium (IV) oxide nanoparticles appeared to have formed more and larger aggregation clumps (approximately 2.5 times larger than the erythrocyte aggregates present in the control sample). From these findings it may be said that although the malformations are present, it cannot be said with certainty that the nanomaterials possess erythrocyte incompatibility that will result in an *in vivo* complication.

6. The results obtained following ESR testing showed that the impact of the nanomaterials on erythrocyte aggregation was not as large as the impact that would be seen in the presence of a diseased state, as all the ESR values obtained were within the clinically normal range. Therefore, it is unlikely for the observed effects on erythrocyte aggregation to result in an *in vivo* complication related to blood viscosity and tissue perfusion.

4.4 Concluding Remarks

Based on the results obtained in this research, it can be said that from the five nanomaterials investigated the nanoliposomes exhibited the greatest compatibility with the erythrocytes, in terms of erythrocyte morphology and aggregation behaviour. Therefore, from a preliminary *in vitro* perspective nanoliposomes may be the most promising candidate to further investigate in the design of a biohybrid platelet-based hitchhiker nano system, for the potential penetration of the BBB. An *in vivo* model should be used to test the system.

As it was observed that all of the nanomaterials had some impact in altering normal erythrocyte behaviour, and as the exact mechanisms of interaction between the erythrocytes and the nanomaterials are not properly known, it was believed that for the purpose of this research project further investigation should be performed on the clot lytic ability of platelets having undergone direct drug loading as opposed to platelets loaded with hitchhiker nanoparticles containing tPA. Additionally, this decision was made so as to analyse the drug carrying ability of the platelets without the presence of a synthetic material. Therefore, chapter 5 focuses on investigating whether platelets having undergone direct drug loading may achieve clot lysis, for the potential penetration of the BBB and treatment of ischaemic stroke.

4.5 References

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CHAPTER 5

TISSUE PLASMINOGEN ACTIVATOR (tPA)-LOADED PLATELETS FOR CLOT LYSIS IN THE POTENTIAL TREATMENT OF ISCHAEMIC STROKE

5.1 Introduction

The ability of blood to clot is beneficial to prevent excessive bleeding at an injury site. However, when blood clots form in the absence of injury or are unable to dissolve, this can be detrimental as it may lead to thromboembolic disease that requires the dissolution of the blood clot so that normal blood flow can be re-established and not compromise tissue perfusion. Thrombolytic agents are important as the most common therapy for treating thromboembolism [1]. One example is tissue plasminogen activator (tPA) that is the only FDA approved drug for clot dissolution in the treatment of ischaemic strokes [2].

tPA has proven to be effective in clot lysis but has been associated with some limitations specifically because of the non-targeted approach to therapy. [1]. Therefore, different approaches have been investigated to achieve more targeted delivery of tPA, with the vast majority focused on employing bio-inspired drug delivery systems. Research on the use of biological agents, such as platelets, to develop targeted thrombolytic systems has been rare and mostly focused on the use of red blood cells (RBCs) [1].

The hypothesis presented in Chapter 3 provided a theoretical framework of the mechanism of action of using platelets as a drug delivery vehicle that may result in targeted clot dissolution in the treatment of ischaemic stroke. To prove the hypothesis there is a need for adequate *in vitro* and *in vivo* analyses to be conducted. Therefore, the research presented in this chapter focused on the performance of *in vitro* experiments to determine whether natural platelets were able to effectively encapsulate human tPA, through direct drug loading, for the purpose of clot lysis, and to analyse whether these platelets may be potentially used in the treatment of ischaemic stroke. Direct drug loading involves the loading of the drug directly into the

platelets without the presence of an intermediary vesicle used to encapsulate the drug. Chapter 3 mainly discussed loading platelets via the process of direct drug loading.

Natural platelets obtained from the South African National Blood Service (SANBS) were loaded with tPA. Thereafter experiments were performed on the encapsulation efficiency (EE), drug release upon activation, and clot lysis efficacy of the loaded platelets. The morphology and concentrations of the platelet proteins CD 61 and human glycoprotein VI (GP6) present in the native platelets and the tPA-loaded platelets were also investigated. Additionally, two of the novel *in vitro* methods detailed in chapter 4 were employed to investigate the degree of erythrocyte aggregation (DEA) and the blood suspension stability index (SSI) following the addition of the tPA-loaded platelets system to blood.

5.2 Materials and Methods

5.2.1 Materials

Human tPA, phosphate buffered saline (PBS) tablets at physiological pH (7.4), hexamethyldisilizane (HMDS), and human thrombin were purchased from Sigma-Aldrich (St. Louis, MO, USA). In this experiment PBS solution at physiological pH was used. In order to obtain PBS solution at a pH of 7.4, one PBS tablet was dissolved in 200 mL of distilled water at room temperature (25°C) [3]. To ensure sterility, PBS solution was freshly prepared prior to each set of experiments.

Isolated, purified platelets were obtained from the SANBS and a single unit at a time was obtained of human platelet concentrate apheresis from a single donor. One unit had a volume of 200 mL, and the platelet yield for this volume was 4.96×10^{11}. Upon acquisition, the platelets were stored in the dark in an orbital shaker incubator (YIHDER, Taiwan) set at 22°C and 25 rpm to ensure constant agitation of the platelets and to prevent premature platelet activation. Platelets have a lifespan of five days outside of the human body [4], therefore, all experiments were conducted within five days from the time of platelet collection. The experiments were also conducted under sterile conditions and in the dark to prevent premature and unwanted platelet activation caused by exposure to light during experimentation.

5.2.2 Ethics Clearance

In order to purchase platelets from the SANBS a pre-approval later was required. After receiving this letter (Appendix L), ethics approval was obtained from the SANBS Human Research Ethics Committee (ethics clearance certificate number: 2018/-0451), and the Human Research Ethics Committee (Medical) of the University of the Witwatersrand, Johannesburg (ethics clearance certificate number: M180978).

5.2.3 Loading of tPA into the Platelets

The human tPA was dissolved in PBS at an amount of 1.67 µg/mL, and this was placed into sterile tubes with the isolated platelets in the volume ratio of 1.5:1. This ratio was used as it was found to be the ratio for optimal tPA encapsulation. The amount of tPA mixed with the isolated platelets was 2.5 µg/mL. The platelet + tPA mixture was incubated for 1 hour at 37°C in an orbital shaker incubator (YIHDER, Taiwan) set at 100 rpm [5]. This mixture was then passed through a chromatography column containing washed cross-linked Sepharose® CL-2B (Sigma-Aldrich, St. Louis, MO, USA) to purify the platelets by removing free (unencapsulated) tPA [5].

5.2.4 Determination of the tPA Encapsulation Efficiency Within the Platelets

To confirm that tPA was successfully encapsulated within the platelets the concentration of unencapsulated tPA was analysed using a MyBioSource Human Plasminogen Activator, Tissue ELISA Kit (MBS760814). The kit was based on the sandwich ELISA principle. The sandwich ELISA principle involves measuring the concentration of a certain antigen in a sample. This antigen is measured between two antibody layers. A platelet + tPA mixture was prepared as described in section 5.2.3 with the step of passing the mixture through a Sepharose® CL-2B column omitted. The mixture was centrifuged using an Eppendorf 5804 centrifuge (Hamburg, Germany) for 15 minutes at 2000 rpm at room temperature to collect the platelets and remove any free tPA. The supernatant was collected and diluted to perform the ELISA assay. The optical density (OD) values were measured at a wavelength of 450nm using a microplate reader (Victor X3, PerkinElmer, Waltham, MA, USA) to obtain the concentration of tPA present in the sample based on the standard curve.

The EE of the tPA-loaded platelets was calculated using Equation 5.1.

$$EE\ (\%) = [(C_I - C_O) / C_I] \times 100$$

Equation 5.1

Where C_I is the initial concentration (in ng/mL) of tPA added to the platelets, for the volume analysed, and C_O is the concentration (in ng/mL) of unencapsulated (free) tPA observed to be present for the equal volume analysed.

5.2.5 Determination of the *In Vitro* Release of tPA From the Activated Platelets

To determine the release of tPA upon platelet activation, a MyBioSource Human Plasminogen Activator Tissue ELISA Kit (MBS760814) was used. Human thrombin was used to promote platelet activation by firstly dissolving in PBS to obtain a thrombin concentration of 2 NIH units/mL. NIH units are the standard units for thrombin used in the United States. This was added to the tPA-loaded platelets in a ratio 1:10. The mixture was then placed in an orbital shaker incubator for five minutes (37°C; 100rpm). Thereafter, the mixture was centrifuged and mixed with PBS at 5000 rpm at room temperature for five minutes. The supernatant was removed and used to perform the ELISA assay with the OD values measured at a wavelength of 450nm using a microplate reader. The OD values were used to obtain the concentration of tPA present in the sample based on a standard curve.

The tPA release efficacy from the activated platelets was calculated using Equation 5.2.

$$\text{Drug Release Efficacy}\ (\%) = (C_R / C_E) \times 100$$

Equation 5.2

Where C_R is the concentration (in ng/mL) of tPA released from the platelets upon activation, and C_E is the concentration (in ng/mL) of tPA encapsulated within the platelets.

5.2.6 Morphological Characterisation of the Native and tPA-Loaded Platelets

To determine the effect of tPA loading on platelet morphology, distribution, and platelet activation and aggregation behaviour, light microscopy and scanning electron microscopy were performed prior to, and following the loading of tPA into the platelets.

5.2.6.1 Light Microscopy

A total of 50 µL of the native platelets and 50 µL of tPA-loaded platelets were mounted onto sterile blank glass microscope slides with sterile cover slips in place causing an even distribution of sample over the microscope slide. The samples were then viewed using an Olympus Microscope (CK2, Shibuya-ku, Tokyo, Japan) and images were captured at a magnification of 40x.

5.2.6.2 Scanning Electron Microscopy

Before characterisation occurred, samples were dried. The native and tPA-loaded platelets were dried by gradient dehydration using 100% ethanol. Solutions with a volume of 10 mL, containing 50%, 60%, 70%, 80%, 90% and 100% of ethanol were prepared by mixing the required volume of 100% ethanol and distilled water. Table 5.1 depicts the volumes of 100% ethanol, and distilled water that were used to make each solution.

Table 5.1: Preparation of ethanol dilutions used to dehydrate the platelet samples

Concentration of Solution (%)	Volume of 100% Ethanol Used (mL)	Volume of Distilled Water Used (mL)
50	5	5
60	6	4
70	7	3
80	8	2
90	9	1
100	10	0

A volume of 2 mL of the native platelets and 2 mL of the tPA-loaded platelets were washed with 10 mL of PBS. This was performed by placing the platelets and the PBS in a centrifuge tube and centrifuging the mixture for 10 minutes at 2000 rpm at room temperature. Thereafter, the supernatant was discarded and the pellet was washed two more times with PBS in the same way as described above. After the third washing of the pellet with PBS, the supernatant was discarded and the remaining pellet was washed with 10 mL of the 50% ethanol solution for 15 minutes at 2000 rpm at room temperature. Thereafter, the supernatant was discarded and the same process of washing the pellet was repeated with the remaining ethanol solutions, beginning with the solution containing the lowest concentration of ethanol and ending with the solution containing 100% ethanol. The pellet was washed twice with the 100% ethanol solution. After the final discarding of the supernatant the pellets containing the native platelets

and the tPA-loaded platelets were placed in sterile petri dishes. Three drops of HMDS were placed on each sample and the samples were placed in a fume hood for 10 minutes, after which they were completely dry. The samples were then placed on double sided tape on an SEM stub and taken to the Council for Scientific and Industrial Research (CSIR) for characterisation. At CSIR, the samples were coated and viewed using a Field Emission Scanning Electron Microscope (FE-SEM, JSM-7500F, JEOL, Japan).

5.2.7 Analysis of the *In Vitro* Effect of tPA Loading on Platelet Proteins CD 61 and GP6

To determine whether loading the platelets with tPA had any impact on platelet integrity and ability to respond to vascular injury, the concentrations of CD 61 and GP6 present in the native and tPA-loaded platelets were analysed using ELISA kits. The Elabscience E-EL-H2203 ELISA kit was used to determine the concentration of CD 61 present in the samples and the Elabscience E-EL-H1992 ELISA kit was used to determine the concentration of GP6 present in the samples. The kits were based on the sandwich ELISA principle. Sample preparation and the analyses were conducted by following the manufacturer's detailed protocol instructions. The OD values were measured at a wavelength of approximately 450nm using a microplate reader. These OD values were used to obtain the concentrations of the proteins present in the samples based on the standard curve, as the OD values are proportional to the concentrations of the proteins present.

5.2.8 *In Vitro* Investigation of the Clot Lytic Ability of the tPA-Loaded Platelets Using an Activated Platelet Clot

Before tests could be conducted on the efficacy of the tPA-loaded platelets in causing clot lysis of a whole blood clot, a preliminary qualitative observational analysis was performed on a clot composed only of activated platelets. This was conducted in order to obtain a general idea of whether the tPA-loaded platelets were able to induce clot lysis at the site of action. The positive results that were observed led to investigating the clot lytic efficacy of the tPA-loaded platelets in a whole blood clot model.

Human thrombin was dissolved in PBS to obtain a thrombin concentration of 2 NIH units/mL, and this was added to native platelets in a ratio 1:10. This mixture was then

placed in the orbital shaker incubator for five minutes at 37°C and at a speed of 100 rpm, in order to induce platelet activation and the subsequent formation of a platelet clot. Two platelet clots were formed and placed in separate sterile petri dishes. PBS was added to the first clot to act as a negative control, and the tPA-loaded platelets at a tPA concentration of 1.575 µg/mL were added to the second clot. The petri dishes were incubated at 37°C for two hours. Thereafter, a visual observation was made of the petri dishes. Photographs were taken to show the clot lytic action of the tPA-loaded platelets.

5.2.9 Collection of Blood That Was Used in the Experiments Mentioned in Sections 5.2.11 – 5.2.13 Below

Fresh blood was collected via clean venepuncture from healthy donors. Half of the blood samples were collected into potassium (K^2) ethylenediaminetetraacetic acid anticoagulant (EDTA) vacutainer tubes. EDTA was chosen as the anticoagulant due to its ability to preserve the cells present in the blood [6]. The tubes were inverted 10 times to ensure that the blood and the anticoagulant were adequately mixed. The blood was immediately refrigerated and experiments were initiated within two hours of collection [7]. These anticoagulated blood samples were used in the analyses described in sections 5.2.12 and 5.2.13. The other half of the blood samples were collected into tubes without any anticoagulant present. These samples were used in the analysis described in section 5.2.11, and these samples were utilised immediately following collection.

5.2.10 Ethics Clearance

Before blood collection could take place, the donors read the participant information sheet (Appendix J) and thereafter signed an informed consent form (Appendix K). This process was performed with approval from the Human Research Ethics Committee (Medical) of the University of the Witwatersrand, Johannesburg. Ethics clearance certificate number: M180978.

5.2.11 Analysis of the Efficacy of the tPA-Loaded Platelets in Causing Clot Lysis Using an *In-Vitro* Whole Blood Clot Model

The method used in this study to obtain the whole blood clots was modified from the published method used by Elnager and co-workers [8]. Briefly, immediately following

blood collection the blood was transferred into sterile glass tubes that had been previously weighed. The tubes were covered to prevent contamination and they were left in an upright position and allowed to clot for approximately 20 minutes at room temperature. Three clots were formed. Thereafter, the tubes containing the clots were weighed to obtain the weight of each clot using Equation 5.3.

Clot weight = Weight of the clot and the tube − Weight of the tube alone [8]

Equation 5.3

The method used to analyse clot lysis was slightly modified from the method used by Huang and co-workers [9]. Briefly, PBS was added to the first clot in the tube and this was designated as the negative control sample. Free tPA at a concentration of 1.575 µg/mL was added to the second clot in the tube and designated as the positive control sample. The tPA-loaded platelets, having an equivalent tPA concentration of 1.575 µg/mL was added to the third clot in the tube. This concentration was selected as it was the concentration of tPA encapsulated within the platelets. The three tubes were incubated at 37°C for two hours. Thereafter, any liquid present in the tubes was removed using a pipette and the clots were weighed again. The percentage clot lysis of each sample was calculated, and the efficacy of the tPA-loaded platelets in causing whole blood clot lysis was determined by comparing its percentage clot lysis with that of the positive control.

Percentage clot lysis was calculated using Equation 5.4:

$$\text{Clot lysis (\%)} = [(W_I - W_F) / W_I] \times 100$$

Equation 5.4

Where W_I is the initial weight of the clot (g) before the analyses, and W_F is the final weight of the clot (g) after the two-hour testing period.

5.2.12 Investigating the Suspension Stability Index of Blood Post Addition of the tPA-Loaded Platelets

In an attempt to analyse the effects of the tPA-loaded platelets on the colloidal suspension stability of blood, a Turbiscan instrument was used to measure the intensities of transmission and backscattering of emerging light passed through each sample. The anticoagulated blood was placed into 10 mL sterile glass bottles and

mixed with the tPA-loaded platelets. For every 10 mL of blood, 25 µL of tPA-loaded platelets were added. Blood with no added platelets was designated as the control sample. The samples were incubated at 37°C in an orbital shaker incubator (YIHDER, Taiwan) for one hour at 50 rpm [10].

Following incubation, 10 mL of each sample was placed in individual sample vials that were sealed and placed individually in the Turbiscan^{LAB} (Formulaction, L'Union, France) platform with the parameter settings maintained at a constant temperature of 37°C throughout the analyses, over a period of one hour. The process of analysis of the stability of the samples consisted of the instrument performing scans of the entire length of the sample vial, for a period of one hour. Thereafter, data illustrating the transmission and backscattering profiles of the emerging light as a function of the height of the glass sample vials were obtained.

5.2.13 Investigating the Effects of the tPA-Loaded Platelets on the Degree of Erythrocyte Aggregation

In order to determine the effects of the tPA-loaded platelets on the DEA, analyses using the ElastoSens^{TM}Bio2 instrument (Rheolution, Inc., Montreal, Quebec, Canada) was performed to compute the shear storage modulus values of blood prior to, and following the addition of the tPA-loaded platelets. The tPA-loaded platelets were incorporated and incubated with blood using the same method as described above, in section 5.2.12, and blood with no added platelets was again designated as the control sample. Following incubation, 5 mL of each sample was placed in individual sample holders using a syringe. A small drop, the size of a pin head, of silicone oil was added to the top of each sample to prevent dehydration of samples during the analysis as this would invalidate the results. The instrument was set for the analyses of soft samples at 37°C over a period of 5.5 hours (+-20 000s), after which the shear storage modulus values of the samples were obtained.

5.3 Results and Discussion

5.3.1 Analysis of the tPA Encapsulation Efficiency Within the Platelets

tPA was encapsulated within the platelets through the open canalicular system (OCS) [5]. The OCS is an intracellular structure that facilitates the flux of substances across platelets [11]. Upon platelet activation, the OCS allows for substances to be released

from the alpha granules of the platelets to the exterior environment [12]. The average calculated EE (n=3) for the tPA-loaded platelets was 63±1.2 % as presented in Table 5.2. The average calculated concentration of encapsulated tPA that was recorded and further used in this study was 1575 ng/mL. This result was important as it indicated that the platelets successfully encapsulated tPA without modifying the tPA, therefore the tPA remained detectable within the sample. Additionally, this result confirmed that the method employed in tPA loading was successful.

Table 5.2: Results obtained following the analyses of the tPA-loaded platelets EE, and drug release efficacy upon activation. The results are presented as the average percentage ± standard deviation (n = 3).

EE ± SD (%)	Drug Release Efficacy Upon Activation ± SD (%)
63 ± 1.2	15.2 ± 2.7

*EE: encapsulation efficiency; SD: standard deviation

5.3.2 Assessment of the tPA Release Efficacy from the Activated Platelets

The average percentage drug release efficacy ± SD of the tPA-loaded platelets upon activation was 15.2 ± 2.7 %, as presented in Table 5.2. As has been described in chapter 3, one of the key elements that are essential for the success of the tPA-loaded platelets system to function as a targeted clot lytic therapy is that the tPA-loaded platelets need to release the encapsulated drug upon activation. For this reason, drug release efficacy of the tPA-loaded platelets was analysed upon *in vitro* platelet activation. Before testing the ability of the tPA-loaded platelets to get activated and release the drug in the presence of a platelet clot, human thrombin was used to activate the tPA-loaded platelets in order to establish their drug release ability upon activation by a known stimulus.

5.3.3 Morphological Characterisation of the Native and tPA-Loaded Platelets

As mentioned in chapter 3, it is extremely important to analyse the morphology of platelet carriers following tPA loading, as an alteration of the normal platelet morphology may increase the likelihood of immunogenicity taking place. Although certain limitations are associated with the use of light microscopy for the purpose of imaging tiny structures like platelets, in medical and research settings light microscopy

is often the first method of imaging used to obtain a general idea of the platelets structure [13]. Therefore, in this research light microscopy was performed before SEM analyses in order to obtain a general idea of the platelets structure, and distribution and aggregation behaviour prior to and following the loading of tPA.

Figure 5.1 presents the images obtained by light microscopy of the platelets prior to tPA loading (A and B), and of the tPA-loaded platelets (C and D). The black structures seen in the images are the platelets. The images obtained present the platelets as being circular to slightly irregular shaped, and they can be seen distributed uniformly over the entire microscope slide. This is the normal structure and distribution pattern of platelets when they are viewed using light microscopy [13, 5]. It is clear that there is no significant distinction between the images presented in Figure 5.1, in terms of platelet structure and distribution patterns. This indicates that loading the platelets with tPA did not significantly alter the general platelet morphology as seen by light microscopy, nor did it result in unintended premature platelet activation, which would translate into a change in platelet distribution patterns.

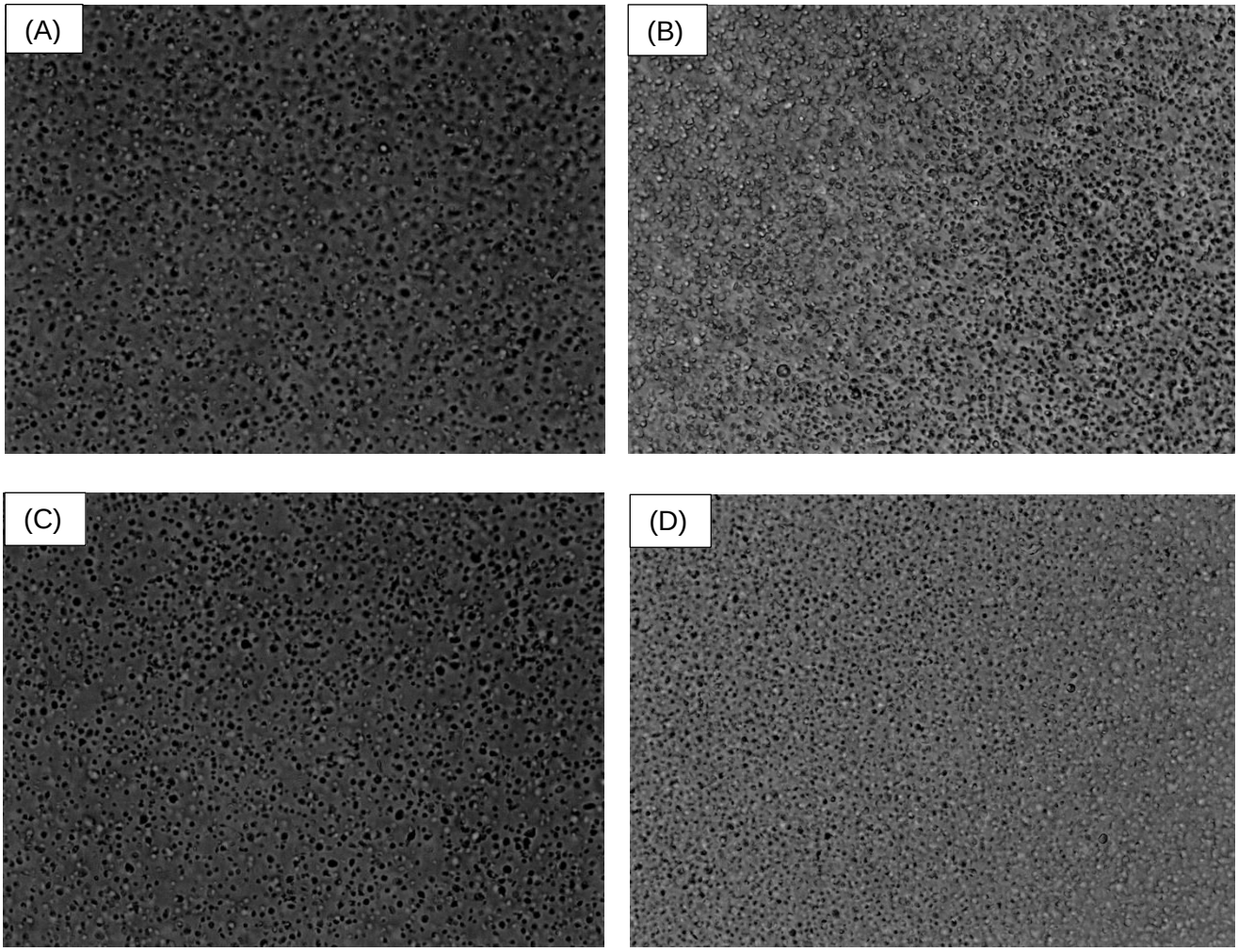

Figure 5.1: Images obtained by light microscopy of the platelets prior to tPA loading (**A** and **B**), and of the tPA-loaded platelets (**C** and **D**).

In an attempt to obtain more detailed images of the surface morphology of the platelets prior to and following tPA loading, SEM analyses were performed. Figure 5.2 presents the images obtained by SEM analyses of (A) the platelets prior to tPA loading, and (B) the tPA-loaded platelets. Some of the platelets have been encircled in red to clearly show the structure of the platelets seen in the images. It is clear that there were no significant differences in the surface morphology of the platelets observed in Figure 5.2 (A) and (B). Both images show the platelets possessing smooth discoid shapes, without any pseudopods. This is the normal platelet structure when viewed using a SEM [14]. This indicated that loading the platelets with tPA did not have a significant impact on normal platelet morphology, nor did it result in unintended premature platelet activation, as activated platelets possess pseudopods that can be clearly seen under the SEM [14]. This confirms the results presented in the images obtained from the light

microscope. Figure 5.2 (B) also does not show any drug particles present on the surface of the platelets. This indicated that the drug particles did not become adherent to the cell membranes in any abnormal manner, as it at times occurs with drug loading in other biological agents.

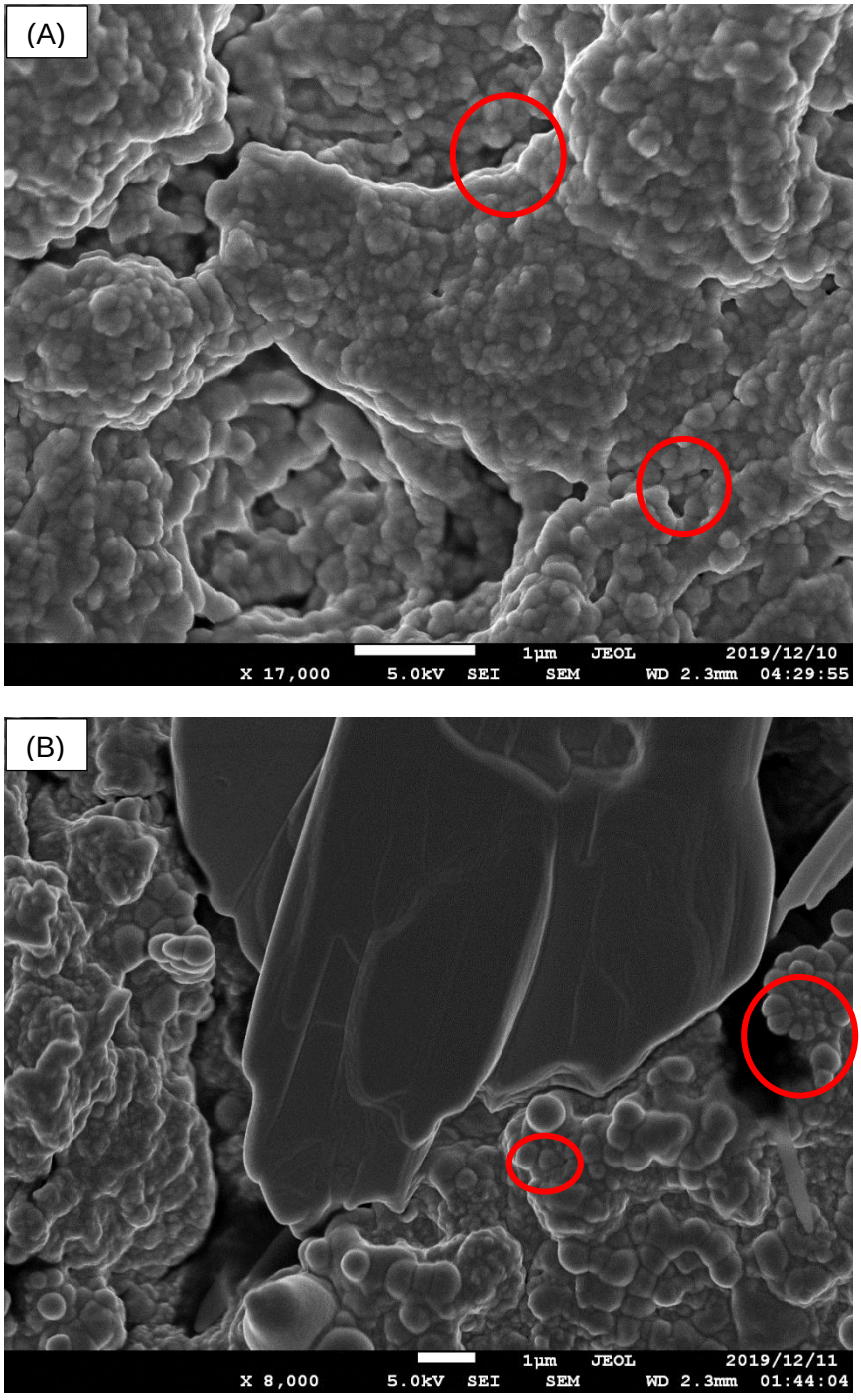

Figure 5.2: Images obtained by SEM analysis of **(A)** the platelets prior to tPA loading, and **(B)** the tPA-loaded platelets. Some of the platelets have been encircled in red for clarity.

5.3.4 The *In Vitro* Effect of tPA Loading on Platelet Proteins CD 61 and GP6

CD 61 (also called integrin beta 3) is a platelet surface protein that forms a complex with CD 41. This complex is important for platelet activation and aggregation to occur successfully, due to the complex binding plasma proteins that contribute to platelet aggregation [15]. CD 61 is one of the proteins that is often used as a typical marker to identify platelets [16]. For this reason, it was important to analyse CD 61 prior to and following tPA loading of the platelets, as the analyses provided an indication of whether the tPA-loaded platelets still maintained their platelet integrity.

GP6 is a platelet membrane specific glycoprotein that acts as a receptor for the binding of collagen. When damage occurs to the wall of a vessel, the exposed collagen is able to interact with the platelets via the GP6 receptor. Therefore, this protein is important for the adhesion, activation and aggregation of platelets, making GP6 extremely important in platelet haemostatic functions [17]. It was important to analyse GP6 prior to and following tPA loading of the platelets, as the analyses provided an indication of whether the tPA-loaded platelets were able to maintain one example of their normal platelet functions, and whether they maintained their ability to respond to vascular injury. In this research the concentrations of CD 61 and GP6 present in the native and the tPA-loaded platelets were analysed. Figure 5.3 provides comparative graphic representations of the concentrations of (A) CD 61, and (B) GP6 present in the native (unloaded) and the tPA-loaded platelets. The results are presented as the average ± SD (n = 3).

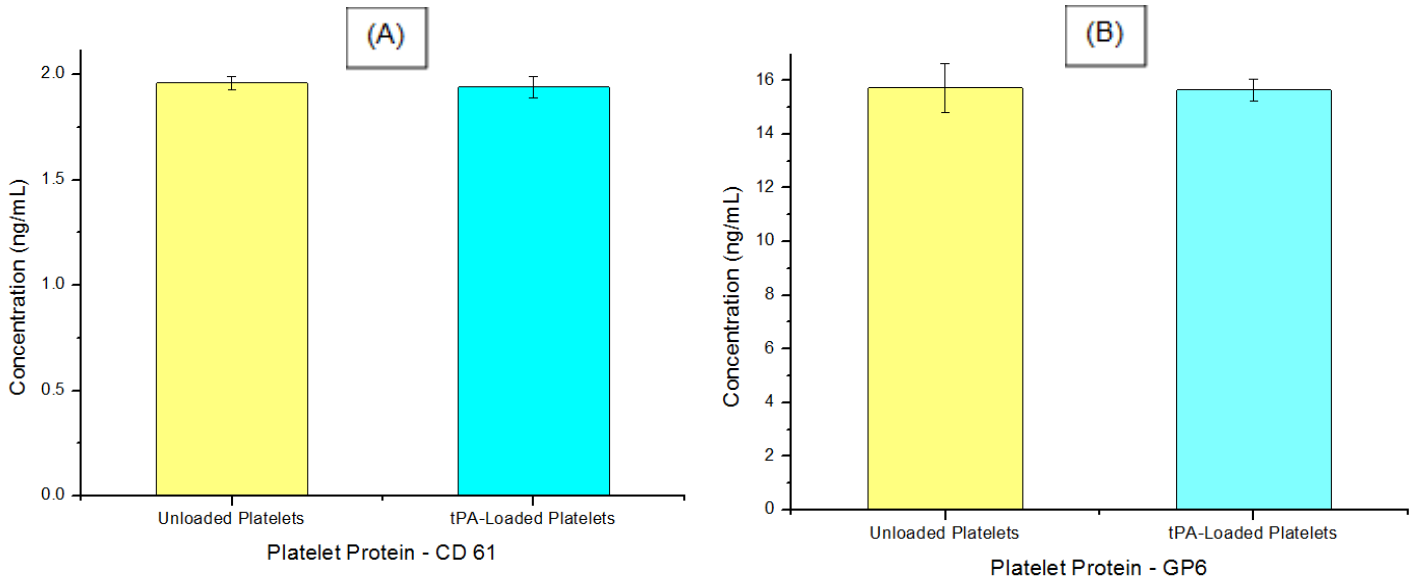

Figure 5.3: Comparative graphic representations of the concentrations of (A) CD 61, and (B) GP6 present in the native (unloaded) and the tPA-loaded platelets. The results are presented as the average ± SD (n = 3).

The average concentration of CD 61 ± SD (n = 3) for the native platelets was 1.96 ± 0.03 ng/mL and that of the tPA-loaded platelets was 1.94 ± 0.05 ng/mL. The average concentration of GP6 ± SD (n = 3) for the native platelets was 15.72 ± 0.90 ng/mL and that of the tPA-loaded platelets was 15.64 ± 0.40 ng/mL. It is clear that there were no significant differences in the concentrations of both CD 61 and GP6 present within both samples. Therefore, loading the platelets with tPA did not significantly alter platelet expression of CD 61 and GP6, as an alteration of the proteins expression would result in an alteration of the amounts present within the samples. The finding associated with GP6 is consistent with the findings published by Lu and co-workers [18].

The observed CD 61 concentrations indicate that the tPA-loaded platelets may be identified as normal, native platelets, as they maintain their biological marker. The observed GP6 concentrations indicate that loading the platelets with tPA will not affect the ability of the platelets to respond to vascular injury. This shows that the encapsulated tPA and the process of drug loading did not alter one of the most crucial platelet functions, i.e., responding to exposed collagen. Although there are other platelet proteins that may be analysed to assess platelet integrity and function, the results obtained from the analyses of CD 61 and GP6 are important for the functioning of this delivery system, as the tPA-loaded platelets need to maintain their integrity and retain their normal platelet functions in order to be signalled to, and to respond at the site of a clot. Additionally, the ability of the tPA-loaded platelets to maintain their integrity helps to postulate their biocompatibility, as the surveillance system of the body will be likely to identify these platelets in the same way as it does the normal, native platelets.

5.3.5 Qualitative Observation of the *In Vitro* Ability of the tPA-Loaded Platelets to Induce Clot Lysis of a Platelet Clot

It was important to perform a preliminary investigation into the ability of the tPA-loaded platelets in causing clot lysis at the site of action, as it was not established whether the tPA-loaded platelets could get activated at the site of a clot and thereby release their encapsulated drug at this site of action. Therefore, a clot composed of activated platelets was used before testing the clot lytic ability of the tPA-loaded platelets using a whole blood clot model. By merely looking at the clot following incubation with the tPA-loaded platelets, it was clear that clot lysis had occurred. Therefore, the qualitative

analysis alone proved the need to test the tPA-loaded platelets system in an *in vitro* whole blood clot model.

Figure 5.4 presents the photographs taken of (A) the platelets before clotting, (B) a clot newly formed and prior to incubation, (C) the clot after incubation with PBS, and (D) the remains of the clot following incubation with the tPA-loaded platelets. Incubation took place for two hours at 37°C. The images show that no clot lysis occurred upon incubation with PBS, as expected. It is clear that the tPA-loaded platelets did have a clot lytic effect. Therefore, there was a need to perform a quantitative assessment of the efficacy of the tPA-loaded platelets in a whole blood clot model, and to use the model to compare the clot lytic effects of the tPA-loaded platelets with free tPA, as whole blood provides a more accurate model of testing compared to a clot only formed by isolated platelets.

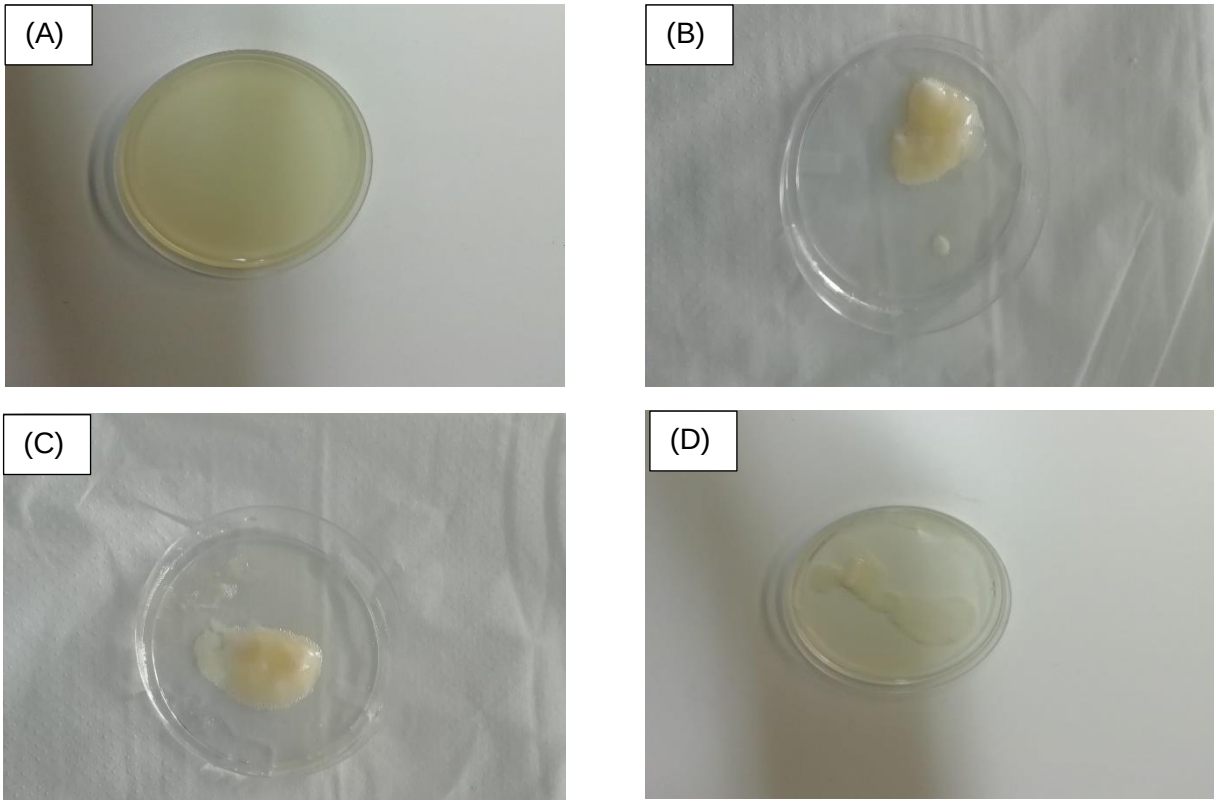

Figure 5.4: Photographs taken of **(A)** the platelets before clotting, **(B)** a platelet clot newly formed and prior to incubation, **(C)** the clot after incubation with PBS, and **(D)** the remains of the clot following incubation with the tPA-loaded platelets. Incubation took place for two hours at 37°C.

5.3.6 The *In Vitro* Efficacy of the tPA-Loaded Platelets in Causing Clot Lysis of a Whole Blood Clot Model

It was important to investigate the efficacy of the tPA-loaded platelets in causing *in vitro* whole blood clot lysis, due to the observed effect of the tPA-loaded platelets on the clot formed by the isolated platelets, shown in section 5.3.5 above. The efficacy of the tPA-loaded platelets was determined by comparing the percentage clot lysis caused by the tPA-loaded platelets with the percentage clot lysis caused by free tPA after two hours. This was achieved by measuring the weight of the clots prior to and following incubation. The analyses were conducted over an incubation period of two hours because research conducted by Absar and co-workers [19] proved that the levelling off time for the activity of tPA was two hours.

Figure 5.5 depicts the average percentage clot lysis ± SD (n = 3) of the three samples after an incubation period of two hours. The negative control contained PBS and the positive control was the sample containing the free tPA. The concentration of tPA used was the same for the free drug and the encapsulated drug. Free tPA exhibited an average percentage clot lysis of 88.5 ± 2%. In comparison the tPA-loaded platelets exhibited an average percentage clot lysis of 72 ± 1.35%. Although the average clot lytic efficacy of the tPA-loaded platelets was over 15% lower than that of free tPA, it can be said that the tPA-loaded platelets were still effective in causing clot lysis, as the remaining clot was considerably small, indicating a high efficacy. The ability of the tPA-loaded platelets to cause clot lysis indicates that the encapsulated tPA still maintained its enzymatic activity and therefore, was able to perform its function as a thrombolytic agent. This is an important finding as it shows that platelet encapsulation did not appear to alter the pharmacological action of the drug. The lowered clot lytic activity of the tPA-loaded platelets could be a result of incomplete release of the drug from the platelets upon activation. It is also possible that the tPA-loaded platelets were not fully and effectively activated, thereby not able to exhibit complete release of their internal contents, which includes the drug.

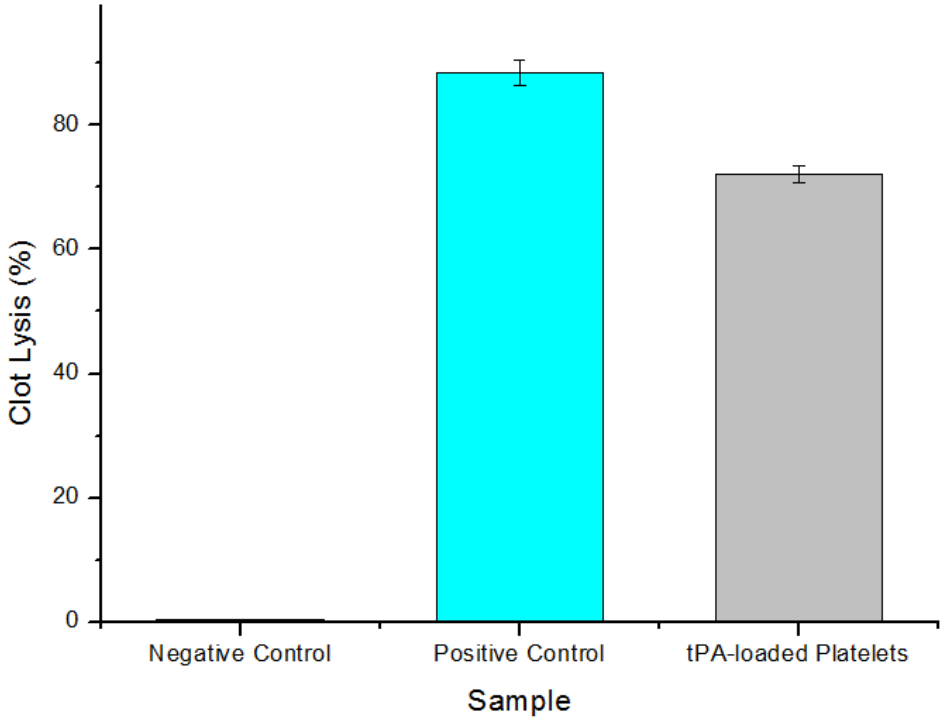

Figure 5.5: The average percentage clot lysis ± SD (n = 3) of the three samples after an incubation period of two hours. The negative control contained PBS and the positive control was the sample containing the free tPA.

5.3.7 Determination of the Colloidal Suspension Stability of Blood Post Addition of the tPA-Loaded Platelets

It is important to assess erythrocyte aggregation behaviour as part of haemocompatibility studies as erythrocyte aggregation plays a major role in haemorheological characteristics. One *in vitro* method that may be used to study erythrocyte aggregation is analysing light transmission and backscattering through the erythrocytes [20]. Light transmission refers to the amount of light that is able to pass through the sample, and backscattering of light refers to the reflection of the light off an object or particle in the direction of the original light wave [21]. The intensities of both transmission and backscattering of the emerging light are dependent on the concentration of the sample and the size of the particles within the sample being analysed [22]. The Turbiscan^{LAB} (Formulaction, L'Union, France) analyser was used to analyse the effects of the tPA-loaded platelets on the colloidal suspension stability of blood over a period of one hour, using photo-deflection.

Figure 5.6 depicts the transmission and backscattering profiles of (A) the control sample and (B) the sample containing the tPA-loaded platelets. For each data set the

transmission and backscattering profiles shown before approximately 2 mm (on the x-axis) and after approximately 23 mm (on the x-axis) represents the transmission and backscattering data of the emerging light passed through the sample bottle alone and not the actual blood sample, as the sample bottles were not filled in their entirety with the blood samples. Therefore, it is only the data shown between 2 mm and 23 mm that was relevant for the investigation.

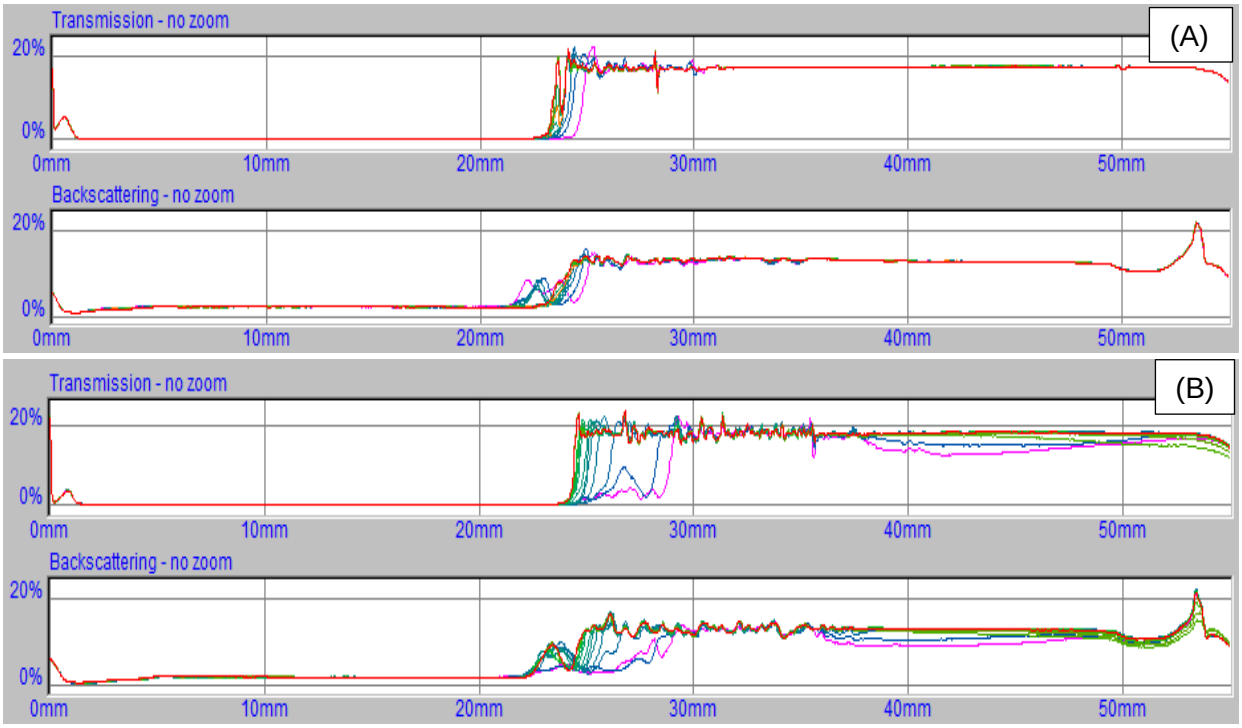

Figure 5.6: Transmission and backscattering profiles as indicators of the colloidal suspension stability of: (A) the control sample, and (B) the sample containing the tPA-loaded platelets. The x-axes represent the height of the sample bottle from the bottom to the top, and the y-axes represent the % transmission and % backscattering of the emerging light obtained over a period of one hour. The different coloured graphs represent the data obtained at different time intervals within the one-hour testing period.

It is clear from Figure 5.6 that there were no significant changes in both the transmission and backscattering profiles, over the one-hour testing period for the samples. This is evident by the superimposed profiles of the data obtained. Blood is opaque in nature, therefore, no transmission of the emerging light occurred through the samples, as expected. The intensities of backscattering were low and similar for the control sample and the sample containing the tPA-loaded platelets. This indicated that no significant changes in the concentration of the sample and the size of the particles was detected. Therefore, the addition of the tPA-loaded platelets to blood did not alter the colloidal suspension stability of blood.

5.3.8 The Effects of the tPA-Loaded Platelets on the Degree of Erythrocyte Aggregation

Figure 5.7 provides graphic representations illustrating the shear storage modulus values of (A) the blood control sample, (B) the sample containing the tPA-loaded platelets, and (C) a comparative representation of the shear storage modulus values of both samples, over a period of 5.5 hours (+-20 000s). The graph in Figure 5.7 (A) shows that as time passed the shear storage modulus of blood steadily increased, indicating that the elastic nature of blood was increasing as a result of aggregation of the erythrocytes [23, 24]. As erythrocyte aggregation took place, sedimentation occurred and the sample became progressively more solid in nature [24]. This is indicated by the increase in the shear storage modulus values with time. A higher shear storage modulus value is associated with increased erythrocyte aggregation.

For approximately the first 2.75 hours (9900 s) of the analysis the shear storage modulus values for the tPA-loaded platelets sample were slightly lower than that of the control sample. This could indicate that the rate of erythrocyte aggregation was slightly decreased. After this period, the graphs obtained for both samples were almost identical. This indicates that the addition of the tPA-loaded platelets did not significantly alter the rate at which erythrocyte aggregation took place in the long term. The results obtained in this section indicate that the tPA-loaded platelets exhibited compatibility with the erythrocytes in terms of their degree of aggregation.

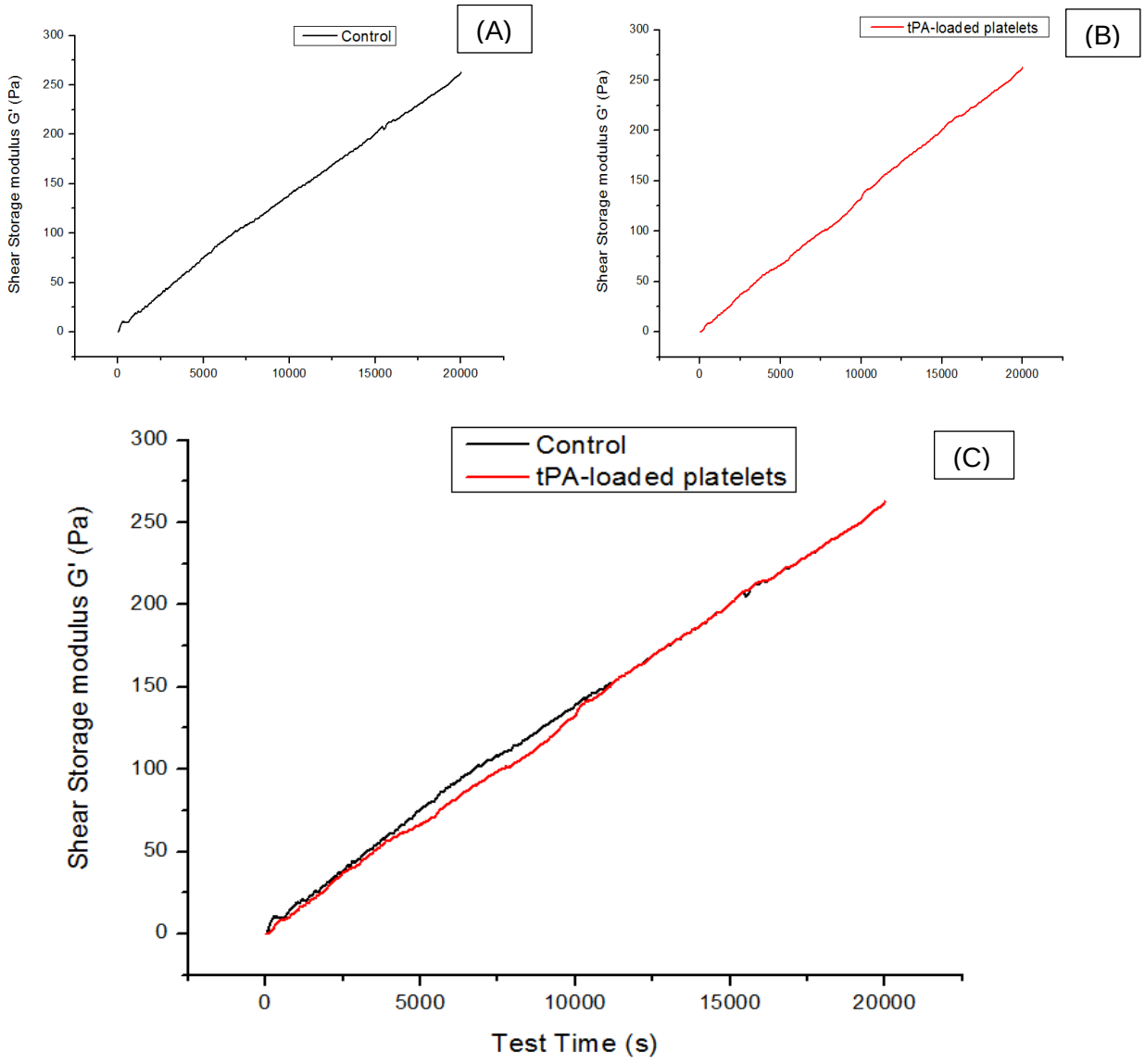

Figure 5.7: Graphic representations illustrating the shear storage modulus values of (A) the blood control sample, (B) the sample containing the tPA-loaded platelets, and (C) a comparative representation of the shear storage modulus values of both samples, over a period of 5.5 hours (+-20 000 s).

5.3.9 Proofs to Support the Use of the tPA-Loaded Platelets in the Treatment of Ischaemic Stroke

The ability of platelets to encapsulate tPA and to release it at the site of a clot, thereby causing effective clot lysis proves that biological agents can play a critical role in the development of a targeted thrombolytic treatment approach. This is an important development in the pharmaceutical and biological sciences, as it provides new insights into platelets and their potential role as drug delivery vehicles. Additionally, this novel development may provide opportunities for further research to be undertaken. The

previous chapters have outlined the potential benefits of the use of the tPA-loaded platelets, and they have performed well in the *in vitro* experiments that were conducted and presented in this chapter. The following findings support the use of the tPA-loaded platelets system in the treatment of ischaemic stroke:

1. The platelets were able to encapsulate tPA with a high percentage EE addressing the first inquiry on platelets as a suitable delivery vehicle for tPA, presented in chapter 3 Figure 3.3.
2. The platelets released the encapsulated tPA upon activation. This is important as it proved that the tPA did not remain entrapped within the platelet structure and was released when a platelet response was evoked. This proves the basis upon which the hypothesis in chapter 3 was developed.
3. Loading the platelets with tPA did not alter the morphology, distribution and activation behaviour of the platelets. This result addresses the second inquiry presented in chapter 3 Figure 3.3. This is an important finding as it indicated that from a structural perspective, the tPA-loaded platelets were completely unchanged. If platelet structure is intact, it is more likely that the platelets will naturally cross the BBB easily during an ischaemic stroke to deliver tPA at the target site.
4. Loading the platelets with tPA did not alter its integrity and biological protein marker. The maintenance of platelet integrity further indicated that the possibility of the tPA-loaded platelets to cross the BBB remains high. Additionally, maintenance of platelet morphology and integrity will not provoke an immune response.
5. The ability of the tPA-loaded platelets to cause clot lysis. This finding indicated that the platelets were able to cloak the tPA resulting in targeted treatment.
6. The tPA-loaded platelets did not affect the colloidal suspension stability of blood nor did the tPA-loaded platelets alter the degree of erythrocyte aggregation, as observed by the shear storage modulus values of blood following the addition of the tPA-loaded platelets system. This promotes their erythrocyte compatibility profile.

5.4 Concluding Remarks

Based on the results obtained, it is evident that tPA may be successfully encapsulated within platelets with no alteration of normal platelet morphological structure or integrity. Although free tPA had a greater clot lytic efficacy, the tPA-loaded platelets exhibited the ability to release the drug at the site of action, as well as being successful in causing

in vitro clot lysis in a whole blood model. Additionally, the tPA-loaded platelets did not affect the suspension stability of blood nor did the tPA-loaded platelets alter the rate of erythrocyte aggregation, indicating that the tPA-loaded platelets are unlikely to affect blood viscosity and tissue perfusion. The results presented in this chapter indicate that from an *in vitro* perspective the tPA-loaded platelets may prove to be a promising treatment strategy to achieve targeted thrombolysis for the potential treatment of ischaemic stroke.

5.5 References

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CHAPTER 6

CONCLUSION, LIMITATIONS AND RECOMMENDATIONS

6.1 Conclusion

Despite the many advancements made in medicine and the pharmaceutical sciences, ischaemic stroke is still associated with a great deal of morbidity and mortality. This may be due to either the difficulties associated with early detection of the ischaemic stroke or the shortcomings associated with the current conventional methods employed for ischaemic stroke treatment. Therefore, there is a great need for alternative mechanisms of early diagnosis and effective treatment. The current COVID-19 pandemic has shed greater light on the need for more effective ischaemic stroke treatments, as many sufferers of COVID-19 even those who are young and wouldn't normally be at risk, are affected by ischaemic strokes.

The challenges associated with ischaemic stroke treatments may be due to the drawbacks and adverse effects related to the use of tissue plasminogen activator (tPA). Some of the drawbacks limiting the use of tPA include non-targeted treatment, possible damage to the blood-brain barrier (BBB), and severe allergic events that may occur as a result of an immune response. As a result, it is believed that the development of a targeted treatment approach may help to mitigate some of the limitations associated with tPA treatment and thereby improve the likelihood of positive patient outcomes. Greater amounts of research are being undertaken on the use of biological agents as drug carriers to allow for targeted treatment, due to the biocompatibility of these agents as well as their inherent abilities to respond to certain sites of injury. Platelets have shown promise as therapeutic agents due to their multiplicity of roles in the body. Platelets are a key component in the development of ischaemic stroke, due to their role in clot formation.

In this research, the role of platelets as possible drug delivery vehicles was investigated in an attempt to develop a targeted system for the potential treatment of ischaemic stroke. Different elements related to this targeted delivery system were presented in this dissertation. A comprehensive literature review was conducted in order to better

understand how platelets function as targeted therapeutic agents in different platelet-inspired therapeutic modalities. A hypothesis was developed that demonstrated from a theoretical perspective how platelets may be used as drug delivery vehicles for the penetration of the BBB. Two methods of drug loading the platelets were mentioned in the hypothesis. The first method involved direct drug loading of the platelets and the second method involved loading a drug-encapsulated nanoparticle acting as a "hitchhiker" into the platelets. The system designed when platelets are loaded using the second method of drug loading was referred to as a biohybrid system.

A preliminary investigation was conducted on the suitability of five commonly used nanomaterials in the design of a biohybrid platelet-based hitchhiker nano system. This investigation was conducted to determine which nanomaterial would be the most suitable in terms of erythrocyte compatibility to encapsulate the drug tPA for loading into platelets. The effects of the nanomaterials on erythrocyte morphology and aggregation behaviour were analysed using light microscopy and three novel *in vitro* methods. From the nanomaterials tested, the nanoliposomes exhibited the greatest erythrocyte compatibility. Therefore, it is believed that nanoliposomes should be further investigated in the design of a biohybrid platelet-based hitchhiker nano system. For the purpose of this research project, it was decided that further research be conducted on the clot lytic ability of natural platelets that had undergone direct tPA loading, instead of platelets loaded with a tPA-encapsulated nanoparticle.

tPA was able to be encapsulated within natural platelets with a relatively high encapsulation efficiency, and these platelets were able to release the encapsulated tPA upon activation. It was also observed that loading the platelets with tPA did not result in any morphological changes to the platelets, nor did it alter the integrity and ability of the platelets to respond to vascular injury. The tPA-loaded platelets exhibited a high percentage efficacy of clot lysis in an *in vitro* whole blood clot model. Additionally, the tPA-loaded platelets did not alter the degree of erythrocyte aggregation (DEA) and the colloidal suspension stability of blood. This promoted their erythrocyte compatibility. Thus, the results of this research have provided novel data regarding the *in vitro* use of platelets as carriers for tPA that may potentially be successful in the treatment of ischaemic stroke. However, before a proper prediction can be made it is important for the tPA-loaded platelets system to be tested in an *in vivo* stroke model.

6.2 Limitations of this Study

When a novel drug delivery system is designed, *in vitro* analyses are crucial as they provide important information regarding the efficacy of the system under controlled conditions. However, *in vitro* analyses are limited as it is not possible to fully mimic the conditions present in the human body. The following limitations associated with *in vitro* analyses have been identified from this study:

1. One of the key elements required for the success of the tPA-loaded platelets system in the treatment of ischaemic stroke is that the tPA-loaded platelets will release the encapsulated drug upon activation at the site of the clot in the brain. It was hypothesised that the activated platelets that are present in the clot will cause the tPA-loaded platelets to become activated due to the release of signalling molecules. This study did show that the tPA-loaded platelets were able to release the encapsulated drug and cause a sufficient degree of clot lysis. However, due to the nature of *in vitro* analyses it cannot be said with certainty that the activation of the tPA-loaded platelets was caused completely by being in contact with the clot. Although all known precautions were taken, due to the sensitivity of platelets an external force may have induced activation of the tPA-loaded platelets resulting in the release of the encapsulated tPA.

2. It is difficult to analyse whether the tPA-loaded platelets will be effective in providing targeted tPA delivery by using the *in vitro* testing model, as *in vitro* analyses are unable to take into account the systemic circulation of the body.

3. In the original protocol of this study, the use of a BBB Kit™ was proposed to analyse the *in vitro* ability of the tPA-loaded platelets in crossing the BBB. This kit is produced by PharmaCo-Cell Company Ltd, which is in Japan. The BBB Kit™ provides a means of *in vitro* drug testing of the BBB. It also can be used to determine and further investigate the pathophysiology of the BBB. Primary cultures of monkey (*Macaca irus*) or rat (Wistar rat) brain capillary endothelial cells, brain pericytes and astrocytes are used in this model. Due to the reconstitution of this kit, the *in vivo* features of the BBB can be observed. The main applications of this kit include: BBB permeability testing, brain cell toxicology testing, and disease modelling assays of the BBB.

Although every effort was made to obtain the kit, there was a problem with the import permits of the company as they are not a regular supplier and the kit could not be purchased. Additionally, a suitable alternative could not be found. Therefore, the ability of the system to penetrate the BBB, and its subsequent impacts on the BBB was not investigated.

The only experimental model that may address and overcome all of the limitations that have been mentioned is an *in vivo* model. All other models that are tested outside of a living organism may be influenced by external forces and are unable to fully mimic the conditions present in the body at the time of an ischaemic stroke. An *in vivo* model is also necessary to determine whether the differences observed in the clot lytic activity of the free tPA and the tPA-loaded platelets is clinically meaningful. Additionally, the functioning of the tPA-loaded platelets in the presence of a concurrent clot can only be analysed using an *in vivo* model.

6.3 Recommendations for Future Studies

6.3.1 Nanomaterial Haemocompatibility Studies

1. The research presented in chapter 4 only examined the impact of the nanomaterials on erythrocyte morphology and aggregation behaviour. Therefore, before an absolute prediction can be made regarding the suitability of a nanomaterial in the design of a biohybrid platelet-based hitchhiker nano system further research on both *in vitro* and *in vivo* models should be undertaken on other aspects of biocompatibility to ensure that the nanomaterials do not provoke any immune response.

2. As the aim of the analyses conducted in chapter 4 was to get a preliminary understanding of the interaction between the nanomaterials and the blood, a shorter time frame was selected for the experiments. However, experiments based on the DEA and SSI analyses, described in chapter 4, may be designed to test the effects of the nanomaterials over a longer period of time, like 24 hours. This may provide a better indication of the interaction between the nanomaterials and blood.

3. The data collected in chapter 4 may be used to design *in vivo* experiments to determine the exact impact of these nanomaterials specifically on tissue perfusion and the cardiovascular system.

4. As novel methods were employed for the analyses undertaken in chapter 4, it contributes to increasing the applications of each method and equipment employed. The new applications may be included in studies performed by researchers who have access to the equipment used.

5. Majority of the studies that investigate erythrocyte compatibility focus more on the assessment of haemolysis. There is potential for researchers to incorporate analyses on erythrocyte aggregation behaviour as well.

6. Further research in the form of SEM analyses or analyses of blood smears may be performed to determine with certainty whether the nanomaterials analysed in chapter 4 resulted in the formation of echinocytes or acanthocytes.

7. As the exact mechanisms by which nanomaterials and erythrocytes interact are not properly known, there is great scope for the performance of *in vitro* and *in vivo* studies that may determine the mechanisms of interaction. This will provide a clearer picture regarding the erythrocyte compatibility of nanomaterials.

8. The hypothesis outlined in chapter 3 excluded the use of synthetic platelet-like particles as potential drug delivery vehicles for the penetration of the BBB. The exclusion was made on the basis that synthetic platelet-like particles may not be biocompatible due to the foreign nanomaterial used in their synthesis and therefore, their presence in the body will result in the occurrence of immunogenicity. There is scope for future studies to be performed in order to test this claim made in the hypothesis and to determine to what extent it is correct.

6.3.2 Studies on Platelet Drug Delivery Vehicles

1. *In vitro* and *in vivo* experiments should be conducted on the use of platelet drug delivery vehicles for the potential treatment of glioblastoma multiforme, as is described in chapter 3.

2. Alternative methods may be employed to analyse platelet proteins. These include flow cytometry and western blot testing.

3. Apart from the platelet protein CD 61, CD 41 may also be analysed as it is also a platelet marker that may provide information regarding the integrity of platelets.

4. Further studies should increase the number of biological and technical replicates used.

5. Translation of this research into *in vivo* studies:
All life forms are precious. Therefore, when suitable alternatives are present, they should be utilised to prevent animal testing. However, in the field of novel drug delivery research animal testing models provide important and comprehensive information on the pharmacokinetic and pharmacodynamic profiles of the tested system [1]. The translation of this study into an *in vivo* model will provide an indication of whether the tPA-loaded platelets system has potential to be used in humans [2]. A few brief points are mentioned below regarding the *in vivo* studies of an ischaemic stroke model.

As the system being tested is novel, a pilot study should be conducted first to optimise the procedure and dosing requirements. The cranial circulation and ischaemic stroke patterns of humans are highly similar to that of Sprague-Dawley rats [3]. Therefore, these rats should be the animal model of choice. Male rats should be chosen instead of female rats as oestrogen is neuroprotective [4]. An induction of stress may cause unwanted effects in the rats, therefore, the rats should be handled with care and given time for acclimatisation to their new environment before tests are conducted [5]. Ischaemic stroke may be induced in the rats via occlusion of the middle cerebral artery [6]. The neuro-behavioural patterns of the rats should be observed closely prior to and following administration of the tPA-loaded platelets. It is extremely important to monitor whether any unwanted bleeding occurs after the administration of the tPA-loaded platelets. In an attempt to observe the effects of the tPA-loaded platelets in the presence of a subsequent clot, a clot may be induced in another part of the rat's body and the action of the tPA-loaded platelets may be observed. Following euthanisation, the organs of the rat should be analysed to determine whether the tPA-loaded platelets had any effect on them.

6.4 References

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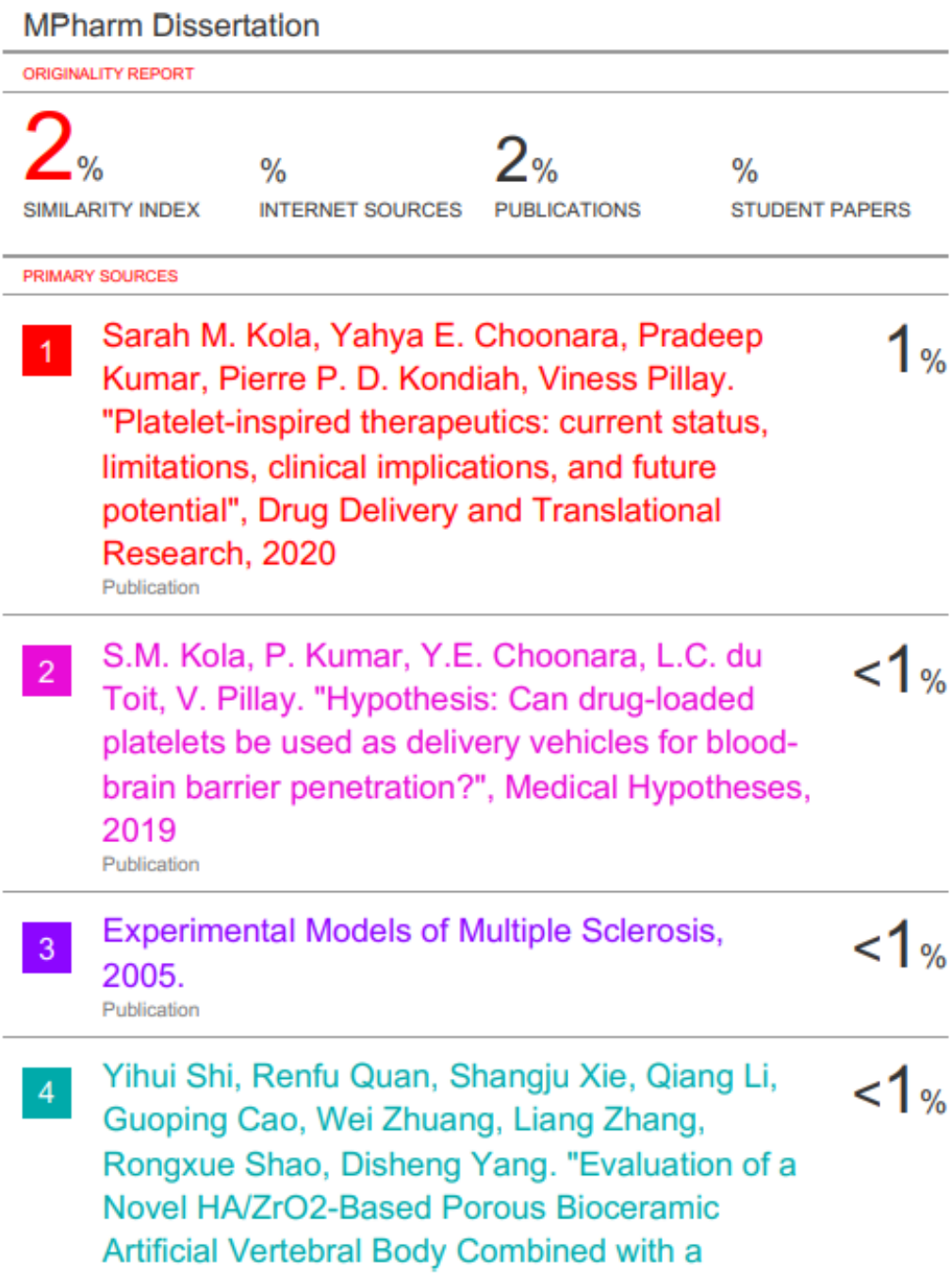
APPENDICES

APPENDIX A – Turnitin Originality Report



MPharm Dissertation

ORIGINALITY REPORT

2%	%	2%	%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	Sarah M. Kola, Yahya E. Choonara, Pradeep Kumar, Pierre P. D. Kondiah, Viness Pillay. "Platelet-inspired therapeutics: current status, limitations, clinical implications, and future potential", Drug Delivery and Translational Research, 2020 Publication	1%
2	S.M. Kola, P. Kumar, Y.E. Choonara, L.C. du Toit, V. Pillay. "Hypothesis: Can drug-loaded platelets be used as delivery vehicles for blood-brain barrier penetration?", Medical Hypotheses, 2019 Publication	<1%
3	Experimental Models of Multiple Sclerosis, 2005. Publication	<1%
4	Yihui Shi, Renfu Quan, Shangju Xie, Qiang Li, Guoping Cao, Wei Zhuang, Liang Zhang, Rongxue Shao, Disheng Yang. "Evaluation of a Novel HA/ZrO2-Based Porous Bioceramic Artificial Vertebral Body Combined with a	<1%

APPENDIX B – WITS Human Research Ethics Committee Clearance Certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

R14/49 Ms Sarah Kola

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M180978

NAME: (Principal Investigator)	Ms Sarah Kola
DEPARTMENT:	Pharmacy and Pharmacology
PROJECT TITLE:	Development of a biohybrid, platelet based drug delivery vehicle for the potential treatment of ischaemic stroke
DATE CONSIDERED:	28/09/2018
DECISION:	Approved Unconditionally
CONDITIONS:	
SUPERVISOR:	Prof V. Pillay, Dr P. Kumar and Prof Y. Choonara
APPROVED BY:	Doctor CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	16/11/2018

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on the Third Floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in **September** and will therefore be due in the month of **September** each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature

Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

APPENDIX C – SANBS Ethics Clearance Certificate

Head Office or Zone
1 Constantia Boulevard
Constantia Kloof Ext 22, 1709

Postal Address: Private Bag X14, Weltevreden Park, 1715
Tel: 011 761 9000 Fax: 0866747666
Email: customerservice@sanbs.org.za

SOUTH AFRICAN NATIONAL BLOOD SERVICE NPC
Human Research Ethics Committee

OHRP Number : IORG0006278
FWA Registration Number : IRB00007553
SA NHREC Registration Number : REC-270606-013

Secretariat: Tel: 011 761 9096 | Valencia.Simmadari@sanbs.org.za

To : Ms Sarah Kola
E-mail : sarah.kola@student.wits.ac.za

Dear, Ms Kola

DATE OF COMMITTEE MEETING : 06 November 2018
PROJECT TITLE : **DEVELOPMENT OF A BIOHYBRID, PLATELET-BASED DRUG DELIVERY VEHICLE FOR THE POTENTIAL TREATMENT OF ISCHAEMIC STROKE**
DECISION OF THE COMMITTEE : Approved
CLEARANCE CERTIFICATE NO : 2018/-0451

1. Execution of the study must be compliant with applicable guidelines and policies.
2. Any amendment, extension or any other modifications to the protocol must be submitted to this Ethics Committee for approval prior to implementation.
3. The Committee must be informed of any serious adverse event, planned and unplanned termination of the study.
4. A progress report should be submitted yearly for studies longer than a year and a final report at completion of the study for both short term and long term studies.
5. Kindly refer to the SANBS HREC clearance certificate number on all future correspondence on this study to the HREC secretariat.
6. This approval is valid for 5 years from the date stated above.

COMMITTEE GUIDANCE DOCUMENTS:

- International Conference on Harmonization (ICH) Good Clinical Practices (GCP) Guideline (ICH, 1996), Ethics in Health Research: Principles, Structures and Procedures (SA Department of Health, 2015); Guidelines for Good Practice in the Conduct of Clinical Trials in Human Participants in South Africa (SA Department of Health, 2006); Ethical Principles for Medical Research Involving Human: Declaration of Helsinki (World Medical Association, 2013); Reviewing Clinical trials: A Guide For Ethics Committees (Karlberg and Speers, 2010).

23 May 2019

CHAIRPERSON: Prof J.N. Mahlangu **DATE**

APPENDIX D – Review Paper Abstract

Drug Delivery and Translational Research
https://doi.org/10.1007/s13346-020-00751-2

REVIEW ARTICLE

Platelet-inspired therapeutics: current status, limitations, clinical implications, and future potential

Sarah M. Kola[1] · Yahya E. Choonara[1] · Pradeep Kumar[1] · Pierre P. D. Kondiah[1] · Viness Pillay[1]

Abstract

Recent research has been successful in demonstrating the importance of the addition of platelets to the field of cell-mediated therapeutics, by making use of different platelet forms to design modalities able to positively impact a wide range of diseases. A key obstacle hindering the success of conventional therapeutic interventions is their inability to produce targeted treatment, resulting in a number of systemic side effects and a longer duration for the onset of action to occur. An additional challenge facing current popular therapeutic interventions is biocompatibility of the system, resulting in the decline of patient compliance to treatment. In an attempt to address these challenges, the past few decades have been witness to the discovery and innovation of precision therapy, in order to achieve targeted treatment for an array of conditions, thereby superseding alternative mechanisms of treatment. Platelet-mediated therapeutics, as well as employing platelets as drug delivery vehicles, are key components in advancing precision therapy within research and in clinical settings. This novel approach is designed with the objective that the platelets retain their original structure and functions within the body, thereby mitigating biocompatibility challenges. In this article, we review the current significant impact that the addition of platelet-inspired systems has made on the field of therapeutics; explore certain limitations of each system, together with ideas on how to overcome them; and discuss the clinical implications and future potential of platelet-inspired therapeutics.

Keywords Platelets · Platelet-inspired therapeutics · Drug delivery systems · Precision therapy

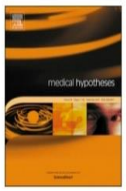
APPENDIX E – Hypothesis Paper Abstract

Medical Hypotheses 125 (2019) 75–78

Contents lists available at ScienceDirect

Medical Hypotheses

journal homepage: www.elsevier.com/locate/mehy



Hypothesis: Can drug-loaded platelets be used as delivery vehicles for blood-brain barrier penetration?

S.M. Kola, P. Kumar, Y.E. Choonara, L.C. du Toit, V. Pillay*

Wits Advanced Drug Delivery Platform Research Unit, Department of Pharmacy and Pharmacology, School of Therapeutic Sciences, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, 7 York Road, Parktown 2193, South Africa

ARTICLE INFO

Keywords:
Platelet drug-delivery
Blood-brain barrier
Glioblastoma multiforme
Ischemic stroke
Tumour cell-induced platelet aggregation (TCIPA)

ABSTRACT

Neurovascular conditions are disorders associated with the blood vessels of the brain that are extremely difficult to treat successfully due to the selectivity and fastidious nature of the blood- brain barrier. Consequently, the efficacy of the pharmacological treatments for these conditions are greatly reduced thereby resulting in large amounts of neurovascular-related morbidity and mortality. Platelets are an important component of blood that actively respond to neurovascular distress in the body. Recent research has proven the effectiveness of platelets as drug delivery vehicles, during circumstances where the body naturally elicits a platelet response. This hypothesis highlights the theoretical use of platelets as drug delivery vehicles, able to penetrate the blood-brain barrier, for the treatment of two neurovascular conditions; glioblastoma multiforme and ischemic stroke. The success of the hypothesised system may lead to the development of a novel and extremely necessary delivery mechanism.

APPENDIX F – Chapter 4 Research Paper Abstract

AN INVESTIGATION INTO THE SUITABILITY OF COMMONLY USED NANOMATERIALS IN THE DESIGN OF A BIOHYBRID PLATELET-BASED TISSUE PLASMINOGEN ACTIVATOR DELIVERY SYSTEM

Sarah M Kola, Pradeep Kumar, Yahya E Choonara, Viness Pillay

Wits Advanced Drug Delivery Platform Research Unit, Department of Pharmacy and Pharmacology, School of Therapeutic Sciences, Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, 7 York Road, Parktown 2193, South Africa

Abstract

tPA-encapsulated nanomaterials may be loaded into platelets as a “hitchhiker” nano system. The platelets, acting as drug delivery vehicles, may then carry these particles across the blood-brain barrier to the site of an ischaemic stroke. However, before this system may be designed it is important to select the correct nanomaterial that will be used to encapsulate the tPA. An extremely important aspect that needs to be assessed when selecting the correct nanomaterial is biocompatibility. One element of biocompatibility is haemocompatibility. In an attempt to determine the preliminary suitability of five nanomaterials in the design of a biohybrid platelet-based hitchhiker system the erythrocyte compatibility of the nanomaterials was analysed. Erythrocyte morphology and aggregation behaviour were investigated following the addition to blood of poly methyl methacrylate particles, carbon nanotubes, cerium (IV) oxide nanoparticles, nanoliposomes, and silica nanoparticles. Light microscopy was employed to observe changes in erythrocyte morphology. Erythrocyte aggregation behaviour was analysed by investigating erythrocyte sedimentation rates (ESR), by employing the ElastoSens^{TM}Bio2 to determine the degree of erythrocyte aggregation (DEA) of the samples, and the suspension stability index (SSI) of each sample was analysed using a Turbiscan analyser. Morphological results indicated that all of the nanomaterials altered the morphology of the erythrocytes, and all of the nanomaterials with the exception of nanoliposomes, altered the size and shape of the erythrocyte aggregates. The nanoliposomes did not alter the rouleaux characteristics of the aggregates. The results obtained from the aggregation analyses indicate that the addition of each nanomaterial alters the rate at which erythrocyte aggregation occurs in both the short and long term, in different ways. This was seen by the changes in the shear storage modulus values of the samples tested and the changes in the ESR values obtained. Although the addition of the nanomaterials to the blood altered the rate of erythrocyte aggregation and the morphology of the erythrocytes, this change did not impact the colloidal suspension stability of the blood. It can be concluded that nanoliposomes exhibit the greatest erythrocyte compatibility but there is scope for further studies to be conducted on all of the tested nanomaterials. The results obtained in this research may be used to design *in vivo* experiments to further analyse the effects of these nanomaterials on other elements of biocompatibility.

APPENDIX G – Chapter 5 Research Paper Abstract

TISSUE PLASMINOGEN ACTIVATOR (tPA)-LOADED PLATELETS FOR CLOT LYSIS IN THE POTENTIAL TREATMENT OF ISCHAEMIC STROKE

Sarah M Kola, Pradeep Kumar, Yahya E Choonara, Viness Pillay

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Abstract

Tissue plasminogen activator (tPA) is a thrombolytic agent that is used for the purpose of clot dissolution in the treatment of ischaemic strokes. Although the use of tPA is associated with advantages, there are a number of adverse effects that occur following its administration into the body. Therefore, research is being undertaken on new methods of delivering tPA into the body that may mitigate some of these adverse effects and provide a more targeted delivery of the medication. Natural platelets have shown promise as biological drug delivery vehicles due to their innate ability to target sites in the body that elicit a platelet response. In this study, tPA was encapsulated within platelets to investigate whether this system will result in clot lysis. The results obtained showed that the platelets were able to encapsulate tPA with a relatively high encapsulation efficiency, and that the platelets were effective in releasing the drug upon activation. It was also observed that loading the platelets with tPA did not result in any morphological changes to the platelets, nor did it alter the integrity and ability of the platelets to respond to vascular injury. The tPA-loaded platelets on average were able to cause 72% clot lysis when tested using an *in vitro* whole blood clot model. Additionally, the tPA-loaded platelets were not seen to alter the degree of erythrocyte aggregation and the colloidal suspension stability of blood, thereby promoting their erythrocyte compatibility. Thus, there is *in vitro* evidence to support the use of tPA-loaded platelets in the potential treatment of ischaemic stroke.

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APPENDIX J – Participant Information Sheet

Department of Pharmacy and Pharmacology
School of Therapeutic Sciences, Faculty of Health Sciences
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Participant Information Sheet

Title of research project: The development of biohybrid, platelet-based drug delivery vehicles for the potential treatment of ischaemic stroke.
Name of researcher: Ms. Sarah Kola

Good day. You are being invited to take part in this research project. Before you decide whether to do so, it is important that you understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. Thank you for reading this information sheet.

This research project aims to use blood platelets, from human donors, in order to develop a novel drug carrier. The platelets will be used as a vehicle to encapsulate an appropriate drug, forming the novel delivery system that can potentially be used to treat ischaemic stroke.

Please note the following minimum requirements to be a blood donor, as stipulated by the South African National Blood Service (SANBS):

- You are between the ages of 16 and 65 years old, for first time donors.
- You weigh a minimum of 50 kgs, and 55 for this research as it requires platelets.
- You are in good health.
- You lead a low risk lifestyle.
- You consider your blood safe for transfusion.
- You have had a balanced meal within four hours of donating blood.
- You have not donated blood in the last 56 days, and for this research in the last 14 days as it involves platelets.
- Your pulse is between 50-100 regular beats per minute.
- Your blood pressure is below 180/100mmHg, and above 100/60mmHg.
- Your haemoglobin level is 12.5 g/d L or above.

*Note: We will have equipment available on site to conduct the necessary wellness tests.

Please provide us with more information if:

- You are on any medication such as antibiotics.
- You are from, or have travelled to, a malaria area.

- You have had cancer, heart disease, epilepsy, a bleeding disorder or any other chronic medical condition.
- You are involved in a "hazardous" occupation or sport.

Please self-exclude from donating blood for this study if:

- You are being treated for HIV/AIDS.
- There is any chance that you may have been exposed to HIV/AIDS; or if you are donating blood only to be tested for HIV/AIDS.
- You are being treated for a sexually transmitted infection (STI).
- You are pregnant or breastfeeding.
- You have had surgery in the last 6 months or are due for an operation within the next 6 weeks.

The blood donation process will take place at The University of the Witwatersrand Medical School, 8th floor, WADDP Main Laboratory, on the 15th and 30th day of each month. You will be asked for a brief medical history, in order to ensure that you are eligible to donate blood. Approximately 100 ml of blood will be drawn from a vein in your arm into a heparin tube. A sterile needle, directly out of its packaging, will be used to prick you. You are likely to feel slight discomfort by the prick of the needle into your vein. The discomfort will disappear as soon as the needle and the collection tubes are removed. Blood donors will receive an appropriate snack and an orange juice following donation in an attempt to provide a source of folate and thereby reduce the risk of dizziness and syncope. The blood will be collected by a trained medical doctor, who will ensure that all the necessary precautions for the safe collection of blood samples are adhered to. It is unlikely for any major complications or side effects to occur by you donating blood, however blood donation may place you at risk of syncope or slight dizziness. Blood donation does not put you at risk or disadvantage you in any way. It is highly recommended that you eat and drink something after donating blood. In the event of a complication occurring due to the blood withdrawal procedure, the medical doctor on site will tend to you. No further information will be taken from you, and you will not be photographed or recorded with any media device. Final results of the research project will be published. You will not be identified in any report or publication. If you wish to be given a copy of any reports resulting from the research, please ask us to put you on our circulation list.

Your participation in donating blood for this study is entirely voluntary. If you choose to participate, you will not receive any remuneration for your blood sample donation. Your participation will be kept strictly confidential. If you decline participation, you will not face any negative consequences. Please do not participate if you have any medical condition that does not permit you to donate blood and/or if you do not comply with the requirements set out by the SANBS.

There is no direct benefit to you as the donor for taking part in this study. However, your participation will be greatly appreciated as it will contribute to the research of a much-needed drug delivery system that has the potential to positively impact the lives of many patients, and to give rise to further research opportunities and knowledge exploration.

Every effort has been made to ensure that the blood donation process runs smoothly. It is unlikely for any negative events to occur. If you do however have any complaints about the blood withdrawal in the first instance you can contact any member of the research team. If you feel your complaint has

not been handled to your satisfaction you can contact Prof. V. Pillay to take your complaint further (see below).

The project has been organised by the Wits Advanced Drug Delivery Platform (WADDP); a division of the Department of Pharmacy and Pharmacology. This project is funded by the National Research Foundation of South Africa. This study has been approved by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand, Johannesburg ("Committee"). A principal function of this Committee is to safeguard the rights and dignity of all human subjects who agree to participate in a research project and the integrity of the research.
If you have any concern over the way the study is being conducted, please contact the Chairperson of this Committee who is Professor Clement Penny, who may be contacted on telephone number 011 717 2301, or by e-mail on Clement.Penny@wits.ac.za. The telephone numbers for the Committee secretariat are 011 717 2700/1234 and the e-mail addresses are Zanele.Ndlovu@wits.ac.za andRhulani.Mukansi@wits.ac.za.

For further information please feel free to contact any member of the research team mentioned below.

Thank you for considering taking part in this research.

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Chairman and Head of Department
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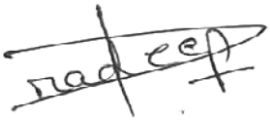

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NRF Research Chair
Personal Professor in Pharmaceutics
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Faculty of Health Sciences
University of the Witwatersrand
Email: Viness.Pillay@wits.ac.za

APPENDIX K – Informed Consent Form

Department of Pharmacy and Pharmacology
School of Therapeutic Sciences, Faculty of Health Sciences
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Informed Consent Form

Title of research project: The development of biohybrid, platelet-based drug delivery vehicles for the potential treatment of ischemic stroke.
Name of researcher: Ms. Sarah M. Kola

I, …… agree to participate in this research project. The research has been explained to me and I understand what my participation will involve.

I agree that my participation will remain anonymous: YES NO (please circle)

I am eligible to donate blood according to the requirements for being a blood donor as stipulated by the SANBS: YES NO

I do not suffer from any medical condition that does not permit me to donate blood: YES NO

…… (Name of participant)

…… (Signature of participant)

…… (Date)

APPENDIX L – SANBS Pre-Approval Letter

Toll Free: 0800 11 9031

Head Office or Zone
Head Office
1 Constantia Boulevard
Constantia Kloof Ext 22
1709

Postal Address: Private Bag X14, Weltevreden Park, 1715

Tel: 011 761 9000 Fax: 0866747666

Email: customerservice@sanbs.org.za www.sanbs.org.za

Registration No. 2000/026390/08

30 August 2018

To SRC / HREC

Re: subject of study: Development of a biohybrid, platelet-based drug delivery vehicle for the potential treatment of ischaemic stroke.

Subject to approval, this letter is to confirm that SANBS will allow:

Ms. Sarah Kola from Department of Pharmacy and Pharmacology Faculty of Health Sciences University of the Witwatersrand to purchase 8 units of the Platelet Conc. Single Donor Apheresis (nappi code: 708804-001), every 1-2 months for the research project

Signed:

30 August 2018

Michael Lennards

National Manager: Processing & Issuing, Processing and Issuing