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DEVELOPMENT OF AN ULTRASTRUCTURE PRESERVATION PROTOCOL FOR PLATELET AND FIBRIN NETWORKS UTILIZING HIGH PRESSURE FREEZING AND SUBSEQUENT COMPARISON OF NEW AND ESTABLISHED PROTOCOLS

by

Antonia Vergina Buys

26092345

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Degree: MSc (Anatomy with specialization in Cell Biology)

ABSTRACT

Abnormalities in the structure of fibrin fibers and blood platelets have been broadly studied and well correlated to functional abnormalities in the coagulation system due to disease, genetics or environmental factors. The importance of these and further ultrastructural investigations of fibrin fiber networks and platelets is therefore paramount in understanding

the aetiology of haemorrhagic or thrombogenic tendencies and disorders. To study structural abnormalities and variations of fibrin fibers and platelets, electron microscopy is essential; unfortunately the use of electron microscopy necessitates several preparation steps to make a biological specimen stable enough to withstand the high vacuum environment of an electron microscope and also electron beam irradiation. The two most common procedures to accomplish this is chemical fixation and freeze fixation. Chemical fixation entails the chemical alteration of a specimen by means of the introduction of chemical bonds and cross-links that keep molecules and structures in place, followed by several rinsing and dehydration steps. Freeze fixation preserves biological specimens by the removal of thermal energy from the specimen at an extremely fast rate (> 10^5K/s) allowing the water in a biological specimen to reach a super-cooled stabilized state (vitrification). The general consensus in the scientific community is that ultrastructural preservation by high pressure freeze fixation is superior to that of chemical fixation, although the facts are that different fixation methodologies have dissimilar chemical and physical interactions with different specimens and as a result different artefact introductions. Therefore the best possible specimen preparation method to ensure an accurate likeness of the fixated specimen to its *in vivo* condition needs to be ascertained and used.

In this study, methods to fixate fibrin fiber and platelet networks by freeze fixation was developed, optimised and subsequently compared to chemical fixation methods to ascertain the optimum preparation technique for transmission and scanning electron microscopy for ultrastructural studies of platelets and fibrin networks.

Ultimately it was found that high pressure freezing coupled with freeze substitution is a superior method for fine structure preservation of fibrin fiber networks and platelets when utilizing transmission electron microscopy. Contrastingly for scanning electron microscopy ultrastructural studies it was found that chemical fixation is the more optimal method for the preparation of fibrin networks and platelets.

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DECLARATION

Full names of student: Antonia Vergina Buys

Student number: 26092345

Topic of work: “Development of an Ultrastructure Preservation protocol for Platelet and Fibrin networks utilizing High Pressure freezing and subsequent comparison of new and established protocols”

Declaration

1. I understand what plagiarism is and am aware of the University’s policy in this regard.

2. I declare that this dissertation is my own original work. Where other people’s work has been used (either from a printed source, internet or any other source), this has been properly acknowledged and referenced in accordance with departmental requirements.

3. I have not used work previously produced by another student or any other person to hand in as my own.

4. I have not allowed, and will not allow, anyone to copy my work with the intention of passing it off as his or her own work.

SIGNATURE.....

ACKNOWLEDGEMENTS

I would like to acknowledge with gratitude the following people and institutions:

Firstly and foremost I would like to thank the Lord my God and heavenly Father for the patience to see this project through and for the knowledge needed for interpretation of the results.

The Anatomy Department of the University of Pretoria, for granting me the opportunity to undertake and complete my studies. Also for the funding and hosting of the project.

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Last, but most definitely not least, my family. Very special thanks to my husband Riaan, for granting me the opportunity to further my studies, for all the times during the last five years you gave me emotional and financial support, I will always love you and treasure our time together. To my mother and father, thank you for all your unwavering love and support throughout my life and all my studies, not just this thesis. And to both my sisters; Aletia and Marisa thank you your love and resolute support, and an extra thanks to Marisa for the help with all the images in this thesis. I love you all so very much.

LIST OF PUBLICATIONS

Full articles:

Buys, AV and Pretorius, E 2011, 'Comparing different preparation methods to study human fibrin fibers and platelets using TEM', *Microscopy Research and Technique*, vol. 75, no. 6, pp. 801–806.

Articles in preparation:

Buys, AV and Pretorius .... 'The internal structure of the fibrin fibre visualised by TEM'

Table of Content

List of Tables and Figures

Table of Abbreviations, Symbols and Chemical Formulae

pm	picometer
nm	nanometer
TEM	transmission electron microscope
SEM	scanning electron microscope
HPF	high pressure freezing
DNA	deoxyribonucleic acid
mm^3	cubic millimeter
$C_5H_8O_2$	glutaraldehyde
mm	millimeter
CH_2O	formaldehyde
%	percentage
$HO\text{-}CH_2\text{-}OH$	methylene hydrate
$()_n$	$()_{number}$
OsO_4	osmium tetroxide
Tm	minimum melting temperature
K	Kelvin
Tn	minimum nucleation temperature
K/sec	Kelvin per second
µm	micrometer

ms	millisecond
MPa	megapascal
DDSA	dodecyl succinic anhydride
NMA	nadic methyl anhydride
NSA	nonenyl succinic anhydride
BDMA	benzyldimethylamine
DMP-30	tri(dimethylaminomethyl)phenol
DMAE	dimethylaminoethanol
DP	dibutyl phthalate
HMDS	hexamethyldisilazane
psi	pounds per square inch
CO_2	carbon dioxide
°C	degrees centigrade
Pa	Pascal
α	alpha
g	gram
g/l	grams per liter
β	beta
γ	gamma
ER	endoplasmic reticulum
kDA	kiloDalton
HMWK	high molecular weight kininogen

Factor XI	Plasma Thromboplastin Antecedent
Factor XII	Hageman Factor
Factor III	Tissue Factor
Factor VII	Stable Factor
Factor X	Stuart-Prower Factor
Factor II	Prothrombin
Factor I	Fibrinogen
Factor XIII	Fibrin stabilizing factor
FPA	fibrinopeptide A
FPB	fibrinopeptide B
factor XIII	fibrin stabilizing factor
ADP	adenosine diphosphate
PAS	plasminogen activators
tPA	tissue plasminogen activator
uPA	urokinase-type plasminogen activator
α2-AP	alpha2-antiplasmin
PAI-1	plasminogen activator inhibitor-1
PAI-2	plasminogen activator inhibitor-2
TAFI	thrombin-activatable fibrinolysis inhibitor
factor VIII	anti-hemophilic factor
factor IX	christmas factor
DTS	dense tubular system

EC	exterior coat
Me	cell membrane
SMF	sub-membrane filaments
MT	microtubules
G	glycogen
AG	α granules
MC	mitochondria
D	dense bodies
OCS	open canalicular system
GP	glycoprotein
VLA-6	very late activation antigen 6
IP3	inositisol-1,4,5-triphosphate
Ca^{2+}	calcium ion
TXA2	thromboxane A2
Mg^{2+}	magnesium ion
PDGF	platelet-derived growth factor
CTAP III	connecting tissue activating peptide III
factor V	pro-accelerin / labile factor
HPS	Hermansky-Pudlak syndrome
CHS	Chediak-Higashi syndrome
VWF	Von Willebrand factor
PAR	protease-activated receptor

HIV	human immunodeficiency virus
CD4	cluster of differentiation 4
AIDS	acquired immune deficiency syndrome
pm	integrin αIIβ3
PRP	PG-endoperoxide-H-synthase-1
GTP	guanosine triphosphate
PRP	platelet rich plasma
$CaCl_2$	calcium chloride
L	longitudinal
T	transverse
A	association
TF	thick fibers
B	branching
tF	thin fibers
IF	intermediate fibers
DC	dilated channel
P	pseudopodia
O	oligomers
BP	band pattern
Pl	platelet

Chapter 1:
Introduction

1.1. Introduction

Coagulation or thrombogenesis is of utmost importance to haemostasis and overall health as any dysfunction in coagulation may lead to either bleeding tendencies and events or thrombotic tendencies and events. The two most vital participants in thrombogenesis are the platelets and the fibrin network; with dysfunction in either of these two components leading to severe haematological implications. Abnormalities and dysfunction in fibrin networks and platelets have been shown to be well correlated to an altered morphology and ultrastructure of the particular component (Pretorius 2008). The fibrin network structure is a fundamental determinant in the progression of coagulation, when network rigidity and strength is increased above the norm which could be seen as either a dense network of numerous thin fibers or longitudinally fused thick fibers (Pretorius *et al.,* 2006) it is predisposed to thrombosis (Collet *et al.,* 2000 and Standeven *et al.,* 2005). Correspondingly a loose or flimsy fibrin network with increased porosity, made up from either thin or thick fibers leads to bleeding tendencies (Weisel 2005). Changes in platelet size, granularity and level of activation have been noted in several diseased conditions such as cancer, diabetes and HIV (Pretorius 2008; Pretorius *et al.,* 2009b and Zhuge *et al.,* 2009).

To study the ultrastructure of platelets and fibrin fibers high resolution imaging equipment is needed; at a potential resolution in the sub-nanometer scale, electron microscopes are the most powerful tools available for ultrastructural studies of biological materials. The resolving power of an electron microscope is due to the exploitation of energized electrons that has a significantly shorter wavelength of illumination (approximately 2-4pm (picometer), depending on the accelerating voltage) than that of a light microscope (approximately 400nm (nanometer)) (Bozzola & Russel 1999). For electron microscopy of

biological specimens, the specimen needs to be prepared by one of several methods, each comprised of a number of steps. The process begins with a living hydrated tissue and ends with tissue that is free of water and preserved in a static state within a resin matrix for transmission electron microscopy (TEM) and dehydrated state for scanning electron microscopy (SEM). Two of the main methods to achieve the aforementioned preparation of biological specimens for examination with an electron microscope are chemical and cryo fixation. Chemical fixation involves the chemical alteration of a specimen by means of the introduction of chemical bonds and cross-links that keep molecules and structures in place and stabilize the specimen. Several rinsing and dehydration steps, and ultimately infiltration and polymerization with an embedding medium followed by thin sectioning for TEM and drying for SEM, follow fixation. The second, freeze fixation involves the immobilization of all the molecules and the arrest of cellular metabolism in a biological specimen by the removal from the thermal energy in the system, facilitated by rapidly lowering the temperature to subzero temperatures (Vanhecke *et al.,* 2008) (high pressure freezing (HPF) or plunge freezing). This is called vitrification and is followed by the substitution of the vitrified water at low temperatures, and then similarly to chemical fixation, infiltration, polymerization and sectioning for TEM or freeze drying for SEM.

Attempting to stabilize a biological specimen in any way, may it be by the introduction of chemical linkages or by vitrification, is a process of alteration of the native state of the specimen, and with any alteration in state a modification in ultrastructure is expected. Alterations and the introduction of artefacts caused by the chemical fixation procedure is well known; in general the aldehydes and organic solvents used, lead to aggregation of proteins, collapse of hydrated carbohydrates, the removal and loss of lipids (Kellenberger *et al.,* 1992 and Studer *et al.,* 2008) and tissue shrinkage or swelling (Murk *et al.,* 2003 and Studer *et al.,* 1992). The general consensus in the scientific community is that ultrastructure preservation by HPF fixation is superior to that of chemical fixation (Giddings 2003; Porta & López-Iglesias 1998 and Studer *et al.,* 2008). Even though this method is believed to be superior, limitations such as the size limit of the sample that is capable of being effectively frozen, and also, possible artefact introduction should be noted. The

effect of the high pressures used in this method on biological structures is also a concern. It has been demonstrated that certain specimens such as the liquid crystal phase of deoxyribonucleic acid (DNA) is destroyed when frozen under high pressure (Leforestier *et al.,* 1996) and some lipid mixtures are structurally altered during HPF (Semmler *et al.,* 1998). Different fixation methodologies have different chemical and physical interactions with a specimen and therefore diverse artefact introduction; these artefacts could be useful as well as harmful (Griffiths *et al.,* 1993). The bottom-line with either choice of fixation technique is that consideration to the possible chemical and/or physical changes introduced is important to take into account.

1.2. Purpose of this Study

It is clear that the ultrastructure of the fibrin network and of the blood platelets is able to provide insight into and information about several diseased conditions. Preparation of these platelets and fibrin networks for electron microscopy is critically important since any distortions or artefacts introduced by the preparation process will ultimately be represented in the final images taken with the microscope. This could lead to conclusions that is at worst, erroneous and at best, interpreted in such a way that the uncertainty is taken into account. Therefore in this study freeze fixation techniques will be developed for the ultrastructural preservation for TEM and SEM applications and compared to equivalent chemical methods to determine the best possible processing technique. The different techniques will be compared with relation to their quality of preservation, financial cost, time, repeatability, expertise required and their safety of use.

1.3. Ethical Clearance

Blood was obtained by venipuncture from healthy volunteers without any known medical condition, who do not smoke and in the case of women, were not on contraception. Ethical

clearance was obtained from The Research Ethics Committee, Faculty Health Sciences, University of Pretoria, who complies with ICH-GCP guidelines and has US Federal wide Assurance; ethics clearance number: 151/2006, re-approved 2009.

Chapter 2:
Literature Review

2.1. Introduction

In this thesis different techniques of fixation for ultrastructural studies are being investigated. The current chapter includes an overview of chemical and HPF fixation together with further processing steps like dehydration and embedding for TEM and critical point drying and freeze-drying for SEM (Figure 2.1). Furthermore the coagulation and fibrinolytic pathways of the fibrin network and the function and aggregation process of blood platelets are described together with abnormalities of the aforementioned coagulation components.

2.2. Fixation

Anatomy has evolved from simple macroscopic studies of organisms to complex microscopic histological and ultrastructural studies at the cellular and even molecular level. When studying tissues or cells at these microscopic levels, it is necessary to prepare the tissue or cell for microscopy, the process begins with a living hydrated tissue and ends with tissue that is virtually water free and preserved in a static state within a plastic resin matrix for TEM and dehydrated state for SEM (see Figure 2.1). The first and most important part of this process is called fixation.

Several different techniques or methods of fixation are available depending on the desired results. Examples include; chemical fixation, freeze fixation and heat fixation. No matter which method is employed, the aim of fixation remains the same:

- To preserve the tissue or cell as close to its natural state as possible.
- To arrest any biological activity and metabolic processes of the cell.
- To prevent autolysis, by stopping intracellular enzyme activity.

- To prevent microbial attack and bacterial decomposition.
- To prevent loss and diffusion of soluble substances.
- To increase the tissue or cell's mechanical strength or stability.

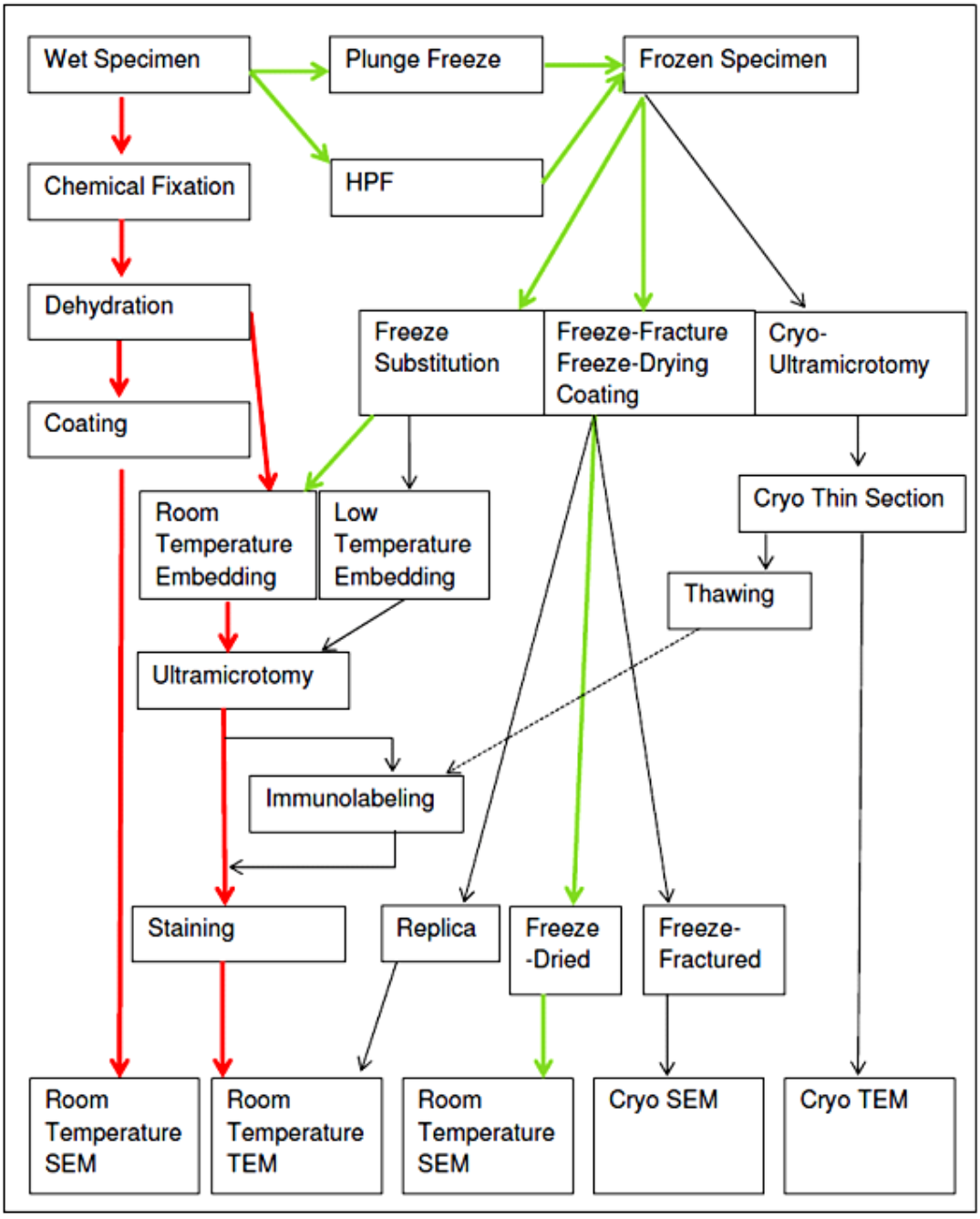


Figure 2.1: Preparation pathways for electron microscopy.

2.2.1. Chemical Fixation

Chemical fixation remains the most widely used fixation method for microscopic studies due to its well-known and documented parameters, wide variety of usages, comparative ease of use and relatively low cost.

Chemical fixation is achieved by employing chemical agents that permeate tissues and cells by diffusion and then by either covalently binding with their major biochemical constituents (lipids, proteins and carbohydrates) or denaturing and aggregating the proteins in the tissues, “fix” the components into place. Several such agents exist and the choice of which to use is usually made on the basis of the type of tissue, the type of research and the results required.

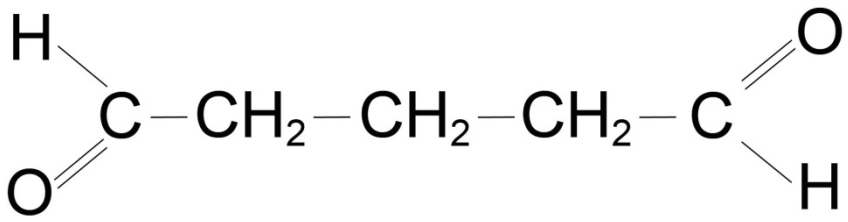
Regardless of the type of fixative employed, several factors affect the quality of preservation and to achieve good quality preservation certain parameters has to be met:

- Tissue specimen size; a tissue block of 0.5 cubic millimeters (mm^3) or less is a good standard (Hayat 2000).
- Osmolarity and osmolality; the fixative solution should be at the correct osmolarity and osmolality for the specific type of specimen (Glauert 1981 and Hayat 2000).
- Ionic composition of the fixative solution; the fixative’s ionic composition should be similar to the extracellular fluids of the specimen (Glauert 1981; Griffiths *et al.,* 1993 and Hayat 2000).
- Fixative pH; the pH of the fixative must remain close to the pH of the specimen (Glauert 1981 and Hayat 2000).
- Fixative penetration speed; an ideal fixative should penetrate the specimen rapidly (Hayat 2000).
- Temperature of fixation; the optimum fixation temperature should be used, this is dependent on the specimen and the fixative used (Griffiths *et al.,* 1993 and Hayat 2000).

- Duration of fixation; the optimum duration of fixation should be used, this is dependent on the specimen and the fixative used (Coetzee 1985; Griffiths *et al.,* 1993 and Hayat 2000).
- Concentration of the fixative; the optimum concentration is dependent on the duration, temperature and type of fixation (Coetzee 1985; Griffiths *et al.,* 1993 and Hayat 2000).
- The effects of added substances; when adding substances, such as dextran for the improved preservation of myelin, the osmolarity, osmolality, pH, penetration time of the fixative and the ionic composition of the fixative solution should be taken into consideration (Coetzee 1985; Griffiths *et al.,* 1993; Hayat 2000).

For ultra-structural studies, cross-linking agents such as the aldehydes are the most commonly used, usually with post fixation by agents such as osmium tetroxide, which enhance fine structure preservation and lipid fixation (Glauert 1981). For this reason; only glutaraldehyde, formaldehyde and osmium tetraoxide will be discussed in more detail.

2.2.1.1. Glutaraldehyde

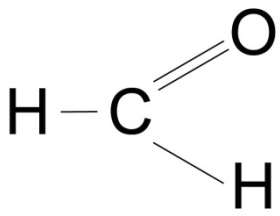


Glutaraldehyde ($C_5H_8O_2$) is a five carbon aliphatic dialdehyde with a molecular weight of 100, in which a hydrocarbon chain link two aldehyde moieties (Griffiths *et al.,* 1993 and Hayat 2000). The ability of glutaraldehyde for fine structure preservation is attributed to the terminal aldehyde groups that make it a bifunctional molecule being able to link two protein molecules (Hayat 2000 and Maunsbach & Afzelius 1999). This ability to crosslink proteins

together with the fact that it does so effectively and irreversibly before extraction by the buffer is able to occur, makes glutaraldehyde the preferred fixative for fine structure preservation of basically all living entities (Coetzee 1985; Griffiths *et al.,* 1993; Hayat 2000). One of the major drawbacks of glutaraldehyde is its slow rate of penetration, roughly 1 millimeter (mm) per hour into, especially compact, tissues (Maunsbach & Afzelius 1999).

The general reaction of glutaraldehyde with proteins is that the aldehyde group reacts with the protonated amine groups, forming pyridines and pyridine polymers of which the polymers forms cross-links that bridge randomly spaced primary amino groups in cells (Griffiths *et al.,* 1993 and Hayat 2000). Lysine is the most important protein component involved in the cross-linking reaction of glutaraldehyde, although glutaraldehyde reacts with other amino acids as well, including; tyrosine, tryptophan, histidine, phenylalanine and cysteine (Griffiths *et al.,* 1993 and Hayat 2000). Reactions with lipid and nucleic acids have not been extensively explored, although it is believed to react with amino groups in the lipid bilayer, such as phosphatidylserine, and in DNA, such as cytidine and guanine, and the polyhydroxyl compounds in carbohydrates is believed to be responsible for polymer formation in carbohydrate containing specimens (Hayat 2000).

2.2.1.2. Formaldehyde

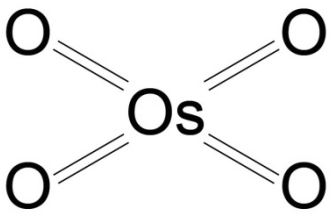


Formaldehyde (CH_2O) is a small molecule occurring naturally as a colourless, soluble gas (Griffiths *et al.,* 1993; Hayat 2000 and Kiernan 2000), it is dissolved in water to saturation at 37 – 40 percent (%) and combines chemically to form methylene hydrate ($HO\text{-}CH_2\text{-}OH$). Methylene hydrate has the same chemical reactivity as that of formaldehyde (Hayat 2000), further polymerization to dimers, trimers, etc with the formulae ($HO\ (CH_2O)_n$) also occur

(Griffiths *et al.,* 1993 and Hayat 2000). In reacting with proteins in a specimen, the functional aldehyde groups react with the uncharged, protonated amine groups of proteins to form methylol compounds, which condense with other groups [amino, amido, guanidine, thiol, phenolic, imidazolyl, lysine and indolyl (Hayat 2000)] to form methylene bridges, which in turn form cyclic compounds (Griffiths *et al.,* 1993 and Hayat 2000). These bridges are more slowly formed and less stable than cross links formed by glutaraldehyde (Griffiths *et al.,* 1993). It has been shown that formaldehyde react, to some extent, with unsaturated fatty acids, but tissues fixed with formaldehyde fail to preserve lipids after dehydration and embedding, suggesting a very weak interaction (Hayat 2000). Carbohydrates are not fixed by formaldehyde, whereas nucleic acids are very well preserved through covalent bonds of closely related DNA molecules (Hayat 2000).

The cross-linking abilities of formaldehyde is far less than glutaraldehyde, because formaldehyde contains only one functional aldehyde group (Griffiths *et al.,* 1993; Kiernan 2000 and Maunsbach & Afzelius 1999), but the small size of the molecule encourages high penetration rates (Glauert 1981 and Hayat 2000). For this reason, formaldehyde is generally used in combination with glutaraldehyde, as their respective chemical reactions complement each other, taking advantage of the rapid penetration of the small molecules of formaldehyde, and initiating structural stabilization together with the rapid and thorough cross-linking of the glutaraldehyde, better fixation is achieved (Glauert 1981 and Kiernan 2000).

2.2.1.3 Osmium Tetroxide



Osmium tetraoxide (OsO_4) is a tetrahedral, symmetrical, and therefore, non-polar molecule containing four double bonded oxygen molecules (Hayat 2000 and Maunsbach &

Afzelius 1999). The non-polarity of this molecule facilitates its diffusion through charged surfaces of tissues, cells and organelles (Hayat 2000). Osmium tetroxide oxidizes unsaturated double bonds of fatty acids; it is then reduced and deposited at the polar end of lipid molecules (Hayat 2000). The reduced osmium is a black metallic compound which is electron dense and adds contrast to biological tissues (Bozzola & Russel 1999 and Hayat 2000). This fixative also reacts with the sulfhydryl groups of certain amino acid side chains, acting as an inter-protein and / or intra-protein cross-linking agent (Bozzola & Russel 1999 and Hayat 2000). Protein–lipid cross-linking in lipid bilayers also occur which may build bridges between aliphatic chains of lipids and certain peptide bonds of proteins, immobilizing molecular motion in the lipid bilayer (Hayat 2000).

Osmium tetroxide is mainly used as a secondary fixative as it penetrates and reacts with tissue components very slowly, and considerable changes in the structures can occur before fixation is complete (Bozzola & Russel 1999 and Glauert 1981). Never the less, as a secondary fixative it has an essential role in fine structure preservation since it reacts with components of tissues, particularly lipids, that are not fixed by aldehydes. A further advantage is that osmium tetraoxide acts as an electron stain, as mentioned above, imparting high contrast to the osmiophilic structures in the specimen (Glauert 1981 and Hayat 2000).

2.2.2. Freeze Fixation

Chemical fixation has long been providing exceptionally useful information regarding biological structure and function, and it will continue to do so. The study of rapidly frozen or cryo-fixed specimens has confirmed much of the information derived from analysis of chemically fixed specimens, and although many features in chemically fixed and rapidly frozen samples resemble each other, there are some important differences, as chemical fixation has its limitations. Chemical fixatives has a normal delay in diffusion, making it impossible to capture certain dynamic processes that occur in milliseconds, additionally fixation with aldehydes involves oxygen consumption and hydronium ion production, thereby depriving the tissue of oxygen before fixation is completed. For example, the crystal-like configuration of mitochondria is considerably altered when fixated with

chemical compounds (Hayat 2000). Further chemical (fixation and dehydration) induced artefacts includes, but is not limited to, shrinkage or swelling of organelles, membrane fusion, alteration of fracture planes of membranes, rearrangement of intramembranous particles and membrane junction changes (Hayat 2000; Studer *et al.,* 2001 and Vanhecke *et al.,* 2008).

Cryo-fixation is a physical fixation procedure used to preserve the distribution and structure of all components in a biological system. The principal aim and advantage is the near instantaneous arrest of the cellular metabolism whereby soluble cell constituents is stabilized and retained in the native three dimensional structures allowing for analysis of specimens very close to the living state.

As with chemical fixation certain parameters have to be met to ensure good fixation. If summarized to one word, it would be vitrification: the instant solidification of a solution by the extreme increase of viscosity during cooling without ice crystallization.

Crystallization is limited to the temperature between the melting point (Tm) (273 Kelvin (K)) and the de-vitrification or recrystallization point (173K) below which the energy is not sufficient for the formation of new crystals (Moor 1987 and Shimoni & Müller 1998). When water is cooled below its freezing point (273K), it doesn't freeze immediately, but remains in a super-cooled liquid state until about 233K (nucleation point (Tn)), at which molecules act as seeds for crystallization. This crystallization is accompanied by the release of latent heat of fusion, which heats the water to its melting point producing a mixture of ice and water (Morphew 2000 and Quintana 1994). The system will then remain at the same temperature until all the water is completely transformed into one of three solid states; hexagonal crystals, cubic crystals or the vitreous or amorphous state; depending on crystallization kinetics of the specific system. The third vitrified state is believed to be a faithful representation of the liquid state (Morphew 2000 and Quintana 1994). The vitreous or amorphous state is achieved when the liquid state is cooled to below the recrystallization point, and the latent heat of fusion is removed from the specimen more quickly than it is produced, avoiding phase separation and leading to a solid state without any crystal formation (Hayat 2000; Shimoni & Müller 1998 and Quintana 1994), in other

words avoiding or more accurately passing immensely quickly through the two temperatures (melting point and recrystallization / de-vitrification point) between which ice crystal formation occur. The rate of cooling is therefore very important; for pure water a rate of $3x10^6$ Kelvin per second (K/sec) is needed. In biological samples with low concentrations of free water, local cooling rates may be higher, 10^4-10^5K/sec, because water crystallizes by heterogeneous nucleation and therefore the zone of crystallization is reduced (Moor 1987; Quintana 1994 and Vanhecke *et al.,* 2008).

One of the major problems encountered during the freezing of tissues is not the rate of cooling but the poor thermal conductivity of the tissue, which impede the cooling rate towards the centre of the sample, resulting in ice crystal formation. Most biological samples can rarely be vitrified to a depth exceeding a few micrometres (µm), resulting that the necessary freezing rates can only be achieved for very thin layers <20µm (Morphew 2000 and Vanhecke *et al.,* 2008).

2.2.2.1. High Pressure Freezing

Improved vitrification or vitrification of thicker samples can only be achieved by changing the physical properties of the water in the sample, for example; by lowering the melting point, reducing the nucleation temperature and rate of ice crystal nucleation or slowing the growth of ice crystals (Shimoni & Müller 1998). At high pressures (about 2.050 bar) the melting temperature of water drops to about 251K, water in the sample does not begin to crystallize until the temperature reaches 183K at which the rate of ice crystal formation is also reduced (Bozzola & Russel 1999; Dahl & Staehelin 1989; Hayat 2000 and Shimoni & Müller 1998) (Figure 2.2). The depth of vitrification is about 10 times larger when freezing at high pressures. Theoretically specimens up to 0.6mm thick can be immobilized (Hayat 2000 and Humbel & Müller 1984).

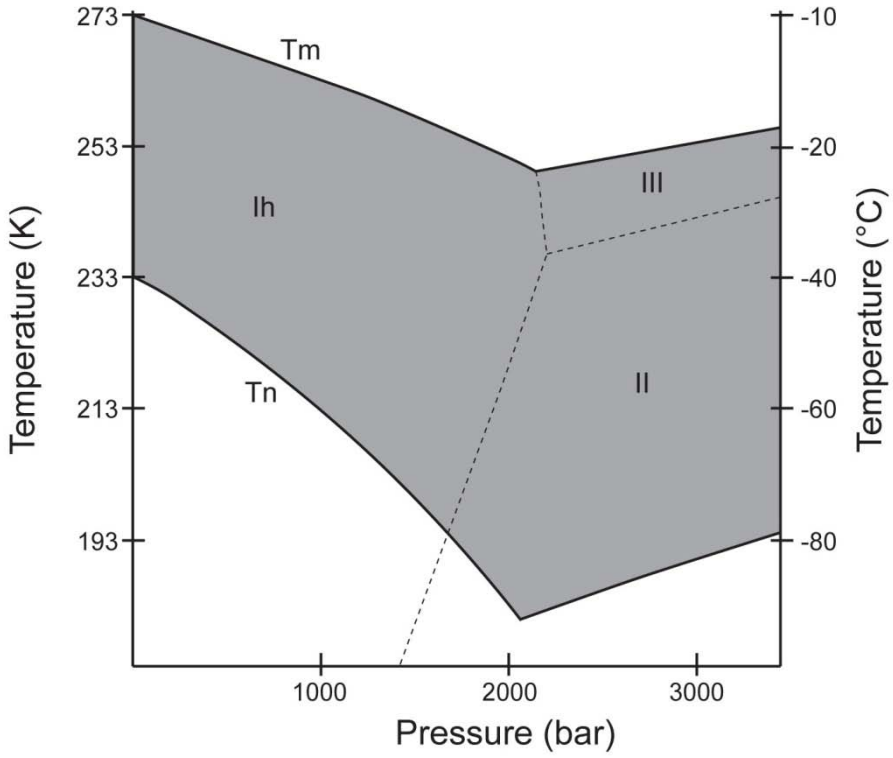


Figure 2.2: Part of the phase diagram of water.

The phase diagram shows the minimal melting temperature (Tm) and the minimal nucleation temperature (Tn) around 2000 bar. The grey area indicates at what temperatures and pressures super-cooled water can exist. The best situation for high pressure freezing is at about 2000 bar, where the melting point is at a minimum and the possibility for super-cooling is maximized (Vanhecke *et al.,* 2008).

When using HPF apparatus the specimen is transferred into a specimen carrier (several types of carriers exist and the choice of which to use depends on the type of specimen and experiment). This specimen carrier is secured onto a loading device which in turn is inserted into the HPF instrument. The specimen and its carrier is inserted into a pressure chamber filled with methylcyclohexane, which is, through a series of valves and pistons, firstly pre-cooled with liquid nitrogen and then a compression is applied generating over 2000 bar of pressure in less than 10 milliseconds (ms) (Studer *et al.,* 2001 and Vanhecke

et al., 2008). Once the required pressure is achieved the temperature is decreased by propelling a jet of liquid nitrogen onto the specimen, cooling the specimen down to approximately liquid nitrogen's boiling point, 77K (Figure 2.3) (Vanhecke *et al.,* 2008). The sample is then automatically brought into a liquid nitrogen bath (this procedure is true for the Leica EMPACT, different instrument's procedure may vary) (Studer *et al.,* 2001). This method has the highest ability to avoid ice crystal artefacts.

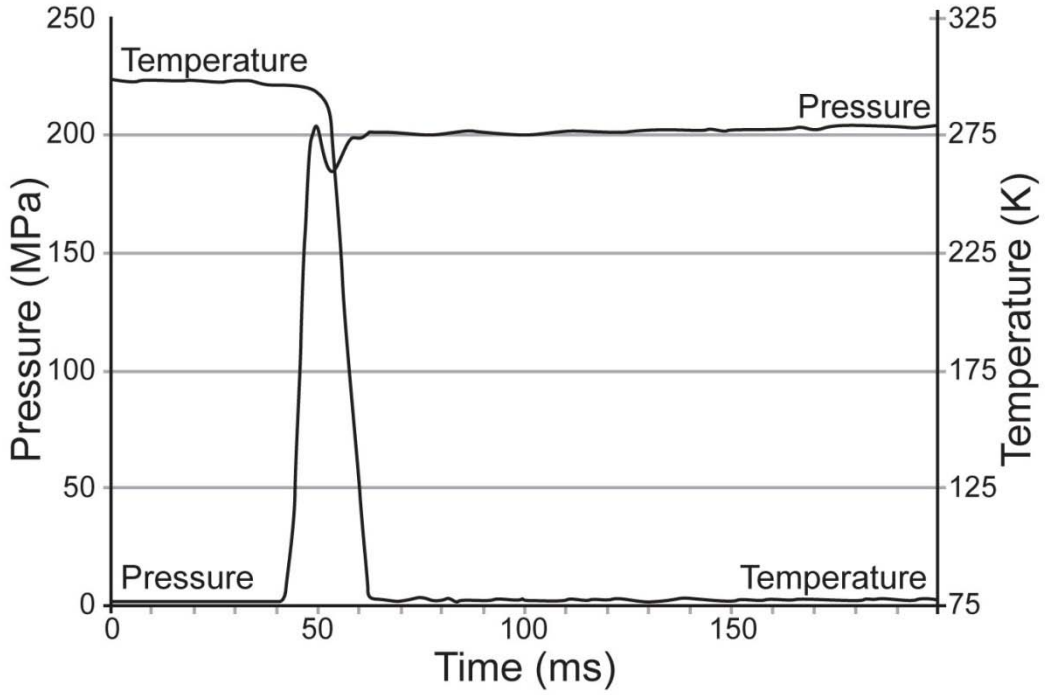


Figure 2.3: The process of HPF (Leica EMPACT 2).
The pressure is increased to 200 megapascal (MPa) or 2000 bar within 10ms, followed by a temperature drop to about 77K (Vanhecke *et al.,* 2008).

2.2.2.2. Plunge Freezing

The plunge-freezing method involves dipping the specimen into a cryogen by hand or by plunging the specimen with the aid of a spring loaded or gravity-driven device. This method is the simplest, least expensive and most widely used procedure when referring to freeze fixation (Bozzola & Russel 1999 and Hayat 2000).

An effective cryogen must have high thermal conductivity and heat capacity, a low melting point at atmospheric pressures, a high specific heat, and a wide temperature range between its freezing and boiling points (Hayat 2000). Several different cryogens has been used for plunge freezing, including; liquid propane, mixtures of liquid propane and isopentane, liquid nitrogen, solid-liquid nitrogen slush, ethane and freons (Hayat 2000). Liquid ethane and propane, cooled by liquid nitrogen is one of the best coolants for general use. It has a low melting point (84K), high boiling point (231K) and good thermal conductivity and specific heat. It is also readily available and relatively inexpensive. Apart from the choice of cryogen the size of the specimen and the rate of plunging are the most important parameters. Plunging should be performed as fast as possible through sufficient depth of cryogen producing a freezing progression that is completed during the plunging motion by continuous cryogen renewal all around the specimen (Hayat 2000).

2.3. Rinsing, Dehydration and Embedding

For examination with a TEM, biological specimens need to be sectioned into thin sections, roughly between a 50nm to 100nm range, to permit electrons to travel through the sample. Unfixed samples or chemically fixed samples is too fragile for ultramicrotomy, and therefore need to be strengthened and hardened by infiltration and embedment in a suitable medium. To accomplish this all the traces of the fixative and also all the water in the sample needs to be removed as most of the common embedding media is not miscible with water and react with certain chemical compounds. Likewise, when preparing a sample for SEM inspection, the excess chemical fixatives and water need to be removed as a specimen must be dry to be viewed in a SEM.

2.3.1. Rinsing

After primary and secondary fixation, it is important to remove all unreacted aldehydes and oxides to avoid any possible reaction with the dehydrating agent. In double fixation traces of aldehyde left in the specimen will be oxidised by osmium tetroxide and produce a dark

precipitate of reduced osmium, obscuring valuable structural data (Bozzola & Russel 1999 and Hayat 2000), similarly osmium may react with alcohol.

It is important to use the same buffer vehicle for rinsing the sample than was used as a fixative vehicle to ensure similar osmolarity for the specimen, as even fixated tissue membranes retain differential permeability to a certain extent. Drastic changes in the environment of the specimen such as using a buffered fixative vehicle and then rinsing with water is very destructive to ultrastructure preservation, as dissolution and progressive disintegration of any unfixed materials will occur if electrolytes or non electrolytes are removed and not rapidly replaced (Bozzola & Russel 1999 and Hayat 2000).

Generally two to three rinses for a total period of roughly 20 minutes is adequate for most specimens, although care should be taken as some tissues is known to require as much as 24 hours of washing (Hayat 2000).

2.3.2. Chemical Dehydration

Bozzola and Russel (1999) described dehydration as the process of replacing water in a biological specimen with a fluid that acts as a solvent between the aqueous environment of the cell and the hydrophobic embedding media. Most embedding media are not soluble in water, implying that a resin will not be able to infiltrate the specimen and polymerize in the presence of water, all the water in the sample needs to be removed. This is accomplished by chemically displacing the water by substituting it with an organic compound that is both miscible with water and with the embedding monomer (Bozzola & Russel 1999; Glauert 1981; Hayat 2000 and Maunsbach & Afzelius 1999). The most commonly used dehydrating agents are ethanol and acetone, with ethanol being the most widely used (Bozzola & Russel 1999). The replacement of water by the dehydrating agent is a gradual process and is accomplished by using a graded series of the dehydrating agent with decreasing amounts of water. When ethanol is used, the use of a transitional solvent that is highly compatible with the embedding media is beneficial to interface with the embedding media, as ethanol is not as readily miscible with embedding resins (Bozzola & Russel 1999; Glauert 1981; Hayat 2000 and Maunsbach & Afzelius 1999). Propylene oxide is normally used for this purpose, and is used at the last stages of dehydration

(Bozzola & Russel 1999 Hayat 2000 and Maunsbach & Afzelius 1999). Propylene oxide has the added advantage that it doesn't separate from the embedding media during polymerization; therefore if small amounts of propylene oxide are left in the embedding media, polymerization will not be affected (Hayat 2000). When using acetone, the use of propylene oxide as an intermediary solvent is not necessary as acetone is completely miscible with most embedding mediums (Hayat 2000).

Two typical protocols for dehydration are (Bozzola & Russel 1999 and Glauert 1981);

1. 30% ethanol in water 10 minutes
2. 50% ethanol in water 10 minutes
3. 70% ethanol in water 10 minutes
4. 90% ethanol in water 10 minutes
5. 100% ethanol 15 minutes
6. 100% ethanol 15 minutes
7. 100% ethanol 15 minutes
8. 100% propylene oxide 15 minutes
9. 100% propylene oxide 15 minutes

1. 30% acetone in water 10 minutes
2. 50% acetone in water 10 minutes
3. 70% acetone in water 10 minutes
4. 90% acetone in water 10 minutes
5. 100% acetone 15 minutes
6. 100% acetone 15 minutes
7. 100% acetone 15 minutes

The highly hygroscopic nature of acetone, ethanol and propylene oxide poses a difficulty as these solvents absorb water from the atmosphere (Bozzola & Russel 1999). This dilutes the concentration of dehydration agents rendering them incapable of complete dehydration and subsequently interferes with polymerization of the resin (Bozzola &

Russel 1999 and Hayat 2000). Special care should be taken to avoid unnecessary exposure to air.

Because all the above-mentioned dehydrating agents are extremely strong organic solvents, volume changes of the specimen and extraction of cell constituents is regrettably inevitable (Bozzola & Russel 1999; Glauert 1981; Hayat 2000 and Maunsbach & Afzelius 1999). During dehydration, a noteworthy loss of tissue components such as carbohydrates, even proteins and especially lipids occur, and shrinkage from 5-70% has been shown (Hayat 2000). The extent of the undesirable effects of dehydration is dependent on several factors, including; the size and type of specimen, the fixative used, the effectiveness of the stabilization by the fixative, the dehydrating agent employed, the duration of dehydration and also the temperature at which dehydration takes place (Bozzola & Russel 1999 and Hayat 2000). To minimize these effects, the specimen should be kept small, fixation should be optimized, the correct dehydration agent should be chosen for the specific specimen, the duration of dehydration should be carried out as rapidly as possible (however, long enough to ensure complete dehydration), and dehydration should be performed at low temperatures (Bozzola & Russel 1999; Glauert 1981 and Hayat 2000).

2.3.3. Freeze Substitution

Rapid freezing procedures such as HPF are followed by its complimentary dehydration step, called freeze substitution. Similar to chemical dehydration, freeze substitution is essentially the replacement of water in the form of amorphous ice with an organic solvent, only at low temperatures (Glauert 1981; Hayat 2000; Humbel & Müller 1984; McDonald & Webb 2011 and Shiurba 2001). The most commonly used solvent for freeze substitution is acetone, but other solvents used include, methanol, ethanol, heptane and diethyl ether (Hayat 2000). The frozen specimen and solvent is kept at low temperatures of about 193K to 178K over a certain period of time during which the organic solvent dissolves and substitutes the ice (Bozzola & Russel 1999 and Hayat 2000). These temperatures are below the critical temperature of crystallization; at which the crystal growth of water within cells will distort and tear the structures of the specimen (Glauert 1981; Steinbrecht &

Müller 1987 and Wild *et al.,* 2011). After completion of freeze substitution the temperature can be raised without danger of damage, given that the water is now replaced by the organic solvent (Steinbrecht & Müller 1987).

With water miscible solvents such as the aforementioned, macromolecules precipitate into coarse aggregates at room temperature when the solvent content of the substitution mix reach a specific threshold, situated between 40 to 70% (Bohrmann & Kellenberger 2001). The solubility of biological macromolecules is determined by the ordered arrangement of the water dipoles at the surface of macromolecules or so-called hydration shells (Kellenberger 1987). In normal room temperature dehydration, these hydration shells are removed, leading to precipitation and aggregation (Bohrmann & Kellenberger 2001 and Kellenberger 1991). In this situation macromolecules are cross-linked by fixatives prior to dehydration, transforming the solution into a gel able to withstand the aggregation caused by dehydration. In freeze substitution the theory is that these hydration shells are completely or only partially removed during the freeze substitution process, but due to the low temperatures, the surrounding solvent is increasingly viscous and only allow exceedingly slow thermal movement, so cross-links will occur at a reduced rate and the "gel" will build up very slowly resulting in the prevention of coarse aggregation (Bohrmann & Kellenberger 2001). This reasoning is sound when applied to fibrous material, like proteins and nucleic acids, but when applied to more globular material as found in the cell cytoplasm it is less convincing (Bohrmann & Kellenberger 2001).

It has been shown that freeze substitution with only an organic solvent present is possible to ensure fine structure preservation and avoid precipitation (Bohrmann & Kellenberger 2001; Humbel & Müller 1984; Steinbrecht & Müller 1987 and von Schack *et al.,* 1993); however satisfactory results are difficult to reproduce repeatedly. Because of the ambiguity in the research community of "fixation" during freeze substitution using only an organic solvent, especially when working with complex structures such as cells, a fixative is usually added to the substitution media (Bohrmann & Kellenberger 2001; Giddings 2003; Hawes *et al.,* 2007; Humbel & Müller 1984 and Wild *et al.,* 2011). Several fixatives, alone or in combination, has been employed for this purpose. A few of these include; osmium

tetroxide, glutaraldehyde, tannic acid and uranyl acetate (Giddings 2003 and Hawes *et al.,* 2007). Further adaptations to the freeze substitution mixture for the preservation of specific cell constituents have also been suggested, for example Walther & Ziegler (2002) found that the addition of 1–5% water enhanced the preservation of plasma membranes.

Many different protocols for freeze substitution exists, varying in the solvent used, the fixative used, the duration of substitution [from 6 days (Edelmann 1991) to just a few hours (McDonald & Webb 2011)], the rate of temperature rise [typically in the range of 1-10°C (degrees Celsius) per hour (McDonald & Webb 2011)] and the final temperature [(243K to 277K) (Wild *et al.,* 2011)].

A few basic protocols for freeze substitution for ultrastructure preservation and some of its variations are (Morphew 2000):

- 1% Osmium tetroxide in acetone.
- Transfer frozen samples into frozen fixative under liquid nitrogen.
- Transfer frozen vials with samples to pre-cooled (-90°C) substitution vessel.
- Substitute for 3 days at -90°C.
- Warm samples to room temperature over 18 hours - two days.

- 0.1% Uranyl acetate + 1% osmium tetroxide + 0.1% water + acetone (optimized for membrane contrast).
- Transfer frozen samples into frozen fixative under liquid nitrogen.
- Transfer frozen vials with samples to pre-cooled (-90°C) substitution vessel.
- Substitute for 3 days at -90°C.
- Warm samples to room temperature over 18 hours - two days.

- 0.1% Tannic acid + acetone (optimized for filament preservation).
- Transfer frozen samples into frozen fixative under liquid nitrogen.
- Transfer frozen vials with samples to pre-cooled (-90°C) substitution vessel.

- Substitute for 1 day at -90°C.
- Rinse three times with acetone at -90°C.
- Add pre-cooled 1% osmium tetroxide + 0.1% uranyl acetate + acetone and leave for two days at 90°C.
- Warm samples to room temperature, and rinse three times with acetone.

- 0.1% Tannic acid + 0.5% glutaraldehyde + acetone (optimized for ultrastructure preservation of thin monolayers of cultured cells).
- Transfer frozen samples into frozen fixative under liquid nitrogen.
- Transfer frozen vials with samples to pre-cooled (-90°C) substitution vessel.
- Substitute for 1 day at -90°C.
- Rinse three times with acetone at -90°C.
- Add pre-cooled 1% osmium tetroxide + 0.1% uranyl acetate + acetone and leave for two days at 90°C.
- Warm samples to room temperature, and rinse three times with acetone.

2.3.4. Embedding for TEM

The sole purposes of embedding a specimen in embedding media or resin are to strengthen and harden the specimen to such an extent that it is possible to cut the specimen sufficiently thin for viewing in a TEM. This is accomplished by infiltrating the specimen with a liquid embedding medium which is then polymerized to form a hard block.

Numerous types of embedding media have been experimented with and several are currently in general use (Glauert 1981 and University of Georgia, Center for Ultrastructural Research 2011).
Ideally embedding media should have the following properties:

- The media should be readily available (Glauert 1981).
- The media should have uniformity from one batch to another (Glauert 1981).
- The media should be stable during storage.

- The monomer of the media should have low viscosity and atomic weight (Glauert 1981 and University of Georgia, Center for Ultrastructural Research 2011).
- The media should be soluble in the dehydrating agents (Glauert 1981).
- The media should polymerize smoothly and uniformly (Glauert 1981 and Hayat 2000).
- The media should polymerize with as little as possible volume changes, such as shrinkage (Glauert 1981 and Hayat 2000).
- The media should not chemically alter the specimen, by extraction or modification of the histochemical properties (University of Georgia, Center for Ultrastructural Research 2011).
- The polymerized resin should bind tightly with the specimen as to ensure it doesn't pull away from the specimen during sectioning (Glauert 1981 and Hayat 2000)
- The mechanical properties (hardness, plasticity, etcetera) of the resin should enhance ease of sectioning (Glauert 1981).
- The final polymer's density should be low as to be transparent to the passage of electrons (University of Georgia, Center for Ultrastructural Research 2011).
- The resin should be stable in the electron beam (Glauert 1981 and University of Georgia, Center for Ultrastructural Research 2011).

The three main types of embedding media are the epoxy resins, the polyester resins and the methacrylates (Bozzola & Russel 1999; Glauert 1981; Hayat 2000 and University of Georgia, Center for Ultrastructural Research 2011). None of these resins possess all the desired qualities and in choosing a specific resin certain desired properties will be sacrificed. For example a resin which causes little shrinkage during polymerization usually has a high initial viscosity (Glauert 1981).

Epoxy resins are the most commonly used as they have most of the required properties, and it is also the embedding medium of choice for this study, for this reason only epoxy resins will be discussed in detail.

Epoxy resins are poly-aryl ethers of glycerol with a terminal epoxy group which is capable, in the presence of a curing agent, of reacting indiscriminately with available hydrogen molecules from any source. This leads to cross-links of the epoxy group with other epoxy groups (homo-polymerization) but also with hydroxyl molecules (addition) in the specimen itself (Bhatnagar 1996; Bozzola & Russel 1999; Glauert 1981; Hayat 2000 and University of Georgia, Centre for Ultrastructural Research 2011). Unpolimerized epoxy monomers exists in a highly associated state, this fact together with the afore mentioned reactions, leads to a thermoset product with very uniform polymerization and little shrinkage (Bhatnagar 1996; Glauert 1981 and Hayat 2000). These qualities in addition to the fact that the epoxy resins are relatively stable under an electron beam are the main reasons they are the most popular types of resins used (Glauert 1981). Epoxy resin's biggest disadvantage is the high initial viscosity, which necessitating long infiltration times (Bozzola & Russel 1999; Glauert 1981 and University of Georgia, Center for Ultrastructural Research 2011).

The curing agents normally used are either organic anhydrides (cross-linkers or hardeners) or tertiary amines (catalyst), in most laboratories both are employed (Hayat 2000). The anhydrides participate directly in the reaction and are absorbed in the resin chain while forming cross-links between the resin molecules, whereas the amines only promote epoxy-epoxy and epoxy-hydroxyl reactions, never serving as a direct cross-linking agent itself (Hayat 2000). The reactions with curing agents leave the subsequent resin block hard and rigid, so several modifiers are employed to optimize the qualities of the resin. Plasticizers prevent excessive brittleness and increase the softness of the block, flexibilizers increase the flexibility of the resin, and both increase the ease of ultra-thin sectioning (Glauert 1981 and Hayat 2000).

Many different types of epoxies, hardeners, catalysts, plasticizers, flexibilizers are available (see Table 2.1 for some common products), all of which has different properties and effects on the final product.

Table 2.1: Commercially available epoxy resins and their modifiers.
(Bozzola & Russel 1999; Glauert 1981; Hayat 2000 and University of Georgia, Center for Ultrastructural Research 2011)

Epoxies	Araldite
	Agar 100 (Agar)
	Quetol 812 (Nishin)
	Maraglas 655
	Epon 812 (Shell)
	ERL 4206 (Spurr)
Hardeners	Dodecyl succinic anhydride (DDSA)
	Hexahydrophtalic anhydride
	Nadic methyl anhydride (NMA)
	Nonenyl succinic anhydride (NSA)
Catalysts	Benzyldimethylamine (BDMA)
	Tri(dimethylaminomethyl)phenol (DMP-30)
	Azodiisobutyronitril
	Dimethylaminoethanol (DMAE)
Plasticizers	Dibutyl phthalate (DP)
Flexibilizers	Cardolite NC-513
	Thiokol LP-8
	DER 736

The desired properties of the final thermoset resin block can be controlled by the choice of one type of constituent over another and the ratios in which they are employed, extensive

tests and experiments have been conducted to establish recipes for specific purposes (for a comprehensive review, see Bozzola & Russel 1999, and Glauert 1981).

Epoxy resins are infiltrated into the sample through a series of graded solution of the resin and dehydrating agent, until all the dehydrating agent is replaced by the embedding media.

A typical protocol for this procedure is:

1. 3:1 dehydrating agent: embedding medium - 60 minutes.
2. 1:1 dehydrating agent: embedding medium - 60 minutes.
3. 1:3 dehydrating agent: embedding medium - 60 minutes.
4. 0:1 dehydrating agent: embedding medium - 240 minutes.
5. Place specimen in embedding container containing fresh embedding medium.
6. Polymerize at 60°C for 48 hours

Freshly prepared embedding media should be used as the mixture increases its viscosity over time and may also become unstable. It is advantageous to agitate the mixture in steps one to four, as this will increase the infiltration of the viscous media into the specimen. When placing the specimen into the embedding container it is important to make sure that the container is clean and dry, and the orientation of the specimen must be kept in mind to expedite the ease of sectioning. Also, a label should be placed in the container to keep track of the specimen. This procedure should be carried out with care, as epoxy resins and their additives may cause dermatitis and the vapours are often toxic (Glauert 1981).

Methacrylates has a very low viscosity, making infiltration easy and rapid, they are also inexpensive, easy to use and relatively non-toxic (University of Georgia, Center for Ultrastructural Research 2011). The biggest disadvantage of the methacrylates is that it

polymerizes unevenly as swelling and distortion of the structure of the specimen may occur. They are also strong lipid solvents and are unstable under electron bombardment (Glauert 1981 and University of Georgia, Center for Ultrastructural Research 2011), which has rendered them unsuitable for ultrastructural research. Polyester resins has similar good properties to that of epoxy resins, but is less readily available, not stable during storage and more unstable under electron bombardment than epoxy resins (Glauert 1981 and University of Georgia, Center for Ultrastructural Research 2011). Several other types of embedding media exist, including but not limited to, water soluble resins, acrylic resins, polyethylene glycol resins and aldehyde resins. Most of these resins are used for very specific types of research such as immunocytochemistry (Bozzola & Russel 1999; Glauert 1981; Hayat 2000 and University of Georgia, Center for Ultrastructural Research 2011).

2.4. Sectioning for TEM

Fixation, dehydration and embedding has al lead up to this final step of ultra thin sectioning or ultramicrotomy. It is known to be the most difficult preparatory step of sample preparation. The goal of ultramicrotomy is to cut sections from the embedded specimen thin enough for electrons in the TEM to penetrate, which should be in the range of 50-100nm but still able to withstand the high vacuum and electron bombardment. The thickness of the section is not the only challenge as the quality of the section is of utmost importance.The section should be of uniform thickness, free from chatter marks, wrinkles, breaks and folds. To accomplish this takes practise, patience and skill.

The tool used for ultramicrotomy is aptly called an ultramicrotome. The ultramicrotome is a highly specialised instrument that advances a specimen by thermal or mechanical movement in precise repeatable steps towards a cutting surface (Bozzola & Russel 1999; Hayat 2000 and University of Georgia, Center for Ultrastructural Research 2011) (Figure 2.4b). The cutting surfaces used during this process are glass knife edges, which can be made with the correct equipment, and specialized sharpened diamond knifes, which has to

be bought.

The fundamental process will be briefly explained and illustrated (Figure 2.4):

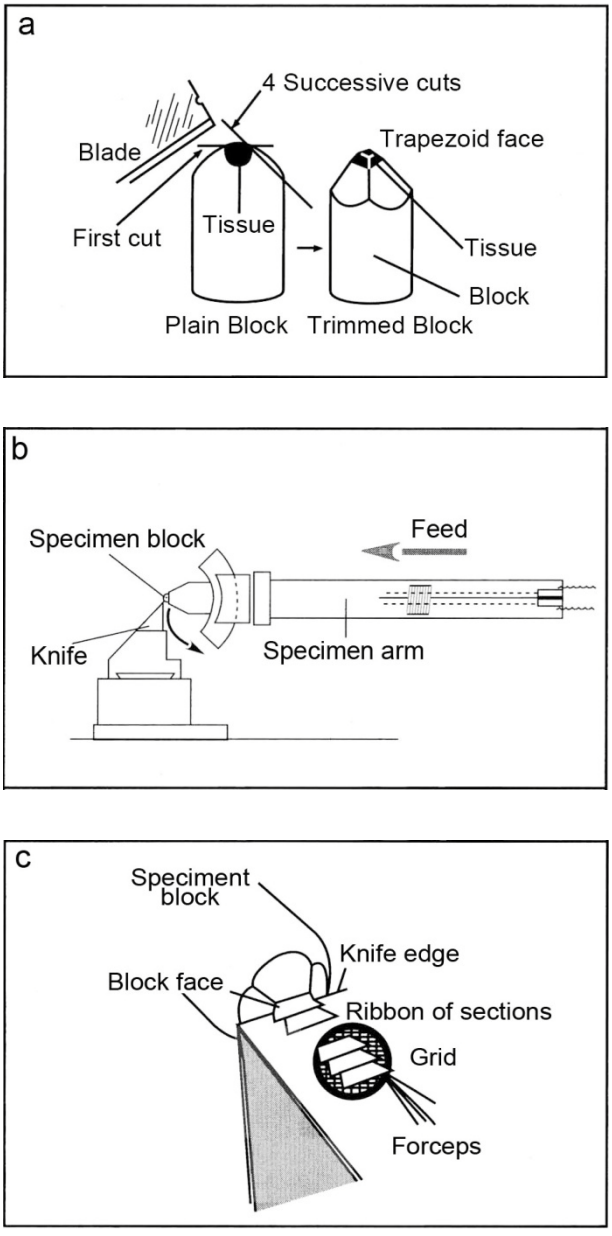


Figure 2.4: Illustration of steps involved in the ultramicrotomy process.

a: Trimming of the resin block to expose the specimen and form a trapezoid cutting face. b: The trimmed specimen block is placed into the ultramicrotome for thick and thin sectioning. c: Fine sectioning of the specimen, forming a ribbon of thin sections, which are picked up with a TEM grid (redrawn and adapted from Bozzola & Russel 1999).

Shaping the specimen block

The excess resin has to be trimmed away and the block face should be shaped into the correct size (0.5mm or less) and form (trapezoidal to rectangular) (Bozzola & Russel 1999 and University of Georgia, Center for Ultrastructural Research 2011). The first step is hand trimming where the specimen block is secured and a one-edged razor blade is used to carefully and thinly slice away excess resin leaving a pyramidal (side-view) trapezoid (top-view) shape (Figure 2.4a). Mechanical trimming using a tilted glass knife, can also be used if available (Bozzola & Russel 1999 and Hayat 2000).

Thick sectioning

Thick sectioning is done with a glass knife if orientation or a preview of the sample is needed and it also accomplish flattening of the block face. The sample is mounted into the ultramicrotome, aligned with the knife (Figure 2.4b), and sections of between 0.5 – 2µm are cut. The mounting of the sample and its alignment with the knife is of critical importance as unnecessary forces or stress and strain on both the knife and the resin block should be avoided. Also, the bases of the trapezoid resin block should be parallel to the cutting surface of the knife. Thick sections are floated of from the knife edge on a water surface, picked up and placed on a glass microscope slide for inspection with a light microscope.

Fine trimming

After the thick sections have been surveyed, the resin block can be trimmed further around the area of interest if needed in the same way as the initial shaping of the block face as described above. This is also the time to ensure the block face geometry is correct.

Thin sectioning

Fine sectioning is performed with a diamond knife, which is fixed in its holder that incorporates a trough (Hayat 2000) filled with distilled water. The sample is again aligned with the knife edge and is then automatically moved forward and up and down to cut sections of approximately 100nm. If the alignment and block geometry is correct, serial sections will form a ribbon on the water which is easily picked up with a grid held by a pair of sharp-tipped forceps (Figure 2.4c).

This is a very basic explanation of the procedure and do not take into consideration other very important factors of thin sectioning, such as preparation and maintenance of the knifes, sectioning angles, cutting range adjustments, cutting speed, thickness assessment, artefact trouble-shooting and environmental factors influencing sectioning, to name only a few. For a more detailed review see Bozzola & Russel 1999; Hayat 2000 and University of Georgia, Center for Ultrastructural Research 2011).

2.5. Drying Specimens for SEM

To study the surface morphology of biological specimens with a SEM, the specimens needs to be prepared for the high vacuum environment they will encounter in the microscope. The first preparation steps (fixation, rinsing and dehydration) have already been discussed, but one of the final steps is the complete desiccation of the specimen. When drying a specimen, special care needs to be taken to avoid deformation and collapse of the structures. The principal cause of such damage is surface tension (Quorum Technologies 2009).

Surface tension is a property of liquids due to the effect of unbalanced intermolecular attraction forces at the boundary of the liquid. The water molecules within a liquid biological environment associate with each other and also with polar biological molecules. These interactions form a hydration shell around the biological molecules and when the liquid evaporates the liquid-air interface or phase boundary will at some point encounter the surface of the specimen. The ensuing surface tension and removal of hydration shells exerts substantial forces on the specimen which presses the tissue flat, producing collapse of the structures and artefact introduction (Anderson 1951; Smith & Finke 1972, and Quorum Technologies 2009). The main concern therefore in drying specimens for SEM investigation is to lower or completely remove these surface tension forces on the specimen.

The only way to accomplish this is to chemically or thermodynamically alter the drying process (Figure 2.5), and thereby transitioning from one phase (liquid, vapour or solid) to another with greater ease.

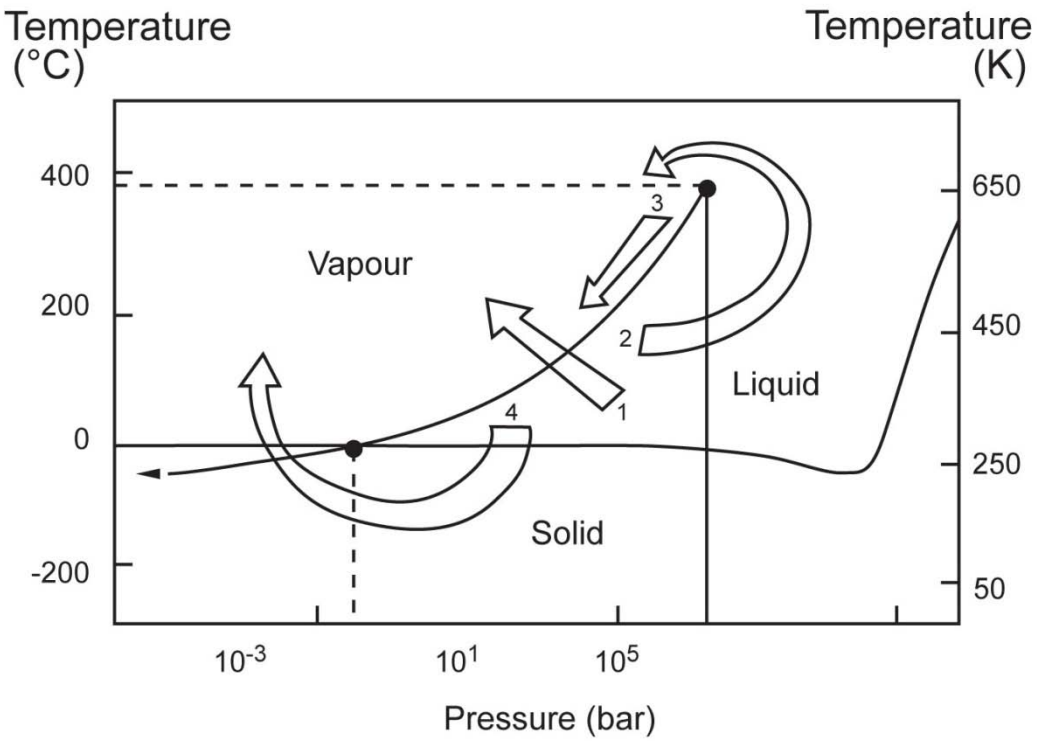


Figure 2.5: Pressure-temperature phase diagram of water.
The different ways to transform from a liquid phase to a vapour phase: 1-air drying, 2-critical point drying, 3- Hexamethyldisilazane (HMDS) drying, 4-freeze drying (redrawn and adapted from CNRS (CENBG) 2010).

There is a few techniques to accomplish the above mentioned;

1. Air-drying at atmospheric pressure and ambient temperature.
2. Critical point drying after the replacement of water with an organic solvent.
3. Air-drying at atmospheric pressure and ambient temperature with the use of an organic solvent.
4. Freeze-drying of hydrated specimen (lyophilisation).

The choice of method to use depends on the preceding preparatory steps and also the specimen type. The first three options concern specimens chemically fixated. Firstly air drying, where a specimen is just left out to dry, represented by arrow 1 (Figure 2.5) which crosses the vaporisation curve above the triple point (CNRS (CENBG) 2010). This method is known to induce drastic structural changes, and won't be discussed further. Critical point drying involves going around the critical point and moving directly from a liquid to a gaseous phase (arrow 2 in Figure 2.5). The critical point for water is not easily accessible thus a solvent with a reachable critical point is used as an intermediary (CNRS (CENBG) 2010 and Quorum Technologies 2009). Air drying by employing an organic solvent such as hexamethyldisilazane (HMDS), endeavours to chemically lower the critical point, which reduces the surface stresses during drying (arrow 3 in Figure 2.5) (Braet 2003 and Slížová *et al.,* 2003). Finally, using freeze drying (arrow 4 in Figure 2.5), the water crosses the melting curve and is transformed to a solid and then sublimated to a vapour by going below the triple point (CNRS (CENBG) 2010; Schwartz *et al.,* 1993).

2.5.1. Critical Point Drying

On the premise of the existence of "continuity of state", which is defined as the transition between two states where there is no abrupt changes in the physical properties, suggests a technique in which surface tension can be fully eliminated (Bozzola & Russel 1999 and Quorum Technologies 2009). If liquefied gas's temperature is increased, the contact angle of the meniscus of the fluid surface becomes smaller, which indicate a reduction in surface tension. When the surface tension is reduced to a certain point the liquid surface becomes exceedingly unstable and eventually it disappears (Smith & Finke 1972 and Quorum Technologies 2009). At this point the liquid turns to gas, and the specimen would go from a wet to a dry state, this point is termed the critical point (Quorum Technologies 2009). It is this quality of gas and liquid phase transition that critical point drying is based on. If water was employed for critical point drying, biological specimens would be extremely damaged as the critical point for water is 647K and 3203.7 pounds per square inch (psi) (CNRS (CENBG) 2010). Numerous solvents was tested and experiments of increasing temperature and the associated volume and pressure changes revealed carbon dioxide

(CO_2) to be a suitable agent for critical point drying (Figure 2.6) (Quorum Technologies 2009).

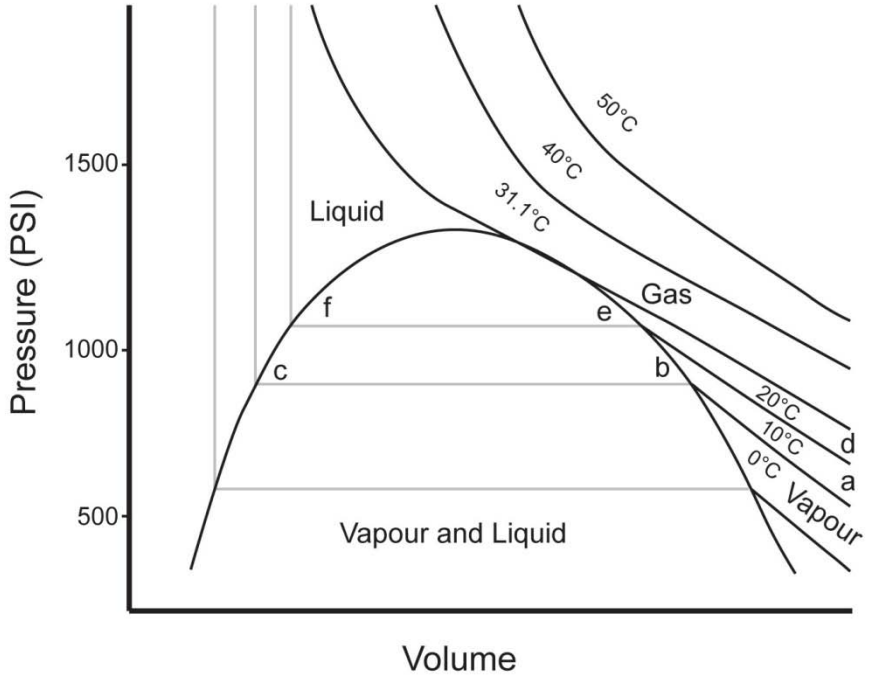


Figure 2.6: Isotherms of CO_2.
(Redrawn and adapted from Quorum Technologies 2009)

In Figure 2.6 it can be seen that at 10 degrees centigrade (°C) CO_2 is in a gaseous phase (or more correctly: vapour phase), with an increase of pressure (Figure 2.6: a-b) the CO_2 becomes a mixture of vapour and liquid and a slight increase of the pressure causes the vapour to transforms completely into a liquid (Figure 2.6: c) and reach a state of saturation (Anderson 1951 and Quorum Technologies 2009). When repeating the experiment at 20°C similar characteristics are seen, however the saturation phase is smaller (Figure 2.6: e-f), which indicates that the densities of the vapour and liquid are approaching each other and that their properties are becoming similar (Quorum Technologies 2009). At 31.1°C the vapour and liquid phase are in equilibrium (Figure 2.6), and their physical properties are

identical, this is termed the critical temperature and has an associated critical pressure (1072 psi) (Dykstra & Reuss 2003; Smith & Finke 1972 and Quorum Technologies 2009).

After dehydration of a specimen with ethanol or acetone the specimen is placed in a chamber together with liquefied CO_2. The dehydrating agent and CO_2 are miscible, so they will blend. The chamber is purged several times of this dehydrating agent- CO_2 mixture, while CO_2 continuously replaces the purged mixture, until all traces of the dehydrating agent is removed. Temperature is increased in the chamber by applying heat and pressure is then built up inside the chamber in response to the heat. This is continued until the critical temperature and pressure is reached. The liquefied CO_2 will cross the boundary to gas leaving the specimen dry. The chamber may then be vented at a slow rate to avoid a subsequent temperature drop and recondensation of the gas (Smith & Finke 1972).

Critical point drying is not a perfect technique, shrinkage still occurs in most specimens (Bozzola & Russel 1999 and Smith & Finke 1972) and additional extraction of some cell constituents may also occur (Bozzola & Russel 1999). The extent of shrinkage and extraction is reasonably tissue specific and can also be minimized by correct and effective tissue processing prior to critical point drying (Bozzola & Russel 1999 and Smith & Finke 1972).

2.5.2. Hexamethyldisilazane

The use of HMDS to dry biological specimens for SEM is a tried and tested method, having been successfully employed to process specimens varying from vascular tissue (Slížová *et al.,* 2003) and other tissue cultures (Bray *et al.,* 1993), to entomological (Kennedy *et al.,* 1989) and microbiological samples (Araujo *et al.,* 2003). In most of these experiments HMDS drying was found to produce similar results as that of the critical point drying technique (Araujo *et al.,* 2003; Braet 2003 and Slížová *et al.,* 2003). The precise mechanism of action of HMDS is not known, but it has been shown that HMDS has exceptionally low surface tension properties and additional cross-linking properties; these are likely the central factors contributing to HMDS's success (Braet 2003 and Slížová *et*

al., 2003). Furthermore HMDS drying is inexpensive, fast and not limited to a chamber in which only a certain number of specimens can be inserted, meaning more specimens can be dried simultaneously and also the process does not need much expertise (Braet 2003 and Oshel 1997).

For HMDS drying the specimen in the dehydrating agent, must be transferred to 100% HMDS through a graded series of ethanol-HMDS mixtures. When the final transition step is reached one last change of HMDS is performed and the sample is left overnight in a fume hood to dry (Oshel 1997). The number of changes and the duration of the changes is dependent on the specimen; with dense, impermeable specimens both the number and duration of changes should be increased (Oshel 1997).

A typical protocol for HMDS drying is (Oshel 1997);

- 1. 3:1 Ethanol: HMDS 30 minutes
- 2. 1:1 Ethanol: HMDS 30 minutes
- 3. 1:3 Ethanol: HMDS 30 minutes
- 4. 100% HMDS 60 minutes

As with critical point drying, HMDS causes a degree of shrinkage and may introduce artefacts which were noted particularly in plant materials (Slížová *et al.,* 2003 and Oshel 1997).

Care should be taken when using HMDS, as it may irritate the eyes, skin and respiratory system. The vapours are toxic therefore it is important to always work in a fume hood (Oshel 1997 and SUPELCO 1997).

2.5.3. Freeze Drying

Freeze drying, also called lyophilisation, is a process where water is gently removed from a specimen by reducing the pressure and adding heat, allowing the frozen water to

sublimate (CNRS (CENBG) 2010; Dudek *et al.,* 1982 and Lee & Chow 2012). It is similar to critical point drying in the fact that a smooth transition from one phase to another is desired, only with freeze drying the transition is between a solid phase and a gaseous phase.

The specimens are kept at a temperature below the recrystallization point, by placing them in a liquid nitrogen cooled chamber. The pressure in the chamber is then decreased in order to keep the partial pressure of the water in the system lower than the vapour pressure (CNRS (CENBG) 2010; Hanzon & Hermodsson 1960 and Schwartz *et al.,* 1993). The chamber serves as a cold trap, ensuring that the temperature of the chambers walls is lower than that of the specimen, in this way the water is condensed onto the liquid nitrogen cooled surface. It is important to always maintain this temperature gradient (CNRS (CENBG) 2010 and Hanzon & Hermodsson 1960). The use of desiccants such as zeolites or synthetic zeolites are also occasionally used to remove the vapour released from the specimen (CNRS (CENBG) 2010).The drying proceeds at temperature below 193K and high vacuum with pressures between 10^2 to 1 Pascal (Pa) for 48 hours, after which the temperature is raised to room temperature, and the vacuum is released (Hanzon & Hermodsson 1960; Lee & Chow 2012 and Schwartz *et al.,* 1993).

Freeze drying is in most cases a two-step process; nearly 90% of the water in a specimen is removed during the first phase by direct sublimation of the ice formed during freezing, leaving behind a porous “ghost” of the initial hydrated sample. The second phase, sometimes referred to as isothermal desorption, removes unfreezable water associated with macromolecules (Echlin 1992)

In principle, the sublimation of ice avoids the high surface tensions at the liquid-air interface which causes disruptions in the structure of the specimen (Lee & Chow 2012). Freeze fixation coupled with freeze drying, is a purely physical process, without the use of chemical agents that may introduce structural and biochemical changes (Grunbaum & Wellings 1960 and Hanzon & Hermodsson 1960). For this reason the method is often very successfully used for ultrastructural and immunocytochemical studies (CNRS (CENBG) 2010 and Dudek *et al.,* 1982).

2.6. Contrasting and Coating

For visualisation of biological specimens in an electron microscope an additional process is required for electrons to be used as an imaging tool. For TEM, this process is called contrasting or staining and for SEM, it is termed coating.

2.6.1. Contrasting

Visualisation with a TEM occurs by electron scattering induced by the atoms in a sample. Since biological specimens are composed mainly of light elements, the amount of scattering is very small and therefore very little contrast is created. To increase the electron scattering ability of a biological specimen, heavy metal salts are used. This can be done either by staining and imparting contrast to the specimen itself (positive staining) or the areas surrounding the specimen (negative staining), while leaving the specimen more translucent. Negative staining is specifically used for particulate specimens such as viruses or isolated proteins, and won't be discussed in any further detail.

The two most commonly used stains used for positive staining, are uranyl acetate and lead citrate; and by mainly binding to phosphate and hydroxyl groups respectively impart contrast in cellular components. Staining is accomplished by floating the grid with the thin sections on a drop of aqueous solution of uranyl acetate, gentle but thorough rinsing and repeating these steps with an aqueous solution of lead citrate. As with most of the preparation steps several variations of positive staining exists, such as elevating the temperature during staining and using alcoholic solutions of the metal salts or even staining prior to embedding. The choice of method is usually based on the type of resin used and the required results.

2.6.2. Coating

When biological, non-conducting specimens are viewed in a SEM, a conductive coating must (in most cases) be applied to the surface to simplify operation. This is because the electron beam that strikes the specimen surface requires a conducting path to earth in order to remove any electron charge. If a surface charge builds up, the secondary

(imaging) electrons are unable to penetrate this charge, distorting the image form and the signal level higher secondary electron yield which equates to a higher resolution image.

The specimen can be coated by a variety of materials. Metal compounds such as gold, palladium or platinum can be sputtered onto a sample or carbon can be evaporated onto the sample. Briefly; with sputter coating the metal target is bombarded with ionised gas atoms and the metal atoms ejected from the target cross the discharge and is deposited onto the surface of a specimen. Carbon fibres or rods will evaporate if heated to above 2000°C in a vacuum, which will form a very thin carbon film over the specimen creating a conducting path over the specimen.

The multi-step process of preparing samples for electron microscopy have now been discussed with the details involved in each step to ensure optimum sample preparation for obtaining high quality micrographs. As this study uses fibrin networks and blood platelets as a model to development a method of rapidly vitrifying platelet and fibrin networks utilizing high pressure freezing coupled with freeze substitution or freeze drying, for the use in ultrastructural studies, the next section of this chapter will focus on the fibrin network and platelet structure and formation.

2.7. The Fibrin Network

The normal physiological response that prevents significant blood loss following vascular injury is called haemostasis. This finely tuned process serves to preserve the integrity of the circulatory system. The reactions that govern haemostasis can be separated into five distinct phases for analysis: initiation of coagulation, propagation of α (alpha) thrombin formation, termination of the pro-coagulant response, elimination of the fibrin clot, and tissue repair and regeneration. One of the key factors in this process is a blood protein called fibrinogen. Fibrinogen, through the coagulation cascade, leads to the formation of a fibrin network and ultimately a haemostatic plug or a blood clot.

2.7.1. Fibrinogen

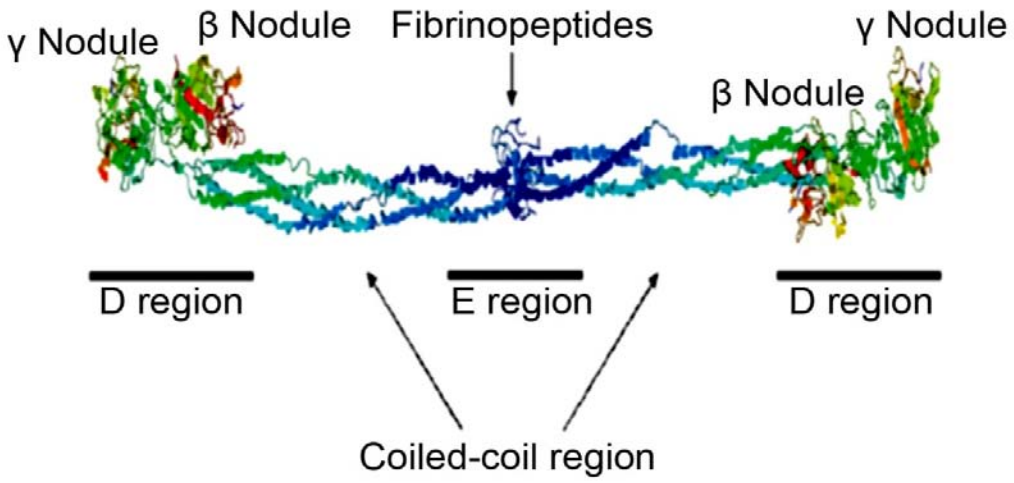


Figure 2.7: The human fibrinogen molecule.
Crystal structure of human fibrinogen showing distal globular D regions connected by three-stranded coiled-coils to the central E region (Cilia la Corte *et al.,* 2011).

Human fibrinogen is a plasma glycoprotein (Standeven *et al.,* 2005; Weisel 2005 and Woodhead *et al.,* 1984), synthesized mainly in liver hepatocytes (Blombäck 1996; Pretorius *et al.,* 2006; Standeven *et al.,* 2005 and Weisel 2005), but also in megakaryocytes (Leven *et al.,* 1985; Takeda 1966 and Uzun *et al.,*), at an approximate rate of 1.7-5 grams (g) per day (Standeven *et al.,* 2005 and Takeda 1966), resulting in a circulating plasma concentration of 2–3 grams per liter (g/l) plasma (Weisel 2004 and Weisel 2005).

It consists of three pairs of non-identical polypeptide chains, designated α, beta (β), and gamma (γ) (Langer *et al.,* 1988; Pretorius *et al.,* 2006; Standeven *et al.,* 2005; Weisel 2005 and Weisel *et al.,* 1987), which is the products of three separate, closely linked genes found in a cluster on the distal third of the long arm of chromosome four (Blombäck 1996; Kant *et al.,* 1985 and Pretorius *et al.,* 2006). Each gene is separately transcribed and translated, after which the newly synthesized chains are discharged into the endoplasmic

reticulum (ER) where methodical assembly into mature fibrinogen occurs (Yu *et al.,* 1983; Yu *et al.,* 1984; Yu *et al.,* 1986; and Standeven *et al.,* 2005). There is a progression from single chains to two-chain complexes to the trimetric half molecule (Weisel 2005), ending with the combination of half molecules to a dimeric molecule by the introduction of disulphide bonds (Blombäck 1996). The result is an elongated 45nm tri-nodular structure, consisting of two outer D domains, connected by a coiled-coil segment to a central E domain (Figure 2.7) (Blombäck 1996; Fowler *et al.,* 1981; Hantgan & Hermans 1979; Mosseson *et al.,* 1993; Mosseson *et al.,* 2001; Pretorius *et al.,* 2006; Ryan *et al.,* 1999; Weisel 2004 and Weisel 2005), with a molecular weight of 340 kiloDalton (kDa) (Blombäck 1996; Mosseson *et al.,* 2001; Standeven *et al.,* 2005 and Weisel *et al.,* 1987). The pair of three polypeptide chains, each pair forming identical halves of the fibrinogen molecule is linked by 29 disulphide bonds (Standeven *et al.,* 2005; Weisel 2005 and Woodhead *et al.,* 1984). Protein folding results in the N-terminal of each chain being located in the globular central E domain linked by three inter-chain disulphide bonds, or a so called “disulphide knot” (Blombäck 1996; Cilia la Corte *et al.,* 2011; Mosseson *et al.,* 1993; Mosseson *et al.,* 2001; Weisel 2005; Weisel *et al.,* 1987 and Woodhead *et al.,* 1984), and rod-like regions extending from either side of this central domain, which is comprised of three stranded α-helical coiled-coils that terminate in two distal D region nodules (Ryan *et al.,* 1999; Standeven *et al.,* 2005 and Woodhead *et al.,* 1984). The D domain’s proximal (C-terminal) and distal (αC-domain) regions is comprised of the carboxy terminal portions of the β and γ chains respectively (the carboxy terminal portion of the α chain depart from the end domains and fold into a conformation that stretches back towards the central domain) (Blombäck 1996; Weisel *et al.,* 1985 and Weisel *et al.,* 1987).

2.7.2. Formation of the Fibrin Network

The formation of a fibrin network is initiated by the coagulation cascade, which is a sequential process of biochemical reactions involving plasma proteins, phospholipids and calcium ions (illustrated in Figure 2.8) (Cilia la Corte *et al.,* 2011 and Furlan 1984). The cascade is initiated by two pathways; the intrinsic (contact activation) and extrinsic (tissue factor) pathways, following injury or trauma, after which a common point is reached and is

then called the common pathway. The intrinsic pathway is initiated when inactive enzymes or zymogens involved in this pathway (prekallikrein, high-molecular-weight-kininogen (HMWK), plasma thromboplastin antecedent (factor XI) and Hageman factor (factor XII)) is exposed to a negatively charged surface (Sherrif 2004). For example interaction with the phospholipids of circulating lipoprotein particles and the surface of bacteria, this is termed the contact phase (McDonagh *et al.,* 2001). The contact phase initiates a number of reactions involving the conversion of inactive zymogens into active enzymes (see Figure 2.8). The extrinsic pathway is initiated at the site of injury in response to the release of tissue factor (factor III), which together with active proconvertin (factor VIIa) catalyses the activation of Stuart-Prower factor (factor X) (Sherrif 2004); the common pathway is reached when factor Xa activates thrombin (factor II) (Mosseson *et al.,* 2001 and Standeven *et al.,* 2005).

Fibrinogen (factor I) is activated by the action of thrombin; a serine protease, proteolytic enzyme (Blombäck 1996; Di Stasio *et al.,* 1998; Mosseson *et al.,* 2001; Ryan *et al.,* 1999 and Weisel 2005; Wolberg 2007 and Woodhead *et al.,* 1984), with a specificity for fibrinogen's fibrinopeptides. This interaction is due to hydrophobic and structure-dependent interactions between the fibrinopeptides and thrombin's catalytic sites, and non-catalytic binding of enzyme to substrate (Weisel 2005). When thrombin interacts with fibrinogen it cleaves two pairs of acidic fibrinopeptides from the N-terminal portions of the α and β chains, called fibrinopeptide A (FPA) and fibrinopeptide B (FPB), which exposes binding sites and results in an altered charge distribution in the fibrin monomer (Blombäck 1996; Hantgan & Hermans 1979; Cilia la Corte *et al.,* 2011; Pretorius *et al.,* 2006; Ryan *et al.,* 1999 and Woodhead *et al.,* 1984). Altered charges in the molecule facilitate electrostatic interactions between adjacent fibrin molecules allowing the exposed polymerization sites (*A*) and (B), due to cleavage of FPA and FPB respectively, in the central E domain of the molecule to non-covalently interact with complimentary binding pocket (*a* and b) in the outer D domain (Da and Db sites) of neighbouring fibrin monomers (Cilia la Corte *et al.,* 2011; Di Stasio *et al.,* 1998; Pretorius *et al.,* 2006; Ryan *et al.,* 1999; Weisel 2005; Weisel *et al.,* 1987 and Woodhead *et al.,* 1984). The intermolecular interactions between the complimentary binding sites A-Da causes the monomers to

associate leading to oligomers and ultimately double stranded protofibrils in which monomers align in an end-to-middle, half staggered, overlapping arrangement (Blombäck 1996; Cilia la Corte *et al.,* 2011; Collet *et al.,* 1993; Di Stasio *et al.,* 1998; Fowler *et al.,* 1981; Mosseson *et al.,* 1993; Pretorius *et al.,* 2006; Ryan *et al.,* 1999; Standeven *et al.,* 2005; Weisel *et al.,* 1987 and Woodhead *et al.,* 1984). The cleavage of FPB occurs simultaneously, but it has been shown that the rate of cleavage only becomes significant after the polymerization of fibrin monomers has reached a certain point (Hantgan & Hermans 1979 and Weisel 2005), which suggest a conformational change after polymerization that enhances release or preferential cleavage from the fibrin polymer (Di Stasio *et al.,* 1998, and Weisel 2005). With the release of FPB and exposure of polymerization site *B*, lateral aggregation of protofibrils is enhanced, and fibrin fibers is formed (Blombäck 1996; Cilia la Corte *et al.,* 2011; Di Stasio *et al.,* 1998; Fowler *et al.,* 1981; Hantgan & Hermans 1979; Weisel 2005 and Woodhead *et al.,* 1984).

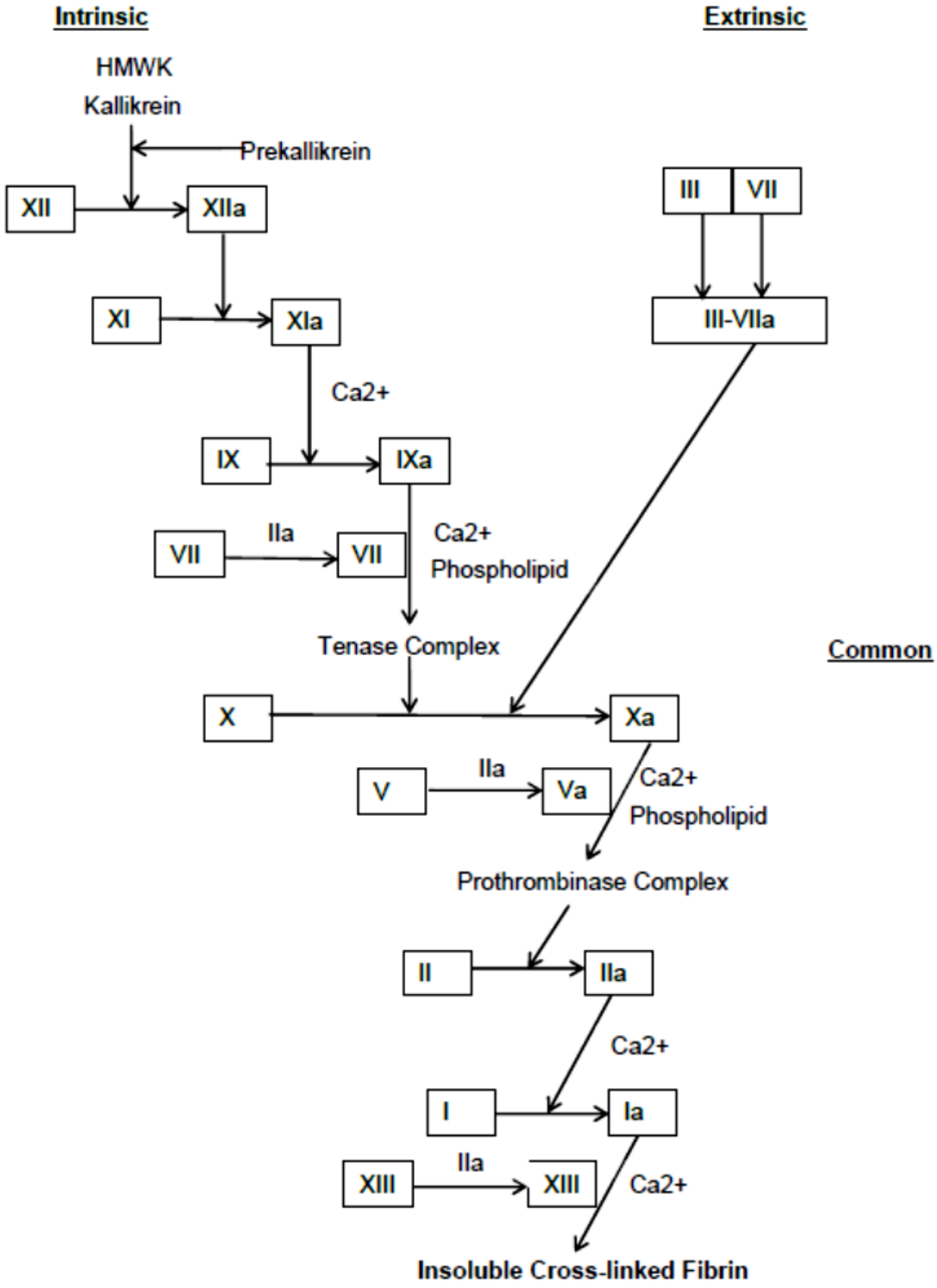


Figure 2.8: The coagulation cascade.

(Adapted from Cilia la Corte *et al.*, 2011)

The structure of fibrin is further covalently stabilized by a plasma transglutimase, called fibrin stabilizing factor (Factor XIII), an enzyme which introduces isopeptide bonds or cross-links between the glutamine and lysine residues in the α and γ chains of fibrin monomers (Blombäck 1996; Hantgan & Hermans 1979; Ryan *et al.,* 1999 and Weisel 2005). Different opinions exists on the orientation of the cross-links, some believing the cross-links to be longitudinally arranged between two monomer D domains in the same strand, whereas others find evidence for transverse cross links to be between fragment D domains of opposing strands (Blombäck 1996; Mosseson *et al.,* 2001 and Standeven *et al.,* 2005).

Protofibrils are intrinsically twisted and when they aggregate to make a fiber. They twist around each other while maintaining the periodicity of 22.5nm [this periodicity can be seen as the well-published band pattern (Fowler *et al.,* 1981 and Weisel 2005)]. This causes the protofibrils near the surface to be stretched relative to those in the centre, as the path length increases with fiber diameter (Blombäck 1996; Standeven *et al.,* 2005; Weisel 2004; Weisel 2005 and Weisel *et al.,* 1987). When the energy required to stretch an additional protofibril exceeds the energy required for binding that protofibril, lateral association will end (Blombäck 1996; Collet *et al.,* 1993; Standeven *et al.,* 2005; Weisel 2004; Weisel 2005 and Weisel *et al.,* 1987). At this point fiber growth cease and fibers will branch out to the form of a complex network of fibers (Standeven *et al.,* 2005). Two types of branching has been shown; a tetramolecular or bilateral branch (Baradet *et al.,* 1995), where double stranded fibrils converge side to side, and tri-molecular or equilateral branching (Mosseson *et al.,* 1993), which forms by coalescence of three fibrin molecules that connect three double stranded fibrils of equal width (Mosseson *et al.,* 2001 and Weisel 2005). Branching is essential for the properties of fibrin, since it provides a structural framework for the intravascular thrombus, a space filling gel with the mechanical strength to “plug” lesions and to withstand the pressure of arterial blood flow (Mosseson *et al.,* 1993; Weisel 2004 and Weisel 2005).

2.7.3. Factors Affecting the Fibrin Network's Ultrastructure

All molecules directly participating in the assembly and formation of fibrin networks availability, quantities and concentrations alter the final outcome of the fibrin networks' structure, for example fibrinogen, thrombin and calcium. Higher thrombin concentrations correspond to finer fibers and decreased turbidity (Wolberg 2007 and Wolberg *et al.,* 2003), whereas elevated fibrinogen levels correspond to an increase in the number, branching and length of the fibers which results in lower porosity (Fatah *et al.,* 1992; Ryan *et al.,* 1999 and Weisel & Nagaswami 1992). Thrombin is also incorporated into the fibrin clot and remains bound to fibrin in the E and D domains, which has been shown to be dependent on the quality of the fibrin network; with more thrombin being trapped by networks made up of thinner fibers (Wolberg 2007). This previously mentioned trapped thrombin is active and potentially has haemostatic and pro-thrombotic roles shortening the time required to re-establish a clot that has failed for mechanical or other reasons, it also increase the risk of re-thrombosis (Wolberg 2007). Calcium ions are of importance as a basic physiological modulator of fibrin polymerization (Blombäck 1996), capable of controlling fiber size (Vindigni & Di Cera 1996). When bound to fibrin, calcium promotes lateral aggregation, limit the extent of plasmin digestion, and protect against denaturation by heat and pH (Cilia la Corte *et al.,* 2011 and Yamamoto *et al.,* 1994).

Several other factors plays a role in the structure of fibrin networks *in vivo*, for example blood proteins, cells, pH and ionic strength (Standeven *et al.,* 2005 and Weisel 2005).
Plasma proteins such as fibronectin and albumin interact with fibrin, the former becomes cross-linked with fibrin, influencing fiber size, density and network strength (Blombäck 1996 and Weisel 2005), while the latter does not crosslink fibrin, but has been shown to influence lateral aggregation of protofibrils and subsequently fiber thickness (Torbet 1986). Other proteins such as thrombospondin, released from α-granules of stimulated platelets, are integrated into the fibrin network forming a reversible non-covalent complex affecting the network properties (Dixit *et al.,* 1984). Von Willebrand factor and Fibulin-1 support platelet adhesion to fibrin under blood flow conditions (Weisel 2005). The ion, magnesium, has also been shown to affect fibrinogen binding (Blombäck 1996).

Platelets also bind fibrinogen which encourage adenosine diphosphate (ADP) induced aggregation (Blombäck 1996), and the organization of fibrin fibers around platelet aggregates (Carr & Carr 1995 and Standeven *et al.,* 2005).
Demographic and environmental factors such as diet, age, drug use, smoking, alcohol consumption, body mass, gender, physical exercise, race and season has also been shown to affect the network structure, mainly by raising plasma fibrinogen levels (Standeven *et al.,* 2005).

The structure of the network consecutively affects other components which takes part in wound healing. Thick fiber networks promote a pro-fibrinolytic and anti-thrombotic environment, stimulate migration and alignment of endothelial cells along the length of the fiber and also encourage ingrowths of tubular structures consistent with angiogenesis, suggesting that a thick fiber network promotes wound healing (Wolberg 2007).

2.7.4. Fibrinolysis

Just as important as the formation of the fibrin network is the subsequent breakdown of the network, this process is called fibrinolysis, where fibrin actively regulates its breakdown through several interactions with fibrinolytic and antifibrinolytic components (Collet *et al.,* 2000).

Fibrinolysis' primary fibrinolytic components are a series of protein serine proteases and their antifibrinolytic inhibitors, known as SERPINS (Cilia la Corte *et al.,* 2011), which tightly control the process (Figure 2.9). The central molecule of the fibrinolytic system is a pro-enzyme called plasminogen, which is converted to its active form, plasmin by the action of two different plasminogen activators (PAS); tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) (Blombäck 1996; Cilia la Corte *et al.,* 2011; Collen & Lijnen 1991 and Weisel 2005).

Plasminogen circulates in the blood and preferentially binds to polymerized D-regions of fibrin, which is its major substrate. In addition, fibrin also binds plasminogen's activating factors such as tPA forming a ternary complex between fibrin, plasminogen and t-PA

thereby regulating its own degradation (Blombäck 1996; Cilia la Corte *et al.,* 2011 and Weisel 2005). Activated plasmin lyses fibrin into typical fragments of fibrinogen, by cleaving bonds in the C terminal portion of the α chain, and in the C and N terminal portions of the β chain, this lyses act to expose new plasminogen-binding sites, leading to a further enhancement in plasmin generation and fibrin degradation (Cilia la Corte *et al.,* 2011; Furlan 1984 and Weisel 2005). Plasmin also hydrolyse the γ chain but at a very slow rate (Furlan 1984). uPA activates plasminogen and generates plasmin as well, but does so in a fibrin-independent manner (Cilia la Corte *et al.,* 2011).

The regulation of plasmin-stimulated fibrinolysis is very important to prevent bleeding; this is accomplished by several plasmin inhibitors (Cilia la Corte *et al.,* 2011). Alpha2-antiplasmin (α2-AP) is the main physiological inhibitor of plasmin, synthesized in the liver and found in the circulatory system. Its inhibitory effect is achieved by forming a cross-link with plasmin; an irreversible enzyme-inhibitor complex (Cilia la Corte *et al.,* 2011). Plasminogen activator inhibitor-1 (PAI-1) and plasminogen activator inhibitor-2 (PAI-2) are inhibitors of tPA and uPA, by binding the plasminogen activators and forming a stable complex it inhibits plasmin generation (Cilia la Corte *et al.,* 2011). PAI-1 is the stronger inhibitor of these two enzymes; it is synthesized and secreted by endothelial cells and platelets resulting in a high local concentration protecting the clot from premature lyses. Thrombin-activatable fibrinolysis inhibitor (TAFI) expressed by the liver and present in platelets, is activated by the action of thrombin in the presence of thrombomodulin. It binds fibrin by active factor XIII via its activation peptide, which inhibits the binding of plasminogen and tPA, thus reducing plasmin generation (Redlitz *et al.,* 1995).

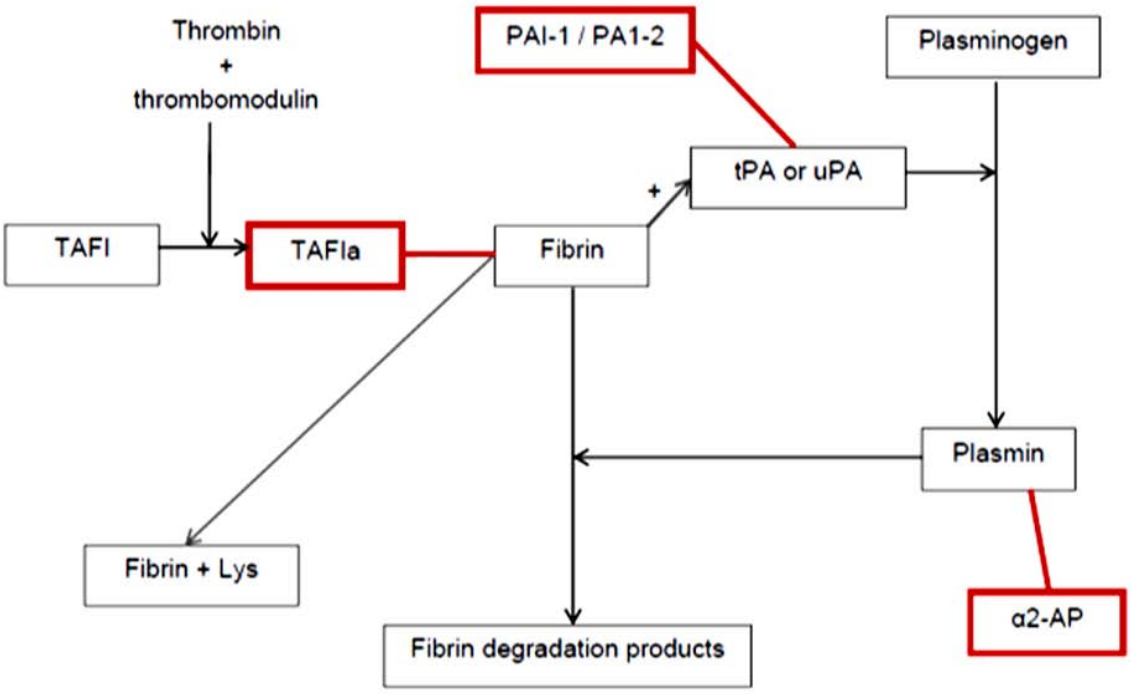


Figure 2.9: Schematic overview of fibrinolysis.
Clot dissolution is mediated by plasmin, which is produced by the actions of tPA or uPA. The bold red lines indicate where the fibrinolysis pathway is inhibited by PAI-1 and PAI-2, and α2-AP. TAFI is activated (TAFIa) by the thrombin: thrombomodulin complex and inhibits fibrinolysis by cleaving C-terminal lysine residues on fibrin preventing tPA and plasminogen binding (redrawn from Cilia la Corte *et al.,* 2011).

Fibrin network structure is very important to the digestion of the clot. For effective lyses plasmin has to degrade fibrin in the coiled-coil region between the D and E regions; this process is hindered by the presence of α-chain cross-links (Francis *et al.,* 1980). Generally, fibers are dissected laterally rather than progressively, resulting in loose thick fibrin conformations being broken down at a faster rate than tight networks of thin fibers (Collet *et al.,* 2000). However, individually, thick fibers are cleaved at a slower rate (Cilia la Corte *et al.,* 2011 and Standeven *et al.,* 2005), this could possibly be explained by the

ternary complex between fibrin, plasminogen and t-PA differing between network types and also the permeation of fibrinolytic agents through different density networks may be a limiting factor (Blombäck 1996; Cilia la Corte *et al.,* 2011 and Standeven *et al.,* 2005).

2.7.5. Abnormal Fibrin Networks in Disease

There is a dynamic equilibrium between clotting and fibrinolysis, so that the conversion of fibrinogen to fibrin and the dissolution of the clot must be carefully regulated. Any disparity can result in either haemorrhage or thrombosis. Several disease conditions, environmental factors and chemical interactions can cause alterations in the fibrin network structure, caused by altered protein levels, structure, function or interactions with other proteins, which leads to irregular or ineffective polymerization of fibrinogen to fibrin, and often to irregular fibrinolysis. Emerging evidence shows that the structure of the fibrin clot may determine the risk of thrombotic and bleeding diathesis diseases (Standeven *et al.,* 2005), and that genetic variants may impart alterations in the fibrin structure (Standeven *et al.,* 2005).

2.7.5.1. Dysfibrinogenemia

Dysfibrinogenemia is a large group of either congenital or acquired conditions, associated with abnormal clot formation leading to bleeding tendencies and/or thrombotic disorders. Acquired dysfibrinogenemia is mostly associated with hepatic conditions, such as advanced liver disease, cirrhosis and hepatitis (Standeven *et al.,* 2005 and Weisel 2005). In these types of conditions structural defects in fibrin are caused by increased carbohydrate content; impairing polymerization of the fibrin molecule. Congenital dysfibrinogenemias is a qualitative abnormality of the fibrin molecule, inherited in an autosomal dominant or co-dominant fashion, in which a single base change, results in an amino acid substitution or truncation (Weisel 2004). Defects may occur in several steps of coagulation, although the most common defects involve polymerization of the fibrin monomer. An example is Dusart syndrome; where lateral aggregation of protofibrils is impaired due to a single base substitution in the α chain, believed to cause a conformational change in the protein (Collet *et al.,* 1993), and accompanying disulphide

attachment of albumin (Collet *et al.,* 1993 and Weisel 2004). This alteration results in a network made up of very short, thin and highly branched fibers (Collet *et al.,* 1993 and Weisel 2004), shown to be six fold stiffer with subsequent heightened resistance to fibrinolysis. This resistance is due to decreased permeability and less of the fibrin surface being exposed to fibrinolytic agents (Collet *et al.,* 1993), which in turn cause thrombotic tendencies (Carr & Carr 1995; Francis *et al.,* 1980; Standeven *et al.,* 2005 and Weisel 2004).

2.7.5.2. Cardiovascular disease / Myocardial infarction

Epidemiological studies of cardiovascular diseases, mainly comprising coronary artery disease leading to myocardial infarction have shown a distinct and significant relationship between myocardial infarction and elevated fibrinogen levels (Ryan *et al.,* 1999; Standeven *et al.,* 2005 and Weisel 2004), and subsequent altered mechanical clot properties. These changes can be clearly seen as a densely packed, tight and rigid network with a decreased fiber mass-length ratio (Fatah *et al.,* 1992 and Scott *et al.,* 2004). Meaning, a network made up of highly branched thin fibers with small pores, and subsequent increased resistance to fibrinolysis (Ryan *et al.,* 1999 and Standeven *et al.,* 2005).

2.7.5.3. Haemophilia

Haemophilia is a bleeding disorder resulting from deficiency of factors VIII or IX (Haemophilia A or B respectively). Biochemically, this results in an abnormality in the intrinsic or propagation in platelet tenase (the activating complex) (Wolberg 2007), caused by delayed onset and also reduced rate of thrombin generation. This in turn causes delayed fibrinopeptide release and fibrin polymerization (Wolberg 2007). Structurally these alterations result in clots made up of thicker fibers with a slight deposition of thin fibers, and an increased porosity which leaves the network more susceptible for fibrinolysis (Weisel 2005 and Wolberg 2007).

2.7.5.4. Diabetes

It is believed that alterations in the fibrin clot contribute to diabetics pro-atherogenic tendencies, likely through molecular interaction between glucose and fibrinogen by the non-enzymatic glycation of lysine residues (Standeven *et al.,* 2005 and Weisel 2005). As lysine residues are involved in both cross-linking and plasmin induced lyses, it is feasible that glycation would alter fibrin polymerization. Ultrastructural studies of ambient glucose levels showed that glucose independently affect the structure of fibrin networks, with increased glucose levels resulting in a network consisting of thin fibers with increased branching and an overall decreased porosity (Standeven *et al.,* 2005). Together with the glycation, independent studies have shown increased thrombin generation which also result in a network made up of thinner fibers with an increased resistance to fibrinolysis (Wolberg 2007), also decreased plasmin activation and reduced binding of plasminogen and tPA to the fibrin clot, reducing the rate of fibrinolysis even further has been reported (Cilia la Corte *et al.,* 2011).

2.7.5.5. Asthma

Fibrin networks presents with an abnormal structure in asthma, where the thick fibers seem to be fused longitudinally, have a matted appearance, and numerous thin fibers forming a thick, tangled layer over a thick fiber deposit (Pretorius *et al.,* 2007). This is possibly due to the inflammatory response, which is known to up-regulate fibrinogen production (Cilia la Corte *et al.,* 2011).

2.7.5.6. Pregnancy

Although pregnancy is not a diseased condition, it is a well-studied and established fact that pregnancy constitutes a hypercoagulable state (Hellgren 2003 and Weiner *et al.,* 1984), with an increased prevalence of venous thromboembolism (Lockwood 2006) considered to be mainly the result of increased oestrogen levels (Bonnar *et al.,* 1969). Among several alterations in factors affecting coagulation and fibrinolysis, the following is observed during pregnancy; gradual increase in plasma fibrin monomers (Bonnar *et al.,*

1969; Bremme *et al.,* 1992; Hellgren 2003 and Pretorius *et al.,* 2009b) coagulation factor VII, anti-hemophilic factor (factor VIII), christmas factor (factor IX), factor X and factor XII, and also increase in thrombin activity (Bremme *et al.,* 1992; Pretorius *et al.,* 2009b and Weiner *et al.,* 1984), raised pro-enzyme plasminogen (Hellgren 2003), and a decrease in fibrinolytic activity (Bonnar *et al.,* 1969 and Bremme *et al.,* 1992).

Pretorius *et al.,* (2008) reported the morphological result of the above mentioned changes as a fiber network of evenly distributed major fibers, and a fine, dense net of minor fibers distributed evenly over the major fibers, suggesting that a fine net deposition followed the thick fiber deposition. This denser clot morphology would result in a lower fibrinolytic efficiency, leading to the increased tendency toward thromboembolism.

2.7.5.7. Drugs

Certain drug interactions have been shown to alter fibrin clot structure and properties. The antihyperglycemic drug metformin inhibits cross-linking of fibers by factor XIII resulting in decreased fiber diameter and pore size (Dhall & Nair 1994). The anti-diabetic drug gliclazide increases fiber diameter, which creates a less permeable network (Dhall & Nair 1994). Aspirin cause acetylation of lysine residues leading to antithrombotic alterations in the fibrin structure, whereby the clot is less dense, with increased fiber thickness, large pores, reduced rigidity and enhanced lyses properties (Ajjan *et al.,* 2009; Cilia la Corte *et al.,* 2011 and Standeven *et al.,* 2005). Increased plasminogen affinity for fibrin has also been reported (Standeven *et al.,* 2005), which has been associated with a risk of arterial thrombotic disease (Ajjan *et al.,* 2009).

2.7.5.8. Smoking

Smoking has been shown to result in fibrin clots with thin dense fiber networks resulting in increased resistance to fibrinolysis (Barua *et al.,* 2010 and Pretorius *et al.,* 2010). Inflammation (20-25% increase in peripheral leukocyte count in smokers) is believed to be one of the major contributors in the abnormal structure of the fibrin network and subsequent thrombotic tendencies (Ambrose & Barua 2004), oxidative stress and reduced

tPA mediated plasmin generation has also been shown to contribute (Ambrose & Barua 2004 and Barua *et al.,* 2010).

2.8. Platelets

Platelets are small, membrane bound fragments of cytoplasm derived from megakaryocytes, passively circulating in the blood stream and containing all the organelles found in normal mammalian cells except for a nucleus and mitotic apparatus (Becker 2008; Kuter 1997; Michelson 2007 and Spyropoulos 2002). The main role of platelets is to maintain haemostasis by contributing to blood coagulation (Becker 2008 and Michelson 2007), although it has been shown that platelets also possess rudimentary bactericidal and phagocytic activity (Michelson 2007).

2.8.1. Structural Anatomy

Resting platelets present with a flattened disc shaped structure with dimensions of approximately 2.0 to 5.0µm. Surface structures are featureless, lacking protrusions, apart from small membrane infolding or pores, which are associated with the open canalicular system (Becker 2008 and Michelson 2007). When damage is detected platelets change shape. This shape change starts with the platelet losing its discoid shape and becoming rounded. This is followed by finger-like protrusions or pseudopodia propagating from the periphery followed by a flattening of the platelet and a broad lamellae being extended (Michelson 2007). Membrane convolutions with extensive infolding can also be seen at this point.

Platelets are a structurally complex element, which can be anatomically divided into four zones:

The peripheral zone that consists of the membrane and its invaginations, forming the open canalicular system. This zone can be further divided into the exterior coat, which comprises of a 10 to 20nm thick glycolax rich in glycoproteins, the unit membrane, a lipid bilayer similar in structure and function to other cell membranes and the sub-membrane

area beneath the unit membrane consisting of a network of microfilaments associated with membrane proteins and the cytoplasmic filament system (Becker 2008; Michelson 2007 and Spyropoulos 2002). The peripheral zone of a platelet does not only serve as a barrier to separate the platelet contents from the external milieu, it is the site of first contact, sensing the changes in the vascular compartment requiring haemostatic response (Michelson 2007). The glycoproteins forming part of the exterior glycolax facilitate adhesion to the damaged area and provide receptors for stimuli triggering platelet activation. In turn, the unit membrane is an essential surface for interaction with coagulant proteins and has been shown to accelerate coagulation (Becker 2008 and Spyropoulos 2002). The sub-membrane area, together with upper two zones participates in shape change and translocation of receptors and particles.

The sol-gel zone is the matrix of the cytoplasm and consists of two fiber systems. The outermost system is a circumferential tightly coil of tubulin microtubules which is a cytoskeletal support system maintaining the resting shape of the platelet (Becker 2008 and Michelson 2007). The second set is made up of various stages of polymerized actin-myosin filaments, involved in shape change, internal transformation and ultimately the contraction of the haemostatic plug (Becker 2008 and Michelson 2007).

The organelle zone is an indistinct zone containing storage granules, dense bodies, peroxisomes, lysosomes and mitochondria, which all play a part in the metabolic processes and storage of nutrients (Becker 2008 and Spyropoulos 2002).

The final zone consists of a membrane structure, a dense tubular system (DTS) located in the cells interior derived from the parent cells rough-ER. This system acts as a storage site for calcium and enzymes (Becker 2008 and Spyropoulos 2002).

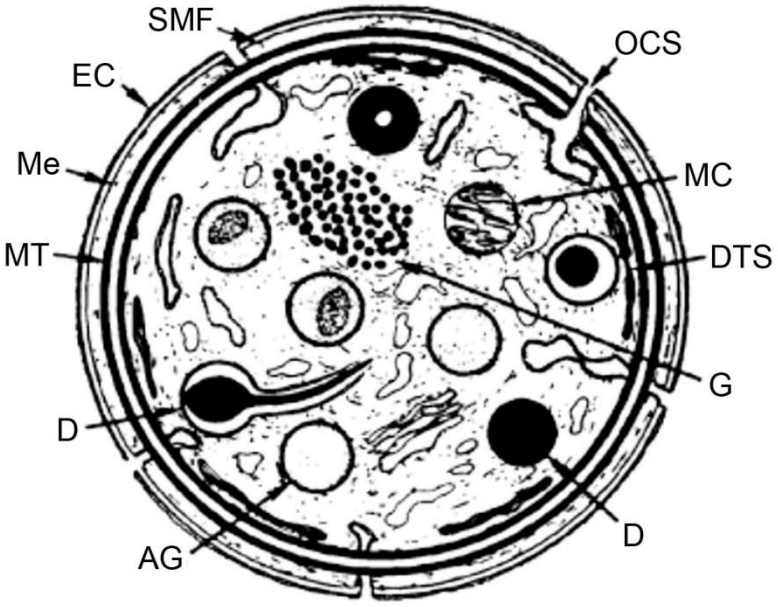


Figure 2.10: The platelet morphology.
Features of a discoid platelet: exterior coat (EC), cell membrane (Me), sub-membrane filaments (SMF), Sol-gel zone contains microfilaments, microtubules (MT) and glycogen (G); formed elements; α-granules (AG), dense bodies (D) and mitochondria (MC); DTS and the open canalicular system (OCS) are part of the membrane system (redrawn and adapted from Michelson 2007)

2.8.2. Functional Anatomy

In response to vascular injury platelets undergo a complex series of biochemical and cellular processes to ultimately take part in clot formation. This can be loosely divided into four categories; adhesion, activation, secretion and aggregation.
The primary haemostatic response to damaged, disrupted or dysfunctional vascular endothelium is called, adhesion. Adhesion is initiated either by contact with high molecular weight proteins or foreign surfaces (Rao 1999). The platelet stops and is held to the site, mediated by platelet membrane glycoproteins acting as adhesive protein receptors. Several glycoproteins (GP) essential for adhesion and their ligand have been identified (Table 2.2), mostly consisting of non-covalent complexes of individual glycoproteins.

Adhesion is further also a strong stimulus for platelet activation via pathways mediated by membrane glycoprotein receptors.

Table 2.2: Surface membrane glycoprotein that function as adhesion receptors.

(Very Late Activation Antigen 6 (VLA-6)) (Becker 2008 and Spyropoulos 2002)

Receptor	**Ligand**	**Biological action**
GPIa/IIa	Collagen	Adhesion
GPIb/IX	Von Willebrand factor	Adhesion
GPIc/IIa	Fibronectin	Adhesion
GPIIb/IIIa	Collagen, Fibrinogen, Fibronectin, Vitronectin Von Willebrand factor	Aggregation (secondary role in adhesion)
GPIV	Thrombospondin, Collagen	
GPIVI	Collagen	Adhesion
Vitronectin receptor	Vitronectin, Thrombospondin	Adhesion
VLA-6	Laminin	Adhesion

Activation of platelets is in turn triggered by several biochemical and/or mechanical stimuli. The biochemical agonist are mainly produced or released by the platelets themselves after adhesion. The aforementioned agonist binds surface glycoproteins and stimulates signal transduction across the membrane via G-coupled messenger proteins, which in turn trigger one of two intracellular pathways (Becker 2008 and Spyropoulos 2002). The phosphoinositoside pathway is initiated with activation of phospholipase C, producing second messengers inositisol-1,4,5-triphosphate (IP3) and diacylglycerol which in turn increase in cytosolic calcium ions (Ca^{2+}) due to external influx and release from intracellular stores (Becker 2008 and Spyropoulos 2002) ultimately causing, amongst others, fibrinogen receptor expression (Becker 2008). The second pathway involves

phospholipase A, which following activation liberates arachidonate, and is further converted to thromboxane A_2 (TXA_2) which is another agonist of platelet activation, creating a positive feedback mechanism (Becker 2008).

The third stage, secretion, is stimulated by the activation of platelets, leading to cytoskeletal reorganization, membrane fusions, exteriorization and secretion of the contents of three different types of storage granules; lysosomes, α granules and dense bodies (Becker 2008 and Rao 1999). Lysosomes contain a number of enzymes (almost exclusively acid hydrolases) that hydrolyse the glucidic moiety of glycol proteins and endocytosed material (Becker 2008 and Spyropoulos 2002). The dense bodies, so called as they are osmophilically electron dense (Becker 2008 and Spyropoulos 2002), contain a large amount of non-metabolic adenines (e.g. ADP which promote recruitment and activation of additional platelets to the site of vascular injury), bivalent cations (Ca^{2+}, magnesium ions (Mg^{2+}), serotonins and pyrophosphates (Becker 2008 and Spyropoulos 2002). α Granules contain platelet-specific proteins [e.g. platelet-derived growth factor (PDGF)] which is believed to modulate smooth muscle cell proliferation, connecting tissue activating peptide III (CTAP III) and platelet factor-4 both of which is involved in fibroblast proliferation) (Becker 2008). In addition α-granules contains coagulation factors [e.g. pro-accelerin (factor V) which is critical to assembly of prothrombinase] and a variety of glycoproteins (e.g. thrombospondin which is thought to play a role in smooth muscle cell proliferation) (Becker 2008).

Following activation, a conformational change in GPIIb/IIIa receptor occurs. This allows fibrinogen and the GPIIb/IIIa receptor to interact and to form multiple cross-links between adjacent platelets or aggregation. Fibrinogen is required as a cofactor for aggregation and because it is a large dimeric molecule, it is likely that it binds to receptors on adjacent activated platelets, cross-linking them into aggregates (Rao 1999).

2.8.3. Factors affecting Platelet Function and Ultrastructure

As with fibrin networks; there are various environmental factors that affect the function and in several cases the ultrastructure of the platelet, including, age of the platelet, diet,

smoking, clinical history, fasting status, systemic diseases, the concentration and availability of plasma proteins, extracellular matrixes, chemicals and other molecules directly involved in platelet adhesion, activation, secretion or aggregation. Dysfunction and abnormal morphology of platelets due to the absence or increase of inherent molecules or factors directly participating in coagulation is usually associated with disease, and will be discussed in the following section. See Table 2.3 for a few examples of certain food and environmental factors that affect platelet function and ultrastructure.

Table 2.3: Pharmaceuticals and their effect on platelet function and ultrastructure.

FACTOR	Affected Function	Affected Ultrastructure
Omega-3 fatty acids, fish oil	Reduction in TXA_2 synthesis	Prolonged bleeding time, decreased aggregation (Shanttil *et al.,* 2012)
Vitamin E	Inhibition of protein kinase C	Decreased pseudopodia formation and adhesion (Steiner 1999)
Onion, cumin, turmeric, clove	Decrease platelet thromboxane production	Decreased activation and aggregation (Shanttil *et al.,* 2012)
Garlic	Inhibit cyclooxygenase activity and TXA_2 formation, by suppressing mobilization of intra-platelet Ca2+	Decreased aggregation (Rahman 2007)
Smoking	Mechanism uncertain	Increase platelet reactivity (Quinn & Fitzgerald 2005). Increased adhesion (Pittilo *et al.,* 1984)
High fat diet	Mechanism uncertain	Fusion between adjacent platelet membranes (Nathaniel *et al.,* 1972)

2.8.4. Abnormal platelets in disease

Platelet's importance in haemostasis has long been recognized and it is a well known fact that platelets play a significant role in blood coagulation, tissue repair and also inflammation and phagocytosis. Any abnormality or disease of blood platelets impairs the

platelet function. Several of these well-known dysfunctions or thrombocytopathies, has been well correlated with morphological abnormalities of blood platelets.

2.8.4.1. Hereditary Thrombocytopenia

Thrombocytopenia is a decrease in the number of platelets in circulating blood and this decrease can have a number of causes. One of these hereditary conditions; Micro-thrombocytopenia is caused by a mutation in the short arm of the X chromosome which leads to platelets that are functionally defective and morphologically abnormally small. These defective platelets are removed by the spleen which leads to a further decrease in clotting functionality (Dunphy 2010 and Spyropoulos 2002).
Macro-thrombocytopenia, as the name suggests, presents with abnormally large platelets, and can be caused by an X recessive disorder due to a missense mutation in the X chromosome. These platelets present with a more spherical shape, abnormal membrane complexes and clusters of smooth endoplasmic reticulum (Dunphy 2010 and Spyropoulos 2002). Macro-thrombocytopenia can also be caused by an autosomal dominant disorder in the MYH9 gene, which presents with giant platelets and basophilic Döhle-like inclusions in the leukocytes (Greinacher & Mueller-Eckhardt 1990 and Spyropoulos 2002). Clinical symptoms related to platelet function are variable with mild bleeding tendencies being the norm, but easy bruising, menorrhagia, heavy post-operative and postpartum bleeding and hematomas have been reported (Greinacher & Mueller-Eckhardt 1990).
An autosomal recessive inherited disorder (Bernard Soulier Syndrome) has also been found that presents with giant platelets lacking granules and/or a disorganized system of microtubules. Clinically severe bleeding disorders, decreased platelet adhesion and reduced platelet survival is seen (Dunphy 2010; Spyropoulos 2002 and White & Gerrard 1972). This has been shown to be due to defects in the glycoprotein GPIb/V/IX receptor complexes (Dunphy 2010; Spyropoulos 2002 and White & Gerrard 1972).

2.8.4.2. Thrombasthenia

Thrombasthenia is a rare autosomal recessive disorder, characterized by the complete absence of platelet aggregation, leading to prolonged bleeding time. This lack of

aggregation is due to defective or absent glycoprotein, GP IIb and IIIA, on the platelet surfaces (Nurden & Caen 1974 and White & Gerrard 1972). Although normal internal morphology has been reported, lack of activation with the inability to spread or form pseudopodia is characteristically seen with scanning electron micrographs (White & Gerrard 1972).

2.8.4.3. Disorders associated with abnormal organelles

In Hermansky-Pudlak syndrome (HPS) the population of electron dense bodies is greatly reduced and in a number of cases completely absent (White & Gerrard 1972 and Witkop *et al.,* 1987). Biochemical studies has shown intrinsic low levels of serotonin and adenine nucleotides, defective availability of platelet factor III and decreased calcium (White & Gerrard 1972 and White *et al.,* 1971). The cell's morphological abnormalities seen, are due to insufficient secretion, leading to decreased platelet-platelet aggregation and functionally results in prolonged bleeding times (White & Gerrard 1972). Chediak-Higashi syndrome (CHS) is a rare autosomal inherited disorder in which platelets has been shown to include large cytoplasmic granules never seen in healthy platelets (White & Gerrard 1972), decreased number of dense bodies (Buchanan & Handini 1976 and Rendu *et al.,* 1983) and abnormal platelet aggregation (Buchanan & Handini 1976 and Rendu *et al.,* 1983). Functionally, clinically prolonged bleeding times is found (Buchanan & Handini 1976 and White & Gerrard 1972). In the congenital bleeding disorder, Gray platelet syndrome, platelets show decreased aggregations and retention rates, and morphologically the platelets are large and lacking α granules (Levy-Toledano *et al.,* 1981; Mori *et al.,* 1984 and White & Gerrard 1972).

2.8.4.4. Diabetes mellitus

Individuals suffering from diabetes mellitus have been shown to have hyperactive platelets with increased aggregability and adhesiveness (Colwell & Nesto 2007; Sower & Epstein 1995 and White & Gerrard 1972). Functionally several changes has been identified including; reduced membrane fluidity, altered calcium and magnesium homeostasis (Sower & Epstein 1995), increased arachidonic acid metabolism and decreased

prostacyclin production (Colwell & Nesto 2007). Ultrastructurally changes are seen as highly activated shrunken platelets with membrane blebbing and typical apoptotic morphology (Pretorius *et al.,* 2011).

2.8.4.5. Anaemia

Certain types of anaemia have been shown to exhibit abnormal platelet morphology (Doll 1982 and Michelson 2007). In spur-cell anaemia platelets present with irregularly distributed bulbous like projections (Doll 1982), and in anaemia associated with chronic liver disease thrombocytopenia, impaired aggregation and reduced adhesiveness is found (Michelson 2007).

2.8.4.6. Hypertension

It is well known that hyperactive platelets, with increased sensitivity to ADP induced aggregation (Nityanand *et al.,* 1993), are associated with hypertension (White & Gerrard 1972), with the degree of activation of platelets being well correlated to the level of severity of hypertension (Pande *et al.,* 1993). Morphological alterations include an increased mean platelet volume and platelet mass, decreased platelet granularity and an increased platelet size. (Haouari & Rosado 2009)

2.4.8.7. Asthma

Asthma is a chronic airway disorder, characterized by airway inflammation, in which platelets has been shown to play an important role by participating in bronchoconstriction, airway hyper-responsiveness and bronchial wall remodelling (Pretorius 2008; Pretorius & Oberholzer 2009 and Stafforini *et al.,* 1999). Platelet function has been shown to be altered in response to allergen exposure in patients suffering from asthma by partaking in the activation and chemotaxis of adaptive immune cells through the release of inflammatory markers, platelets also migrate to the lungs and may contribute to airway remodelling (Morley *et al.,* 1984; Pitchford *et al.,* 2007; Pretorius 2008 and Stafforini *et al.,* 1999). Pretorius and Oberholzer have shown ultrastructural alterations in platelets from

asthmatic patients, where platelet granularity is increased and platelets form abnormally loose connected aggregates (Pretorius & Oberholzer 2009).

2.8.4.8. Cancers

Cancer is by nature a hypercoagulable state and platelets have therefore been clinically associated with this disease for more than 200 years (Gupta *et al.,* 2004). Enhancement of tumour cell proliferation, increased tumour cell interaction with the extracellular matrix and also promoted tumour extravasations and angiogenesis are also attributed to platelet mediated means (Bambace & Holmes 2011; Gupta *et al.,* 2004 and Honn *et al.,* 1992). Generally, platelet interaction with tumour cells is achieved by adhesion (membrane bound) molecules and secreted compounds, see Table 2.4 for examples of platelet related molecules that interact with tumours (Bambace & Holmes 2011 and Honn *et al.,* 1992).

Table 2.4: Key platelet components and their contribution to haemostasis and malignancy. (Adapted from Bambace & Holmes 2011)

Platelet component	**Principal role in thrombus formation**	**Role in malignancy (in vivo)**
GPIIb/IIIa	Activation allows fibrinogen binding and platelet plug reinforcement	Decreased pulmonary metastasis following inhibition of receptor antagonists (in vivo)
GP Ib-IX-V	Binding of von Willebrand factor (VWF); anchors platelet to subendothelium	Decreased pulmonary metastasis following inhibition of receptor by antibody and receptor antagonists
GPVI	Platelet adhesion to collagen	50% reduction in pulmonary metastases in GPVI-deficient mice
P-selectin	Mediates platelet-leukocyte tethering; triggers leukocyte activation	Deficiency or blockade of P-selectin inhibits the formation of melanoma metastases
Purinergic Receptor 2Y	ADP-mediated platelet aggregation	ADP depletion associated with reduced metastases
Protease activated Receptor (PAR)	Thrombin mediated platelet activation	Promote metastases
Alpha granules	Storage of proteins that enhance adhesive process	Maintenance of intra-tumour vascular integrity
Platelet micro-particles	Enhances thrombosis and secondary haemostasis	Increased chemo-invasiveness and metastases in lung cancer models

Platelet ultrastructural changes has been seen in most common cancers, including colorectal, lung and breast cancer, as well as gastric, renal and most urogenital malignancies present with thrombocytosis (Bambace & Holmes 2011). In clear cell renal carcinoma Pretorius *et a*, (2009a) demonstrated platelet aggregates with several breakages in the platelet membrane and a pockmarked, prune like appearance as opposed to smooth rounded membranes of control platelets. Transmission electron microscopy of non-small cell lung cancer, platelets appear swollen, with increased α-granules, vesicles, and glycogenosome, whereas scanning electron microscopy show increased surface processes and furrows (Zhuge *et al.,* 2009).

2.8.4.9. HIV

Thrombocytopenia occurs in about 40% of patients suffering from Human Immunodeficiency Virus (HIV) (Littleton 2007 and Pretorius 2009c) and is directly correlated with increased morbidity and mortality, enhanced deterioration of the cluster of differentiation 4 (CD4) counts and progression to full-blown acquired immune deficiency syndrome (AIDS) (Littleton 2007). Thrombocytopenia is believed to be caused by a defect in platelet production by HIV infected megakaryocytes, or a peripheral destruction of the platelets, resulting in decreased aggregation of platelets. Ultrastructurally platelets present with membrane blebbing and membrane breakages, this morphology support the theory of destruction of the platelets as an alternative to abnormal thrombopoiesis (Pretorius 2008 and Pretorius *et al.,* 2009c).

2.8.4.10. Drugs

Several drugs have been shown to affect platelet function and ultrastructure. Below are a few examples:

- Oral Integrin αIIβ3 (IIbαβ3) inhibitors and fibrinolytic agents inhibit GPIIb/IIIa-fibrinogen interaction (Sharathukumar & Shapiro 2008), and is morphologically seen as an absence of platelet aggregation in response to agonists such as collagen, ADP, and epinephrine (Lisman *et al.,* 2004).

- Ticlopidine and clopidogrel cause inhibition of ADP receptors which leads to impairment of platelet aggregation (Shanttil *et al.,* 2012).
- Methylxanthines and related drugs inhibit phosphodiesterase, which in turn produce reduced aggregation (MacIntyre *et al.,* 1997).
- Aspirin acetylate and irreversibly inactivate the enzyme PG-endoperoxide-H-synthase-1 (PGHS-1) (Shanttil *et al.,* 2012), reducing aggregation and a complete loss of pseudopodia (Lisman *et al.,* 2004; Rao 1999 and White & Gerrard 1972).
- Nonsteroidal anti-inflammatory drugs inhibit the thromboxane pathway, which may impair platelet secretion and second wave aggregation (Shanttil *et al.,* 2012).
- Calcium blockers, blocks the necessary calcium influx, which inhibit platelet aggregation (Johnson *et al.,* 1986).
- Tri-cyclic antidepressants inhibit serotonin uptake which leads to an impairment of platelet aggregation and a decreased platelet count (Shah *et al.,* 2005).
- Statins interferes with the guanosine triphosphate (GTP) signalling pathways, which give rise to inhibition of platelet adhesion and reactivity (Di Napoli *et al.,* 2002).
- Antibiotics such as β-lactam and penicillin, reduce platelet aggregation and secretion as well as ristocetin-induced platelet agglutination (Shanttil *et al.,* 2012), although the mechanisms is uncertain.
- Anaesthetics, for example halothane, produce impairment in platelet aggregation which is believed to be due to the effects of calcium signalling (Shanttil *et al.,* 2012).
- Radiologic contrast agents, antihistamines and oncologic drugs impair platelet aggregation by unknown mechanisms (Shanttil *et al.,* 2012).
- Diclofenac sodium or more commonly known as voltaren increase activation and the elongation of pseudopodia (Djaldetti et a. 1982 and White & Gerrard 1972), it also increases the phagocytic activity, while decreasing protein synthesizing ability of the platelets (Djaldetti et a. 1982).

2.9. Research Objective

The literature clearly indicates that abnormalities of fibrin network and platelet ultrastructure have definite clinical implications, leading to bleeding tendencies or increased thrombotic events. The use of the best potential preparation methods of these specimens for ultrastructural investigation is of utmost importance as any of the necessary steps in sample preparation affect the quality of the final electron micrographs. Artefact introduction and structural alterations due to preparation process may lead an investigator to misinterpret the results and ultimately erroneous conclusions. Therefore the following research objectives will be investigated in the current dissertation:

- The development of a method of rapidly vitrifying platelet and fibrin networks utilizing high pressure freezing and freeze substitution, for the use in TEM ultrastructural studies.
- The comparison of chemical and high pressure freezing preparation methods to study human fibrin fibers and platelets using TEM.
- The development of a method of rapidly vitrifying platelet and fibrin networks utilizing high pressure freezing and freeze drying for the use in SEM ultrastructural studies.
- The comparison of chemical and high pressure freezing preparation methods to study human fibrin fibers and platelets using SEM.

Chapter 3:

Development of a method of rapidly vitrifying platelet and fibrin networks utilizing HPF and FS for the use in TEM ultrastructural studies

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Research Question 1:

What method of rapidly vitrifying platelets and fibrin networks utilizing high pressure freezing and freeze substitution is the most repeatable and gives the most informative results in TEM ultrastructural studies?

3.1. Introduction

Coagulation or thrombogenesis is of utmost importance to haemostasis and overall health, any dysfunction in coagulation may lead to either bleeding or thrombotic tendencies. The two most vital participants in thrombogenesis are the platelets and the fibrin network, with dysfunction in either of these two components leading to severe haematological implications.

Many dysfunctional and diseased states of either platelets or the fibrin network are known to be well correlated to corresponding ultrastructure abnormalities (Pretorius 2008). Ultrastructural studies with TEM have shown several morphological abnormalities of platelets in disease. For example; in hypertension decreased platelet granularity and an increased platelet size is seen (Haouari & Rosado 2009) and in asthma platelets form loose aggregates and has increased granularity (Pretorius & Oberholzer 2009). Furthermore, non-small cell lung cancer, platelets appear swollen, with increased α-granules, vesicles, and glycogenosome (Zhuge *et al.,* 2009), and in HPS there is a decrease or even an absence in electron dense bodies (White & Gerrard 1972 and Witkop

et al., 1987). Ultrastructural studies of fibrin fibers and fiber networks in disease is as a rule realised using a scanning electron microscope and transmission electron microscopy examples are scarce, leaving a certain void to be filled in this kind of research. Two ultrastructural abnormalities studied by TEM are; the congenital dysfibrinogenemia presenting with Metz-fibrinogen that forms fragile networks made up mostly of thin fibers (Mosseson *et al.,* 1987) and in another congenital fibrinogen abnormality, Paris I, the fibrin network is characterized with non-fibrous clumps of material connected by irregularly sized to thin fibrous strands (Hohenberg *et al.,* 1994).

At a potential resolution of less than one nanometer, TEM's are the most powerful tools available for ultrastructural studies of biological materials. The resolving power of an electron microscope is due to the exploitation of energized electrons that has significantly shorter wavelengths of illumination (approximately 2-4pm, depending on the accelerating voltage) than that of a light microscope (approximately 400nm) (Bozzola & Russel 1999). In very basic terms, the principle process of a TEM is; an electron source emits and accelerates electrons through a vacuum; the electrons are then focused onto the sample by a series of electromagnetic lenses and apertures. As electrons travel through the specimen, the electrons interact with molecules in the sample and an image forms when transmitted electrons hit a fluorescent screen or a digital camera. For electrons to be able to travel through a specimen to the image-forming screen, the specimen has to be suitably thin; to meet this specific requirement (and others such as stability under vacuum), extensive alteration of the specimen by several preparatory steps is required.

Currently the two main methods of preparation of biological specimens for examination with TEM are chemical and freeze fixation. Chemical fixation involves the chemical alteration of a specimen by means of the introduction of chemical bonds and cross-links that keep molecules and structures in place and stabilize the specimen. Several rinsing and dehydration steps, and ultimately infiltration and polymerization with an embedding medium follow fixation. The hardened block is then cut into thin sections (50 - 100nm), by the use of an ultramicrotome. The second, freeze fixation requires ultra-rapid freezing (typically under high pressure - HPF) of the specimen, followed by the substitution of the

vitrified water at low temperatures, and then similarly to chemical fixation, infiltration, polymerization and sectioning.

Several authors have highlighted the superiority of ultra-rapid freezing of biological tissues for ultrastructural studies to that of conventional chemical methods (Kellenberger 1991; Murk *et al.,* 2003 and Quintana 1994). Although the conventional chemical preparation of specimens has been and will continue to be invaluable for ultrastructural studies, it has severe limitations. Chemical fixation has been shown to cause (to name a few) distorted membranes and organelles (McDonald & Morphew 1993), shrinkage and deformation in certain specimens (Murk *et al.,* 2003), extraction of proteins and lipids from the cytosol (Kellenberger 1991), and because it is a relatively slow process, it allows the loss or redistribution of diffusible constituents (Quintana 1994).

When using HPF, several different techniques, depending on tissue type and desired results exists: The general method used for the majority of specimens, is to transfer the specimen into a specialised flat specimen carrier (0.5mm thick, 1.5mm inner diameter, 200µm deep) (Figure 3.1). No air should be present during the freezing process, so it is wise to either pre-fill the specimen carrier or add a droplet of a cryoprotectant, such as 1-Hexadene, on top of the sample.

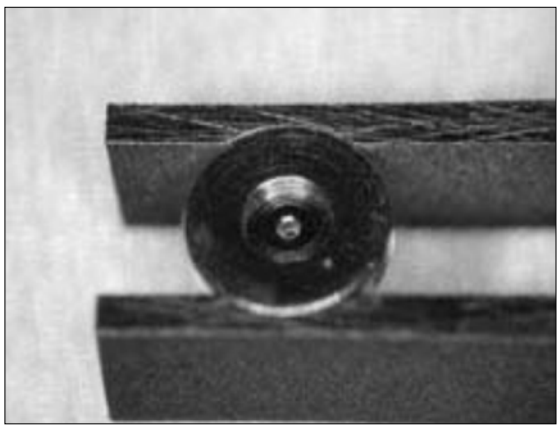


Figure 3.1: Flat specimen carrier in the transfer slider tool.
(Leica Microsystems Vienna, Austria).

Hohenberg et al (1994) instituted the use of cellulose capillary tubes with an inner diameter of 200µm for HPF (Figure 3.2). For samples that are normally difficult to handle or very fragile, such as cell suspension, this method has proven to be extremely successful. A piece of capillary tubing is divided into small pieces, 10-20mm in length, and is held with forceps at one end while the other is placed into the specimen suspension. The suspension is drawn up by capillary action into the tube (Hohenberg *et al.,* 1994 and Tiedemann *et al.,* 1998); some choose to use micro-aspiration to "pull" the specimen in when working with sparse or extremely delicate specimens (see Triffo *et al.,* 2008). The capillary tube is cut into a smaller piece and sealed using a crimping tool; this is to maintain the specimen within the tubing and to be able to fit into the specimen carrier for freezing.

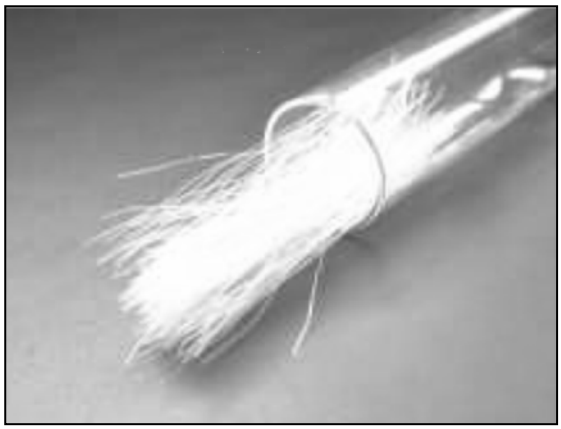


Figure 3.2: Cellulose micro-capillary tubes.
(Leica Microsystems Vienna, Austria).

Other types of specimen carriers and accessories also exist for the use of specific model systems. Specimen carriers exclusively for biopsies, carriers specifically for freeze-fracture, sapphire cover slips and Aclar sheets for adherent cells and finder grids masks for ease of microscopy, are available from the manufacturer; these will not be discussed further.

HPF is followed by a process called freeze substitution, where specimens in vitreous ice is exposed to mixtures of organic solvents, fixatives and contrasting agents at low

temperatures (< -80°C) in order to dissolve the ice, replace the water and stabilize the material (Glauert 1981; Hayat 2000; Humbel & Müller 1984; McDonald & Webb 2011 and Shiurba 2001). The diffusion of the reagents into the ice at these types of temperatures is awfully slow, so the substitution is usually conducted over several days. Protocols for freeze substitution media and time protocols are numerous, variations is usually based on the required results and the nature and thickness of the sample. Similarly, to the chemical method the specimens need to be infiltrated with an embedding medium and polymerized to form a hard resin block suitable for thin sectioning. Several types of embedding media exists (epoxy -, methacrylate - and polyester resins), the consensus is that epoxy resins are superior for ultrastructural research. Within the “family” of epoxy resins, there are numerous types of resin, all with different properties and features, this coupled with all the different types of modifiers (hardeners, accelerators, plasticisers and flexibilizers) available has led to hundreds of different recipes for embedding media. Two of the most widely used epoxy resins are Quetol and Epon resins; both are low viscosity resins, capable of relative rapid infiltration (Epon’s viscosity is a tad lower), both polymerize uniformly and without big volume changes and has relatively stable polymers (Hayat 2000). After resin polymerization, ultrathin (50 – 100nm) sections are cut with an ultramicrotome, subsequent thin sections are then typically stained with heavy metal salts to increase contrast in the TEM.

In the current chapter, several different approaches of freeze fixation, employing HPF and freeze substitution, are carried out to determine the optimum preparation method for transmission electron microscope ultrastructural studies of platelets and fibrin networks. To accomplish this several factors in the preparation procedure was varied; these factors are the;

- Coagulation agent
- Coagulation agent-platelet-rich-plasma (PRP) ratio
- Coagulation time
- Centrifugation time
- Gelation temperature

- HPF technique
- Freeze substitution protocol
- Embedding media
- Staining

3.2. Materials and Methods

3.2.1. Samples

Healthy individuals, without any known medical condition, who do not smoke and in the case of women, were not on contraception, were chosen for this study. Whole blood obtained by venipuncture was collected in citrate tubes [for the inhibition of calcium-mediated coagulation by the addition of sodium citrate (Sabbatani 1901a and Sabbatani 1901b)], followed by 2 minutes of centrifugation to separate the plasma, containing the platelets and fibrinogen from the erythrocytes and leukocytes.

3.2.2. Sample Preparation

From the next stage onward, the process is varied at predetermined coagulation factor points and sample preparation steps (see Table 3.1). The basic methodology will be explained next, followed by a detailed explanation towards each variant.

The supernatant liquid (PRP) was separated from the pellet (erythrocytes and leukocytes) by pipetting the liquid off into a polyethylene holder (eppendorf). The PRP was then mixed with a coagulation agent, centrifuged (in some experiments) and left for coagulation to occur. The platelet and fibrin coagulation was then high pressure frozen (EMPACT2 (Figure 3.3a), Leica Microsystems, Vienna, Austria) and kept under liquid nitrogen conditions until such a time that freeze substitution was done. For freeze substitution the specimens were transferred into a pre-cooled sample container (Leica Microsystems, Vienna, Austria) filled with freeze substitution-media consisting of 2% osmium tetroxide, 0.1% uranyl acetate in acetone, and 1% distilled water. FS was carried out with the Leica

EM AFS2 (Leica Microsystems, Vienna, Austria). After freeze substitution the specimens were rinsed three times with acetone for 10 minutes per rinse, and the specimens removed from the specimen carriers, followed by embedding media infiltration. Firstly, the specimens were placed into a mixture of 1:1 ratio of 100% acetone to embedding media and left for 30 minutes, the mixture was drawn off, new embedding media was added and allowed to infiltrate for 3 hours. The specimens were then placed in fresh resin and allowed to polymerize for 36 hours at 60°C. The hard resin blocks were sectioned using an ultramicrotome (Reichart-Jung Ultracut E, Vienna, Austria) into 80nm sections.

Not all of the possible variations were performed as this would result in almost 3000 different experiments to prepare and analyse, with financial, laboratory time and equipment limitations this was not feasible.

The optimum coagulation properties and HPF technique was determined using only the two coagulation agents, the different coagulation agent PRP ratios, the three HPF techniques, variable centrifugation times and the first and third coagulation times. These experiments were carried out at least three times to ascertain their reproducibility. The variables giving the most promising results were further varied with regards to the mid-range coagulation times, freeze substitution and embedding media (see Figure 3.4 for a flow diagram of initial experiments and Figure 3.5 for a flow diagram of subsequent experiments). To accomplish the last variation in technique (staining), a few additional sections were cut and viewed separately without staining, meaning that a “complete” experiment was not performed in order to analyse this variable.

Table 3.1: The variants of coagulation factors and specimen preparation factors used in this study.

Coagulation or Preparation Factor	**Variation1**	**Variation 2**	**Variation 3**	**Variation 4**	**Variation 5**
Coagulation agent	Thrombin	$CaCl_2$	-	-	-
Coagulation agent-(PRP) ratio	1:1 (T)	1:5 (T)	5:1 (C)	10:1 (C)	100:3 (C)
HPF technique	C1	C2	C3	-	-
Centrifugation time (min)	0 (C1)	2 (C1)	5 (C1)	-	-
Coagulation time (min)	1	10	20	60	-
Coagulation temperature	RT	37°C			
Freeze substitution protocol	90 hours	36 hours	-	-	-
Embedding media	Quetol	Epon	-	-	-
Staining	Y	N	-	-	-

Key: (T) – Thrombin, (C) – $CaCl_2$, C1 – Specimen Carrier Method 1, C2 –Specimen Carrier Method 2, C3 – Cellulose Capillary Method, RT – Room Temperature, Y – Yes, N – No.

3.2.2.1. Coagulation Agent

The two coagulation agents used were thrombin and calcium chloride ($CaCl_2$). Thrombin is a serine protease, proteolytic enzyme (Blombäck 1996; Di Stasio *et al.,* 1998;

Mosseson *et al.,* 2001; Ryan *et al.,* 1999 and Weisel 2005; Wolberg 2007 and Woodhead *et al.,* 1984), which initiates fibrinogen polymerization by structure-dependent interactions between the fibrinopeptides and thrombin's catalytic sites, and noncatalytic binding of enzyme to substrate (Weisel 2005). Thrombin also promotes platelet activation and aggregation via G-coupled messenger proteins, which in turn trigger one of two intracellular pathways (Becker 2008 and Spyropoulos 2002).

The South African National Blood Service supplied human thrombin used in this study; it was prepared from a single donor by the calcium chloride activation of a euglobulin fraction of the plasma.

$CaCl_2$ is often used as a coagulation agent in blood clotting studies, as chloride ions are known to be a basic physiological modulator of fibrin polymerization (Standeven *et al.,* 2005) and are known to initiate platelet activation by stimulation of glycoprotein G expression (Phillips *et al.,* 1980).

3.2.2.2. Coagulation Agent – PRP Ratio

Different ratios of the coagulation agent to PRP (see Table 3.1), was loosely based, on concentrations regularly used in literature and considering physiologically relevant concentrations.

Thrombin: PRP – 1:1 (Pretorius *et al.,* 2006; Pretorius *et al.,* 2007; Pretorius *et al.,* 2009b and Pretorius *et al.,* 2011)

Thrombin: PRP – 1:5 (Pretorius *et al.,* 2007b)

$CaCl_2$: PRP - 5:1 (Born & Cross 1963 and Chargraff & Olson 1937)

$CaCl_2$: PRP - 10:1 (Metassan *et al.,* 2009)

$CaCl_2$: PRP - 100:3 (Lim *et al.,* 2010)

3.2.2.3. HPF Technique

Three different approaches were used for high pressure freezing (see Figure 3.4); for lack of more descriptive phrasing they are called Specimen Carrier Method 1, Specimen Carrier Method 2 and Cellulose Capillary Method.

Specimen Carrier Method 1;

1. PRP was prepared as described above.

2. Coagulation was initiated with one of the two coagulation agents as described above.

3. The mixture was centrifuged for one of the periods above (centrifugation time).

4. The mixture was left and time periods above (gelation time), at one of the two temperature options above (gelation period).

5. The subsequent clot was removed from the holder with sterilized sharp-pointed tweezers and a small piece was dissected off (±0.5-1mm).

6. The specimen were transferred to a specimen carrier, which was pre-fitted into the transfer slide (Figure 3.3b), and prefilled with 1-Hexadene (Figure 3.3c).

7. The specimen carrier with specimen was loaded onto the loading device (Figure 3.3d), and securely screwed into the freezing bayonet pod (Figure 3.4e) with the help of a specialized torque wrench (Figure 3.3f). The bayonet pod was then mounted onto the bayonet-loading device (Figure 3.3g).

8. The bayonet assembly was placed into the high-pressure freezing platform (Figure 3.3h) and subsequently high pressure frozen (Figure 3.3i) by compression generation and liquid nitrogen spraying of the specimen, after which the specimen was automatically brought into a liquid nitrogen bath (Figure 3.3j).

Specimen Carrier Method 2;

1. PRP was prepared as described above.

2. Coagulation was initiated with one of the two coagulation agents by placing minute volumes of the PRP and coagulation agent into the specimen carrier and gently mixing.

3. The mixture was left and time periods above (gelation time), at one of the two temperature options above (gelation period).

4. The specimen holder with the specimen was fitted into the transfer slide (Figure 3.3c).

5. Further processing was identical to steps seven and eight in Specimen Carrier Method 1

Cellulose Capillary Method;

1. PRP was prepared as described above.

2. Coagulation was initiated with one of the two coagulation agents as described above.

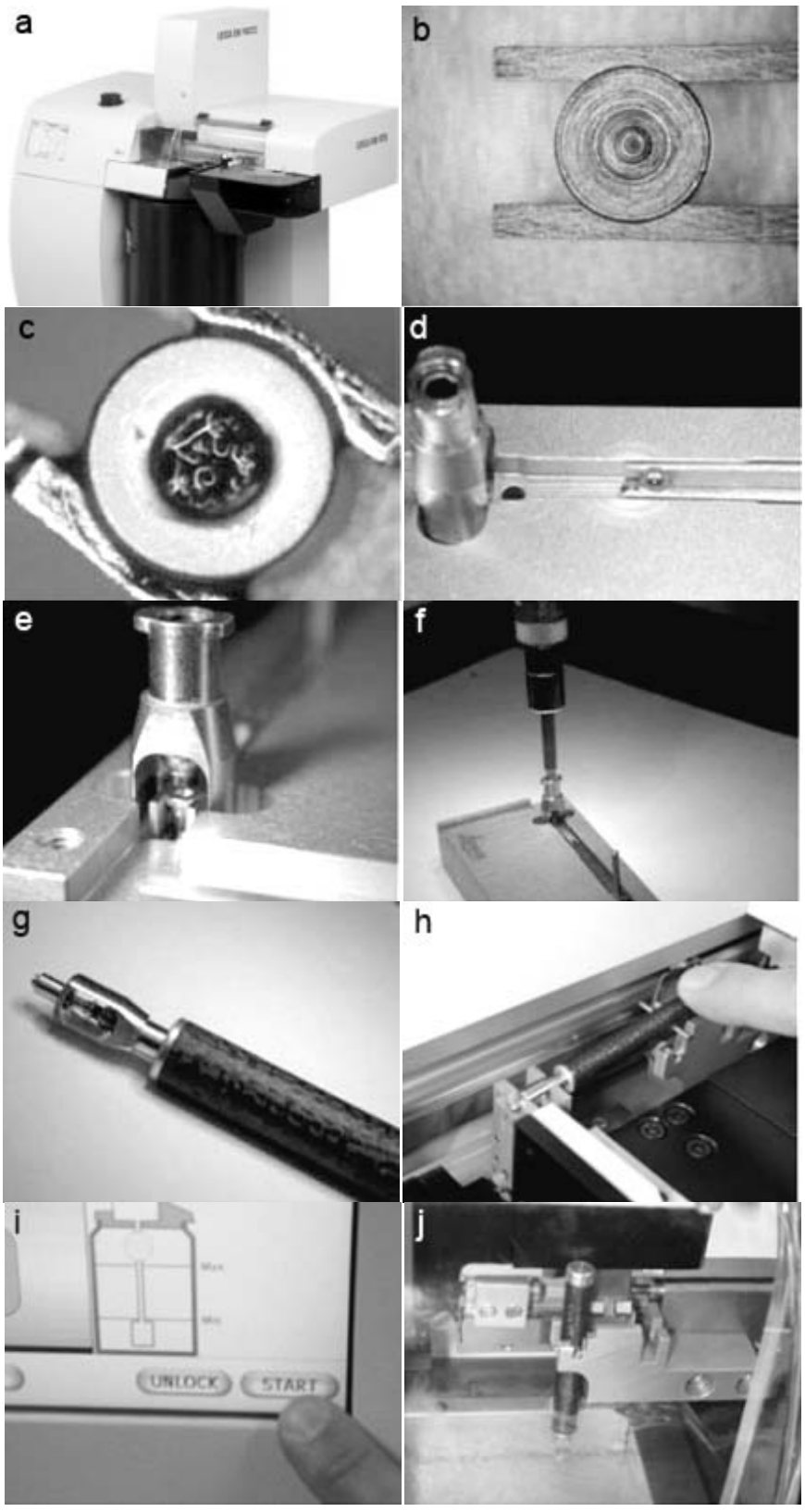
3. A pre-cut piece (10-20nm) of cellulose capillary tube was held with forceps at one end while the other was placed into the PRP-coagulation agent mixture, the mixture was allowed to be drawn up by capillary forces until the whole tube was filled.

4. The mixture in the capillary tube was left for the periods above (gelation time), at one of the two temperature options above (gelation period).

5. The central part of the capillary tube was cut out (±1mm) and sealed by using a crimping tool (a blunted scalpel was used for this purpose).

6. The pieces of capillary tubing containing the specimen were transferred to a specimen carrier, which was pre-fitted into the transfer slide (Figure 3.3b), and prefilled with 1-Hexadene (Figure 3.3c).

7. Further processing was identical to steps seven and eight in Specimen Carrier Method 1.



(Legend on next page)

Figure 3.3: The high-pressure freezing process.
a: Leica EMPACT2 high pressure freezer. b: Specimen carrier, fitted into the transfer slide. c: Specimen carrier in the transfer slide containing a specimen. d: Transfer slide with the specimen carrier in the loading device. e: The freezing bayonet pod. f: The torque wrench, securing the specimen carrier in the bayonet pod. g: The bayonet pod mounted on the bayonet-loading device. h: The bayonet assembly in the high-pressure freezers freezing platform. i: Leica EMPACT2 touch screen control. j: Bayonet assembly with the pod containing the frozen specimen in the liquid nitrogen bath, directly after high-pressure freezing.

(These procedure are true for the Leica EMPACT2, different instrument's procedures may vary)

3.2.2.4. Centrifugation Time

Centrifugation time was varied between zero and five minutes (see Table 3.1). Centrifugation was used to create a platelet and fibrin clot that is similar in density and ease of handling than that of a tissue block and additionally to further mix the PRP with the coagulation agent. Samples that were prepared in this method were mainly for the use of high pressure freezing in a specimen carrier.

3.2.2.5. Coagulation Time

The time allowed for coagulation of the fibrin and subsequent blood clot was varied between one and 60 minutes (see Table 3.1).

3.2.2.6. Coagulation Temperature

Studies of the coagulation of fibrin and platelets has been performed in this laboratory previously, in these studies coagulation was allowed to continue at room temperature, and proved to be completely adequate. A gelation temperature of 37°C was added as a variable for the sake of physiological relevancy.

3.2.2.7. Freeze Substitution Protocol

The freeze substitution protocol was only varied in the time schedule, as the freeze substitution mixture (2% osmium tetroxide, 0.1% uranyl acetate in acetone) used is a well-known recipe for the enhancement of membranes and fibrous molecules. This was done to try to ascertain if a more time efficient schedule gave equally satisfying results than that of the generally used schedule.

The first time schedule was (total of 90 hours):

- -90°C for 42 hours.
- Heated to -60°C over 15 hours (2°C per hour).
- -60°C for eight hours.
- Heated to -30°C over 15 hours (2°C per hour).
- -30°C for eight hours.
- Heated to 0°C over 2 hours (15°C per hour).
- Allowed to stabilize at room temperature.

The second time schedule was (total of 36 hours):

- -90°C for 16 hours.
- Heated to -70°C over two hours (10°C per hour).
- -70°C for eight hours.
- Heated to -30°C over eight hours (5°C per hour).
- Heated to 0°C over two hours (15°C per hour).
- Allowed to stabilize at room temperature over (±12 hours).

3.2.2.8. Embedding Media

The first embedding media used was a Quetol (Nishin) epoxy resin commonly used in this lab and sufficient for most TEM studies conducted here, it consists of (for 15g);

- Quetol (epoxy) 5.83g

- NMA (hardener) 6.69g
- DDSA (hardener) 2.49g
- Araldite RD2 (epoxy) 0.3g
- DMAE (catalyst) 0.15g

The second is a well-known and used Epon (Shell) epoxy resin, with a slightly lower viscosity than Quetol, which was employed to determine if infiltration and polymerization could possibly be bettered, this resin consisted of (for 15g);

- EMBED 812 (epoxy) 7.85g
- NMA (hardener) 5.3g
- DDSA (hardener) 2.05g
- DMAE (catalyst) 0.152g

3.2.2.9. Staining

After thin sectioning, the sections were stained with uranyl acetate for five minutes followed by two minutes of staining with lead citrate. Staining is known to occasionally cause artefacts on the sectioned material due to stain precipitation, to make sure any features observed, when the sections was stained, are not artefacts, sections was viewed without any staining. Sections without stain were also viewed to determine if the inherent contrast of the specimen and the contrast given by the osmium is similar to a stained section, and that all features are observable and clear. If unstained sections give the same amount of information than stained sections, an unstained section would be used, as staining is just another step further away from the specimen's natural state.

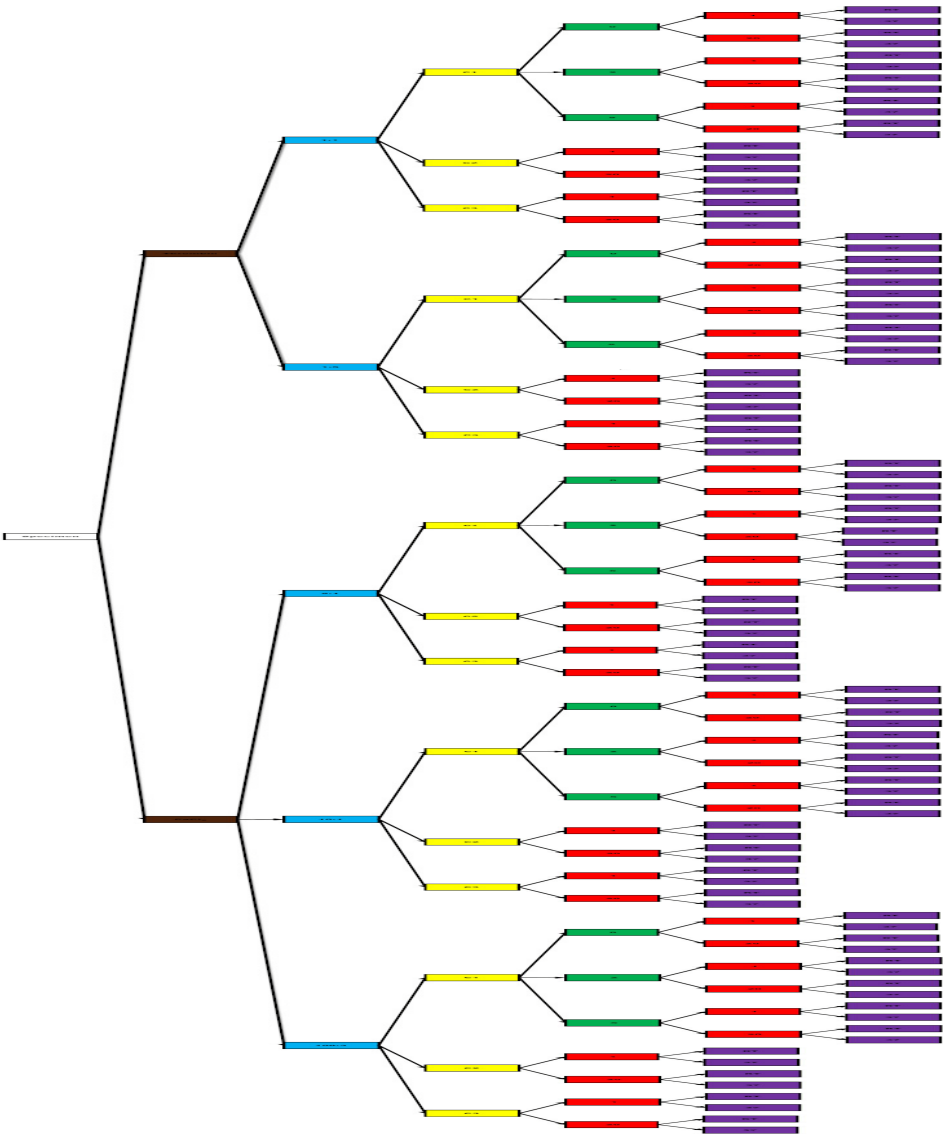


Figure 3.4: Flow diagram of experimental setup.

Key: white – specimen, grey – coagulation agent (thrombin / $CaCl_2$), blue – coagulation agent PRP ratio (thrombin 1:1 / 1:5, $CaCl_2$ 1:5 / 1:10 / 3:100), yellow – HPF technique (C1 / C2 / C3), green – centrifugation time (0 / 2 / 5), red – coagulation time (1 / 20), purple – coagulation temperature (room temperature / 37°C).

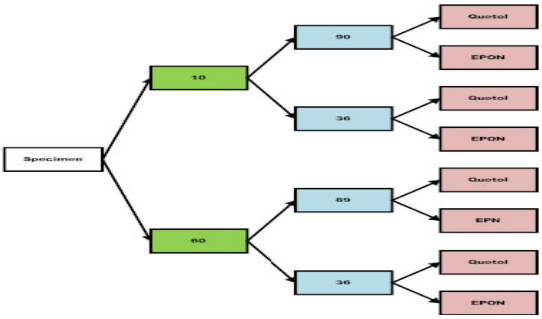


Figure 3.5: Flow diagram of subsequent experiments based on the results from the initial experiments.
Key: light green – coagulation time (10 / 60), light blue – freeze substitution protocol (90 / 36), light pink – embedding medium (Quetol / EPON).

3.3. Results

Individual variables will be discussed separately.

3.3.1. Coagulation Agent

Both thrombin and $CaCl_2$ were satisfactory as an activating agent for coagulation and in the formation of a fiber network.

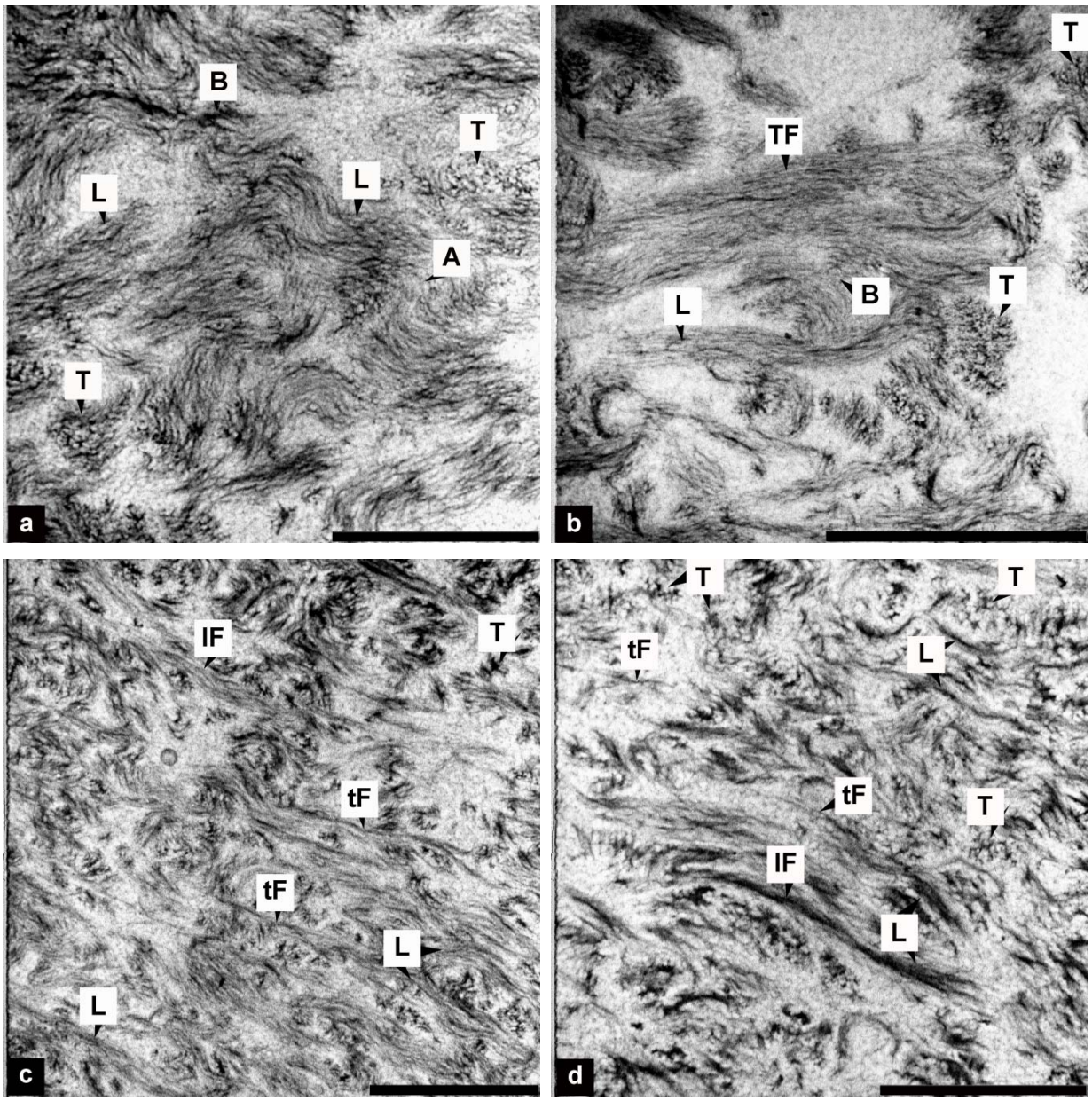


Figure 3.6: Fibrin networks activated by either thrombin (a-b) or $CaCl_2$ (c-d).
All scale bars = 1µm. a: Thrombin activated network showing (L) longitudinal as well as (T) transverse sectioned fibers, with increased (A) association between the fibers and ambiguous (B) branching. b: Thrombin activated network showing (L) longitudinal as well as (T) transverse sectioned (TF) thick fibers with (B) branching. c: CaCl2 activated network showing (L) longitudinal as well as (T) transverse sectioned (tF) thin and (IF) intermediate fibers. d: CaCl2 activated network showing (L) longitudinal as well as (T) transverse

sectioned (tF) thin and (IF) intermediate fibers.

The use of $CaCl_2$ as a coagulation agent resulted in a finer network, predominately made up from thin to intermediate fibers, where lateral associations between the fibers was decreased and branching of the thicker fibers was not clearly seen (Figure 3.6: c & d). The platelets activated by $CaCl_2$ was more activated than platelets activated by thrombin, presenting with organelles ranging from being disorganized to centralized with occasional pseudopodia formation, increased secreted vesicles and granules in the surrounding matrix was noted (Figure 3.7: b). Thrombin activation lead to a network made up of thicker fibers with a more condensed structure, with increased associations between the fibers and visible branching (Figure 3.6: a & b). More consistent results between repetitions of the same experiment, of network structure and fiber morphology were seen with thrombin coagulation, than compared to $CaCl_2$ coagulation.

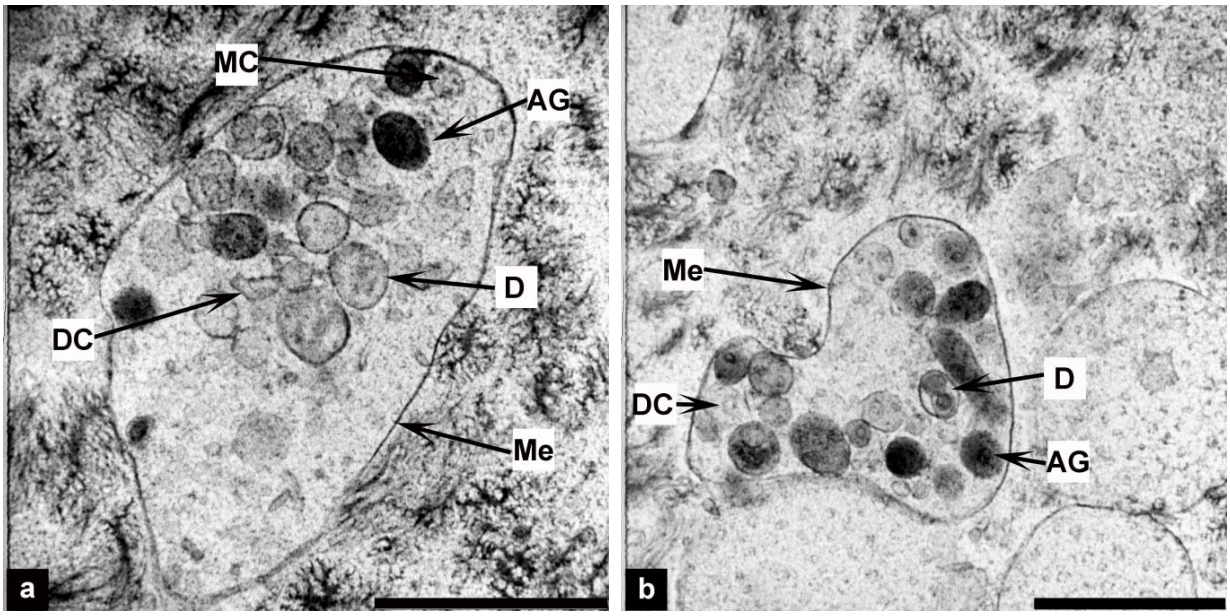


Figure 3.7: Platelets activated by either thrombin (a) or $CaCl_2$ (b).
All scale bars = 1µm. a: Bulbous platelet with organelles; (AG) alpha granules, (MC) mitochondria, (D) dense body, (DC) dilated channel and well defined (Me) membrane, surrounded by the fibrin network. b: Structurally reorganized platelet with organelles; (AG) alpha granules, (D) dense body, (DC) dilated channel and well defined (Me) membrane.

3.3.2. Coagulation Agent – PRP Ratio

An increase in concentration of $CaCl_2$ cause an unambiguous increase of fiber diameter, density and the amount (Figure 3.9: a, b & c), however the level of activation seen in the platelets does not differ significantly. The opposite is seen with increasing concentration of thrombin, the level of activation of the blood platelets was increased, whereas the fiber diameter is mostly the same (Figure 3.8: a & b).

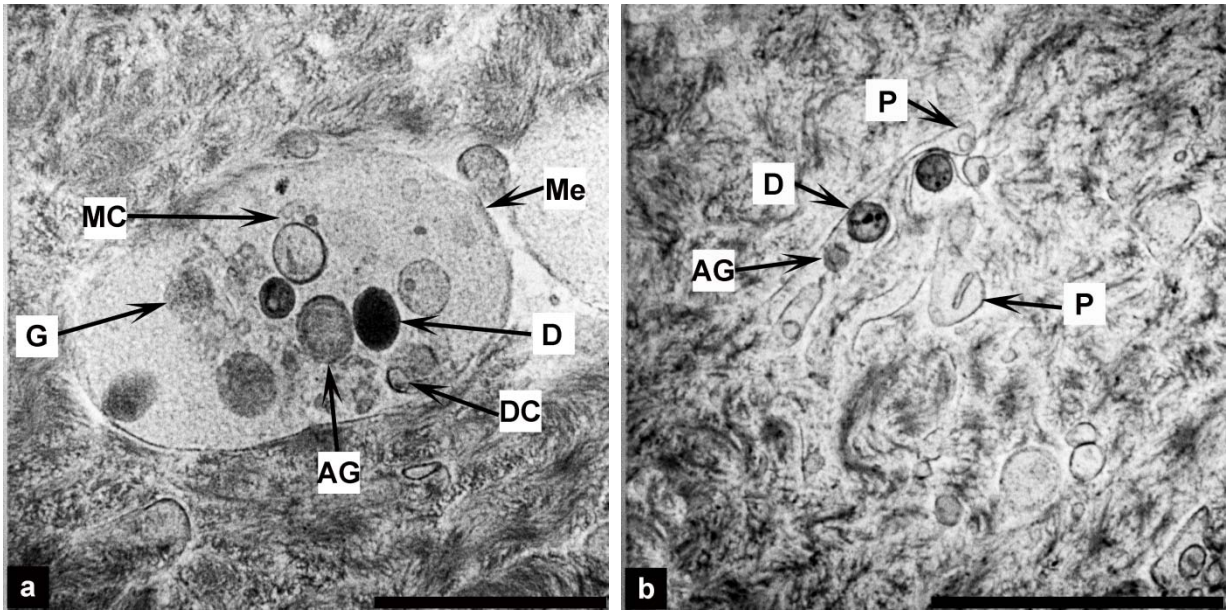


Figure 3.8: Fibrin networks and platelets activated by a PRP: thrombin ratio of either 5:1(a) or 1:1 (b).
All scale bars = 1µm. a: Bulbous platelet with a granular cytoplasm, surrounded by the fibrin network, with organelles; (AG) alpha granules, (D) dense body, (G) glycogen (DC) dilated channel and (Me) membrane. b: Activated platelet, surrounded by the fibrin network, with (P) pseudopodia formation, organelles; (AG) alpha granules and (D) dense body.

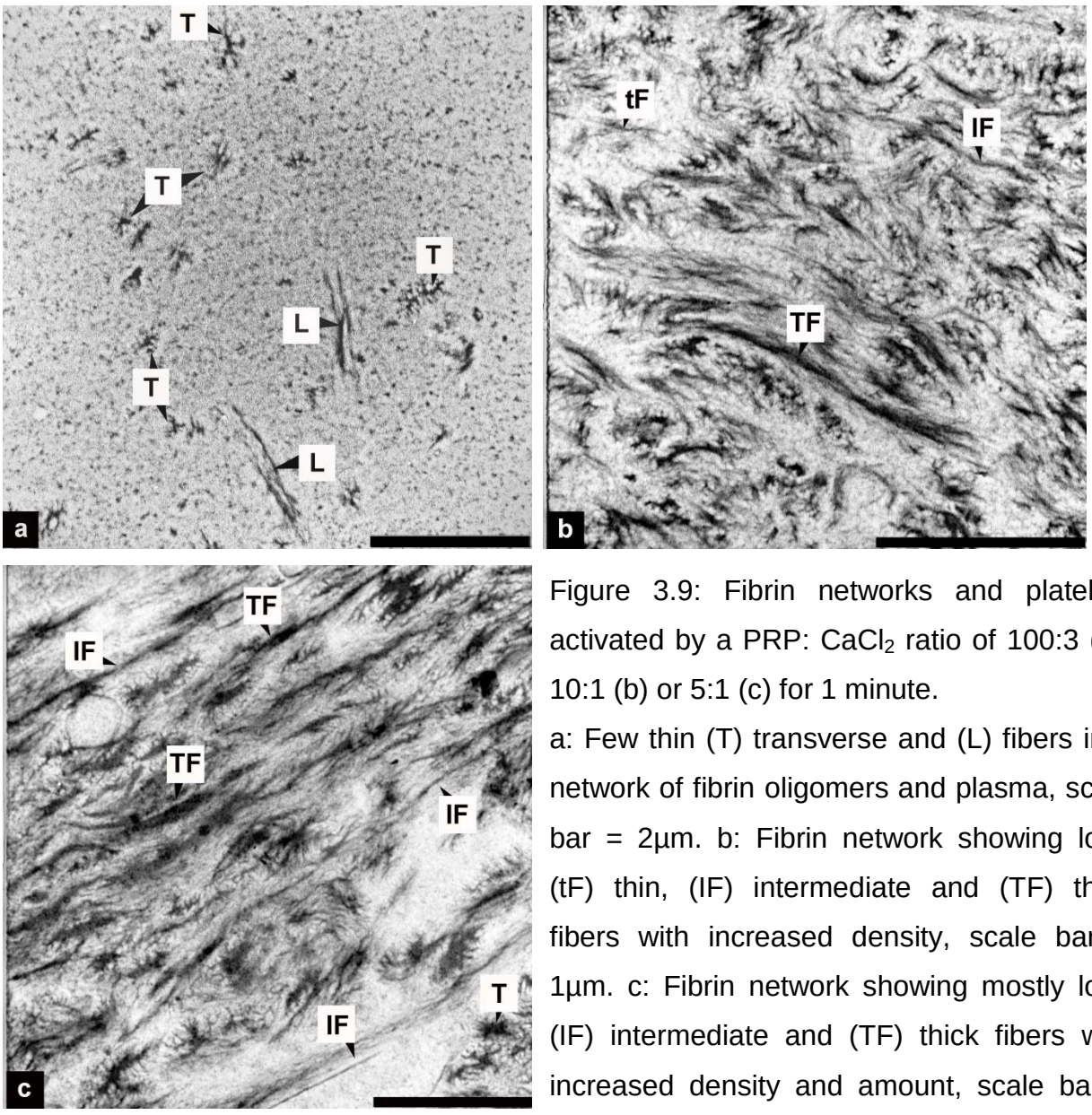


Figure 3.9: Fibrin networks and platelets activated by a PRP: $CaCl_2$ ratio of 100:3 (a), 10:1 (b) or 5:1 (c) for 1 minute.
a: Few thin (T) transverse and (L) fibers in a network of fibrin oligomers and plasma, scale bar = 2µm. b: Fibrin network showing long (tF) thin, (IF) intermediate and (TF) thick fibers with increased density, scale bar = 1µm. c: Fibrin network showing mostly long (IF) intermediate and (TF) thick fibers with increased density and amount, scale bar = 2µm.

3.3.3. HPF Technique

3.3.3.1. Specimen Carrier Method 1

This method proved to be easy to execute, reliable and repetitive, with all the different coagulation preparations, as can be seen in all the images except Figures 4.10, 4.13 and 4.14.

3.3.3.2. Specimen Carrier Method 2

Specimen Carrier Method 2 proved to be problematic, when viewing these samples it was found that platelets and fibrin networks were extremely sparse and often completely absent.

3.3.3.3. Cellulose Capillary Method

Visualization of platelets and fibrin networks coagulated with thrombin was not successful with this method; the coagulation occurred so fast in the majority of experiments (even at reduced thrombin concentrations) that the mixture of PRP and thrombin was too viscous to be drawn up by the cellulose capillary tube. Even though mixtures of $CaCl_2$ and PRP were successfully drawn into the tubes and coagulation proceeded inside the tubing, the further processing (cutting and sealing of the tube) is exceedingly difficult and the specimen was "lost" on several occasions. Sectioning if resin blocks proved to be somewhat precarious, as the consistency of the cellulose tube caused a difference in hardness in the final block which caused the tube to often "pull away" from the surrounding resin (Figure 3.10: a), if this phenomena was not to severe, the section was still usable.

When the specimen was successfully retained in the capillary cellulose tube during freezing and the following preparation steps (Figure 3.10 b), the specimen was still quite sparse and mostly only single thick, loosely arranged, fibrin fibers transversely sectioned and surrounded by a fine fibrin network was seen. Progression of coagulation seems to be retarded in the capillary tubes as fibrin and platelet comparisons with Specimen Carrier Method 1 with similar coagulation times yield significantly different results. Although the platelets and fibrin networks were sparse, platelet preservation was especially good quality with this method, as was that of the transversely sectioned fibrin fibers (Figure 3.10: c & d).

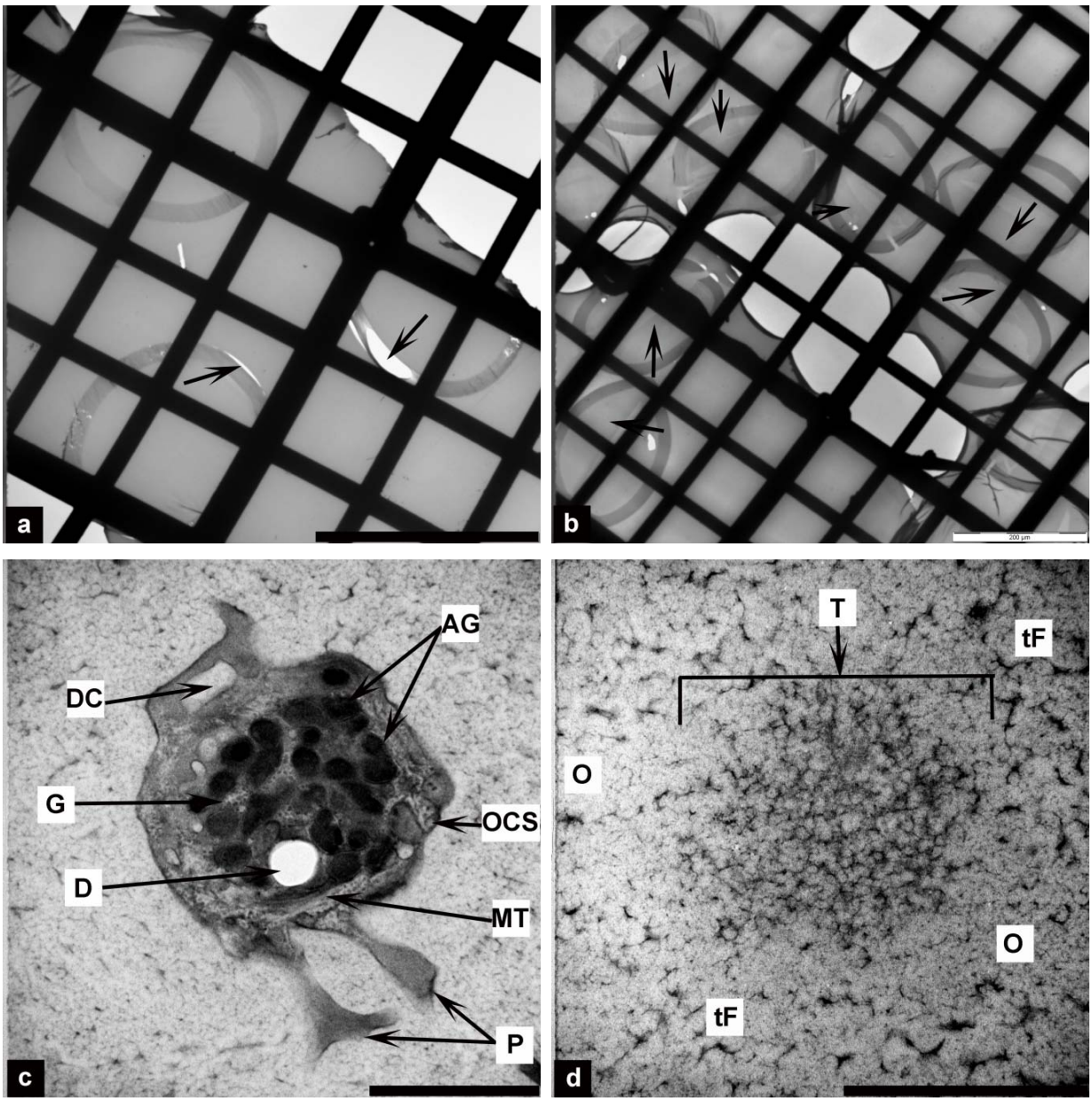


Figure 3.10: The Cellulose Capillary Method.

a: Empty capillary tubes in resin, the capillary can be seen to detach from the surrounding resin (arrows), scale bar = 200µm. b: Capillary tubes in resin, containing specimen (arrow), scale bar = 200µm. c: An activated platelet prepared by the capillary tube method, with a well defined (Me) cell membrane long (P) pseudopodia, a darkly stained granular cytoplasm, and centralized cytoplasmic organelles ((AG) Alpha granules, (D) dense body, (OCS) open canalicular system, (G) glycogen and (DC) dilated channel) surrounded by

(MT) microtubules, scale bar = 1µm. d: Transverse sectioned fibrin fiber surrounded with (tF) thin fibers and (O) fibrin oligomers in plasma, scale = 1µm.

3.3.4. Centrifugation Time

Centrifugation increases the compactness of the coagulation clot (Figure 3.11; a & b), facilitating the ease of handling and ensured an adequate amount of material for examination in the microscope. Longer centrifugation times did not significantly increased the density from the middle range, centrifugation time.

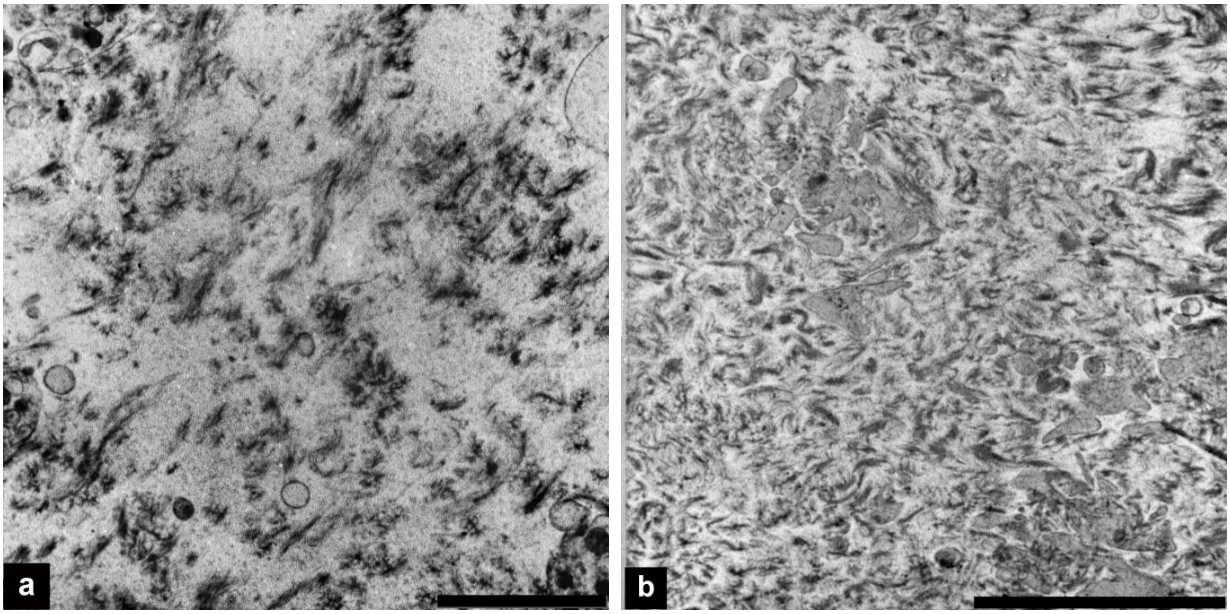


Figure 3.11: Fibrin networks formed at different centrifugation times.
All scale bars = 1µm. a: Fibrin networks and platelets centrifuged for 0 minutes, show more sparsely distributed material. b: Fibrin networks and platelets centrifuged for 5 minutes, show more densely compacted material.

3.3.5. Coagulation Time

Coagulation is a progressive process, so as expected the coagulation time is an important contributing factor to the clot structure and fiber size. An increase in coagulation time when initiating the reaction with thrombin showed a marked increase in fiber size and density

(Figure 3.12: a-d), platelets appear to be in a similar to slightly higher state of activation with increased concentrations of thrombin. The same trend is seen with the fibrin network when employing $CaCl_2$ as a coagulation agent (Figure 3.13: a-d).

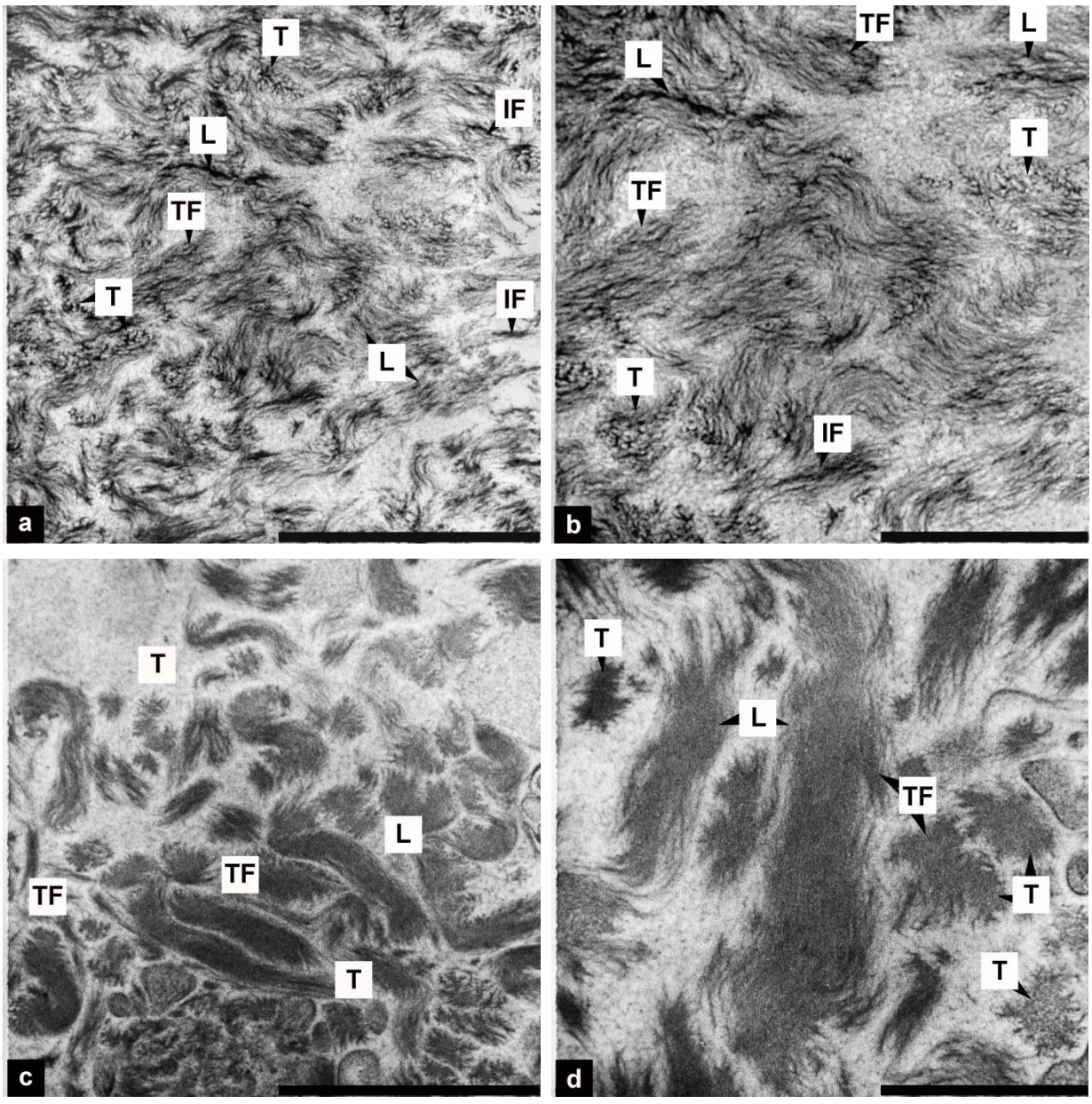


Figure 3.12: Fibrin networks formed by thrombin addition and left to coagulate for different lengths of time.

All scale bars = 1µm. a & b: 1 minute coagulation show (tF) thin and (TF) thick (L) longitudinal and (T) transverse fibers with a disperse structure. c &d: 60 minutes

coagulation shows mostly (TF) thick (L) longitudinal and (T) transverse fibers with a compact structure.

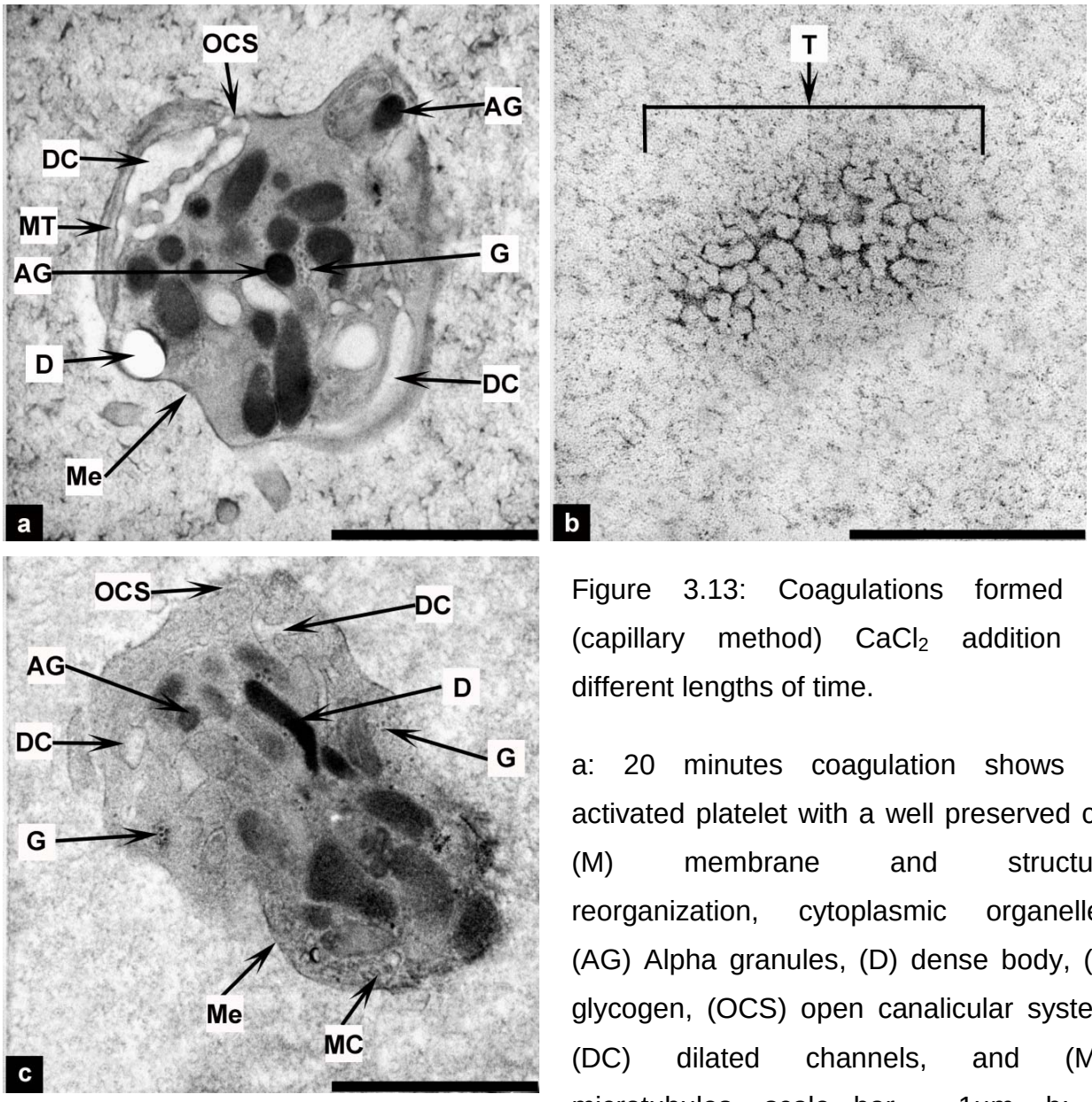


Figure 3.13: Coagulations formed by (capillary method) $CaCl_2$ addition by different lengths of time.

a: 20 minutes coagulation shows an activated platelet with a well preserved cell (M) membrane and structural reorganization, cytoplasmic organelles; (AG) Alpha granules, (D) dense body, (G) glycogen, (OCS) open canalicular system. (DC) dilated channels, and (MT) microtubules, scale bar = 1µm. b: 20 20 minutes coagulation shows a very diffuse (T) transversely sectioned fibrin fiber, scale bar = 500nm. c: 60 minutes coagulation shows a structurally reorganized, activated platelet with a slightly more disrupted membrane, and cytoplasmic organelles, (AG) granules, (D) dense body, (G) glycogen, (MC) mitochondria, (OCS) open canalicular system and (DC) dilated channels, scale bar = 1µm. d: 60 minutes coagulation shows a

thicker more dense (T) transversely sectioned fibrin fiber, scale bar = 500nm.

3.3.6. Coagulation Temperature

When coagulation is initiated using $CaCl_2$ and allowed to gelate at 37°C, clot formation occurs at a slightly more rapid rate than that of coagulation at room temperature, the fiber networks were thicker and more dense, with less thin fibers (Figure 3.14: a-d). Other than that, little difference was seen between the two temperatures. Interestingly enough when thrombin is used and coagulation continuous at 37°C, occasionally the fibers appeared more uncondensed, with a similar (although more dense) structure as seen with $CaCl_2$ coagulation. These micrographs appeared more grainy (Figure 3.15: a & b).

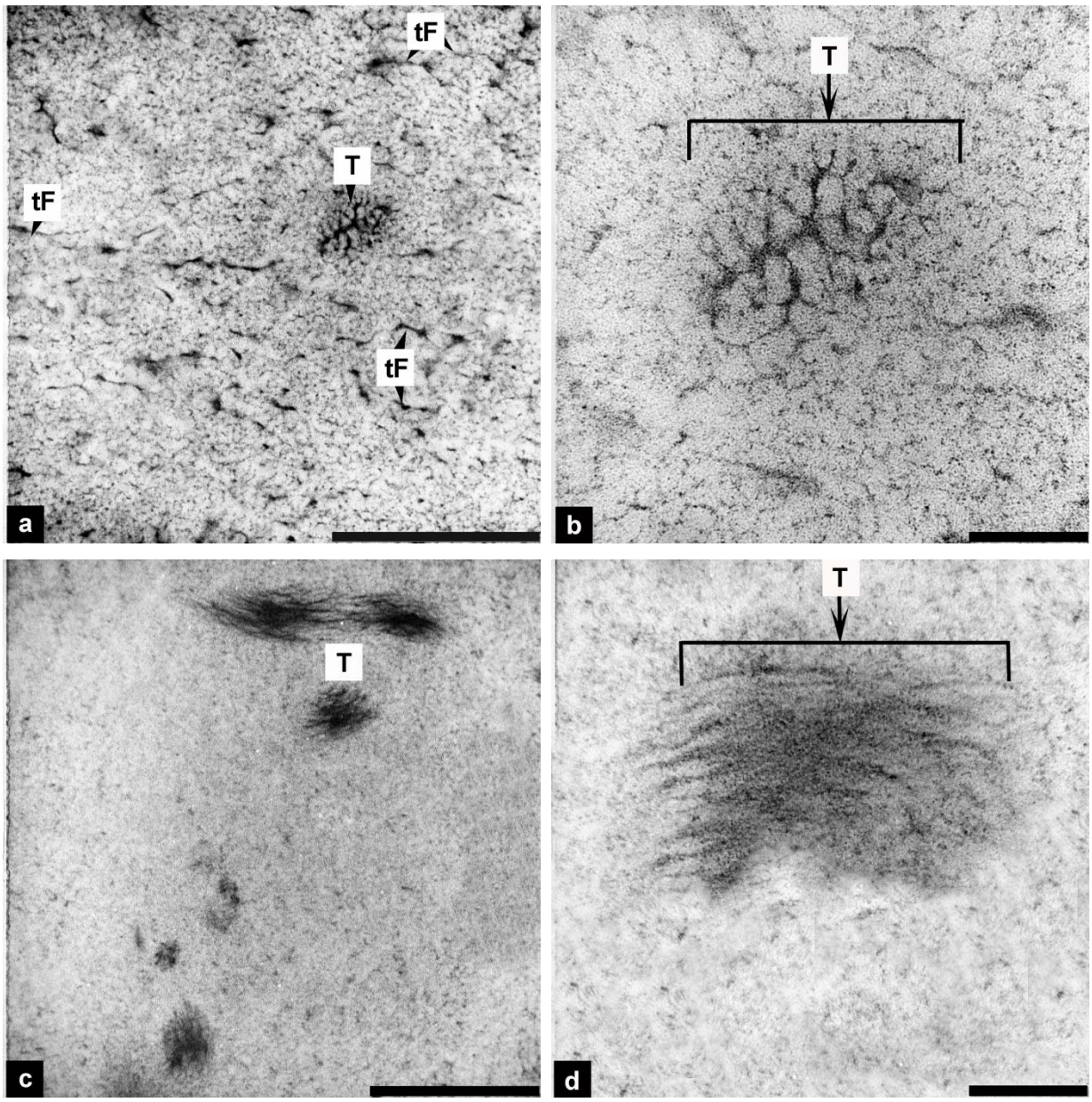


Figure 3.14: Coagulations formed by $CaCl_2$ (capillary method), at either room temperature or at 37°C.

a: Coagulation at room temperature shows a very diffuse (T) transversely sectioned fibrin fiber surrounded by a (tF) thin fiber network, scale bar = 1µm. b: A higher magnification of coagulation at room temperature shows a very diffuse (T) transversely sectioned fibrin fiber, scale bar = 500nm c: Coagulation at 37°C shows dense (T) transversely sectioned fibrin fibers, scale bar = 1µm. d: A higher magnification of coagulation at 37°C shows a

denser (T) transversely sectioned fibrin fiber, scale bar = 500nm.

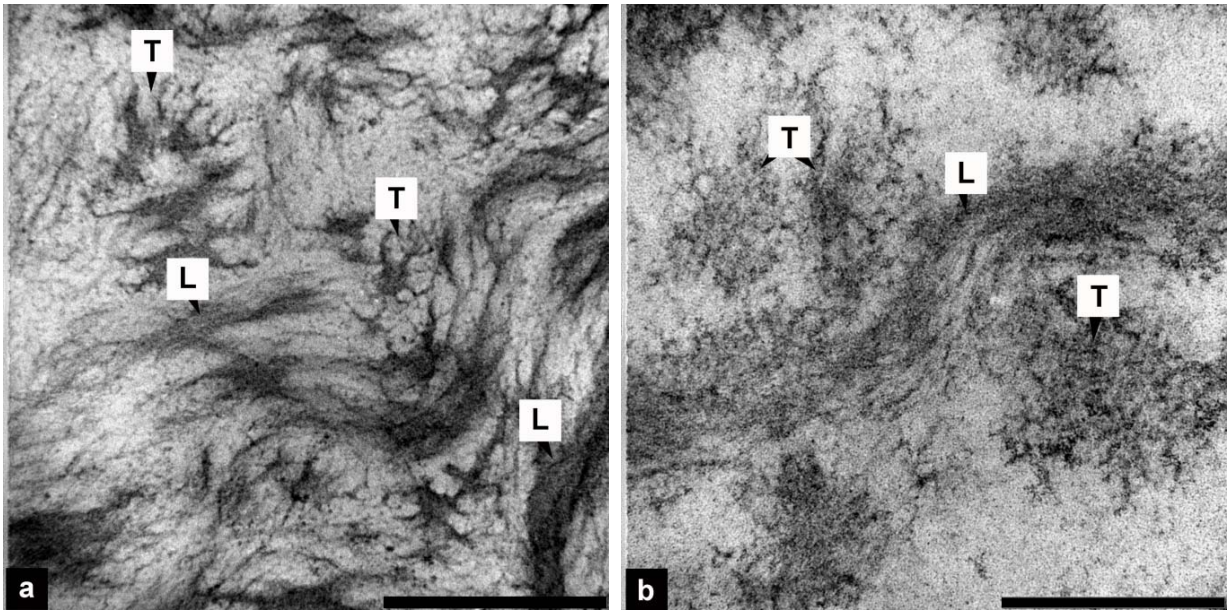


Figure 3.15: Coagulations formed by thrombin at either room temperature or at 37°C. All scale bars = 500nm. a: Fibrin fibers formed at room temperature shows (T) transverse and (L) longitudinal fibers with a clear filamentous nature. b: Fibrin fibers formed at 37°C shows a grainy matrix with (T) transverse and (L) longitudinal fibers that is more diffuse and larger, with a similar structure as seen with $CaCl_2$ coagulation.

3.3.7. Freeze Substitution Protocol

On few occasions, the shorter 36-hour freeze substitution protocols produced odd results. With a small number of the specimens, a portion of the specimen seemed extremely dense and blotchy (similar to spread ink), to such a degree that the internal organization of a fibrin fiber cannot be seen, whereas the rest of the specimen appeared as expected (Figure 3.16: a & b).

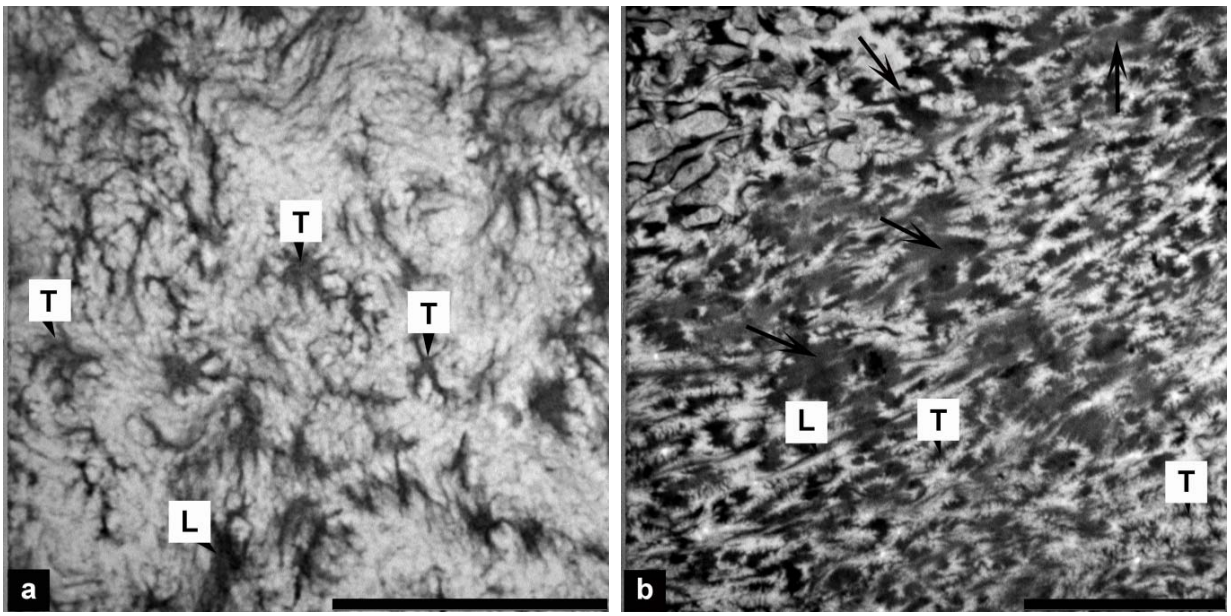


Figure 3.16: Freeze substitution effects on different areas on the thin section of the same specimen.
All scale bars = 1µm. a: The fibrin network as it is expected to appear, diffuse filamentous (T) transverse and (L) longitudinal fibers. b: A blotchy (arrows) fibrin network, where it is sometimes difficult to distinguish (T) transverse and (L) longitudinal fibers.

3.3.8. Embedding Media

Quetol and EPON epoxy resins both produced good quality embedding, and no shrinkage, swelling, extraction of cellular components or any other distortions was more discernible from one embedding media to the next.

3.3.9. Staining

Staining of the thin sections to visualise fibrin networks and platelets is undeniably required for optimal ultrastructure visualisation (Figure 3.17: a). Without staining, the specimens appear featureless and certain structures cannot be discerned, fine fibrin fiber detail is especially difficult to observe (Figure 3.17: b). Staining should be carried out correctly as faulty staining masks the ultrastructural characteristics of the specimens, as

can be seen when the stain precipitates onto the sample (usually due to the age of the stain), staining artefacts can occur in this type in varying degrees (Figure 3.17: c & d).

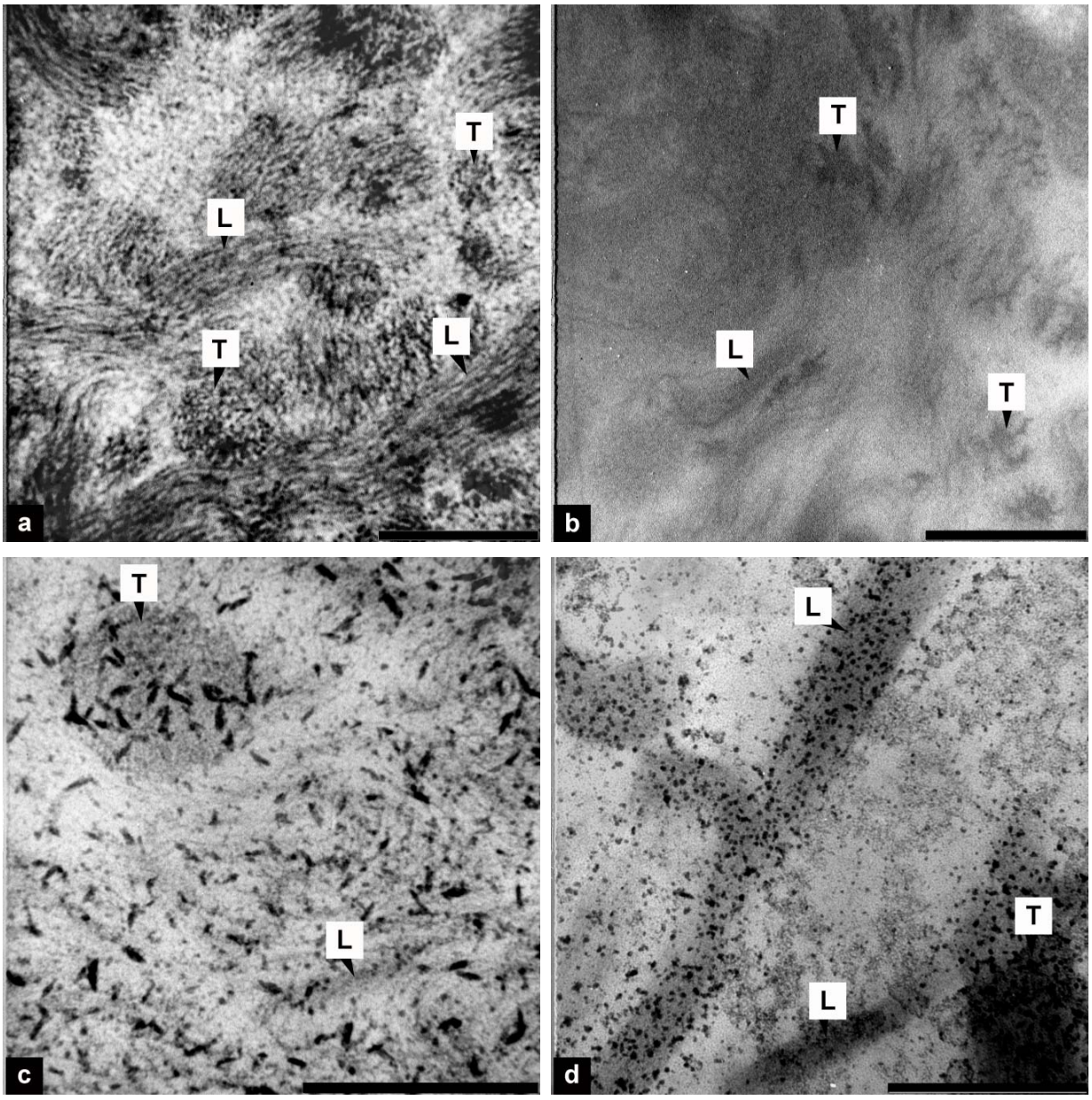


Figure 3.17: Staining of thin sections.

All scale bars = 500nm. a: An example of a section that is stained well, the fibrous nature and detailed structure of (T) transverse and (L) longitudinal sections of the fibrin fiber can be seen. b: An example of a section that is not stained, the fine structure cannot be well observed in the (T) transverse and (L) longitudinal fibers. c: Staining artefact on a thin

section, some details are observable such as fibrous (L) longitudinal section detail, but details on the (T) transverse section the information is obscured. d: Staining artefacts on a thin section, (T) transverse and (L) longitudinal fibers can be recognized, but all the specimen details are obscured.

3.4. Discussion

Collection of blood in citrate containing tubes inactivates the coagulation system that would normally occur if blood components were to be removed from their native environment. Coagulation can be re-activated by several mechanisms. The first is by the addition of thrombin, which stimulates fibrinogen coagulation, leading to the formation of a fibrin network. The citrated blood may also be recalcified by the addition of $CaCl_2$ which reverses the effect of the citrate, and initiate coagulation by the generation of thrombin due to the intrinsic clotting mechanisms. The ultrastructural differences seen between these two techniques is most likely mainly due to the rate limiting platelet activating phospholipids for thrombin generation (limited by the amount available in a closed system) (Kumar *et al.,* 1995). During fibrin fiber formation, FPA and FPB cleavage, initiated by thrombin, occur simultaneously but FPB cleavage only becomes significant after polymerization of FPA has reached a certain point (Hantgan & Hermans 1979 and Weisel 2005). FBP cleavage is known to be a key regulator of lateral association between fibers (Blombäck 1996; Cilia la Corte *et al.,* 2011 and Di Stasio *et al.,* 1998), resulting that a decrease of FPB cleavage (as would be expected with decreased thrombin) will result in a thinner network. The introduction of chloride ions (Cl^-) by $CaCl_2$ is also believed to affect the size distribution of fibrin fibers, as experiments by Di Stasio *et al.,* (1998) has shown that in the presence of Cl^- longer, but thinner fibers are formed.

The fact that lower PRP: thrombin ratios used in this study was unsuccessful to demonstrate this afore mentioned phenomena is believed to be due to the fact that even at the lower ratio used, the concentration is still considerably higher than is seen with intrinsic thrombin formation by $CaCl_2$ introduction. Increasing fiber diameter and fiber density as

seen with increasing concentrations of $CaCl_2$ correlate well to what Okada and Blombäck (1983) found, this is believed to be due to the binding of calcium to low affinity binding sites that enhances FPB release and lateral association.

Thrombin generation during blood coagulation is hypothesized to be a dependant on diffusion transport whereas thrombin inactivation is chemical limited reaction, this would equate to a temperature rise from 25°C to 35°C increasing the formation reaction by about 3%, but the inactivation reaction by 50% (Hemker *et al.,* 2005). The fact that fibrin fibers are more diffuse when formed at 37°C is therefore most likely due to the increased inactivation of thrombin molecules at this temperature. This was not seen with $CaCl_2$ coagulation, the reason for the discrepancy is unsure, but might be due to the effects of calcium binding enhancing lateral association of fibers. The results on platelets seen when $CaCl_2$ is added to PRP in this study is in agreement with literature: initiation of activation of platelets by the addition of exogenous calcium has been shown to enhance several steps in the platelet activation, including sensitivity of arachidonate induced aggregation (Kerry & Paton 1984) and platelet secretion (Detwiler 1987).

When considering the technique of HPF; it is tricky due to the size of the consumable and takes some practise and a steady hand, especially when working with cellulose capillary tubes. Fulfilling the technical requirements, high pressure freezing of the specimens, with the exception of Specimen Carrier Method 2, all produced interesting results of high quality.

3.5. Conclusion

Based on the experimental results, three methods for TEM ultrastructural studies of platelets and fibrin networks, utilizing HPF and freeze substitution was selected, based on their repeatability, the quality of the results and informativeness.

The first method is:

Coagulation of PRP initiated by thrombin at a ratio of 1:1, followed by two minutes centrifugation (at 37°C) and a minute's gelation time at 37°C. HPF utilizing "Specimen Carrier Method 1", freeze substitution for 90 hours, Quetol epoxy resin infiltration and polymerization, sectioning and ultimately staining with uranyl acetate and lead citrate.

The second method is:

Coagulation of PRP initiated by $CaCl_2$ at a ratio of 10:1, followed by two minutes centrifugation, and 20 minutes gelation time. HPF utilizing "Specimen Carrier Method 1", freeze substitution for 90 hours, Quetol epoxy resin infiltration and polymerization, sectioning and ultimately staining with uranyl acetate and lead citrate.

The third method is:

Coagulation of PRP initiated by $CaCl_2$ at a ratio of 100:3, followed by the mixture being drawn up into a cellulose capillary tube and 20 minutes gelation time. HPF utilizing "Cellulose Capillary Method", freeze substitution for 90 hours, Quetol epoxy resin infiltration and polymerization, sectioning and ultimately staining with uranyl acetate and lead citrate.

Specimen Carrier Method 1 was chosen as the main HPF technique as this method gave consistent results the majority of the time and as thrombin and $CaCl_2$ initiation of coagulation both produced satisfactory but dissimilar results, both coagulation agents were selected. Only coagulation by thrombin was performed at 37°C as it yielded significantly different results from room temperature coagulation, this choice was made considering the physiological relevancy of thrombin kinetics. The centrifugation and coagulation time choice was largely based on the amount and density of tissue that was obtained; enough for thorough investigation but not so much as to obscure any features. The longer freeze substitution protocol was selected, as the shorter protocol on occasion gave irregular results, although it cannot be said with any certainty that this was the fault of the protocol, the longer freeze substitution protocol was preferred, as to "stay on the safe side". As both

epoxy resins gave similar results, the most convenient resin was employed and ultimate staining of the thin section was deemed essential.

Additionally HPF of coagulated plasma in capillary tubes was selected as the third technique despite its difficulty, due to the well-preserved nature of platelets and transverse sectioned fibrin fibers seen with this method.

In conclusion, we believe that HPF combined with freeze substitution is a high-quality preservation method for the selected specimen model, that it may provide new information on platelet structure and function, and especially on the fibrin network and the assembly of fibrin monomers forming the fiber.

Chapter 4:

Comparison of chemical and HPF preparation methods to study human fibrin fibers and platelets using TEM

Research Question 2:

What method of sample preparation is superior for TEM ultrastructural studies, rapidly vitrifying platelets and fibrin networks utilizing high pressure freezing and freeze substitution or the well-established chemical preparation method, and what is the difference between the results obtained from these two methods?

4.1. Introduction

With the radical increase in resolution of the TEM from the resolution achievable with a light microscope the discovery (Palade & Porter 1954) and further study of cellular organelles and other tissue substructures has become not only possible but essential for the advancement of ultrastructural studies of biological tissues and materials. The shortcoming of transmission electron microscopy is the requirement that the specimen needs to meet several criteria for it to be able to be studied with a transmission electron microscope;

- The specimen must not contain any volatile elements such as water.
- The specimen must remain unchanged under high vacuum conditions.
- The specimen needs to be able to withstand thermal and physical stress (electron beam radiation).
- The specimen needs to be adequately thin (electron transparency).
- The specimen needs to be small enough to be inserted and viewed in the TEM.

When biological material is the specimen of interest, the material needs to be stabilized (biochemical processes such as autolysis must be halted), all the water in the system have to be removed and in the majority of cases the specimen needs to be altered in size and thickness. In practical terms this means the specimen needs to be fixated, dehydrated, infiltrated by an embedding medium and polymerised and finally sectioned into ultra-thin sections. Many methods exist to achieve the aforementioned, including chemical fixation, heat fixation and freeze fixation; all followed by their subsequent related processing steps. Currently the two main methods of preparation of biological specimens for examination with TEM are chemical and freeze fixation.

Chemical fixation involves the chemical alteration of a specimen by means of the introduction of chemical bonds and cross-links between the specimen's major constituents (lipids, proteins and carbohydrates) that keep molecules and structures in place and ultimately stabilize the specimen. The most widely used chemical fixatives for this purpose are aldehydes which are non-coagulating cross-linking agents, most notably glutaraldehyde and formaldehyde. Glutaraldehyde, introduced by Sabatini and co-workers in 1963 is a highly soluble aliphatic dialdehyde and has become the fixative of preference for routine electron microscopy of nearly all cells and tissues. Glutaraldehyde rapidly cross biological membranes (Korn *et al.,* 1972) and react with protein nitrogen (Griffiths *et al.,* 1993; Walker 1964), most notably amines, producing a large network of cross-links. Formaldehyde, naturally occurring as an unstable gas at room temperature, is dissolved in water and used in a concentrated liquid state (Griffiths *et al.,* 1993; Walker 1964). It fixates mainly (not solitary) by reacting with protonated amino groups forming methylene bridges (Griffiths *et al.,* 1993; Puchtler and Meloan 1985; Walker 1964). These two aldehydes are generally used in combination, as their respective chemical reactions complement each other, by taking advantage of the rapid penetration of the small CH_2O molecules of formaldehyde, initiating structural stabilization, and rapid and thorough cross-linking of the glutaraldehyde oligomers (Kiernan, 2000), better fixation is achieved. Fixation by aldehydes is usually followed by secondary fixation with an agent such as osmium tetroxide, which oxidizes unsaturated double bonds of fatty acids, is reduced and deposited at the polar end of lipid molecules (Hayat 2000), thereby enhancing lipid

preservation.

After chemical fixation the specimen is rinsed with a graded series of an organic solvent (acetone or ethanol), which replaces all the water in the specimen, enable infiltration of the embedding medium and subsequent polymerization of the medium resulting in a hard resin block containing the stabilized specimen. The hardened block is then cut into thin sections (50 - 100nm), by the use of an ultramicrotome.

Freeze fixation involves the immobilization of all the molecules and the arrest of cellular metabolism in a biological specimen by the removal of the thermal energy in the system, facilitated by rapidly lowering the temperature to sub-zero temperatures (Vanhecke *et al.,* 2008). In the freezing process, liquid water (Figure 4.1a and d) is converted to solid ice by crystallization initialized at a nucleation site (Figure 4.1b), herein lays a problem when utilizing freezing for ultrastructural preservation. The predominant forms of ice seen during normal freezing are that of hexagonal and cubic crystals, and as these crystals grow they will break and tear the specimen (Figure 4.1e). Thus the freezing process needs to be modified in such a way that the less known crystal form of ice, amorphous ice, is formed. This form of ice is not an “authentic” type of crystal and not formed by crystallization, but rather by super-cooling (Figure 4.1c) and increasing of viscosity of water to such an extent that the water molecules becomes stabilized in a solid form of the liquid phase (Figure 4.1f) which is called vitrification (Advanced Microscopy, University of Utah 2011; Morphew 2000 and Quintana 1994).

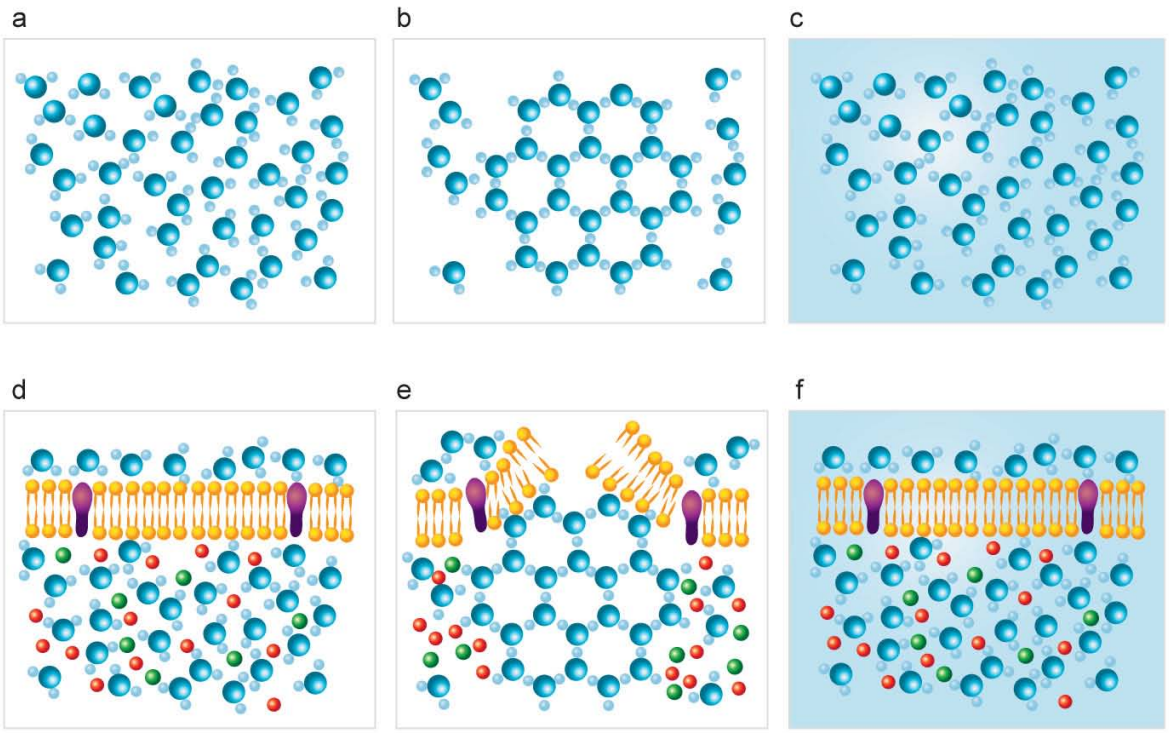


Figure 4.1: Crystallization and vitrification of water.
a: Highly mobile water molecules in liquid. b: At 0ºC, water starts to crystallize if there is a nucleation site. c: Stabilized vitrified water. d: Mobile water molecules in a cell. e: Ice crystals formation and growth can break the cell membrane. f: Vitrification of cellular water enables preservation of the native state of a cell (Redrawn from Advanced Microscopy, University of Utah 2011)

Vitrification requires the extremely high cooling rates, which can only be accomplished in very thin layers of biological specimens (< 20µm) (Shimoni & Müller 1998), as heat can only be efficiently removed from the surface of a sample. For the use and establishment of vitrification as an ultrastructural preservation method, effective freezing of thicker samples needed to be possible. It was found that the application of high pressures (~2000 bar), changes the physical properties of water (freezing point and the rate of ice crystal nucleation and growth) to such a degree that the required cooling rates are reduced and vitrification of thicker samples can be achieved (Dahl and Staehelin 1989; Moor & Hoechli

1970 and Shimoni & Müller 1998). This process coupled with the next preparation step. Dehydration at low temperatures (freeze substitution) (Simpson 1941) made cryofixation methods for ultrastructural studies a feasible approach for solving real cell biological questions. During Freeze substitution the solidified water in the frozen sample is replaced by an organic solvent, such as acetone or methanol, after which the temperature can be raised without risk, since the water is now absent (Steinbrecht and Muller 1987). Further processing steps are similar to that of chemical preparation methods; infiltration and polymerization of an embedding medium and thin sectioning.

Attempting to stabilize a biological specimen in anyway, may it be by the introduction of chemical linkages or by vitrification, is a process of alteration to the native state of the specimen, and with any alteration in state a modification in ultrastructure is expected. Alterations and the introduction of artefacts caused by the chemical fixation procedure are well known. In general, the aldehydes and organic solvents used, lead to aggregation of proteins, collapse of hydrated carbohydrates, the removal and loss of lipids (Kellenberger *et al.,* 1992 and Studer *et al.,* 2008), and tissue shrinkage or swelling (Murk *et al.,* 2003 and Studer *et al.,* 1992).

The general consensus in the scientific community is that ultrastructure preservation by HPF fixation is superior to that of chemical fixation (Giddings 2003; Porta & López- Iglesias 1998 and Studer *et al.,* 2008). Even though this method is believed to be superior, it has its limitations such as the size limit of the sample that is capable of being effectively frozen, and artefact introduction is a possibility. The effect of the high pressures on biological structures is a concern. It has been demonstrated that certain specimens such as the liquid crystal phase of DNA is destroyed when frozen under high pressure (Leforestier *et al.,* 1996) and some lipid mixtures are structurally altered during HPF (Semmler *et al.,* 1998).

Different fixation methodologies have dissimilar chemical and physical interactions with a specimen and therefore different artefact introduction; these artefacts could be useful as well as harmful (Griffiths *et al.,* 1993). The bottom-line with either choice of fixation

technique is that consideration to the possible chemical and/or physical changes introduced is important to take into account.

Blood platelets and fibrin networks are both integral components of the coagulation system, responsible for the maintenance of homeostasis, wound healing and overall health. Any abnormality of either platelets or fibrin, leads to dysfunctionality in coagulation, which may result in several pathological phenomena, ranging from a slight bleeding tendency to thrombosis to death. Abnormalities and dysfunction in fibrin networks and platelets have been shown to be well correlated to an altered morphology and ultrastructure (Pretorius 2008). The fibrin network structure is a fundamental determinant in the progression of coagulation, when network rigidity and strength is increased above the norm which could be seen as either a dense network of numerous thin fibers or longitudinally fused thick fibers (Pretorius *et al.,* 2006) it is predisposed to thrombosis (Collet *et al.,* 2000 and Standeven *et al.,* 2005). Correspondingly a loose or flimsy fibrin network with increased porosity, made up from either thin or thick fibers, leads to bleeding tendencies (Weisel 2005). Changes in platelet size, granularity and level of activation have been noted in several diseased states such as, cancer, diabetes and HIV (Pretorius 2008; Pretorius *et al.,* 2009c and Zhuge *et al.,* 2009)

It is clear that the ultrastructure of fibrin networks and blood platelets is able to give us insight into and provide information to several diseased conditions. Preparation of these platelets and fibrin networks for TEM is critically important as any distortions or artefacts introduced by the preparation process will ultimately be represented in the final images taken with the microscope. This could lead to conclusions that is at worst, erroneous and at best, interpreted in such a way that the uncertainty is taken into account.

Therefore in the current chapter three techniques developed in this lab for ultrastructural preservation of platelets and fibrin networks by freeze fixation (HPF and freeze substitution) and three chemical fixation methods, comparable in the parameters, apart from the fixation and dehydration steps, are carried out. These methods are then compared with relation to their quality of preservation, financial cost, time, repeatability, expertise required and their safety of use.

4.2. Materials and Methods

4.2.1. Samples

Healthy individuals, without any known medical condition, who are not smokers and in the case of women, were not on contraception, were chosen for this study. Whole blood obtained by venipuncture was collected in citrate containing tubes (for the inhibition of calcium-mediated coagulation by the addition of sodium citrate (Sabbatani 1901a and Sabbatani 1901b)), followed by 2 minutes of centrifugation to separate the plasma, containing the platelets and fibrinogen from the erythrocytes and leukocytes. All experiments were conducted with freshly prepared platelet rich plasma (PRP), within two hours of the blood being drawn.

4.2.2. Sample Preparation

A total of six techniques were carried out, see Table 4.1 for a summary.

Table 4.1: Summary of fixation techniques employed.

#	Technique	Coagulation agent	PRP : Coagulation agent	Centrifugation time	Gelation Time
1	HPF – Sample Carrier Method 1	Thrombin	1:1	2	1
2	Chemical	Thrombin	1:1	2	1
3	HPF – Sample Carrier Method 1	$CaCl_2$	10:1	2	1
4	Chemical	$CaCl_2$	10:1	2	1
5	HPF – Capillary Method	$CaCl_2$	100:3	0	20
6	Chemical – Capillary Method	$CaCl_2$	100:3	0	20

4.2.2.1. Technique 1

The supernatant liquid (PRP) was separated from the pellet (erythrocytes and leukocytes) by pipetting the liquid off and mixed with thrombin at a 1:1 ratio in an eppendorf tube. This was then centrifuged for 2 minutes and left for 2 minutes at 37°C for gelation to occur. The coagulant was removed by the use of sterilized sharp-pointed tweezers and a small piece was dissected off (±0.5-1mm). The specimen was transferred to a specimen carrier, which was pre-fitted into the transfer slide and pre-filled with 1-Hexadene (cryoprotectant). The specimen carrier containing the specimen was loaded onto the loading device, and securely screwed into the freezing bayonet pod with the help of a specialized torque wrench; this was then mounted onto the bayonet-loading device, placed into the high-pressure freezing platform and subsequently high pressure frozen (EMPACT2. Leica

Microsystems, Vienna, Austria), after which the specimen was automatically brought into a liquid nitrogen bath. The frozen specimen was transferred into a pre-cooled sample container (Leica Microsystems, Vienna, Austria) filled with freeze substitution-media consisting of 2% osmium tetroxide and 0.1% uranyl acetate in acetone. Freeze substitution was carried out with the Leica EM AFS2 (Leica Microsystems) for 90 hours, specimens were kept at -90°C for 42 hours, heated to -60°C over a 15 hour period (at 2°C per hour), kept at-60°C for eight hours, heated to -30°C over 15 hours (2°C per hour) and kept a further eight hours at -30°C after which the specimen was heated to 0°C over 2 hours (15°C per and allowed to stabilize at room temperature. After freeze substitution the specimens were rinsed three times with acetone for 10 minutes per rinse, and removed from the specimen carriers, followed by embedding media (Quetol Epoxy) infiltration. Firstly, the specimens were placed into a mixture of 1:1 ratio of 100% acetone to embedding media and left for 30 minutes, the mixture was drawn off, new embedding media was added and allowed to infiltrate for 3 hours. The specimens were then placed in fresh resin and allowed to polymerize for 36 hours at 60°C. The hard resin blocks were sectioned using an ultramicrotome (Reichert-Jung Ultracut E, Vienna, Austria) into 80 - 100nm sections and stained with uranyl acetate for five minutes followed by two minutes of staining with lead citrate. The samples were then subsequently viewed with a JEOL, JEM 2100F Field Emission Electron Microscope (JEOL, Tokyo, Japan).

4.2.2.2. Technique 2

The supernatant liquid (PRP) was separated from the pellet (erythrocytes and leukocytes) by pipetting the liquid off and mixed with thrombin at a 1:1 ratio in an eppendorf tube. This was then centrifuged for 2 minutes and left for 2 minutes at 37°C for gelation to occur. The coagulant was removed by the use of a sterilized sharp-pointed tweezers and a small piece was dissected off (±0.5-1mm). The specimen was transferred to a glass vial containing a 2.5 % glutaraldehyde-formaldehyde fixative and left for one hour. After which the sample was rinsed three times with (0.075M) sodium phosphate buffer (pH 5 7.4), for 15 minutes before being placed in secondary fixative, 1% osmium tetraoxide solution for one hour. Samples were then dehydrated with a series of ethanol, 30%, 50%, 70%, 90%,

and three changes of 100% for 10 minutes each followed by embedding media (Quetol Epoxy) infiltration. This was accomplished by leaving the samples in a mixture of 100% ethanol and media for 30 minutes (1:1 ratio), followed by three hours in pure embedding media, specimens were then placed in fresh media and allowed to polymerize for 36 hours at 60°C. Ultrathin (80 -100nm) sections were cut with a Reichert-Jung Ultracut E microtome (Vienna, Austria) and stained with uranyl acetate for five minutes followed by two minutes of staining with lead citrate. The samples were then subsequently viewed with a JEOL, JEM 2100F Field Emission Electron Microscope (JEOL, Tokyo, Japan).

4.2.2.3. Technique 3

The supernatant liquid (PRP) was separated from the pellet (erythrocytes and leukocytes) by pipetting the liquid off and mixed with $CaCl_2$ at a 10:1 ratio in an eppendorf tube, this was then centrifuged for 2 minutes and left for a minute at room temperature for gelation to occur. The coagulant was removed by the use of a sterilized sharp-pointed tweezers and a small piece was dissected off (±0.5-1mm). The specimen was transferred to a specimen carrier, which was pre-fitted into the transfer slide and pre-filled with 1-Hexadene (cryoprotectant). The specimen carrier containing the specimen was loaded onto the loading device, and securely screwed into the freezing bayonet pod with the help of a specialized torque wrench; this was then mounted onto the bayonet-loading device, placed into the high-pressure freezing platform and subsequently high pressure frozen (EMPACT2. Leica Microsystems, Vienna, Austria), after which the specimen was automatically brought into a liquid nitrogen bath. The frozen specimen was transferred into a pre-cooled sample container (Leica Microsystems, Vienna, Austria) filled with freeze substitution-media consisting of 2% osmium tetroxide and 0.1% uranyl acetate in acetone. Freeze substitution was carried out with the Leica EM AFS2 (Leica Microsystems) for 90 hours, specimens were kept at -90°C for 42 hours, heated to -60°C over a 15 hour period (at 2°C per hour), kept at-60°C for eight hours, heated to -30°C over 15 hours (2°C per hour) and kept a further eight hours at -30°C after which the specimen was heated to 0°C over 2 hours (15°C per and allowed to stabilize at room temperature. After freeze substitution the specimens was rinsed three times with acetone for 10 minutes per rinse,

and removed from the specimen carriers, followed by embedding medium (Quetol Epoxy) infiltration. Firstly, the specimens was placed into a mixture of 1:1 ratio of 100% acetone to embedding media and left for 30 minutes, the mixture was drawn off, new embedding medium was added and allowed to infiltrate for 3 hours. The specimens were then placed in fresh resin and allowed to polymerize for 36 hours at 60°C. The hard resin blocks were sectioned using an ultramicrotome (Reichert-Jung Ultracut E, Vienna, Austria) into 80 - 100nm sections and stained with uranyl acetate for five minutes followed by two minutes of staining with lead citrate. The samples were then subsequently viewed with a JEOL, JEM 2100F Field Electron Microscope (JEOL, Tokyo, Japan).

4.2.2.4. Technique 4

The supernatant liquid (PRP) was separated from the pellet (erythrocytes and leukocytes) by pipetting the liquid off and mixed with $CaCl_2$ at a 10:1 ratio in an eppendorf tube, this was then centrifuged for 2 minutes and left for one minute at room temperature for gelation to occur. The coagulant was removed by the use of a sterilized sharp-pointed tweezers and a small piece was dissected off (±0.5-1mm). The specimen was transferred to a glass vial containing a 2.5 % glutaraldehyde-formaldehyde fixative and left for one hour. After which the sample was rinsed three times with (0.075M) sodium phosphate buffer (pH 5 7.4), for 15 minutes before being placed in secondary fixative, 1% osmium tetroxide solution for one hour. Samples were then dehydrated with a series of ethanol, 30%, 50%, 70%, 90%, and three changes of 100% for 10 minutes each followed by embedding medium (Quetol Epoxy) infiltration. This was accomplished by leaving the samples in a mixture of 100% ethanol and resin for 30 minutes (1:1 ratio), followed by three hours in pure embedding medium, specimens were then placed in fresh resin and allowed to polymerize for 36 h at 60°C. Ultrathin (80 -100nm) sections was cut with a Reichert-Jung Ultracut E microtome (Vienna, Austria) and stained with uranyl acetate for five minutes followed by two minutes of staining with lead citrate. The samples were then subsequently viewed with a JEOL, JEM 2100F Field Emission Electron Microscope (JEOL, Tokyo, Japan).

4.2.2.5. Technique 5

The supernatant liquid (PRP) was separated from the pellet (erythrocytes and leukocytes) by pipetting the liquid off and mixed with CaCl2 at a 100:3 ratio in an eppendorf tube. A pre-cut piece (10-20mm) of cellulose capillary tube (wall thickness (dry) 8µm, inner diameter (dry) 200µm; Leica Microsystems, Vienna, Austria) was held with forceps at one end while the other was placed into the PRP-coagulation agent mixture, the mixture was allowed to be drawn up by capillary forces until the whole tube was filled. The mixture in the capillary tube was left for 20 minutes in a humid environment, at room temperature to allow coagulation to occur. The central part of the capillary tube was subsequently cut out (±1mm) and sealed by using a crimping tool (a blunted scalpel was used for this purpose). The pieces of capillary tubing containing the specimen were transferred to a specimen carrier, which was pre-fitted into the transfer slide, and prefilled with 1-Hexadene (cryoprotectant). The specimen carrier containing the specimen was loaded onto the loading device, and securely screwed into the freezing bayonet pod with the help of a specialized torque wrench; this was then mounted onto the bayonet-loading device, placed into the high-pressure freezing platform and subsequently high pressure frozen (EMPACT2. Leica Microsystems, Vienna, Austria), after which the specimen was automatically brought into a liquid nitrogen bath. The frozen specimen was transferred into a pre-cooled sample container (Leica Microsystems, Vienna, Austria) filled with freeze substitution-media consisting of 2% osmium tetroxide and 0.1% uranyl acetate in acetone. Freeze substitution was carried out with the Leica EM AFS2 (Leica Microsystems) for 90 hours, specimens were kept at -90°C for 42 hours, heated to -60°C over a 15 hour period (at 2°C per hour), kept at-60°C for eight hours, heated to -30°C over 15 hours (2°C per hour) and kept a further eight hours at -30°C after which the specimen was heated to 0°C over 2 hours (15°C per hour) and allowed to stabilize at room temperature. After freeze substitution the specimens was rinsed three times with acetone for 10 minutes per rinse, and removed from the specimen carriers, followed by embedding media (Quetol Epoxy) infiltration. Firstly, the specimens was placed into a mixture of 1:1 ratio of 100% acetone to embedding medium and left for 30 minutes, the mixture was drawn off, new embedding media was added and allowed to infiltrate for 3 hours. The specimens were then placed in

fresh resin and allowed to polymerize for 36 hours at 60°C. The hard resin blocks were sectioned using an ultramicrotome (Reichert-Jung Ultracut E, Vienna, Austria) into 80 - 100nm sections and stained with uranyl acetate for five minutes followed by two minutes of staining with lead citrate. The samples were then subsequently viewed with a JEOL, JEM 2100F Field Emission Electron Microscope (JEOL, Tokyo, Japan).

4.2.2.6. Technique 6

The supernatant liquid (PRP) was separated from the pellet (erythrocytes and leukocytes) by pipetting the liquid off and mixed with CaCl2 at a 100:3 ratio in an eppendorf tube. A pre-cut piece (10-20mm) of cellulose capillary tube (wall thickness (dry) 8µm, inner diameter (dry) 200µm; Leica Microsystems, Vienna, Austria) was held with forceps at one end while the other was placed into the PRP-coagulation agent mixture, the mixture was allowed to be drawn up by capillary forces until the whole tube was filled. The mixture in the capillary tube was left for 20 minutes in a humid environment, at room temperature to allow coagulation to occur. The edges of the tubing was sealed using a crimping tool (blunted scalpel) and transferred to a glass vial containing a 2.5% glutaraldehyde-formaldehyde fixative and left for one hour. After fixation the sample was rinsed three times with (0.075M) sodium phosphate buffer (pH 5 7.4), for 15 minutes before being placed in secondary fixative, 1% osmium tetroxide solution for one hour. Samples were then dehydrated with a series of ethanol, 30%, 50%, 70%, 90%, and three changes of 100% for 10 minutes each followed by embedding medium (Quetol Epoxy) infiltration. This was accomplished by leaving the samples in a mixture of 100% ethanol and media for 30 minutes (1:1 ratio), followed by three hours in pure embedding medium, specimens were then placed in fresh resin and allowed to polymerize for 36 h at 60°C. Ultrathin (80 - 100nm) sections were cut with a Reichert-Jung Ultracut E microtome (Vienna, Austria) and stained with uranyl acetate for five minutes followed by two minutes of staining with lead citrate. The samples were then subsequently viewed with a JEOL, JEM 2100F Field Emission Electron Microscope (JEOL, Tokyo, Japan).

4.3. Results

4.3.1. Technique 1

Fibrin networks coagulated and formed by thrombin introduction and fixated by HPF and subsequent freeze substitution shows the fibrin fiber as a filamentous structure loosely arranged (longitudinal section), growing outward from a central point (transverse section) (Figure 4.2: a-c). The fibrin fiber can clearly be seen to be assembled from sub-units or monomer / oligomers complexes arranged in a highly organized repeated structure (Figure 4.2: b). This repetitive structure is seen in various different sized fibers and also in the surrounding matrix made up from thinner fibers, protofibrils and fibrin oligomers (Figure 4.2: b). Branching points of the longitudinal fibrin fiber are also occasionally visible (Figure 4.2: a). Platelets prepared with this method appear rounded with a granular cytoplasm containing the expected cellular constituents (open canalicular system, dilated channel, alpha granules, mitochondria and dense bodies), that is well-defined, with a clear cell membrane (Figure 4.2: d).

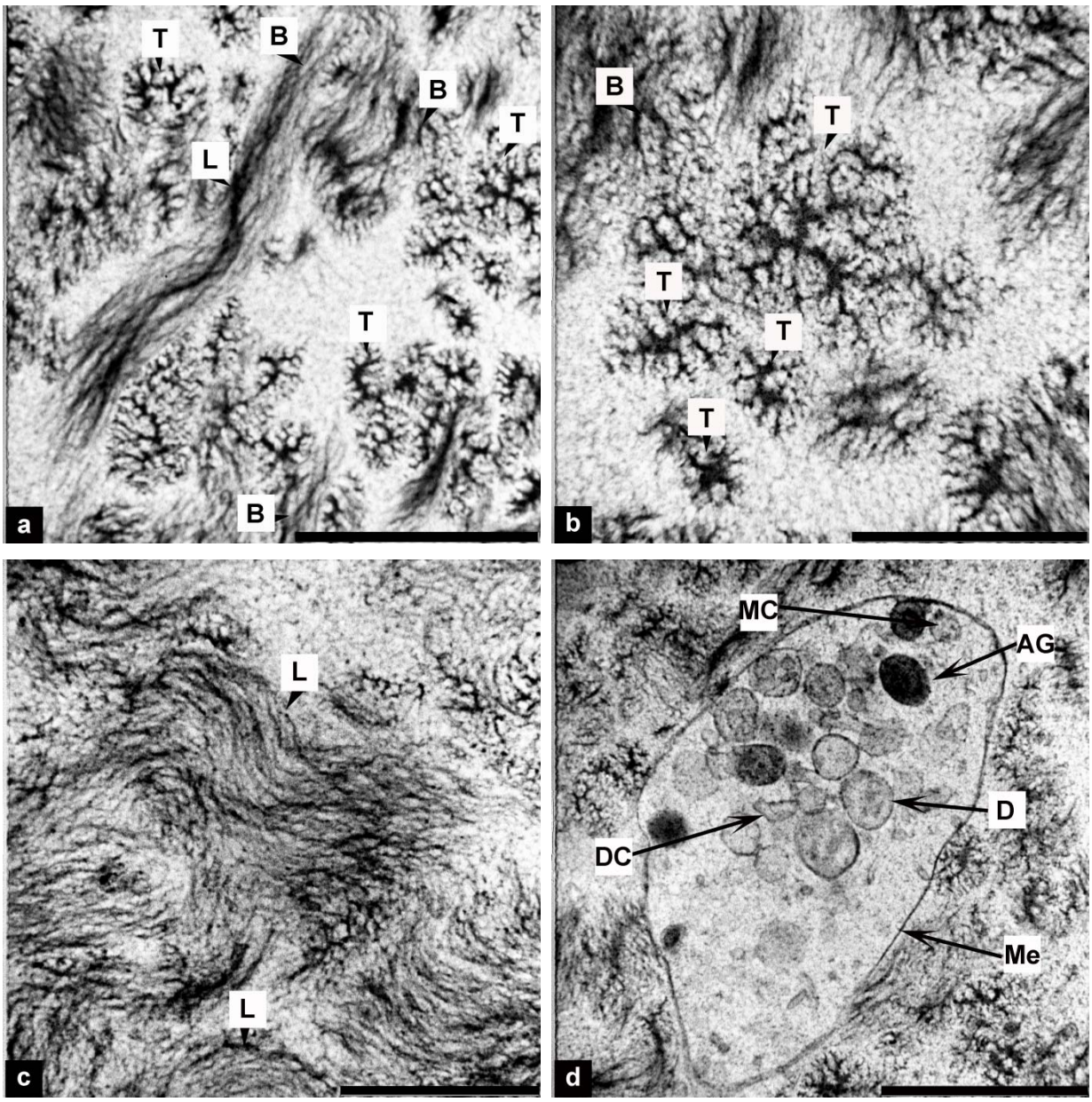


Figure 4.2: Fibrin fibers and platelets preserved by technique 1.

a: Fibrous (L) longitudinal and (T) transverse sections through fibrin fibers are seen, with occasional (B) branching, scale bar = 1µm. b: (T) transverse sections through several different sizes of fibrin fibers are seen growing outward from a central point in a crystalline-like structure, occasional (B) branching is seen in (L) longitudinal section, scale bar = 500nm. c: (L) Longitudinally arranged fibrin fibers can be seen to be made up of diffuse filamentous elements, scale bar = 500nm. d: Bulbous platelet with organelles; (AG) alpha

granules, (D) dense body, (M) mitochondria (DC) dilated channel and well-defined (Me) membrane, scale = 1µm.

4.3.2. Technique 2

When a fibrin fiber's coagulation is initiated by thrombin and the network is fixated by chemical fixation, the fiber can be seen as fibrous structure (longitudinal section) (Figure 4.3: a), but in contrast to technique 1, the fiber is condensed forming a tightly packed agglomerate made up of thinner fibers. The longitudinal section shows the well published band pattern (BP), believed to be protein density along the fiber (Hall, 1949) (Figure 4.3: b). A transverse section shows the same development; a compactly packed polymer that with high magnification can be seen to be made up from smaller elements (Figure 4.3: c). Platelets prepared in the same way appear rounded with a granular cytoplasm containing the expected cellular constituents, but when compared to technique 1, the cell, its components (open canalicular system, dilated channel) and organelles (alpha granules, mitochondria, dense bodies, glycogen) and the membrane is more vague and undefined (Figure 4.3: d).

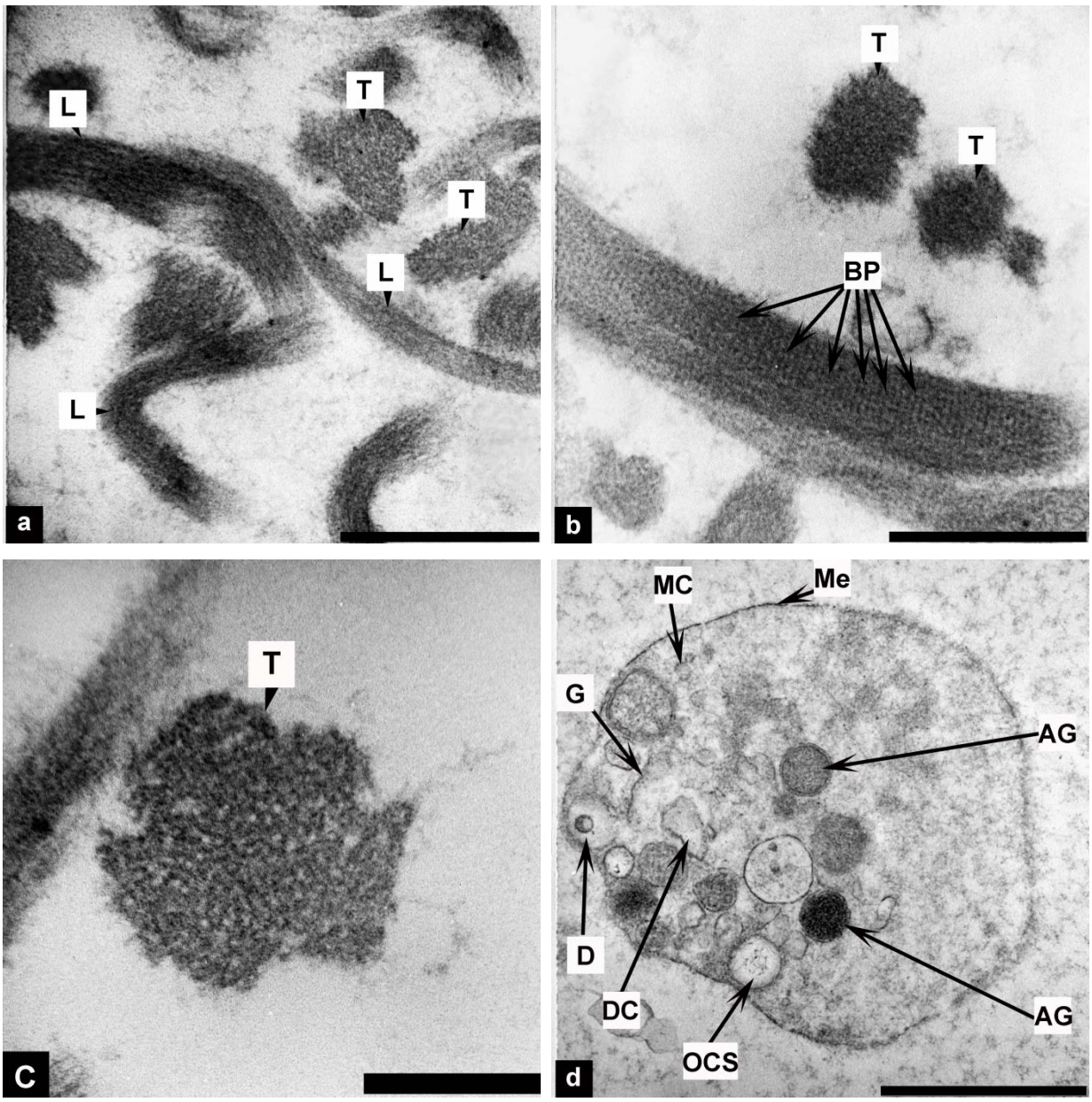


Figure 4.3: Fibrin fibers and platelets preserved by technique 2.

a: (L) Longitudinal and (T) transverse sections through thick fibrin fibers showing a highly condensed fibrous structure, scale bar = 500nm. b: A (T) transverse section through a thick fibrin fiber with the clearly identifiable (BP) band pattern, scale bar = 500nm. c: (T) Transversely sectioned fibrin fiber, showing that it is a compactly packed unit constructed from smaller elements, scale bar = 200nm. d: Platelets with a granular cytoplasm, with less clear organelles ((AG) alpha granules, (D) dense body, (G) glycogen, (DC) dilated

channel) and cell (Me) membrane, scale = 1µm.

4.3.3. Technique 3

Initiation of the fibrin network coagulation by $CaCl_2$ and fixation by HPF followed by freeze substitution shows similarly to technique 1 the fibrin fiber, in a transverse section, as a filamentous structure loosely arranged (Figure 4.4: a-c). In contrast to technique 1 the fibers are thinner and more highly dispersed throughout the matrix. Less transverse sections through the fibers are seen; most probable just due to the fact that they are diffuse, smaller and less obvious, the few transverse sections that can be clearly identified are composed of a similar repetitive structure than seen in technique 1 (Figure 4.4: c). No definite branching points of the longitudinal fibrin fiber are seen. Platelets activated by $CaCl_2$ appear more highly activated than their counterparts activated by thrombin, the platelets structure is restructured with pseudopodia formation and organelles ranging from being disorganized to centralized (Figure 4.4: d). The expected cellular constituents (open canalicular system, dilated channel, alpha granules, mitochondria and dense bodies), are also seen and the cell in general is relatively well-defined, with a clear cell membrane.

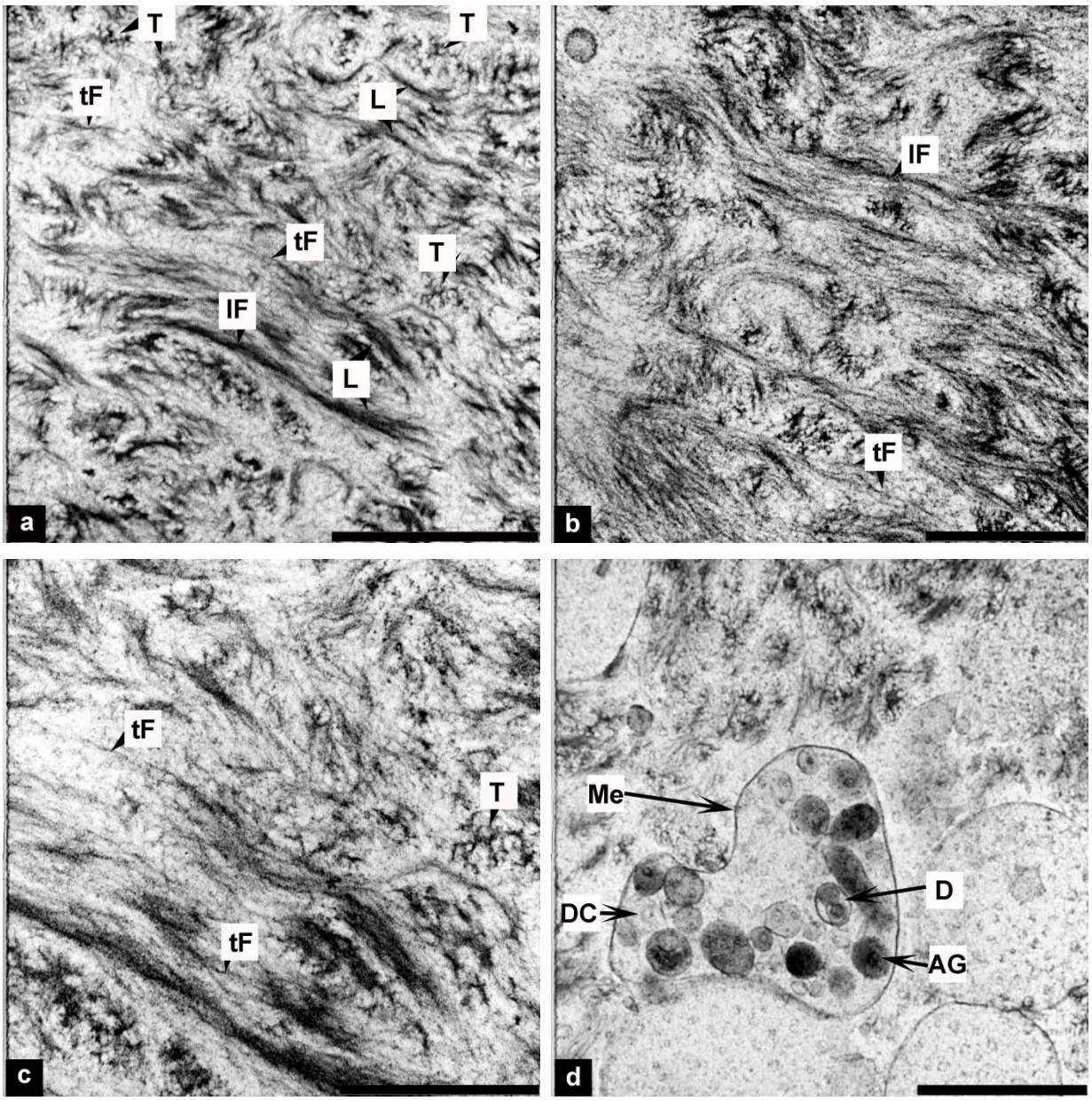


Figure 4.4: Fibrin fibers and platelets preserved by technique 3.

a: Coagulation of (T) transverse and (L) longitudinal (tF) thin, (IF) intermediate and (TF) thick fibrin fibers, scale bar = 1µm. b: Highly dispersed coagulation of (tF) thin and (IF) intermediate fibers, scale = 500nm. c: Higher magnification image showing that the coagulation has a dispersed (tF) thin fiber network between the bigger fibers, also (T) transverse fibers are seen to have a similar (although smaller) structure to those seen in technique 1, scale bar = 500nm. d: Structurally reorganized platelet with organelles; (AG)

alpha granules, (D) dense body, (DC) dilated channel and well-defined (Me) membrane scale bar = 1µm.

4.3.4. Technique 4

With the same coagulation kinetics as used in technique 3 but followed by chemical fixation and subsequent ethanol dehydration similar results as to technique 2 is seen (Figure 4.5: a & b). Fibers are more densely packed as individual fibers as seen with $CaCl_2$ activation and HPF, but more disperse than is seen with thrombin coagulation coupled with chemical fixation. The fiber is evidently a cumulative structure made of filamentous components as can be seen in longitudinal and transverse sections (Figure 4.5: a & b), organized protein density, the band pattern can also be seen in the longitudinal sections of fibers (Figure 4.5: c), with possible ambiguous branching points. In contrast to technique 3 several transverse sections of a fiber is clearly distinguishable, most likely due to their increased agglomeration and dense packing making them more pronounced (Figure 4.5: a). Platelets prepared in this method, corresponding to method 3 appear more activated, with converged organelles and evident pseudopodia formation (Figure 4.5: c). The expected cellular constituents (open canalicular system, dilated channel, alpha granules and dense Bodies), is also seen but the cell is, contrary to method 3, less well-defined, presenting with an indistinct appearance and cell membrane.

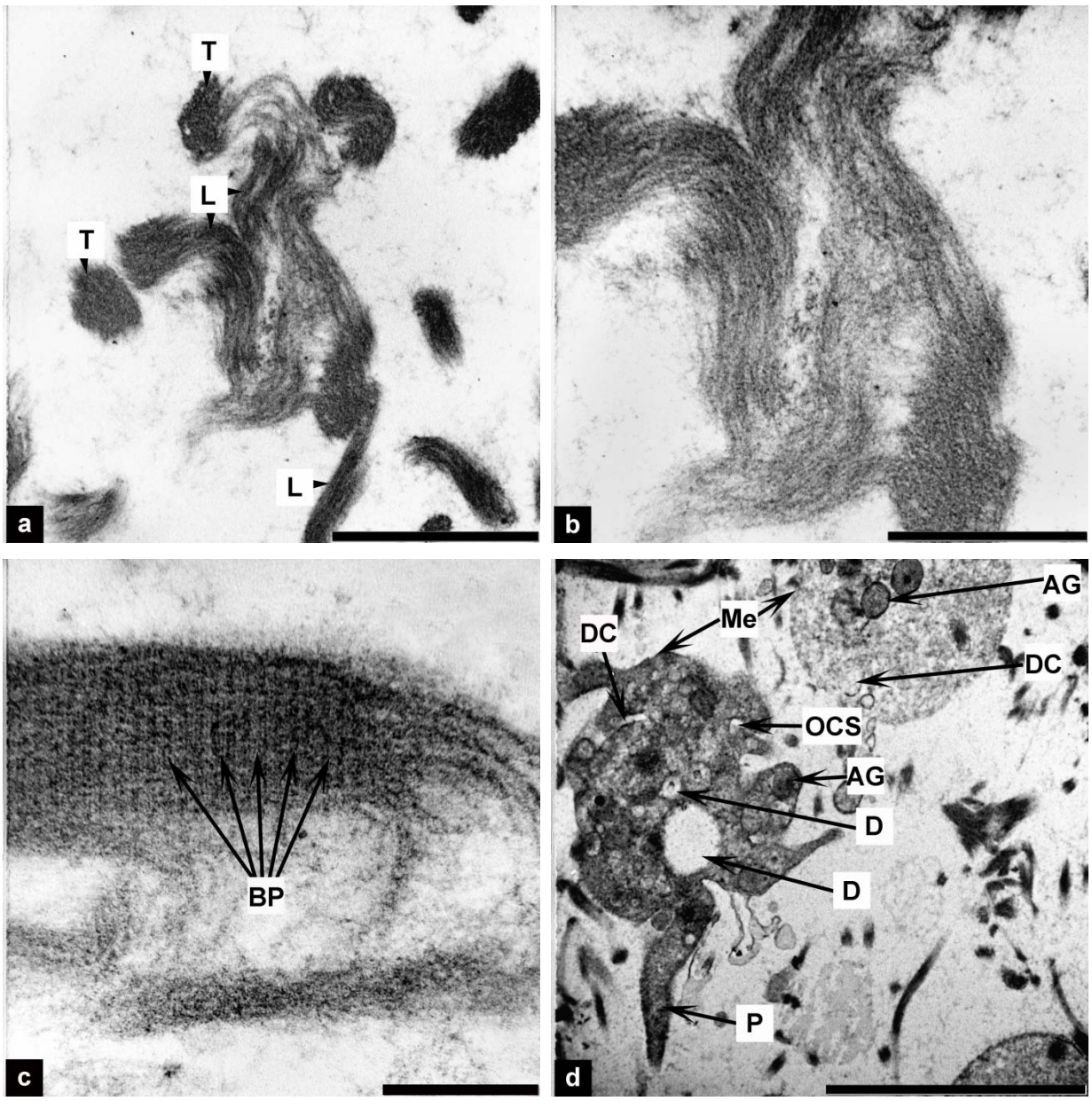


Figure 4.5: Fibrin fibers and platelets preserved by technique 4.

a: Thick and dense (T) transverse and (L) longitudinal fibrin fibers, scale bar = 1µm. b: Transversely sectioned fibrin fibers, highlighting their filamentous nature, scale bar = 500nm. c: Higher magnification image of a transverse section of a fiber with the (BP) band pattern, scale bar = 200nm. d: Activated platelet with a darkly stained cytoplasm and pseudopodia formation, and a (BP) bulbous platelet with a poorly defined (Me) membrane; organelles are seen in both platelets, ((AG) Alpha Granules, (D) Dense Body,

(DC) Dilated Channel), scale bar = 1µm.

4.3.5. Technique 5

PRP and $CaCl_2$ mixtures drawn up into a cellulose capillary tube and high pressure frozen, present with very interesting and informative results. Fibrin fibers is mostly seen in transversely sectioned orientation, and are less agglomerated and dense, the fibers are also polymerized to a lesser degree than fibers seen in any other method, which enables one to clearly see the lateral associations of fibrin oligomers in the construction of a fiber (Figure 4.6: a-c). The internal structure of the fiber can be seen to be of a highly ordered, crystalline nature. Occasional transverse sections that are seen resemble fibers seen in technique 3 although they are smaller in diameter and more disperse. Platelets prepared by this technique are found individually and are markedly activated showing centralized organelles and extensive pseudopodia formation. The cell is extremely well preserved with clear membranes and distinctly and easily discernible cellular elements (open canalicular system, dilated channel, alpha granules, glycogen, mitochondria and dense bodies) (Figure 4.7: a-d).

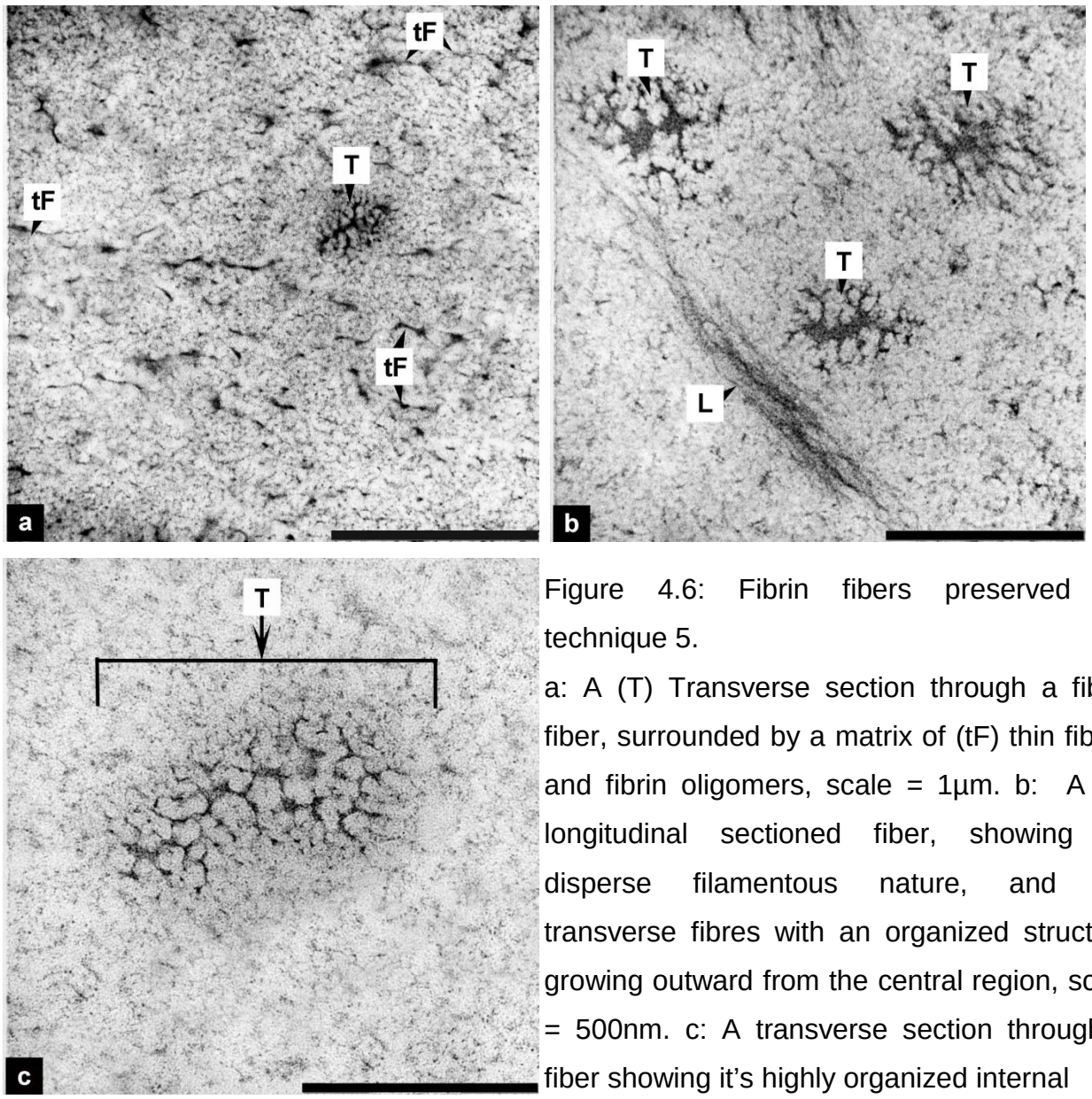


Figure 4.6: Fibrin fibers preserved by technique 5.

a: A (T) Transverse section through a fibrin fiber, surrounded by a matrix of (tF) thin fibers and fibrin oligomers, scale = 1µm. b: A (L) longitudinal sectioned fiber, showing its disperse filamentous nature, and (T) transverse fibres with an organized structure growing outward from the central region, scale = 500nm. c: A transverse section through a fiber showing it's highly organized internal structure and the lateral associations of fibrin oligomers in the construction of a fiber, scale bar = 500nm.

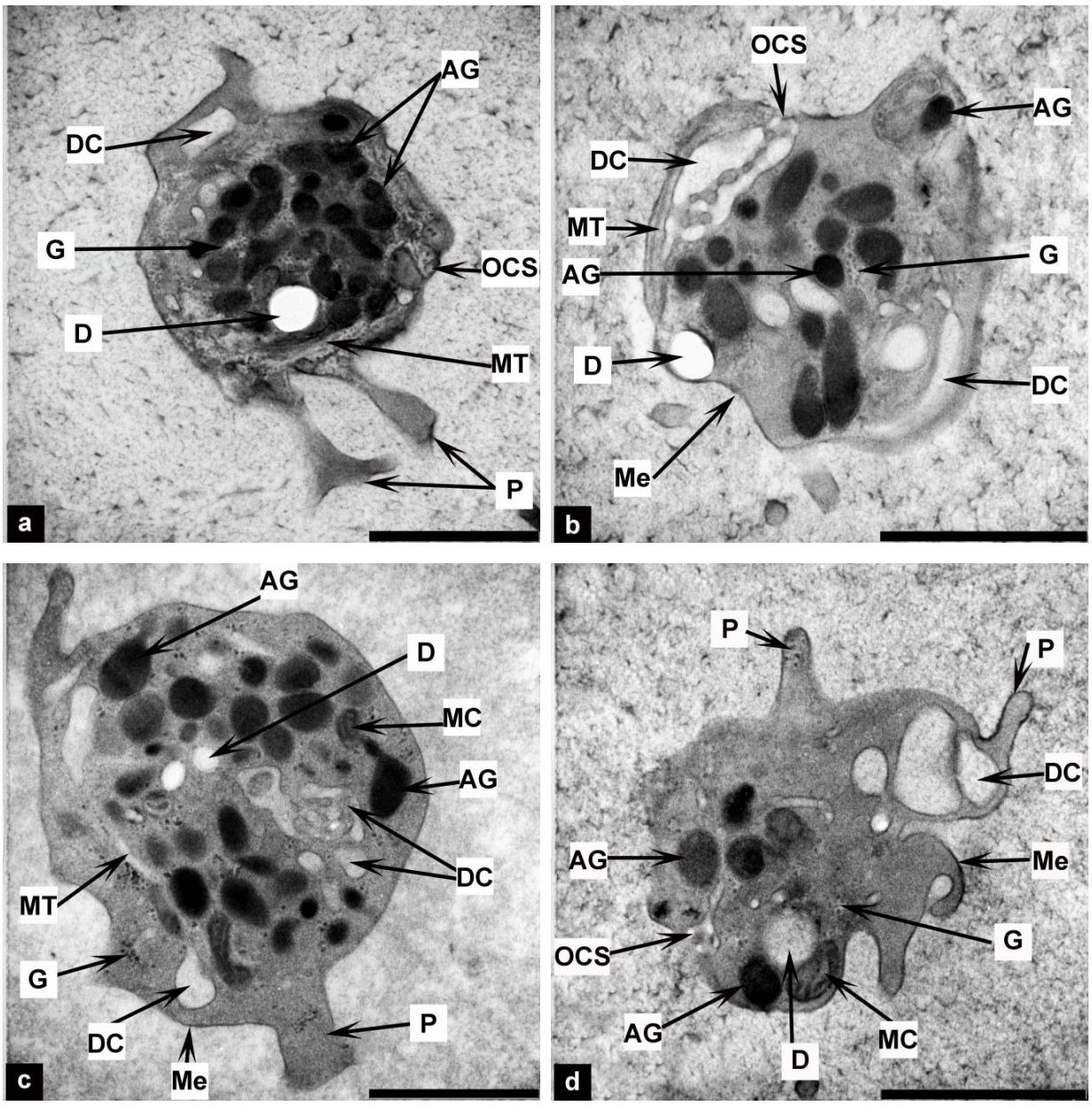


Figure 4.7: Platelets prepared by technique 5.

a-d: All scale bars = 1µm. Extremely well preserved individual platelets, are distinctly activated showing centralized organelles and extensive (P) pseudopodia formation. The cell (Me) membranes are clear and the organelles are distinctly and easily visible (OCS) open canalicular system, (DC) dilated channel, (AG) alpha granules, (MC) mitochondria, (D) dense bodies, (G) glycogen and (MT) microtubules.

4.3.6. Technique 6

PRP mixtures prepared in the same way as method 5 but followed by chemical fixation and ethanol dehydration shows densely packed aggregate fibers similar to results seen in technique 2 and 4 (Figure 4.8: a & b). The fiber diameter is significantly smaller to fibers seen in technique 5. As with technique 5, mostly transversely sectioned fibers are found, with rare transverse sectioned fibers present (Figure 4.8: b). Platelets seen are similar to those seen with technique 5, with the only dissimilarity in the quality of preservation and sharp features (Figure 4.9: a-d).

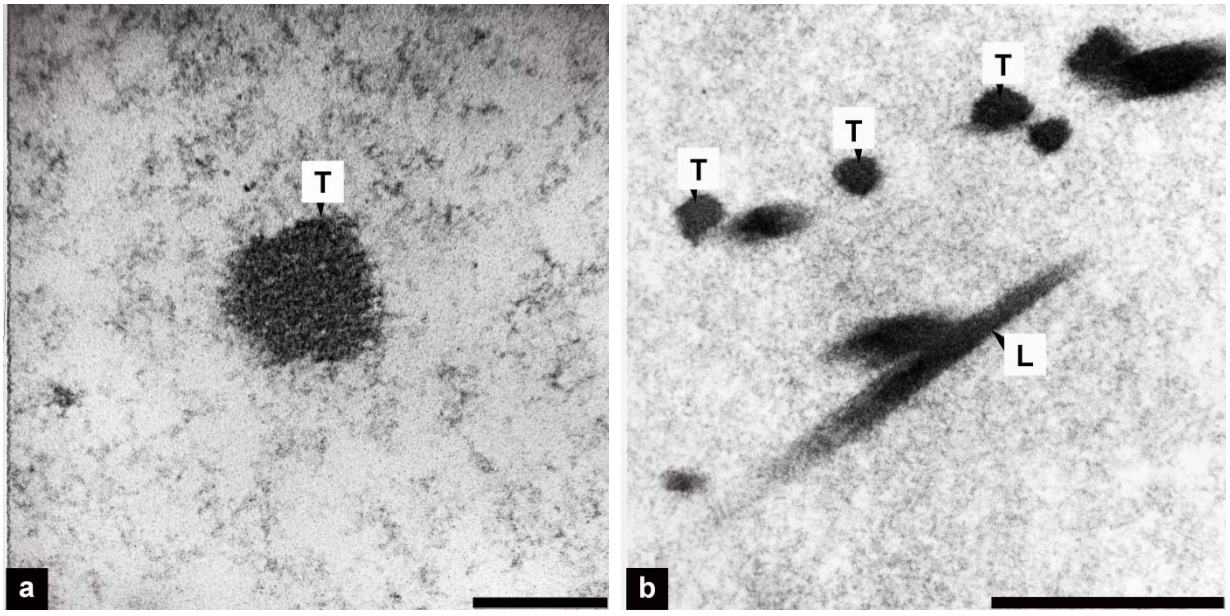


Figure 4.8: Fibrin fibers preserved by technique 5.
a: High magnification of a (T) transverse section through a tightly packed fibrin fiber, surrounded by a granular matrix, scale = 200nm. A (L) longitudinal sectioned compact fiber, and condensed (T) transverse fibers, scale = 1µm.

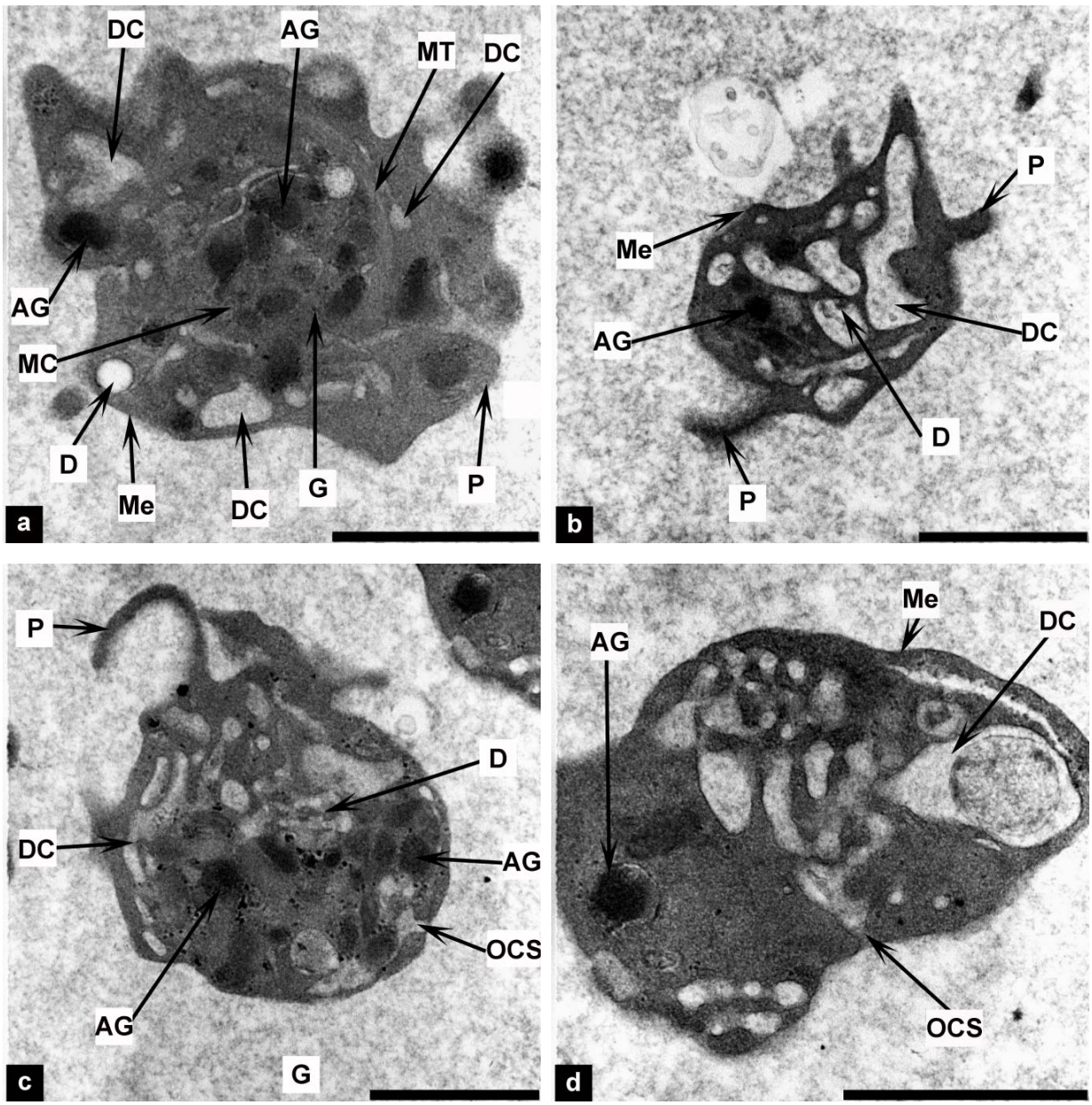


Figure 4.9: Platelets prepared by technique 6.

All scale bars = 1µm. a-d: Well preserved darkly stained individual platelets are distinctly activated showing centralized organelles and extensive (P) pseudopodia formation. The cell (Me) membranes are much less clear than those seen in technique 5, the organelles easily visible but less distinct ((OCS) open canalicular system, (DC) dilated channel, (AG) alpha granules, (MC) mitochondria, (D) dense bodies, (G) glycogen and (MT)

microtubules).

4.4. Discussion

4.4.1. Thrombin and Calcium Chloride

Differences seen between the coagulation and activation of fibrin networks and platelets initiated by thrombin or by $CaCl_2$ can be attributed to several factors.
With the addition of thrombin, cleavage of FPA and FPB from the N-terminal portions fibrinogen monomer is activated, exposing binding sites and resulting in intermolecular interactions (Blombäck 1996; Cilia la Corte *et al.,* 2011; Di Stasio *et al.,* 1998; Mosseson *et al.,* 2001; Ryan *et al.,* 1999 and Weisel 2005). Interactions between complimentary binding sites causes fibrinogen monomers to associate which leads to oligomers and ultimately double stranded protofibrils in which monomers are aligned in an end-to-middle, half staggered, overlapping arrangement (Blombäck 1996; Cilia la Corte *et al.,* 2011; Collet *et al.,* 1993; Di Stasio *et al.,* 1998; Fowler *et al.,* 1981; Mosseson *et al.,* 1993; Pretorius *et al.,* 2006; Ryan *et al.,* 1999; Standeven *et al.,* 2005; Weisel *et al.,* 1987 and Woodhead *et al.,* 1984). Although cleavage of both peptides occur simultaneously, it has been shown that the cleavage of FPB only becomes significant after polymerization of fibrin monomers has reached a certain point (Hantgan & Hermans 1979 and Weisel 2005). FBP is known to enhance lateral aggregation of the protofibrils, thus when the construction of protofibrils is actualized, FBP is cleaved causing aggregation of the protofibrils and leading to the formation of thicker fibrin fibers (Blombäck 1996; Cilia la Corte *et al.,* 2011; Di Stasio *et al.,* 1998; Fowler *et al.,* 1981; Hantgan & Hermans 1979; Weisel 2005 and Woodhead *et al.,* 1984).

When coagulation of PRP is activated by $CaCl_2$ without supplementation of thrombin, the calcium activates platelets which lead to several biochemical changes, such as protein expression on the platelet surface (Phillips *et al.,* 1980), cytoskeleton reorganization and membrane fusions, exteriorization and secretion of the contents of different storage granules (Becker 2008 and Rao 1999). During this process platelets supply factors that enhance the activation of prothrombin into its active form, thrombin (Michelson 2007). In

the techniques used, thrombin generation occur in a closed system, resulting in that a limited amount of platelet activating phospholipids for thrombin generation are available, and a limited amount of thrombin is formed (Kumar *et al.,* 1995). Apart from the limited amount formed, the formation of thrombin takes time, which naturally results in a lag phase decreasing actual coagulation time and ultimately altering the fiber structure. Di Stasio *et al.,* (1998) additionally showed that the changes in pH and ionic strength due to ion introduction affect the fiber lateral association and the ultimate diameter and structure; an increase in chloride ions opposes lateral aggregation and results in thinner, longer and more curved fibers (Di Stasio *et al.,* 1998).

When platelets are activated by thrombin, intracellular calcium is discharged from the dense tubular system (Ebbeling *et al.,* 1992). The free calcium then regulates several regulatory events in platelet activation, including the centralization of granular organelles, arachidonic acid liberation, phospholipase A_2 stimulation and platelet aggregation (Ebbeling *et al.,* 1992; Nesbitt *et al.,* 2003 and Rao 1993). If exogenous calcium is added to the coagulation it is rapidly taken up by the platelets, this surge in calcium has been shown to affect platelet function and morphology; platelet activation is enhanced (Detwiler 1987), the sensitivity of arachidonate induced aggregation is increased (Kerry & Paton 1984) and platelet secretion is up-regulated (Detwiler 1987).

4.4.2. High Pressure Freezing and Chemical Fixation

4.4.2.1 Platelets

Both fixation techniques gave good preservation of ultrastructural details. However, HPF together with freeze substitution gave superior preservation and better overall specimen contrast. Platelets prepared by the freeze fixation technique showed enhanced preservation of the cell membrane and distinct cellular features such as the open canalicular system, microtubule coils, α-granules, glycogen, mitochondria and dense bodies. The afore-mentioned, is especially true for platelets freeze fixated by technique 5.
In technique 5 and 6 all the platelets observed are increasingly activated as seen by the high degree of structural reorganization and pseudopodia extension. As these cells were

incubated for a longer period of time, an experiment was done in the form of technique 3 with longer coagulation time, as to ascertain if any other factors contributed to this observation (not shown). This experiment failed to produce the same result (a high degree of activation in all platelets); we suggest that the cellulose capillary tube might enhance activation of platelets, as platelets are known to be reactive in varying degrees to different types of materials (Ramström 2005).

4.2.2.2. Fibrin Networks

The process of chemical fixation, dehydration and the subsequent preparation steps is well known to cause adverse structural changes to a biological specimen; coagulation and precipitation of proteins are one of these effects (Kellenberger 1991 and Kiernan 2000). Highly aqueous specimens are especially sensitive to the precipitation reaction caused by the agents employed during chemical fixation, most notably the dehydration medium (Figure 4.14). In solution, molecules are freely mobile, after cross linking the movement is restricted and the solution becomes a gel. When the water in the system is removed, the cross-links should ideally preserve the original gel structure, but it has been shown that in solutions with less than 50% protein content, gelation is inadequate which leads to aggregation and precipitation (Figure 4.14b). Even with adequate cross-link formation, some aggregation is still expected (Figure 4.14c) (Kellenberger 1991).

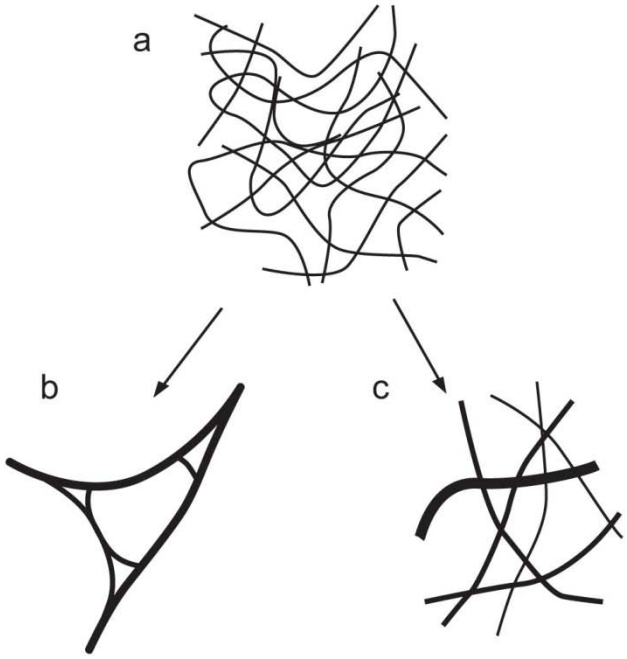


Figure 4.10: Schematic representation of the aggregation of fibrous bio-macromolecules in aqueous solution in organic solvents.
a: Fibrous bio-macromolecules in aqueous solution. b: Aggregation by organic solvent without prior cross-linking or with inadequate cross-linking. c: Reduced aggregation by efficient previous cross-linking (Redrawn from Kellenberger 1987).

The solvent content of a fibrin fiber is known to be between 70 and 80% (Weisel 1986 and Yeromonahos et al 2010). This unquestionably constitutes this macromolecule as an aqueous specimen, sensitive to aggregation and precipitation. The detrimental effect of this phenomenon can be seen when diameter measurements between turbidimetry, light-scattering and electron microscopy methods are compared. Average turbidimetry and light-scattering measurements are three times larger than what is seen in the electron microscope (Yeromonahos et al 2010). The results found in this electron microscopy study support measurements obtained from turbidimetry and light-scattering methods; of higher solvent concentrations and a dispersed fiber formation.

Several studies have attempted to study the internal structure of the fibrin fiber and several theoretical models have been built to explain the high solvent content of the fiber seen with other techniques (Hermans 1979; Weisel 1986 and Yang *et al.,* 2000). To name a few theories, Yang *et al.,* (2000) suggested a quasi-crystalline structure with tetragonal cells, Guthold (2004) and Hunziker *et al.,* (1990) support a random lateral structure, whereas Yeromonahos *et al.,* 2010 found fibrin fibers to have a disjointed structure with local crystalline arrangement. The results seen in HPF fixation of fibrin fibers, in particular with the capillary technique, show a disperse fiber formation of a highly ordered, crystalline nature. Further analysis and modelling of the results is needed to ascertain the type of crystalline structure that is predominant.

4.4.3. Comparison of Operational and Practical parameters

Comparison of several parameters was done to determine the practical aspects connected to each technique tested in this study. The comparisons and subsequent “score” of each technique does not determine which technique is superior, the superiority is based on the quality of the results alone.

The quality of the results was discussed in previous sections. The cost involved with chemical fixation and processing is relatively low. The method additionally requires only the chemicals for fixation, rinsing, dehydration and embedding, and consumables such as pipettes. Controversially freeze fixation requires the HPF machine and the freeze substitution machine with regards to equipment. Also, the consumables used for these processes are usually costly as they are specific to the equipment. Together with the chemicals necessary for freeze substitution and the liquid nitrogen consumed, this technique is very expensive. Freeze substitution of the frozen specimens, account for the HPF technique to also be the most costly technique in relation to time. Most of the above mentioned chemicals are graded as either toxic, harmful or as an irritant, as chemical techniques and freezing techniques both employ these chemicals but only HPF additionally use liquid nitrogen (can cause frostbite, an explosion of a sealed container and may displace oxygen causing asphyxiation). Thus, the HPF technique is the more dangerous of the two.

When freeze fixation is selected for fixation, the samples need to be extremely small for optimal vitrification to take place. The consumables (specimen carriers) used in the HPF process are also exceedingly small, which is difficult to work with. The cellulose capillary tubes are especially challenging, as these tubes with a total diameter of roughly 220µm, needs to be cut down to a 1mm length and sealed simultaneously. This process tends to press out the small amount of specimen that was in there initially. This governs the fact that this technique requires a certain amount of practise and expertise and is also the most difficult technique to repeat successfully.

Table 4.2: Summary of operational and practical parameters comparisons.

Technique	Quality	Cost	Time	Rpt	Exp	Safety	Total
1	4	2	2	5	2	3	**18**
2	2	4	4	5	4	4	**23**
3	4	2	2	4	2	3	**17**
4	2	4	4	4	4	4	**22**
5	5	2	2	2	2	3	**16**
6	3	4	4	3	3	4	**21**

Key:			
	Quality of preservation	1 = worst	5 = best
	Financial cost	1 = high	5 = low
	Time	1 = high	5 = low
	Repeatability (Rpt)	1 = high	5 = low
	Expertise required (Exp)	1 = high	5 = low
	Safety of use	1 = dangerous	5 = not dangerous

4.5. Conclusion

Despite the fact HPF coupled with freeze substitution is the most expensive, most difficult and least repeatable method for fixation purposes it is the most suitable method for fine structure preservation of fibrin fiber networks and platelets, in particular technique 5.

As a final note, the detailed nanostructure of the fibrin fiber such as whether it is crystalline or largely random, is still unknown. This is likely of particular interest for the mechanical properties of the fibrin network and subsequent clot. The results found with HPF fixation in this study could therefore be significant in providing a more accurate structure of the native state fibrin fiber, revealing information on the radius, density and internal organization of the fibrin fiber.

Chapter 5:

Development of a method of rapidly vitrifying platelet and fibrin networks utilizing freeze fixation and freeze drying for use in SEM ultrastructural studies

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Research Question 3:

Which method of rapidly vitrifying platelets and fibrin networks utilizing cryo fixation and freeze drying is the most repeatable method and gives the most informative results in SEM ultrastructural studies?

5.1. Introduction

As discussed in the preceding chapters (chapters three and four), the importance of ultrastructural investigations of fibrin fiber networks and platelets is paramount in understanding the aetiology of diseased states. Firmly linked to this is the best possible specimen preparation method to ensure an accurate resemblance of the fixated specimen to its *in vivo* condition. Freeze fixation preserves biological specimens by the removal of thermal energy from the specimen. This is done at an extremely fast rate (> 10^5K/s) allowing the water in a biological specimen to reach a super-cooled state (vitrification) (see Figure 4.1, chapter 4, page 103) (Moor 1987; Quintana 1994 and Vanhecke *et al.,* 2008). In this state the water's viscosity is so high that the water molecules become stabilized and is unable to rearrange into a lattice formation of a crystal, meaning the water is in a solid form of the liquid phase (Moor 1987; Morphew 2000 and Quintana 1994).

Freeze fixation is generally carried out by one of several methods, two of which is used in this study. The first being high pressure freezing which has been discussed in previous chapters (chapters three and four).

The second method is plunge-freezing. This process involves plunging a biological specimen into a cryogen such as liquid nitrogen or cooled propane, either by hand or with the aid of a plunge-freezing device. For plunge freezing to be an effective fixation method, the specimen size, the choice of cryogen, the plunging speed and the plunging depth are the most important parameters (Hayat 2000). The specimen size needs to be as small as possible as it is estimated that the layer of well frozen material is only between 10 and 20 µm (Humbel & Müller 1986 and Shimoni & Müller 1998). The choice of cryogen is based on the substance's thermal conductivity and its melting, freezing and boiling points (Hayat 2000). For example liquid nitrogen, which is a cheap and relatively safe cryogen, is not often used in plunge freezing because of its boiling point. It boils at room temperature meaning that the insertion of a warm body, like a biological specimen or the tweezers holding it, will cause the nitrogen to boil adjacent to the body and form an insulating layer around it, thereby reducing the rate of cooling. Ethane and propane on the other hand is used at temperatures far below their boiling points, meaning the cryogen will only heat up around the sample due to the specimen's energy dissipation (Von Schack and Fakan 1993). Other considerations such as the speed and depth of plunging needs to be optimal. If the speed of plunging is too slow or the plunging depth too little the cryogen around the specimen will not be renewed continuously, leading to a cooling rate which is inadequate and only partial vitrification will be accomplished.

With either freeze fixation method a substrate onto which the biological specimen is applied is typically needed. Several such substrates exist, for example sapphire discs, Aclar films and grids.

Sapphire disc with a diameter of 1.4mm was first used as a coverslip material for high pressure freezing of adherently growing cells (Figure 5.1). It was found that sapphire had excellent physical and chemical properties that are needed for cryo-fixation (for example,

high heat conductance rate). This coupled with sapphire's natural inert and smooth surface provided a perfect platform for biological applications (Reipert *et al.,* 2003).

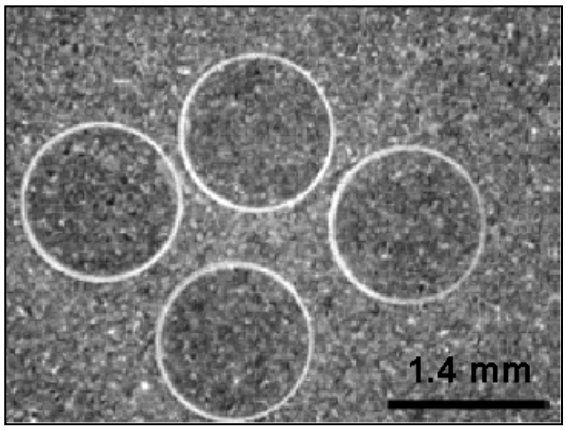


Figure 5.1: Sapphire discs of 1.4mm diameter.
(Leica Microsystems, Vienna, Austria)

A copolymer plastic film made from fluorinated-chlorinated resin, commercially known as Aclar, has been shown to be a superb substrate for the use of HPF in biological applications (Jiménez *et al.,* 2006). Aclar is non-toxic, chemically inert (Kingsley and Cole 1988), and it remains flexible at very low temperatures (Burry and Lasher 1978), but probably its best characteristic when used in HPF is the fact that large sheets of it can be used for the initial preparation of the specimen and just prior to freezing a piece can be punched or cut out of the sheet to the required shape and size, easing handling of the generally very small objects (Jiménez *et al.,* 2006) (Figure 5.2).

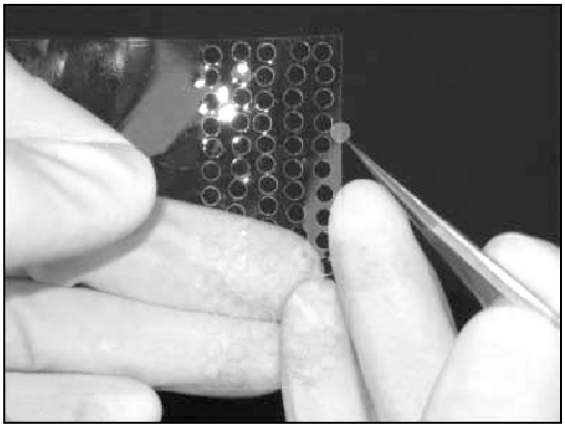


Figure 5.2: Aclar sheet with several punched out sections.
(Jiménez *et al.,* 2006).

Leica (Leica Microsystems, Vienna, Austria) also supplies a specialised grid that is small enough to fit into the standard specimen carrier (Figure 5.3). The grid is usually used as a "finder" grid to help an investigator with specimen orientation and area identification but may be used as a specimen substrate.

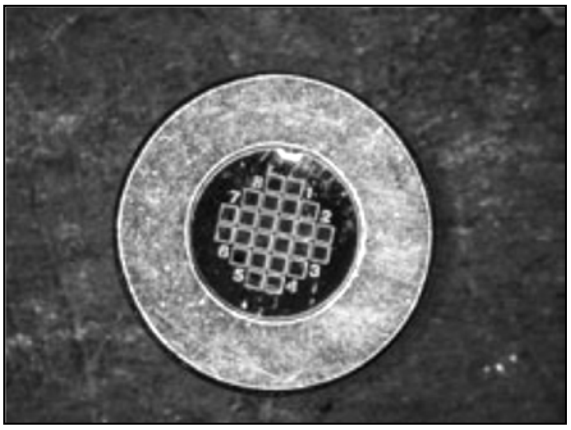


Figure 5.3: Specialised grid in HPF specimen carrier
(Leica Microsystems, Vienna, Austria).

For ultimate investigation with a SEM, biological samples needs to be completely free of any volatile substances and stable when radiated with an electron beam, necessitating the removal of the super-cooled water in the frozen specimen. This is done by a process

called freeze drying. Freeze drying removes the vitrified water by sublimation. A frozen specimen is placed in a chamber where the pressure is reduced to keep the partial pressure of the water in the specimen lower than the vapour pressure (CNRS (CENBG) 2010; Hanzon & Hermodsson 1960 and Schwartz *et al.,* 1993) and the specimen is heated somewhat (but still below the temperature of recrystallization) while the chamber walls are cooled, creating a temperature gradient. In this way the water is condensed onto the cooled surface (CNRS (CENBG) 2010 and Hanzon & Hermodsson 1960).

As scanning electron microscopy is extensively used in ultrastructural research of abnormal or diseased blood platelets and fibrin fiber networks (Ajjan *et al.,* 2009; Barua *et al.,* 2010; Pretorius *et al.,* 2006; Pretorius *et al.,* 2007; Pretorius *et al.,* 2009b; Pretorius *et al.,* 2010; Wolberg 2007 and Zhuge *et al.,* 2009), in the current chapter, several different approaches of freeze fixation, employing HPF, plunge freezing and freeze drying, are carried out to determine the optimum preparation method for scanning electron microscope ultrastructural studies of platelets and fibrin networks. To accomplish this, several factors in the preparation procedure was varied. These factors are the:

- Coagulation agent
- Coagulation agent - platelet rich plasma (PRP) ratio
- Freezing substrate
- Freezing substrate application
- Coagulation time
- Coagulation temperature
- Freezing Technique

5.2. Materials and Methods

5.2.1. Samples

Healthy individuals, without any known medical condition, who are not smokers and in the case of women, were not on contraception, were chosen for this study. Whole blood obtained by venipuncture was collected in citrate containing tubes (for the inhibition of calcium-mediated coagulation by the addition of sodium citrate (Sabbatani 1901a and Sabbatani 1901b)), followed by 2 minutes of centrifugation to separate the plasma, containing the platelets and fibrinogen from the erythrocytes and leukocytes.

5.2.2. Sample Preparation

From the next stage onward, the process is varied at predetermined coagulation factor points and sample preparation steps (see Table 5.1), the basic methodology will be explained next, followed by a detailed explanation towards each variation.

The supernatant liquid (PRP) was separated from the pellet (erythrocytes and leukocytes) by pipetting the liquid off into a polyethylene holder (eppendorf). The PRP was then mixed with a coagulation agent on the chosen surface (substrate) or applied to the chosen surface (substrate), and left for coagulation to occur in a humid environment. This was followed by a washing step in (0.075M) sodium phosphate buffer (pH 7.4), using a micro-plate shaker to remove plasma proteins trapped in the network. The platelet and fibrin coagulation was then high pressure frozen (EMPACT2 (Figure 3.3a, Chapter 3 page 76), Leica Microsystems, Vienna, Austria) or frozen by plunging the specimen into liquid propane cooled by liquid nitrogen, and subsequently kept under liquid nitrogen conditions until such a time that freeze drying was done. For freeze drying the specimens were put into a pre-cooled copper sample container and transferred to the drying chamber of the freeze drying apparatus (self-built in this lab) which is connected to a vacuum evaporator and a liquid nitrogen dewar. The specimens were then freeze dried under high vacuum while gradually heating the stage from liquid nitrogen temperatures to room temperature, over the period of 72 hours. The dry specimens were mounted on aluminium stubs, coated

by carbon evaporation and subsequently viewed with a Zeiss, Field Emission Scanning Electron Microscope (Zeiss, Germany).

Not all of the possible variations were performed as this would result in over 700 different experiments to prepare and analyse, with financial, laboratory time and equipment limitations (such as a limited amount of specimens that can be freeze dried at one time) this was not feasible.

The optimum coagulation properties and HPF technique was determined using only the two coagulation agents, one coagulation agent – PRP ratio (thrombin = 1:1, $CaCl_2$ = 1:10), the four freezing substrates, the three freezing substrate application techniques, one coagulation time (10 minutes), one coagulation temperature (37°C) and the two freezing techniques. These experiments were carried out at least three times to ascertain their reproducibility. The recipes showing the most promising results were further varied with regards to the coagulation agent – PRP ratios, the coagulation times and the coagulation temperatures (see Figure 5.4 for a flow diagram of initial experiments and Figure 5.5 for a flow diagram of subsequent experiments).

Table 5.1: The variants of coagulation factors and specimen preparation factors used in this study.

Coagulation or Preparation Factor	Variation1	Variation 2	Variation 3	Variation 4	Variation 5
Coagulation Agent	Thrombin	$CaCl_2$	-	-	-
Coagulation Agent-(PRP) ratio	1:1 (T)	1:5 (T)	5:1 (C)	10:1 (C)	100:3 (C)
Freezing Substrate	Sapphire	Aclar	Millipore	Grid	-
Freezing Substrate Application	SA1	SA2	SA3		
Coagulation Time (min)	1	10	30	-	-
Coagulation Temperature	RT	37°C	-	-	-
Freezing Technique	HPF	Plunge			

Key: (T) – Thrombin, (C) – $CaCl_2$, SA1 – Substrate Application Method 1, SA2 – Substrate Application Method 2, SA3 – Substrate Application Method 3, RT – Room Temperature.

5.2.2.1. Coagulation Agent

The two coagulation agents used were thrombin and $CaCl_2$.

For a more comprehensive discussion see Chapter 3, section 3.2.2.1, pages 72-73.

5.2.2.2. Coagulation Agent – PRP Ratio

As in Chapter 3; different ratios of the coagulation agent to PRP (see Table 5.1), was loosely based, on concentrations that is physiologically relevant and concentrations frequently used in literature, for the rationalization and references see chapter 3, section 3.2.2.2, page 73.

5.2.2.3. Freezing Substrate

Four different substrates were used to form the fibrin fiber network and platelet coagulates on;

Sapphire Discs

As platelet and fibrin fiber networks are routinely created on glass coverslips in this laboratory, the use of a similar application method for freeze fixation was employed by the use of sapphire discs with a diameter of 1.4mm (Figure 5.1).

Aclar Membrane (Figure 5.2)

The Aclar membrane was used to benefit from the ease of handling of a larger sheet, adequately sized areas was partially punched out of the sheet and after the platelet and fibrin fiber network was formed on the sheet, the sections was fully removed and freeze fixated.

Millipore Membrane

Millipore membranes have proven to be good substrates for platelet and fibrin fiber network formation, so the membrane was cut down to the appropriate size and used for formation of the platelet and fiber network and ensuing freeze fixation.

Grids

Grids have been successfully used as a substrate for especially fibrin network formation by several investigators (Karges and Kuhn 1970 and O'Brien *et al.,* 2008). Specialised grids

from Leica Microsystems (Vienna, Austria) were used as they are small enough to fit into the HPF specimen carriers (Figure 5.3).

(NOTE: As only HPF requires specimen substrates of extremely small size (1.4mm diameter), which is difficult to handle; plunge freezing experiments were performed with normal glass coverslips (10mm), larger sections of Aclar film, uncut Millipore membranes (10mm) and normal copper TEM grids (± 3mm).)

5.2.2.4. Freezing Substrate Application

Substrate Application Method 1 (Figure 5.4)

1. PRP was prepared as described above.

2. Coagulation was initiated with one of the two coagulation agents by mixing the agent and the PRP, placing a drop of the mixture on the substrate and leaving it for a period of time (first set of experiments was left for 10 minutes, and subsequent experimentations was varied, see 5.2.2.4) in a humid environment for coagulation to occur.

3. The substrate with the applied specimen was then washed as described above.

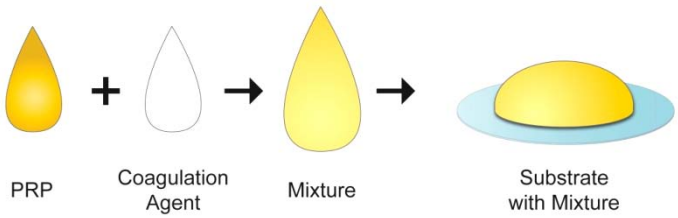


Figure 5.4: Substrate Application Method 1.

Substrate Application Method 2 (Figure 5.5)

1. PRP was prepared as described above.

2. Coagulation was initiated with one of the two coagulation agents by mixing the agent and the PRP on top of the substrate and spreading the mixture over the substrate and substrate edges in an effort to create a thin network, this was then left for a period of time (first set of experiments was left for 10 minutes, and subsequent experimentations was varied, see 5.2.2.4) in a humid environment for coagulation to occur.

3. The substrate with the applied specimen was then washed as described above.

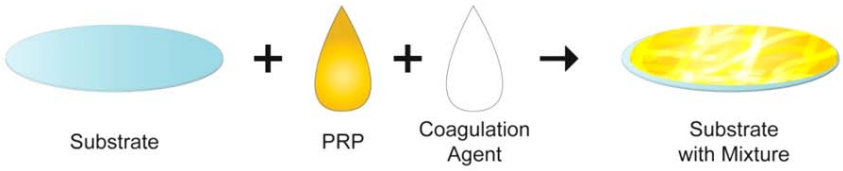


Figure 5.5: Substrate Application Method 2.

Substrate Application Method 3 (Figure 5.6)

1. PRP was prepared as described above.

2. Coagulation was initiated with one of the two coagulation agents as by mixing the agent and the PRP and placing a drop of the yet un-gelled mixture on a flat surface and then placing the inverted specimen substrate to the surface of the drop, the substrate and specimen was then placed in a humid environment and left for a period of time (first set of experiments was left for 10 minutes, and subsequent experimentations was varied, see 5.2.2.4) for coagulation to occur. After which the substrate was removed from the coagulation mixture by pulling it from the clot in a tangential direction to the clot surface.

3. The substrate with the applied specimen was then washed as described above.

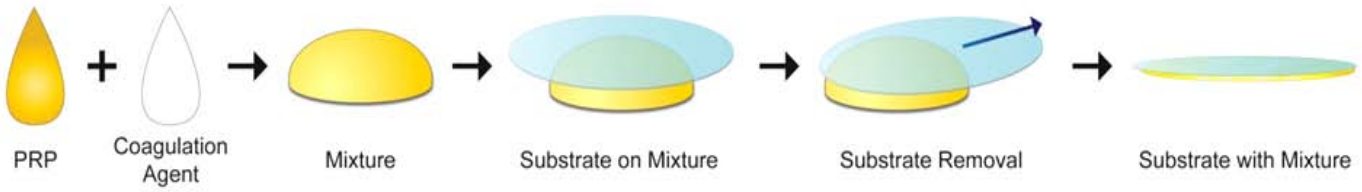


Figure 5.6: Substrate Application Method 3.

5.2.2.5. Coagulation Time

The time allowed for coagulation of the fibrin and subsequent blood clot was varied between one and 30 minutes (see Table 5.1).

5.2.2.6. Coagulation Temperature

Studies of the coagulation of fibrin and platelets has been performed in this laboratory previously, in these studies coagulation was allowed to continue at room temperature, and proved to be completely adequate. A gelation temperature of 37°C was also used for physiological relevancy.

5.2.2.7. Freezing Technique

Specimens where frozen either by HPF or plunge freezing.

HPF

The freezing substrate used, was transferred to a sample carrier, and subsequently high pressure frozen (Leica EMPACT2, Leica, Vienna, Austria). For a complete description of the freezing process, please see Chapter 3, section 3.2.2.3, Specimen Carrier Method 1, steps 6 – 8, pages 73-74.

Plunge Freezing (Figure 5.7)

The specimen was formed on the freezing substrate as described above. The specimen applied to the chosen substrate was plunged into liquid nitrogen cooled propane by hand and placed in a pre-cooled specimen tube until further processing (freeze drying) was required.

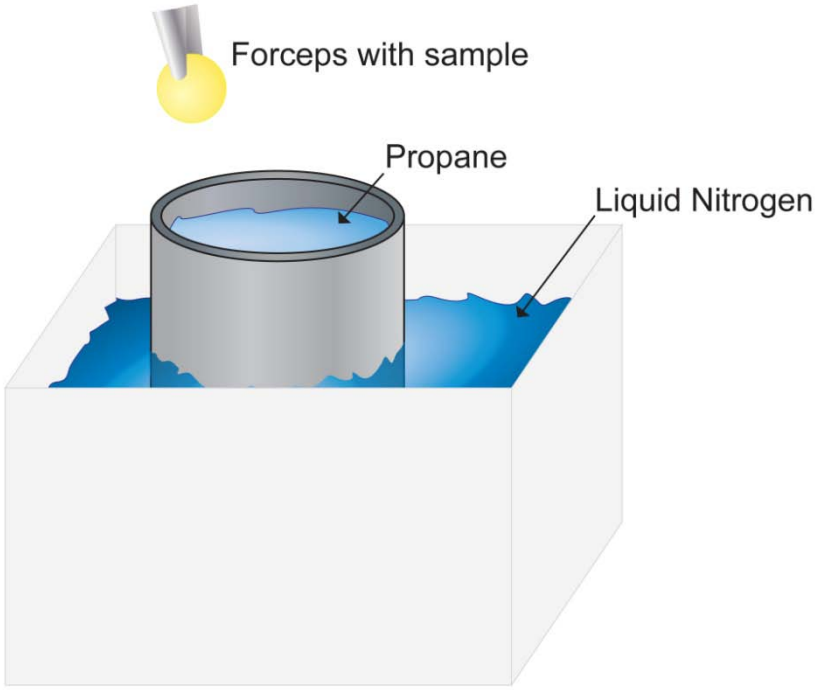


Figure 5.7: Plunge freezing; experimental setup.

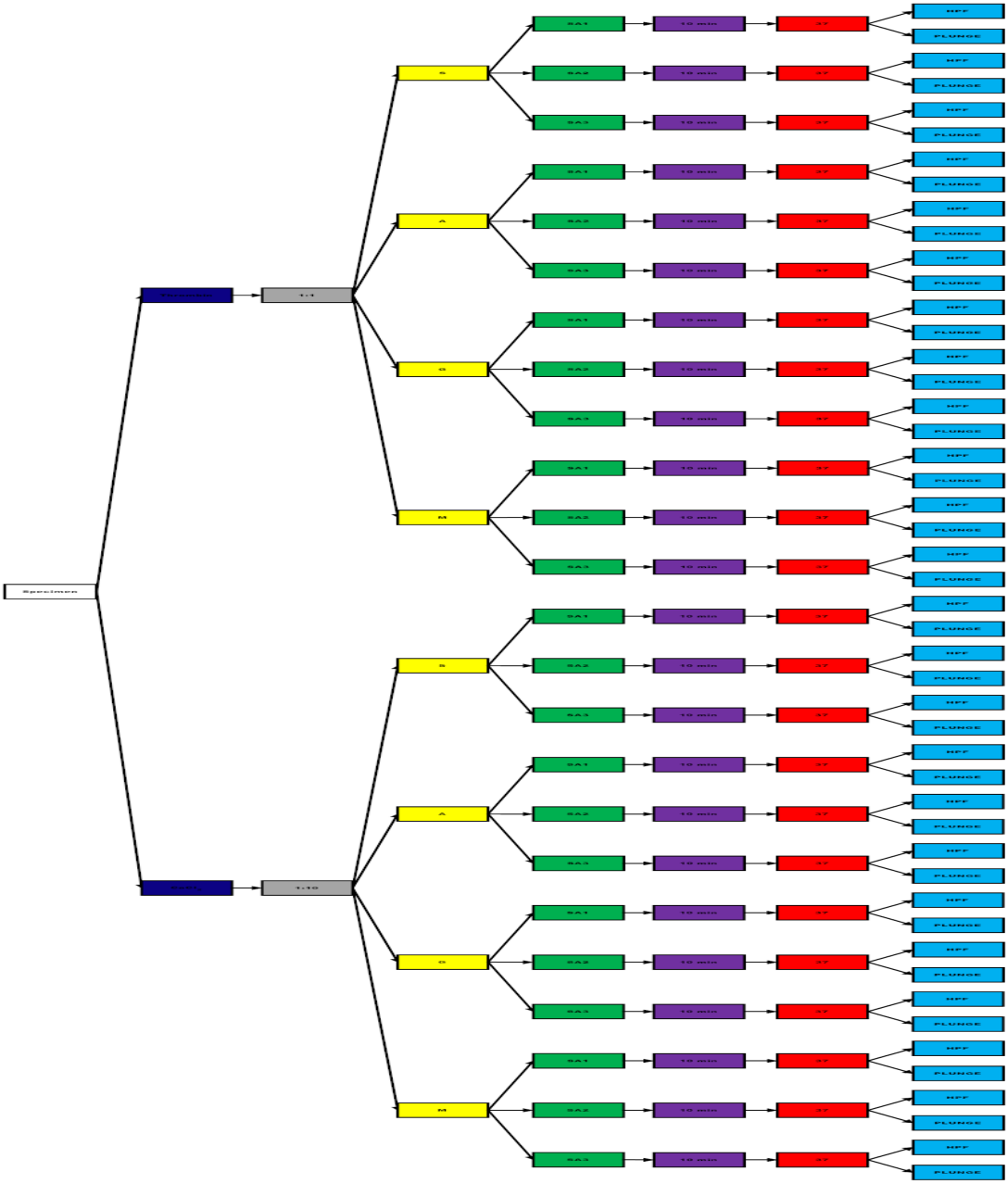
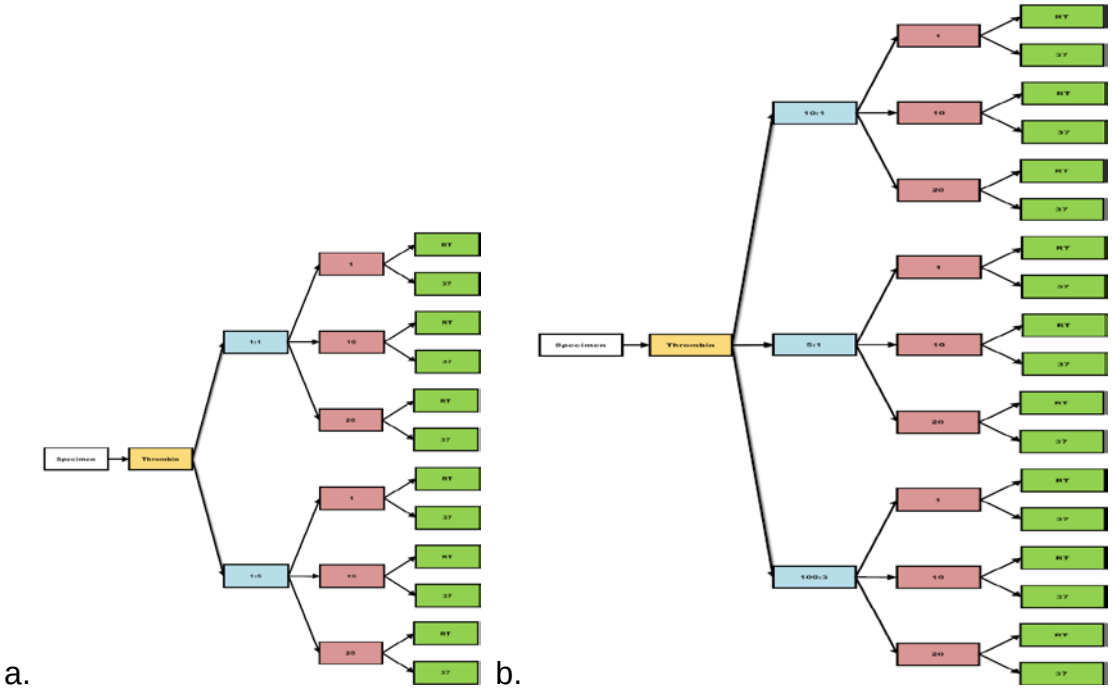


Figure 5.8: Flow diagram of experimental setup.

Key: white – specimen, dark blue – coagulation agent (thrombin / $CaCl_2$), grey – coagulation agent PRP ratio (thrombin 1:1, $CaCl_2$ 1:10), yellow –freezing substrate (sapphire, Aclar, grid, Millipore membrane), green – freezing substrate application method (SA1 / SA2 / SA3), purple – coagulation time (10), re – coagulation temperature (37°C), blue – freezing technique (HPF / plunge freezing).



a. b.

Figure 5.9: Flow diagram of subsequent experiments based on the results from the initial experiments.

a: Experiments with thrombin. b: Experiments with $CaCl_2$. Key: light orange – coagulation agent, light blue – coagulation agent: PRP ratio (1:1 / 5:1 / 1:5 / 1:10 / 3:100), pink – coagulation time (1 / 10 / 30), light green – coagulation temperature (RT / 37°C).

5.3. Results

Individual variables will be discussed separately.

5.3.1. Coagulation Agent

The coagulation of fibrin fiber networks by addition of thrombin results in an extensive fiber network made up of thick to thin fibers (Figure 5.10: a-b). Platelets activated by thrombin are found as dense coagulates highly associated with the fiber network (Figure 5.10: c).

Coagulation with $CaCl_2$ resulted in an elevated number of platelets in various stages of activation (Figure 5.11: c), but a decreased number and density of fibrin fibers. In general, the fibers are sparser, and appear thinner (Figure 5.11: a-b).

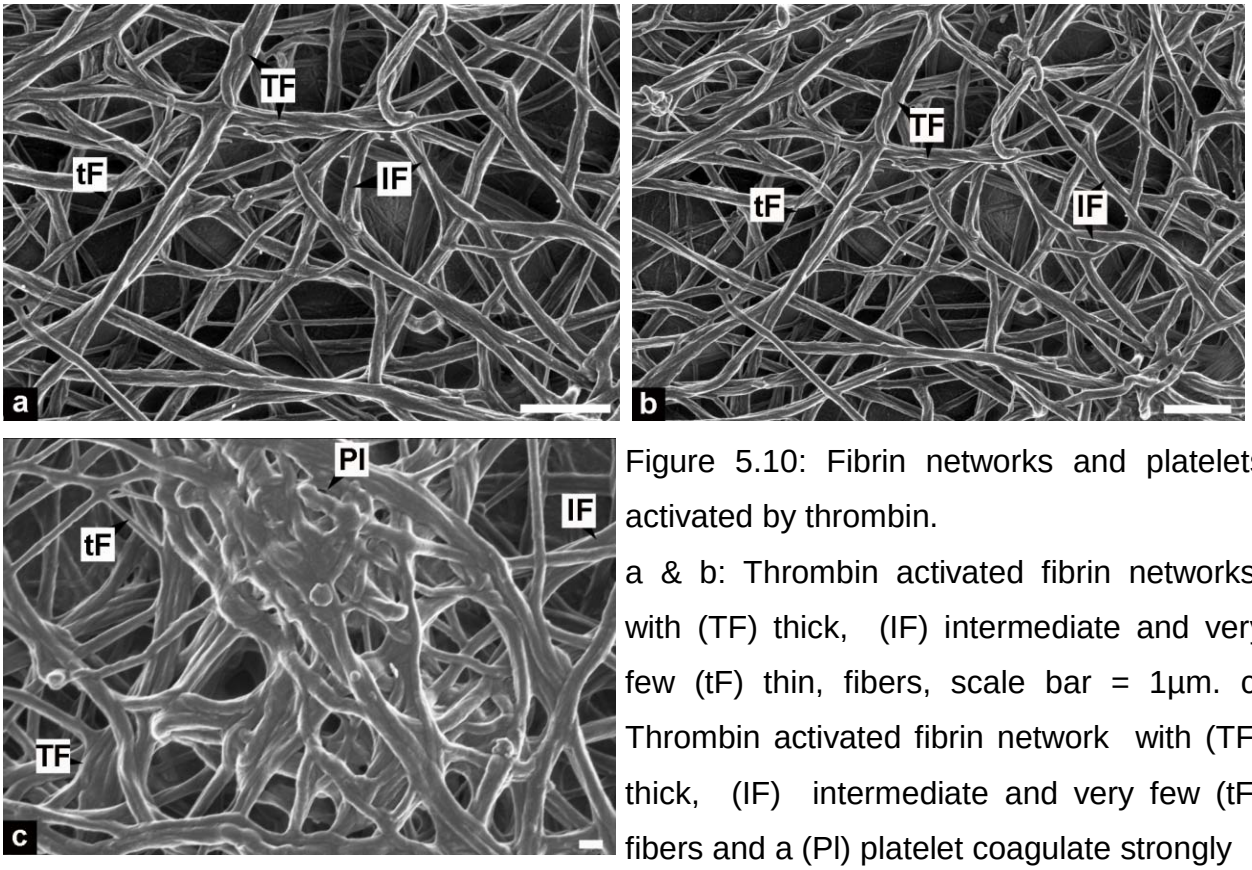
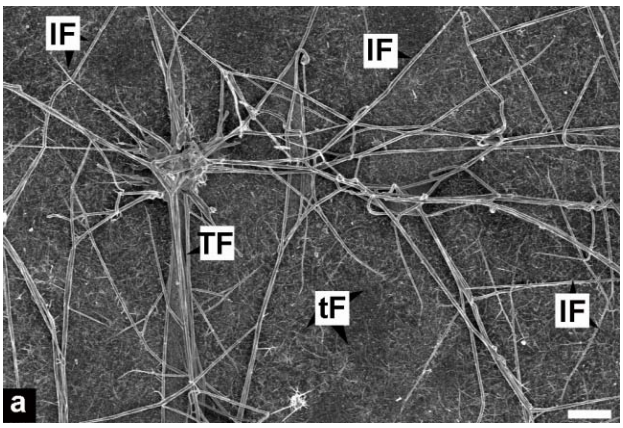
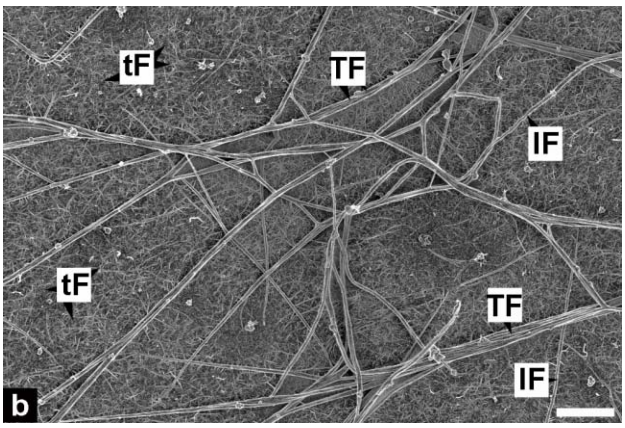


Figure 5.10: Fibrin networks and platelets activated by thrombin.
a & b: Thrombin activated fibrin networks, with (TF) thick, (IF) intermediate and very few (tF) thin, fibers, scale bar = 1µm. c: Thrombin activated fibrin network with (TF) thick, (IF) intermediate and very few (tF) fibers and a (Pl) platelet coagulate strongly associated with the fiber network, scale bar = 200nm.





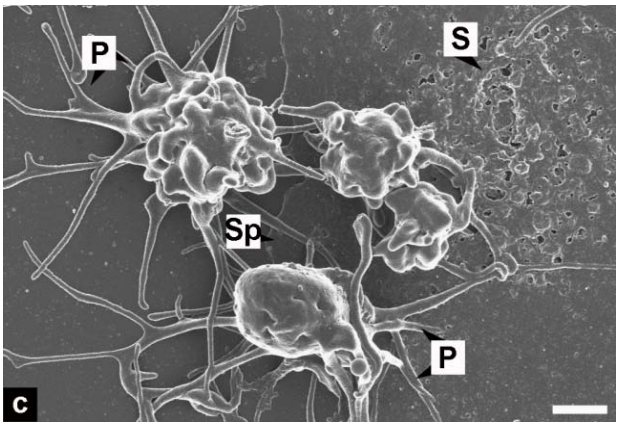


Figure 5.11: Fibrin networks and platelets activated by $CaCl_2$.
a & b: Fibrin networks, with mostly (tF) thin and (IF) intermediate fibers and very few (TF) thick, fibers, scale bar = 2µm. c: $CaCl_2$ activated platelets in various stages of activation, (P) pseudopodia, (Sp) spreading and (S) secretion with subsequent breakdown is seen, scale bar = 1µm.

5.3.2. Coagulation Agent – PRP Ratio

An increase in thrombin and $CaCl_2$ both resulted in an increased density and thickness of the fibrin fiber network; with thrombin longitudinal fusing is also apparent (Figure 5.12: a-b, Figure 5.13: a-b, Figure 5.14: a-c). Platelets activated by increasing amounts of thrombin showed increasing levels of activation (Figure 5.13: b) whereas increasing amounts of $CaCl_2$ did not affect the level of activation of the blood platelets significantly.

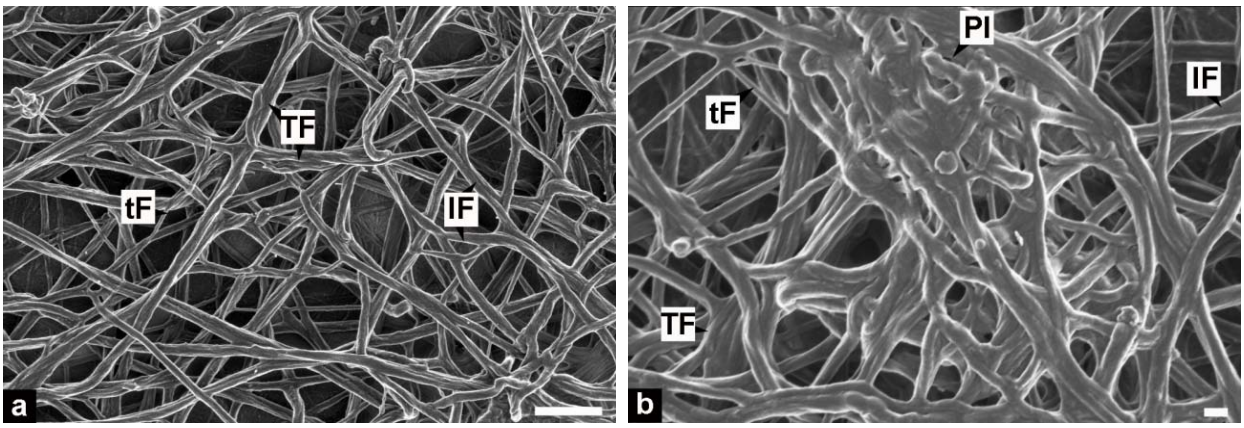


Figure 5.12: Fibrin networks and platelets activated by a PRP: thrombin ratio of 1:1.
a: Thrombin activated fibrin networks, with (TF) thick, (IF) intermediate and very few (tF) thin, fibers, scale bar = 1µm. b: Thrombin activated fibrin network with TF) thick, (IF) intermediate and very few (tF) thin, fibers and a (Pl) platelet coagulate strongly associated with the fiber network, scale bar = 200nm.

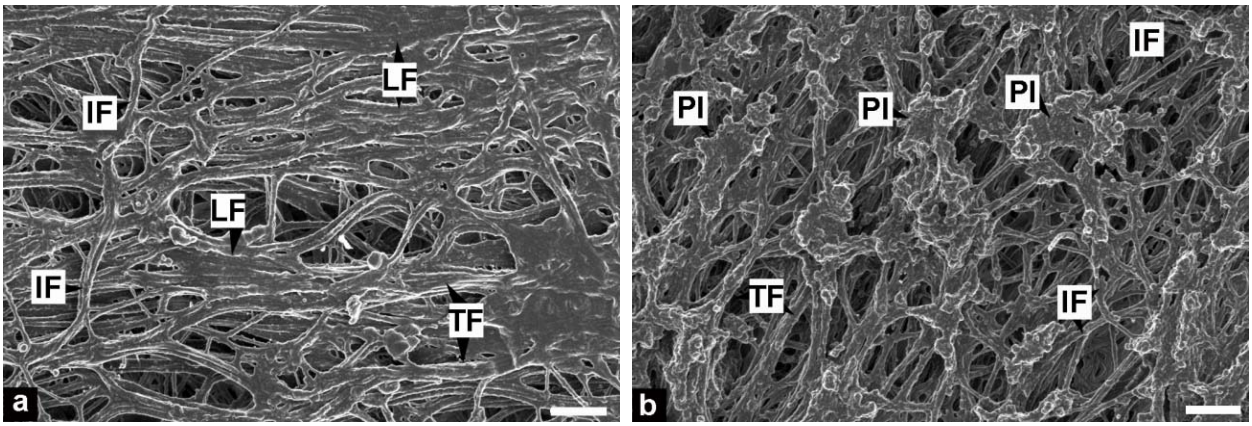


Figure 5.13: Fibrin networks and platelets activated by a PRP: thrombin ratio of 1:5.
a: Thrombin activated fibrin networks, with (TF) thick and (IF) intermediate with numerous (LF) longitudinally fused fibers, scale bar = 1µm. b: Thrombin activated fibrin network with (TF) thick and (IF) intermediate fibers and several (Pl) platelet coagulates strongly associated with the fiber network, scale bar = 1µm.

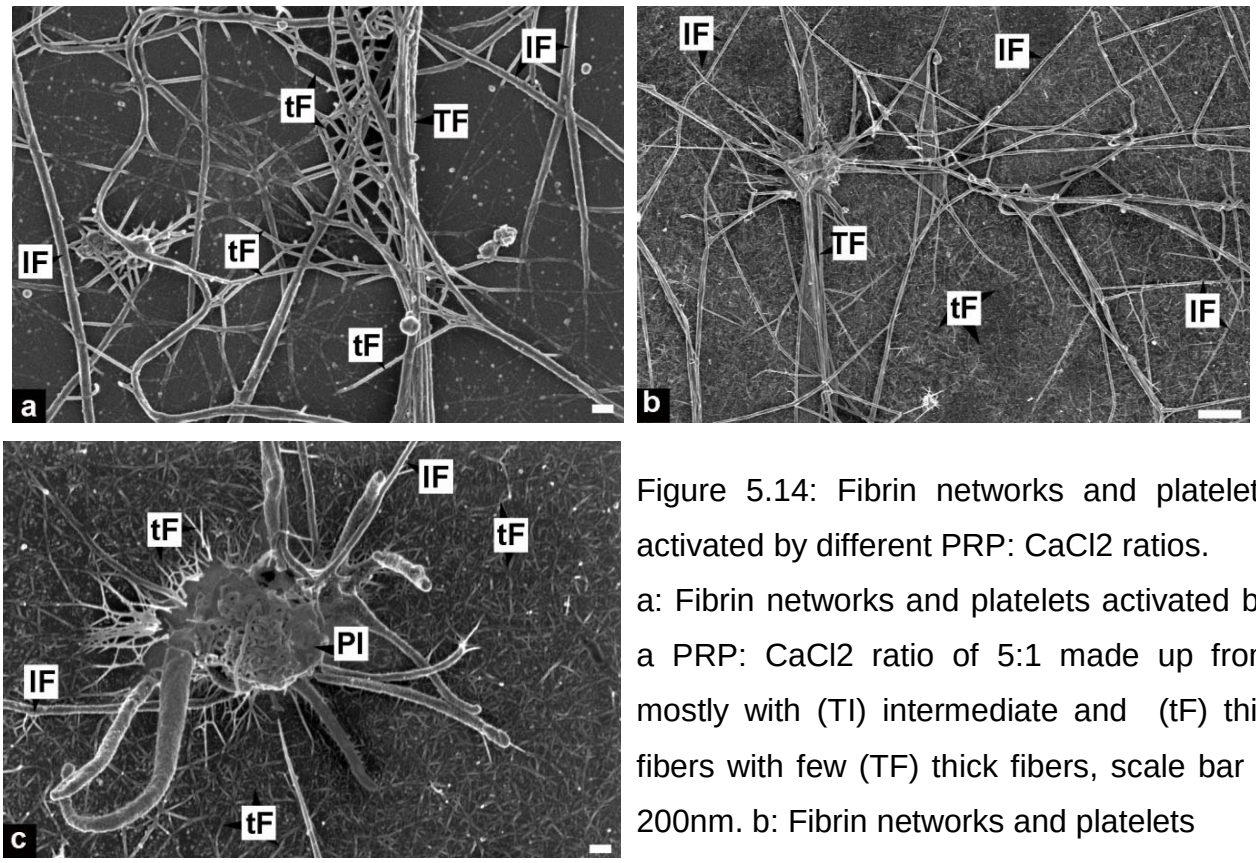


Figure 5.14: Fibrin networks and platelets activated by different PRP: CaCl2 ratios. a: Fibrin networks and platelets activated by a PRP: CaCl2 ratio of 5:1 made up from mostly with (TI) intermediate and (tF) thin fibers with few (TF) thick fibers, scale bar = 200nm. b: Fibrin networks and platelets activated by a PRP: $CaCl_2$ ratio of 10:1 made up from mostly with (TI) intermediate and (tF) thin fibers with few (TF) thick fibers, scale bar = 200nm. c: Fibrin networks and a platelet activated by a PRP: $CaCl_2$ ratio of 100:3 with (tF) thin fibers with few (IF) intermediate fibers, scale bar = 200nm.

5.3.3. Freezing Substrate

After the first three sets of freezing experiments, Aclar sheets and Millipore membranes as freezing substrate was promptly eliminated as freezing substrates for the formation of platelet and fibrin fiber networks. Firstly, both these materials was frequently found to result in a failure of the high pressure freezing process, most probably due to small cutting / punching defects on the sides of the materials, hindering the complete sealing of the HPF specimen carrier and the bayonet pod.

Secondly the materials (especially the Millipore membranes) had a tendency to twist and curl, creating difficulties with SEM imaging.

Sapphire discs and grids proved adequate substrates for the formation of fibrin fiber and platelet networks (Figure 5.15: a-b).

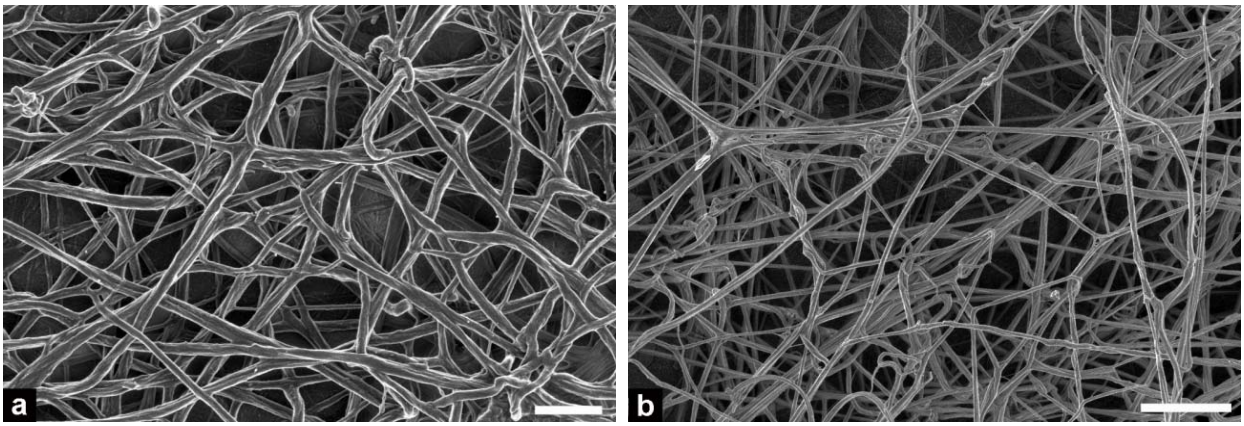


Figure 5.15: Fibrin fiber networks formed on different substrates.
a: Fibrin fiber network formed on a sapphire disc, scale = 1µm. b: Fiber network formed on a grid, scale bar = 2µm.

5.3.4. Freezing Substrate Application

Substrate Application Method 1 primarily gave dense, thick coagulates (Figure 5.16: a-c) with un-coagulated proteins and a plasma matrix forming part of the network (Figure 5.16: a), the whole network presented with an entangled and flattened appearance (Figure 5.16: b-c).

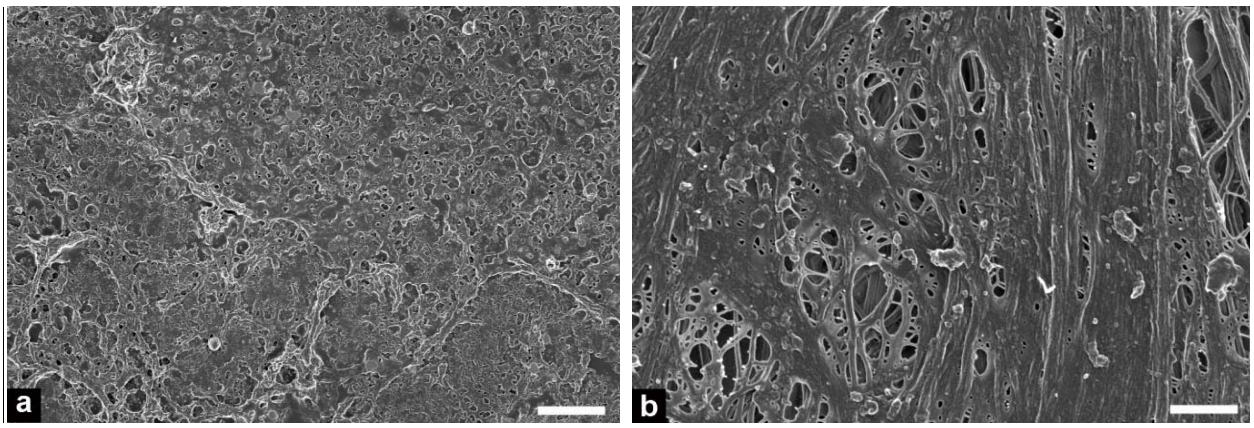


Figure 5.16: Fibrin and platelet coagulates formed by substrate application method 1. a-c: Fibrin and platelet coagulates shows thick matted coagulates, scale bars = 1µm.

Substrate Application Method 2 gave well preserved fiber and platelet networks when activated by either thrombin or $CaCl_2$ (Figure 5.17: a-d).

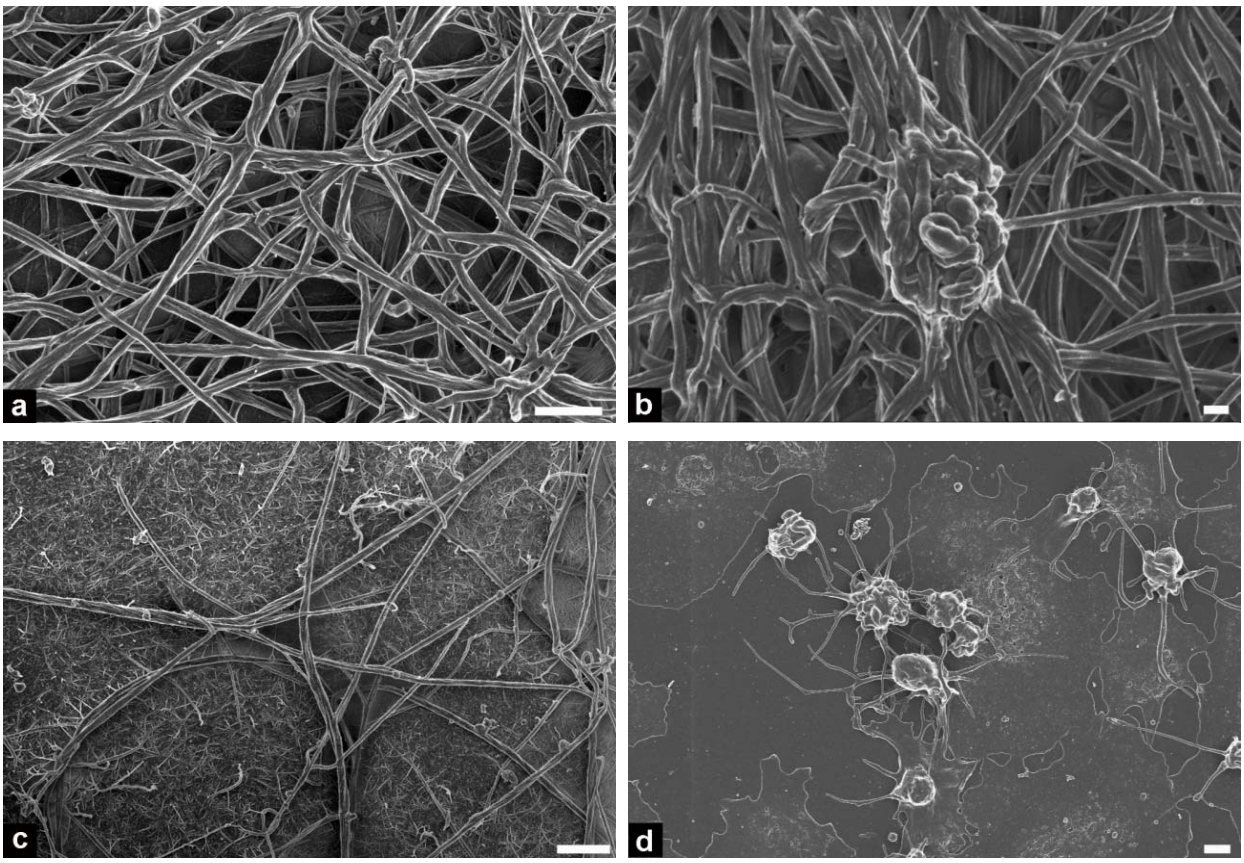


Figure 5.17: Fibrin and platelet coagulates formed by substrate application method 2.
a: Fibrin and platelet coagulates formed by substrate application method 2 and addition of thrombin, showing a well preserved fiber network, scale bar = 1µm. b: Fibrin and platelet coagulates formed by substrate application method 2 and addition of thrombin, showing a well preserved fiber network and a platelet closely associated with the fibers, scale bar = 200nm. c: Fibrin fibers formed by substrate application method 2 and addition of $CaCl_2$, showing a well preserved fiber network, scale bar = 1µm. d: Platelets formed by substrate application method 2 and addition of $CaCl_2$, showing platelets in various stages of activation, scale bar = 1µm.

Substrate application method 3 showed more consistent results with when using grids as a substrate (Figure 5.18: a & c), strangely in some specimens a thin fiber network was seen covering a thicker network (Figure 5.18 a & c).

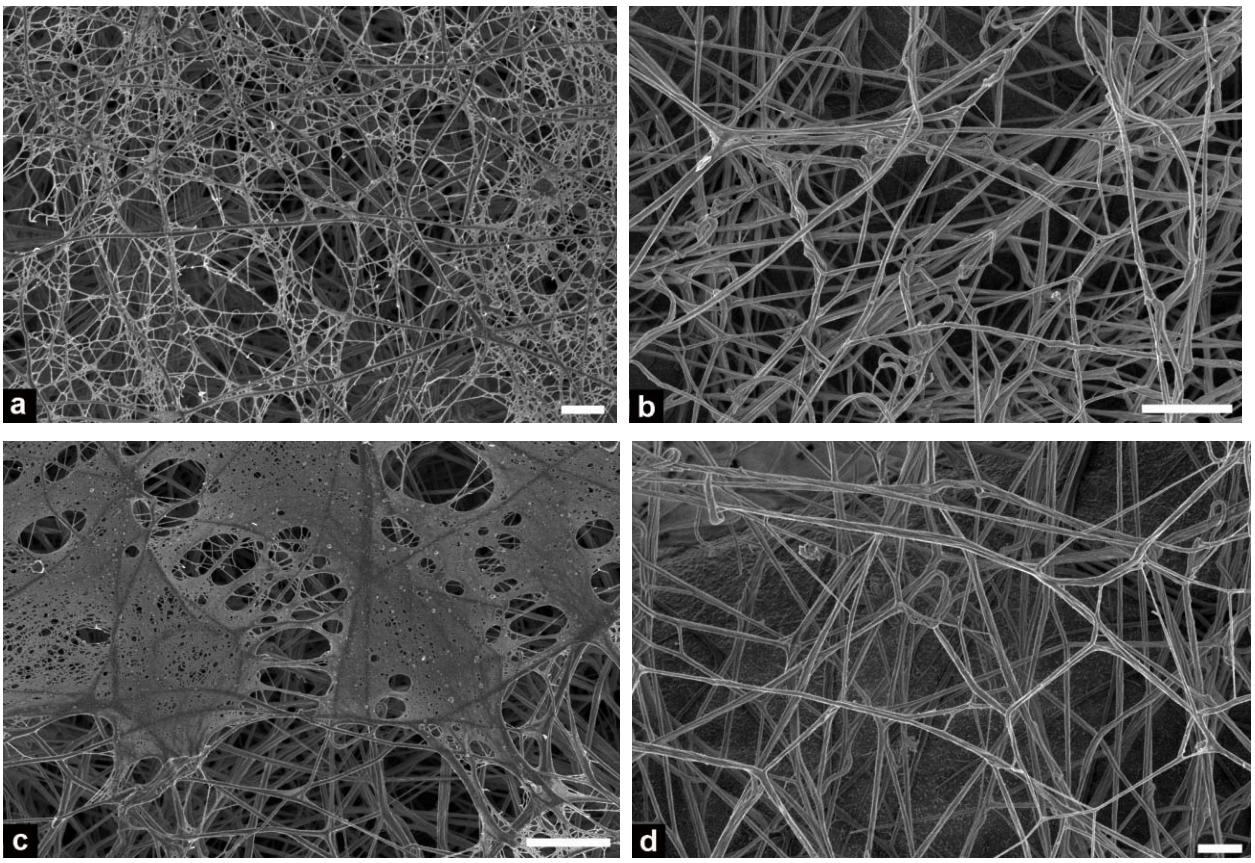


Figure 5.18: Fibrin and platelet coagulates formed by substrate application method 3.
a: Fibrin and platelet coagulates formed by substrate application method 3, showing a well preserved fiber network with fine network mesh, scale bar = 1µm. b: Fibrin fibers formed by substrate application method 3, showing a well preserved fiber network, scale bar = 2µm. c: Fibrin fibers formed by substrate application method 3, showing a well preserved fiber network with a thin fiber network covering thicker fibers, scale bar = 2µm. b: Fibrin fibers formed by substrate application method 3, showing a well preserved fiber network, scale bar = 1µm.

5.3.5. Coagulation Time

Similar to the results seen with TEM studies in chapter 3, an increase in coagulation time when initiating the reaction with thrombin showed a marked increase in fiber size and density with evident lateral fusion of fibers (Figure 5.19: a-b). Platelets appear to be in a similar to slightly higher state of activation with increased concentrations of thrombin. An

increase in the amount of fibers is seen when employing $CaCl_2$ as a coagulation agent (Figure 5.20: a-b).

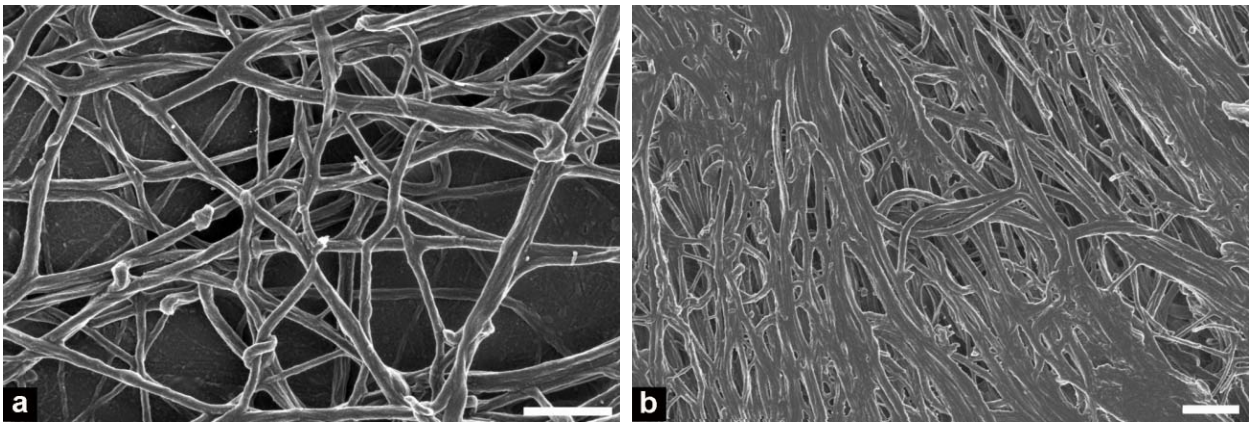


Figure 5.19: Fibrin and platelet coagulates formed by thrombin addition and left to coagulate for different time periods.
a: Fiber network, activated with thrombin and left to coagulate for 10 minutes, scale bar = 1µm. b: Fiber network, activated with thrombin and left to coagulate for 30 minutes, with an increased fiber density and lateral fusion of fibers, scale bar = 1µm

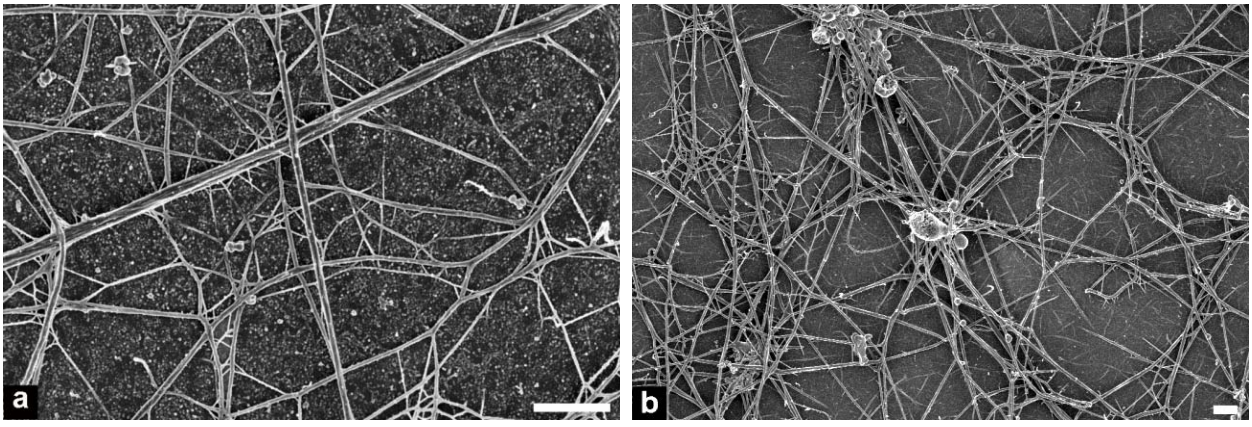


Figure 5.20: Fibrin and platelet coagulates formed by CaCl2 addition and left to coagulate for different time periods.
a: Fiber network, activated with CaCl2 and left to coagulate for 10 minutes, scale bar = 1µm. b: Fiber network, activated with CaCl2 and left to coagulate for 30 minutes, with an

increased fiber density and amount, scale bar = 2µm.

5.3.6. Coagulation Temperature

When coagulation is initiated using $CaCl_2$ and allowed to gelate at 37°C, clot formation occurs at a slightly more rapid rate than that of coagulation at room temperature, resulting in an increase of thicker fibers, fiber density and amount. Other than that, little difference was seen between the two temperatures. Coagulation initiated by thrombin showed an increase in the lateral fusion of fibers between specimens coagulated at room temperature and 37°C (Figure 5.21: a-b).

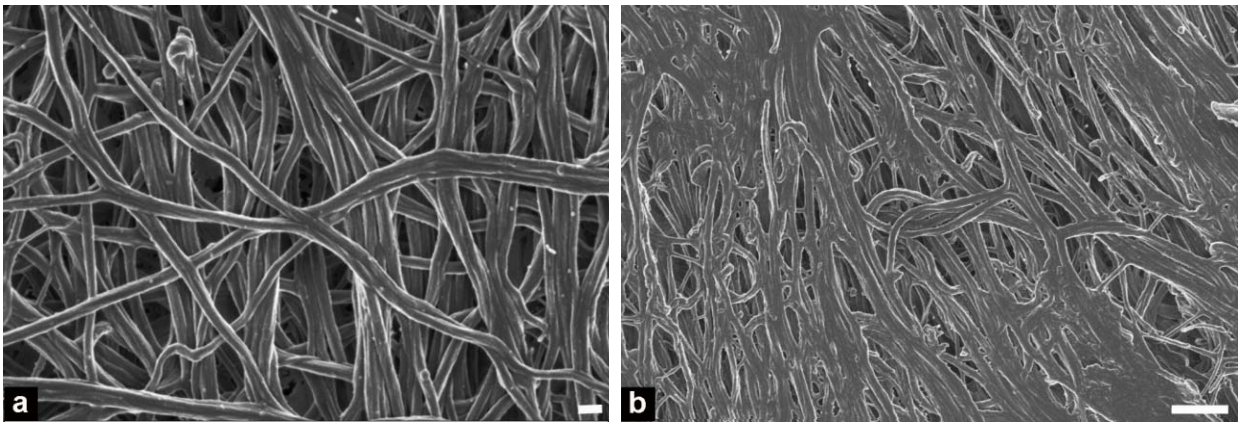


Figure 5.21: Fibrin and platelet coagulates formed by thrombin addition and left to coagulate at different temperatures.
a: Fiber network, activated with thrombin and left to coagulate for 30 minutes at room temperature, scale bar = 200nm. b: Fiber network, activated with thrombin and left to coagulate for 30 minutes at 37°C, with an increased fiber density and lateral fusion of fibers, scale bar = 1µm.

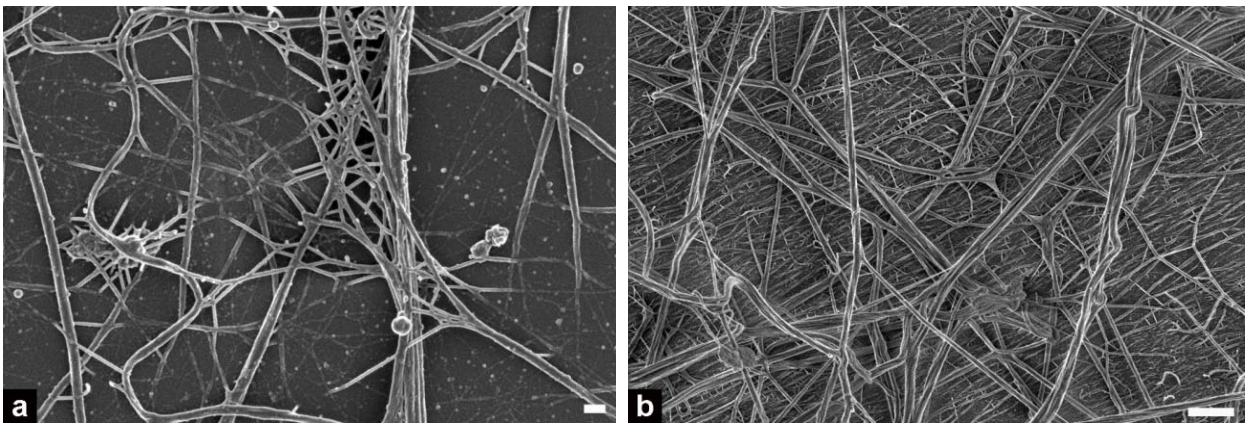


Figure 5.22: Fibrin and platelet coagulates formed by $CaCl_2$ addition and left to coagulate at different temperatures.
a: Fiber network, activated with CaCl2 and left to coagulate at room temperature, scale bar = 200nm. b: Fiber network, activated with CaCl2 and left to coagulate at 37°C, with an increased fiber density and amount, scale bar = 1µm.

5.3.7. Freezing Technique

Both techniques resulted in well preserved fibrin fiber and platelet networks. Regrettably good preservation was not constant, several specimens showed inadequate vitrification or damage from freeze drying.

Plunge freezing with grids obtained more invariable well preserved fibrin fiber and platelet (Figure 5.23: e) networks, although thick networks coagulates with a matted appearance was also seen (Figure 5.23: c) and fibrin networks occasionally presented with uncharacteristic thick regions (Figure 5.23: b). Plunge freezing with glass coverslips predominantly presented with thick networks coagulates with a matted appearance (Figure 5.24: a-b), with occasional networks with a more standard appearance being seen (Figure 5.24: c).

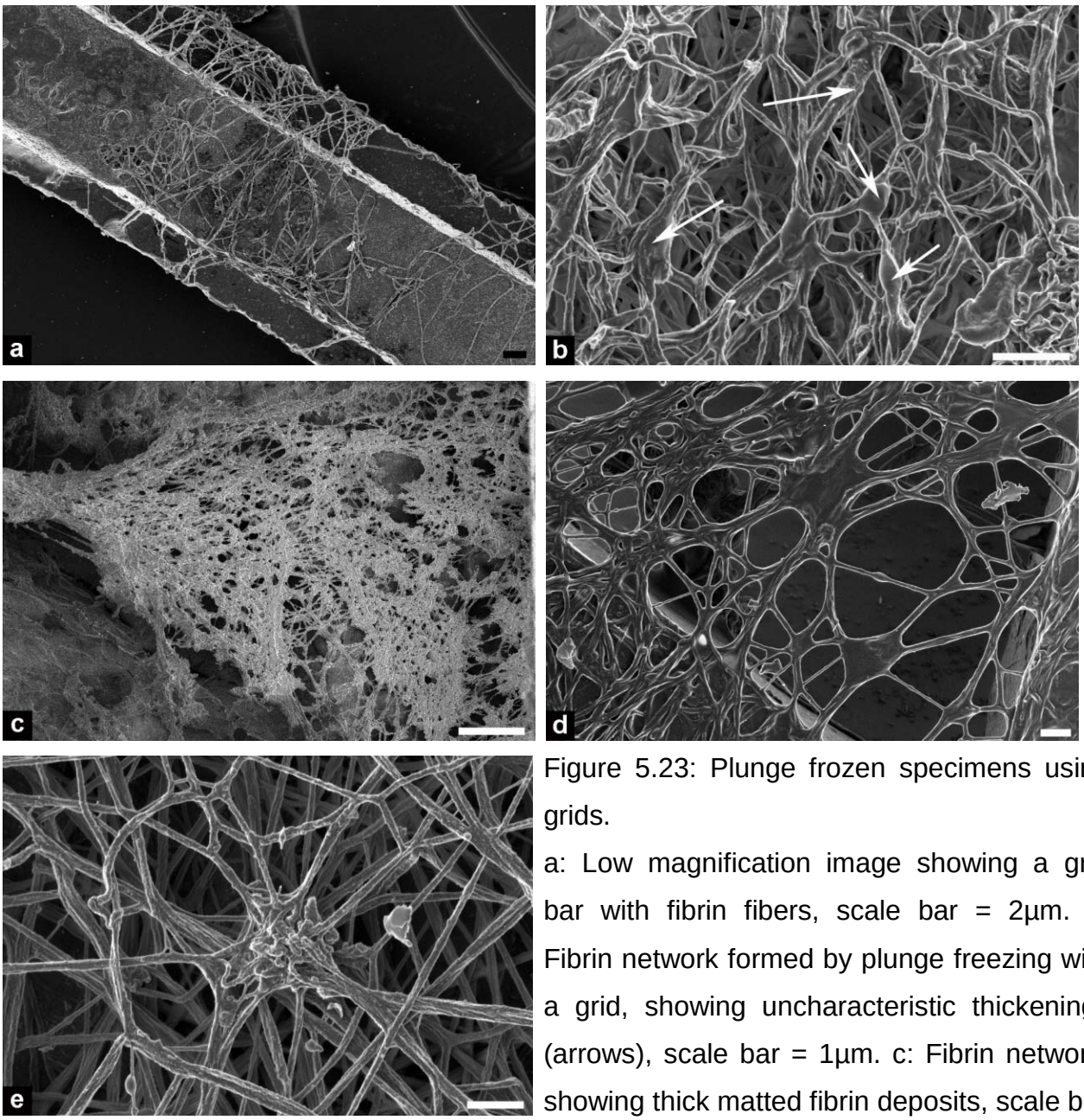


Figure 5.23: Plunge frozen specimens using grids.

a: Low magnification image showing a grid bar with fibrin fibers, scale bar = 2µm. b: Fibrin network formed by plunge freezing with a grid, showing uncharacteristic thickenings (arrows), scale bar = 1µm. c: Fibrin network, showing thick matted fibrin deposits, scale bar = 10µm. d: Network, showing a platelet and fibrin fiber coagulate, scale bar = 1µm. e: Fibrin network with a platelet associated with the fibers, scale bar = 1µm.

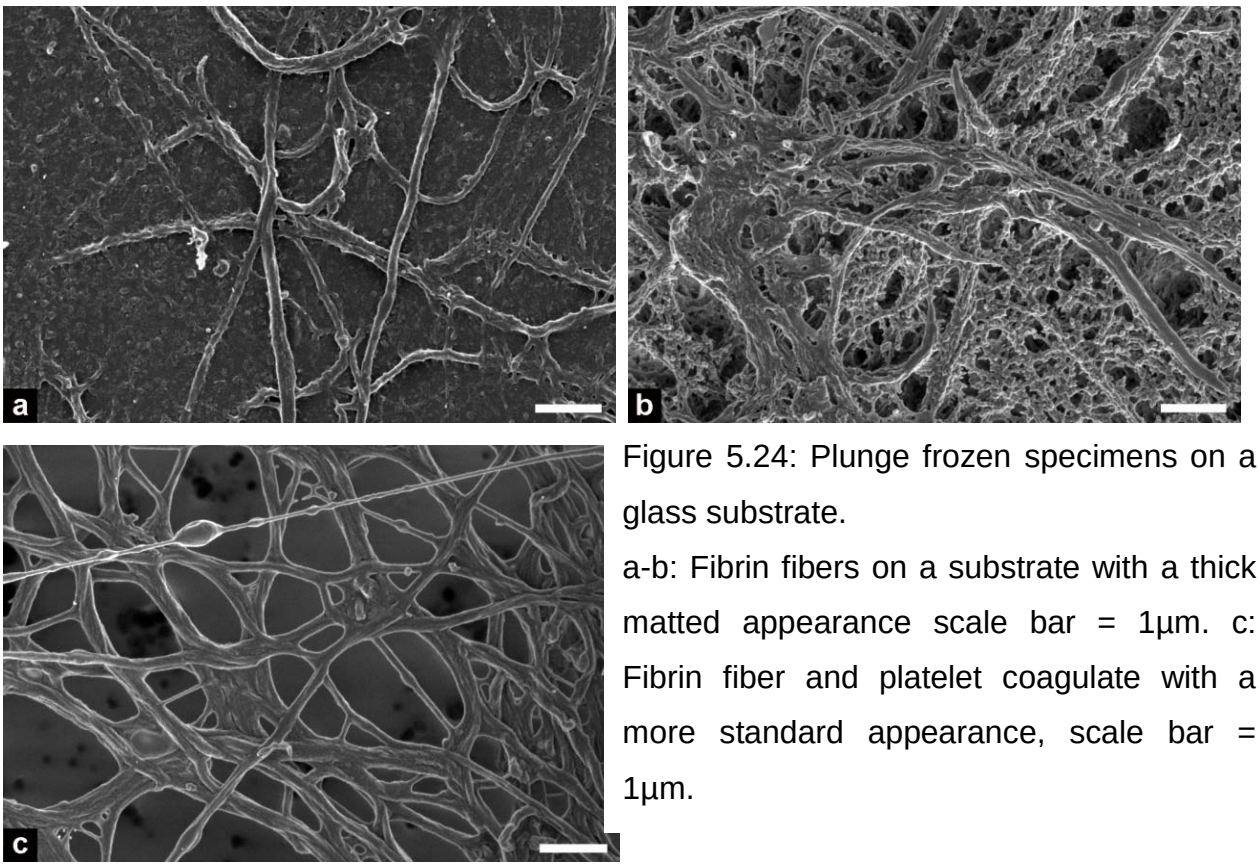


Figure 5.24: Plunge frozen specimens on a glass substrate.
a-b: Fibrin fibers on a substrate with a thick matted appearance scale bar = 1µm. c: Fibrin fiber and platelet coagulate with a more standard appearance, scale bar = 1µm.

Fibrin fiber and platelet networks preserved by HPF showed well preserved specimens (Figures 5.25: a, 5.10: a-c and 5.11: a-c) though occasionally freeze damage (Figures 5.25: b), specimens with a squashed and flattened appearance (Figures 5.25: c), mostly empty substrates with isolated platelets with an abnormal appearance (Figures 5.25: d) and specimens that appear dirty, most probably from inadequate washing (Figures 5.25: e) were seen.

The use of grids for HPF gave predominantly poor results, with specimens that appeared poorly washed with contaminants on the fibers and platelets (Figure 5.26: a & b). Also, crystal formation was seen. This happened predominantly when $CaCl_2$ was used as a coagulation agent. It is therefore likely that the crystals are calcium chloride or calcium phosphate crystals as indicated by the arrows in Figure 5.26: a & b.

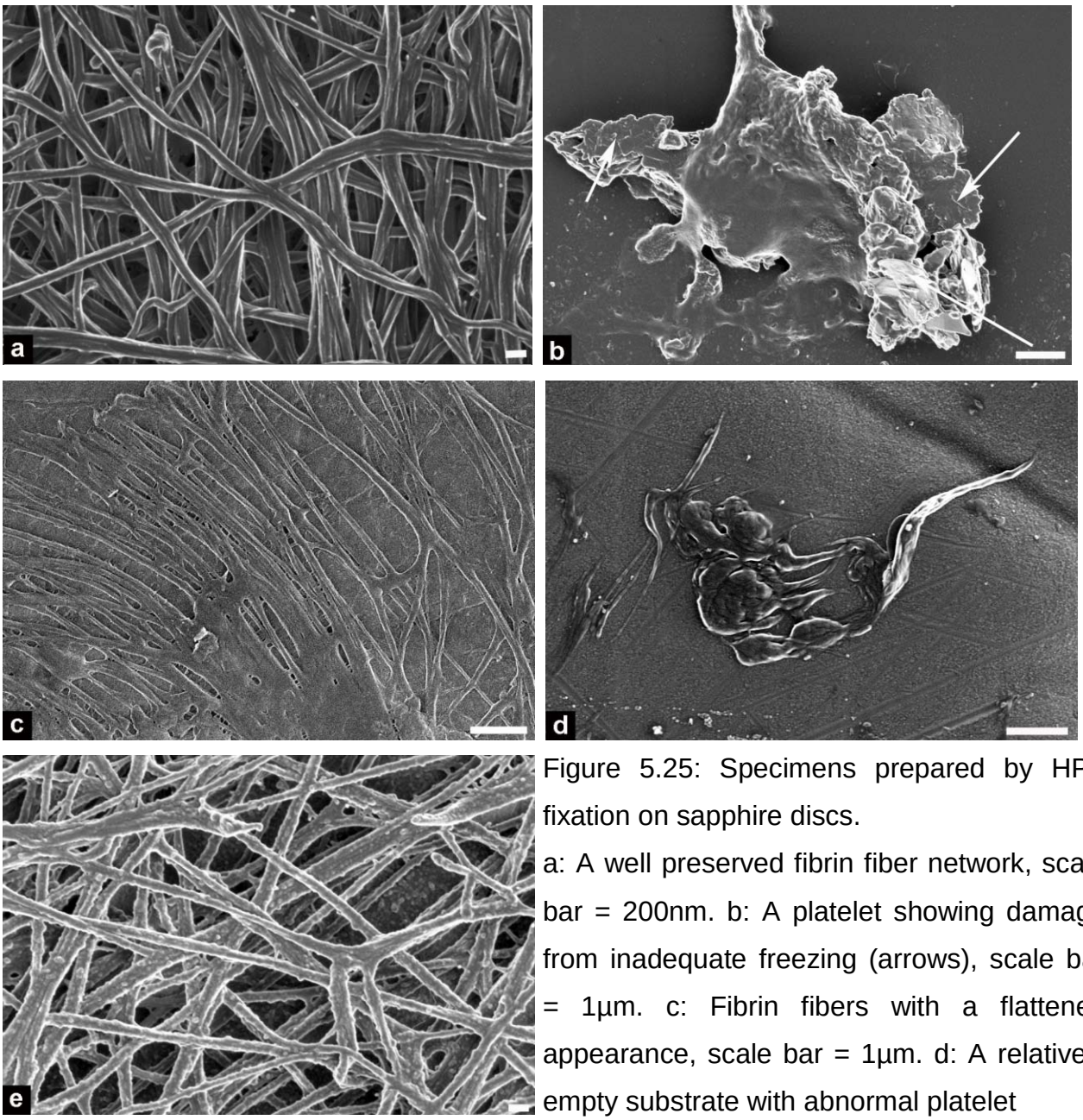


Figure 5.25: Specimens prepared by HPF fixation on sapphire discs.
a: A well preserved fibrin fiber network, scale bar = 200nm. b: A platelet showing damage from inadequate freezing (arrows), scale bar = 1µm. c: Fibrin fibers with a flattened appearance, scale bar = 1µm. d: A relatively empty substrate with abnormal platelet coagulates, scale bar = 1µm. e: Fibrin fiber network with a “contaminated” appearance, scale bar = 200nm

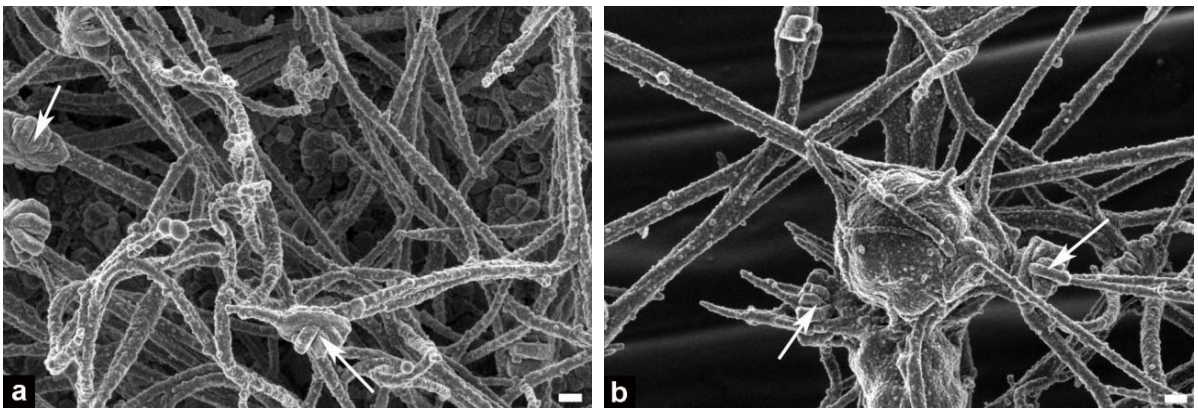


Figure 5.26: Specimens prepared by HPF fixation using a grid.
a-b: Fibrin fiber and platelet network that appears “contaminated”, with crystal formation (arrows), scale bar = 200nm.

5.4. Discussion

Variations in factors of the fibrin fiber and platelet formation and subsequent preparation steps for SEM, imparted big variations in the results. Unfortunately, occasional differences were seen between specimens prepared with the same method as well.

When comparing the differences between thrombin and $CaCl_2$ coagulation, it is obvious that fibrin fiber formation is significantly higher with thrombin coagulation. This is similar to what is seen in chapter 4 (see chapter 4, section 4.4.1, and pages 125-126 for a detailed discussion). An increase in thrombin and $CaCl_2$ concentration both resulted in an increased density and thickness of the fibrin fiber and platelet (only thrombin) network. This result concurs with literature that found that the ultimate clot structure is influenced by the relative concentration of the factors involved in coagulation (Standeven *et al.,* 2005). Increasing the coagulation time or temperature resulted in the expected changes in fibrin fiber and platelet network as it is well known that coagulation is a time dependant process and as any other biological function, physiological temperature provides the optimum environment for the coagulation reaction.

The failure of Millipore membranes and especially Aclar as substrates was largely due to the substrates tendency to deform and the failure of complete freezing. Most probably due to small cutting / punching defects which hindered the sealing of the HPF specimen carrier and the bayonet pod. Both these problems could most probably be rectified by more careful cutting and handling of the substrate, but as sapphire discs and grids did not present with these difficulties it was decided to discard Millipore membranes and Aclar as potential freezing substrates. Differences observed in results between the methods of application to the substrate demonstrated tendencies but were still highly variable. Method one resulted in thick coagulates, making discrimination of the fibers and platelets difficult. Method two gave the most promising and unvarying results of well-developed and preserved fibrin networks and platelets. Application of the fibrin and platelets by method three, showed well preserved fiber networks, mostly when using a grid as a substrate, however unanticipated results such as the absence of blood platelets and a network of thin fibers covering the thicker fiber network was frequently found.

Variations in the quality of preservation when using plunge freezing as a fixation method is believed to be mainly due to human deviation and error, relating to plunging speed and sample handling. More consistent results are possible if the use of an automatic plunging apparatus would have been employed. The occasional freeze damage and poor results seen when using HPF is believed to be mostly attributed to specimen application and subsequent handling as these small consumables are very delicate and difficult to manage.

5.5. Conclusion

Based on the experimental results, two methods for SEM ultrastructural studies of platelets and fibrin networks, utilizing HPF and freeze drying were selected, based on their repeatability and the quality of the results.

The first method is:
Coagulation of PRP initiated by thrombin at a ratio of 1:1, by mixing the two components on a sapphire disc and spreading the mixture over the substrate and substrate edges in an effort to create a thin network, followed by ten minutes coagulation time at 37°C, a washing step, HPF and ultimately freeze drying to remove the vitrified water.

The second method is:
Coagulation of PRP initiated by $CaCl_2$ at a ratio of 10:1, by mixing the two components on a sapphire disc and spreading the mixture over the substrate and substrate edges in an effort to create a thin network, followed by 30 minutes coagulation time at 37°C, a washing step, HPF and ultimately freeze drying to remove the vitrified water.

In conclusion, we believe that HPF combined with freeze drying is a high-quality preservation method for the selected specimen model, and may be useful in ultrastructural research.

Chapter 6:

Comparison of chemical and HPF preparation methods to study human fibrin fibres and platelets using SEM

Research Question 4:

Which method of sample preparation is superior for SEM ultrastructural studies, rapidly vitrifying platelets and fibrin networks utilizing freeze fixation and freeze drying or the well-established chemical preparation method, and what is the difference between the results obtained from these two methods?

6.1. Introduction

The scanning electron microscope first became commercially available in the 1960's, and has since then been widely used as a tool in biological research, providing seemingly three-dimensional images of the sample surface. With recent technological advances the study of exceedingly small specimens such as biological macromolecules has become possible, rivalling TEM resolution and use in biological applications.

Similarly to the TEM, the SEM use a beam of electrons to image a specimen, the electrons are produced at the top of the microscope by an electron gun, and follow a vertical path down the column of the microscope, the beam is then directed and focused on the sample surface by a series of electromagnetic lenses. Electrons are transmitted through a sample in a TEM, however the electrons in a SEM are scanned in a raster pattern over the surface of the specimen by a pair of scanning coils. The electrons interact with atoms at or near the surface of the specimen, generating a variety of signals; including secondary electrons, back-scattered electrons, characteristic x-rays, cathode-luminescence, specimen current and transmitted electrons. In biological research secondary electrons are mostly employed

to view the surface topography and morphology of a specimen, this signal is obtained by low energy electrons that are ejected from the k-orbital's of atoms by inelastic scattering interactions of the electron beam. Due to the low energy of these secondary electrons, only electrons originating within a few nano-meters of the sample surface are able to escape and be detected, resulting in a very high resolution image of the sample surface. The high resolution, coupled with the depth of field which yields a three-dimensional appearance of the surface, is very useful in understanding the fine surface structures of biological materials.

SEM has been widely used in the ultrastructural study of fibrin networks and platelets in health and disease, which are two factors of utmost importance in blood coagulation and haemostasis. Increasing evidence shows that the coagulation clot ultrastructure varies significantly between individuals due to genetics, environmental factors and disease. This has an effect on the stability of the coagulation clot and its susceptibility to lyses, which in turn is associated with thrombotic or bleeding tendencies (Cilia la Corte *et al.,* 2011 and Pretorius 2008). In studies by Pretorius *et al.,* (2006; 2009b; 2009c; 2010 and 2011) abnormal fibrin fiber network and platelet ultrastructure was observed in several conditions such as pregnancy and smoking, and also diseases such as dysfibrinogenemias, diabetes and HIV. Abnormal platelet or fibrin network ultrastructure has also been shown in anaemia (Doll 1982), cardiovascular disease (Ryan *et al.,* 1999 and Standeven *et al.,* 2005), asthma (Pretorius *et al.,* 2007) and certain cancers (Bambace & Holmes 2011 and Pretorius *et al.,* 2009a). These types of studies have proven to be valuable in augmentation of the knowledge of disease patterns and may ultimately assist in enhancing treatment regimes of these illnesses.

The main disadvantage for using electron microscopes such as the SEM in biological research is the fact that the microscope operates in a high vacuum environment, which means that specimens need to be completely free of any volatile substances such as water. This necessitates several steps including fixation, to preserve and stabilize the structure and drying to remove the water from the biological tissue, before viewing. The two most generally used methods to achieve the afore mentioned is chemical fixation

coupled with either critical point drying or HMDS drying and freeze fixation coupled with freeze drying (see chapter 4, pages 101-104 for a detailed discussion).

For biological research it is optimal to view a specimen as close to its natural state as possible, to be certain that any and all observations are purely due to the structure and function of the specimen itself and not to the introduction of artefacts by the processing of the specimen. Unfortunately all of the necessary stabilization and drying methods are bound to impart certain changes in the specimen; therefore optimization and comparison of these methods are key in understanding the effect of the processing and ultimately interpretation of the micrographs.

Therefore, in the current chapter two techniques developed in this lab for ultrastructural preservation of platelets and fibrin networks are compared. Freeze fixation by HPF coupled with freeze drying, and two chemical fixation methods, comparable in the parameters, apart from the fixation and drying steps. These methods are then compared with relation to their quality of preservation, financial cost, time, repeatability, expertise required and their safety of use.

6.2. Materials and Methods

6.2.1. Samples

Healthy individuals, without any known medical condition, who are not smokers and in the case of women, were not on contraception, were chosen for this study. Whole blood obtained by venipuncture was collected in citrate tubes (for the inhibition of calcium-mediated coagulation by the addition of sodium citrate (Sabbatani 1901a and Sabbatani 1901b)), followed by 2 minutes of centrifugation to separate the plasma, containing the platelets and fibrinogen from the erythrocytes and leukocytes. All experiments were conducted with freshly prepared PRP, within two hours of the blood being drawn.

6.2.2. Sample Preparation

Table 6.1: Summary of fixation techniques employed.

#	Freezing Technique	Substrate	Application Method	Coagulation agent and PRP ratio	Coagulation Time	Coagulation Temperature
1	HPF	Sapphire	2	Thrombin 1:1	10	37°C
2	Chemical	Sapphire	2	Thrombin 1:1	10	37°C
3	HPF	Sapphire	2	$CaCl_2$	10	37°C
4	Chemical	Sapphire	2	$CaCl_2$	10	37°C

6.2.2.1. Technique 1

The supernatant liquid (PRP) was separated from the pellet (erythrocytes and leukocytes) by pipetting the liquid off into a polyethylene holder (eppendorf tube). The PRP was mixed with thrombin at a one to one ratio on the sapphire disc and spread over the substrate and substrate edges in an effort to create a thin network (see figure 5.5, page 139). The substrate and mixture was then left for a 10 minutes at 37°C in a humid environment for coagulation to occur. This was followed by a washing step in (0.075M) sodium phosphate buffer (pH 7.4), using a micro-plate shaker to remove plasma proteins trapped in the network. The sapphire disc with the platelet and fibrin coagulation was then transferred to a specimen carrier, which was pre-fitted into the transfer slide, and prefilled with 1-Hexadene (cryoprotectant). The specimen carrier containing the specimen was loaded onto the loading device, and securely screwed into the freezing bayonet pod with the help of a specialized torque wrench; this was then mounted onto the bayonet-loading device, placed into the high-pressure freezing platform and subsequently high pressure frozen (EMPACT2. Leica Microsystems, Vienna, Austria), after which the specimen was automatically brought into a liquid nitrogen bath (see Figure 3.3, Chapter 3 page 76), and

subsequently kept under liquid nitrogen conditions until such a time that freeze drying was done. The specimens were then freeze dried under high vacuum while gradually heating the stage from liquid nitrogen temperatures to room temperature, over a period of 72 hours. The dry specimens were mounted on aluminium stubs, coated by carbon evaporation and subsequently viewed with a Zeiss Ultra, Field Emission Scanning Electron Microscope (Zeiss, Germany).

4.2.2.2. Technique 2

The supernatant liquid (PRP) was separated from the pellet (erythrocytes and leukocytes) by pipetting the liquid off into a polyethylene holder (eppendorf tube). The PRP was mixed with thrombin at a one to one ratio on the sapphire disc and spread over the substrate and substrate edges in an effort to create a thin network (see figure 5.5, page 139). The substrate and mixture was then left for a 10 minutes at 37°C in a humid environment for coagulation to occur. This was followed by a washing step in (0.075M) sodium phosphate buffer (pH 7.4), using a micro-plate shaker to remove plasma proteins trapped in the network. The washed specimen was transferred to a glass vial containing a 2.5 % glutaraldehyde-formaldehyde fixative and left for one hour. After which the sample was rinsed three times with (0.075M) sodium potassium phosphate buffer (pH 5 7.4), for 15 minutes before being placed in secondary fixative, 1% osmium tetroxide solution for one hour. Samples were then dehydrated with a series of ethanol, 30%, 50%, 70%, 90%, and three changes of 100% and dried using critical point drying. . The dry specimens were mounted on aluminium stubs, coated by carbon evaporation and subsequently viewed with a Zeiss Ultra, Field Emission Scanning Electron Microscope (Zeiss, Germany).

4.2.2.3. Technique 3

The supernatant liquid (PRP) was separated from the pellet (erythrocytes and leukocytes) by pipetting the liquid off into a polyethylene holder (eppendorf tube). The PRP was mixed with $CaCl_2$ at a 10:1 ratio on the sapphire disc and spread over the substrate and substrate edges in an effort to create a thin network (see figure 5.5, page 139). The substrate and mixture was then left for a 10 minutes at 37°C in a humid environment for

coagulation to occur. This was followed by a washing step in (0.075M) sodium phosphate buffer (pH 7.4), using a micro-plate shaker to remove plasma proteins trapped in the network. The sapphire disc with the platelet and fibrin coagulation was then transferred to a specimen carrier, which was pre-fitted into the transfer slide, and prefilled with 1-Hexadene (cryoprotectant). The specimen carrier containing the specimen was loaded onto the loading device, and securely screwed into the freezing bayonet pod with the help of a specialized torque wrench; this was then mounted onto the bayonet-loading device, placed into the high-pressure freezing platform and subsequently high pressure frozen (EMPACT2. Leica Microsystems, Vienna, Austria), after which the specimen was automatically brought into a liquid nitrogen bath (see Figure 3.3, Chapter 3 page 76), and subsequently kept under liquid nitrogen conditions until such a time that freeze drying was done. The specimens were then freeze dried under high vacuum while gradually heating the stage from liquid nitrogen temperatures to room temperature, over the period of 72 hours. The dry specimens were mounted on aluminium stubs, coated by carbon evaporation and subsequently viewed with a Zeiss Ultra, Field Emission Scanning Electron Microscope (Zeiss, Germany).

4.2.2.4. Technique 4

The supernatant liquid (PRP) was separated from the pellet (erythrocytes and leukocytes) by pipetting the liquid off into a polyethylene holder (eppendorf tube). The PRP was mixed with $CaCl_2$ at a 10:1 ratio on the sapphire disc and spread over the substrate and substrate edges in an effort to create a thin network (see figure 5.5, page 139). The substrate and mixture was then left for a 10 minutes at 37°C in a humid environment for coagulation to occur. This was followed by a washing step in (0.075M) sodium phosphate buffer (pH 7.4), using a micro-plate shaker to remove plasma proteins trapped in the network. The washed specimen was transferred to a glass vial containing a 2.5 % glutaraldehyde-formaldehyde fixative and left for one hour. After which the sample was rinsed three times with (0.075M) sodium phosphate buffer (pH 7.4), for 15 minutes before being placed in secondary fixative, 1% osmium tetroxide solution for one hour. Samples were then dehydrated with a series of ethanol, 30%, 50%, 70%, 90%, and three changes

of 100% and dried using critical point drying. . The dry specimens were mounted on aluminium stubs, coated by carbon evaporation and subsequently viewed with a Zeiss Ultra, Field Emission Scanning Electron Microscope (Zeiss, Germany).

6.3. Results

6.3.1. Technique 1

Fibrin and platelet networks prepared by technique 1 shows the fibrin fiber networks made up from thick, intermediate and thin fibers (Figure 6.1: a-d). Activated platelets are seen to be highly associated with fibrin fibers (Figure 6.1: e).

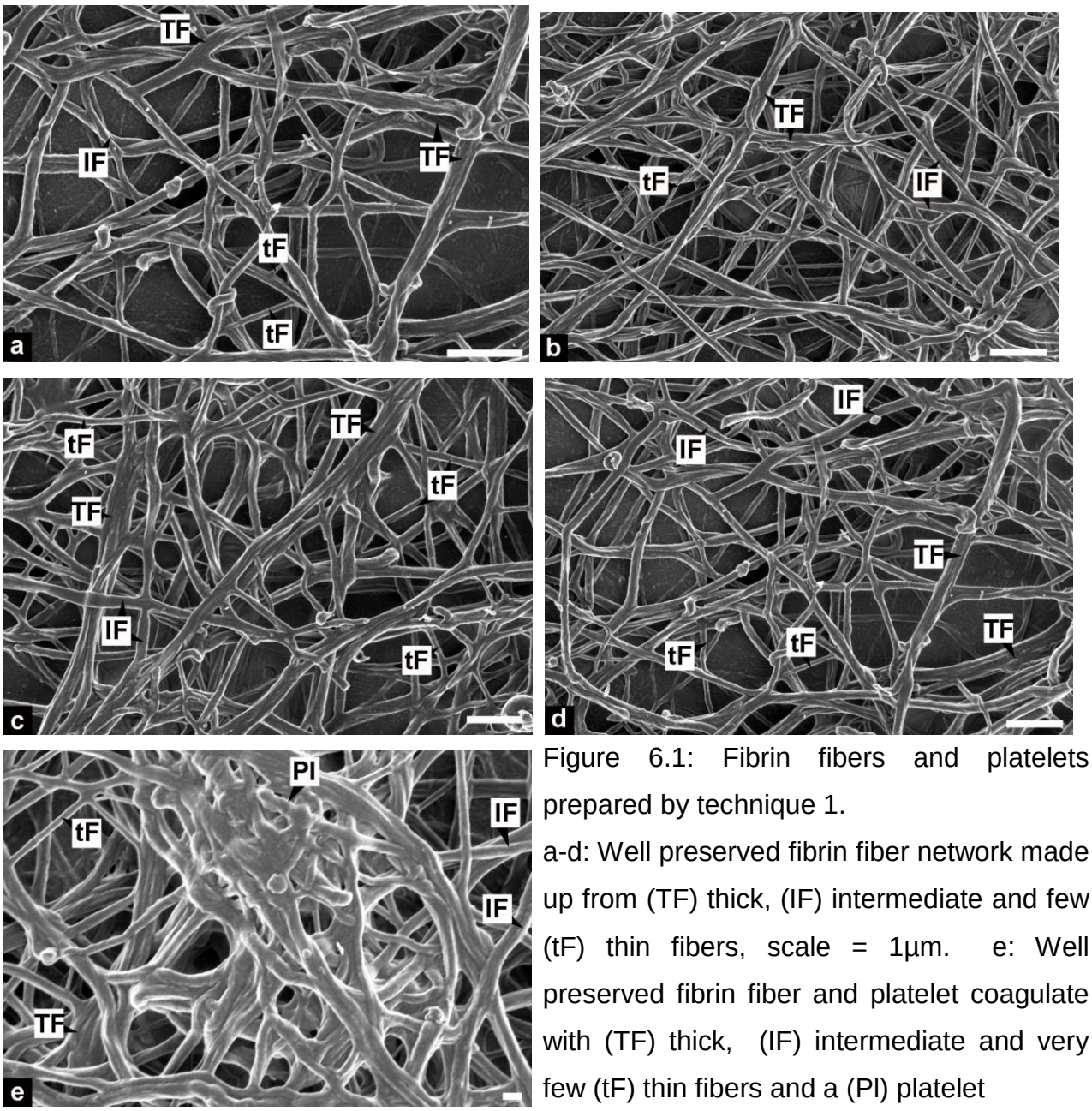


Figure 6.1: Fibrin fibers and platelets prepared by technique 1.
a-d: Well preserved fibrin fiber network made up from (TF) thick, (IF) intermediate and few (tF) thin fibers, scale = 1µm. e: Well preserved fibrin fiber and platelet coagulate with (TF) thick, (IF) intermediate and very few (tF) thin fibers and a (Pl) platelet coagulate strongly associated with the fiber network, scale bar = 200nm.

6.3.2. Technique 2

Fibrin and platelet networks prepared by technique 2 shows the fibrin fiber networks made up from thick, intermediate and thin fibers (Figure 6.2: a-d), activated platelets are seen to be highly associated with fibrin fiber network (Figure 6.2: e).

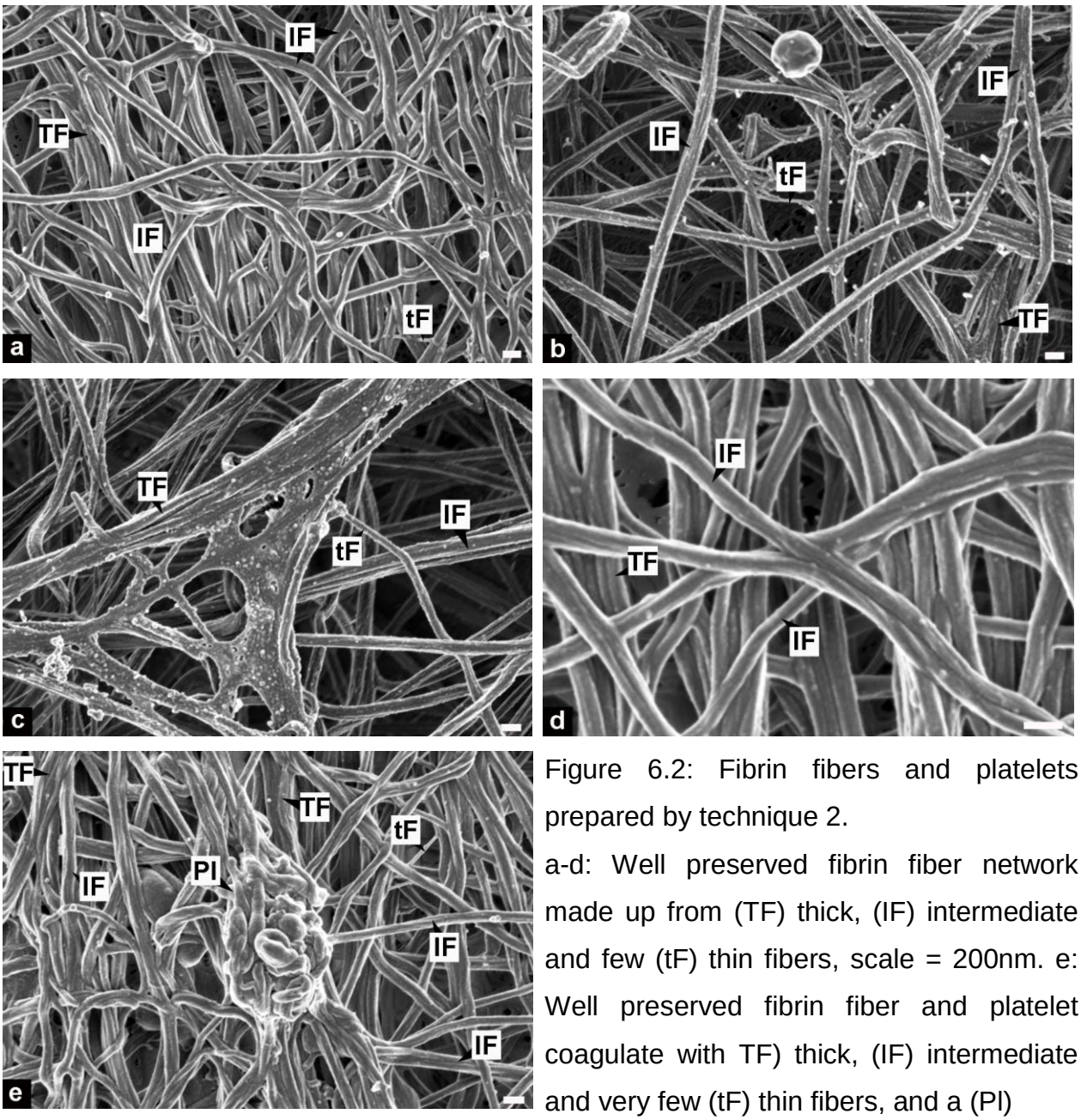


Figure 6.2: Fibrin fibers and platelets prepared by technique 2.
a-d: Well preserved fibrin fiber network made up from (TF) thick, (IF) intermediate and few (tF) thin fibers, scale = 200nm. e: Well preserved fibrin fiber and platelet coagulate with TF) thick, (IF) intermediate and very few (tF) thin fibers, and a (Pl) platelet associated with the fiber network, scale bar = 200nm.

6.3.3. Technique 3

Fibrin and platelet networks prepared by technique 3 shows the fibrin fiber networks made up from mostly thin and intermediate fibers with few thick fibers (Figure 6: a-b, e), activated

platelets with pseudopodia formation, spreading, open canalicular systems and granule secretion are seen. These platelets are often associated with fibrin fibers (Figure 6.4: c-d).

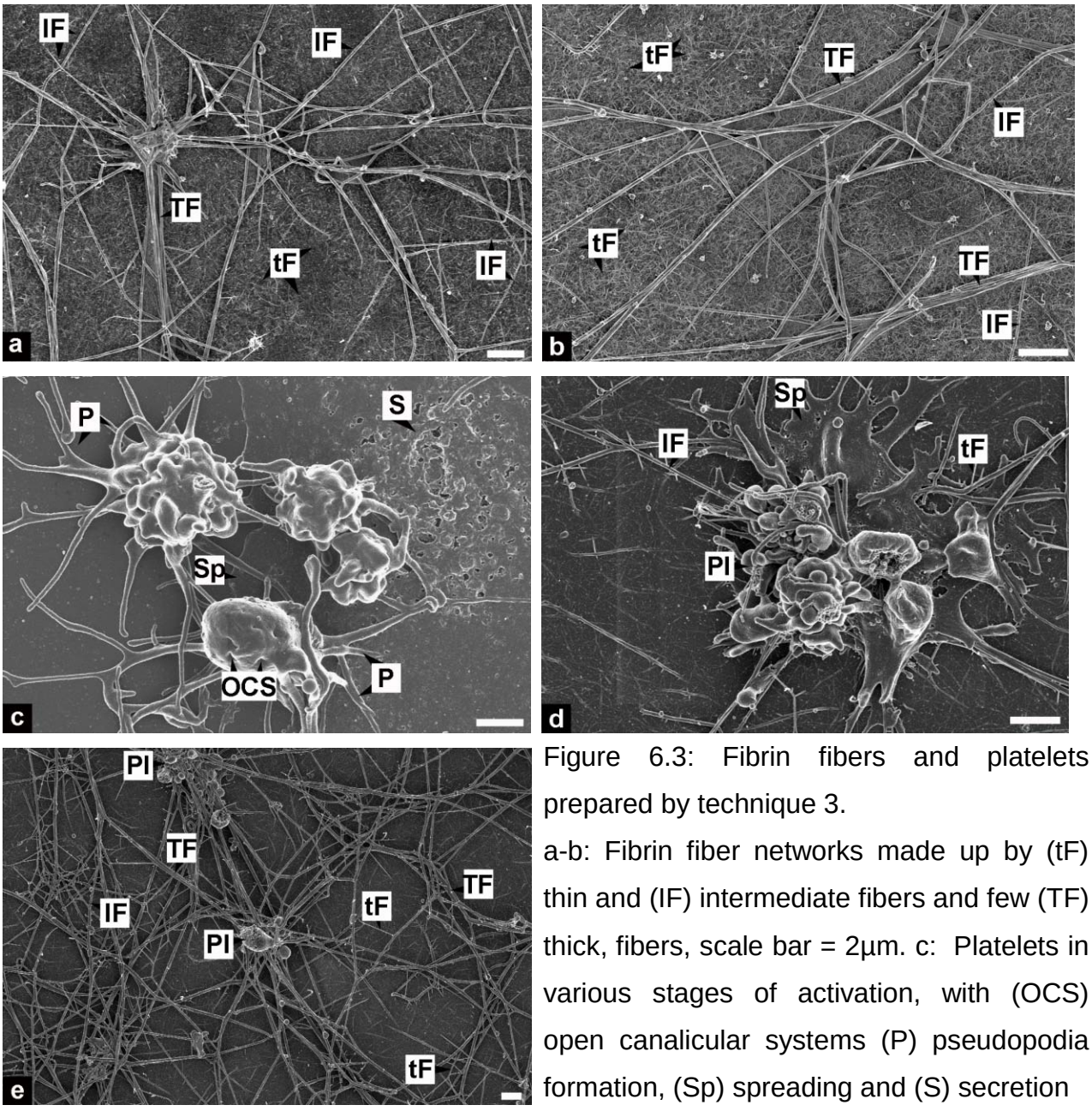


Figure 6.3: Fibrin fibers and platelets prepared by technique 3.
a-b: Fibrin fiber networks made up by (tF) thin and (IF) intermediate fibers and few (TF) thick, fibers, scale bar = 2µm. c: Platelets in various stages of activation, with (OCS) open canalicular systems (P) pseudopodia formation, (Sp) spreading and (S) secretion with subsequent breakdown evident, scale bar = 2 µm. d: Activated (Pl) platelet with skeletal reorganization, pseudopodia formation and (Sp) spreading, association with (tF) thin and (IF) intermediate fibers, scale = 1µm. e: Activated (Pl) platelets associating with the fiber network of (tF) thin, (IF) intermediate and (TF) thick fibers, scale bar = 1µm.

6.3.4. Technique 4

Fibrin and platelet networks prepared by technique 4 shows the fibrin fiber networks made up from mostly thin and intermediate fibers with few thick fibers (Figure 6.4: a-b), activated platelets with pseudopodia formation, spreading, open canalicular systems and granule secretion, are seen, these platelets are often associated with fibrin fibers (Figure 6.4: c-e).

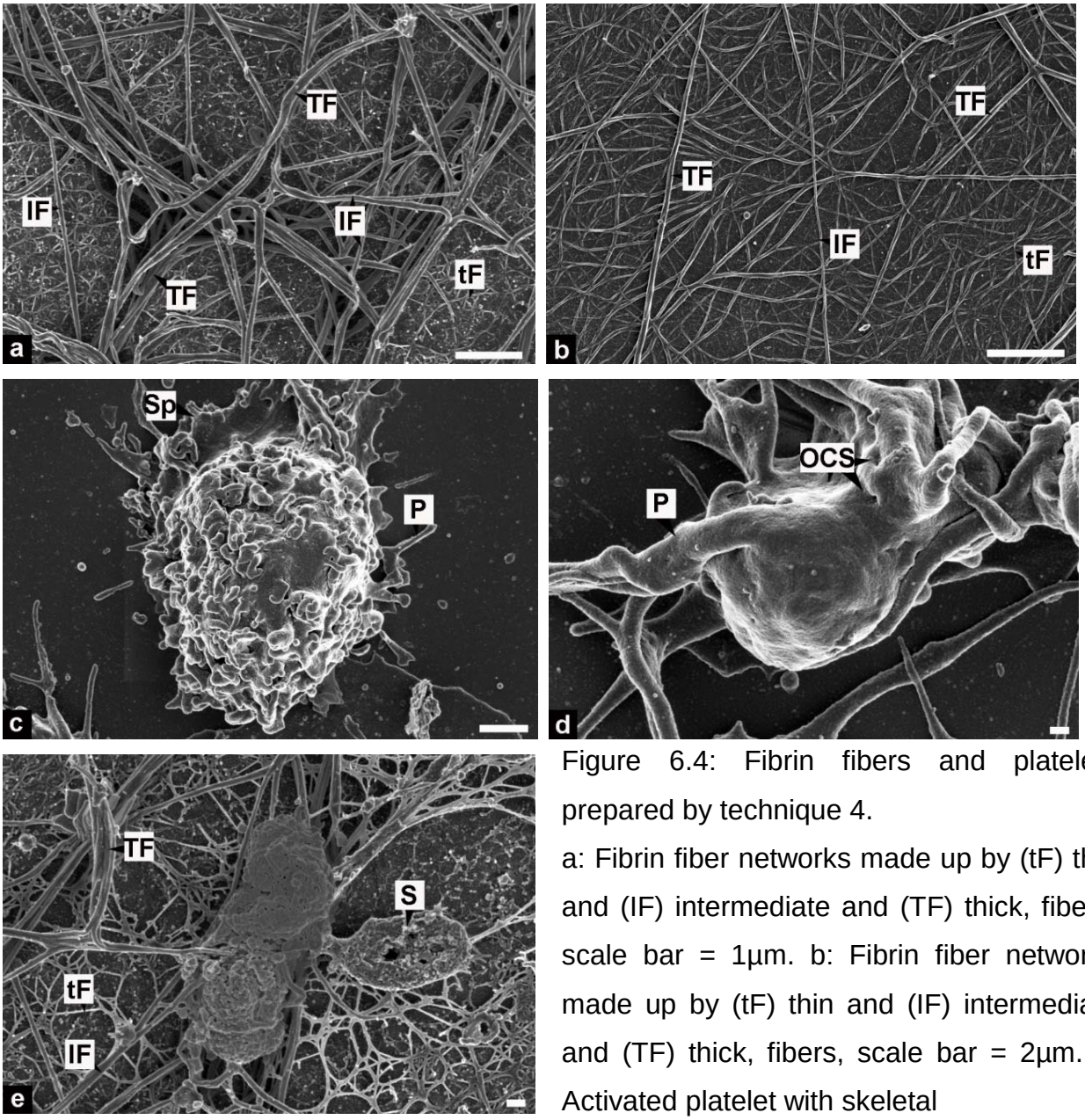


Figure 6.4: Fibrin fibers and platelets prepared by technique 4.
a: Fibrin fiber networks made up by (tF) thin and (IF) intermediate and (TF) thick, fibers, scale bar = 1µm. b: Fibrin fiber networks made up by (tF) thin and (IF) intermediate and (TF) thick, fibers, scale bar = 2µm. c: Activated platelet with skeletal reorganization, (P) pseudopodia formation and (Sp) spreading, scale bar = 1µm. d:

Activated platelet with skeletal reorganization, (P) pseudopodia formation and (OCS) open canalicular system pores visible, scale bar = 200nm. e: Activated platelet with skeletal reorganization and (S) secretion visible, associating with (tF) thin and (IF) intermediate and (TF) fibers, scale bar = 200nm.

6.4. Discussion

6.4.1. Thrombin and Calcium Chloride

When comparing thrombin and $CaCl_2$ coagulation, it is obvious that fibrin fiber formation is significantly higher with thrombin coagulation. This is similar to what is seen in chapter 4 (see chapter 4, section 4.4.1, pages 125-126 for a detailed discussion).

6.4.2. High Pressure Freezing and Chemical Fixation

Both fixation techniques gave good preservation of ultrastructural details. In actual fact, the preservation of the fibrin fiber and platelet between these two fixation methods is in essence equivalent. HPF fixation unfortunately showed quite inconstant results with occasionally freeze damage. Specimens with a flattened appearance as well as specimens that appear contaminated were seen. These effects are most probably due to human error such as inadequate washing or faulty loading of the specimen into the freezing specimen carrier.

In an attempt to extricate differences between these techniques, measurements of the fibrin fibers was made (Technique 1 vs. Technique 2), using Image Tool© Version 3, and compared (see Engelbrecht 2011, for methodology). Measurement showed that fibrin fibers fixated by means of HPF was marginally, but still statistically significantly (95% confidence, analysed by one-way Anova) thicker than that of specimens processed by the chemical method, with frozen fibers having a mean thickness of 0.151 ± 0.093µm and chemically fixed samples 0.13 ± 0.071µm. The distribution pattern seen between the two

fixation methods is similar, but freezing fixation shows a larger range of fibers with exceedingly increased widths.

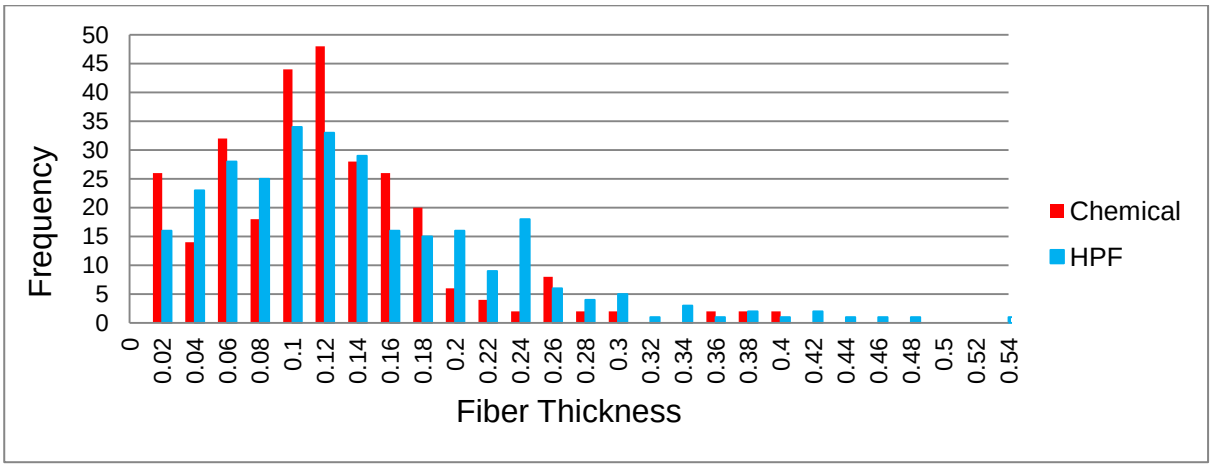


Figure 6.5: Frequency distribution of fiber thickness.

Chemical fixation and processing is known to cause shrinkage and coagulation of biological molecules (see chapter 4, section 4.2.2.2, pages 127-128). Although HPF coupled with freeze drying is considered superior to conventional chemical fixation and processing, freeze drying is known to cause a certain degree of shrinkage depending on the specimen type. Mackenzie (1972) found that irrespective of the drying time and vacuum conditions, freeze drying does not remove all the water from a biological system. The residual water can only be removed by raising the temperature of the specimen under vacuum. This is done at the end of the freeze drying cycle and is believed to cause the “collapse” phenomenon. The fiber thickness inconsistency between specimens prepared by HPF coupled with freeze drying and the conventional chemical processing when compared to fiber thickness measurements in a wet system shows that both these methods result in shrinkage or aggregation of the fibers, but freeze fixation to a lesser degree than chemical methods.

6.4.3. Comparison of Operational and Practical parameters

Comparison of several parameters was done to determine the practical aspects connected to each technique tested in this study. The comparisons and subsequent “score” of each

technique do not determine which technique is superior. The superiority is based mainly on the quality of the results.

The quality of the results was discussed in previous sections. The cost involved with chemical fixation and processing is relatively low, the methods require; apart from the critical point drying machine, only the chemicals for fixation, rinsing and dehydration, and consumables such as pipettes. Controversially freeze fixation requires the HPF machine and the freeze drying machine with regards to equipment; also the consumables used for these processes are usually costly as they are specific to the equipment. Together with the liquid nitrogen consumed, this technique is very expensive. Freeze drying of the frozen specimens, account for the HPF technique to also be the most “costly” technique in relation to time. Most of the chemicals employed are graded as either toxic, harmful or as an irritant, and HPF use liquid nitrogen (can cause frostbite, an explosion of a sealed container and displace oxygen causing asphyxiation) so both these techniques are fairly dangerous.

When freeze fixation is selected as a preservation method, the samples needs to be extremely small for optimal vitrification to take place. The specimen carrier used in the HPF process is also exceedingly small, which is difficult to work with. Applying a fibrin and platelet network to such small substrates proved difficult; this resulted in several specimens either being too thick or mostly absent from the substrate. Further processing such as washing and inserting the substrate into the specimen carrier also presented with difficulties. This governs the fact that this technique requires a certain amount of practise and expertise and is also the most difficult technique to repeat successfully.

Table 6.2: Summary of operational and practical parameters comparisons.

Technique	Quality	Cost	Time	Rpt	Exp	Safety	Total
1	4	2	2	2	2	3	**13**
2	4	4	4	5	4	3	**24**
3	4	2	2	2	2	3	**13**
4	4	4	4	5	4	3	**24**

Key:			
	Quality of preservation	1 = worst	5 = best
	Financial cost	1 = high	5 = low
	Time	1 = high	5 = low
	Repeatability (Rpt)	1 = low	5 = high
	Expertise required (Exp)	1 = high	5 = low
	Safety of use	1 = dangerous	5 = not dangerous

6.5. Conclusion

High pressure freezing coupled with freeze drying failed to significantly increase the quality of morphological preservation when compared to chemical fixation. This method did prove though to cause less aggregation and shrinkage of fibrin fibers. These facts together with other practical parameters such as finance, time and repeatability, leads us to conclude that chemical fixation is the more optimal method for the preparation of fibrin networks and platelets for ultrastructural studies.

Chemical preparation methods are cheaper, faster, and easier and also give comparable quality of ultrastructural preservation of the specimen.

Chapter 7:
Concluding Discussion

Chemical fixation has long been providing exceptionally useful information regarding biological structure and function, and it will continue to do so. However several investigators have highlighted the superiority of freeze fixation of biological tissues for ultrastructural studies (Kellenberger 1991; Murk *et al.,* 2003 and Quintana 1994). The study of rapidly frozen or cryo-fixed specimens has confirmed much of the information derived from analysis of chemically fixed specimens and although many features in chemically fixed and rapidly frozen samples resemble each other, there are some important differences. Chemical fixatives have a normal delay in diffusion, making it impossible to capture certain dynamic processes that occur in milliseconds. Additionally, fixation with aldehydes involves oxygen consumption and hydronium ion production, thereby depriving the tissue of oxygen before fixation is completed.

Cryo-fixation is a physical fixation procedure which preserves the distribution and structure of all components in a biological system. The principal aim and advantage is the near instantaneous arrest of the cellular metabolism whereby soluble cell constituents is stabilized and retained in the native three dimensional structures by the removal of the thermal energy in the system, facilitated by rapidly lowering the temperature to subzero (Vanhecke *et al.,* 2008).

Blood platelets and fibrin networks are both integral components of the coagulation system, responsible for the maintenance of homeostasis, wound healing and overall health. Any abnormality of either platelets or fibrin, leads to dysfunctionality in coagulation, which may result in several pathological phenomena, ranging from a slight bleeding tendency to thrombosis to death. The literature clearly indicates that abnormalities of fibrin network and platelet ultrastructure have definite clinical implications, leading to bleeding tendencies or increased thrombotic events (Collet *et al.,* 2000; Pretorius 2008; Pretorius *et*

al., 2006; Pretorius *et al.,* 2009c; Standeven *et al.,* 2005; Weisel 2005 and Zhuge *et al.,* 2009). The use of the best potential preparation methods of these specimens for ultrastructural investigation is of utmost importance as any of the necessary steps in sample preparation affect the quality of the final electron micrographs. Artefact introduction and structural alterations due to the preparation process may lead an investigator to misinterpret the results and ultimately erroneous conclusions. Therefore, development and optimization of freeze fixation preparation techniques for blood platelets and fibrin fiber networks for ultrastructural studies with electron microscopy was performed and compared to conventional chemical methods.

Several variations concerning the formation of the platelet and fibrin fiber network was carried out, including the coagulation agent, coagulation agent-platelet-rich-plasma (PRP) ratio, coagulation time and temperature. Both the coagulation agents used (thrombin and $CaCl_2$) showed satisfactory although dissimilar results. Thrombin activation resulted in denser networks made up from thicker fibers and aggregated platelets, whereas with $CaCl_2$ activation the number of activated platelets was increased but decreased platelet aggregation was seen. The differences seen between these two techniques is most likely mainly due to the rate limiting platelet activating phospholipids for thrombin generation (limited by the amount available in a closed system) (Kumar *et al.,* 1995). Differences seen when other factors such as time, temperature and component concentration was varied, presented with results that would be expected from a biological time and temperature dependant process such as coagulation.

The freezing technique, different substrates used for freezing and the subsequent processing methods was also varied and it was found that for TEM ultrastructural studies platelet and fibrin coagulates formed in an Eppendorf tube, transferred to an HPF specimen carrier, frozen and freeze substituted for 90 hours, provided outstanding results. Also, activation by $CaCl_2$ and freezing in a cellulose capillary tube gave interesting and informative results. For SEM studies HPF coupled with freeze drying on sapphire coverslips was the best method and substrate to form and preserve platelet and fibrin networks. Ultimately it was found that HPF combined with freeze substitution (for TEM) or

freeze drying (for SEM) is a high-quality preservation method for the selected specimen model.

Comparison between TEM results of HPF and chemical fixation showed that platelets were superiorly preserved by HPF methods, with distinct cell membranes and other cellular features such as the open canalicular system, microtubule coils, α-granules, glycogen, mitochondria and dense bodies. Fibrin fibers are also believed to be preserved closer to their native state when using freeze fixation. The fibers appear as very disperse structures made up in an organized structure from many thin protofibrils, whereas chemically fixated fibers appear as aggregated and condensed structures. Several authors have shown that the solvent content of fibrin fibers is between 70 and 80% (Weisel 1986 and Yeromonahos et al 2010), and we believe the results obtained in this study more closely represents the natural “hydrated” nature of a fibrin fiber.

When SEM is employed to compare freeze fixated and chemical fixated platelet and fibrin fiber specimens, fiber diameter measurements show a slight increase with the HPF method which confirms that freeze fixation coupled with freeze drying results in less aggregation and shrinkage of the fibers, although very little other morphological differences are seen.

Practical considerations for the preservation of ultrastructure for electron microscopy studies also need to be taken into account when superiority of a method is under investigation. These include; financial cost, time, repeatability, expertise required and safety.

Although the quality of preservation of HPF was found to be superior to that of chemical preparation methods, HPF is inferior when graded against practical parameters such as the above-mentioned. The cost involved with chemical fixation and processing is relatively low. The methods require, apart from the critical point dryer (for SEM), only the chemicals for fixation, rinsing, dehydration and embedding, and consumables such as pipettes. Controversially freeze fixation requires the HPF and the freeze substitution or freeze

drying equipment. Also, the consumables used for these processes are usually costly as they are specific to the equipment. Together with the chemicals necessary for freeze substitution and the liquid nitrogen consumed, this technique is very expensive. Freeze substitution and drying are both very time consuming which makes the HPF technique also the most costly technique in relation to time. Most of the chemicals employed are graded as either toxic, harmful or as irritant, as both chemical and freezing techniques employ these chemicals but only HPF additionally use liquid nitrogen (can cause frostbite, an explosion of a sealed container and displace oxygen causing asphyxiation). Thus the HPF technique is the more dangerous of the two.

When utilizing freeze fixation, the samples need to be extremely small for optimal vitrification to take place. The consumables used in the HPF process are also exceedingly small, which is difficult to work with. With TEM specimen preparation the cellulose capillary tubes are especially challenging, as these tubes with a total diameter of roughly 220µm, needs to be cut down to a 1mm size and sealed simultaneously. This process tends to press out the small amount of specimen that was in there initially. When preparing specimens for SEM the application of fibrin and platelet networks to such small substrates proved difficult; which resulted in several specimens either being too thick or mostly absent from the substrate. Further processing such as washing and the insertion of the substrate into the specimen carrier also presented difficulties. This governs the fact that this technique requires a certain amount of practise and expertise and is also the most difficult technique to repeat successfully.

In conclusion, for TEM ultrastructural studies HPF coupled with freeze substitution is the most suitable method for fine structure preservation of fibrin fiber networks and platelets, despite the fact that it is the most expensive, most difficult and least repeatable method. This method may even be significant in providing a more accurate internal organization model and information on the formation of the fibrin fiber. Contrarily for SEM ultrastructural studies it was found that chemical fixation is the more optimal method for the preparation of fibrin networks and platelets, due to the absence of improved preservation by HPF coupled with freeze drying, apart from fiber thickness.

Chapter 8:
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