



# **Enzymatic and chemical modifications of erythrocyte surface antigens to identify *Plasmodium falciparum* merozoite binding sites**

by

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## Declaration

**University of Pretoria**  
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**Subject of the work:** Enzymatic and chemical modifications of erythrocyte surface antigens to identify *Plasmodium falciparum* merozoite binding sites

### Declaration

1. I understand what plagiarism entails and am aware of the University's policy in this regard.
2. I declare that this dissertation is my own, original work. Where someone else's work was used (whether from a printed source, the internet or any other source) due acknowledgement was given and reference was made according to departmental requirements.
3. I did not make use of another student's previous work and submit it as my own.
4. I did not allow and will not allow anyone to copy my work with the intention of presenting it as his or her own work.

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## Abstract

Malaria is a disease caused by the protozoan parasite *Plasmodium* where the species that causes the most severe form of malaria in humans is known as *Plasmodium falciparum*. At least 40% of the global population is at risk of contracting malaria with 627 000 people dying as a result of this disease in 2012. Approximately 90% of all malaria deaths occur in sub-Saharan Africa, where approximately every 30 seconds a young child dies, making malaria the leading cause of death in children under the age of five years old.

The malaria parasite has a complex life cycle utilising both invertebrate and vertebrate hosts across sexual and asexual stages. The erythrocyte invasion stage of the life cycle in the human whereby the invasive merozoite form of the parasite enters the erythrocyte is a central and essential step, and it is during this stage that the clinical symptoms of malaria manifest themselves. Merozoites invade erythrocytes utilising multiple, highly specific receptor-ligand interactions in a series of co-ordinated events. The aim of this study was to better understand the interactions occurring between the merozoite and erythrocyte during invasion by using modern, cutting-edge proteomic techniques. This was done in the hope of laying the foundation for the discovery of new key therapeutic targets for antimalarial drug and vaccine-based strategies, as there is currently no commercially available antimalarial vaccine and no drug to which the parasite has not at least started showing resistance.

In this study healthy human erythrocytes were treated separately with different protein-altering enzymes and chemicals being trypsin, the potent oxidant sodium periodate (NaIO<sub>4</sub>), the amine cross-linker tris(2-chloroethyl)amine hydrochloride (TCEA) and the thiol cross-linker 1,11-bis(maleimido)triethylene glycol (BM(PEG)<sub>3</sub>). The resulting erythrocyte protein alterations were visualised as protein band differences on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE), where treated and untreated control erythrocyte ghost protein fingerprints were visually compared to one another. The protein bands showing differences between treated and control samples were in-gel digested using trypsin then sequenced by liquid chromatography tandem mass spectrometry (LC-MS/MS) and identified using proteomics-based software. In this way, the erythrocyte proteins altered by each enzyme/chemical treatment were identified.

Malaria invasion assays were performed where each treatment group of erythrocytes as well as the control erythrocytes were incubated separately with schizont stage malaria parasites for the duration of one complete life cycle. Using fluorescent staining and flow cytometry, the invasion inhibition efficiency for each treatment group was evaluated. By utilising these methods, the identification and the relative importance of the erythrocyte proteins involved in the invasion process were determined.

Protein fingerprints of control and treated erythrocyte ghosts were visualised and optimised on SDS PAGE where induced protein band differences were successfully identified by LC-MS/MS. It was found that each treatment altered erythrocyte proteins with changes found in Band 3, actin, phosphoglycerate kinase 1, spectrin alpha, spectrin beta, ankyrin, haemoglobin, Bands 4.1 and 4.2, glycophorin A and stomatin. The invasion assays revealed that TCEA inhibited invasion to the greatest extent as compared to the other treatments, followed by BM(PEG)<sub>3</sub> and trypsin. Sodium periodate-treated erythrocytes could not be assessed using the invasion assay due to auto-haemolysis. Band 3, glycophorin A, Band 4.1 and stomatin appear to be of higher relative importance in the invasion process as compared to the other altered erythrocyte proteins. These results confirmed the known roles of spectrin alpha, spectrin beta, glycophorin A, Band 3 and Band 4.1 in invasion, and suggested that ankyrin, Band 4.2 and stomatin may also be involved.

This study highlighted the potential that modern, cutting-edge proteomic techniques provide when applied to previous comparative studies found in older literature, as previously unidentified proteins that can be involved in invasion were revealed.

These results can be used as a foundation in future studies in order to identify new key targets for the development of new antimalarial drug- and vaccine-based strategies, with the hope of preventing the suffering of the millions of malaria-inflicted people worldwide, and ultimately eradicating this deadly disease.

**Keywords:** Malaria, *Plasmodium falciparum*, erythrocytes, invasion, proteomics, gel electrophoresis, LC-MS/MS, trypsin, sodium periodate, TCEA, BM(PEG)<sub>3</sub>.



## Glossary of Abbreviations

°C	Degree Celsius
µl	Microlitre
µm	Micrometre
%	Percent
2-DE	Two-dimensional gel electrophoresis
ACN	Acetonitrile
ACT	Artemisinin-based combination therapy
AgNO <sub>3</sub>	Silver nitrate
ASB-14	Amidosulfobetaine-14, a zwitterionic surfactant
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BM(PEG) <sub>3</sub>	1,11-bis(maleimido)triethylene glycol
BSA	Bovine serum albumin
C-terminal	Carboxyl-terminal
CO <sub>2</sub>	Carbon dioxide
CR1	Complement receptor 1
CuSO <sub>4</sub>	Cupric sulfate
d.H <sub>2</sub> O	Deionised water
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EBA	Erythrocyte binding antigen
EBL	Erythrocyte binding-like
EDTA	Ethylenediaminetetraacetic acid
FA	Formic acid
g	Gram
<i>g</i>	Gravity
g/l	Grams per litre
G-3-PD	Glyceraldehyde -3-phosphate dehydrogenase

H <sub>2</sub> O	Water
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
HCl	Hydrochloric acid
HNO <sub>3</sub>	Nitric acid
Hr	Hour
IAA	Iodoacetamide
IEF	Isoelectric focusing
IHME	Institute for Health Metrics and Evaluation
IPG	Immobilised pH gradient
K <sub>2</sub> Cr <sub>2</sub> O <sub>7</sub>	Potassium dichromate
kDa	Kilodalton
l	Litre
LC-MS/MS	Liquid chromatography tandem mass spectrometry
M	Molar
<i>m/z</i>	Mass-to-charge ratio
mg/ml	Milligram per millilitre
ml	Millilitre
mM	Millimolar
MSP-1	Merozoite surface protein 1
N <sub>2</sub>	Nitrogen
Na <sub>2</sub> C <sub>4</sub> H <sub>4</sub> O <sub>6</sub> .2H <sub>2</sub> O	Disodium tartrate dihydrate
Na <sub>2</sub> CO <sub>3</sub>	Sodium carbonate
Na <sub>2</sub> CO <sub>3</sub> .10H <sub>2</sub> O	Sodium carbonate decahydrate
NADPH	Nicotinamide adenine dinucleotide phosphate
NaHCO <sub>3</sub>	Sodium bicarbonate
NaIO <sub>4</sub>	Sodium periodate
NaOH	Sodium hydroxide
NH <sub>4</sub> HCO <sub>3</sub>	Ammonium bicarbonate
NHS-fluorescein	Fluorescein-5-EX N-hydroxysuccinimide ester
nm	Nanometre

O <sub>2</sub>	Oxygen
PBS	Phosphate buffered saline
<i>Pf</i>	<i>Plasmodium falciparum</i>
PfAMA-1	<i>Plasmodium falciparum</i> apical membrane antigen 1
PfEMP1	<i>Plasmodium falciparum</i> erythrocyte membrane protein 1
PfRh	<i>Plasmodium falciparum</i> reticulocyte binding-like
pH	Negative logarithm of the hydrogen ion concentration
pI	Isoelectric point
PMSF	Phenylmethylsulfonyl fluoride
PRBC	Packed red blood cell
RBC	Red blood cell
RPMI	Roswell Park Memorial Institute
SAO	Southeast Asian Ovalocytosis
SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SH	Sulfhydryl
SWR	Standard working reagent
TCA	Trichloroacetic acid
TCEA	Tris(2-chloroethyl)amine
TCEP	Tris(2-carboxyethyl)phosphine
TCP-1-ε	T-complex protein 1 subunit epsilon
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
UV	Ultraviolet
V	Volts
v/v	Volume per volume
WHO	World Health Organisation

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# Chapter 1: Introduction

## 1.1 Literature Review

### 1.1.1 Introduction

The parasitic *Plasmodium* species is responsible for causing the deadly disease malaria, which relies on both vertebrate and mosquito hosts during its complex life cycle. Malaria is responsible for infecting hundreds of millions of people globally each year, causing the deaths of more than 500 000 people and becoming the leading cause of paediatric deaths in sub-Saharan Africa (Cowman and Crabb, 2006, WHO, 2013).

Despite the large amount of research being done worldwide with the aim of significantly reducing and ultimately eliminating malaria completely, there is no antimalarial drug available today that has not at least started showing signs of resistance, and a long-term antimalarial vaccine is yet to reach the market (WHO, 2012). There is therefore a great need to discover and identify new key therapeutic targets for antimalarial drug and vaccine developments. Ideal therapeutic targets would be those erythrocyte proteins that are required for the invasion stage of the malaria parasite life cycle. Erythrocyte invasion is a central and essential stage in the life cycle, where the invasive form of the malaria parasite (the merozoite) enters the erythrocyte beginning the asexual blood stage where massive increases in parasite numbers occur and at which stage the clinical symptoms of malaria manifest. Thus, inhibiting the invasion process would not only prevent the parasite's life cycle, but would spare the infected individual the suffering caused by the highly unpleasant symptoms of malaria. Erythrocyte invasion involves specific interactions that occur in a multi-step, coordinated series of events between merozoite ligands and erythrocyte receptor molecules, the exact mechanisms of which remain unknown (Riglar et al., 2011). These receptor-ligand complexes serve to be promising antimalarial therapeutic targets. Therefore utilising modern, cutting-edge proteomic techniques to better understand the proteomic interactions occurring between erythrocytes and merozoites during the invasion process, holds promise in identifying new key targets for antimalarial treatment strategies that may be capable of eradicating malaria.

### 1.1.2 The human erythrocyte

One of the most important cells in the blood is the red blood cell, also known as the erythrocyte. These erythrocytes are unique cells within the human body as they are non-nucleated and have a biconcave disk shape, which their structural plasma membrane is responsible for, that plays an important role in cellular transport as well as in antigenic and mechanical characteristics (Kakhniashvili et al., 2004, Mohandas and Gallagher, 2008). Mature erythrocytes consist of a cytoplasm surrounded by a

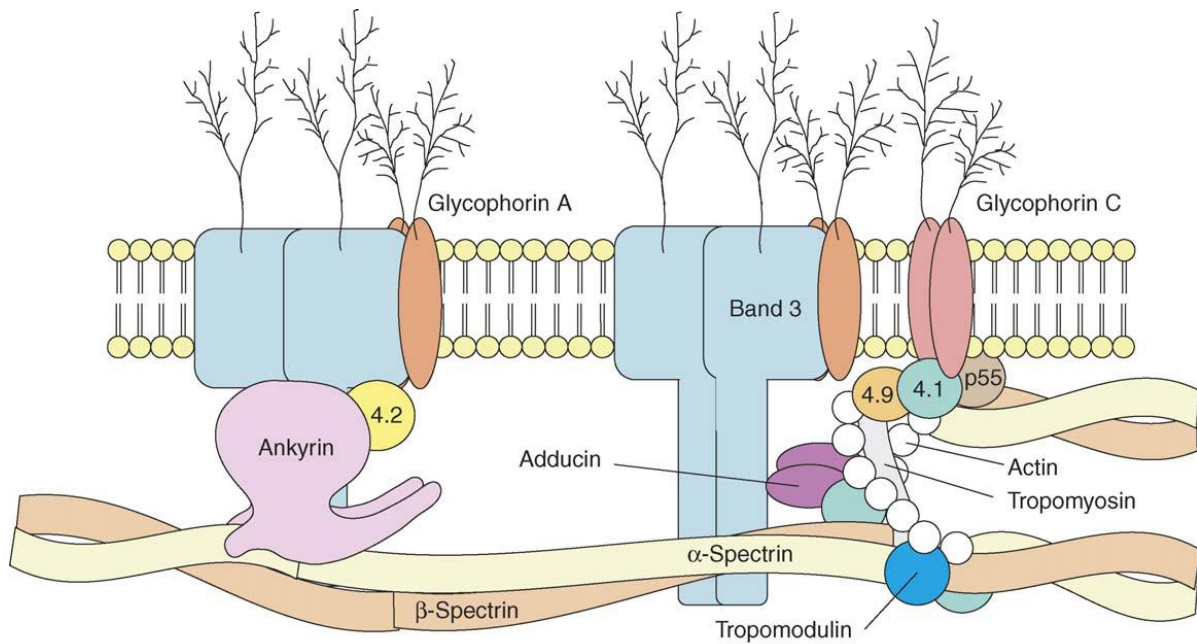


plasma membrane and do not have any internal organelles. The plasma membrane encapsulates high concentrations of haemoglobin, an iron-containing metalloprotein, within the cell that allows for the binding and release of oxygen and carbon dioxide at the lungs and body tissues (Kakhniashvili et al., 2004). In order to succeed in fulfilling this function, the erythrocyte, which is approximately 7  $\mu\text{m}$  in size, needs to squeeze through capillaries as narrow as 1  $\mu\text{m}$  in diameter repeatedly during its 120 day lifespan (Goodman et al., 1988, Mohandas and Gallagher, 2008, Yazdanbakhsh et al., 2000). This is where the discoid shape of the erythrocyte, its unique highly elastic and strong membrane and the cell's ability to respond rapidly to fluid stresses come into play (Mohandas and Gallagher, 2008).

The erythrocyte membrane consists of three layers being the external layer known as the glycocalyx that is carbohydrate rich, followed by a lipid bilayer consisting of cholesterol and phospholipids, which is secured on the cytoplasmic side of the cell to the third layer, a two-dimensional network of intracellular skeletal proteins. Transmembrane proteins passing through the bilipid layer to the exterior surface of the cell and to the cytoplasm are located where they help to secure and anchor the bilipid layer to the elastic skeletal protein network. These skeletal proteins allow for the deformability, elasticity and durability of the erythrocyte (Mohandas and Gallagher, 2008, Yazdanbakhsh et al., 2000).

There are more than fifty known transmembrane proteins found on the erythrocyte membrane, copies of which are found in varying proportions that range from hundreds to millions per cell surface. These proteins have various functions such as transporter proteins, signalling receptors and adhesion proteins. Several proteins' activities remain unknown. Well-defined membrane proteins include Band 3 (anion transport), intercellular adhesion molecule-4 (adhesion), aquaporin 1 (water transport) and the glycophorins (sialoglycoproteins acting as antigenic determinants) (Mohandas and Gallagher, 2008).

The main erythrocyte proteins making up the meshwork of the membrane skeleton include spectrin (comprised of spectrin alpha and spectrin beta subunits), actin, protein 4.1, adducin, dematin, tropomyosin and tropomodulin (Bennett, 1989, Bennett and Baines, 2001, Mohandas and An, 2006). The deformability and mechanical strength of the erythrocyte is mainly due to the unique structural feature of spectrin whose subunits lie in an anti-parallel arrangement. These spectrin alpha and beta subunits through self associations form tetramers, which are at the core of the erythrocyte cytoskeletal network (Yazdanbakhsh et al., 2000). Hence spectrin is said to be responsible for erythrocyte membrane integrity and cellular shape (Kirkpatrick, 1976, Marchesi et al., 1976). Spectrin forms interactions with actin, protein 4.1, ankyrin, adducin, tropomyosin and tropomodulin. Cytoskeletal proteins interact with the bilipid layer and transmembrane proteins, for example spectrin with Band 3 via an ankyrin connection and spectrin with glycophorin C via protein 4.1 (Figure 1.1) (Liem and Gallagher, 2006, Mohandas and Gallagher, 2008, Yazdanbakhsh et al., 2000).



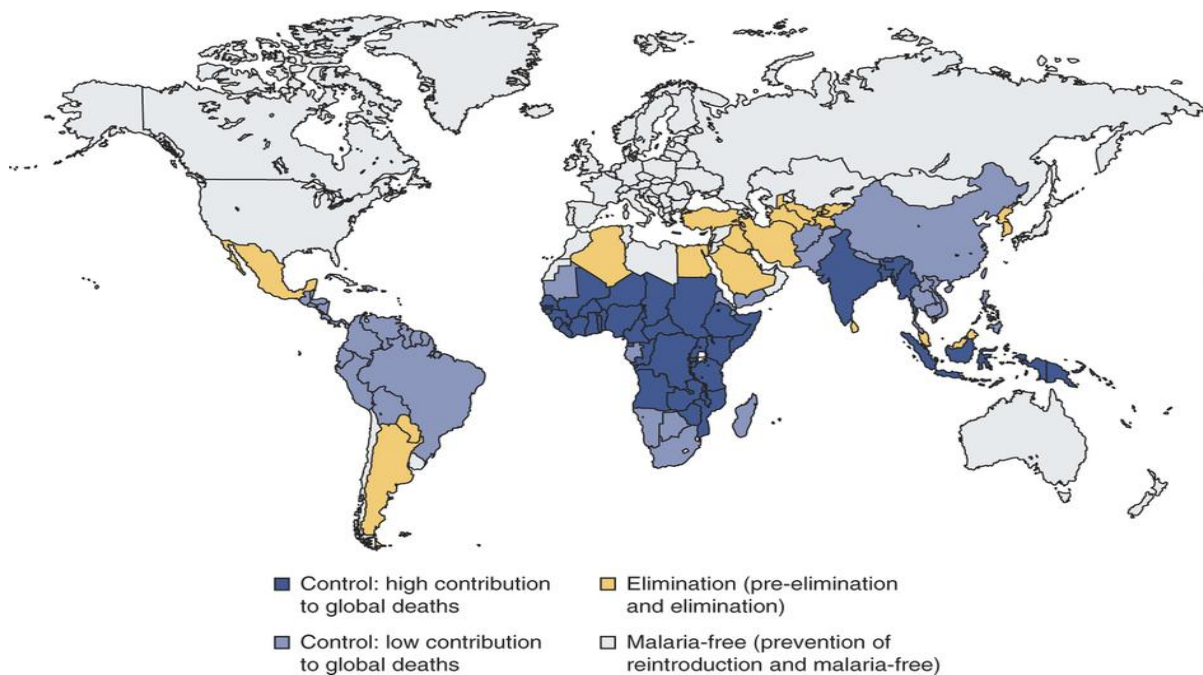
**Figure 1.1: A diagram depicting the organisation of proteins associated with the human erythrocyte membrane.** The human erythrocyte has a unique structural organisation of membrane and cytoskeletal proteins allowing for its mechanical strength, deformability and elasticity (Liem and Gallagher, 2006) (with permission).

Erythrocyte ghosts are the erythrocyte membrane and cytoskeleton that remain after hypotonic haemolysis during which the cell content is released. These ghosts remain intact, retaining their original morphology and are often used in surface protein studies as they are 'clean' and free of intracellular protein content (Schwoch and Passow, 1973). Erythrocyte surface proteins are often assayed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE), where optimal erythrocyte ghost protein concentration and protein solubility play important roles in the quality of the resulting protein fingerprint.

### 1.1.3 Incidence of malaria

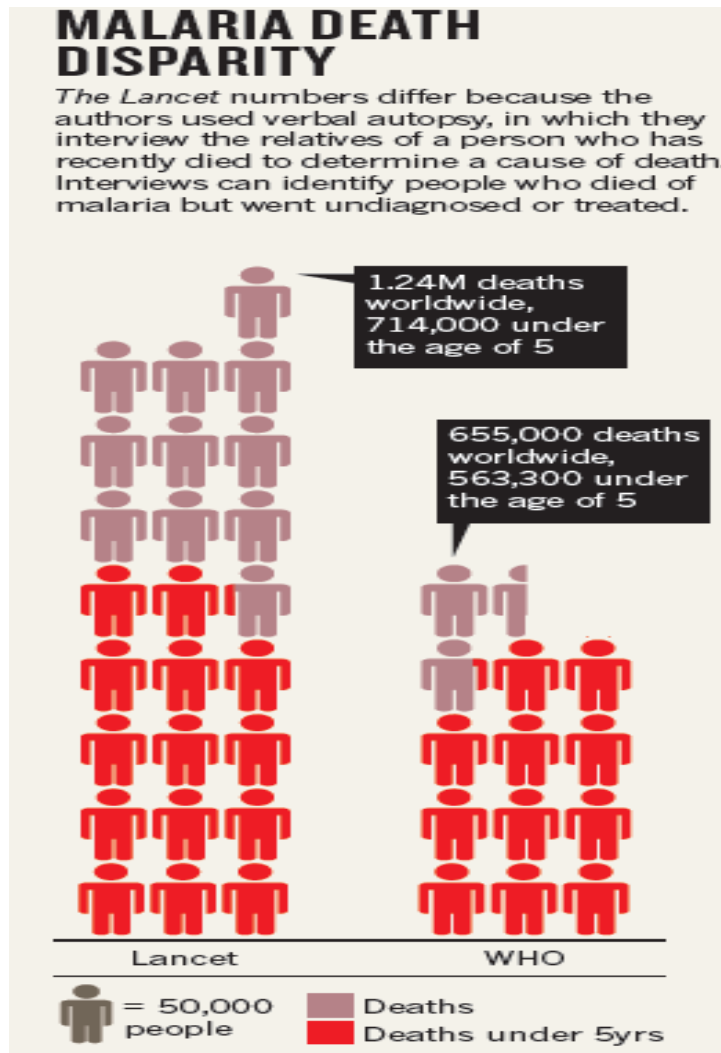
Malaria is a parasitic infection that has been recognised as a disease that has affected humans for at least 10 000 years, yet the cause was only identified about 100 years ago and the complete life cycle of the responsible parasite finally understood less than 30 years ago. The world distribution of malaria is restricted to the tropical regions where infection rates are high as shown in Figure 1.2. There are very few diseases in the world that have had comparable effects on the economy and global health that malaria has had (Snow et al., 2005). Malaria, alongside tuberculosis and HIV, is considered one of the world's three most deadly diseases (Goldberg et al., 2012). At least 40% of the global population is at risk of malaria with over 200 million people affected worldwide annually, and approximately 0.6 million people dying as a result of this disease (Ghansah et al., 2014, Smit et al., 2010,

WHO, 2013). Malaria deaths are most prevalent in sub-Saharan Africa where 80% of global malaria-associated deaths occur and where a malaria-inflicted child is estimated to die every 30 seconds, making malaria the leading cause of death in children under the age of 5 (Figure 1.2) (Florens et al., 2002, Smit et al., 2010). In the year 2000 global malaria deaths stood at 890 340 people, decreasing to 627 000 global malaria deaths in 2012. This serves to indicate that although there has been a decline in malaria mortality in recent years due to increased antimalarial strategies and international support, the annual global malaria death figures are still disturbingly high (WHO, 2013).



**Figure 1.2: World map indicating the global distribution of malaria and the contribution to malaria mortality by region.** Visualisation of global malaria distribution and to what degree antimalarial interventions have contributed towards the control or elimination of malaria (Alonso and Tanner, 2013) (with permission).

Using their global model, Snow, Guerra, Noor *et al.* estimated 515 million cases of clinical malaria caused by *Plasmodium falciparum* in 2002 which was almost 50% higher than what was estimated by the World Health Organization (WHO) for the same year (Snow et al., 2005). In addition, according to the Institute for Health Metrics and Evaluation (IHME) at the University of Washington, Malaria was responsible for 1.24 million deaths globally in 2010, that being double the figure of what was estimated by the WHO, which was 655 000 deaths (Figure 1.3) (Lancet, 2012, Shetty, 2012). The malaria associated death statistics although already shocking, are most likely underestimated and have been so for years. Malaria can be considered as an epidemic and urgently needs to be eradicated (Snow et al., 2005).



**Figure 1.3: Global malaria death statistics- The Lancet versus World Health Organization (WHO).** The number of malaria deaths worldwide in 2010 as stated by The Lancet and the WHO differ by up to almost 100%. This difference can be at least partially attributed to a significant amount of people dying due to malaria, but going undiagnosed (Shetty, 2012) (with permission).

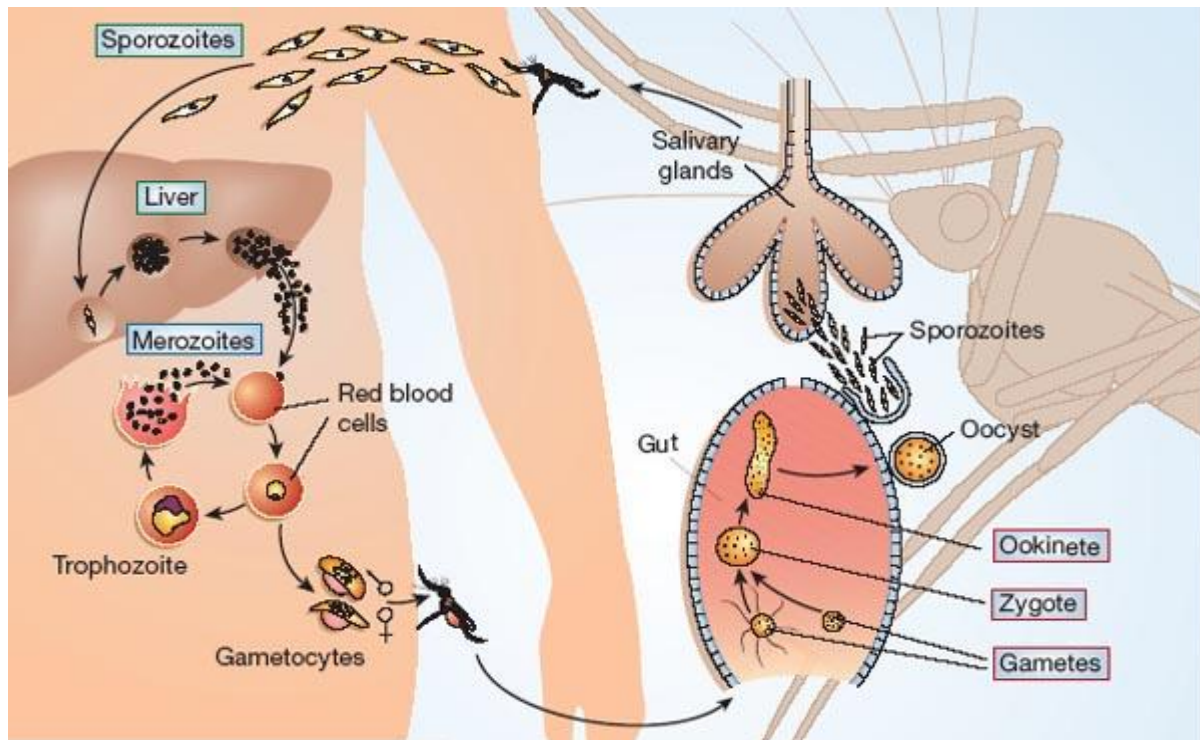
#### 1.1.4 *Plasmodium falciparum* life cycle and pathogenesis

Malaria is caused by a protozoan parasite known as *Plasmodium* of which four different species cause human malaria. These are: *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium falciparum*. The *Plasmodium* species that causes the most severe form of malaria in humans and is responsible for the largest number of deaths is *Plasmodium falciparum* (Cowman and Crabb, 2006, Dubovsky and Rabinovich, 2004).

The *Plasmodium falciparum* parasite has a complex life cycle that utilises both an invertebrate and a vertebrate host. This form of malaria is primarily spread to the human host via a female *Anopheles* mosquito which serves as a compulsory vector

in the parasite's life cycle. Upon biting a human, an infected mosquito injects sporozoite forms of *Plasmodium falciparum*, residing in the mosquito's salivary glands, into this human host. The sporozoites are injected into the subcutaneous tissue most of the time, but are also injected directly into the blood stream. These sporozoites migrate to the liver where they evade the immune protection of the kupffer cells, and invade hepatocytes. Only tens to hundreds of hepatocytes are invaded by the mosquito-borne sporozoites and thus the parasite does not cause any disease symptoms to the host at this stage (Miller et al., 1994). Within the hepatocytes, the sporozoites multiply and develop into tens of thousands of merozoites which are subsequently released into the blood stream where they seek and invade healthy erythrocytes. This marks the beginning of the asexual blood-stage life cycle of the parasite. Within the erythrocyte, merozoites go through the sequential developmental stages of ring, trophozoite and schizont forms respectively, dividing to form at least 16 daughter merozoites within 48 hours. The increasing numbers of intraerythrocytic merozoites cause the infected erythrocytes to burst. The subsequent egress of these merozoites into the blood stream results in the rapid invasion of new, healthy erythrocytes, where the cycle of development is repeated. This developmental process of *Plasmodium falciparum* daughter merozoites takes 48 hours. These merozoites are the smallest extracellular form of the parasite, measuring a mere 1-2  $\mu\text{m}$  in length. The asexual blood stage of *Plasmodium falciparum* is responsible for the majority of the clinical symptoms and pathologies that humans experience upon contracting malaria (Cowman and Crabb, 2006, Gaur et al., 2004, Kuss et al., 2012, Miller et al., 2002, Riglar et al., 2011).

A small proportion of intraerythrocytic parasites develop into gametocytes (being male or female) which are the sexual forms of the parasite. Gametocytes are essential for the transmittance of the malaria infection, and are taken up by a female *Anopheles* mosquito upon taking a blood meal from an infected human. These gametocytes cause no harm to the insect. Within the mosquito's gut the gametocytes develop into mature gametes, fertilise to form zygotes and subsequently develop into ookinetes (the motile invasive form of the parasite) that form oocysts in the gut lining of the mosquito. Growth and division of these oocysts produce thousands of active haploid sporozoites that burst out and migrate to the mosquito's salivary glands. These sporozoites in the mosquito's salivary glands are then injected into a human host when the infected mosquito bites during its blood meal (Figure 1.4) (Cowman and Crabb, 2006, Lasonder et al., 2002, Miller et al., 2002). Sexual development and fertilisation are vital processes of the malaria parasite's life cycle.

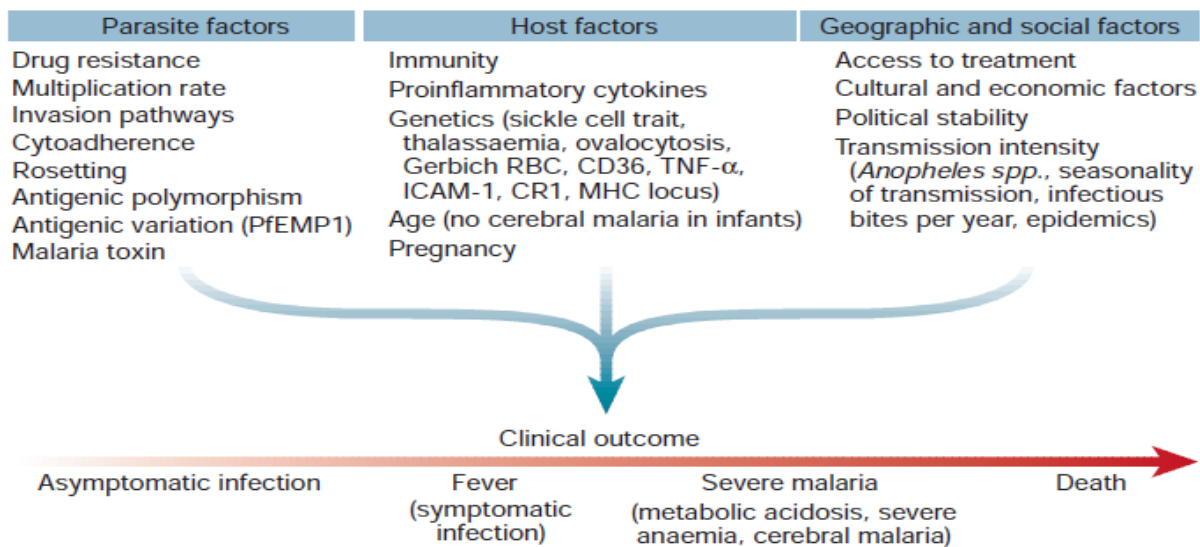


**Figure 1.4: The life cycle of *Plasmodium falciparum* in the human host and *Anopheles* mosquito.** The malaria parasite has a complex life cycle utilising both invertebrate and vertebrate hosts across sexual and asexual stages (Ménard, 2005) (with permission).

An erythrocyte that has been infected with *Plasmodium falciparum* is radically modified when it comes to the cell's morphology as well as adhesive and mechanical properties. The parasite, although in a parasitophorous vacuole upon invading the erythrocyte, has the ability to release and transport parasite proteins to the surface of the erythrocyte in order to enhance the transmission and ultimately the survival of the parasite. *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) is an example of such a surface parasite protein. It is a highly variant molecule that is expressed on the surface of mature infected erythrocytes (Baruch, 1999, Papakrivovs et al., 2005, Pei et al., 2005). PfEMP1 allows vascular sequestration as well as rosetting of infected erythrocytes. Sequestration refers to the attachment of erythrocytes infected with mature stage parasites to the endothelial cells that line post-capillary venules, and this occurs mainly in the lungs, kidneys, heart and liver in humans. However, this sequestration can occur in brain capillaries (the patient is then said to be suffering from cerebral malaria) which can bring about a coma and even death. Rosetting is the phenomenon where malaria-infected erythrocytes surround a central infected erythrocyte, forming what can be described as an erythrocyte cluster. Both erythrocyte sequestration and rosetting aid parasite survival by helping to evade the immune system. That is, infected erythrocytes cluster and bind to endothelial cells in specific organs, and therefore are not present in peripheral circulation where they can be removed and destroyed in the spleen. While these parasitic properties aid in malaria survival, they also contribute to severe

pathologies experienced by the human host (Aikawa et al., 1990, Miller et al., 2002, Pongponratn et al., 1991, Sherman et al., 1992).

The signs and symptoms of malaria result in multiple outcomes and pathologies, where the severity of the disease can range from asymptomatic infection to death. The degree of severity of the disease experienced by an individual depends on multiple factors including age, pregnancy status and the status of the individual's immune system. The geographical origin, the species, the genotype of the parasite and the degree of endemicity of malaria in the region also play a role in the patterns of pathology. The clinical outcome of malaria encompassing such variables has been well studied in African children (Figure 1.5) (Miller et al., 1994, Weatherall et al., 2002).



**Figure 1.5: The clinical outcome of malarial infection is dependent on multiple factors.** The degree of severity of malaria in an individual depends on multiple parasite, host, geographic and social factors, the combination of which result in a number of clinical outcomes ranging from asymptomatic infection to death (Miller et al., 2002) (with permission).

Over the past few decades research dedicated to improve current knowledge of what exactly constitutes the disease malaria, specifically severe malaria which leads to the loss of millions of lives has been performed. It takes between 7-18 days after initial infection with malaria parasites for symptoms of the disease to occur. In uncomplicated or mild cases of malaria, symptoms may include muscular aches, headaches, fever and chill cycles which can be accompanied by nausea, vomiting, abdominal pain and diarrhoea. These symptoms usually reoccur every 48 hours which is synchronised with the egress of new daughter merozoites from infected, lysed erythrocytes. In cases of severe malaria, more serious disorders are experienced such as respiratory distress, acute renal failure accompanied by hypotension and shock, hypoglycaemia, severe anaemia and even coma and death.

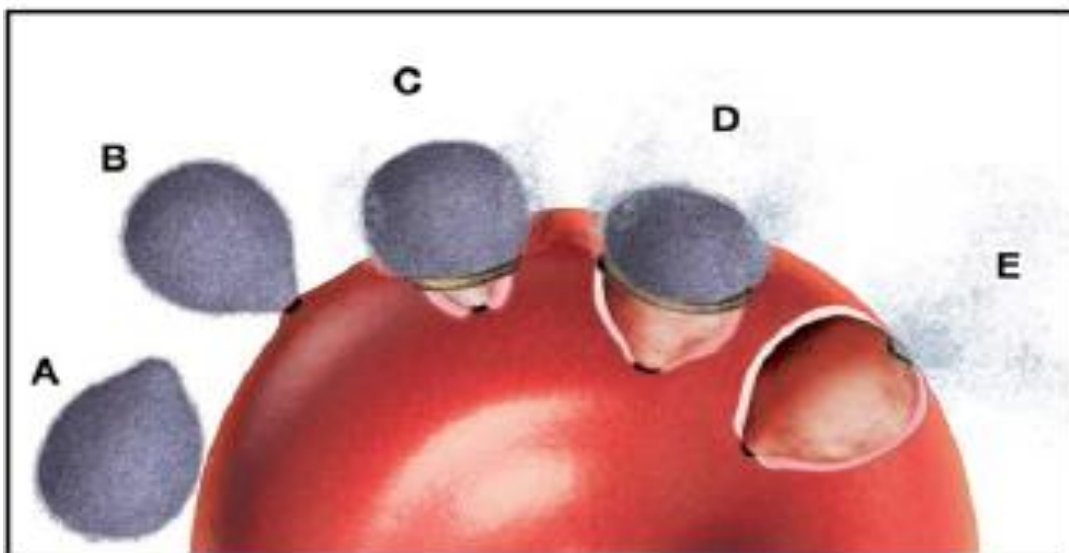
These symptoms mostly occur in children or in people who have no immunity to malaria e.g. outside visitors to a malaria-inflicted area. Children who suffer from severe malaria usually die within 72 hours after acquiring symptoms. Those that are fortunate enough to survive severe malaria usually experience negative effects when it comes to their mental and physical development (Bremner et al., 2001, Chen et al., 2000, Girard et al., 2007, Miller et al., 2002, Weatherall et al., 2002). The signs and symptoms of severe malaria include pathological changes in the neurological, renal and pulmonary systems of the individual as well as a high parasitemia (Phillips and Solomon, 1990). Severe malaria is a complex, multisystem disorder that may appear to affect a single organ (for example the brain), but involves several different organs and tissues. For example metabolic acidosis is observed in cerebral malaria and malarial anaemia (Marsh et al., 1995). The molecular and cellular events that occur during the life cycle of *Plasmodium falciparum* are responsible for multiple pathogenic activities that combine to cause severe malaria. Such events include rapid accumulation of erythrocyte mass, destruction of healthy and infected erythrocytes, the adhesion of infected erythrocytes to the endothelium leading to microvascular obstruction, and over stimulation of inflammatory responses which accumulatively lead to a reduction in tissue perfusion. These events can occur in specific organs, like in the placenta during pregnancy or the brain in cases of cerebral malaria (Miller et al., 2002).

### 1.1.5 Invasion

Invasion of the erythrocyte by the malaria parasite is a central and essential step in the development of malaria, and it is the speed (invasion can occur anywhere between 20 seconds and 1 minute) and efficiency of this process that has contributed to the evolutionary success of *Plasmodium falciparum* (Pasvol, 2003, Perkins, 1981). The invasion process of *Plasmodium* merozoites into erythrocytes has been studied and visualised by both live cell imaging and electron microscopy, thus rendering a good overall picture of this complex process (Aikawa et al., 1978, Dvorak et al., 1975, Riglar et al., 2011). Merozoite invasion utilises multiple receptor-ligand interactions and can be broken down into five basic steps: (1) initial recognition and reversible binding by any part of the parasite to the erythrocyte; (2) reorientation of the bound merozoite with its apical region facing towards the erythrocyte membrane in an irreversible bond (merozoite is now committed to invasion); (3) tight junction formation between the erythrocyte surface and the merozoite apical region; (4) movement of the parasite through the tight junction from the apical to the posterior region of the merozoite using an intracellular actin-myosin motor while shedding the merozoite surface protein coat at the junction; and (5) complete internalisation of the parasite into the erythrocyte by the creation of a parasitophorous vacuole (Figure 1.6) (Cowman and Crabb, 2006, Gaur et al., 2004). The apical end of the merozoite contains membrane-bound organelles known as the rhoptries and micronemes, and the reorientation of the merozoite (step 2) allows the



content of these organelles to interact with the erythrocyte surface. Key merozoite ligands involved in the binding of erythrocyte receptors are localised in these apical organelles. The release of parasite ligands from micronemes and rhoptries to the apical surface of the merozoite has to be critically timed for successful erythrocyte and merozoite receptor-ligand interaction (Gaur and Chitnis, 2011). For invasion to be successful merozoite ligands must recognise and bind erythrocyte receptor molecules in a multi-step, highly specific coordinated series of molecular events, however the exact molecular mechanisms that occur during invasion are unknown. Numerous surface proteins involved in the invasion process have been identified on both the erythrocyte and the merozoite, however the functions of most of these proteins remain unknown and the knowledge of exact receptor-ligand protein interactions are lacking. These erythrocyte and parasite receptor-ligand proteins have potential as promising targets for antimalarial drugs or vaccines that may prevent the invasion process from occurring (Chitnis, 2001, Cowman and Crabb, 2006, Crosnier et al., 2011, Gaur et al., 2004).

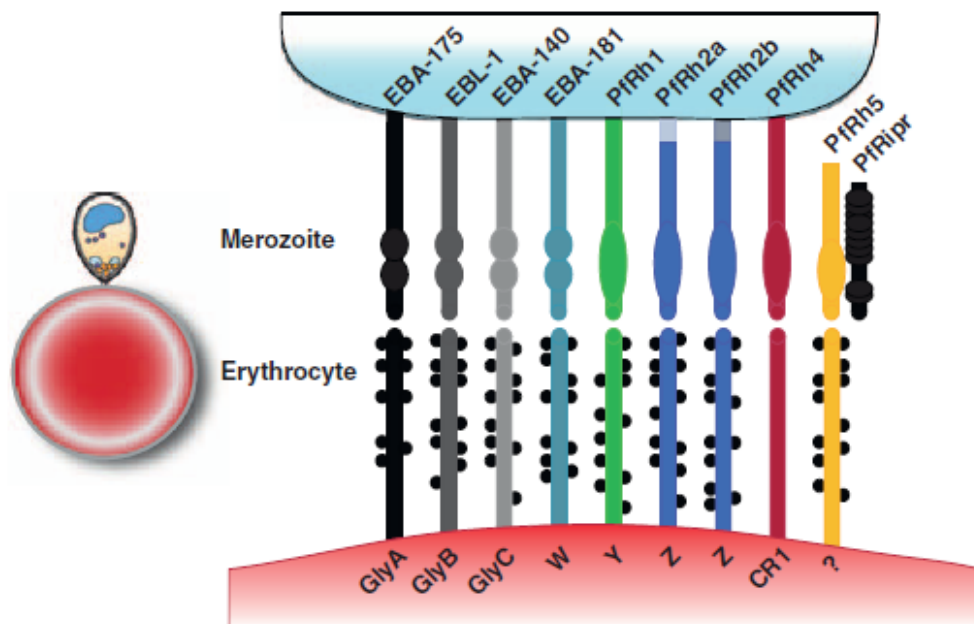


**Figure 1.6: Invasion of the erythrocyte by the *Plasmodium falciparum* merozoite.** (A) initial recognition and low-affinity, reversible binding of the merozoite ligands to erythrocyte surface receptors; (B) reorientation of merozoite with its apical region irreversibly bound to the erythrocyte membrane; (C) tight junction formation through high affinity bonds between apical merozoite ligands and erythrocyte surface receptors; (D) tight junction movement and parasite surface coat shedding from the apical to the posterior region of the parasite, powered by an actin-myosin motor; and (E) complete internalisation of the merozoite within a parasitophorous vacuole (Cowman and Crabb, 2006) (with permission).

Parasite ligands that are involved in the invasion process by binding to erythrocyte receptors belong to two critical protein families, being the erythrocyte binding-like (EBL) protein family and the *Plasmodium falciparum* reticulocyte binding-like (PfRh) protein family. Proteins of the EBL family include the erythrocyte binding antigen

(EBA) proteins: EBA-140 (also known as BAEBL), EBA-181 (also known as JESEBL), EBL-1 and EBA-175 (Camus and Hadley, 1985, Gilberger et al., 2003, Maier et al., 2002, Mayer et al., 2001, Thompson et al., 2001). Where the proteins of the PfRh family include: PfRh1, PfRh2a, PfRh2b, PfRh4 and PfRh5 (Duraisingh et al., 2003, Rayner et al., 2001, Stubbs et al., 2005, Triglia et al., 2005, Triglia et al., 2001). To date there are no reports where these EBL and PfRh proteins have been detected on the surface of the merozoite but they are stored within parasitic apical organelles, principally being in the micronemes and in the rhoptries for the EBL and PfRh protein families respectively (Ord et al., 2012). When merozoite ligands initially interact with erythrocyte receptors, signalling cascades are triggered that result in the release of EBL and PfRh proteins from the micronemes and rhoptries to the merozoite apical surface. In this way the proteins have limited exposure to the host's immune system and prove to be elusive antimalarial drug and vaccine targets (Adams et al., 1990). Merozoite surface antigens that have dominated recent malaria research include *Plasmodium falciparum* apical membrane antigen 1 (PfAMA-1) and merozoite surface protein 1 (MSP-1). PfAMA-1 is another parasite protein that is localised within the apical region of the merozoite in the rhoptry organelles and seems to be essential in apical interaction with the erythrocyte. PfAMA-1 is a leading antimalarial vaccine target (Chitnis, 2001). Due to the fact that MSP-1 is evenly distributed across the merozoite's surface, it was thought for a long time that this protein may possibly play a critical role in the initial interaction between the merozoite and erythrocyte during invasion (Perkins and Rocco, 1988). MSP-1 has in fact been reported to form a co-ligand complex with Band 3, a highly abundant transmembrane transport glycoprotein present on the erythrocyte surface. This was confirmed when it was found that two extracellular regions of Band 3, being regions 5ABC and 6A, functioned as crucial host receptors for MSP-1 carboxyl-terminal (C-terminal) products MSP-1<sub>42</sub> and MSP-1<sub>19</sub> binding (Goel et al., 2003, Li et al., 2004). The reason why no successful antibody has been made to block MSP-1 is that there is a large antigenic diversity of this protein between parasite clones (Miller et al., 1993).

Previous studies have repeatedly confirmed that glycophorin plays an important role in the attachment of *Plasmodium falciparum* merozoites to erythrocytes in the invasion process. The glycophorins are the relatively abundant and main sialoglycoproteins of the erythrocyte membrane. Glycophorin is present in different molecular forms on the erythrocyte surface, predominantly being glycophorins A, B, C and D. The receptor-ligand interactions between the merozoite and the erythrocyte glycophorins have been established. Erythrocyte binding antigens EBA-175, EBL-1 and EBA-140 have been found to bind glycophorin A, glycophorin B and glycophorin C erythrocyte surface proteins respectively. PfRh4 binds to complement receptor 1 (CR1) on the erythrocyte membrane, where the erythrocyte receptors for the other PfRh proteins remain unknown (Figure 1.7) (Camus and Hadley, 1985, Gaur and Chitnis, 2011, Gaur et al., 2004, Pasvol et al., 1982a, Pasvol et al., 1982b, Spadafora et al., 2010).

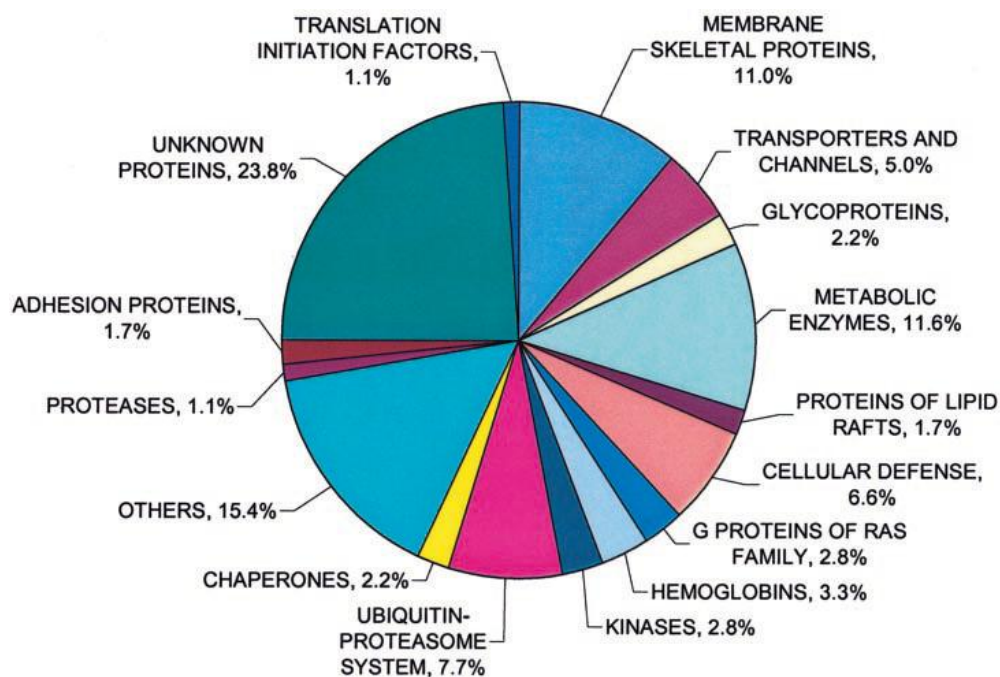


**Figure 1.7: Specific protein binding between erythrocyte surface receptors and merozoite ligands.** Highly specific binding occurs between erythrocyte membrane proteins and merozoite ligands during invasion. The EBL (EBA-175, EBA-140, EBA-181 and EBL-1) and the Pfrh (Pfrh1, Pfrh2a, Pfrh2b, Pfrh4 and Pfrh5) protein families play vital roles in the attachment of the merozoite to the erythrocyte. These parasite proteins bind to erythrocyte membrane proteins such as the glycoproteins and CR1. GlyA, B and C refer to glycoproteins A, B and C; CR1 refers to complement receptor 1; and W, Y and Z represent unknown erythrocyte receptor proteins. The black dots on the erythrocyte membrane proteins represent sialic acid (Tham et al., 2012) (with permission).

### 1.1.6 Characterising and identifying erythrocyte membrane proteins involved in invasion

Many of the erythrocyte surface proteins involved in the merozoite invasion of erythrocytes during the *Plasmodium falciparum* life cycle remain unknown. In addition to this there is a significant percentage of detected erythrocyte proteins that are unidentified (Figure 1.8) (Kakhniashvili et al., 2004). Previous studies were conducted where various enzymes and chemicals were utilised to modify the cytoskeletal and surface proteins of erythrocytes. The effects of these erythrocytic modifications on merozoites' ability to invade erythrocytes were then assessed via *in vitro* invasion assays. Invasion assays involve isolating either trophozoite or schizont stages of malaria parasites and incubating them in the wells of a microtiter plate with complete medium and enzymatically or chemically modified erythrocytes, with untreated, healthy erythrocytes plated as a control. Incubation times vary depending on the life stage of the parasites plated, but are calculated so as to ensure the release of new merozoites and allow time for invasion and growth to occur at least until the ring stage. Using blood films fixed with methanol and stained with Giemsa

followed by microscopic analysis, or more recently using SYBR Green staining and flow cytometric analysis, the percentage of newly infected erythrocytes per well is determined. By calculating the number of infected erythrocytes as a percentage of the control, the effect of specific chemical and enzyme treatments on erythrocyte invasion efficiency can be determined. Erythrocytes that have been modified either chemically or enzymatically have shown significant deficiencies in parasite invasion efficiency (Bates et al., 2010, Breuer et al., 1983, Gaur et al., 2003, Gilberger et al., 2003, Hadley et al., 1987, Miller et al., 1977, Mitchell et al., 1986). These erythrocyte modification type studies serve to help in determining characteristics of or even identifying erythrocyte proteins involved in the parasite invasion process (Table 1.1). For example it was found that full length *Plasmodium falciparum* MSP-1 is dependent on the presence of sialic acid to bind erythrocytes (Perkins and Rocco, 1988). These studies are possible because the target and the modification brought about by each enzyme and chemical is known, although it cannot be claimed that each and every specific site that can be modified by an enzyme or chemical on the erythrocyte proteins has been established.



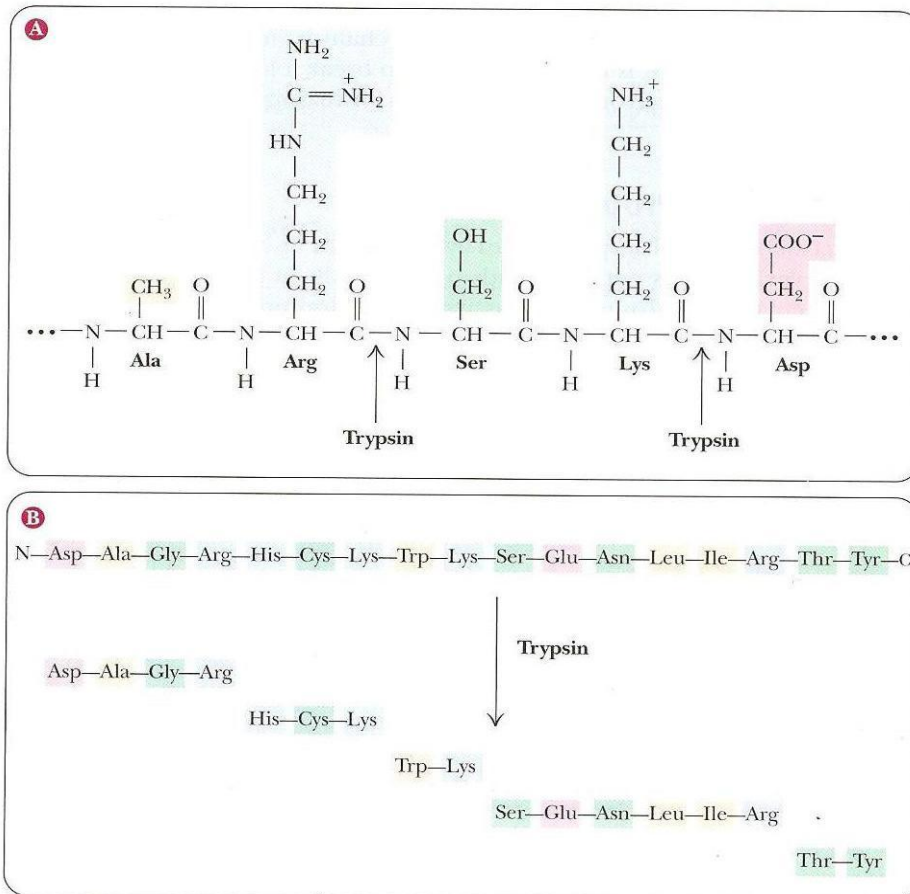
**Figure 1.8: The percentage of unidentified and identified erythrocyte proteins.** A pie chart indicating the functional categories of 181 identified erythrocyte proteins expressed as a percentage of the total amount of detected erythrocyte proteins. Almost a quarter of all detected erythrocyte proteins remain unidentified (Kakhniashvili et al., 2004) (with permission).

**Table 1.1: Interactions of EBL and PfRh proteins with host receptors**

(Gaur and Chitnis, 2011).

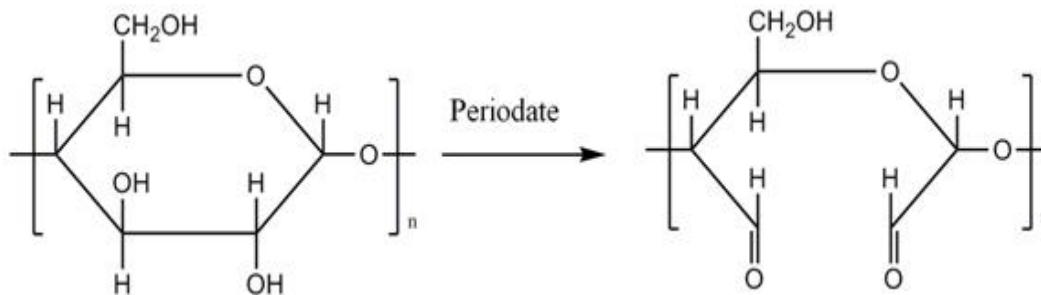
Parasite ligand	Erythrocyte receptor	Binding phenotype
<u>EBLs</u>		
EBA-175	Glycophorin A	Ns Ts Cr
EBL-1	Glycophorin B	Ns Tr Cs
EBA-140 <sup>a</sup>	Glycophorin C	Ns Tr Cr
EBA-181 <sup>a</sup>	Band 4.1	Ns Tr Cs
<u>PfRhs</u>		
PfRh1	Receptor Y <sup>b</sup>	Ns Tr Cr
PfRh2	Receptor Z <sup>b</sup>	Nr Tr Cs
PfRh4	Complement Receptor 1	Nr Ts Cs
PfRhs5 <sup>a</sup>	Receptor W <sup>b</sup>	Nr Tr Cr
N: neuraminidase treatment of erythrocytes; T: trypsin treatment of erythrocytes; C: chymotrypsin treatment of erythrocytes; s: sensitive; r: resistant. <sup>a</sup> Polymorphisms are known to affect receptor specificity. <sup>b</sup> Receptor unknown.		

Trypsin is a pancreatic serine protease enzyme that is found in the digestive systems of many vertebrates, and that has the ability to cleave specific peptide bonds of proteins through hydrolysis. Trypsin preferentially cleaves peptides at the carboxyl side of the positively charged arginine and lysine residues, except for when either of these amino acids is followed by a proline residue. When trypsin is used to digest a protein into smaller peptide fragments, the protein is said to have been trypsinised. Trypsinisation of a protein results in peptides that have Arginine or Lysine residues at their C-terminal ends and which are therefore positively charged (Figure 1.9) (Brown and Wold, 1973, Campbell and Farrell, 2008). On the erythrocyte surface, trypsin is known to cleave sialoglycopeptides from for example the glycophorins (Miller et al., 1977).



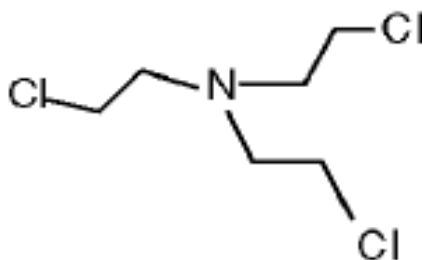
**Figure 1.9: The trypsinisation of a protein into smaller peptide fragments.** (a) Trypsin catalyses the hydrolysis of peptide bonds at the C-terminal of Arginine and Lysine amino acids. (b) Trypsinisation results in proteins being hydrolysed to form multiple polypeptides with C-terminal Lysine or Arginine residues (Campbell and Farrell, 2008) (with permission).

Sodium periodate ( $\text{NaIO}_4$ ) is an oxidising agent that is the sodium salt of periodic acid (Wee et al., 2006). Sodium periodate has the ability to cause oxidative stress in biological membranes and hence bring about modifications. As a potent oxidant, the periodate ion can modify phospholipids, sugars and amino acids in biological membranes (Heller et al., 1984). Sodium periodate has the ability to cleave carbon-carbon covalent bonds between vicinal diol groups and thus opens saccharide rings exposing aldehyde groups (Figure 1.10). This action occurs in the carbohydrate moiety of glycoproteins present on the surface of erythrocytes (Furthmayr, 1977). It has been found that all alpha-amino acids are modified by the oxidative power of periodate, however this occurs at different rates and to varying extents depending on the specific amino acid. Methionine and cysteine have been found to be the amino acids most extensively affected by periodate in soluble proteins. Sodium periodate has been shown to cause the complete cross-linking of the erythrocyte cytoskeletal protein spectrin, and the partial cross-linking of other erythrocyte polypeptides (Clamp and Hough, 1965, Gahmberg et al., 1978, Gorin and Godwin, 1966, Rippa et al., 1981, Yamasaki et al., 1982).



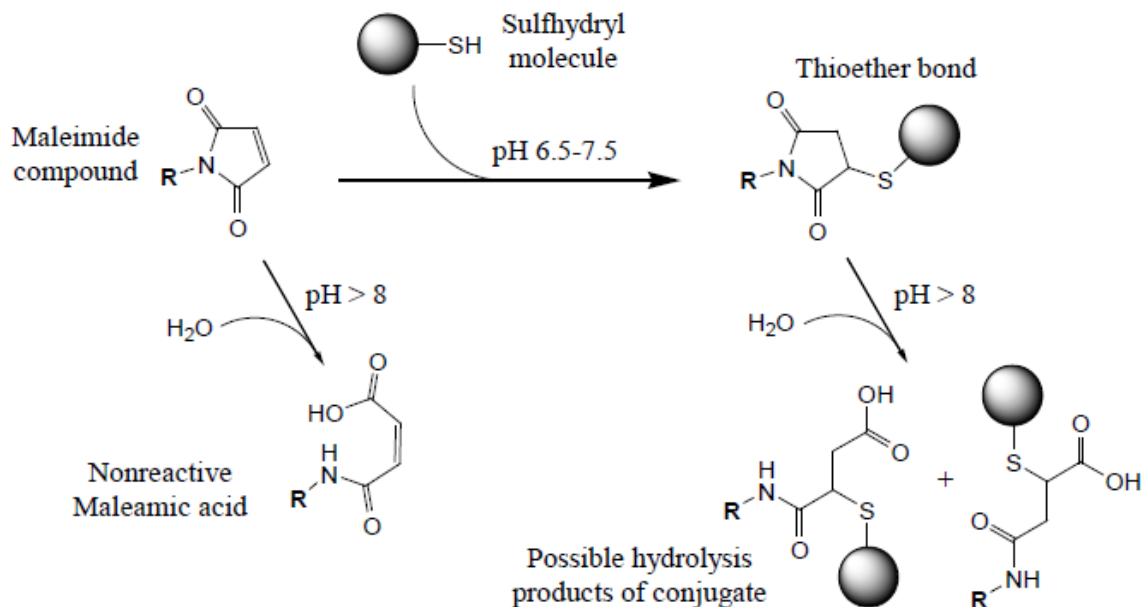
**Figure 1.10: Sodium periodate oxidises sugars with vicinal hydroxy groups and this results in the formation of aldehyde groups** (Song et al., 2009).

Tris(2-chloroethyl)amine (TCEA) is a cytotoxic, antitumor alkylating agent (Figure 1.11). This amine cross-linker prevents changes in cell shape, including that of the erythrocyte. TCEA has been found to alter the spectrin network bringing about spectrin polymerisation and the cross-linking of various other erythrocyte membrane proteins. This leads to a fixed and rigid erythrocyte membrane. Furthermore, TCEA has the ability to permeate the erythrocyte membrane and enter the cytoplasm causing the alkylation of haemoglobin to haemoglobin dimers (Breuer et al., 1983, Wildenauer and Weger, 1979, Wildenauer et al., 1980) (with permission).



**Figure 1.11: Structure of trifunctional tris(2-chloroethyl)amine** (Gresham et al., 2000) (with permission).

The cross-linker 1,11-bis(maleimido)triethylene glycol (BM(PEG)<sub>3</sub>) selectively conjugates the sulfhydryl (SH) groups of proteins. This homobifunctional, bismaleimide cross-linker reacts with SH groups via its maleimide group to form a stable thioether linkage. At pH 6.5-7.5 the reaction between sulfhydryls and maleimide is highly specific, however at pH values greater than 8, although at a significantly reduced reaction rate, maleimides have the ability to react with primary amines (Figure 1.12) (Smyth et al., 1964, Thermo Fisher Scientific, 2012).



**Figure 1.12: BM(PEG)<sub>3</sub> is a selective thiol cross-linker which conjugates proteins through their respective sulfhydryl groups** (Thermo Fisher Scientific, 2012) (with permission).

Studies involving the modification of erythrocyte proteins by utilising enzymes and chemicals followed by *in vitro* invasion assays have the potential to identify unknown erythrocyte surface or skeletal proteins involved in merozoite invasion, as well as possibly discover an erythrocyte protein that is vital for invasion across all *Plasmodium* species. Studies carried out 30-40 years ago used similar surface modification approaches but showed little success due to limited modifications that could be detected using the techniques available at that time. Therefore applying advanced scientific techniques available today to these studies together with new selective labelling utilising different chemicals and enzymes, offers new insights and opportunities for identifying erythrocyte proteins involved in *Plasmodium falciparum* invasion (Gilberger et al., 2003, Hadley et al., 1987, Miller et al., 1977, Mitchell et al., 1986, Orlandi et al., 1992, Perkins, 1981).

### 1.1.7 The 'intelligence' of the malaria parasite

The fact that malaria is caused by the *Plasmodium* parasite was discovered over a hundred years ago and it has been almost forty years since Dvorak *et. al.* made groundbreaking descriptions of the malaria parasite's invasion of the human erythrocyte (Cowman and Crabb, 2006, Dvorak et al., 1975). Furthermore millions of U.S dollars have been spent on malaria research, just one example being the \$750 million donated by the Bill & Melinda Gates Foundation to the Global Alliance for Vaccines and Immunisation. Yet still the disease's mechanisms of action are not completely understood and as such malaria is yet to be globally eliminated (Murray et al., 2012).



*Plasmodium falciparum* is an 'intelligent' parasite and is difficult to hinder due to a number of factors. Upon egress from an infected erythrocyte, the merozoite recognises and invades a healthy erythrocyte within seconds to minutes at most. The merozoite is thus exposed to the immune system in the blood stream for a very short period of time, and therefore even though the immune system is capable of recognising and responding to antigens on the merozoite surface, the time is too short to mount a full immunological response to effectively destroy the merozoite (Dvorak et al., 1975).

Different strains of the *Plasmodium falciparum* parasite are each capable of utilising different erythrocyte receptor-merozoite ligand pairs during invasion and therefore trying to block the few known receptor-ligand interactions between the erythrocyte and parasite is proving ineffective, as no interactions have found to be crucial in all *Plasmodium* strains (Boyle et al., 2010, Crosnier et al., 2011, Riglar et al., 2011). Furthermore merozoites of any one such *Plasmodium falciparum* or *Plasmodium knowlesi* strain do not depend solely on a single invasion pathway and are capable of utilising multiple redundant invasion pathways (Chitnis, 2001, Crosnier et al., 2011). This means the parasite is able to adapt to proteomic variations in erythrocyte receptors via post-transcriptional regulation. Through this mechanism the parasite has the ability to make use of alternative invasion pathways. For example *Plasmodium falciparum* has the ability to use both a sialic acid-dependent and sialic acid-independent pathway, and this is reported to be due to the transcriptional activation of a single parasite ligand. When using the sialic acid-dependent pathway the erythrocyte receptor-parasite ligand pairing would be for example glycophorin A with EBA-175 or glycophorin C/D with EBA-140. The identity of the erythrocyte receptor-parasite ligand pair utilised in the sialic acid-independent pathway remains unknown, however the erythrocyte receptor has been dubbed 'Receptor X' in literature (Ganczakowski et al., 1995, Gaur et al., 2004, Mgone et al., 1996). There are also changes in parasite protein expression which occur during invasion pathway switching, that are not regulated during the transcription process and are controlled by an unknown mechanism (Kuss et al., 2012).

Different *Plasmodium falciparum* strains have different levels of PfRh protein expression, which affects the invasion pathway used. For example up regulation of a neuraminidase (a sialidase that catalyses the hydrolysis of terminal sialic acid residues) resistant parasite ligand (e.g. PfRh4) results in a switch from sialic acid-dependent to sialic acid-independent invasion pathway utilisation, and *vice versa* for high expression levels of neuraminidase sensitive parasite ligands (e.g. PfRh1) (Duraisingh et al., 2003, Iyer et al., 2007, Stubbs et al., 2005). Some *Plasmodium falciparum* laboratory strains have the ability to utilise sialic acid residues on different sialoglycopeptides during invasion i.e. switch from binding glycophorin A to binding glycophorin B. The significance of this is that the parasite is not totally dependent on one sialoglycopeptide, in this case being glycophorin A. Furthermore, making use of genetically deficient erythrocytes, studies have found that *Plasmodium falciparum* is

not completely dependent on any individual receptor or on the sialic acids of the human erythrocyte for invasion to occur. This means that none of the receptor-deficient erythrocytes tested were completely resistant to parasite invasion. Therefore the malaria parasite has a great advantage when it comes to circumventing obstacles such as receptor heterogeneity in various host populations, as well as the host's immune system response (Chitnis, 2001, Dolan et al., 1990, Gaur et al., 2004, Hadley et al., 1987, Mitchell et al., 1986, Perkins and Holt, 1988).

By having the ability to use multiple invasion pathways the *Plasmodium falciparum* parasite is able to evade the human immune system. This is typically achieved in a number of ways. Firstly, if an immune response is built up against any of the immuno-dominant epitopes of the EBL proteins, there are alternative proteins to which there is no immune response and thus little chance of invasion being completely blocked. Secondly the parasite has the ability to invade erythrocytes of different ages, as the quantity and presence of erythrocyte surface proteins change throughout the cell's lifespan. Lastly, it can therefore be stated that in spite of the large amount of polymorphisms found in human erythrocyte surface proteins, there have been no known erythrocytes that are completely resistant to *Plasmodium falciparum* (Cowman and Crabb, 2006, Gaur et al., 2004, Miller et al., 2002). Utilising complete invasion pathways as antimalarial drug or vaccine targets may be impractical, however targeting the multiple molecular mechanisms that make up the pathways offers hope for identifying targets for therapeutic interventions (Pasvol, 2003).

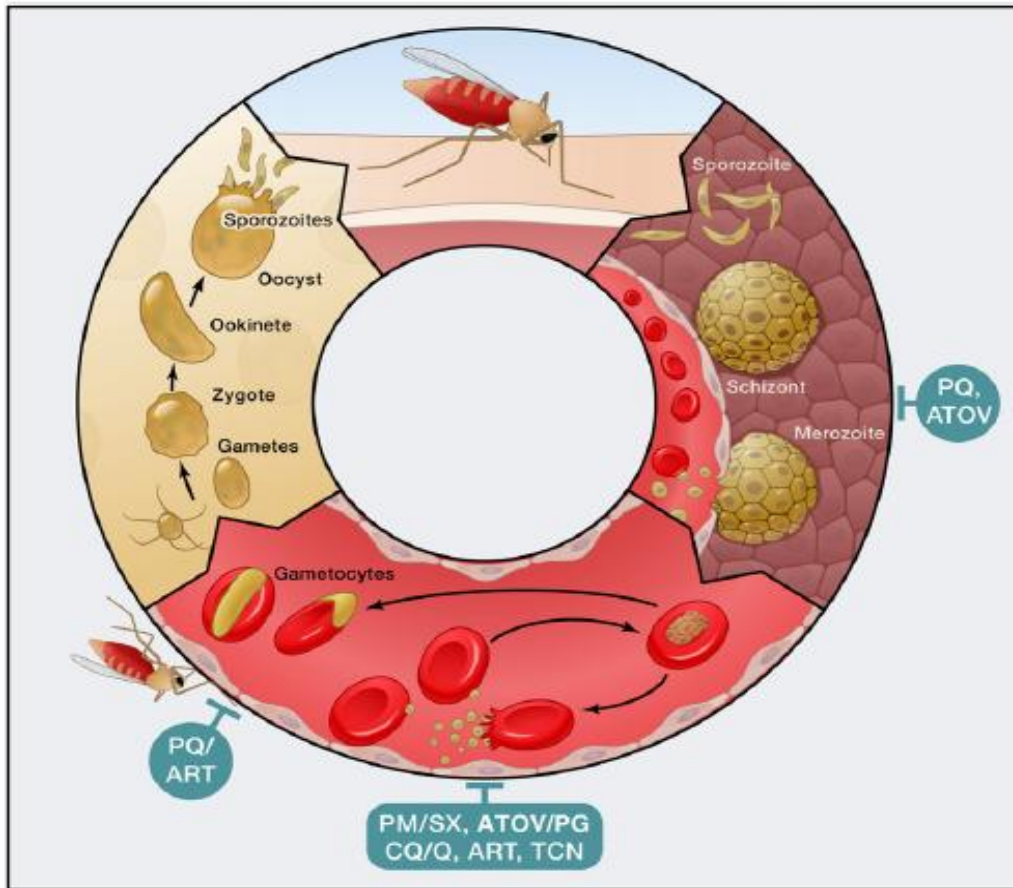
The 'intelligence' of the malaria parasite is further witnessed in the fact that it targets erythrocytes, cells that are abundant and fragile, and does so without damaging the erythrocyte in any way. This allows the parasite to utilise the erythrocyte to 'hide' from the immune system, as well as multiply. Another evasive tactic of the parasite is to shed the merozoite surface antigens during entry into the erythrocyte, leaving a high concentration of surface proteins in the blood stream to act as a decoy mechanism for the host's immune system.

### **1.1.8 Malaria prophylaxis**

The best way to prevent malaria is to prevent people being bitten by infected mosquitoes, i.e. vector control, and therefore avoid the disease completely by utilising methods such as mosquito nets and insecticides. This however is not always possible as can be seen in the WHO World Malaria Report of 2011, where only 11% of the African population considered to be at risk of contracting malaria, had access to indoor residual insecticide spraying. Thus antimalarial drugs remain vital in both the prevention and the acute treatment of malaria (Murray et al., 2012, WHO, 2012).

There have previously been antimalarial chemotherapies that were successful, however the *Plasmodium falciparum* parasite has become resistant to almost every

antimalarial drug used in the chemoprophylaxis and the treatment of malaria. Different antimalarial drugs act at different stages of the parasite's life cycle, where drugs used to treat a malaria infection typically act on parasites in the blood (i.e. on intraerythrocytic parasites) and chemoprophylactic drugs act on merozoites emerging from the liver (Figure 1.13). Antimalarial drugs in use today include the quinolone derivatives, the antifolates and the artemisinin derivatives. The quinolone derivatives include mefloquine, quinine and chloroquine which are blood schizonticidal agents, and primaquine which has gametocidal action (Rang et al., 2007). Chloroquine was once a mainstream drug prescribed for both the prophylaxis and treatment of malaria, which is now outdated in most endemic malaria areas due to parasite resistance that has spread rapidly (Becker et al., 2010, Goldberg et al., 2012). Sulfadoxine-pyrimethamine and atovaquone-proguanil make up the antifolates. Sulfadoxine-pyrimethamine is a blood schizonticide and sporontocide, while atovaquone-proguanil inhibits mitochondrial electron transportation in the parasite (Finkel et al., 2009). A drug known as artemisinin, a blood schizonticide, is currently the only drug still effective against *Plasmodium falciparum*. The artemisinin derivatives artesunate (a water-soluble derivative), artemether and artether (synthetic analogues) are the preferred antimalarials, as they have a higher activity and are more easily absorbed than artemisinin which is a poorly soluble chemical (Rang et al., 2007). The first signs of resistance against artemisinin however have recently been noted in Cambodia, Myanmar, Thailand and Vietnam and although the evidence is not yet compelling, artemisinin should no longer be used as a monotherapy (WHO, 2012). To support this statement is the occurrence of the 'dormancy phenomenon' where after effective treatment with artemisinin, there is a recrudescence of *Plasmodium falciparum* parasites.



**Figure 1.13: Antimalarial drugs acting at different stages during the *Plasmodium falciparum* life cycle.** The different stages of the life cycle of the malaria parasite are shown where, red: intraerythrocytic stages; brown: liver stages; yellow: mosquito stages. The malaria parasite has shown some degree of resistance to all of these drugs except artemisinin. PQ: primaquine; ATOV: atovaquone; PM: pyrimethamine; SX: sulphadoxine; PG: proguanil; CQ/Q: 4-aminoquinolines like chloroquine and quinine and related compounds; ART: artemisinin and related compounds; TCN: tetracyclines (Goldberg et al., 2012) (with permission).

The mutation rate of the malaria parasite, the amount of mutations required to bring about resistance, pharmacokinetic and pharmacodynamic aspects, as well as the quality, misuse and abuse of antimalarial drugs all contribute to the rate at which antimalarial drug resistance develops (Goldberg et al., 2012, White, 2004). Due to drug resistance the WHO recommends that monotherapies no longer be used even in the treatment of uncomplicated malaria infections and hence, most malaria-endemic countries have adopted artemisinin-based combination therapy (ACT) treatment policies. ACTs are the first line of treatment for *Plasmodium falciparum* recommended by the WHO, where artemisinin and its derivatives are used in combination with other antimalarial drugs in order to reduce the chances of drug resistance and improve the overall effectiveness of the treatment. The antimalarial drugs that are combined with artemisinin and its derivatives should have variant mechanisms of action, as well as longer half-lives than that of artemisinin and the

derivatives. This serves to eliminate any remaining parasites in the blood after the initial mass removal of malaria parasites by artemisinin (WHO, 2001, WHO, 2010a, WHO, 2012). Combination therapy reduces the probability for the selection of drug resistant malaria parasites because the chances of a mutation occurring in the parasites that allows for the resistance against two antimalarial drugs with different mechanisms of action are very low (White, 1999). The following five antimalarial drug combinations are currently recommended: Artemether-lumefantrine, artesunate-amodiaquine, artesunate-mefloquine, artesunate-sulfadoxine-pyrimethamine and dihydroartemisinin-piperaquine (WHO, 2010b). Some newer antimalarial treatments have failed, where in some instances drug resistance has occurred almost simultaneously with the drug's introduction (Hyde, 2005). Rapid development of the drug-resistant forms of the malaria parasite highlights the urgency for constant research and innovative strategies to discover and provide effective antimalarial drugs before resistant *Plasmodium* variants make malaria virtually untreatable. This relies greatly on the identification of new potential drug targets (Smit et al., 2010).

Malaria has been said to be a 'disease of poverty' due to being widespread in developing countries. Previously, new and effective antimalarial drugs such as chemical derivatives of artemisinin have been unaffordable and limited in access when it comes to the poverty-stricken countries and villages that heavily rely on them. These expensive and sometimes inaccessible treatments are partly the reason as to why malaria-associated mortality remains high in Africa (Girard et al., 2007, Miller et al., 2002). Furthermore in these developing countries, especially in the rural areas where the majority of the people that should be taking antimalarial drugs reside, there is a lack of health professional access. This means that many of the antimalarial drugs are consumed by self-treatment and are done so inadequately. For example, in Brazil it was found that recurrent malaria correlated with poor antimalarial drug adherence (Duarte and Gyorkos, 2003). Previous studies found that in Burkina Faso 69% of febrile patients suffering from malaria were self-treated, and such was the case in 67% of febrile patients in Ethiopia (Deressa et al., 2003, Müller et al., 2003). Thus convenient packaging, easy to administer and well tolerated drug regimens, as well as education concerning symptoms and various antimalarial drug treatments is imperative when it comes to the successful treatment of self-treating rural populations of endemic malaria regions (Baird, 2005).

During the past few years progress has been reported in the development of a malaria vaccine. Yet even though this progress has been significant, a licensed vaccine is still not commercially available. This is in part due to the difficulty in identifying appropriate antigen vaccine targets. Contributing to this difficulty is differential protein expression by the parasite. That is throughout the life cycle of the malaria parasite, surface proteins (potential vaccine targets) and metabolic proteins (potential drug targets) change significantly and therefore for antimalarial vaccines and drugs to be effective, they will have to target proteins expressed at multiple

specific stages during the parasite's life cycle (Florens et al., 2002, Girard et al., 2007).

The current antimalarial medicines, equipment and techniques are beginning to fail within certain subpopulations. For example women in their first trimester of pregnancy have very limited options when it comes to protecting themselves from malaria (Goldberg et al., 2012). In addition the antimalarial drugs currently available all have adverse effects and contraindications, with some even carrying the risk of severe adverse events (Table 1.2) (Baird, 2005). It is imperative that new antimalarial agents and treatment strategies are developed which have the ability to avoid resistance and are easily accessible, affordable and safe (Miller et al., 2002).

**Table 1.2: Safety and tolerability of available antimalarial drugs**

(Baird, 2005, Phillips-Howard and Wood, 1996, Taylor and White, 2004, Wernsdorfer, 2004).

Drug	Adverse Effects	Contraindications
Chloroquine	Gastrointestinal upset, itching, dizziness	Epilepsy
Sulfadoxine-pyrimethamine	n/a	Pregnancy, renal disease
Quinine	Tinnitus, vertigo, headache, fever, delirium, nausea	Pregnancy, optic neuritis, tinnitus, blackwater fever
Mefloquine	Vomiting, headache, insomnia, vivid dreams, anxiety, dizziness	Depression, schizophrenia, anxiety disorder, any psychosis
Atovaquone-chloroguanide	Gastrointestinal upset, headache, stomatitis	Weight <11kg in children, pregnancy, breast-feeding, renal impairment
Artemether-lumefantrine	Dizziness, palpitations	Pregnancy, severe malaria
Artesunate-mefloquine	Vomiting, anorexia, diarrhoea	Depression, schizophrenia, anxiety disorder, any psychosis
Halofantrine	Gastrointestinal upset, prolonged QTc	Conduction abnormalities, pregnancy, breast-feeding, infancy, use of mefloquine
Primaquine	Gastrointestinal upset, elevated levels of methemoglobin	Pregnancy, breast-feeding, G6PD deficiency

G6PD: glucose-6-phosphate dehydrogenase  
 QTc: QT interval corrected for heart rate

### 1.1.9 Gel electrophoresis and proteomics

Two-dimensional gel electrophoresis (2-DE) has been prominent in proteomic studies since the 1970's, where this technique is used to separate proteins based on both their size and isoelectric point (pI) values (Klose, 1975, Macgillivray and Wood, 1974, O'Farrell, 1975). Proteins are first separated based on their pI values by a process known as isoelectric focusing (IEF), after which they are separated

according to size by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). The power of 2-DE in proteomics however was only completely revealed when it was linked with the microanalytical technique of mass spectrometry, which has the high sensitivity and specificity required to accurately and reliably, quantitate, sequence and identify proteins using various protein databases, including the characterisation of those proteins with post-translational modifications (Lahm and Langen, 2000, Wilkins et al., 1999). Despite the great advantages of 2-DE coupled with mass spectrometry, this technique too has its disadvantages. These include the lengthy sample preparation and run times, poor protein extraction of certain classes of protein, solubility limits during IEF especially when it comes to lipophilic proteins such as membrane proteins, the apparent “loss” of proteins that have extreme pI values and the lack of dynamic range of detectable proteins. Another complicating factor of 2-DE is that resulting protein spots can be due to more than one protein (Rabilloud, 2002, Tannu and Hemby, 2006). The weaknesses of 2-DE have forced researchers to consider alternative proteomic options, whereby IEF would not be utilised. The most apparent option is to simplify the process, eliminating the first dimension completely and analysing proteins only by SDS PAGE (Rabilloud, 2002).

SDS PAGE coupled with mass spectrometry has been found to be a very successful proteomic technique. However in complex protein fractions where the likelihood of an apparently single band containing more than one protein is high, protein quantitation is not possible. In comparison if the aim is to identify all the proteins present in a sample then this approach has previously been found to be highly reliable and accurate (Bell et al., 2001, Husi et al., 2000, Rabilloud, 2002). In addition, membrane proteins (including erythrocyte membrane proteins) which are known to be difficult to analyse when in native form, can be extracted from lipids effectively during denaturing conditions as used in SDS PAGE. This results in a more accurate representation on SDS PAGE than on 2-DE with improved protein representation (Low et al., 2002, Rabilloud, 2009).

### **1.1.10 In summary**

The *Plasmodium falciparum* parasite is a complex and well developed organism that has been able to co-evolve and survive in both vertebrates and mosquitoes for thousands of years. In order to potentially stop this deadly parasite with new innovative therapies, its biology needs to be more properly understood and in this area, proteomic analysis of its surface proteins and protein interactions is vital. The advantage of determining the surface proteomics of merozoites and erythrocytes during invasion is the possible treatment strategies that may eradicate the parasite before it has even entered the erythrocyte and manifested clinical symptoms. Determining the relative importance of the different receptor-ligand interactions between the parasite and erythrocyte as well as obtaining a better understanding of the molecular mechanisms during a central process such as invasion, may lay a

foundation for the discovery of new key targets for both drug- and vaccine-based antimalarial strategies which should be a global health priority (Florens et al., 2002, Riglar et al., 2011).

## 1.2 Scope of the Study

### 1.2.1 Study motivation

Malaria affects hundreds of millions of people globally each year, leading to almost a million deaths per year and making malaria the number one killer of children under the age of five in sub-Saharan Africa (Cowman and Crabb, 2006, Florens et al., 2002, Smit et al., 2010).

As of yet there is no successful antimalarial vaccine available on the market, nor is there an antimalarial drug available to which the *Plasmodium* parasite has not started to show signs of resistance (Girard et al., 2007, Goldberg et al., 2012, WHO, 2012). In addition, new and effective antimalarial drugs tend to be expensive and not always easily accessible, if accessible at all, to the rural populations that so desperately require them (Girard et al., 2007, Miller et al., 2002).

New key targets are required for the development of successful antimalarial drug- and vaccine-based treatment strategies. Proteomic analysis of the specific interactions occurring between merozoite ligands and erythrocyte receptors when the *Plasmodium* merozoite invades a healthy erythrocyte, offers the opportunity of discovering such key targets. The possibility of identifying a yet unexplored antimalarial drug or vaccine target that is vital for the invasion of erythrocytes across all *Plasmodium* species, has the potential to eradicate malaria in a manner that is affordable, simple and easily accessible to the public. Blocking the target could potentially alleviate the unbearable clinical symptoms one suffers after contracting malaria (because it would potentially block the invasion process and the clinical symptoms manifested post-invasion), and could be the cure to malaria that is in such dire need (Cowman and Crabb, 2006).

### 1.2.2 Study aim

To confirm known and possibly identify new erythrocyte surface antigens involved in the initial recognition and binding of the *Plasmodium falciparum* merozoite ligands during parasite invasion by applying protein modification and labelling techniques followed by mass spectrometric proteomic methods.



### 1.2.3 Study objectives

- i. To culture sufficient numbers of highly synchronised *Plasmodium falciparum* parasites
- ii. To optimise erythrocyte ghost isolation and sample preparation to ensure reproducible protein fingerprints on SDS PAGE gels
- iii. To use enzyme and chemical treatments to effectively alter erythrocyte proteins prior to ghost preparation and parasite invasion assays
- iv. Assess protein fingerprints of SDS PAGE separation of erythrocyte ghosts to determine the effects of the selected enzymes and chemical modifications on surface and cytoskeletal erythrocyte proteins by LC-MS/MS
- v. Assess the effects of enzyme treatments and chemical modifications on parasite invasion of treated erythrocytes using fluorescence techniques
- vi. Identification of possible erythrocyte proteins acting as *Plasmodium falciparum* receptors during the initial recognition and binding phases of invasion using sequential electrophoretic and mass spectrometric methodologies

## Chapter 2: Materials and Methods

### 2.1 Erythrocyte Ghost Preparation

#### 2.1.1 Erythrocyte ghost isolation

##### Aim

To optimise erythrocyte ghost preparation methods to perform erythrocyte membrane and cytoskeletal protein studies.

##### Materials

i. **Phosphate buffered saline (PBS)/Ethylenediaminetetraacetic acid (EDTA) solution**

FTA Hemagglutination buffer was purchased from BD Biosciences (Sparks, USA). A solution was made up at 9.23 g/l as per product instructions, with deionised water. The pH of the PBS solution was increased to pH 8 using a 4 M sodium hydroxide (NaOH) solution. EDTA was purchased from BDH Chemicals (Poole, England) and made up to 1 mM in PBS. The solution was stored at 4°C until used.

ii. **Phenylmethylsulfonyl fluoride (PMSF)**

PMSF was purchased from Sigma-Aldrich (St Louis, USA). Stock solutions were made up at 100 mM in 96% ethanol purchased from Sigma-Aldrich (St Louis, USA). Stock solution aliquots of 500 µl were stored in 1.5 ml microcentrifuge tubes at -80°C until used.

iii. **Deionised water/EDTA solution**

Deionised water was adjusted to pH 8 using a 0.5 M NaOH solution, after which 1 mM EDTA was added. The solution was stored at 4°C until used.

##### Methods

The erythrocyte ghost isolation method was based on the method described by Dodge *et. al.* (1963).

A volume of 8 ml of blood was collected into two 4 ml EDTA BD Vacutainer® tubes from healthy, consenting volunteers. The blood was centrifuged at 1800 x g for 10 minutes, after which the plasma and buffy coat were removed. The remaining packed red blood cell (PRBC) pellets were washed thrice in triple their volume of a PBS/EDTA/PMSF solution re-sedimenting the cells at 450 x g for 10 minutes each time. Stock solutions of PMSF were added to the PBS/EDTA solution just before washing (a 1/100 dilution into solution), as PMSF is only stable for 30 minutes in aqueous solutions.

The PRBC pellets were transferred to 50 ml tubes and haemolysed by filling the tubes with deionised water (d.H<sub>2</sub>O)/EDTA/PMSF solution (PMSF stock solutions were added just before use). The 50 ml tubes were left on ice for 10 minutes to allow for complete haemolysis, after which they were centrifuged at 3000 x g for 10 minutes. The resulting supernatants were transferred to new 50 ml tubes and the remaining erythrocyte ghost pellets were again resuspended in the d.H<sub>2</sub>O/EDTA/PMSF solution. Tubes containing supernatant were centrifuged at 3000 x g for 10 minutes and the resulting pellets were combined with the original RBC ghost cells.

The ghost pellets were transferred to a single 1.5 ml microcentrifuge tube, resuspended in a PBS/EDTA/PMSF solution and centrifuged at 16 000 x g for 10 minutes. The resulting ghost pellet was removed from the protease-rich granules and fibrous pellets at the bottom of the microcentrifuge tube. The ghost pellet was then washed with a PBS/EDTA/PMSF solution until the haemoglobin was removed and only a 'clean', white ghost pellet remained. The resulting 100 µl ghost pellet was resuspended in 200 µl of a PBS/EDTA/PMSF solution and stored at -80°C until used.

### 2.1.2 Bicinchoninic acid (BCA) protein assay

#### Aim

To determine the protein concentration of solutions derived from erythrocyte ghosts in mg/ml.

#### Materials

##### i. Reagent A

A volume of 100 ml solution in deionised water was made up by dissolving 1 g bicinchoninic acid (BCA) disodium salt hydrate purchased from Sigma-Aldrich (St Louis, USA), 5.4 g sodium carbonate decahydrate (Na<sub>2</sub>CO<sub>3</sub>·10H<sub>2</sub>O) purchased from UniLab (Krugersdorp, RSA), 0.21 g disodium tartrate dihydrate (Na<sub>2</sub>C<sub>4</sub>H<sub>4</sub>O<sub>6</sub>·2H<sub>2</sub>O), 0.40 g sodium hydroxide (NaOH) both purchased from Merck (Darmstadt, Germany) and 0.95 g sodium bicarbonate (NaHCO<sub>3</sub>) purchased from Sigma-Aldrich (St Louis, USA). The pH of the solution was adjusted to 11.25 with 10 M NaOH. Reagent A was stored at 4°C until use.

##### ii. Reagent B

An amount of 0.26 g cupric sulfate (CuSO<sub>4</sub>) purchased from Rochelle Chemicals (Johannesburg, RSA) was dissolved and made up to 10 ml with deionised water. Reagent B was stored at 4°C until used.

iii. **Phosphate buffered saline (PBS)**

FTA Hemagglutination buffer was purchased from BD Biosciences (Sparks, USA). A solution was made up at 9.23 g/l as per product instructions, with deionised water. The solution was stored at 4°C until used.

iv. **Protein standards**

Bovine serum albumin (BSA) was purchased from Santa Cruz Biotechnology, Inc, (Dallas, USA). A 2.5 mg/ml (37.6 mM) BSA stock solution was prepared by adding 7.5 mg of BSA into 3.0 ml of PBS. A range of BSA protein standards of varying concentrations: 2.5 mg/ml, 2.0 mg/ml, 1.0 mg/ml, 0.5 mg/ml, 0.4 mg/ml, 0.25 mg/ml, 0.2 mg/ml and 0.1 mg/ml were prepared individually from the 2.5 mg/ml BSA stock solution to a volume of 1 ml using PBS. A quality control BSA protein standard was also prepared in PBS independently of the stock solution at 2.1 mg/ml. Protein standards were stored at 4°C until used for up to 1 month.

## Methods

The bicinchoninic acid (BCA) protein assay as first described by Smith *et. al.* (1985) was used to determine the protein concentration of erythrocyte ghosts. Briefly, a standard working reagent (SWR) was made by mixing 50 volumes of reagent A with 1 volume of reagent B. Using a round-bottomed 96-well plate, 250 µl of SWR and 5 µl of each BSA protein standard (or quality control) as well as the ghost sample were added to the wells of the plate in triplicate. The plate was shaken gently for 10 minutes and was then incubated at 37°C for 30 minutes. The samples were cooled at room temperature and the absorbance, at a single wavelength of 562 nm, of the wells were measured using an ELx800 UV universal microplate reader (Bio-Tek Instruments Inc., Vermont, USA).

Using GraphPad Prism version 5.00 (GraphPad Software, San Diego California, USA) the absorbance values of the BSA protein standards were used to plot a calibration curve of absorbance ( $A_{562}$ ) versus protein concentration (mg/ml). The protein concentrations of the erythrocyte ghost solutions were determined from the linear regression of the standard curve (mg/ml).

### 2.1.3 Erythrocyte ghost solubilisation

#### Aim

To assess the ability of different protein solubilisation treatments to produce high quality erythrocyte ghost protein fingerprints on SDS PAGE.

#### Materials

i. **Solubilisation solution 1**

Amounts of 2.1 g (7 M) urea purchased from BDH Chemicals (Poole, England), 760 mg (2 M) thiourea, 38.55 mg (50 Mm) dithiothreitol (DTT), 100 mg (2%) amidosulfobetaine-14 (ASB-14) all purchased from Sigma-Aldrich (St Louis,

USA) and 20  $\mu$ l (0.4%) ampholytes (pH 3-10) purchased from Bio-Rad Laboratories, Inc. (Berkeley, USA) were weighed out, added to a volumetric flask and brought to a volume of 5 ml with deionised water. The solution was sonicated for 5 minutes and stored at 4°C until used. The solution could be stored for up to a week but required gentle warming to ensure it was completely dissolved before use.

ii. **Solubilisation solution 2**

A volume of 5 ml solution in deionised water was made up by dissolving 50 mM DTT, 2% ASB-14 and 0.4% ampholytes. The solution was sonicated for 5 minutes and stored at 4°C until used. The solution could be stored for up to a week.

iii. **Solubilisation solution 3**

A volume of 5 ml solution in deionised water was made up by dissolving 7 M urea, 2 M thiourea, 50 mM DTT and 0.4% ampholytes. The solution was sonicated for 5 minutes and stored at 4°C until used.

The solution could be stored for up to a week but required gentle warming to ensure it was completely dissolved before use.

## **Methods**

An erythrocyte ghost sample was prepared according to the methods described in Section 2.1.1, with a total packed volume of approximately 300  $\mu$ l. The sample was aliquoted into four microcentrifuge tubes containing 70  $\mu$ l of sample each. Only PBS (50  $\mu$ l) was added to the first tube (the control tube), 50  $\mu$ l of Solubilisation solution 1 was added to tube 2, 50  $\mu$ l of Solubilisation solution 2 was added to tube 3 and 50  $\mu$ l of Solubilisation solution 3 was added to tube 4. The tubes were briefly sonicated and incubated at room temperature for 1 hour. All four samples were then prepared for SDS PAGE as described in the methods of Section 2.3.

## **2.2 Enzyme and Chemical Treatments**

### **2.2.1 Enzyme treatments**

#### **Aim**

To alter erythrocyte surface proteins by enzymatic cleavage to assess the effect on parasite invasion.

## Materials

- i. **Phosphate buffered saline (PBS)/Ethylenediaminetetraacetic acid (EDTA) solution**  
As described in Section 2.1.1 sub-paragraph i.
- ii. **Phenylmethylsulfonyl fluoride (PMSF)**  
As described in Section 2.1.1 sub-paragraph ii.
- iii. **Deionised water/EDTA solution**  
As described in Section 2.1.1 sub-paragraph iii.
- iv. **Trypsin/versene solution**  
A trypsin/versene solution (for cell culture applications) containing 0.25% trypsin and 0.1% EDTA in calcium and magnesium free PBS was purchased from Highveld Biological (Johannesburg, RSA). The solution was stored at 4°C.
- v. **N-hydroxysuccinimide (NHS)-Fluorescein/dimethyl sulfoxide (DMSO) solution**  
Fluorescein-5-EX N-hydroxysuccinimide ester was purchased from Sigma-Aldrich (St Louis, USA) and stored at 4°C. Just before use a solution was made up by dissolving 3 mg of fluorescein derivative in 300 µl of DMSO purchased from Sigma-Aldrich (St Louis, USA).
- vi. **Glycine solution**  
Glycine was purchased from Sigma-Aldrich (St Louis, USA) and dissolved in PBS/EDTA solution (as described in sub-paragraph i) at a concentration of 13 mM just before use.

## Methods

The erythrocyte ghost isolation methods described in Section 2.1.1 were followed with modifications for trypsin treatment.

A volume of 8 ml of blood was collected into two 4 ml EDTA BD Vacutainer<sup>®</sup> tubes from a healthy, consenting volunteer. The blood was centrifuged at 1800 x g for 10 minutes, after which the plasma and buffy coat were removed. The remaining packed red blood cell (PRBC) pellets were washed thrice in triple their volume of a PBS/EDTA/PMSF solution at 450 x g for 10 minutes. No stock solutions of PMSF were added to the PBS/EDTA solution before washing for the trypsin treatments, as PMSF is an inhibitor of trypsin.

### Trypsin treatment:

To each PRBC pellet 8 ml of a trypsin/versene solution was added, resulting in a final concentration of 1 mg/ml of trypsin in the sample. The samples were gently shaken and incubated for 1 hour at 37°C, after which the erythrocytes were

harvested by centrifugation at 300 x g for 10 minutes to remove the trypsin/versene solution. For NHS-fluorescein tagging (done on selected PRBC samples), the PRBC pellet was washed thrice in triple its volume using PBS/EDTA/PMSF solution. A volume of 2 ml of PBS/EDTA solution was added to the PRBC pellet, followed by 100 µl of the NHS-fluorescein/DMSO solution. The contents of the tube were mixed well by gentle shaking, and the PRBC pellet was incubated at 37°C for 1 hour with intermittent gentle inversion. Following the incubation period, the PRBC pellet was centrifuged at 300 x g for 10 minutes and was washed once with PBS/EDTA/PMSF solution at 450 x g for 10 minutes. Subsequent washes with glycine solution were done at 450 x g for 10 minutes until the supernatant was clear and contained no traces of NHS-fluorescein.

Erythrocyte ghost isolation was then performed as described in the methods of Section 2.1.1.

## 2.2.2 Chemical treatments

### Aim

To alter erythrocyte surface and cytoskeletal proteins through chemical treatments to assess the effect on parasite invasion.

### Materials

#### i. **Triethanolamine/chloroform solution**

Triethanolamine purchased from Merck (Darmstadt, Germany) was dissolved in chloroform purchased from Sigma-Aldrich (St Louis, USA) at a concentration of 14.7 mM. The solution was made up just before use.

#### ii. **Thionyl chloride/chloroform solution**

All work with thionyl chloride was performed in a flow hood. Thionyl chloride purchased from Merck (Darmstadt, Germany) was dissolved in chloroform purchased from Sigma-Aldrich (St Louis, USA) at a concentration of 12.6 mM. The solution was made up just before use.

#### iii. **TCEA/tris base solution**

TCEA (synthesised in-house as described below in the Methods section) and tris(hydroxymethyl)aminomethane purchased from Merck (Darmstadt, Germany) were dissolved together in PBS at concentrations of 2.3 mM and 2.1 mM respectively. The solution was made up just before use.

#### iv. **PBS/EDTA/glycine solution**

Glycine purchased from Sigma-Aldrich (St Louis, USA) was dissolved in PBS/EDTA solution (as described in sub-paragraph vii below) at a concentration of 2 mM. The solution was made up just before use.

- v. **Solubilisation solution 3**  
As described in Section 2.1.3 sub-paragraph iii.
- vi. **Trichloroacetic acid (TCA) stock solution**  
A mass of 1.9 g of TCA purchased from Merck (Darmstadt, Germany) was added to deionised water to make up a total volume of 6.25 ml of a 30% stock solution of TCA. The solution was stored at 4°C until used
- vii. **Phosphate buffered saline (PBS)/Ethylenediaminetetraacetic acid (EDTA) solution**  
As described in Section 2.1.1 sub-paragraph i.
- viii. **Phenylmethylsulfonyl fluoride (PMSF)**  
As described in Section 2.1.1 sub-paragraph ii.
- ix. **Deionised water/EDTA solution**  
As described in Section 2.1.1 sub-paragraph iii.
- x. **Phosphate buffered saline (PBS)**  
As described in Section 2.1.2 sub-paragraph iii.
- xi. **Sodium periodate solution**  
Sodium periodate was purchased from BDH Chemicals (Poole, England) and dissolved in 37°C PBS at a concentration of 5 mM. The solution was made up just before use.
- xii. **N-ethylmaleimide solution**  
N-ethylmaleimide was purchased from Merck (Darmstadt, Germany) and dissolved in PBS at a concentration of 2 mM. The solution was made up just before use.
- xiii. **Sucrose/glycine solution**  
Sucrose purchased from UniLab (Krugersdorp, RSA) and glycine purchased from Sigma-Aldrich (St Louis, USA) were dissolved in PBS at concentrations of 88 mM and 13.3 mM respectively. The solution was made up just before use.
- xiv. **Tris(2-carboxyethyl)phosphine (TCEP) solution**  
TCEP was purchased from Sigma-Aldrich (St Louis, USA) and dissolved in PBS at a concentration of 5 mM. The solution was made up just before use.
- xv. **EDTA solution**  
EDTA was purchased from BDH Chemicals (Poole, England) and dissolved in PBS at a concentration of 10 mM. This solution was made up just before use.



xvi. **1,11-bis(maleimido)triethylene glycol (BM(PEG)<sub>3</sub>)/DMSO solution**

BM(PEG)<sub>3</sub> was purchased from ThermoFisher Scientific (Massachusetts, USA) and dissolved in DMSO at a concentration of 20 mM. This solution was made up just before use.

### Methods

Tris(2-chloroethyl)amine hydrochloride (TCEA) synthesis was performed in a fume hood as the starting materials and products are strong lachrymators. This synthesis of TCEA was based on the method of Ward (1935). Briefly, the method is as follows: 10 ml of a triethanolamine/chloroform solution was added drop wise to 28 ml of a thionyl chloride/chloroform solution which was constantly stirring while kept cool in a water bath. The reaction mixture was stirred for a further 1 hour under reflux before most of the volatile components were distilled off. This resulted in a solid product being left behind, which was recrystallised from acetone. The newly formed TCEA crystals were air dried then stored in a dark bottle at room temperature. The identity and purity of the TCEA crystals were confirmed by liquid chromatography mass spectrometry using an Agilent 1100 HPLC system, and a 4000 QTrap triple quadrupole mass spectrometer from Applied Biosystems (Concord, Canada).

A volume of 8 ml of blood was collected into two 4 ml EDTA BD Vacutainer<sup>®</sup> tubes from a healthy, consenting volunteer. The blood was centrifuged at 1800 x g for 10 minutes, after which the plasma and buffy coat were removed. The remaining packed red blood cell (PRBC) pellets were washed thrice in triple their volume of a PBS/EDTA/PMSF solution at 450 x g for 10 minutes. Stock solutions of PMSF were added to the PBS/EDTA solution just before washing (a 1/100 dilution into solution).

i. Tris(2-chloroethyl)amine hydrochloride (TCEA) treatment:

The treatment of erythrocytes with TCEA was based on the methods described by Breuer, Ginsburg and Cabantchik (1982) and Wildenauer, Reuther and Remien (1980).

The two separate 2 ml PRBC pellets (originating from each of the two BD Vacutainer<sup>®</sup> tubes) were transferred to 15 ml tubes using an autopipette and were each diluted with 9 volumes (18 ml) of TCEA/tris base solution. The tubes were mixed well by gentle shaking while incubated at 37°C for 30 minutes. The tubes were then centrifuged at 500 x g for 10 minutes and the resulting supernatant quenched in a glycine solution and then discarded. The remaining pellets were washed thrice in triple their volume of a PBS/EDTA/glycine solution at 450 x g for 10 minutes.

ii. Sodium periodate treatment:

The treatment of erythrocytes with sodium periodate was based on the method described by Heller, Poser, Haest *et. al.* (1984).

The PRBC pellets prepared as described above were resuspended in PBS/EDTA solution in order to attain a haematocrit of 40%, that being the approximate haematocrit of the original whole blood samples. One millilitre aliquots of the samples were then transferred to eight 15 ml tubes. Three millilitres of N-ethylmaleimide solution was added to each tube in order to attain approximately a 10% haematocrit, and the total volume of sample in each tube was marked. The PRBC pellets were gently shaken and incubated in the N-ethylmaleimide solution for 15 minutes at 37°C. N-ethylmaleimide was added to the PRBC pellets in order to protect the erythrocytes against possible periodate-induced oxidative damage, by covalently and irreversibly blocking exposed thiol groups. The tubes were centrifuged at 300 x g for 10 minutes and the supernatants discarded. The PRBC pellets were then washed once in triple their own volume with PBS/EDTA solution. A dilution to approximately 10% haematocrit was achieved in each tube by adding PBS/EDTA solution up to the mark on each tube.

Each of the eight samples were transferred from 15 ml tubes into 50 ml tubes and 36 ml of sodium periodate solution was added to each tube. The samples were mixed well by gentle inversion, and the tubes were left on ice for 1 hour. The eight tubes were then centrifuged at 300 x g for 10 minutes and the resulting supernatants discarded. Five millilitres of PBS was added to each PRBC pellet and the eight samples were combined into two 50 ml tubes (four tubes each). The tubes were left on the bench top in order to reach room temperature, after which 20 ml of sucrose/glycine solution was added to each tube to quench any remaining periodate in the solution. The tubes were incubated for 10 minutes at room temperature and then centrifuged at 300 x g for 10 minutes and the supernatant discarded. The PRBC pellets were washed twice more in the same manner with the sucrose/glycine solution.

iii. 1,11-bis(maleimido)triethylene glycol (BM(PEG)<sub>3</sub>) treatment:

A volume of 178 µl of TCEP solution, prepared as described above, was added to each of the PRBC pellets. Samples were incubated for 20 minutes at room temperature in order to reduce all peptide disulfide bonds. The PRBC pellets were washed twice in triple their volume with PBS at 450 x g for 10 minutes. A 1:1 dilution of each PRBC pellet was then done in EDTA solution in order to prevent the reoxidation of disulfide linkages, followed by the addition of 60 µl of BM(PEG)<sub>3</sub>/DMSO solution. The tubes were mixed well by gentle inversion and were incubated at 37°C for 1 hour. The tubes were then centrifuged at 450 x g for 10 minutes and the supernatant was removed and discarded. The PRBC pellets were washed thrice in triple their volume of PBS/EDTA solution at 450 x g for 10 minutes.

Ghost erythrocyte isolation protocol was then resumed for the erythrocytes treated with TCEA, sodium periodate and BM(PEG)<sub>3</sub> as described in the methods of Section 2.1.1.

For the sodium periodate-treated ghosts, the resulting 100 µl ghost pellet was resuspended in 200 µl of a PBS/EDTA/PMSF solution and stored at -80°C until use.

For both the TCEA and BM(PEG)<sub>3</sub> treated erythrocytes the resulting 150 µl ghost pellet was resuspended in 200 µl of Solubilisation solution 3 and was incubated at room temperature for 1 hour. Protein precipitation of the ghost sample was performed using 300 µl of 30% stock trichloroacetic acid (TCA). The sample was vortex mixed for 1 minute and incubated on ice for 15 minutes, after which it was centrifuged for 10 minutes at 16 000 x g. The supernatant was discarded and the remaining pellet was washed three times with ice cold acetone and the protein recovered by centrifugation at 16 000 x g for 5 minutes each time. The pellet was then resuspended in 200 µl of a PBS/EDTA/PMSF solution and stored at -80°C until use. Additionally, the BM(PEG)<sub>3</sub>-treated ghost pellets were homogenised using an ultrasonic homogeniser from BioLogics, Inc.

## 2.3 Two-dimensional Gel Electrophoresis (2-DE)

### Aim

To produce characteristic erythrocyte ghost protein fingerprint patterns based on respective protein isoelectric point values and protein mass.

### Materials

#### i. 2-D Starter Kit

A ReadyPrep 2-D Starter Kit was purchased from Bio-Rad Laboratories, Inc. (Berkeley, USA) which contained ReadyPrep Rehydration/Sample Buffer, Nanopure Water, Equilibration Buffer I, Equilibration Buffer II, 30% Glycerol Solution, Iodoacetamide and Overlay Agarose. The kit was stored at 4°C.

#### ii. Immobilised pH gradient (IPG) strips

ReadyStrip IPG strips (pH 3-10, 11cm) were purchased from Bio-Rad Laboratories, Inc. (Berkeley, USA) and were stored at -20°C.

#### iii. Mineral oil

Mineral oil was purchased from Bio-Rad Laboratories, Inc. (Berkeley, USA) and was stored at room temperature.

#### iv. Protein mass standards

Precision Plus Protein™ Standards Unstained were purchased from Bio-Rad Laboratories, Inc. (Berkeley, USA) covering a reference mass range from 250 kilodaltons (kDa) to 10 kDa. The standards were stored at -20°C until used as per product instructions.

**v. Precast gels**

For all SDS PAGE 4-20% linear gradient, 11cm IPG+1 well, Criterion™ TGX Stain-Free™ Precast Tris-HCl Gels purchased from Bio-Rad Laboratories, Inc. (Berkeley, USA) were used. Gels were stored at 4°C until used as per product instructions.

**vi. 10 x Running buffer**

An amount of 5 g SDS and 15 g tris(hydroxymethyl)aminomethane both purchased from Merck (Darmstadt, Germany) and 72 g glycine purchased from Sigma-Aldrich (St Louis, USA), were added to a volumetric flask and brought to 500 ml with deionised water. The flask was sonicated for 5 minutes and the stock solution was stored at room temperature.

**vii. Fixing solution**

Methanol and glacial acetic acid was purchased from Merck (Darmstadt, Germany). A solution containing 50% methanol, 10% glacial acetic acid and 40% deionised water was made up just before use.

## **Methods**

Erythrocyte ghost samples were prepared as described in the methods of Section 2.1.1, were removed from the -80°C freezer, allowed to thaw at room temperature and clarified by centrifugation at 16000 x g for 10 minutes just before loading onto an IPG strip (pH 3 – 10) for 2-DE. A volume of 200 µl of ghost solution was pipetted along a channel of a rehydration tray, after which an IPG strip was placed gently gel side down onto the sample. This was done in such a manner so that the sample was distributed equally along the gel surface of the IPG strip and no air bubbles were present. Mineral oil (2-3 ml) was then pipetted slowly over the exposed plastic back of the IPG strip in order to prevent evaporation of sample during the rehydration process. The rehydration tray was covered with a lid and was left overnight to allow for full rehydration of the IPG strips with ghost sample.

The following day the IPG strip was removed from the dehydration tray, the mineral oil was drained by holding the strip vertically against paper towel, and the strip was transferred to a channel on a focusing tray where it was placed gel side up. Damp paper electrode wicks were placed on the ends of the strip and under the electrodes of the isoelectric focusing (IEF) cell as receptacles for salts and other non-amphoteric constituents of the loaded sample. The IPG strip was then covered with 2-3 ml of fresh mineral oil and any trapped air bubbles beneath the strip were removed. The focusing tray was placed in and connected to a Hoefer IEF100 cell. The IEF cell was programmed using the protocol in Table 2.1.

**Table 2.1: IEF cell protocol**

Step	Voltage	Time	Volt-Hours	Ramp
1	250	20 min	n/a	Linear
2	250 - 8 000	2.5 hr	n/a	Linear
3	8 000	n/a	20 000	Rapid
<b>Total</b>	-	~5.3 hr	~30 000	-

Once the IEF electrophoresis run was completed, the IPG strip was removed from the tray, the mineral oil was again drained and the strip was placed in a clean channel of an equilibration tray. Preparations for IPG strip equilibration and the second dimension of gel electrophoresis, SDS PAGE, then began. Both equilibration buffers I and II supplied with the 2-D starter kit were prepared according to the enclosed instructions. Included within the equilibration buffers I and II are chaotropic, reducing and alkylating agents as well as detergents, which denature and add negative charge to the proteins in the separated sample and thus increase sample solubility. A volume of 4 ml of equilibration buffer I was added to the channel containing the IPG strip and the tray was gently shaken on a shaker for 10 minutes. Equilibration buffer I was then discarded carefully by decanting the liquid from the tray. Four millilitres of equilibration buffer II was then added to the channel, and again the tray was gently shaken for 10 minutes, after which the buffer was carefully decanted.

The overlay agarose solution provided in the kit was melted in a microwave at low heat in 20 second intervals where the bottle was swirled until it was completely liquefied. The IPG strip was briefly rinsed in 1 x running buffer, by filling a volumetric flask with enough solution to completely immerse the strip.

The precast gel along with the standard and IPG wells were rinsed with deionised water followed by 1 x running buffer. Any excess water or running buffer remaining in the IPG well was blotted away using Whatman Grade 1 filter paper. Using a Pasteur pipette, agarose solution was pipetted along the IPG well. Then using forceps, the IPG strip, gel side forward, was placed against the plastic backing of the precast gel, and was gently pushed down into the agarose overlay of the IPG well. Care was taken in order to ensure that no air bubbles were trapped beneath the strip. The agarose overlay was left to solidify for 5 minutes, after which the precast gel was placed in the electrophoresis tank supplied by Bio-Rad Laboratories, Inc. The tank was then filled with 1 x running buffer. A volume of 10 µl of protein mass standards was pipetted into the allocated end well of the gel. The electrophoresis tank was connected to a Hoeffer PS300-B power source and was run at a constant 200 volts (V). After the gel had run to completion, as indicated by the bromophenol blue tracking dye present in the overlay agarose solution, the gel cassette was removed from the tank, the plastic casing was cracked open and the gel was removed and placed on a Stain-Free Sample Tray of a Gel Doc EZ Imager supplied by Bio-Rad

Laboratories, Inc. The gel was scanned and analysed using Bio-Rad Laboratories “Image Lab” version 3.0 build 11 software. Gels were then stored in fixing solution.

## 2.4 Sodium Dodecyl Sulfate (SDS) Polyacrylamide Gel Electrophoresis (PAGE)

### Aim

To produce characteristic protein fingerprint patterns of erythrocyte ghosts based on the respective protein sizes.

### Materials

**i. 10 x Running buffer**

As described in Section 2.3 sub-paragraph vi.

**ii. 3 x Sample/Laemmli buffer**

An amount of 148 mg tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) purchased from Research Organics Inc. (Cleveland, USA), 300 mg SDS, 750  $\mu$ l  $\beta$ -Mercaptoethanol purchased from Sigma-Aldrich (St Louis, USA) and 1.5 ml glycerol purchased from Sigma-Aldrich (St Louis, USA) were added to a volumetric flask and brought to a final volume of 5 ml with deionised water. An amount of 16 mg bromophenol blue purchased from Merck (Darmstadt, Germany) was dissolved in 2.5 ml of deionised water, and 50  $\mu$ l of this was then added to the prepared buffer solution. This resulted in a sample buffer solution that contained 0.1875 M Tris-HCl, 6% SDS, 15%  $\beta$ -Mercaptoethanol, 30% glycerol and 0.006% bromophenol blue. The sample buffer was aliquoted into microcentrifuge tubes and stored at  $-80^{\circ}\text{C}$  until used.

**iii. Protein mass standards**

As described in Section 2.3 sub-paragraph iv.

**iv. Precast gels**

For all SDS PAGE 4-20% linear gradient, 12+2 well, Midi size Criterion™ TGX Stain-Free™ Precast Tris-HCl Gels purchased from Bio-Rad Laboratories, Inc. (Berkeley, USA) were used. Gels were stored at  $4^{\circ}\text{C}$  until used as per product instructions.

**v. Fixing solution**

As described in Section 2.3. sub-paragraph vii.

**vi. Coomassie Blue**

Aqua colloidal Coomassie Blue stain was purchased from Vacutec (Johannesburg, RSA). The solution was stored at 4°C as per product instructions.

**vii. Ethanol/acetic acid solution**

Glacial acetic acid purchased from Merck (Darmstadt, Germany) and 96% ethanol purchased from Sigma-Aldrich (St Louis, USA) were used to make up a solution containing 10% ethanol and 5% glacial acetic acid in deionised water. The solution was made up just before use.

**viii. Oxidiser reagent**

Potassium dichromate ( $K_2Cr_2O_7$ ) and nitric acid ( $HNO_3$ ) purchased from Merck (Darmstadt, Germany) were added to deionised water at concentrations of 3.4 mM and 3.2 mM respectively. The solution was made up just before use.

**ix. Silver nitrate solution**

Silver nitrate ( $AgNO_3$ ) purchased from Merck (Darmstadt, Germany) was added to deionised water at a concentration of 20 mM. The solution was made up just before use.

**x. Developer solution**

Sodium carbonate ( $Na_2CO_3$ ) purchased from Merck (Darmstadt, Germany) was dissolved in deionised water at a concentration of 0.28 M, after which formaldehyde purchased from Merck (Darmstadt, Germany) was added so as to reach a concentration of 0.008% in solution. The solution was made up just before use.

## Methods

To prepare erythrocyte ghost samples for SDS PAGE, the protein concentration was determined using the BCA protein assay and diluted accordingly to  $\pm 1.5$  mg/ml protein with PBS before each sample was further diluted with Laemmli sample buffer in a 1:2 ratio (sample buffer:sample). This resulted in a protein sample of approximate concentration of 1 mg/ml. Each sample was heated in the sample buffer for 10 minutes at 100°C by floating the microcentrifuge tube containing the dissolved sample in a beaker of boiling water. The sample was then removed from the boil and centrifuged for 5 minutes at 16 000 x g to remove any insoluble products from the erythrocyte ghosts.

The precast gel and the sample wells were rinsed with deionised water. The sample wells were further rinsed and then filled with 1 x concentration of running buffer, after which the precast gel was placed in the electrophoresis tank supplied by Bio-Rad Laboratories, Inc. The tank was then filled with the running buffer. A volume of 10  $\mu$ l

of protein mass standards was pipetted into the two allocated end wells of the gel, and 30  $\mu$ l (unless otherwise stated) of prepared erythrocyte ghost sample was then pipetted into the remaining sample wells. The electrophoresis tank was connected to a Hoeffer PS300-B power source and was run either at a constant 60 volts (V), or at 60 V for the first 30 minutes and then at 200 V for the remainder of the run.

After the gel had run to completion, as indicated by the bromophenol blue tracking dye, the gel cassette was removed from the tank, the plastic casing was cracked open and the gel was placed on a Stain-Free Sample Tray and visualised using a Gel Doc EZ Imager from Bio-Rad Laboratories, Inc. An Ultraviolet Sample Tray and a Blue Sample Tray were additionally utilised in the case of the NHS-fluorescein-treated trypsin sample. The gel scans were analysed using Bio-Rad Laboratories "Image Lab" version 3.0 build 11 software. Gels were then stored in fixing solution and were stained overnight in colloidal Coomassie Blue to indicate the protein band positions to enable band removal for preparation for mass spectrometric analysis.

For selected gels a double-staining technique, silver/Coomassie Blue staining, was applied for comparison against unstained and NHS-fluorescein techniques. The silver staining method was based on the methods of Merril *et. al.* (1981) and Dzandu *et. al.* (1984). The gel was removed from the fixing solution it had been stored in and was washed twice for 30 minutes in ethanol/acetic acid solution to remove any residual SDS. The gel was then equilibrated with 200 ml of oxidiser reagent for 10 minutes and this was followed by 3 washes with deionised water. The gel was then covered with silver nitrate solution for 30 minutes, after which three 1 minute rinses with deionised water were performed to remove any unbound silver. The complete removal of unbound silver was verified by the addition of one drop of 1 M hydrochloric acid (HCl) to the water wash and a resulting absence of cloudiness. Developer solution was warmed to 40°C and 200 ml was added to the gel to develop and visualise the bands. The developer solution was changed when it became a light brown colour, which occurred twice. Within 1 hour bands were visualised with positively-stained bands appearing yellow/brown and bands that did not stain appearing as negative optical images. The developer solution was then removed from the gel and the gel was rinsed with deionised water. Two hundred millilitres of a 10% (v/v) acetic acid solution was added to the gel in order to enhance the yellow/brown colour of the positively-stained bands. The gel was then photographed. For the second part of the double staining process, the silver-stained gel was counterstained with colloidal Coomassie Blue and was again photographed from the same distance to be able to compare the gel images.



## 2.5 Proteomic Analysis

### 2.5.1 In-gel digestion of protein bands

#### Aim

To prepare protein samples for mass spectrometric analysis by utilising destaining, reduction, alkylation, digestion and extraction techniques.

#### Materials

- i. **Ammonium bicarbonate ( $\text{NH}_4\text{HCO}_3$ ) solution**  
A 50 mM solution was made by dissolving 0.2 g of  $\text{NH}_4\text{HCO}_3$  purchased from Sigma-Aldrich (St Louis, USA) in 50 ml of deionised water. The solution was stored at 4°C and was used within 2 weeks.
- ii.  **$\text{NH}_4\text{HCO}_3$ /50% methanol solution**  
Two hundred milligrams of  $\text{NH}_4\text{HCO}_3$  was dissolved in 25 ml of deionised water and 25 ml of 100% mass spectral grade methanol obtained from Romil (Cambridge, Great Britain) was added. This resulted in a 50 mM  $\text{NH}_4\text{HCO}_3$  solution. The solution was stored at 4°C and was used within 2 weeks.
- iii.  **$\text{NH}_4\text{HCO}_3$ /50% acetonitrile solution**  
One millilitre of the 50 mM aqueous  $\text{NH}_4\text{HCO}_3$  solution as described above (i) was dissolved in 1 ml of 100% mass spectral grade acetonitrile obtained from Merck (Darmstadt, Germany), resulting in a 25 mM  $\text{NH}_4\text{HCO}_3$  solution. The solution was sealed and stored at 4°C and was used within 2 weeks.
- iv. **Acetonitrile solution**  
A 75% acetonitrile solution was made by adding deionised water to 7.5 ml of 100% acetonitrile to exactly 10 ml. The solution was sealed to avoid evaporation and stored at 4°C and was used within 2 weeks.
- v. **Trypsin digest working solution**  
Sequencing Grade Modified Trypsin was purchased from Promega (Madison, USA). A volume of 200  $\mu\text{l}$  of the buffer supplied with the lyophilised enzyme was added to a single vial containing 20  $\mu\text{g}$  of lyophilised trypsin. The vial was gently vortex mixed to ensure complete dissolution of the trypsin. Twenty microlitre aliquots were prepared in 1.5 ml microcentrifuge tubes. The aliquots were stored at -20°C until used.
- vi. **Dithiothreitol (DTT) stock solution**  
One hundred and fifty four milligrams of DTT purchased from Sigma-Aldrich (St Louis, USA) was dissolved in 1 ml of deionised water, resulting in a 1 M DTT stock solution. This solution was freshly prepared just before use.

vii. **DTT/ NH<sub>4</sub>HCO<sub>3</sub> solution**

Ten microlitres of the 1 M DTT stock solution described above (vi) was added to a solution which contained 495 µl of the 50 mM NH<sub>4</sub>HCO<sub>3</sub> solution as described in (i) and 495 µl of deionised water. This resulted in a 10 mM DTT/25 mM NH<sub>4</sub>HCO<sub>3</sub> solution. This solution was freshly prepared just before use.

viii. **Iodoacetamide (IAA)/NH<sub>4</sub>HCO<sub>3</sub> solution**

An amount of 10.2 mg of IAA purchased from Sigma-Aldrich (St Louis, USA) was dissolved in a solution made up of 500 µl of 50 mM NH<sub>4</sub>HCO<sub>3</sub> solution as described in (i) and 500 µl of deionised water. This resulted in a 55 mM IAA/25 mM NH<sub>4</sub>HCO<sub>3</sub> solution. This solution was freshly prepared just before use and was stored in a dark container.

## Methods

In-gel trypsinisation of protein bands was based on the methods of Shevchenko *et al.* (2007).

All gel manipulations were carried out using new glass or plastic ware and on cleaned surfaces while using gloved hands to avoid contamination with extraneous protein. After analysis of the Coomassie stained SDS PAGE results in Section 2.3, protein bands of interest were identified from the gel scans. Each of the selected bands was carefully cut from the gel using a new sharp scalpel blade, diced into small pieces (approximately 1 mm x 1 mm), and placed in a 1.5 ml microcentrifuge tube. Approximately 200 µl (or enough to cover the gel pieces) of 50 mM NH<sub>4</sub>HCO<sub>3</sub>/50% methanol was added to each tube, which was then vortex mixed for 20 minutes to destain the protein. The resulting supernatant was discarded and the wash step was repeated. After discarding the supernatant approximately 100 µl (or enough to cover the gel pieces) of 75% acetonitrile was added to each tube, which was again vortex mixed for 20 minutes. The supernatant was once again removed and discarded. The gel pieces were then dried using a Labconco CentriVap at 37°C for 20 minutes.

Approximately 25 µl (or enough to cover the gel pieces) of freshly prepared 10 mM DTT in 25 mM NH<sub>4</sub>HCO<sub>3</sub> was added to the dry gel pieces. Each tube was vortex mixed and briefly centrifuged (75 x *g*, 5 minutes). To allow the reduction reaction to proceed, the tubes were incubated in a water bath at 60°C for 1 hour, after which the tubes were cooled to room temperature (22-23°C). Five hundred microlitres of acetonitrile was then added to each tube and the tubes were incubated for 10 minutes at room temperature. After the incubation the supernatant was removed, discarded and approximately 25 µl (or enough to cover the gel pieces) of 55 mM IAA in 25 mM NH<sub>4</sub>HCO<sub>3</sub> was added to the tubes. Each tube was vortex mixed and centrifuged briefly, and the alkylation reaction was allowed to proceed in the dark for 20 minutes at room temperature. After the incubation period the supernatant was

removed, discarded and the gel pieces were washed with 100  $\mu\text{l}$  of 25 mM  $\text{NH}_4\text{HCO}_3$  in deionised water. The tubes were vortex mixed for 10 minutes and centrifuged for 5 minutes at 75 x g, after which the resulting supernatant was removed and discarded. The gel pieces were then dehydrated with the addition of approximately 100  $\mu\text{l}$  (or enough to cover the gel pieces) of 25 mM  $\text{NH}_4\text{HCO}_3$  in 50% acetonitrile, this followed with the tubes being vortex mixed for 5 minutes, centrifuged for 5 minutes at 75 x g and the supernatant being removed and discarded. The dehydration step was repeated once again, after which the gel pieces were dried completely at 37°C for 20 minutes in the CentriVap.

The microcentrifuge tubes were placed on ice, and each gel plug was covered with trypsin solution (approximately 10-50  $\mu\text{l}$ ). After 30 minutes it was checked whether all the trypsin solution had been absorbed and more solution was added if necessary, as gel pieces needed to be covered completely with trypsin. The gel pieces were rehydrated on ice for another 1 hour and were then centrifuged briefly (1 minute at 75 x g). A 25 mM  $\text{NH}_4\text{HCO}_3$  solution in deionised water was then added as needed to cover the gel pieces and keep them wet during enzyme cleavage. The tubes were spun briefly to collect the contents at the bottom of the tube and were incubated at 37°C in a water bath overnight for 16-18 hours.

The following morning the tubes were centrifuged briefly (1 minute at 75 x g) and the digest solution (the aqueous extraction) was transferred into a clean marked microcentrifuge tube. Enough of a 50% acetonitrile/5% formic acid solution to cover the gel pieces ( $\pm 50 \mu\text{l}$ ) was added to the remaining digested gel pieces. These tubes were then vortex mixed for 25 minutes, sonicated for 5 minutes and briefly centrifuged. The new extract (the supernatant) was added to the first extraction. The gel pieces were again covered in the acetonitrile/formic acid solution and the process was repeated once more to allow for maximum peptide extraction from the gel pieces. The gel pieces were then discarded and the combined extracts completely dried using the CentriVap for approximately 3 hours at room temperature. The dried digests were stored at -20°C until cleanup for nano liquid chromatography tandem mass spectrometry (LC-MS/MS) was performed.

## 2.5.2 Nano LC-MS/MS

### Aim

To separate and sequence digested protein bands by nano LC-MS/MS and to compare resulting mass spectrometric sequences to protein databases.

### Materials

#### i. Acetonitrile/formic acid solutions

The acetonitrile (ACN), formic acid (FA) and purified water were all of mass spectrometry grade and were purchased from Fluka Analytical (Basel, Switzerland). For the 2% ACN/0.2% FA solution, 20 ml ACN and 2 ml FA were

added to 978 ml of purified water per litre of solution. For the 80% ACN/0.1% FA solution, 800 ml ACN and 1 ml FA were added to 199 ml of purified water per litre of solution. For the 0.1% FA solution, 1 ml FA was added to 999 ml of purified water per litre.

## Methods

To prepare the samples for Nano LC-MS/MS analysis, the dried in-gel digests were resuspended in 40  $\mu$ l of a 2% acetonitrile/0.2% formic acid solution and were vortex mixed for 1 minute. The tubes were sonicated on ice for 5 minutes and were then centrifuged at 125  $\times$  *g* for 10 minutes. Individual samples of 35  $\mu$ l were transferred to 0.5 ml Eppendorf LoBind tubes for mass spectrometric analysis.

The samples were analysed using a Dionex Ultimate 3000 RSLC system coupled to an AB SCIEX 4000 QTRAP mass spectrometer (MS). Peptides were first de-salted on an Acclaim PepMap C18 trap column (75  $\mu$ m  $\times$  2 cm) for 8 min at 5  $\mu$ l/min using 2% acetonitrile/0.2% formic acid, then separated on an Acclaim PepMap C18 RSLC column (75  $\mu$ m  $\times$  15 cm, 2  $\mu$ m particle size). Peptide elution was achieved using a flow-rate of 500 nl/min with a gradient: 4-60% B in 30 min (A: 0.1% formic acid; B: 80% acetonitrile/0.1% formic acid). Nano-spray was achieved using a MicroLonspray head assembled with a New Objective, PicoTip emitter. An electrospray voltage of 2.5-2.8 kV was applied to the emitter. The 4000 QTRAP mass spectrometer was operated in 'Information Dependant Acquisition' mode. Enhanced MS scans were acquired from *m/z* 400-1500 and the three most intense ions were automatically fragmented using the Q2 collision cell where nitrogen was used as the collision gas. Collision energies were chosen automatically as a function of the precursor peptide *m/z* and charge. The MS/MS spectra were collected and the peak list data exported to ProteinPilot v 4.0.8085 where the amino acid sequences were derived. This sequence data was then analysed for possible protein identification through peptide homology comparisons using a Paragon search engine (AB SCIEX) for comparison of the obtained MS/MS spectra with protein sequences in the Uniprot/Swiss-Prot 2013 database including a dummy database to check for false calls. Only proteins with a confidence threshold of  $\geq$  99.9% were reported.

## 2.6 Enzyme and Chemical Effects on Invasion

### 2.6.1 Schizont isolation

#### Aim

To isolate highly synchronized schizont stage *Plasmodium falciparum* parasites.

## Materials

- i. **Roswell Park Memorial Institute (RPMI) incomplete medium 1640**  
RPMI medium was obtained in powder form from Sigma-Aldrich (St Louis, USA). An amount of 10.4 g of the medium powder was dissolved in 800 ml of sterile, deionised water and 1.8 g of sodium bicarbonate ( $\text{NaHCO}_3$ ) purchased from Sigma-Aldrich (St Louis, USA) dissolved in 50 ml of sterile deionised water, was added to adjust the pH. Sterile HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid), glucose, gentamicin and hypoxanthine were added to the medium to produce final concentrations of 25 mM, 0.2%, 0.048 mM and 0.2 mM respectively. The volume of the medium was then adjusted to 1 l with deionised water. The final solution was filter sterilised twice with 0.22  $\mu\text{m}$  cellulose acetate filters, aliquoted into sterile 500 ml bottles and stored at 4°C until used.
- ii. **RPMI complete medium 1640**  
Five grams of Albumax II was dissolved in 40 ml of RPMI incomplete medium, as described in (i), and the solution was filter sterilised with a 0.22  $\mu\text{m}$  cellulose acetate filter with a 0.8  $\mu\text{m}$  prefilter. The solution was then made up to a final volume of 500 ml using the sterilized RPMI incomplete medium. The RPMI complete medium was stored at 4 °C and was used within 2 weeks.
- iii. **Sorbitol solution**  
D-Sorbitol was purchased from Sigma-Aldrich (St Louis, USA) and a 5% solution was made up by dissolving 25 g of sorbitol in 500 ml of deionised water. The solution was stored at 4°C until used.
- iv. **Giemsa stain**  
Giemsa stain stock solution was purchased from Sigma-Aldrich (St Louis, USA), where 7 ml was diluted in 42 ml of distilled water to make a working solution. Both stock and working solutions were stored at room temperature.
- v. **Methanol**  
Methanol was purchased from Sigma-Aldrich (St Louis, USA) and stored at room temperature.

## Methods

*Plasmodium falciparum* 3D7 malaria parasites were cultured in 175  $\text{cm}^2$  culture flasks, supplied by Lasec while maintaining a 5% haematocrit to an approximate 8% parasitemia in RPMI complete medium. These parasite cultures were initially mixed cultures, that is to say that there were different life stages of the parasite present in the flask. In order to synchronise the parasites all to the ring stage of the life cycle, the culture was transferred to a 50 ml tube, centrifuged at 1000 x g for 5 minutes to harvest the erythrocytes and 15 ml of sorbitol solution added to the pellet which was gently resuspended and incubated in a water bath at 37°C for 15 minutes. The sorbitol crosses the membranes of trophozoite- and schizont-stage-infected

erythrocytes resulting in lysis of the infected erythrocytes and leaving only ring forms of the parasite behind. This therefore results in a “synchronised” culture. The tube was re-centrifuged again at 1000 x g for 5 minutes, the supernatant was removed and the remaining pellet was washed with 10 ml of culture medium. One millilitre of fresh erythrocytes was added to the tube after synchronisation, the culture was returned to a culture flask which was then gassed with 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N and incubated on a shaker at 37°C.

In order to obtain the volume of schizont stage infected erythrocytes required, the cultures must be synchronised to 40-44 hour old parasites with an approximate 10% parasitemia and 2.5% haematocrit. This was determined by making a thin slide of the culture, staining with Giemsa stain and viewing it under a microscope. A monolayer of the culture was prepared on a glass microscope slide which was then dried with a hair dryer, fixed in methanol and stained in Giemsa stain for 2 minutes. Excess stain was removed by gently running water over the slide after which the slide was again dried with a hair dryer. The parasitemia was calculated by counting how many infected RBCs were present out of a minimum total of 1000 RBCs.

Schizont isolation was performed under sterile conditions using magnetic separation in a VarioMACS Separator from Miltenyi Biotec. This is possible due to the magnetic properties of the hemozoin crystals present in late-stage parasites. The CL type MACS column was washed with ethanol, deionised water and incomplete medium, after which the resuspended parasite culture was allowed to percolate through the column while inserted into the magnet. The initial flow through containing uninfected RBCs and early-stage parasites was collected and discarded. Incomplete medium was used to wash the column until the flow-through was clear. The column was then removed from the magnet and clamped into a burette stand. The schizont-stage parasites were then eluted from the column using complete medium. The parasite infected erythrocytes were collected into a 50 ml tube, gassed with 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub> and incubated at 37°C until used in the invasion assays which never exceeded 2 hours after isolation.

## **2.6.2 Invasion assays**

### **Aim**

To assess the effects of enzymatic and chemical treatments of erythrocytes on invasion efficiency by malaria parasites.

### **Materials**

- i. **Roswell Park Memorial Institute (RPMI) incomplete medium 1640**  
As described in Section 2.6.1 sub-paragraph i.
- ii. **RPMI complete medium 1640**  
As described in Section 2.6.1 sub-paragraph ii.

- iii. **Giemsa stain**  
As described in Section 2.6.1 sub-paragraph iv.
- iv. **Methanol**  
As described in Section 2.6.1 sub-paragraph v.
- v. **Glutaraldehyde**  
A 25% glutaraldehyde stock solution was purchased from Sigma-Aldrich (St Louis, USA). A glutaraldehyde working solution of 0.025% was made up by adding 25  $\mu$ l stock solution to 24.975 ml of deionised water. Both stock and working solutions were stored at 4°C until used.
- vi. **PBS**  
FTA Hemagglutination buffer was purchased from BD Biosciences (Sparks, USA). A solution was made up at 9.23 g/l as per product instructions, with deionised water. The solution was stored at 4°C until used.
- vii. **SYBR Green I**  
SYBR Green I was purchased from Life Technologies (Oregon, USA) and stored at -20°C, in the dark before use. A 1/1000 working solution was made up by adding 0.5  $\mu$ l of SYBR Green I to 500  $\mu$ l PBS. The working solution was stored at -20°C in foil enclosed tubes for a maximum of 2 days.

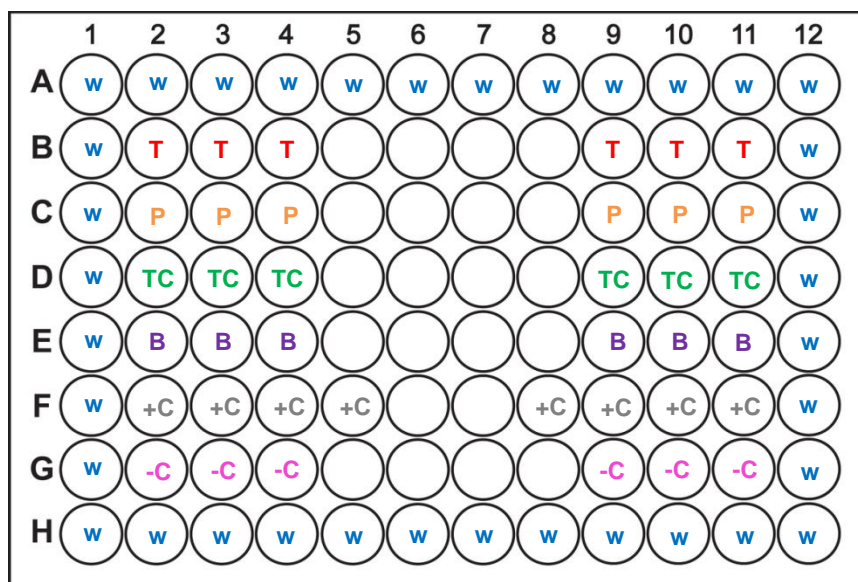
## Methods

All these procedures were performed under sterile working conditions. Blood taken from healthy, consenting volunteers was collected into EDTA Vacutainer blood collection tubes. The blood was centrifuged at 1800 x g for 10 minutes, after which the plasma and buffy coat were removed. The remaining packed red blood cell (PRBC) pellets were split equally in volume and were transferred to 15 ml tubes. This resulted in five tubes that were labelled 'control', 'trypsin', 'sodium periodate', 'BM(PEG)<sub>3</sub>' and 'TCEA', indicating the treatment that each tube of PRBC pellets would receive. Except for the trypsin treated samples, all the pellets were washed thrice in triple their volume of a PBS/EDTA/PMSF solution and collected by centrifugation at 450 x g for 10 minutes. The trypsin sample was washed in a PBS/EDTA solution as PMSF inhibits the action of trypsin. The control was left on crushed ice while the remaining four tubes received their allocated enzyme or chemical treatments. The treatments followed the methods described in Section 2.2, however after the treated PRBC pellets were washed with a PBS/EDTA/PMSF solution post-treatment, they were not haemolysed. Instead, the treated as well as the control PRBC pellets were washed twice in RPMI 1640 incomplete medium for 10 minutes and collected by centrifugation at 450 x g.

The invasion assays were done in sterile, flat-bottomed 96-well microtiter plates. Each plate contained positive controls (complete medium, control erythrocytes and

schizonts), negative controls (complete medium and control erythrocytes) and the four treated erythrocyte samples (complete medium, schizonts and one type of the treated erythrocytes), all of which were plated in triplicate.

A volume of 100 µl of complete medium was pipetted into all test wells on the plate and all the outer wells of the plate were filled with 200 µl of deionised water to prevent the assay from drying out. The isolated schizont stage parasite sample was found to have a 93% parasitemia following isolation with the VarioMACS (as described in Section 2.6.1). Thus, in order to obtain a final 2% haematocrit and 1% parasitemia in each required well, the following procedure was done. Complete RPMI 1640 medium was added to each of the five different PRBC pellets, as well as to the packed schizont pellet in a 1:1 dilution. Then 4.4 µl of diluted schizont pellet, 396 µl of each of the five diluted PRBC pellets and 9.6 ml of complete medium were combined in five separate 50 ml tubes, either containing the control erythrocytes (positive control), or one of the four enzymatically and chemically treated erythrocytes- trypsin, sodium periodate, tris(2-chloroethyl)amine hydrochloride (TCEA) or 1,11-bis(maleimido)triethylene glycol (BM(PEG)<sub>3</sub>). A 50 ml tube was made up in the exact same manner for the negative control, however the 4.4 µl of schizonts was not included and the resulting decreased well volume was made up for with complete medium. A volume of 100 µl of these sample mixtures was then pipetted into the 100 µl of complete medium already present in all the test wells. The plate set up is illustrated in Figure 2.1.



**Figure 2.1: Invasion assay plate setup.** Each sample was plated in triplicate on both sections (left and right) of the plate for the invasion assays, where ‘w’ is deionised water; ‘T’ is trypsin-treated samples; ‘P’ is sodium periodate-treated samples; ‘TC’ is TCEA-treated samples; ‘B’ is BM(PEG)<sub>3</sub>-treated samples; ‘+C’ is the positive control and ‘-C’ is the negative control.



The plate was then placed into an airtight container gassed with 5% CO<sub>2</sub>, 5% O<sub>2</sub> and 90% N<sub>2</sub>, and incubated for 48 hours at 37°C. After 24 hours into the incubation period, 100 µl from an additional positive control well was used to make a slide which was fixed with methanol, stained with Giemsa and analysed under the microscope. This was done in order to ensure parasite survival up until this point, as newly formed rings would be visible by this time in the lifecycle. The container was re-gassed, sealed and incubated for a further 24 hours. After the total 48 hour incubation period, 50 µl from each well was removed, transferred to a microcentrifuge tube and fixed with 1 ml of a 0.025% glutaraldehyde solution. These fixed samples were stored at 4°C and were used for flow cytometry analysis within a week.

To prepare for flow cytometry analysis the glutaraldehyde fixed samples were washed twice with 1 ml PBS by centrifugation, for 5 minutes each at 500 x g, and the supernatant discarded each time to remove the glutaraldehyde solution. The sample volumes were then all made up to 100 µl using PBS and were resuspended. To this, a volume of 10 µl of a 1/1000 dilution of SYBR Green I was added, and the tubes were incubated at room temperature in the dark for 30 minutes. Following the incubation the samples were centrifuged at 500 x g for 5 minutes, after which they were washed with 500 µl of PBS. The sample pellets were finally resuspended in 400 µl of PBS and analysed by flow cytometry. The negative control was used to set gating limits. The samples were analysed using a Beckman Coulter FC500 Series flow cytometer. At least 35 000 events of infected erythrocytes were counted and the channel used was FL1 with a 488 nm excitation wavelength and a 525 nm emission wavelength. Analysis of results was performed using Cyflogic software.

### **Statistical Analysis**

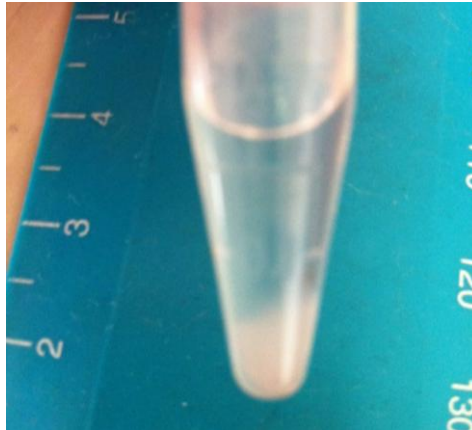
Invasion efficiency was defined as the rate of invasion achieved with enzymatically/chemically-treated erythrocytes expressed as a percentage of the rate of invasion achieved with untreated (control) erythrocytes. The invasion assays were performed in triplicate and were repeated four times. Outliers present in the resulting data were identified by performing the Grubbs' test, and were removed from the data set. The data used to determine invasion efficiency for each treatment (as a percentage of the mean) was normalised and the standard error of the mean (SEM) calculated. Results of the invasion assay were compared to one another using the Kruskal-Wallis test with a post-hoc Dunn's Multiple Comparison Test. Significance was set as  $P < 0.05$ . All statistical analyses were performed using GraphPad Prism version 5.00 software.

## Chapter 3: Results and Discussion

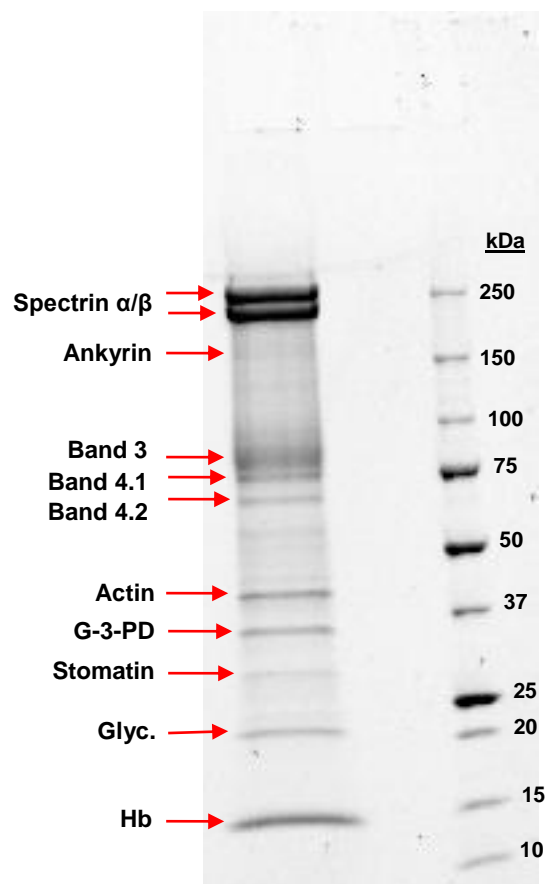
### 3.1 Erythrocyte Ghost Isolation

#### 3.1.1 Results and discussion

Erythrocytes were separated from whole blood and the method to prepare, isolate and collect clean erythrocyte ghosts was optimised. That is, clean, white erythrocyte ghost samples with minimal haemoglobin present, were successfully prepared, isolated and collected from whole blood as illustrated in Figure 3.1. The volume of whole blood required to provide high enough erythrocyte ghost concentrations for gel electrophoresis was investigated, and was determined to be approximately 8 ml. Utilising the bicinchoninic acid (BCA) protein assay it was determined that a final concentration of approximately 1 mg/ml protein and a volume of 30  $\mu$ l (i.e. 30  $\mu$ g of protein) of erythrocyte ghost sample protein was required to be loaded onto the gel to provide a high quality protein fingerprint, as is illustrated in Figure 3.2. The concentration of erythrocytes that were isolated per millilitre of whole blood in this experiment, is in line with previous literature (Anselstetter and Horstmann, 1975, Fairbanks et al., 1971). The resulting erythrocyte ghost protein fingerprint was consistent with the SDS PAGE pattern reported in previous research, with many of the membrane associated cytoskeletal and membrane protein bands being easily identifiable on the gel. In previous research fixed polyacrylamide concentrations in a range of 5% to 13% gels were commonly utilised, as opposed to the 4-20% gradient polyacrylamide gels utilised in this study. The erythrocyte ghost protein fingerprint was virtually identical across various fixed polyacrylamide gels and when compared to gradient polyacrylamide gels, with differences only being present in the spacing between the protein bands (Barker, 1991, Dzandu et al., 1985, Fairbanks et al., 1971, Kakhniashvili et al., 2004, Low et al., 2002, Marczak and Józwiak, 2008, Orlacchio et al., 2007, Rabilloud et al., 1999).



**Figure 3.1: Packed erythrocyte ghost pellet.** The method to isolate clean erythrocyte ghosts free of haemoglobin from whole blood samples was optimised.

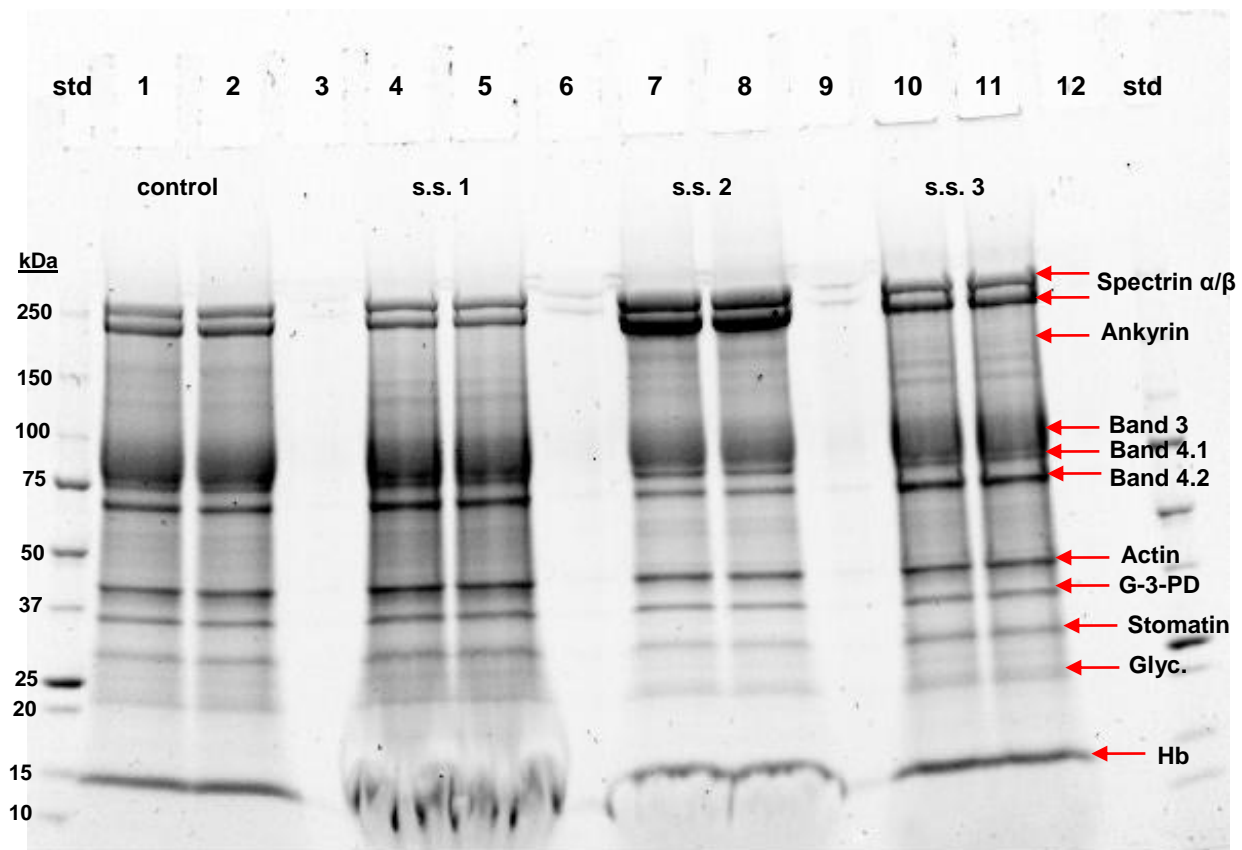


**Figure 3.2: SDS PAGE protein fingerprint of erythrocyte ghosts.** Multiple erythrocyte membrane and cytoskeletal proteins are successfully visualised using SDS PAGE and are identifiable by their molecular masses. The right lane contains molecular mass standards annotated with their kilodaltons (kDa) masses. G-3-PD is glyceraldehyde-3-phosphate dehydrogenase; Glyc. is glycoporphin; Hb is haemoglobin.

## 3.2 Erythrocyte Ghost Solubilisation

### 3.2.1 Results

Isolated erythrocyte ghost samples were treated with three different solubilisation solutions in order to determine which solubilisation treatment provided the highest quality protein fingerprint on SDS PAGE as compared to a control sample that did not receive any additional pre-solubilisation treatment. The results are displayed in Figure 3.3.



**Figure 3.3: SDS PAGE of control and solubilised erythrocyte ghost samples in duplicate.** Erythrocyte ghost samples were solubilised with three different solubilisation solutions (s.s.), being s.s. 1, s.s. 2 and s.s. 3. Lanes 1-2: control erythrocyte ghosts (only Laemmli sample solubilisation treatment); Lanes 4 – 11 are from samples initially treated with one of three solubilisation buffers before the standard Laemmli sample solubilisation buffer. Lanes 4-5: erythrocyte ghosts treated with s.s 1 (7 M urea, 2 M thiourea, 50 mM DTT, 2% ASB-14, 0.4% ampholytes); lanes 7-8: erythrocyte ghosts treated with s.s 2 (50 mM DTT, 2% ASB-14, 0.4% ampholytes); and lanes 10-11: erythrocyte ghosts treated with s.s 3 (7 M urea, 2 M thiourea, 50 mM DTT, 0.4% ampholytes). The two outer lanes are molecular mass standards annotated in kDa. Each sample well contained 30 µg of protein. The gel was a 4-20% Stain-Free gradient gel and was run at a constant 60 V. G-3-PD is glyceraldehyde-3-phosphate dehydrogenase; Glyc. is glycophorin; Hb is haemoglobin.

The resolution of the protein bands as well as differences in detectable protein bands between the control wells and the wells containing pre-solubilised erythrocyte ghosts, are evident on the gel. The presence of band differences occurred mainly in the 125-200 kDa region, where band differences can be observed throughout the gel, specifically when looking at the spectrin bands, Band 3 and Bands 4.1 and 4.2. The lanes where the solubilisation solution used contained the detergent ASB-14 (lanes 4 and 5, and lanes 7 and 8), have prominent disturbances near the bottom of the gel and a distortion of the 16 kDa haemoglobin band. Overall pre-solubilising the erythrocyte ghost samples prior to SDS PAGE appears to improve protein fingerprint quality according to the above results. Erythrocyte ghost treatments with Solubilisation solution 2 and Solubilisation solution 3 render the highest quality protein fingerprints. However, due to the fact that Solubilisation solution 2 gives rise to a seriously distorted pattern in the lower mass region, it can be said that erythrocyte ghosts treated with Solubilisation solution 3 prior to gel electrophoresis gives rise to the highest quality protein fingerprint when compared to the other solubilisation solutions and the control erythrocyte ghosts.

### 3.2.2 Discussion

Erythrocyte ghost isolates were treated with 3 different solubilisation solutions that contained a combination of the chaotropic agents urea and thiourea, the reducing agent dithiothreitol (DTT), the detergent amidosulfobetaine-14 (ASB-14) and ampholytes. Chaotropic agents are known to disrupt hydrogen bonds and when in contact with proteins result in the reduction of hydrophobicity and hence increase protein solubility (Salvi et al., 2005). They also disrupt protein aggregation, which can affect protein mobility during gel electrophoresis. Reducing agents cleave disulfide bonds within and between proteins aiding in disaggregation and their unfolding, therefore providing access to the interior of the protein for detergents such as SDS to bring about full denaturation. Detergents disrupt membranes, break hydrophobic interactions, solubilise lipids and release membrane bound proteins. Thus chaotropic agents, reducing agents and detergents all denature proteins and potentially dissociate oligomeric proteins into their subunits, which is crucial for reproducible quantitative and qualitative proteomic analysis. The result of all erythrocyte ghost samples pre-treated with the 3 solubilisation solutions, rendered overall higher quality protein fingerprints of the higher mass region of the gels, as compared to the control erythrocyte ghost sample fingerprint where only the SDS containing Laemmli buffer was used to solubilise the protein prior to electrophoresis.

The different solubilisation solutions rendered slightly different results with varying quality of specific bands found between treatments. Solubilisation solution 1 contained urea, thiourea, DTT, ampholytes and ASB-14. This mixture gave rise to a slightly improved quality protein fingerprint when compared to the control. This can be observed as Band 3, Band 4.1, Band 4.2 and actin appear more intense, but a very distorting effect is observed for the low molecular mass region, probably due to

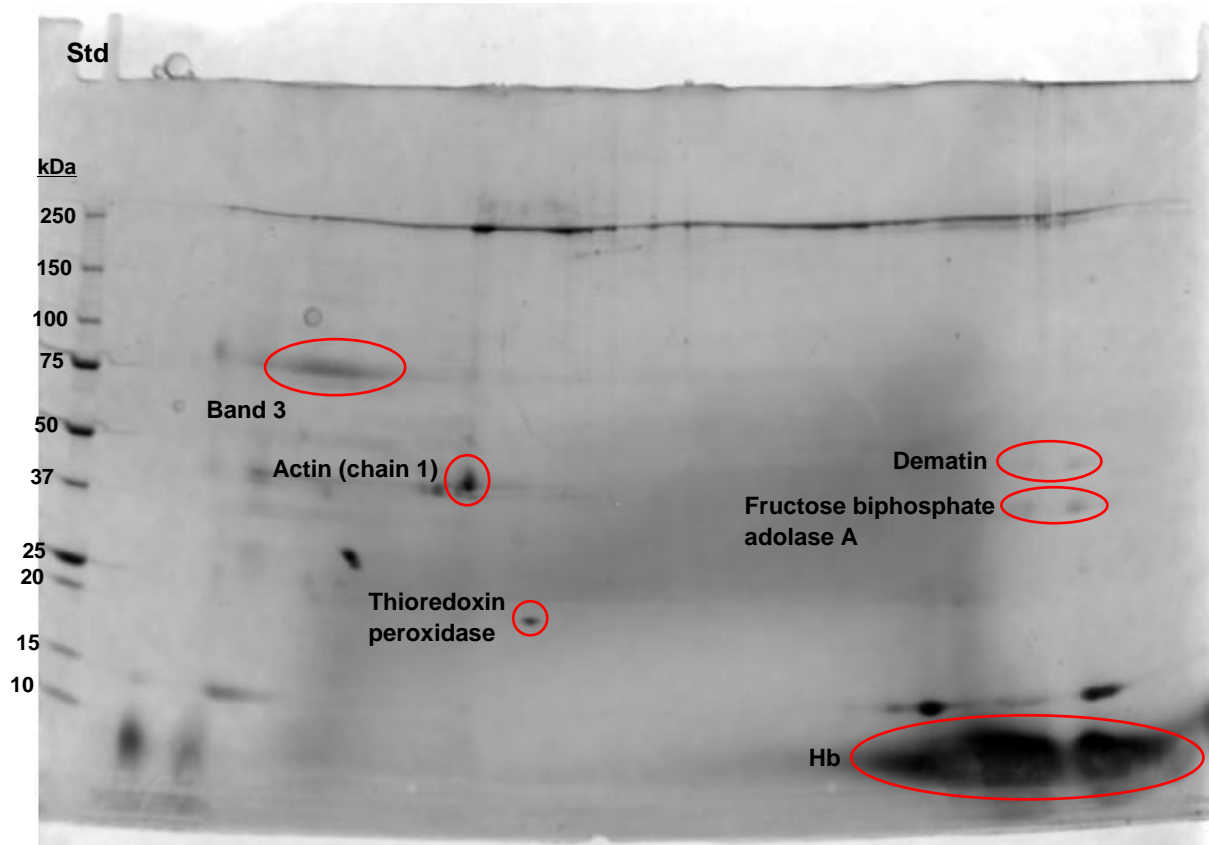
the increased salt concentration of the sample. The major distortion of the haemoglobin band at 16 kDa and distortion in the region from approximately 20 kDa to the bottom of gel was found to be associated with the zwitterionic ASB-14 detergent, and could be due to salt overloading effects. Solubilisation solution 2 contained DTT, ampholytes and ASB-14. Treatment of erythrocyte ghosts with this solution resulted in a protein fingerprint which was of higher quality than that of the control. The faint bands between 125-200 kDa are more pronounced and better visualised when compared to the control. In addition, Bands 3 and 4.1 are sharper and more easily distinguished from one another. However, treatment of the erythrocyte ghosts with this solution, although different in shape and severity, again distorted the haemoglobin band and resulted in a fingerprint of low quality in the low molecular weight region of the gel. In addition, the spectrin bands treated with Solubilisation solution 2 are not as well focused into narrow bands as the spectrin bands of the control and those treated with Solubilisation solutions 1 and 3. This effect could be due to selectively improved solubilisation of the spectrin proteins. Therefore it can be said that the chaotropic agents, urea and thiourea, are required to improve spectrin band quality. The low quality distortion near the bottom region of the gel only occurred when erythrocytes were treated with Solubilisation solutions 1 and 2. It can therefore be concluded that the detergent ASB-14 is responsible for this pattern and the distortion of haemoglobin, as both the control ghost erythrocytes and the treatment of erythrocyte ghosts with Solubilisation solution 3 contained no ASB-14. It is thought that ASB-14 causes this distortion by possibly introducing a high salt concentration into the samples. The third and final solubilisation solution, Solubilisation solution 3 contained urea, thiourea, DTT and ampholytes which when used to treat erythrocyte ghosts resulted in a high quality protein fingerprint on the gel. Compared to the control erythrocyte ghost protein fingerprint, improvements in the Solubilisation solution 3 fingerprint can be seen with better visualisation of the fine bands appearing in the 125-200 kDa region, Band 4.2 being more prominent, and the entire fingerprint demonstrating more focused protein bands and being of a higher visual quality. After analyzing the results in Figure 3.3, it was concluded that treatment of erythrocyte ghost samples with Solubilisation solution 3 renders the highest quality protein fingerprint on SDS PAGE.

These solubilisation techniques have been commonly utilised in two-dimensional gel electrophoresis (Luche et al., 2003, Rabilloud et al., 1999), but have not as yet been applied to erythrocyte ghosts on one-dimensional SDS PAGE.

### 3.3 Two-Dimensional Gel Electrophoresis (2-DE)

#### 3.3.1 Results and discussion

Erythrocytes ghosts were prepared from whole blood and were analysed by 2-DE in order to produce a characteristic protein fingerprint. An example of the resulting gel is shown in Figure 3.4.



**Figure 3.4: Two-dimensional gel electrophoresis of erythrocyte ghosts.** The proteins of erythrocyte ghost samples (250 µg of protein) were separated initially based on their respective isoelectric point (pI) values (first dimension) on an immobilised pH gradient (IPG pH 3 - 10) strip using isoelectric focusing (IEF). The ghost proteins were then reduced, alkylated, denatured in SDS and separated based on their respective sizes (second dimension) by SDS PAGE as is seen in Figures 3.2 and 3.3. The left end lane is a standard (std) lane and contains molecular mass standards annotated in kDa. Hb is haemoglobin. The gel was a 4-20% gradient gel and was run at a constant 200 V.

The resulting protein fingerprint of erythrocyte ghosts following 2-DE does not visualise an accurate representation of prominent membrane and cytoskeletal proteins present in erythrocyte ghosts. That is, compared to known one-dimensional SDS PAGE erythrocyte ghost protein fingerprints the number of identifiable membrane and cytoskeletal proteins on the 2-DE fingerprint is significantly less,

despite the fact that there are proteins visualised on 2-DE (Thioredoxin peroxidase, dematin and fructose biphosphate adolase A) that are not visible on SDS PAGE alone (Low et al., 2002, Marczak and Józwiak, 2008, Olivieri et al., 2001, Orlacchio et al., 2007). The major difficulty when it comes to the characterisation of membrane proteins by 2-DE is their relatively low representation on gels due to their low aqueous solubility and strong association with membrane lipids which often reduces their penetration into IPG strip gels. This creates a bias in the apparent abundance of the membrane proteins. Researchers have thus previously utilised zwitterionic detergents to improve membrane protein solubility, however it remains a challenge to achieve the level of solubility required for high quality 2-DE results. It is due to this apparent low abundance and hydrophobicity of these proteins that many of the known erythrocyte ghost proteins are not identified in the two-dimensional gel shown in Figure 3.4. The difficulty of analysing membrane proteins by 2-DE is well-documented and several research groups have attempted to improve upon and resolve this problem (Chae et al., 2012, Luche et al., 2003, Olivieri et al., 2001, Rabilloud, 2009).

In addition to the solubility problems affecting membrane protein analysis by 2-DE, this technique is also a more time consuming and costly process as compared to one-dimensional SDS PAGE. Based on these facts and the results obtained during initial 2-DE ghost protein fingerprint assessment, it was decided to utilise one-dimensional denaturing SDS PAGE for the analysis of erythrocyte ghost proteins in this study, as the erythrocyte ghost proteome was well represented when using the latter technique. In the event that SDS PAGE could not provide the required proteomic analysis, the option was left open to return to 2-DE and optimise the erythrocyte ghost protein fingerprint utilising multiple two-dimensional compatible solubilisation agents and techniques.

## **3.4 Trypsin Treatments**

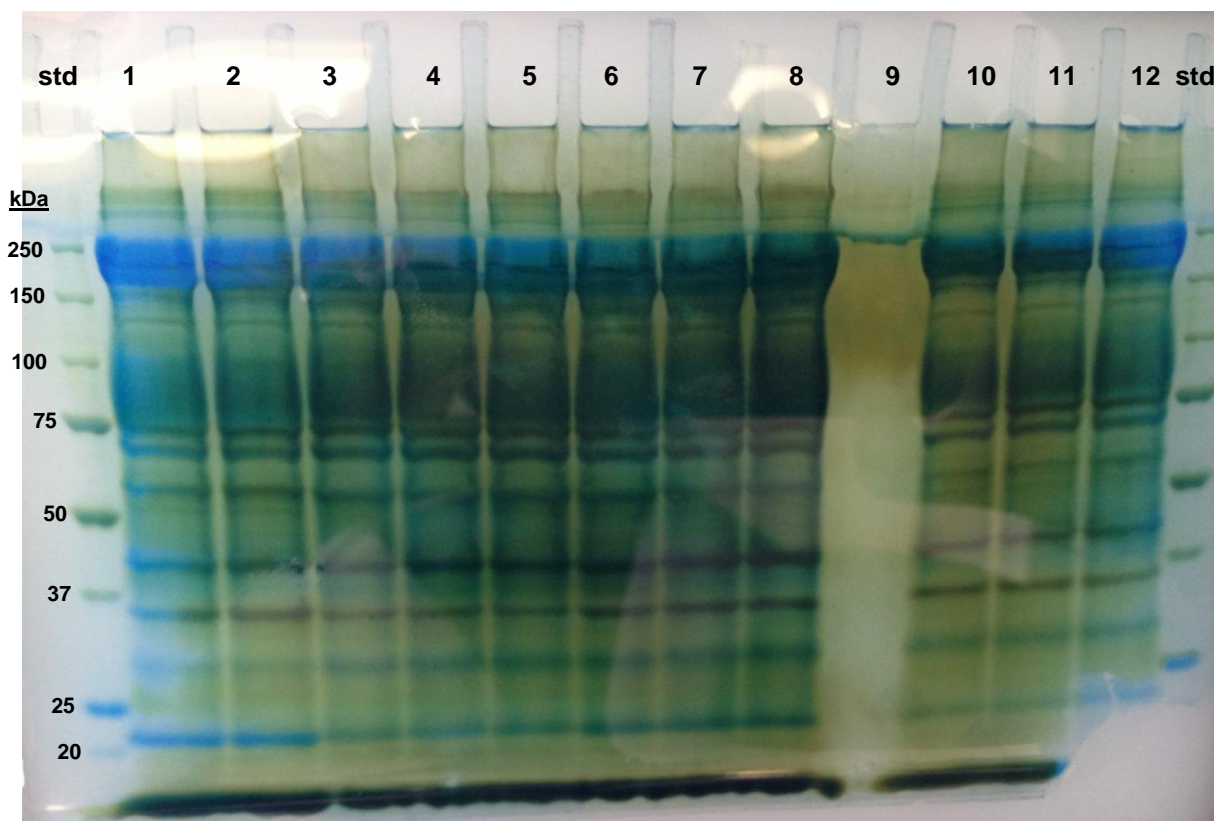
### **3.4.1 Results**

Erythrocytes isolated from whole blood were treated with trypsin prior to haemolysis and erythrocyte ghost preparation. The protein fingerprints of the trypsin-treated erythrocyte ghosts and untreated control erythrocyte ghosts were compared using SDS PAGE. A double-staining technique, fluorescent tagging and solubilisation were all assessed using the resulting SDS PAGE gels.

A double-staining technique that makes use of both silver and Coomassie Blue staining was applied to a repeat of one of the SDS PAGE gels that were run to assess the effect of trypsin cleavage on erythrocyte membrane proteins. This was done in order to better visualise band differences between trypsin-treated erythrocyte



ghosts and control erythrocyte ghosts by differentially staining proteins, lipids and sialoglycoproteins. A typical SDS PAGE gel comparing trypsin-treated erythrocyte ghosts to untreated, control erythrocyte ghosts that was stained with a silver stain and counterstained with Coomassie Blue stain is shown in Figure 3.5.

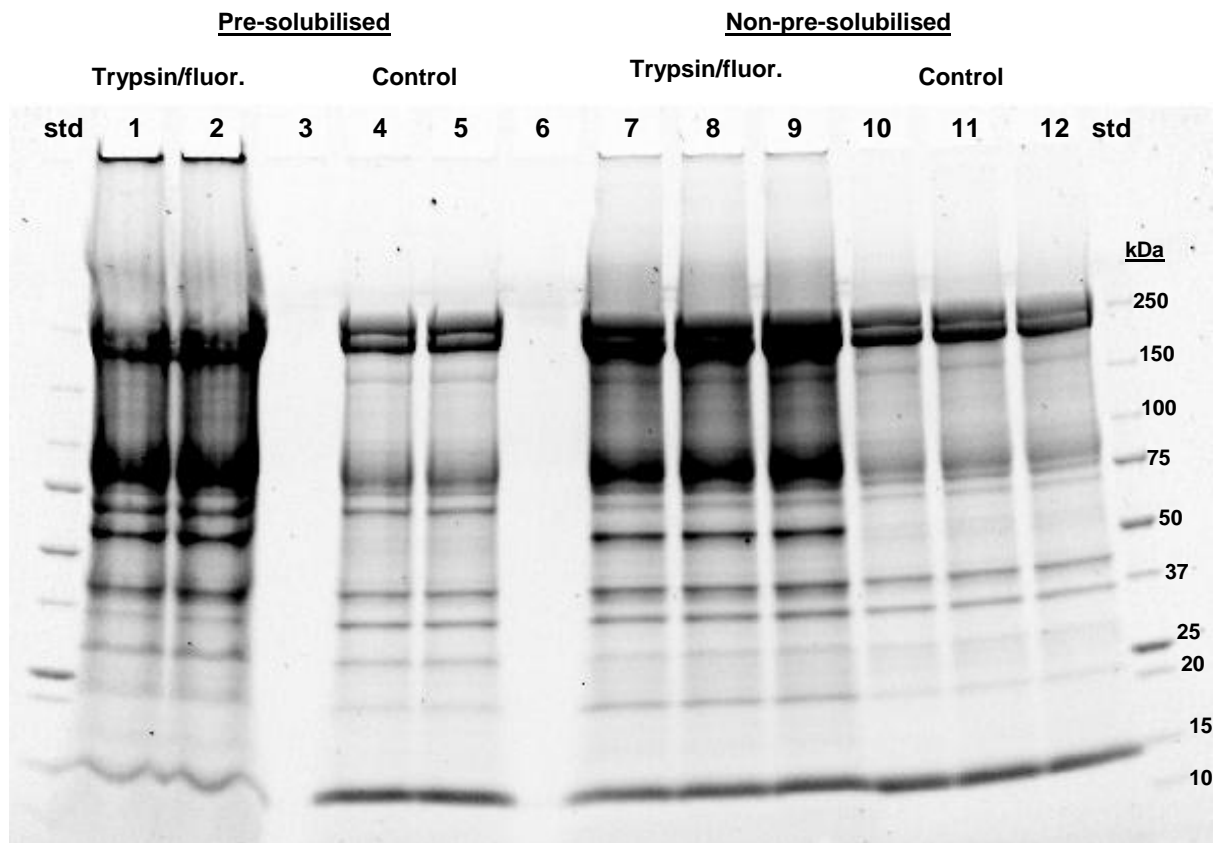


**Figure 3.5: Photograph of a gel of trypsin-treated and control erythrocyte ghosts stained with silver and counterstained with Coomassie Blue G-250 stains.** Erythrocyte ghost protein separations being treated with trypsin or untreated were stained using a double-staining technique. Lanes 1-8: three independently prepared trypsin-treated erythrocyte ghosts with lanes 1-2 in duplicate and lanes 3-8 each in triplicate; lanes 10-12: untreated, control erythrocyte ghost proteins in triplicate. The two outside lanes are standard (std) lanes and contain protein mass standards annotated in kDa. Each sample well contained 30  $\mu$ g of protein. The gel was run at a constant 60 V.

All sample-containing lanes (lanes 1-8 and 10-12) appeared oversaturated when stained using both stains. The double-staining technique is not uniform throughout the gel, as can be seen when comparing the staining patterns of identical samples, for example the comparison of lanes 1 and 2 to each other as well as to lanes 3-8, which contain replicate samples. Lane 1 did not stain as well as lane 2 using the silver stain (yellow stain), and both lanes 1 and 2 are stained to a lesser extent than the comparative sample lanes 3-8. Furthermore, silver staining seemed to stain something that was in the buffer as can be seen in the unfocused region between

250 and 75 kDa in lane 9 which contained no protein sample. The double-staining technique did however visualise bands that were not visible when using the Bio-Rad Stain-Free visualisation system with the Stain-Free gels in both the treated and control lanes. These stained bands generally not seen in the Stain-Free system were most obvious in the upper region of the gel, above the 250 kDa molecular mass standard.

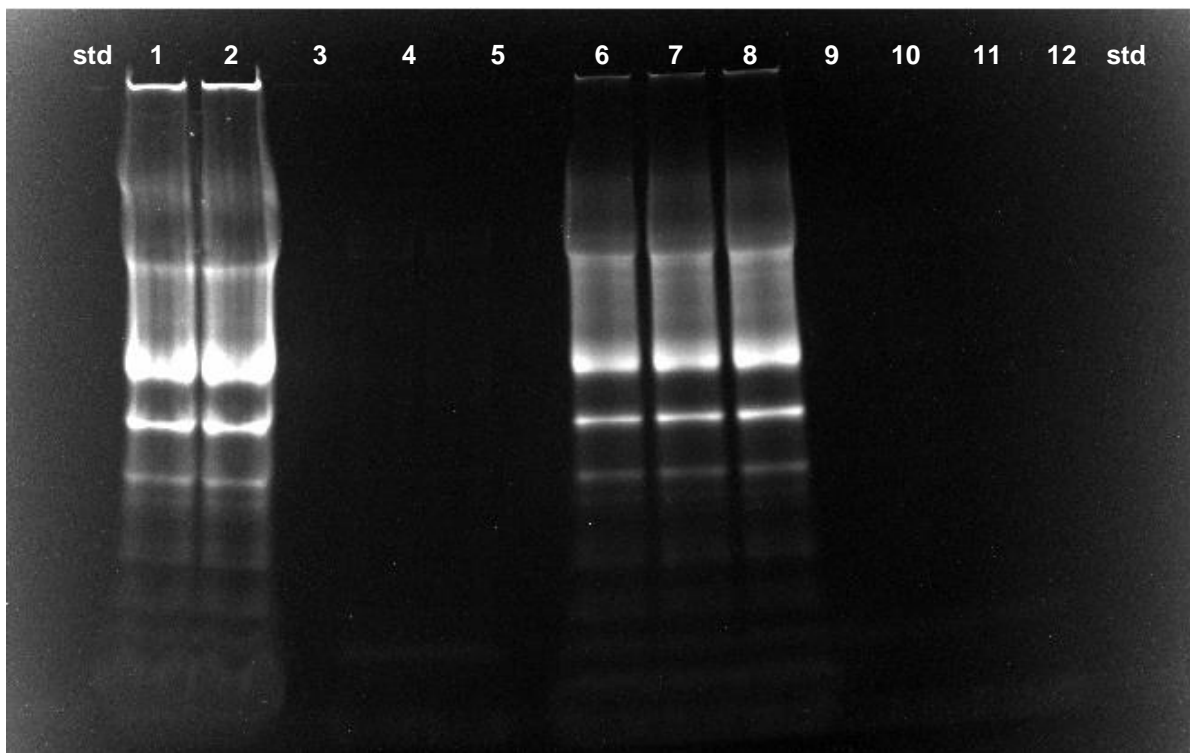
Fluorescein-5-EX N-hydroxysuccinimide ester (NHS-fluorescein) is a fluorescent tag which rapidly reacts with free primary amine groups, such as lysine residues, in aqueous media and was utilised to fluorescently tag proteins that had been cleaved via tryptic digestion where the carboxylic terminal amino acid would present a primary amine that could be derivatised. The effect of Solubilisation solution 3 (urea, thiourea, DTT and ampholytes) on the SDS PAGE protein fingerprint of erythrocyte ghosts formed from trypsin-treated erythrocytes that were subsequently fluorescently tagged, was assessed. The gel used for the analysis was a Criterion TGX Stain-Free gradient gel of 4 – 20% acrylamide and run under a constant 60 V. The SDS PAGE gels were visualised on a Stain-Free Sample Tray using a Gel Doc EZ Imager from Bio-Rad Laboratories, Inc. An Ultraviolet (UV) Sample Tray and a Blue Sample Tray were additionally utilised to assess the fluorescence of the tag. The gel scans were analysed using Bio-Rad Laboratories “Image Lab” version 3.0 build 11 software. The results are shown in Figure 3.6.



**Figure 3.6: SDS PAGE assessing the effect of Solubilisation solution 3 on trypsin-treated, fluorescently-tagged erythrocyte ghosts (scanned on Stain-Free Sample Tray).** The effect of Solubilisation solution 3 (7 M urea, 2 M thiourea, 50 mM DTT and 0.4% ampholytes) on erythrocyte ghost proteins isolated from trypsin-treated and NHS-fluorescein-tagged erythrocytes, is visualised on the gel. Lanes 1-2: trypsinised fluorescently-tagged and solubilised ghost proteins in duplicate; lanes 4-5: solubilised, control ghost proteins in duplicate; lanes 7-9: trypsinised and fluorescently tagged, ghost proteins in triplicate; lanes 10-12: untreated, control ghost proteins. Standard (std) lanes contain mass standards annotated in kDa. Each sample well contained 30  $\mu$ g of protein. The gel was run at a constant 60 V. Note the very high apparent band intensities of the fluorescently tagged samples despite the same protein loading as the controls.

The erythrocyte ghost samples that were derived from trypsin-treated, fluorescently-tagged erythrocytes, and pre-solubilised using Solubilisation solution 3 (lanes 1-2) result in a low quality protein fingerprint on SDS PAGE when visualised using the Stain-Free Sample Tray in the ChemiDoc instrument. These lanes showed sample products that are either too large to penetrate the gel or are insoluble in the base of the wells, vertical streaking common to poor solubility of the sample proteins, and poor resolution of the major high mass protein bands. When comparing this fingerprint to a comparative erythrocyte ghost sample that was not pre-solubilised (lanes 7 - 9), it is evident that the non-solubilised, trypsin-treated, fluorescently-tagged erythrocyte ghosts produce a protein fingerprint of better quality. Significantly less sample products are found in the base of the wells (lanes 7 - 9), no vertical

streaking is evident and the resolution of the protein bands in lanes 7 - 9 is substantially better than those in lanes 1 - 2. By analysing the two erythrocyte ghost sample controls on the gel visually it can be confirmed that unlike the treated erythrocyte ghost samples, the solubilised erythrocyte ghost controls (lanes 4 - 5) gave rise to a protein fingerprint that was of better quality when compared to the non-solubilised erythrocyte ghost controls (lanes 10 - 12). The protein bands in lanes from samples that were fluorescently tagged (lanes 1 - 2 and lanes 7 - 9) appear to be more abundant than lanes containing samples that were not fluorescently tagged, despite the fact that the same amount of erythrocyte ghost protein was loaded into each of the respective wells. When the gel was scanned on a Blue Sample Tray where only fluorescently tagged proteins were visualised, the resulting fluorescent fingerprint matched the bands of higher intensity visualised on the Stain-Free Sample Tray as can be seen in Figure 3.7. To determine whether this darkening in colour/increase in intensity of protein bands could be attributed to the presence of the fluorescent tag, an identical gel that was not loaded with protein samples was removed from the plastic casing and the surface of the gel was directly spotted using a syringe filled with the same concentration of NHS-fluorescein as was used to tag the trypsinised proteins (in DMSO with 1 mg/ml of glycine and no protein present in the sample). After allowing the added solution to soak into the gel it was scanned on a Stain-Free Sample Tray where each band or spot of the NHS-fluorescein tag placed on the gel was clearly visible. The image of the gel spotted with fluorescently labelled glycine is shown in Figure 3.8.

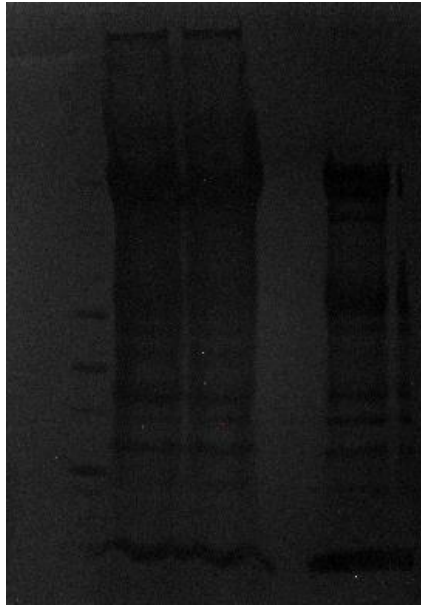


**Figure 3.7:** The image of the same SDS PAGE gel as in Figure 3.6 scanned here on a Blue Sample Tray to visualise only the fluorescently-tagged erythrocyte ghost proteins.



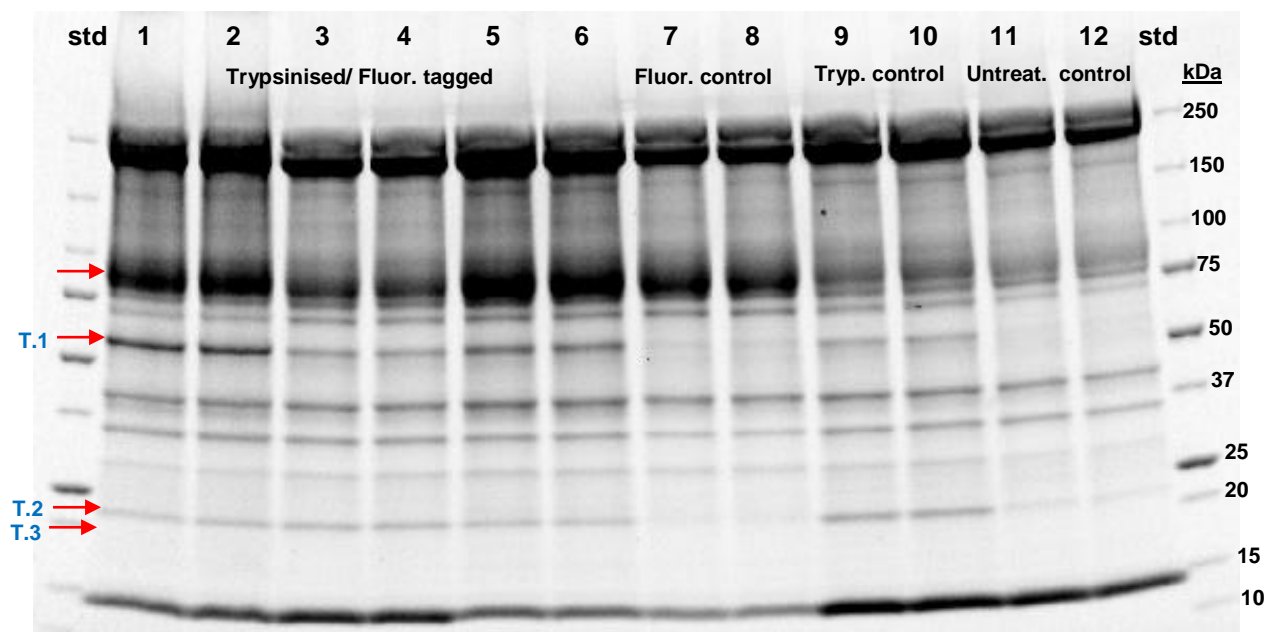
**Figure 3.8: Fluorescein-5-EX N-hydroxysuccinimide ester (NHS-fluorescein) glycine conjugate applied to a Stain-Free gel in the absence of protein demonstrates visualisation when the gel is scanned on a Stain-Free Sample Tray.** Glycine NHS-fluorescein conjugate (10 mg/ml NHS-fluorescein in DMSO with 1 mg/ml glycine) was pipetted directly onto an exposed Stain-Free gel. Lanes 1, 3 and 4 contained bands made up from volumes of 4  $\mu$ l, 2  $\mu$ l and 1  $\mu$ l of NHS-fluorescein glycine conjugate respectively.

Lanes 1 - 4 were cut from the gel in Figure 3.6, with the first two lanes containing fluorescently tagged proteins (lanes 1 - 2), lane 3 being empty and lane 4 containing untagged, control proteins. The gel section was stained with Coomassie Blue in order to assess the effect of this stain on fluorescent tagging and was scanned using a Blue Sample Tray. It was found that Coomassie Blue staining completely quenched all the fluorescence that was previously visualised on the gel in Figure 3.7. The image of the Coomassie Blue stained section of the same gel shown in Figure 3.7 visualised under the same conditions as used in Figure 3.7 is shown in Figure 3.9 below.



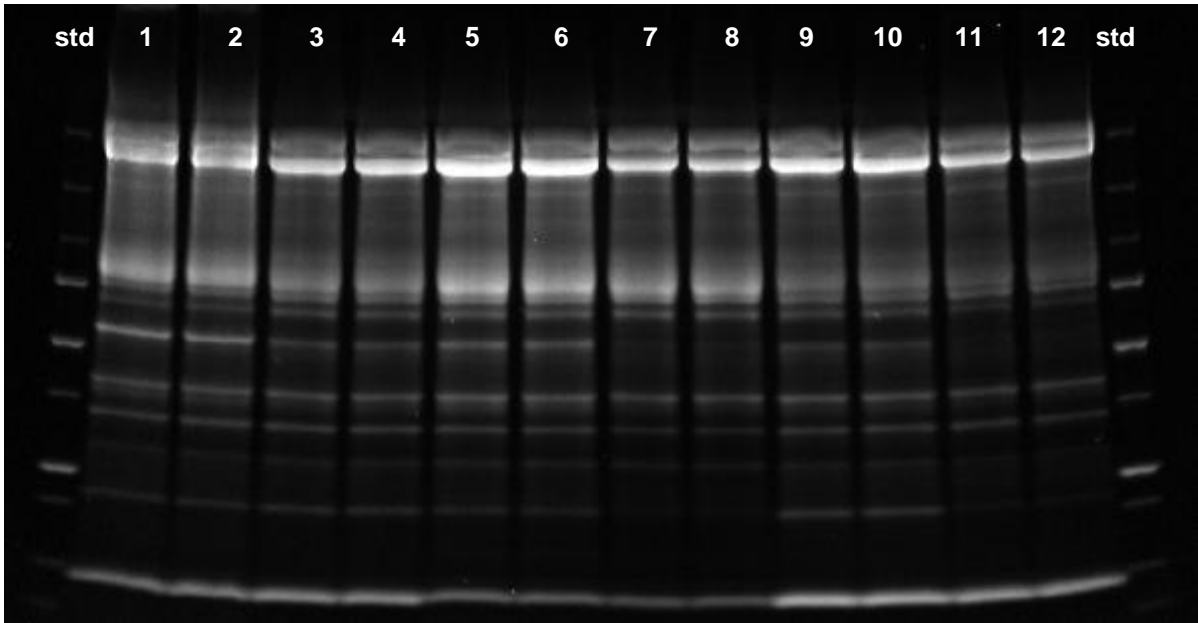
**Figure 3.9: Fluorescence of NHS-fluorescein-tagged SDS PAGE gel is completely quenched after being stained with Coomassie Blue and scanned on a Blue Sample Tray.**

Once the conditions resulting in the highest quality protein fingerprints of trypsinised, fluorescently tagged erythrocyte ghosts had been established, a gel was run in order to analyse band differences between trypsinised erythrocyte ghosts and control erythrocyte ghosts. This was done to track the effects that trypsin cleavage has on erythrocyte surface and cytoskeletal proteins. Three trypsinised then fluorescently tagged erythrocyte ghost samples were prepared independently of one another on separate days, according to exactly the same methods. These three samples along with a NHS-fluorescein tagged erythrocyte ghost control that was not trypsinised, a trypsinised erythrocyte ghost control that was not tagged and an erythrocyte ghost control that underwent no treatment were run on SDS PAGE and sequentially scanned on a Stain-Free Sample Tray, an UV Sample Tray and a Blue Sample Tray. The images of this gel under the different scanning conditions are shown in Figures 3.10, 3.11 and 3.12 respectively.

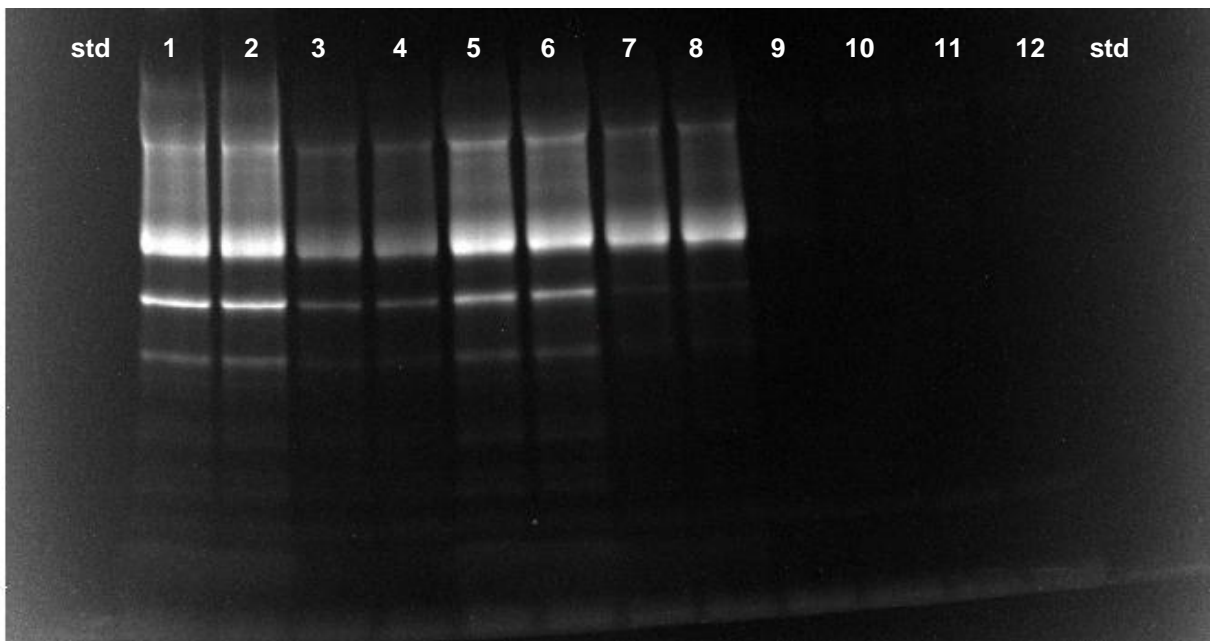


**Figure 3.10: SDS PAGE indicating the effect of trypsinisation on erythrocyte membrane proteins (scanned on a Stain-Free Sample Tray).** Erythrocytes were treated with trypsin and were fluorescently tagged prior to ghost formation. The effects of trypsin on the membrane proteins of the erythrocytes are visualised as protein band differences on the gel. Lanes 1 - 6: three independently prepared erythrocyte ghost samples that were trypsinised, then fluorescently tagged and run in duplicate; lanes 7 - 8: non-trypsinised, fluorescently tagged erythrocyte ghosts (fluorescence control) in duplicate; lanes 9 - 10: trypsinised erythrocyte ghosts in duplicate (trypsin control); lanes 11 - 12: untreated control erythrocyte ghosts in duplicate. Band differences between trypsinised and control erythrocyte ghosts are indicated with red arrows. Blue labels identify bands sequenced by LC-MS/MS. Standard (std) lanes contain mass standards annotated in kDa. Each sample well contained 30  $\mu$ g of protein. The gel was run at a constant 60 V.

Band differences between trypsinised erythrocyte ghosts and untreated, control erythrocyte ghosts are clearly visualised on the SDS PAGE gel. Band differences between the trypsin control erythrocyte ghosts (lanes 9 - 10) and the untreated, control erythrocyte ghosts (lanes 11 - 12) can be seen at 88 kDa, 58 kDa, 23 kDa and a faint band of 18 kDa that is not visible in the printed image of Figure 3.10, but that is visible on the digital scan of the gel with enhanced intensity monitoring. In all fluorescently tagged samples (lanes 1 - 8), a band in the region of 80 - 95 kDa appears darker and more intense.



**Figure 3.11:** An image of the same gel shown in Figure 3.10 indicating the effect of trypsinisation on erythrocyte membrane proteins by fluorescent tagging of resultant cleaved proteins scanned on an UV Sample Tray that shows up proteins with intrinsic fluorescence. Standard (std) lanes contain mass standards. See legend of Figure 3.10 for details of lane identities.



**Figure 3.12:** An image of the same gel seen in Figure 3.10 indicating the effect of trypsinisation on erythrocyte membrane proteins by fluorescent tagging of resultant cleaved proteins scanned on a Blue Sample Tray that excites only the fluorescent tag. Standard (std) lanes contain mass standards. See legend of Figure 3.10 for details of lane identities.



Proteins tagged with NHS-fluorescein are visualised on the Blue and the UV Sample Trays. Bands other than those that were cleaved by trypsin, fluorescently tagged and visualised on the Stain-Free Tray (Figure 3.10) are found to fluoresce. These bands are seen mainly in the 75-260 kDa region on both the UV and Blue Sample Trays. Erythrocyte ghost protein samples that were not fluorescently tagged (lanes 9-12) are found to fluoresce on the UV Sample Tray, most probably due to intrinsic fluorescent properties, whereas the fluorescently-tagged 23 kDa cleavage product of trypsin clearly fluoresces on the UV Sample Tray, but is not visualised on the Blue Sample Tray.

### 3.4.2 Discussion

The silver and Coomassie Blue double-staining technique has previously been utilised in order to improve the analysis of biological membranes on SDS PAGE. This is achieved by using Coomassie Blue to stain the conventional Coomassie Blue-sensitive erythrocyte membrane proteins a blue colour, and silver stain to stain lipids and sialoglycoproteins, which are periodic acid-Schiff reagent-sensitive, a yellow colour. This allows for the easy differentiation between erythrocyte membrane proteins, lipids and sialoglycoproteins on a single gel. This double-staining technique is especially helpful when analysing trypsin-treated erythrocytes, as this enzyme has been reported to cleave sialoglycoproteins present on the erythrocyte membrane (Dzandu et al., 1984).

In this experiment however, the double-staining technique reduced the quality of both the trypsin-treated and the control erythrocyte ghost protein fingerprints as compared to an unstained gel. The gel appeared oversaturated with the combination of stains, which resulted in substantial background interference and loss of visual quality of the protein bands constituting the erythrocyte ghost fingerprints. Protein fingerprints that are not of a high quality are almost impossible to visually analyse and compare. Thus, this double-staining technique did not allow for clear and accurate protein fingerprint comparison between the control and trypsin-treated erythrocyte ghost samples, and band differences were difficult to detect. Making protein fingerprint comparisons more complicated is the fact that the gel was not stained uniformly. That is, certain areas/lanes of the gel appeared to stain more intensely than other areas/lanes despite the fact that the entire gel was exposed to the staining technique that involved gentle agitation to ensure even distribution of the staining solutions. Most notably lanes 1 and 2 (Figure 3.5) were minimally stained with silver when compared to the other lanes (3-8) which contain comparative erythrocyte samples. Differences in stain intensity are even witnessed between virtually identical triplicate samples when comparing lanes 6 and 7 to lane 8. The fact that a lack of silver staining can be seen in the end lanes 1, 2 and 12, suggests a possible 'edge effect' occurring during the staining procedure. Furthermore the silver stain was not very selective as is evident from the area of yellow stain in lane 9,

which contained no erythrocyte ghost sample. Due to the variance in this double-staining technique, reproducibility of gels will most likely be an obstacle.

Despite the reduction in protein fingerprint quality, the double-staining technique did allow for the visualisation of bands with high mass found near the top of the gel, which were not visible on the gel before it was stained with the silver stain. These bands do not appear in the previously known and traditionally accepted erythrocyte protein fingerprint however, and since they are present in all the sample-containing lanes, these bands cannot be attributed to trypsin-related protein cleavage (Barker, 1991, Dzandu et al., 1984, Fairbanks et al., 1971, Low et al., 2002, Yamamoto et al., 1985). The high mass protein bands are thought to be protein aggregates which have no experimental significance in the comparison of untreated erythrocyte proteins to those treated with trypsin (Marczak and Józwiak, 2008).

The oversaturation of staining, the non-specific staining and the lack of uniformity in the double-staining Coomassie Blue/silver stain technique could most likely be improved upon through method optimisation. That is, possibly varying incubation times of the gel in the stains, adding additional water washes and ensuring the complete and uniform coverage of the gel in the various reagents and chemicals throughout the procedure. This procedure however was utilised some 30 years ago and although a cheaper option to use, since then faster and more reproducible methods have become available to visualise the different components in a protein fingerprint. These include the utilisation of Stain-Free gels, as well as fluorescence tagging, which are less time consuming and have better sensitivity and reproducibility when compared to the Coomassie Blue/silver stain technique. Stain-Free options often are the better choice over many gel electrophoresis staining protocols, as there is no background staining, possibly quenching protein bands (Fan et al., 1991, González-Fernández et al., 2014, Susnea et al., 2011, Susnea et al., 2013).

The ability of Solubilisation solution 3 to produce an erythrocyte protein fingerprint of the highest quality was investigated when ghosts formed from erythrocytes that were treated with trypsin and tagged with Fluorescein-5-EX N-hydroxysuccinimide ester (NHS-fluorescein) were solubilised in 7 M urea, 2 M thiourea, 50 mM DTT and 0.4% ampholytes. This was done in the hope that the solubilisation step would improve the quality of the SDS PAGE protein fingerprint of the sample, as was observed with the untreated erythrocyte ghost samples. This however was not the case, as the resulting solubilised protein fingerprint was of a lower quality than that of the non-solubilised, comparatively treated erythrocyte ghost sample. The solubilisation of the trypsin-treated fluorescently tagged ghosts resulted in the presence of insoluble protein aggregates in the wells of the gel, vertical streaking and a decrease in resolution when compared to the non-solubilised sample. The streaking can be said to be due to the insoluble protein aggregates present in the wells, which also cause a reduction in band resolution. That is to say that the NHS-fluorescein used to label

the lysine residues of trypsinised proteins is causing some of the proteins to become insoluble in Solubilisation solution 3.

It was further found that proteins tagged with NHS-fluorescein appeared darker on the SDS PAGE gel when compared to comparative protein bands that were not fluorescently tagged. This suggests that more protein was present in the fluorescently tagged samples than the non-tagged samples despite the fact that the same volume and concentration of erythrocyte ghost sample was loaded in all the wells. When scanning the same gel on a Blue Sample Tray, the visualised fluorescent protein fingerprint matched the darker protein fingerprint visualised on the Stain-Free Sample Tray when it was scanned. This suggested that the higher intensity bands present in the fluorescently-tagged protein samples were due to the presence of NHS-fluorescein. By directly pipetting NHS-fluorescein onto a Stain-Free gel in the complete absence of any proteins and scanning this gel, it can be confirmed that NHS-fluorescein is visualised on the gel and thus contributes to the intensity of the protein bands where these tags are present. This is of concern as the intensity of each protein band visualised by the Stain-Free system on the special SDS PAGE gels can potentially be utilised as a quantitative tool. Therefore using a fluorescent tag on such a gel renders the potential of a quantitative analysis null and void.

When a section of the SDS PAGE gel that contained fluorescently tagged erythrocyte ghost sample as well as control erythrocyte ghost sample with no tag, was stained with Coomassie Blue and scanned using a Blue Sample Tray, it was found that Coomassie Blue quenches the fluorescent tag. That is all the fluorescent protein bands that were visualised on the blue plate prior to Coomassie Blue staining, were no longer visible on the gel after staining.

Three independently prepared trypsinised ghost proteins tagged with NHS-fluorescein were analysed on SDS PAGE in order to track the actions of trypsin on surface erythrocyte proteins by visualising trypsin cleavage proteins. This was done by comparing the SDS PAGE protein fingerprints of these trypsinised and fluorescently tagged erythrocyte ghost proteins to various control erythrocyte ghost SDS PAGE protein fingerprints. The experimental trypsinised and fluorescently-tagged samples were compared to (i) erythrocyte ghosts that were not trypsinised but that were tagged with NHS-fluorescein (fluorescence control), (ii) to trypsinised erythrocyte ghosts that were not tagged (trypsin control) and (iii) to erythrocyte ghosts that were not treated with trypsin or tagged with NHS-fluorescein (untreated control).

The gel was first scanned using a Stain-Free Sample Tray, which in theory does not visualise fluorescence. Comparisons between the protein fingerprints of the three independently but identically prepared, trypsinised and fluorescently tagged samples, revealed fairly obvious band intensity differences. The second sample (Figure 3.10, lanes 3-4) contained bands of a lower intensity when compared to the

same protein bands of the two other samples. This can be attributed to variance in erythrocyte ghost preparation. The erythrocyte ghost pellet is washed multiple times where after centrifugation, the supernatant is removed from the pellet. This process runs the risk of removing ghost sample when trying to remove as much haemoglobin and supernatant as possible from the pellet, and thus erythrocyte ghost sample concentration may vary after preparation. This too explains the variance in the concentration of haemoglobin present between samples prepared according to the same methods. Analysis of the fluorescence control revealed that NHS-fluorescein is responsible for a significant increase in band intensity of erythrocyte ghosts that have not been trypsinised, in the 75-95 kDa region of the gel. Due to this darkened region on the gel which is attributed to the presence of NHS-fluorescein, the trypsin control was required to reveal a trypsin cleavage product of 88 kDa that was otherwise obscured. Besides the 88 kDa cleavage product, the trypsinised, fluorescently-tagged erythrocyte ghost samples and the trypsinised, untagged erythrocyte ghost samples revealed the same protein fingerprint on SDS PAGE when scanned on a Stain-Free Tray. When comparing the trypsinised erythrocyte ghosts tagged with NHS-fluorescein to the controls, additional protein cleavage products are evident. These protein bands appear at 58 kDa, 23 kDa and a very faint band at 18 kDa that is only visible on a digital monitor. Trypsin has previously been shown to cleave the erythrocyte surface sialoglycopeptide, glycophorin A dimer at its N-terminal (Dzandu et al., 1985, Hadley et al., 1987, Perkins, 1981, Sim et al., 1994). However, the reported masses of the glycophorin A dimer and monomers, as well as resulting cleavage proteins after trypsinisation vary in literature. Glycophorin A dimer has been reported to have masses of 90 kDa, 88 kDa and 65.7 kDa, where glycophorin A monomers have been reported to have masses of 60 kDa, 38 kDa, 41 kDa, 31.1 kDa and 27.9 kDa respectively (Dohnal et al., 1980, Dzandu et al., 1984, Dzandu et al., 1985, Perkins, 1981). It has previously been stated that estimates of the mass of the glycophorin A dimer found using SDS PAGE and molecular weight standards, ranged from 53-90 kDa (Banker and Cotman, 1972, Bretscher, 1971, Lenard, 1970, Segrest et al., 1971). Cleavage products of glycophorin A after trypsinisation of erythrocyte surface proteins have previously been found to appear at 40 kDa and 18 kDa, and 58 kDa and 38 kDa (Dzandu et al., 1984, Dzandu et al., 1985). The variation observed in the mass of glycophorin A in both its dimer and monomer forms, can be attributed firstly to the changes and improvements in SDS PAGE technology, for example the improvement of protein band resolution on gels and hence more accurate molecular mass determination. In addition, previous studies have shown and offered explanations to the anomalous migration of membrane proteins, including that of glycophorin A, on SDS PAGE. It was found that the concentration of sodium dodecyl sulfate (SDS), the use of Tris-buffer systems, the protein concentration of the sample to be loaded onto the gel, as well as the temperature at which the sample is incubated in the sample buffer, all play a role in electrophoretic mobility variation. The glycophorin A dimer can dissociate and reassociate when in the presence of detergents, and dimers present in the SDS-containing sample buffer can partially dissociate when heated (Furthmayr and

Marchesi, 1976, Marton and Garvin, 1973). It appears to be common for membrane proteins to not correlate with molecular weight standards on SDS PAGE. This anomaly has been termed “gel shifting” and is yet to be fully understood or explained (Rath et al., 2009).

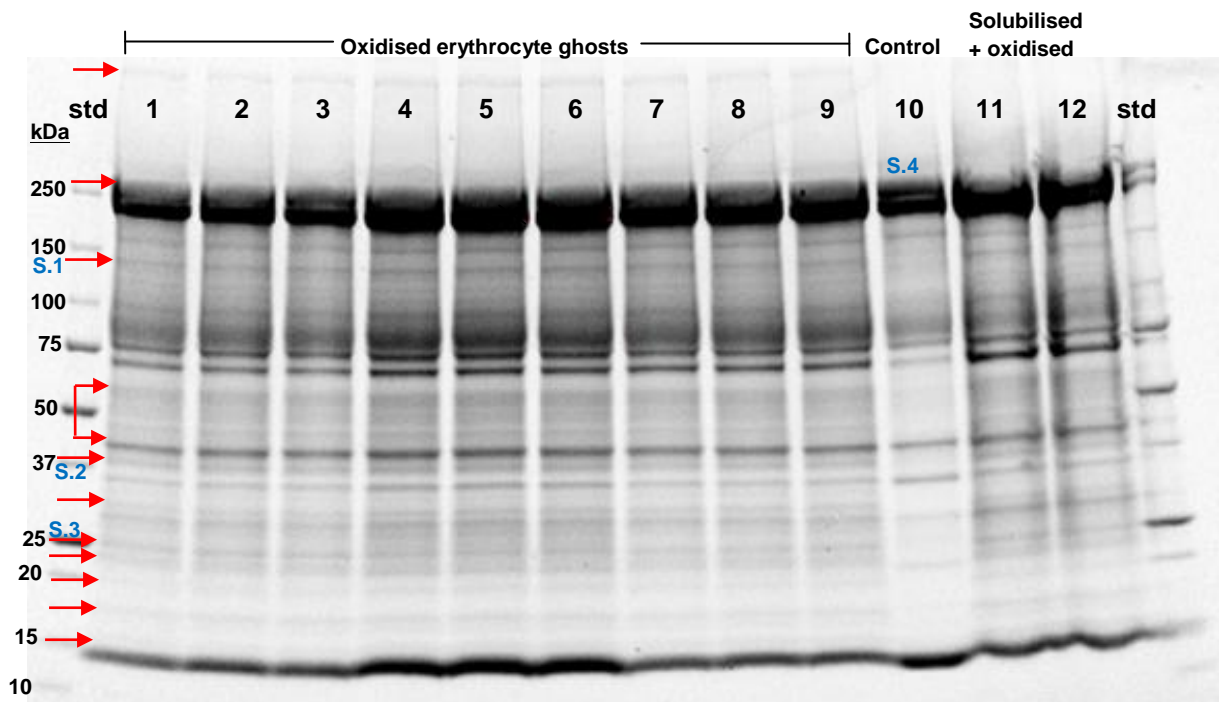
The band differences observed for the trypsin-treated erythrocyte ghost protein fingerprint (Figure 3.10) are thought to be due to the cleavage of glycophorin A as the resultant molecular weights match closely to those in related literature. However, due to the variability noted in the molecular weights of glycophorin dimers, monomers and cleavage products, mass spectrometric analysis of the protein bands in question was performed for absolute identification. There may very well be cleavage products of an erythrocyte surface trypsinised protein that is not glycophorin A, but that has a molecular mass close to that of a previously identified glycophorin A cleavage product. Using cutting edge, modern technology such as LC-MS/MS can reveal such detail that was unidentifiable in previous research, much of which was performed up to four decades ago.

The gel illustrated in Figure 3.10 was scanned on both the Blue Sample Tray and the UV Sample Tray. The Blue Sample Tray is found to be more selective for visualisation of proteins tagged with NHS-fluorescein in the heavier molecular weight region when compared to the UV Sample Tray that can cause auto-fluorescence of many proteins with intrinsic fluorescent ability. The fluorescently-tagged protein bands were easily visualised in the 37-250 kDa region on the Blue Sample Tray, but no bands of less than 37 kDa were observed to fluoresce. Most notably protein bands that did fluoresce on the Blue Sample Tray were those tagged with NHS-fluorescein, whereas bands that were not cleaved (as seen utilising the Stain-Free Sample Tray) fluoresced on the UV Sample Tray. All the prominent bands present in both the control and the trypsinised lanes on the gel that were visualised on the Stain-Free Tray, fluoresced and hence were visualised on the UV Sample Tray. This indicates that the UV Sample Tray is not selective for fluorescent tags, although the proteins that were cleaved by trypsin and tagged with NHS-fluorescein that had molecular masses below 37 kDa were easily observed. Thus, using both the Blue and the UV Sample Trays would be best for protein fingerprint analysis, although neither of these trays are vital, as no bands were visualised using either tray that could not be visualised on the Stain-Free Sample Tray. The UV and Blue Sample Trays need only be utilised for image clarity and protein cleavage confirmation in this specific case.

## 3.5 Sodium Periodate Treatments

### 3.5.1 Results

Erythrocytes isolated from whole blood were treated with sodium periodate prior to haemolysis and erythrocyte ghost formation. The protein fingerprints of the sodium periodate-treated erythrocyte ghosts and control, untreated erythrocyte ghosts were compared using SDS PAGE. The results are shown in Figure 3.13.



**Figure 3.13: SDS PAGE separation of erythrocyte ghost proteins visualising the protein-altering effects of sodium periodate.** Erythrocyte surface proteins were exposed to the oxidative agent sodium periodate which causes cleavage of glycosidic side chains and oxidative stress within the plasma membrane, and thus resulted in erythrocyte protein modifications. These surface protein modifications are clearly visualised on the gel as protein band differences. Lanes 1-9: three erythrocyte ghost samples each in triplicate, independently prepared and treated with sodium periodate; lane 10: untreated, control erythrocyte ghost sample; and lanes 11-12: erythrocyte ghosts treated with sodium periodate then solubilised in Solubilisation solution 3 (7 M urea, 2 M thiourea, 50 mM DTT and 0.4% ampholytes), in duplicate. Band differences between sodium periodate-treated and control erythrocyte ghosts are indicated with red arrows. Blue labels identify bands sequenced by LC-MS/MS. Standard (std) lanes contain mass standards annotated in kDa. Each sample well contained 30 µg of protein. The gel was run at a constant 60 V.

Erythrocyte surface protein differences induced by exposure to the potent oxidant sodium periodate, were visualised as band differences in the ghost protein fingerprint utilising SDS PAGE. Band differences noted in the treated erythrocyte samples as compared to the control erythrocyte samples include the appearance of a high mass protein band above the range of the mass standards (above the 250 kDa region), a band of 145 kDa, many faint bands in the region of 43-60 kDa, followed by band differences at 38 kDa, 30 kDa, 25 kDa, 23 kDa, 20 kDa, 18 kDa and 16 kDa. Solubilising erythrocyte ghosts that have been treated with sodium periodate, resulted in the disappearance of the high mass protein band (located at the top of the gel outside of the mass standard range) present in the non-solubilised sample, and decreased the quality of the erythrocyte ghost protein fingerprint by causing a reduction in band resolution. The solubilisation treatment (urea, thiourea, DTT and ampholytes) of the erythrocyte ghosts in lanes 11-12 contaminated the adjacent standard well, rendering the mass standard fingerprint unreliable.

### 3.5.2 Discussion

Human erythrocyte surface proteins were exposed to oxidative stress after having been treated with 5 mM sodium periodate. Utilising SDS PAGE, which allows for the separation and visualisation of erythrocyte surface and cytoskeletal proteins, it is found that this potent oxidative treatment induces obvious changes in several erythrocyte proteins. The three independently prepared control erythrocyte ghost samples, were prepared according to the same methods and band intensity differences between these three control samples is attributed to minor variation in protein concentrations. Due to the multiple wash steps required in attaining ghosts, unavoidable ghost protein loss may occur. When the protein fingerprint of the untreated, control erythrocyte ghosts is compared to the fingerprint of erythrocyte ghosts prepared in the exact same manner, except for the treatment with sodium periodate, distinctive band differences are observed on the gel. Sodium periodate has previously been shown to cross-link erythrocyte proteins, with spectrin polypeptides being the most susceptible to this treatment (Gahmberg et al., 1978). It is thought that the large protein band visualised in the oxidised ghost samples in Figure 3.13, above the 250 kDa region, is the result of the partial cross-linking of spectrin. The exact mechanism whereby sodium periodate induces the cross-linking of polypeptides remains unknown, however oxidising agents are known to be responsible for tyrosine dimerisation, and this is may possibly be the cause (Waykole and Heidemann, 1976). Periodate is also known to form aldehyde groups from the glucose residues of glycoproteins and the reactive residues could also be the reactive cross-linking moieties linking closely positioned amine residues of nearby proteins. Multiple protein band differences are visualised on the SDS PAGE gel for sodium periodate-treated erythrocytes which includes bands from a wide mass range, with some being more prominent than others and where the majority of differences are found in the 20-60 kDa region. Protein band changes visualised after

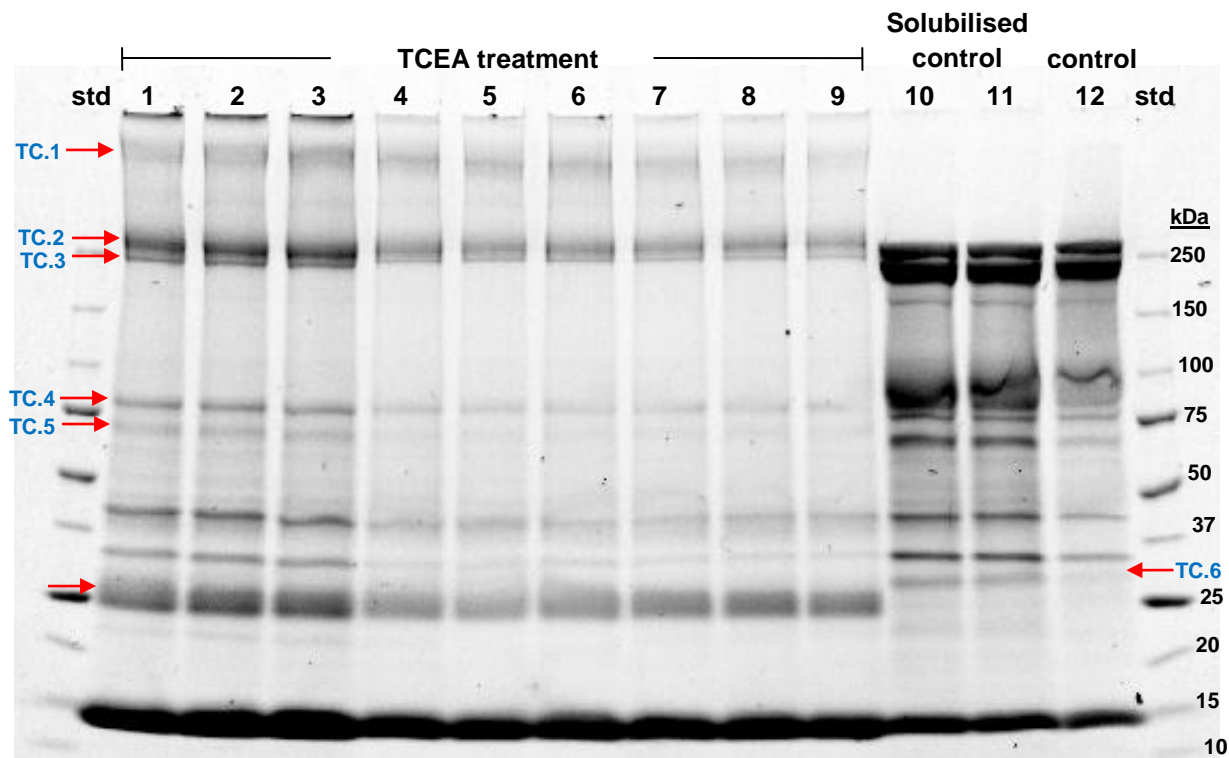
the treatment of erythrocytes with sodium periodate have not previously been reported in literature. The SDS PAGE gels that have been published in literature are some 35 years old where the techniques and tools available at that time, were not sensitive enough to detect the band differences visualised now (Gahmberg et al., 1978). This demonstrates the value of applying the modern and advanced scientific techniques available today (for example precast gradient gels with Stain-Free visualisation and digital analysis) to past research, as results can differ substantially in comparison. The prominent band differences of the oxidised erythrocyte ghost samples require LC-MS/MS analysis for identification of the proteins showing changes in order to better understand the effects that sodium periodate has on the erythrocyte membrane, and more specifically on surface and cytoskeletal proteins.

## **3.6 Tris(2-chloroethyl)amine (TCEA) Treatments**

### **3.6.1 Results**

Erythrocytes isolated from whole blood were treated with the amine cross-linker TCEA prior to haemolysis and erythrocyte ghost formation. The protein fingerprints of erythrocyte ghosts treated with TCEA were compared to the protein fingerprints of control, untreated erythrocyte ghosts utilising SDS PAGE. The results are shown in Figures 3.14 and 3.15.

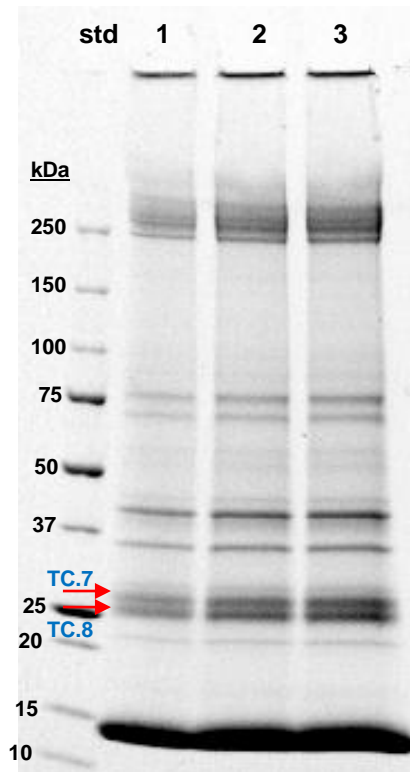




**Figure 3.14: SDS PAGE separation of erythrocyte ghost proteins visualising the protein-altering effects of TCEA.** Erythrocytes were treated with the cross-linking agent TCEA. Resulting surface and cytoskeletal protein modifications are clearly visualised on the gel in the form of protein band differences. Lanes 1-9: three erythrocyte ghost samples each in triplicate, independently prepared, solubilised and treated with TCEA; lane 10-11: untreated, control erythrocyte ghost samples solubilised in Solubilisation solution 3 (7 M urea, 2 M thiourea, 50 mM DTT and 0.4% ampholytes), in duplicate. Lane 12: untreated, control erythrocyte ghost sample. Band differences between TCEA-treated and control erythrocyte ghosts are indicated with red arrows. Blue labels identify bands sequenced by LC-MS/MS. Standard (std) lanes contain mass standards annotated in kDa. Each sample well contained 30  $\mu$ g of protein. The gel was run at a constant 60 V.

TCEA which can diffuse through erythrocyte membranes before causing protein cross-linking, brought about changes to surface and cytoskeletal proteins that are visualised as protein band position and intensity changes on SDS PAGE. The cross-linking agent resulted in the formation of protein products in the erythrocyte ghost samples that were too large in mass to pass through the pores of the gel and thus collected in the bottom of the sample wells of the gel. In the TCEA-treated erythrocyte samples, various protein band differences within a wide molecular mass range can be seen when compared to the control erythrocyte ghost sample protein fingerprint. These TCEA-induced differences include the appearance of a high mass protein band appearing out of the range of the mass standards (i.e. heavier than 250 kDa), the disappearance of the spectrin alpha and spectrin beta bands present in the control protein fingerprint and the appearance of higher mass bands of approximately 270 kDa and 250 kDa respectively in the treated protein fingerprint, the partial disappearance of Band 3, the disappearance of Bands 4.1 and 4.2, the

appearance of an 85 kDa and a 75 kDa band, the disappearance of stomatin (31 kDa) and the appearance of a broad band covering approximately the 23-29 kDa range. Another difference noted between the control erythrocyte ghost protein fingerprint and the fingerprint of erythrocyte ghosts treated with TCEA, is the increased quantity of haemoglobin present in the treated sample.



**Figure 3.15: SDS PAGE of TCEA-treated erythrocyte ghosts run at 60-200 V.** Erythrocytes prepared and treated independently of one another with TCEA were run in triplicate on SDS PAGE at 60 V initially for the first 30 minutes of the run and then increased to 200 V for the remainder. Red arrows indicate band differences as compared to a comparable gel run at a constant 60 V (Figure 3.14). Blue labels identify bands sequenced by LC-MS/MS. Standard (std) lanes contain mass standards annotated in kDa.

Erythrocyte ghosts from TCEA-treated erythrocytes were run on SDS PAGE according to protocol, where the gel was run at an initial 60 V for 30 minutes after which the voltage was increased to 200 V instead of a constant 60 V. This increase in voltage resulted in better resolution of lower mass bands found near the bottom of the gel. This is evident as a single broad band present on the gel run at 60 V in the 23-29 kDa mass region (Figure 3.14), split into two distinct, sharper bands on the gel run at 60-200 V (Figure 3.15). These two distinct bands have masses of 26 kDa and 28 kDa respectively.

### 3.6.2 Discussion

Erythrocytes were treated with the cross-linking agent TCEA, in an attempt to cross-link surface and cytoskeletal erythrocyte proteins. These modifications were visualised when erythrocyte ghost samples, both treated with TCEA and untreated (control erythrocyte ghosts), were run on SDS PAGE. The resulting protein fingerprints were then compared. The cross-linking action of TCEA rendered erythrocyte ghost pellets insoluble in sample buffer and thus could not be loaded uniformly onto the gel. All TCEA-treated erythrocyte ghosts were thus first solubilised in Solubilisation solution 3 (urea, thiourea, DTT and ampholytes) after which protein precipitation was done using trichloroacetic acid (TCA), followed by washes with acetone. Despite solubilisation, protein precipitation and the centrifugation of samples, insoluble products and large, cross-linked protein masses still remained in the TCEA-treated erythrocyte ghost samples which can be seen in the bottom of the sample wells of the gel.

The distinctive pattern present within Band 3 (at around 100 kDa) in the control lanes of Figure 3.14 is thought to be due to the sample having passed through a number of freeze-thaw cycles. This same control erythrocyte ghost sample was utilised on a number of other SDS PAGE gels previously and no such pattern was observed.

All SDS PAGE for the previous treatments and experiments (trypsin and sodium periodate treatments) were performed at 60 V for the first 30 minutes and then 200 V for the remainder of the run, and at a constant 60 V. For both the trypsin and sodium periodate treatments, the SDS PAGE gels proved to be of a better quality when run at a constant 60 V. TCEA was found to be different, where protein bands, particularly those around the 25 kDa mark, proved to have a better resolution when the voltage was increased to 200 V when using these 4 – 20% gradient gels. The high molecular mass band present in Figure 3.14 (run at a constant 60 V) was not however present in Figure 3.15 (run at 60-200 V). Thus, in this case a combination of both the 60 V and the 60-200 V SDS PAGE gels were used for analysis and comparison.

TCEA treatment caused changes to erythrocyte proteins that are clearly visualised on a SDS PAGE gel (Figure 3.14). The appearance of a high molecular mass protein band and the disappearance of the spectrin bands due to suspected polymerisation, indicates that TCEA does react with this intracellular cytoskeletal protein to a large extent. It is thought that TCEA causes spectrin to cross-link to itself as well as to other erythrocyte proteins, thus resulting in the higher mass protein band on the SDS PAGE gel. The disappearance of a band at 31 kDa, that is tentatively identified as stomatin (also known as Band 7), in the control erythrocyte ghost samples (Figure 3.14) and the appearance of two smaller bands (Figure 3.15) in the treated samples at approximately 28 kDa and 26 kDa, is thought to be due to alterations to stomatin induced by TCEA. LC-MS/MS analysis was done to confirm or contradict these theories. The 16 kDa haemoglobin monomer band found at the bottom of the gel is visibly more intense in the TCEA-treated samples as compared to the untreated, control samples. This suggests that TCEA causes the partial cross-linking of

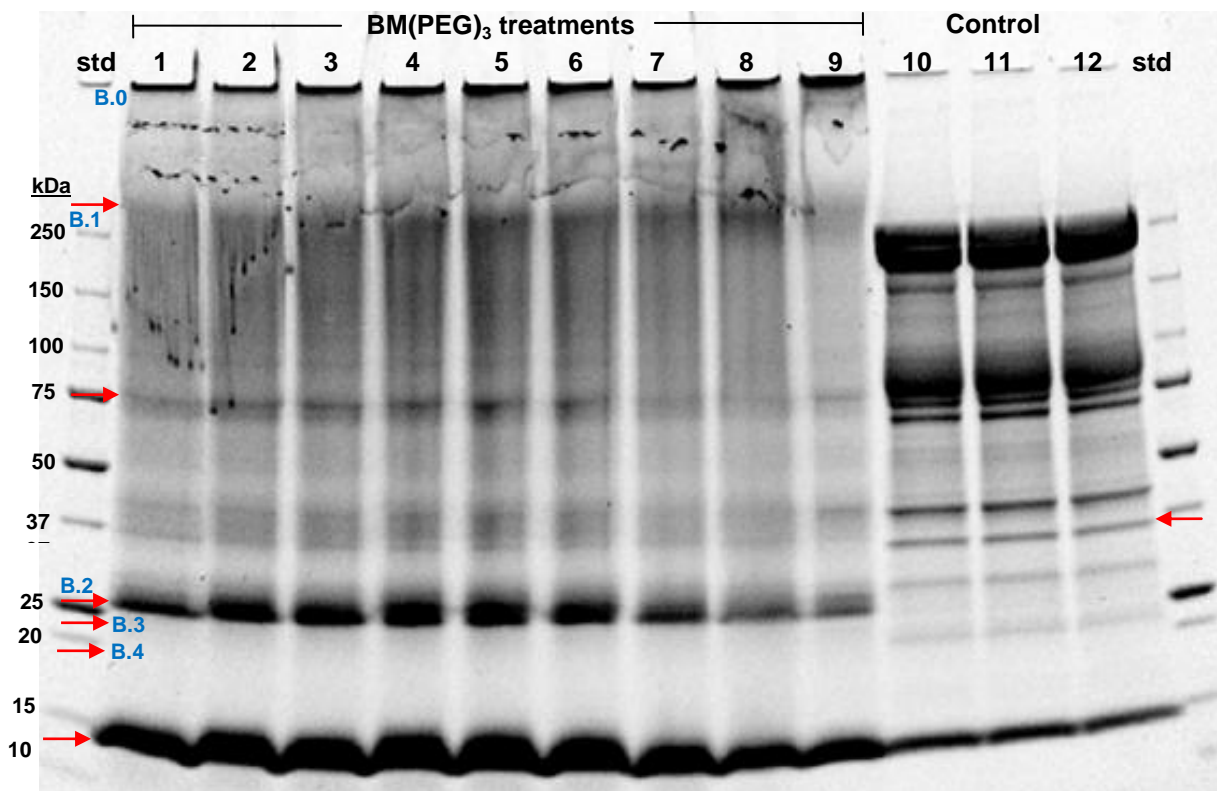
haemoglobin monomers, or the cross-linking of haemoglobin to various other erythrocyte proteins. Furthermore this broader haemoglobin band indicates that TCEA has the ability to cross the erythrocyte membrane and react with cytoplasmic proteins. The effects of TCEA on erythrocyte proteins as seen in this case on spectrin, glycophorin, Band 7 and haemoglobin have previously been noted in literature (Ankel et al., 1985, Wildenauer and Weger, 1979, Wildenauer et al., 1980).

By applying modern and advanced proteomic techniques to analyse the effects TCEA has on erythrocyte proteins, new insight is gained into which erythrocyte proteins are affected by TCEA. That is, erythrocyte ghost protein band differences caused by treatment with TCEA are easily visualised, that were not visible in previously reported literature. This includes the appearance of protein bands at 85 kDa and 75 kDa, and the disappearance of Bands 4.1 and 4.2 (Figure 3.14). This is of interest as no effects on Bands 4.1 and 4.2 due to TCEA exposure have previously been reported in literature. In addition, the extent to which Band 3 is affected by TCEA is grossly underestimated in older literature, where mainly due to poor resolution it seems as if Band 3 is barely affected by TCEA. When making use of modern precast gels, the degree to which Band 3 is affected is clearly visualised where the band has almost completely disappeared due to TCEA exposure (Wildenauer and Weger, 1979, Wildenauer et al., 1980). By utilising LC-MS/MS and sequencing newly visible bands such as the bands at 75 kDa and 85 kDa, and re-evaluating previous bands of interest (bands that were affected by TCEA), potentially more insight into the exact actions of TCEA on erythrocyte proteins can be revealed.

## **3.7 1,11-Bis(maleimido)triethylene glycol (BM(PEG)<sub>3</sub>) Treatments**

### **3.7.1 Results**

Erythrocytes isolated from whole blood were treated with the long spacer, thiol selective BM(PEG)<sub>3</sub> prior to haemolysis and erythrocyte ghost formation. The protein fingerprints of erythrocyte ghosts treated with BM(PEG)<sub>3</sub> were compared to the protein fingerprints of control, untreated erythrocyte ghosts utilising SDS PAGE. The results are shown in Figure 3.16.



**Figure 3.16: SDS PAGE separation of erythrocyte ghost proteins visualising the protein-altering effects of BM(PEG)<sub>3</sub>.** Erythrocytes were treated with the thiol cross-linking agent BM(PEG)<sub>3</sub>. Resulting surface and cytoskeletal protein modifications are clearly visualised on the gel as protein band differences. Lanes 1-9: three erythrocyte ghost samples, independently prepared, treated with BM(PEG)<sub>3</sub>, solubilised and run on the gel in triplicate; lanes 10-12: untreated, control erythrocyte ghost sample in triplicate. Band differences between BM(PEG)<sub>3</sub>-treated and control erythrocyte ghosts are indicated with red arrows. Blue labels identify bands sequenced by LC-MS/MS. Standard (std) lanes contain mass standards annotated in kDa. Each sample well contained 30 µg of protein. The gel was run at a constant 60 V.

The treatment of erythrocytes with BM(PEG)<sub>3</sub> caused significant changes to surface and cytoskeletal proteins, which are visualised as protein bands on SDS PAGE. The sulfhydryl cross-linking agent resulted in the formation of high mass protein products in the erythrocyte ghost samples that were too large to move through the pores of the gel, and thus collected in the bottom of the sample wells as can clearly be seen in lanes 1-9. In the BM(PEG)<sub>3</sub>-treated erythrocyte samples, protein band differences over a wide molecular mass range can be seen when compared to the control erythrocyte ghost sample protein fingerprint. Lanes 1-9 appear as if they have been 'wiped clean' of a number of proteins as compared to the control protein fingerprints in lanes 10-12. Several protein bands in the treated sample lanes were no longer visible, most notably in the region of 25-250 kDa with only a few remaining bands coinciding with those of the control protein fingerprint. Differences include the appearance of a higher mass protein band appearing out of the range of the mass

standards (i.e. heavier than 250 kDa), the disappearance of the spectrin alpha and spectrin beta bands present in the control protein fingerprint and the appearance of a higher mass band ranging from approximately 250-300 kDa respectively in the treated protein fingerprint. In addition, protein bands for ankyrin, Band 4.2, glyceraldehyde-3-phosphate dehydrogenase (G-3-PD) and stomatin are no longer present on the gel, with only remnants of Bands 3 and 4.1 remaining. The formation of new bands in the lanes containing BM(PEG)<sub>3</sub>-treated ghost sample can be seen at approximately 25 kDa and 18 kDa. These faint bands are visualised on the actual stained gel, but unfortunately are not visible in the digital image above (Figure 3.16). The last difference to be noted between the control erythrocyte ghost and the BM(PEG)<sub>3</sub>-treated erythrocyte ghost fingerprints, is that significantly more haemoglobin (around the 15 kDa region) is present in the treated ghost samples as compared to the control ghost samples. A distinct molten wax type pattern is seen obscuring the upper regions of the gel containing the BM(PEG)<sub>3</sub>-treated ghost samples (lanes 1-9). The cause of this pattern is unknown at this stage and it is suggested that it may be related to the type of gel used (Stain-Free, 4-20% gradient gel) in the experiments or may be due to a component present in the protein sample.

### 3.7.2 Discussion

Erythrocyte ghosts were treated with the thiol cross-linker BM(PEG)<sub>3</sub> in order to induce changes to erythrocyte membrane and cytoskeletal proteins. These changes were successfully visualised on a gel using SDS PAGE where the protein fingerprint of BM(PEG)<sub>3</sub>-treated erythrocyte ghosts was compared to an untreated, control erythrocyte ghost fingerprint. Through this comparison it is evident that BM(PEG)<sub>3</sub> has a significant effect when it comes to altering erythrocyte membrane and cytoskeletal proteins, as there are many differences between the treated and untreated, control sample protein fingerprints.

The cross-linking action of BM(PEG)<sub>3</sub> rendered erythrocyte ghost pellets that were insoluble in sample buffer and were thus first solubilised in Solubilisation solution 3 (urea, thiourea, DTT and ampholytes), after which protein precipitation was done using TCA followed by repeated washes with acetone. The ghost samples were then additionally homogenised using an ultrasonic homogeniser. Despite solubilising, precipitating, homogenising and centrifuging the protein samples, insoluble products still remained in the BM(PEG)<sub>3</sub>-treated ghost samples. In addition BM(PEG)<sub>3</sub> cross-links proteins that together will form larger protein complexes, that may be too large to move through the pores of a gel. It is for these reasons that proteins can be seen in the bottom of the wells of the gel containing treated sample (lanes 1-9 Figure 3.16).

As mentioned, BM(PEG)<sub>3</sub> induces multiple alterations to erythrocyte membrane and cytoskeletal proteins, as is visually apparent in Figure 3.16 where many protein bands are missing in the treated fingerprints when compared to the control

fingerprints. When compared to the other three treatments utilised to alter erythrocyte proteins in this research project (trypsin,  $\text{NaIO}_4$  and TCEA),  $\text{BM}(\text{PEG})_3$  seems to have the most profound protein-modifying effect as the most band differences are visualised in this treatment fingerprint when compared to the control erythrocyte ghost fingerprint.  $\text{BM}(\text{PEG})_3$  is found to alter both cytoskeletal and membrane proteins as can be seen by the most notable alterations/disappearances of spectrin, ankyrin, Bands 4.1 and 4.2, and of Band 3, stomatin and glycophorin. Furthermore, it is shown that  $\text{BM}(\text{PEG})_3$  has the ability to cross the membrane and enter the cytoplasm of the erythrocyte as alterations to the enzyme G-3-PD as well as to haemoglobin are evident in the treated lanes of the gel.

A distinct pattern is visualised in Figure 3.16 across the gel in the top regions of the lanes containing  $\text{BM}(\text{PEG})_3$ -treated samples. Although in varying degrees, this molten wax type pattern is found to be present in all three samples that were prepared on different days completely independent of one another. This indicates that the pattern is most likely not due to a contamination, but possibly a component in the sample. This component is suspected to be insoluble lipids, a high salt concentration or some sort of by-product that is not completely solubilised or removed through solubilisation and protein precipitation treatments. Another possible explanation for this pattern is that the specific gel used in the electrophoresis experiments (Stain-free, 4-20% gradient gel) is reacting with residues of the cross-linking agent.

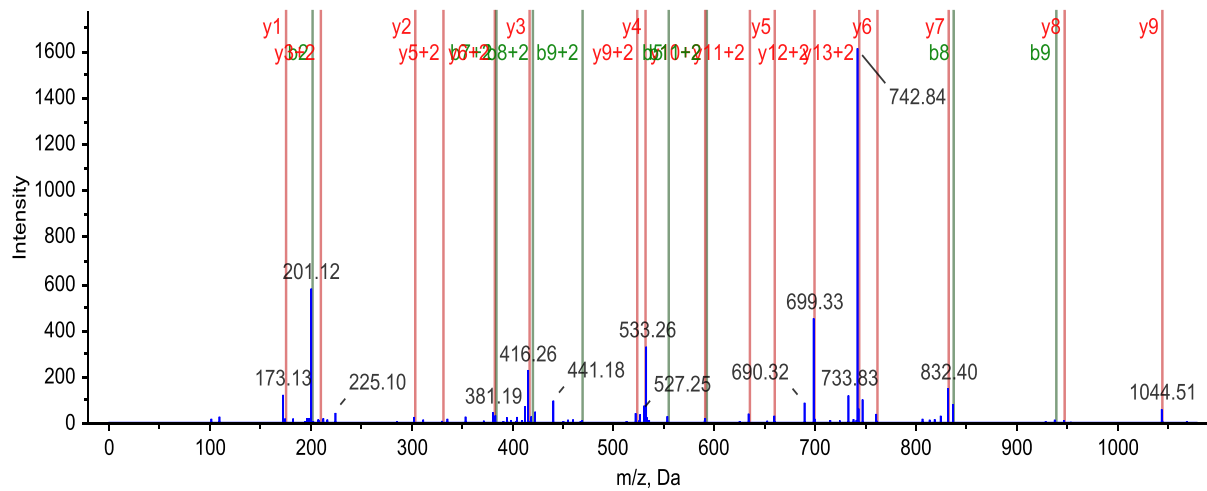
Besides for  $\text{BM}(\text{PEG})_3$  being previously utilised to polymerise thiolated human haemoglobin (Zhang and Palmer, 2010), at present there is no previous research reported whereby  $\text{BM}(\text{PEG})_3$  was utilised to alter erythrocyte proteins that were subsequently analysed by SDS PAGE separation and visualisation. By pairing SDS PAGE with the powerful proteomic analysis that mass spectrometry provides, a clearer understanding of the exact effects that this cross-linker has on erythrocyte proteins should be provided.

## 3.8 Proteomic Analysis

### 3.8.1 Results

Erythrocyte membrane and cytoskeletal proteins were altered by various enzyme and chemical treatments, separated on SDS PAGE gels and visualised as protein fingerprints using Stain-Free and colloidal Coomassie Blue stain. Any obvious changes in protein bands induced by these treatments (as compared to a control) were considered to be of interest. These bands of interest were carefully cut from the gels, destained, reduced, alkylated and in-gel trypsinised. The resulting extracted peptides were separated and analysed using nano LC-MS/MS. Proteins were

identified by matching the peptide fragment spectra, generated by the tandem MS analysis, with spectra from a protein database. Typically results are displayed below in Figures 3.17, 3.18, 3.19 and Table 3.1.

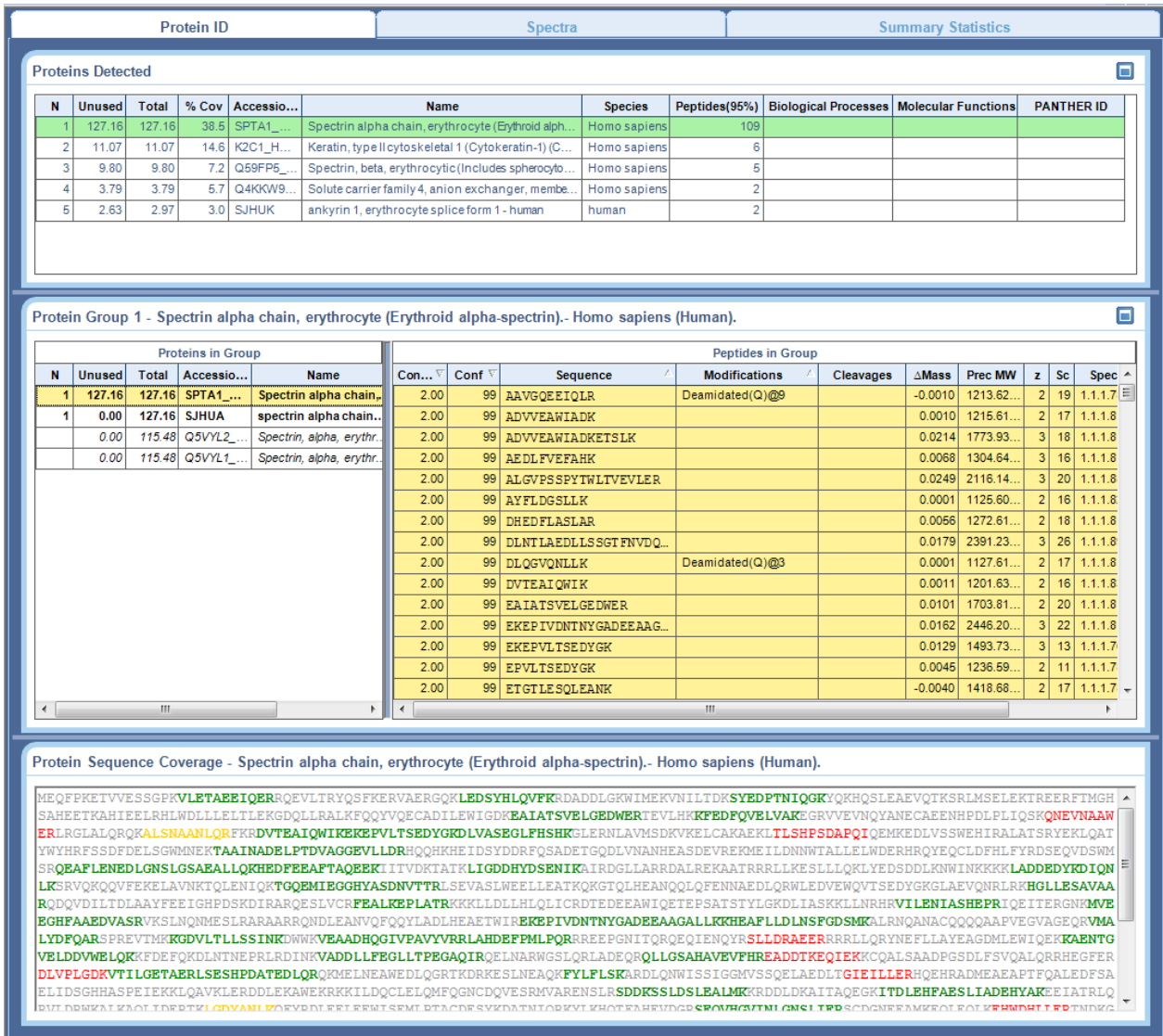


**Figure 3.17: MS/MS spectrum of trypsinised Spectrin alpha peptides indicating b and y ions.** After in-gel protein digestion, chromatographically separated peptides are fragmented using Collision Induced Dissociation (CID) resulting in spectra containing typical b and y ions for each peptide. Fragment peaks extending from the amino terminus are termed ‘b ions’ and the resulting carboxyl terminus ions are termed ‘y ions’. In this way peptides can be sequenced both in a forward and backward manner. It is MS/MS spectra like these that are analysed by ProteinPilot software. ‘m/z’ is mass per charge, ‘Da’ is Daltons.

LSESHPDATEDLQR				
Residue	b	b+2	y	y+2
L	114.0913	57.5493	1597.7453	799.3763
S	201.1234	101.0653	1484.6612	742.8342
E	330.1660	165.5866	1397.6292	699.3182
S	417.1980	209.1026	1268.5866	634.7969
H	554.2569	277.6321	1181.5545	591.2809
P	651.3097	326.1585	1044.4956	522.7515
D	766.3366	383.6719	947.4429	474.2251
A	837.3737	419.1905	832.4159	416.7116
T	938.4214	469.7143	761.3788	381.1930
E	1067.4640	534.2356	660.3311	330.6692
D	1182.4909	591.7491	531.2885	266.1479
L	1295.5750	648.2911	416.2616	208.6344
Q	1423.6336	712.3204	303.1775	152.0924
R	1579.7347	790.3710	175.1190	88.0631

**Figure 3.18: Table indicating the amino acid sequence of a spectrin alpha peptide with the corresponding b and y ions.**





**Figure 3.19: Example of ProteinPilot software analysis where the erythrocyte spectrin alpha protein was successfully identified.** The gel band containing the spectrin alpha protein was cut from a SDS PAGE erythrocyte ghost protein fingerprint, digested and analysed by nano LC-MS/MS. ProteinPilot successfully identified this protein by matching the experimentally generated peptide fragment spectra with in-silico constructed spectra from a protein database. The software ranks proteins present in the sample according to the number of uniquely matched peptide sequences per protein as well as their quality i.e. number of b- and y-ions matched and their mass accuracy. The Protein Sequence Coverage map as seen in the lower block displays the protein sequence with matching peptides and is colour coded where green- shows the highest confidence, yellow- moderate confidence and red- the lowest confidence. The grey zones in the protein sequence indicate where no peptide coverage for the protein could be found.

**Table 3.1: Summary of the identity of proteins of in-gel trypsinised bands from SDS PAGE using LC-MS/MS and ProteinPilot analysis. “Band #s” follow the blue labelling on the images of the SDS gels shown in Figures 3.10 and 3.13, 3.14, 3.15 and 3.16.**

Band #.	Proteins Identified*
T.1	<ul style="list-style-type: none"> <li>▪ Band 3 anion transport protein</li> <li>▪ Trypsin (porcine)</li> <li>▪ Catalase</li> <li>▪ T-complex protein 1 subunit <math>\epsilon</math></li> </ul>
T.2	<ul style="list-style-type: none"> <li>▪ Trypsin (porcine)</li> <li>▪ Peroxiredoxin-2</li> </ul>
T.3	<ul style="list-style-type: none"> <li>▪ Trypsin (porcine)</li> </ul>
S.1	<ul style="list-style-type: none"> <li>▪ Band 3 anion transport protein</li> </ul>
S.2	<ul style="list-style-type: none"> <li>▪ Actin, cytoplasmic 2</li> <li>▪ Phosphoglycerate kinase 1</li> </ul>
S.3	<ul style="list-style-type: none"> <li>▪ Trypsin (porcine)</li> </ul>
S.4	<ul style="list-style-type: none"> <li>▪ Spectrin alpha chain (control)</li> </ul>
TC.1	<ul style="list-style-type: none"> <li>▪ Spectrin alpha chain</li> <li>▪ Spectrin beta chain</li> <li>▪ Haemoglobin subunit beta</li> </ul>
TC.2	<ul style="list-style-type: none"> <li>▪ Spectrin alpha chain</li> <li>▪ Spectrin beta chain</li> <li>▪ Haemoglobin subunit beta</li> <li>▪ Ankyrin-1</li> </ul>
TC.3	<ul style="list-style-type: none"> <li>▪ Spectrin beta chain</li> <li>▪ Ankyrin-1</li> </ul>
TC.4	<ul style="list-style-type: none"> <li>▪ Protein band 4.1</li> <li>▪ Glycophorin A</li> </ul>
TC.5	<ul style="list-style-type: none"> <li>▪ Protein band 4.2</li> <li>▪ Protein band 4.1</li> <li>▪ Haemoglobin subunit alpha</li> </ul>
TC.6	<ul style="list-style-type: none"> <li>▪ Erythrocyte band 7 integral membrane protein</li> </ul>
TC.7	<ul style="list-style-type: none"> <li>▪ Haemoglobin subunit beta</li> </ul>
TC.8	<ul style="list-style-type: none"> <li>▪ Haemoglobin subunit beta</li> </ul>
B.0	<ul style="list-style-type: none"> <li>▪ Spectrin alpha chain</li> <li>▪ Spectrin beta chain</li> <li>▪ Haemoglobin subunit beta</li> <li>▪ Band 3 anion transport protein</li> <li>▪ Ankyrin-1</li> <li>▪ Protein band 4.2</li> </ul>
B.1	<ul style="list-style-type: none"> <li>▪ Ankyrin-1</li> <li>▪ Haemoglobin subunit beta</li> <li>▪ Spectrin beta chain</li> <li>▪ Spectrin alpha chain</li> </ul>
B.2	<ul style="list-style-type: none"> <li>▪ Haemoglobin subunit beta</li> </ul>
B.3	<ul style="list-style-type: none"> <li>▪ Flavin reductase</li> <li>▪ Adenylate kinase isoenzyme 1</li> <li>▪ Haemoglobin subunit beta</li> </ul>
B.4	<ul style="list-style-type: none"> <li>▪ Trypsin (porcine)</li> </ul>

\*All proteins identified are of human species unless otherwise stated. The proteins listed were identified with a confidence threshold of  $\geq 99.9\%$ .

### 3.8.2 Discussion

LC-MS/MS coupled to protein data analysis, sequencing and identification were performed on trypsinised SDS PAGE protein bands of erythrocyte protein pre-treatments with the enzyme trypsin and the chemicals  $\text{NaIO}_4$ , TCEA and  $\text{BM}(\text{PEG})_3$ . The proteins within the selected bands were successfully sequenced and identified with high confidence (Table 3.1).

The proteins identified in the first trypsin-induced erythrocyte band difference, T.1 (Figure 3.10), were Band 3, catalase, porcine trypsin and T-complex protein 1 subunit epsilon (TCP-1- $\epsilon$ ). Band 3 is an anion transport protein that is found in the membrane of erythrocytes (Mohandas and Gallagher, 2008); Catalase is a commonly found enzyme in oxygen-exposed organisms, that catalyses the breakdown of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) into water ( $\text{H}_2\text{O}$ ) and oxygen ( $\text{O}_2$ ) (Loew, 1900); TCP-1- $\epsilon$  is a molecular chaperone that is responsible for the folding of proteins via adenosine triphosphate (ATP) hydrolysis (Seo et al., 2010). Both catalase as well as TCP-1- $\epsilon$  are cytoplasmic proteins and since trypsin does not have the ability to cross the erythrocyte membrane it cannot have an effect on these intracellular proteins. That is trypsin only has a superficial effect on erythrocyte surface proteins and thus would not have an effect on either erythrocytic catalase or TCP-1- $\epsilon$ . The explanation for the presence then of these proteins in the trypsin-induced protein band difference T.1, is that these proteins are in fact also present in the erythrocyte ghost control fingerprint, however without the addition of the cleaved Band 3 protein and resultant quantitative protein increase, the control band appears very light and is not easy to visualise. This statement is supported by the fact that a very faint band can be observed in the control erythrocyte protein fingerprint in the same mass region as band T.1, especially when the image is digitally intensified (Figure 3.10). Furthermore the molecular masses of catalase and TCP-1- $\epsilon$  are both approximately 60 kDa, which is an equivalent mass to that of the T.1 band (Sandstrom and Buttke, 1993, Wagner et al., 2004). Based on these facts, it can be said that trypsin most likely has no cleavage effect on either catalase or TCP-1- $\epsilon$ .

In band T.2 (Figure 3.10) the enzyme peroxiredoxin-2 was found to be present. Peroxiredoxin is an enzyme that is involved in regulating redox reactions within the erythrocyte, whereby it reduces peroxides generated during metabolism (Cha and Kim, 1995). Based much on the same arguments as just discussed, trypsin only has the ability to alter erythrocyte extracellular surface proteins and thus cannot alter cytoplasmic peroxiredoxin. When taking a closer look at Figure 3.10 and digitally intensifying the image, a very faint band in the control erythrocyte protein fingerprint is visualised at the same mass as band T.2 in the treated fingerprint. The presence of this band in the control fingerprint is supported by the fact that the peroxiredoxin-2 monomer has a molecular mass of 21 kDa, much the same as that of band T.2 (Peskin et al., 2007). Thus the band intensity difference observed between band T.2 and the control band of equivalent mass, is thought to be due to the presence of porcine trypsin in band T.2 in addition to that of peroxiredoxin which is present in

both control and treated bands. This porcine trypsin is thought to be sourced from the actual trypsin used to induce the erythrocyte protein alterations rather than from the trypsin used during in-gel digestion, as trypsin has a molecular mass of approximately 23 kDa which corresponds to the mass of band T.2 (Barber et al., 1987). In addition, the trypsin utilised for in-gel digestion would not contribute quantitatively to and therefore increase the intensity of band T.2 visualised on the gel. With the only proteins being found in band T.2 being peroxiredoxin-2 and trypsin, it is only trypsin that could then contribute quantitatively to the peroxiredoxin band T.2. Thus, band difference T.2 is purely a quantitative one caused by the addition of porcine trypsin and is not due to any cleavage of erythrocyte proteins by the trypsin treatment.

The porcine trypsin present in band differences T.1 and T.3 (Figure 3.10) is thought to be from the trypsin used during in-gel trypsinisation as the weight of these bands (60 and 18 kDa respectively) do not correspond with the 23 kDa mass of trypsin. Therefore this porcine trypsin is present in bands T.1 and T.3 due to the addition of trypsin autolysis products from in-gel digestion. Ideally protein concentrations of the protein bands on a gel far outweigh the protein concentration of the trypsin enzyme used during in-gel digestion, meaning the potential enzyme interferences are minimal when it comes to mass spectrometric analysis. This however is not always the case, as faint protein bands on a gel may have a low protein content that may further be lost/degraded during peptide processing. This results in a reversal of the ideal enzyme-to-sample protein ratio where the trypsin autolysis products become more significant than the protein sample itself. A perfect example of such a ratio reversal is in band T.3 where the only protein identified within this band was porcine trypsin and the actual protein forming the faint T.3 band on the gel was unable to be identified. It is a well-known fact that trypsin cleaves the amine terminal (N-terminal) section of the glycophorin A dimer present on the surface of erythrocytes, where trypsinised cleavage products have been found to have a mass of 18 kDa (Dzandu et al., 1984, Dzandu et al., 1985, Hadley et al., 1987, Perkins, 1981, Sim et al., 1994). Since the mass of band T.3 is 18 kDa it is thought that the identity of this band is a glycophorin A cleavage product, but this would need to be confirmed. Confirmation could be done by possibly repeating SDS PAGE and mass spectrometric analysis using a higher protein (erythrocyte ghost) concentration, and also by digesting protein bands immediately after running the gel, so as to prevent time-dependent protein diffusion out of the gel and into the fixing solution during storage.

The erythrocyte protein differences induced by  $\text{NaIO}_4$  treatment were identified where band difference S.1 was found to be Band 3 anion transport protein, band S.2 contained both cytoplasmic actin and phosphoglycerate kinase 1, and band S.3 was identified as porcine trypsin (Figure 3.13). With regards to the proteins identified within band S.2, actin is a vital component in the meshwork of the erythrocyte cytoskeleton, and phosphoglycerate kinase 1 is an important enzyme which

catalyses the transfer of a phosphate group during glycolysis and in doing so produces ATP (Chiarelli et al., 2012, Liem and Gallagher, 2006). The trypsin found in band S.3 is the trypsin used during in-gel digestion that was found at a more significant concentration during mass spectrometric analysis than the protein present in the faint S.3 band on the gel. For identification of the original protein present in band S.3, the experiments would need to be repeated at higher protein sample concentrations as described above for band T.3. Spectrin alpha was cut from the gel (band S.4) and analysed as a control. The identity of spectrin alpha was correctly determined by LC-MS/MS sequencing and protein database comparison.

Erythrocyte proteins found to be altered following treatment with TCEA were spectrins alpha and beta, haemoglobin, ankyrin, glycophorin A, Bands 4.1 and 4.2, and stomatin. These results indicate that TCEA causes the cross-linking of erythrocyte proteins to a significant extent, resulting in higher mass erythrocyte bands on the SDS PAGE fingerprint as compared to each respective control (Figure 3.14). The higher mass protein band TC.1 was the result of the cross-linking between proteins spectrin alpha, spectrin beta and haemoglobin, where band TC.2 was the result of the same cross-linking between these three proteins with the addition of ankyrin. The band marked T.C 3 contained the proteins spectrin beta and ankyrin, band TC.4 contained Band 4.1 and glycophorin A, and TC. 5 contained Bands 4.1 and 4.2 with haemoglobin subunit alpha. All of the protein cross-linking occurred between cytoskeletal proteins, except for haemoglobin. Spectrins alpha and beta, ankyrin and Bands 4.1 and 4.2 are main components of the erythrocyte cytoskeleton, which provide the erythrocyte with mechanical strength and help maintain cell shape (Yazdanbakhsh et al., 2000). Thus TCEA mainly targeted and altered the erythrocyte cytoskeleton and therefore most likely brought about changes to erythrocyte elasticity, durability and shape. In addition, TCEA treatment causes erythrocyte haemoglobin subunit beta to cross-link to itself to varying extents as is evident by the presence of bands TC.7 and TC.8 (Figure 3.15), which are both haemoglobin subunit beta but vary acutely in mass and are both heavier than control haemoglobin subunit beta (approximately 27, 25 and 16 kDa respectively) (Su et al., 2012). Protein band T.6 was confirmed to be stomatin (also known as integral membrane protein Band 7) and it can clearly be seen in Figure 3.14 that TCEA treatment of erythrocytes results in the disappearance of this stomatin band. Stomatin is an erythrocyte membrane protein that functions as a cation transport regulator (Stewart et al., 1993).

Treatment of erythrocytes with BM(PEG)<sub>3</sub> caused the cross-linkage of erythrocyte membrane and cytoskeletal proteins to such an extent that the resulting protein mass was too large to pass through the pores of the gel. This cross-linked protein mass, B.0 (Figure 3.16), consisted of 6 proteins being spectrin alpha, spectrin beta, Band 3, ankyrin, haemoglobin and protein band 4.2. Cross-linking between proteins of the erythrocyte cytoskeleton also occurred to a lesser extent where band difference B.1 contained ankyrin, spectrin alpha, spectrin beta, and haemoglobin,

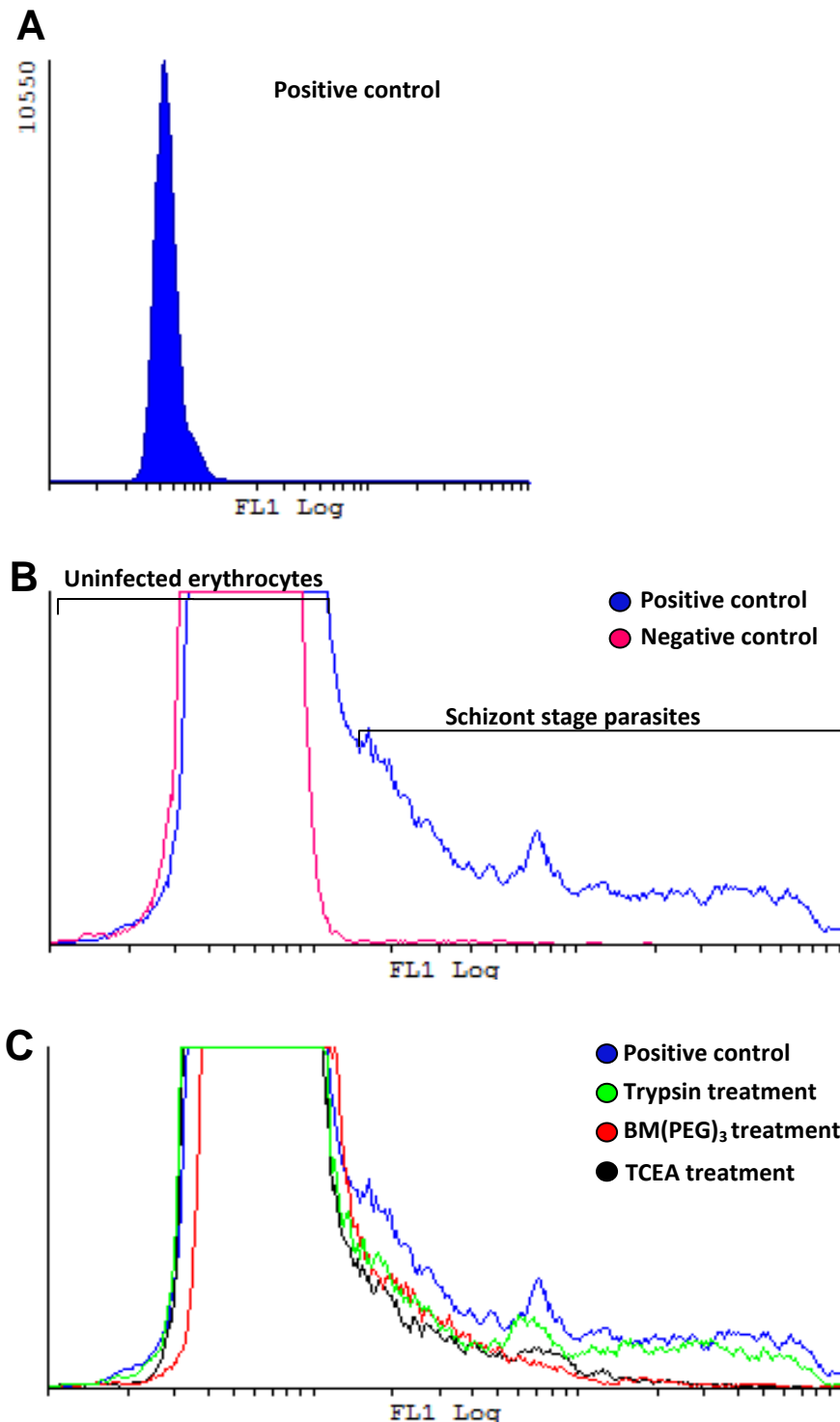
forming a single band of higher mass than each of the equivalent proteins' control bands, indicating that covalent cross-linking had indeed taken place between these proteins. Band B.2 was found to contain haemoglobin subunit beta which cross-linked over itself to some extent as the resulting 27 kDa is heavier than the known 16 kDa haemoglobin monomer band. Band B.3 was found to consist of flavin reductase, which is an enzyme that catalyses the nicotinamide adenine dinucleotide phosphate (NADPH)-dependent reduction of methaemoglobin within erythrocytes; haemoglobin subunit beta and adenylate kinase which plays an important role in regulating energy-dependent processes and overall cellular energy metabolism within the erythrocyte (Dzeja et al., 1998, Yubisui et al., 1980). Band B.3 has an approximate molecular weight of 22 kDa while flavin reductase and adenylate kinase too have a mass of 22 kDa each (flavin reductase has four identical 22 kDa subunits) (Matsubara et al., 2001, Ravera et al., 2007). That being said, it is thought that both these enzymes are in fact present in the control erythrocyte ghost protein fingerprint on SDS PAGE, but are at concentrations that are too low to be visualised on the gel and can only be detected using mass spectrometry. The band difference B.3 is thus only visualised in the BM(PEG)<sub>3</sub> lane on the gel due to the additional presence of cross-linked haemoglobin beta that too has a molecular mass of approximately 22 kDa. The haemoglobin beta therefore increases the overall protein concentration of the 22 kDa protein band resulting in higher band intensity and thus visualisation. Therefore BM(PEG)<sub>3</sub> is not thought to have any effect on the erythrocyte enzymes flavin reductase and adenylate kinase. This can be confirmed by performing in-gel trypsinisation of the 22 kDa region in the erythrocyte control fingerprint and performing mass spectrometric analysis. Regarding the very faint band B.4, the porcine trypsin used during in-gel trypsinisation was found to have a higher concentration during mass spectrometric analysis than the actual protein forming the band on the gel. This is due to a low concentration of the 18 kDa protein on the gel that allows it to be swamped by the added trypsin residue, and therefore results in the trypsin being sequenced and identified instead of the protein forming band B.4. Higher concentrations of BM(PEG)<sub>3</sub>-treated samples would have to be loaded onto the gel for this 18 kDa band to be sequenced in the future.

It can thus be stated that various enzyme and chemical treatments of erythrocytes result in multiple protein alterations. Trypsin was found to alter Band 3; NaIO<sub>4</sub> altered Band 3 along with actin and phosphoglycerate kinase 1; TCEA altered spectrin alpha, spectrin beta, ankyrin, haemoglobin, Bands 4.1 and 4.2, glycophorin A and stomatin; and BM(PEG)<sub>3</sub> altered spectrin alpha, spectrin beta, ankyrin, Band 3, Band 4.2 and haemoglobin. Any of these induced erythrocyte protein alterations could potentially have an effect on the *Plasmodium falciparum* malaria parasite's ability to invade the erythrocyte.

## 3.9 Invasion Assays

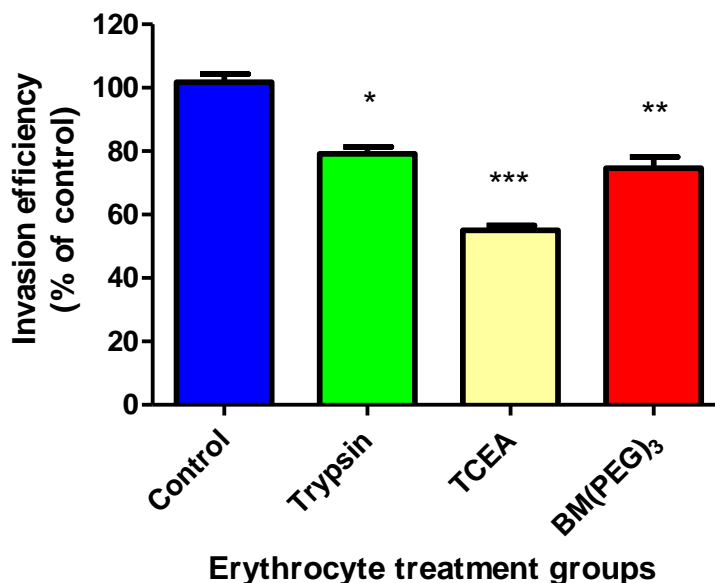
### 3.9.1 Results

Invasion assays were performed in 96-well microtiter plates whereby schizont stage malaria parasites were incubated with untreated, control erythrocytes or those pre-treated with trypsin, sodium periodate ( $\text{NaIO}_4$ ), Tris(2-chloroethyl)amine (TCEA) or 1,11-bis(maleimido)triethylene glycol (BM(PEG)<sub>3</sub>). The incubation lasted 48 hours to allow the malaria parasite to complete at least one complete life cycle i.e. from original schizont parasites to newly formed schizont parasites. The samples were then stained with SYBR Green I dye and analysed by flow cytometry in order to determine invasion efficiency, which was defined as the rate of invasion achieved with enzymatically/chemically-treated erythrocytes expressed as a percentage of the rate of invasion achieved with untreated, control erythrocytes. The  $\text{NaIO}_4$ -treated erythrocytes were extensively haemolysed after as little as 24 hours into the incubation period and thus were excluded from in the invasion assay. The results of the assay are shown in Figures 3.20, 3.21 and Table 3.2.



**Figure 3.20: Flow cytometry histograms depicting invasion assay results.** Two cell populations of uninfected erythrocytes and schizont stage malaria parasites are clearly visible, where different invasion efficiencies are seen for each erythrocyte treatment. (A): Positive control, where only uninfected erythrocyte population is visible as schizont population makes up only 2% of the total sample; (B): Altered scale overlay histogram of negative and positive controls where the schizont population is clearly visible and (C): Overlay histogram of positive control and treated erythrocytes indicating the various resulting invasion efficiencies.





**Figure 3.21: The effect of trypsin, TCEA and BM(PEG)<sub>3</sub> on invasion efficiency expressed as a percentage of the control.** All enzymatically/chemically-treated erythrocytes had a significant effect on decreasing schizont invasion efficiency as compared to the control erythrocytes. \*:  $p < 0.05$ , \*\*:  $p < 0.01$ , \*\*\*:  $p < 0.001$ .

**Table 3.2: Percent inhibition of schizont invasion observed with different erythrocyte treatments.** Results are reported as the mean  $\pm$  standard error of the mean (SEM).

Erythrocyte Treatment	Invasion Inhibition (as percentage of control)
Trypsin	22.61 $\pm$ 2.11
TCEA	46.79 $\pm$ 1.57
BM(PEG) <sub>3</sub>	27.57 $\pm$ 2.70
NaIO <sub>4</sub>	Unattainable - haemolysis

Following data analysis, it was found that TCEA had the greatest effect on inhibiting merozoite invasion of erythrocytes when compared to the other treatments, being BM(PEG)<sub>3</sub> and trypsin (data for NaIO<sub>4</sub> could not be obtained due to haemolysis of the treated erythrocytes before invasion assay completion). TCEA was found to alter erythrocyte proteins and inhibit invasion by 46.79% as compared to the control erythrocytes invasion efficiency, with BM(PEG)<sub>3</sub> and trypsin following with 27.57% and 22.61% inhibition of invasion respectively. The differences in invasion efficiency for each treatment as compared to the control, as well as inter-treatment differences were all found to be significant ( $p < 0.05$ ), except for trypsin and BM(PEG)<sub>3</sub> where no significant difference could be found between these two invasion efficiencies.

### 3.9.2 Discussion

Invasion assays were done in order to determine the effect that altered cytoskeletal and membrane erythrocyte proteins would have on the malaria parasite's ability to complete erythrocyte invasion. It was found that erythrocytes treated with the cross-linking agent tris(2-chloroethyl)amine (TCEA) inhibited parasite invasion to the greatest extent as compared to the cross-linker 1,11-bis(maleimido)triethylene glycol (BM(PEG)<sub>3</sub>) or the enzyme trypsin. TCEA was found to inhibit 3D7 *Plasmodium falciparum* (Pf) parasite invasion of erythrocytes by 46.79%, which is significantly less than the 85% invasion inhibition achieved with the Pf Nigeria strain used in the only previous invasion assay study with comparable conditions (Breuer et al., 1983). Trypsin was found to inhibit invasion of this same 3D7 Pf strain by 22.61% as compared to 45%, 50%, 85%, 91% and 94% in Pf strains Dd2, HB3, MT/S1, 7G8 and Nigeria respectively (Bates et al., 2010, Breuer et al., 1983, Gaur et al., 2003). This serves to indicate that the ability of the malaria parasite to invade protein-modified erythrocytes is strain specific. Furthermore, the 3D7 Pf strain has been reported to be more adaptable when it comes to utilising alternate invasion pathways to enter the erythrocyte (Gaur et al., 2003, Soubes et al., 1999).

It was expected that TCEA and BM(PEG)<sub>3</sub> would have the greatest inhibitory actions on parasite invasion as they are capable of crossing the erythrocyte membrane and thus have the potential to alter both externally expressed surface proteins as well as intracellular membrane and cytoskeletal proteins. TCEA however is a smaller molecule than BM(PEG)<sub>3</sub> (204.53 g/mol and 352.34 g/mol respectively) and has three reactive sites, therefore TCEA was expected to have a greater intracellular effect on erythrocytes presumably resulting in greater inhibition of parasite invasion. This was in fact found to be true as TCEA inhibited invasion by almost 20% more than BM(PEG)<sub>3</sub> did. Trypsin on the other hand cannot penetrate the erythrocyte membrane and therefore only renders surface protein alterations. With trypsin altering erythrocyte proteins to a lesser extent than the two cross-linkers one would expect less interference in the invasion process by the enzyme, however this is not necessarily the case. Although TCEA had a significantly greater inhibitory effect on invasion as compared to trypsin, there was no significant difference found between the inhibitory actions of BM(PEG)<sub>3</sub> and trypsin on parasite invasion of erythrocytes ( $p < 0.05$ ). Thus, although BM(PEG)<sub>3</sub> has an apparently greater effect on erythrocyte protein alteration than trypsin does (as can be seen in Section 3.7.1 Figure 3.16 and Section 3.4.1 Figure 3.10), it can potentially be said that the relative importance of the trypsin-altered proteins in the invasion process is greater than the proteins altered by BM(PEG)<sub>3</sub>.

Following LC-MS/MS analysis, it was found that the erythrocyte proteins that trypsin altered were the major transmembrane protein Band 3 and suspected but yet to be confirmed in this study, glycophorin A. As is evident from the invasion assay results, this suggests that Band 3 alteration results in a decrease in invasion efficiency of the parasite, thus implicating Band 3 in playing a role in the invasion process. This

information supports what is already reported in literature, which is that Band 3 is a crucial receptor for the merozoite protein MSP-1 during invasion, and therefore any alteration to this membrane protein would potentially result in a decrease in invasion efficiency (Goel et al., 2003, Li et al., 2004). Interestingly, it has previously been found that individuals who suffer from a defect known as Southeast Asian Ovalocytosis (SAO) where Band 3 is found to have abnormal structure and function, are resistant to invasion by various malaria parasites including *Plasmodium falciparum*. SAO sufferers are found to have rigid erythrocytes and erythrocyte ghosts, and this lack of fluidity and elasticity in the cytoskeletal network may play a role in decreased invasion efficiency, as cytoskeletal proteins have reduced lateral mobility when invasion occurs (Castelino et al., 1981, Jarolim et al., 1991). Furthermore, it is known that malaria parasite invasion results in tyrosine phosphorylation of Band 3 and this results in Band 3 dissociating from the spectrin-actin cytoskeleton bringing about significant changes to erythrocyte morphology. This again suggests that Band 3 not only acts as a host receptor during merozoite invasion, but also plays an important role in the erythrocyte structure required for successful invasion (Ferru et al., 2011).

The amine cross-linker TCEA was found to have caused distinct alterations to spectrin alpha, spectrin beta, ankyrin, haemoglobin, Bands 4.1 and 4.2, glycophorin A and stomatin. TCEA inhibited parasite invasion by up to almost 50% indicating that alterations to these proteins had a substantial effect on the parasite's ability to invade erythrocytes. The thiol cross-linker BM(PEG)<sub>3</sub> altered similar erythrocyte proteins during treatment as that of TCEA, with the same cytoskeletal proteins being altered, except for that of Band 4.1 where no alteration occurred. Most of the proteins altered by both cross-linker treatments, being spectrin alpha, spectrin beta, ankyrin and Bands 4.1 and 4.2, are important components of the erythrocyte cytoskeleton and are vital in providing mechanical strength, durability and elasticity to the erythrocyte while ensuring the maintenance of its unique biconcave-discoid shape (Mohandas and Gallagher, 2008, Yazdanbakhsh et al., 2000). Thus if alterations occur in these cytoskeletal proteins, it is likely that the erythrocyte will lose some of its elasticity, durability and possibly its discoid shape. The resulting inhibition in invasion efficiency due to treatment of erythrocytes with these cross-linkers serves to suggest that the malaria parasite requires the original erythrocyte cytoskeletal structure and membrane organisation for optimum protein recognition and/or binding ability and therefore overall invasion efficiency. In addition, the cytoskeletal structure of erythrocytes may be vital for the required motile response to the merozoite, and hence successful tight junction formation and eventual creation of a parasitophorous vacuole during the invasion process. Unfortunately not many studies have focused on the biophysical interaction between the parasite and host erythrocyte, especially that of the contribution which the actual erythrocyte membrane and underlying cytoskeleton may have in invasion. This is mainly due to long-standing evidence that the parasite's actin-myosin motor alone drives successful entry of the merozoite into the erythrocyte (Angrisano et al., 2012, Lew and Tiffert, 2007, Miller et al., 1979). It has however recently been shown that the

*Plasmodium* malaria parasite reorganises the erythrocyte cytoskeleton during invasion, and thus any pre-alteration to the cytoskeleton would most likely interfere with this reorganisation and hence the invasion process. Known modifications and resulting rearrangements to the erythrocyte cytoskeleton required for successful merozoite invasion include polymerisation of erythrocyte actin in order to anchor the tight junction and allow for penetration and entry of the parasite into the erythrocyte, as well as dephosphorylation and phosphorylation of erythrocyte cytoskeletal proteins including spectrin beta and Band 4.1 (Dasgupta et al., 2014, Gonzalez et al., 2009, Wasserman et al., 1990). In addition, spectrin has previously been found to play a role in invasion where the invasion of human erythrocytes by *Plasmodium falciparum* was strongly inhibited by anti-spectrin antibodies, as well as by the disulfide cross-linker diamide which was confined to cross-link spectrin. Thus a local rearrangement of the erythrocyte cytoskeleton involving spectrin is suspected to form an important step during parasite invasion (Dluzewski et al., 1983). Furthermore erythrocytes with spectrin mutations or polymorphisms related to hereditary elliptocytosis have shown to be resistant to *Plasmodium falciparum in vitro* (Dhermy et al., 2007). Band 4.1 is also reported to play a vital role in parasite invasion, where this erythrocyte receptor binds to the merozoite ligand EBA-181 during invasion (Gaur and Chitnis, 2011, Lanzillotti and Coetzer, 2006). Thus any alteration to spectrin and/or Band 4.1 is expected to result in a reduction of invasion efficiency.

With regard to the membrane proteins altered by each cross-linker, TCEA was found to have had an effect on glycophorin A and stomatin, where BM(PEG)<sub>3</sub> altered Band 3. Glycophorin A is a sialoglycoprotein found on the surface of the erythrocyte that is vital in the sialic acid-dependent invasion pathway of the malaria parasite, when merozoite EBA-175 binds as a ligand during invasion (Gaur and Chitnis, 2011, Tolia et al., 2005). Therefore any alteration to glycophorin A may result in a decrease in parasite invasion, the extent of which is dependent on whether the parasite utilises the sialic acid-dependent invasion pathway or not. No currently reported study suggests a role for stomatin in the invasion process. As previously mentioned, Band 3 is a crucial receptor for the merozoite protein MSP-1 during invasion, and also plays a role in maintaining the required erythrocyte membrane and cytoskeletal properties for successful invasion (Ferru et al., 2011, Goel et al., 2003, Jarolim et al., 1991, Li et al., 2004).

The invasion assays served to confirm erythrocyte proteins already suspected or known to play a role in malaria parasite invasion of erythrocytes, as well as highlight new erythrocyte proteins that could potentially be involved. Spectrins alpha and beta, Band 3, Band 4.1 and glycophorin A were all confirmed to play a role in the invasion process as modifications to these proteins resulted in a decrease in invasion efficiency. Protein Band 4.2, ankyrin and stomatin could all be involved in invasion, however further studies would have to be done in order to confirm or reject these theories. Interestingly, not one of the enzyme or chemical treatments induced erythrocyte alterations that resulted in complete (or near complete) inhibition of

invasion. This serves to emphasise the invasive ability and adaptability of the 3D7 strain of *Plasmodium falciparum* when it comes to invading erythrocytes and provides some explanation as to why there is such a global struggle to fully eradicate this disease malaria.

## Chapter 4: Final Conclusions and Recommendations

### 4.1 Discussion and Conclusions

The purpose of this study was to confirm known and possibly identify new erythrocyte proteins that are involved in the initial recognition and binding of *Plasmodium falciparum* merozoite ligands during parasite invasion by applying erythrocyte protein modification techniques, followed by mass spectrometric proteomic methods. The main aim was to lay a foundation for the discovery of new key targets for both drug and vaccine-based antimalarial strategies that may prevent the malaria parasite from entering erythrocytes and manifesting clinical symptoms. This in turn could prevent the suffering and potential deaths of the hundreds of millions of people globally affected by malaria.

This study required defined study objectives where highly synchronised cultures of the 3D7 laboratory strain of *Plasmodium falciparum* were successfully maintained; erythrocyte ghost isolation methods and sample preparation were optimised then utilised in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) to provide protein fingerprints of erythrocyte ghosts; methods utilising enzymes and chemicals resulting in modifications of both surface and cytoskeletal erythrocyte proteins were optimised and the erythrocyte ghosts isolated, solubilised and prepared for SDS PAGE where induced protein modifications were clearly visualised. The erythrocyte protein modifications were visualised as protein band differences in the gel fingerprint which were successfully collected, trypsinised and the resulting peptides sequenced by liquid chromatography tandem mass spectrometry (LC-MS/MS). The sequence data was used to identify the proteins in the collected bands, while the effect of these erythrocyte protein modifications on *Plasmodium falciparum* parasite invasion efficiency was assessed using invasion assays monitored by flow cytometry.

Erythrocyte ghosts were successfully isolated from whole blood and prepared for SDS PAGE. These clean ghost samples provided a reproducible protein fingerprint of erythrocyte surface and cytoskeletal proteins on the gel without severe haemoglobin contamination. It was found that a concentration of approximately 1 mg/ml of erythrocyte protein was required to produce a clear, high quality protein fingerprint from which proteins could easily be identified by their mass. Optimisation of this erythrocyte ghost protein fingerprint was assessed by utilising three different protein solubilisation treatments during sample preparation. It was found that Solubilisation solution 3 consisting of urea, thiourea, dithiothreitol (DTT) and ampholytes increased the quality of the erythrocyte ghost protein fingerprint with better visualisation of faint bands and more focused protein bands in general as compared to the non-solubilised fingerprint. Acquiring the best quality erythrocyte

ghost fingerprint possible provides better detail of erythrocyte proteins and thus allows for accurate monitoring of these proteins after exposure to protein-altering enzymes and chemicals.

Two-dimensional gel electrophoresis (2-DE) was also performed using erythrocyte ghosts in the hope that this may produce a more detailed, characteristic ghost fingerprint as compared to one-dimensional SDS PAGE. This however was not found to be the case as 2-DE did not provide an accurate and reproducible representation of erythrocyte membrane or cytoskeletal proteins. That is, compared to the SDS PAGE fingerprint many of the erythrocyte proteins could not be visualised on 2-DE that were found to be prominent bands on SDS PAGE. Erythrocyte membrane and surface proteins were found to be greatly under-represented using 2-DE, which is not unexpected. This is due to the fact that erythrocyte membrane proteins are highly lipophilic and are therefore a challenge to solubilise in the hydrophilic environment that 2-DE utilises. Detergents can be used on membrane protein samples to improve solubility, however it would be a great challenge to obtain the degree of solubility required to match the high quality fingerprint obtained when using the denaturing sample preparation used during SDS PAGE. In addition, 2-DE is a more time consuming and costly process than that of SDS PAGE and taking this into consideration along with the under-representation of membrane proteins, it was decided that 2-DE was not the best suited technique for use in this study. In this case the advantages of and superior quality results found with SDS PAGE far surpassed that of 2-DE.

One enzyme and three different chemicals were used to modify erythrocyte membrane and cytoskeletal proteins, being trypsin, sodium periodate ( $\text{NaIO}_4$ ), tris(2-chloroethyl)amine (TCEA) and 1,11-bis(maleimido)triethylene glycol ( $\text{BM}(\text{PEG})_3$ ). Erythrocytes were treated with the enzyme/chemical prior to ghost formation after which the resulting erythrocyte ghost protein changes were visualised and compared to an untreated control by comparative protein fingerprinting on SDS PAGE. The enzyme, trypsin, was used to alter erythrocyte surface proteins by cleaving accessible proteins at the carboxylic terminus of their lysine and arginine residues. The resulting gel was stained using a silver and Coomassie Blue double-staining technique in the hope of better visualising the trypsin-induced protein alterations on the gel, as Coomassie Blue stains the conventional membrane proteins blue, and the silver stain stains lipids and sialoglycoproteins yellow. This staining technique however did not provide any advantages, as staining was found to be non-specific and lacking in uniformity across the gel. In addition, the gel easily became oversaturated with stain resulting in substantial background interference. All these factors resulted in a gel of low visual quality that would be difficult to reproduce due to high variability.

Fluorescein-5-EX N-hydroxysuccinimide ester (NHS-fluorescein), a fluorescent tagging reagent that reacts with free primary amine groups (including those in lysine residues) was used to track erythrocyte protein alterations induced by trypsin on

SDS PAGE. This tagging technique was only partially successful in the fact that it did fluorescently tag trypsin cleavage sites, however it was not very selective as the majority of the erythrocyte protein fingerprint fluoresced. Thus, this technique was not particularly useful to selectively tag cleaved proteins in this case. In addition, NHS-fluorescein-tagged protein bands appeared more intense than equivalent protein bands that were not fluorescently-tagged when scanned on the Stain-Free Sample Tray (not fluorescent sensitive). This unpredictable increase in band intensities rendered the potential of a quantitative analysis of the protein bands null and void.

The potent oxidant  $\text{NaIO}_4$  was utilised to alter erythrocyte proteins where the resulting protein band differences were easily visualised using SDS PAGE and Stain-Free technology. A large number of protein bands covering the complete mass range of the gel were found to be affected by the  $\text{NaIO}_4$  treatment. When this gel was compared to an equivalent gel reported in literature from 35 years ago, the protein fingerprints varied to large extent where a significant amount of band differences were not visualised on the gel reported in literature. This highlights the importance of reassessing past research results by applying the sensitive and advanced modern proteomic techniques of today, as results may show significant differences not previously identified.

The protein cross-linkers TCEA (amine cross-linker) and  $\text{BM}(\text{PEG})_3$  (thiol cross-linker) both altered erythrocyte proteins with resulting modifications successfully visualised on SDS PAGE using Stain-Free technology. Protein bands of mass in excess of 250 kDa appeared in both the cross-linker-treated protein fingerprints, indicative of the cross-linking of various erythrocyte proteins. All treated as well as control erythrocyte protein fingerprints were found to be of the best visual quality when the gels were run at a constant 60 V, as compared to a much higher 200 V that is claimed to be useful to reduce run times during gel electrophoresis. This was with the exception of the lower mass protein bands of TCEA-treated erythrocyte ghosts being of higher visual quality and achieving better separation when run at 200 V. Thus, new SDS PAGE gels using the new buffer systems must be tested at both high and low voltages to obtain a protein fingerprint of the highest possible quality. The resulting TCEA protein fingerprint using modern proteomics techniques differs significantly to the results reported in literature some 30 years ago, revealing more insight into the actions of TCEA on erythrocyte proteins. This again highlights the importance of reassessing dated results with new cutting-edge, modern techniques.

Solubilisation solution 3 was tested on all four enzymatically/chemically-treated erythrocyte ghosts with the expectation that this treatment would improve the quality of the erythrocyte ghost protein fingerprint on SDS PAGE gels as it did with the control samples. This was found not to be the case, as solubilising erythrocytes treated with trypsin or  $\text{NaIO}_4$  significantly degraded the quality of the resulting protein fingerprints by causing vertical streaking and an overall reduction in band resolution. With regard to the erythrocytes treated with TCEA and  $\text{BM}(\text{PEG})_3$  however, the



opposite was found where it was imperative that the samples be solubilised in Solubilisation solution 3 prior to SDS PAGE, as without this step the erythrocyte ghost samples were found to be insoluble in the Laemmli sample buffer and thus could not be loaded uniformly onto the gel.

Proteomic analysis was done whereby SDS PAGE protein band differences induced by the enzyme/chemical treatments of erythrocytes were selected, cut from the gel and in-gel digested. These tryptic digests were analysed and sequenced using nano LC-MS/MS and identified by ProteinPilot software. It was found that trypsin,  $\text{NaIO}_4$ , TCEA and  $\text{BM}(\text{PEG})_3$  caused changes to the following erythrocyte surface and cytoskeletal proteins: Band 3, actin, phosphoglycerate kinase 1, spectrin alpha, spectrin beta, ankyrin, haemoglobin, Bands 4.1 and 4.2, glycophorin A and stomatin. Any of these induced erythrocyte protein changes can potentially have an effect on the malaria parasite's ability to complete invasion, thereby implicating the proteins showing altered apparent size as being involved in the parasite invasion process.

Invasion assays were done using a highly synchronised 3D7 malaria parasite strain where schizont stage parasites were incubated with untreated, control erythrocytes or erythrocytes exposed to one of the four enzyme/chemical treatments, for the duration of one complete parasite life cycle (48 hours). Invasion efficiency for each treatment was then expressed as a percentage of the control. These assays were done in order to determine the relative importance of the different altered erythrocyte proteins in the invasion process and in doing so, confirm known and potentially identify new proteins involved in the merozoite invasion stages. It was found that erythrocytes treated with TCEA inhibited invasion to the greatest extent, followed by those treated with  $\text{BM}(\text{PEG})_3$  and trypsin. Sodium periodate-treated erythrocytes were not assayed as auto-haemolysis occurred within the 48 hour incubation period. Statistically, no difference was observed between the invasion inhibition of trypsin and  $\text{BM}(\text{PEG})_3$ , even though  $\text{BM}(\text{PEG})_3$  was found to have altered many more erythrocyte proteins than that of trypsin. It can therefore be concluded that the erythrocyte proteins altered by trypsin are of a greater importance in the invasion process than those altered by  $\text{BM}(\text{PEG})_3$ . This then suggests that Band 3 and/or glycophorin A have an important role in invasion. Furthermore, the cross-linkers TCEA and  $\text{BM}(\text{PEG})_3$  were found to have altered similar erythrocyte proteins, however there was a statistically significant difference of almost 20% between the invasion inhibitory actions between these chemicals, with TCEA having the greater inhibition. Therefore the erythrocyte proteins found to be altered by TCEA but not by  $\text{BM}(\text{PEG})_3$  are considered to be of greater importance in the invasion process. These proteins are glycophorin A, Band 4.1 and stomatin. These findings support what is already reported in literature, which is that Band 3, glycophorin A and Band 4.1 are involved in invasion where they bind to the merozoite proteins MSP-1, EBA-175 and EBA-181 respectively (Gaur and Chitnis, 2011, Goel et al., 2003, Lanzillotti and Coetzer, 2006, Tolia et al., 2005). These results also suggest that stomatin may play a role in the invasion process.

Proteomic analysis of control and treated erythrocyte ghosts paired with invasion assays successfully confirmed known and revealed potentially new erythrocyte proteins that are involved in the initial recognition and binding of *Plasmodium falciparum* merozoite ligands during parasite invasion of erythrocytes. The proteins identified as being involved in the invasion process were spectrin alpha, spectrin beta, Band 3, Band 4.1 and glycophorin A. The study results also suggested that the erythrocyte proteins Band 4.2, ankyrin and/or stomatin could also possibly play a role in invasion, however further selective and directed protein research would need to be done to confirm these hypotheses.

This study highlighted the power and potential that modern, cutting-edge proteomic techniques possess, where such techniques revealed a completely new set of results for previous comparative studies found in literature some 30 years ago. The results from this study can be used as a foundation in future studies in order to identify new key targets for the development of new antimalarial drug and vaccine based strategies. This is done with the vision of preventing the suffering endured by the millions of people infected with malaria worldwide, and ultimately completely eradicating this deadly disease.

## 4.2 Study Limitations and Recommendations

Despite the fact that the study aims and objectives were achieved, this study does have limitations and aspects that could be improved upon in future research.

One of the limitations found in the study was that only protein bands on SDS PAGE that were visualised on the Stain-Free gel were taken into account, where protein bands that were of a low abundance and thus present but not clearly visible on the gel were not considered. That is, the exact erythrocyte ghost control protein fingerprint was assessed using only visible and therefore abundant protein bands. This presented as an obstacle when the band differences from the treated erythrocyte ghost fingerprints were analysed and sequenced by LC-MS/MS. The proteins identified within these band differences could not all definitively be said to be altered by the enzyme/chemical treatment, as any of the proteins could have been present in the control erythrocyte ghost fingerprint, but due to low individual concentrations were not visible on the gel and only became known after LC-MS/MS analysis. The only way then to differentiate the proteins sequenced within a band difference as being altered by the enzyme/chemical or not, was by comparing the mass of the protein(s) found in the band difference to its original known mass. Thus in future, the entire erythrocyte ghost protein fingerprint control lane should be cut in small segments, in-gel trypsinised, analysed and sequenced by LC-MS/MS. Having a more detailed and accurate view of the control erythrocyte ghost proteome will allow for more reliable tracking of the protein alterations induced by treatments,

providing a more accurate view of the erythrocyte proteins playing a role in parasite invasion.

Another limitation of the study is that after determining the proteomic changes induced by the protein-altering treatments, and linking these to a decrease in invasion efficiency, it is not known exactly which protein(s) are responsible for this decrease in invasion. That is, if for example three erythrocyte surface/cytoskeletal proteins were altered resulting in a decrease in invasion efficiency, there is no way of knowing whether it was all three of these modifications contributing to this decrease in invasion or if it was due to just one of the protein modifications, whose identity must then be unequivocally determined. Therefore more controlled erythrocyte protein-altering treatments where just one protein of interest is altered at a time would need to be utilised in order to determine exactly which proteins are involved in the invasion process and of what relative importance they have in the process.

Improvements that could be made to this study are utilising erythrocyte-altering treatments that bring about a small number of proteomic changes only, so that these changes can easily be tracked and sequenced. Sodium periodate for example brought such a large number of changes to the erythrocyte proteins, that it was not practical or affordable to track, analyse and sequence every single induced alteration. Thus important erythrocyte proteomic information regarding invasion may easily have been overlooked. In addition, erythrocyte-altering treatments must be able to alter the erythrocyte without inducing haemolysis for at least a 48 hour period, to allow for the completion of invasion assays. Taking these factors into account, it can be said that sodium periodate was not an ideal candidate to be used in this study.

In future studies, protein bands from SDS PAGE must be cut and digested as soon as possible, so as to prevent any loss of protein from the bands e.g. diffusion of proteins from the gel into the fixing solution over time. This step can serve to be critical when it comes to low mass proteins and faint protein bands indicating low abundance, where the identity of the protein can be obscured by contaminating proteins or peptides as was seen in the case of the trypsin utilised during in-gel digestion which obscured low protein band concentrations. Thus, preventing protein loss from the gel post-SDS PAGE must be considered a priority.

This study has the potential to be expanded upon. In future studies different *Plasmodium* species could be utilised and their respective invasion efficiencies compared in order to determine/ confirm whether alternate invasion pathways are utilised by different species and which, if any, erythrocyte proteins serve to be of higher importance to different *Plasmodium* species. Two-dimensional gel electrophoresis could again be attempted with different solubilisation techniques being applied, where if successful a whole new view into the erythrocyte proteome and resulting alterations post-enzyme or chemical treatment could be visualised.

This information in turn could reveal additional insight into the involvement of erythrocyte proteins in parasite invasion.

Lastly, various different erythrocyte-altering treatments could be utilised, where specific treatments could be chosen based on the specific erythrocyte proteins one may want to investigate or confirm as having a role in the invasion process of malaria parasites. With all the potential improvements and recommendations that can be applied to this study if expanded upon in the future, the potential for the discovery of new erythrocyte proteins, as well the determination of the relative importance and confirmation of erythrocyte proteins involved in the malaria parasite invasion process, is viable.

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## Appendix

### Letter of Ethical Approval

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 20 Oct 2016.
- IRB 0000 2235 IORG0001762 Approved dd 13/04/2011 and Expires 13/04/2014.



UNIVERSITEIT VAN PRETORIA  
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Faculty of Health Sciences Research Ethics Committee

7/08/2013

#### Approval Notice New Application

**Ethics Reference No.:** 303/2013

**Title:** Enzymatic and chemical modifications of erythrocyte surface antigens to identify Plasmodium falciparum merozoite binding sites

Dear Ms Kim Baron

The **New Application** for your research received on the 1 July 2013, was approved by the Faculty of Health Sciences Research Ethics Committee on the 7/08/2013.

Please note the following about your ethics approval:

- Ethics Approval is valid for 2 years.
- Please remember to use your protocol number (303/2013) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, or monitor the conduct of your research.

**Ethics approval is subject to the following:**

**Standard Conditions:**

- The ethics approval is conditional on the receipt of 6 monthly written Progress Reports, and
- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes 2004 (Department of Health).

We wish you the best with your research.

Yours sincerely

**Professor Werdie (CW) Van Staden**

MBChB MMed(Psych) MD FCPsych FTCL UPLM

Chairperson: Faculty of Health Sciences Research Ethics Committee

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