A FREE HEME PERSPECTIVE TO SICKLE HEMOGLOBIN POLYMERIZATION

An Abstract

of a

Dissertation

Presented to

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In Partial Fulfillment

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Doctor of Philosophy

in Chemical Engineering

by

Anupam Aich

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A FREE HEME PERSPECTIVE TO

SICKLE HEMOGLOBIN POLYMERIZATION

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To me, pursuing a PhD has seemed more like a “transformation” of life experience in an academic setting (obviously in a positive sense)! This is an experience through which I have evolved as a learner to question concepts, argue hypotheses, find problems, and most importantly, learnt how to solve problems through individual and collective effort. Needless to say that it has been a wonderful experience that has transformed me from just an engineer to a scientist who has learnt how to pursue the challenges of an unknown field of science. My journey as a PhD student has certainly been a rather challenging and difficult endeavor. And this would have never been possible without the guidance of a certain group of people.

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ABSTRACT

Sickle cell hemoglobin (HbS) polymerization is considered to be the primary pathogenic event in the sickle cell anemia. Many cellular and molecular factors have been identified so far as contributor towards the polymerization event. The free heme, prosthetic group of hemoglobin, is one such small molecule which has been previously shown to enhance the polymerization by orders of magnitude and removal of free heme from the supersaturated HbS solution stops the polymerization completely.

In the present study we set out to investigate the free heme concentrations in normal adult and sickle cell erythrocytes. We used an enzymatic chemiluminescence assay for the determination of free heme in erythrocytes. The average free heme concentration in sickle cell patients is 44±9 μM, in sickle trait individuals—33±4 μM, and in healthy adults—20±2 μM. We also found that heme release is autocatalytic and results from spectral determination of methemoglobin percentages over time indicate towards well known higher susceptibility of sickle hemoglobin to autoxidation as mechanism for the release. We propose a link between physiological oxidative stress and autocatalytic heme release through imbalance in the reductase homeostasis in the erythrocytes. Inherent kinetic instability of autocatalytic processes may contribute to the known variability of the patients. Looking at the previous polymerization experiments and combining the current results we propose free heme and its release factors to be new targets for therapeutic and drug discovery for sickle cell anemia disease. We also provide a preliminary design of a cell separation device based on deformability induced margination flow for fractionating sickled and unsickled cells in 100% deoxygenated condition. If completed in future, this device promises a biomarker detection platform for sickle cell disease.

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INTRODUCTION

Sickle cell disease (SCD) is a hemoglobinopathy originating from a genetic mutation which leads to severe acute and chronic hemolytic anemia and other clinical complications [1]. Except for hematopoietic stem cell transplantation with its inherent immunological complicacies, the current treatment options do not offer a cure. Most non-curative management options have associated adverse side effects and thus a search for alternative therapeutic options continue.

In SCD, genetic mutation leads to the expression of a mutant hemoglobin in the erythrocytes of sickle cell patients, termed as sickle hemoglobin (HbS). This sickle hemoglobin undergoes polymerization under deoxygenated conditions, considered primary pathogenic event [2]. These long polymers stretch and rigidify the red blood cells changing their mechanical properties and blood viscosity [3]. Many cellular and molecular factors apart from HbS polymerization have been found to be associated with the pathophysiology of SCD [4]. Out of more than hundred biomarkers associated with SCD, none is directly correlated to HbS polymerization rate [5]. Results with transgenic mice expressing human HbS [6] have demonstrated that delaying polymerization inhibits red blood cell sickling and ameliorates two of the disease symptoms. Thus, it is imperative that therapeutic strategies based on delaying polymerization be sought and biomarkers related to HbS polymerization rate be identified to accelerate such therapeutics development.

Recent findings from our group [7] demonstrated that free heme has a significant effect on inducing HbS polymerization. Due to autoxidation of HbS to unstable met-HbS [8], heme may be excessively released in sickle red cell. The sole determination of the mean concentration of free heme in sickle red cells has yielded a relatively low value of < 1 μM [9], but its variation and response to environmental stress is unknown. The physiological relevance of free heme effect on HbS polymerization will certainly be questioned if those levels found by Liu *et al* are to be trusted. Thus we set out to re-measure the free heme present in red cell cytosols of healthy individuals and sickle cell patients and to examine (if there is a discrepancy in the measured levels) the mechanism of free heme release in red cell cytosol.

To evaluate intraerythrocytic cytosolic free heme level, we developed a novel sensitive method to determine picomolar concentrations of free heme in hemolysate solution; and our measurements indicate toward a more complex physiological mechanism of free heme release than mere autoxidation of HbS. Elucidation of this mechanism and its relation to sickle cell disease pathophysiology is the primary objective of this dissertation. The dissertation also explores the possibility of a new microfluidic technique to efficiently separate sickled and unsickled cell from whole blood and biomarker detection scheme using such technology.

Correspondingly, this dissertation consists of the following nine components:

In Chapter 2, I review the current state-of-the-art in the area of sickle cell hemoglobin polymerization and the recent findings of the effects of free heme on the processes' kinetics.

In Chapter 3, I discuss the design considerations, description and validation of the method for the determination of free heme present in red cell cytosol of healthy individuals and sickle cell patients.

In Chapter 4, I evaluate the results obtained from the heme determination-validation experiments and discuss the possible aggregation states of heme in physiologic conditions. Exploration of the deviation from expected equilibrium amounts of free heme in erythrocytes and the discussion possible equilibrium processes for release of excess heme in red cell cytosol are examined.

In Chapter 5, I propose an autocatalytic reaction mechanism for release of heme as possible non-equilibrium process involved and discuss validation of the proposed mechanism based on the reaction orders.

In Chapter 6, I discuss the involvement of inherent faster autoxidation of HbS and high oxidative stress in relation to the autocatalysis mechanism.

In Chapter 7, I summarize the outcomes from heme determination studies.

In Chapter 8, I discuss the current state-of-art in the area of microfluidics involvement in sickle cell research and propose a microfluidic device for separation of sickled and unsickled red cells from whole blood.

In Chapter 9, I state the design criteria for the microfluidic cell separation device and also discuss the preliminary manufacturing procedure of the device.

In Chapter 10, I propose future work to be pursued for the completion of the microfluidic cell separation device and also propose a line of future experiments which may provide insights for establishing intraerythrocytic free heme as a novel biomarker and therapeutic target in sickle cell disease.

CHAPTER

2

SICKLE HEMOGLOBIN POLYMERIZATION & FREE HEME

Sickle cell anemia is a hemolytic anemia [10] that is caused due to a single point mutation [11, 12] in the human gene responsible for producing oxygen carrying protein hemoglobin – the mutated hemoglobin is called sickle hemoglobin (HbS). **Sickle trait individuals** carry one gene for normal hemoglobin and other on for sickle hemoglobin. People who inherit both genes for HbS show clinical symptoms associated with sickle cell disease – called **sickle cell patients**. Therefore, it is important to review the basic components of hemoglobin molecule which encompasses the prosthetic group heme; the polymerization phenomena of sickle hemoglobin and underlying mechanism influenced by different factors in the surrounding biophysical environment.

2.1 Hemoglobin and heme

Hemoglobin

Hemoglobin - the word comes from conjunction of two words “haima” (blood) and globulin (sphere) [13]. Hemoglobin is a metalloprotein responsible for carrying oxygen from lungs of humans to tissue and cells where the consumption of oxygen takes place [14, 15]. For humans, the difference in the oxygen pressure in human between the lungs and the tissues is approximately 60 torr - with a pressure of 100 torr found in the lungs. Hemoglobin contains four globule chains thus it is a hetero-tetramer. Each of the four globin chains contain one prosthetic molecule, heme. Heme contains at its center an iron

molecule which is responsible for binding to oxygen [15]. This structure enables each hemoglobin molecule to bind up to four oxygen molecules when it is fully saturated with oxygen.

The tetramer protein hemoglobin contains two dimer subunits known as $\alpha_1\beta_1$ and $\alpha_2\beta_2$; the dimers are comprised of two subunits joining together. Depending on the mutations in the hemoglobin gene, there are several variants in hemoglobin – namely, HbF (Fetal Hemoglobin), HbS (Sickle Hemoglobin), HbC, HbD, HbA2, HbO – Arab and others. The β-subunit genes are usually prone to mutation. Fetal hemoglobin, HbF is actually a form of hemoglobin which is present when the fetus is in mother's womb and because of the placental oxygen pressure has to have a different oxygen affinity than normal adult hemoglobin. After the baby is born, normal adult hemoglobin (HbA) starts to be generated and HbF gene stops to produce HbF.

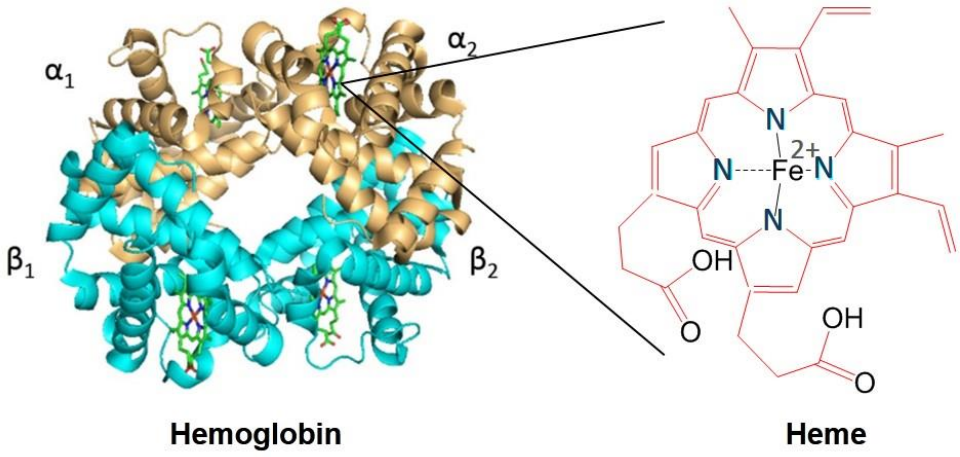

Figure 2.1 Structure of hemoglobin and heme.

Heme

Heme is the prosthetic group present in a group of metalloproteins which are known as *hemoproteins* characterized by its central iron atom in the porphyrin ring (four methine

bridged pyrrole rings). Depending on the side chains, heme is classified by different nomenclatures i.e., heme A, heme B and heme C. These common names are biologically relevant whereas others are chemical derivatives outside the realm of physiological world. The most common heme molecule is protoporphyrin IX (Fig. 2.1) which is found abundantly in human blood as inclusion in the human hemoglobin chains (one in each of the four chains) and is responsible for the red color of blood. The iron atom attached in the center of the heme molecule is responsible for coordinately binding the oxygen with hemoglobin molecule. Heme provides the hemoglobin molecule with oxygen carrying capacity. Except for C-heme, all the hemes usually bind to hemoproteins non-covalently helping them to perform as cell signaling molecules. Heme as part of other hemoproteins is known to play an important role biologically relevant oxidative and reductive enzymology [16].

2.2. Sickle cell anemia – current state

Sickle cell anemia is predominantly found in those of African origin (Benin, Batun, Nigeria), although it is also prevalent in some parts of Saudi Arab and indigenous population in Central India [17, 18]. The haplotypes present among these populations are subdivided among four different genotypes. A recent estimate [19] shows that approximately 312,000 infants are born annually with sickle cell disease across the world. The total death from these births have been approximated to be up to 150,000 by the age of five [20]. Around 100,000 people are affected by sickle cell anemia in the US and about 8% of the African-Americans contain sickle cell gene. According to Center for Disease Control (CDC), one in every five hundred African-American infants is diagnosed with the disease.

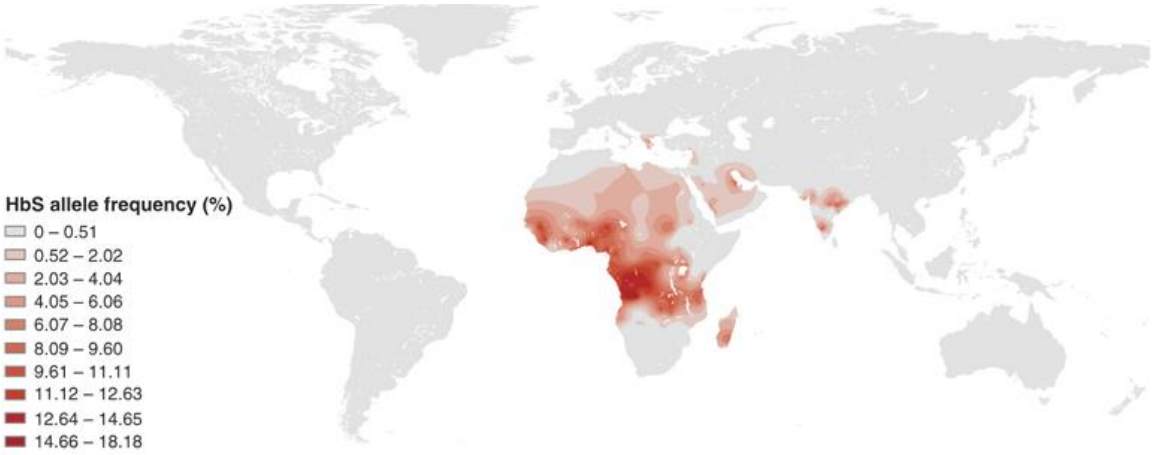

Figure 2.2 Prevalence of HbS allele worldwide.

The introduction of pre-screening and vaccine-antibiotic treatments for neonates have decreased the death rate by almost 50% [21] and the survival rate of the prescreened children for five years is almost reaching 98-99% [17]. This demonstrates the need for new born screening and management procedure which are cheap and accessible to impoverished and underdeveloped countries.

2.3 Sickle cell anemia: symptoms and complications

The mutated sickle hemoglobin (HbS) forms long fibers in deoxygenated condition in physiological conditions which deforms red blood cells. Increased rigidity of the cells coupled with increased viscosity coupled with activation of other blood cells and endothelium adhesion gives rise to the disease pathophysiology. The pathophysiology of sickle cell anemia leads to several symptoms in patients which can be categorized into two classes: 1) related to pain crises and 2) related to anemia. *Anemia* leads to fatigue in the patients which can also be associated with breathing problems, dizziness, jaundice, numbness in the hands and foots and pale skins or nails. Complications originating from

sickle cell anemia involve a span of organs and the consequences can be devastating ultimately leading to organ failure and fatalities.

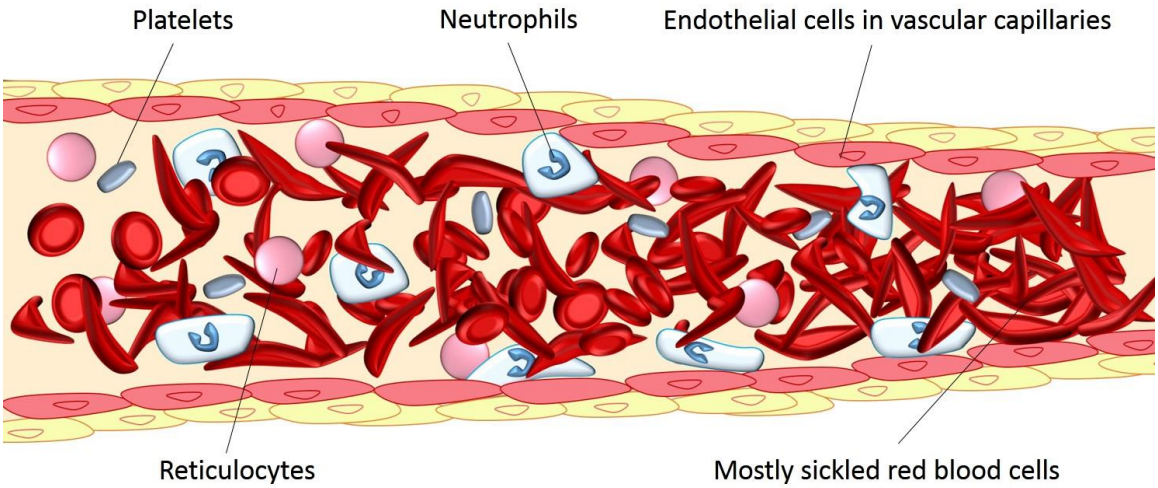

Figure 2.3 Vaso-occlusion in narrow vascular capillaries.

The hallmark of sickle cell anemia and most common reason for hospitalization is *acute pain crises* [22]. The feature of pain crises is sudden excruciating pain experienced in one's limbs or other regions of the body including the abdomen, chest, bones, joints etc. These pain crises are the most common reason for hospitalization of patients. They can last from hours to weeks and the frequency varies among patients. These pain crises, referred to as **sickle cell crises,** are considered the main symptom. The crises result from occlusion in microvessels from sickling of red blood cells during hypoxic condition and their interaction with endothelium and platelets. This occlusion leads to pain in joints, destruction of bones and other organs. Occlusions are characterized by the presence of sharp pain in the abovementioned places in body and requires hydration, blood transfusion, analgesics, hydroxyurea treatment and other opioid administration [23]. They can last from 24 hour up to seven-ten days. The acute pain crises differ from other clinical complications associated with SCD, as acute chest syndrome, pulmonary hypertensions, strokes, bacterial

infections etc., and thus one needs to be very careful to rule out other causes before treating a patient for only pain crisis.

Hemolysis, rupturing of red blood cells, is an important characteristics of sickle cell disease because of the inherent deformation of sickled red blood cells; shortens the life span for erythrocytes from 120 days to around 17 days and increases concentration of free hemoglobin in the plasma [24]. This plasma free hemoglobin has shown to play important roles in vaso-occlusion and other pathologic conditions such as pulmonary hypertension, leg ulcers etc. [24-26] through its action on reducing bioavailability of nitric oxide (NO). It also plays an important role in adhesion activation of endothelial cells which is a contributing factor to further deteriorate vaso-occlusive crises. The plasma free hemoglobin releases heme molecule in the circulation and heme is known to be toxic because of its oxidative properties [24, 27]. The free heme takes part in generating reactive oxygen species (ROS) and these ROS takes part into tissue damage and inflammatory responses. Therefore, hemolysis is a major pathophysiological phenomena over the course of sickle cell disease.

Acute Chest Syndrome (ACS) is one of the leading causes of deaths occurring from sickle cell anemia [28]. This statistic accounts for at least 25% of the deaths occurring from sickle cell anemia [29]. The crises is accompanied by fever, chest pain and infection in the pulmonary tract in parallel with breathing problem, a signature of the syndrome [30-33]. The pathogenesis of ACS differs between adults and children as adults verbalize about the pain experienced in the chest accompanied by breathing problem while children symptoms are overshadowed primarily by pneumococcal infections [31, 34]. Mortality is higher in adults [31] while seasonal variation in children has been also found to be

correlated [33]. Though the pathogenesis of ACS is overwhelmed with different associating factors such as fat embolism [35, 36], bacterial and viral infections [37, 38], *in situ* thrombosis [31], and hypoventilation of the airway [31, 33], it is important to understand that the primary cause for ACS is thought to be pulmonary vaso-occlusion conjoined by adhesion of erythrocytes in the pulmonary tracts and causing pulmonary vasculature and infarction [31-33].

The management of acute chest syndrome (ACS) is quite elaborate ranging from supportive to experimental therapy. Oxygen support in the case of hypoxia [39] and analgesics (without the option of NSAIDs which may worsen the situation) for controlling pain are immediately used during the first response [33]. Saline is administered to ensure hydration of the patient and antibiotics are used to cover the infections associated with the syndrome [39, 40]. Children suffering from asthma require bronchodilator [41] and also in case of extreme respiratory failure this helps to mediate respiration [33]. Transfusions effectively improve ACS condition [41, 42]. Though an NO-dependent vasodilator pathway for remission of ACS or in general sickle cell crises was suggested for more than a decade [43-47], a large multicenter study [48] could not conclude about the efficacy of NO based vasodilators. Another controversial therapeutic avenue is using corticosteroids where the efficacy was tarnished by relapse of the painful crises [41]. Recovered patients, receiving conventional treatments, are required to adhere to the hydroxyurea treatment for lifetime in the future [41].

Stroke is a major complication in sickle cell disease [49, 50]. The neurological complications originating from sickle cell disease can be overwhelming and cerebral infarction is one of the leading cause of deaths in patients which is almost 25% spanning

all ages [51]. The onset of strokes in children typically occurs above the age of two with the severity peaking at the age of five [52]; 11% of the patients experience a stroke by the age of twenty [53]. Cerebral strokes are usually prominent, but silent infarcts [54] can occur continuously showing no symptomatic sign in children [50, 53, 55]. The cerebral infarctions and ischemic strokes are dominant in children while adults usually go through hemorrhagic onset of stroke [52, 53]. The stroke in children even if averted, can leave the child cognitively impaired and with other neurological symptoms. Though small cerebral vessels were thought to be the major location of strokes or infarctions, in early 1970s researchers first established that it can occur in large vessels too [49]. Fat embolism and artery-to-artery embolism is a leading cause of large vessel constriction. Genetic basis for the stroke is under investigation and several locus in human genome that associate with the RBC adhesion molecules and stroke prevalence have been identified [56-58].

Stroke is treated by blood transfusion in sickle cell patients [50, 59, 60]. Three major primary stroke prevention strategies are: blood transfusion coupled with TCD, bone marrow transplantation (only cure for SCD) and hydroxyurea. Blood transfusion therapy increases bioavailability of oxygen in the physiological environment and thus reduces the risk of sickling of red cells. For stroke prevention, Transcranial Doppler (TCD) studies of cerebral blood velocity has been found to be effective [60-63]. The TCD measurements of blood velocities in cerebral vessels have been found to be correlated to the silent infarctions in children and prevention of a first stroke has been since improved by identifying high risk patients using this technique [64-66]. Though TCD has shown very promising results for children [60, 61, 63, 67, 68] and reduced the relative risk of a first stroke by 92% [52], the trials with adults [61] have not found any promising indicator in high risk patients.

Popularly used SCD management drug hydroxyurea has been suggested for improvement and replacement of transfusion treatment [51, 60, 69, 70] as primary prevention of a first stroke, but a multicenter clinical trial called SWiTCH (Stroke With Transfusions Changing to Hydroxyurea) showed that hydroxyurea did not manage relapse of stroke better than chronic transfusion and exchange [71].

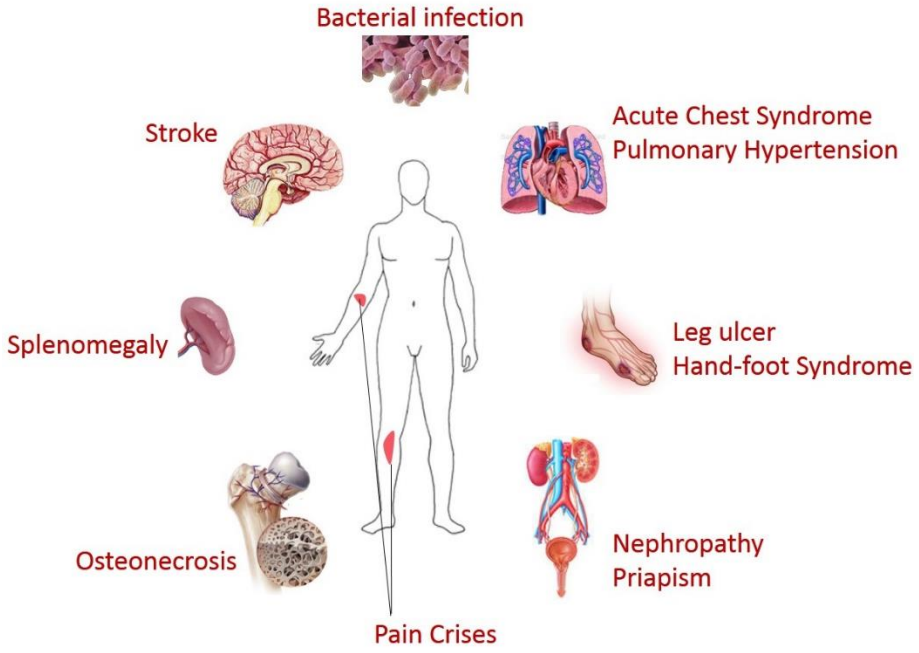

Figure 2.4 Major complications in sickle cell disease arising from vaso-occlusion.

Spleen is one of the earliest organs affected by sickle cell anemia. There are mainly three primary stages of the spleen disorder in sickle cell anemia – namely *Splenomegaly, Hyperslenism and Acute Splenic Sequestration.* Splenomegaly, an abnormal softening of the spleen, is known to occur from recurrent trapping of sickled red blood cells at the very early life and leads to autosplenectomy i.e., absence of spleen in patients [72]. The combination of anemia with splenomegaly is a health condition referred to as Hypersplenism. Preexisting anemia coupled with hypersplenism deteriorates the bone marrow condition and can also affect the transfusion efficiency [72, 73]. **Acute Splenic**

sequestration is caused by the blockage and scarring of deoxygenated sickled blood cells stuck in the spleen [74, 75]. The congestion diverts the flow of blood bypassing the filtering process. A combination of phagocytes and macrophages that attack the abnormal shaped sickled cells increases the blockage in the spleen reducing or completely arresting spleen functionality [74]. This is characterized by increased volume of spleen and low level of hemoglobin from the baseline [72]. Acute splenic sequestration develops in early age after five months up to two years which can also be seen in young age and adults (about 7 to 30%). It is one of leading causes of death of children with SCD [73, 75, 76]. This complicacy can lead to death while other SCD complications has not yet manifested. Thus it portrays a larger threat for children younger than two years [77].

The management of splenic complications has only two major options – blood transfusion and splenectomy i.e., removal of the spleen in its severest form [72, 78, 79]. The hypersplenism and splenomegaly do not require any special management, however, it is important to be checked every six months to make sure that they do not lead to acute sequestration. Acute sequestration is managed by transfusion and in its severe case, splenectomy is the only option [72]. Splenectomy at an early age may provide better outcomes than chronic transfusion and lead to normal life style without recurrence of the crisis episodes [78, 79].

Pulmonary hypertension (PH) is also one of the major causes of death ranging in up to about 40% mortality [80-83] mostly in adult SCD population resulting from failure in the cardiac output [84]. The PH is associated with the increased blood pressure in the arterial blood flow and can lead to minimal cardiac output. The pathogenesis of PH is probably associated with hemoglobin induced NO, arginine and nitric oxide synthase

enzymes scavenging due to intravascular hemolysis [85, 86]. This leads to loss of vaso-dilation, increasing activation of red cell adhesion molecules to the endothelium and platelet activation [81]. Thus higher level of plasma hemoglobin may be indicator of high risk factor for pulmonary hypertension.

The management of PH in SCD patients requires hydroxyurea and blood transfusion therapy to provide patients with increased oxygen saturation in the form of higher fetal hemoglobin and normal adult hemoglobin respectively [85, 87]. Other drugs which increases NO bioavailability such as L-arginine and inhaled NO are also in use for treating PH associated with SCD. Drugs which can control vascular tone independent of NO pathway are also in trial [86].

Infection in sickle cell patients is the major cause of death at very early age (20 to 50% found in several cohort studies over last 25 years) [74]. Even before preventive antibacterial measures are taken, the unnoticed and unscreened children can be infected with encapsulated bacteria such as S*treptococcus pneumoniae, Haemophilus influenzae, Neisseria meningitidis, Salmonella spp* [74, 88, 89]. These pathogens infect the spleen (due to recurrent infarctions) damaging the splenic immune system. Malaria infection and *Salmonellae* infection are also prevalent in the African region at very small age (starting from infant stage up to 9-10 years old). Acute chest syndrome can also be associated with infections in young patients. Infections from blood transfusion such as Hepatitis B, C and HIV are also reasons of death in SCD [33].

Prevention is the first line of defense against infection dominated SCD complication. Good monitoring of hygiene and child infectious agents are important. As pneumonia is the most dangerous consequence of infection for children with SCD,

prophylactic antibiotics administration is important as soon as an infection is noticed. Almost 90% reduction in death from pneumococcal infection has been demonstrated through use of prophylactic medicine. Vaccination against infection causing pathogen is also another strong prevention method sustaining at least for five years and next a booster dose is require to contain the vaccination for future years. Vaccination may not be effective in case of children less than two years old as their immune system may not have developed the response against the antigens. Also, some vaccines with polysaccharide-protein complex are being developed to address the immunity of pathogens against other non-polysaccharide based antigenic responses [90].

Apart from above-mentioned major complications, there are other complications related to SCD which encompass hand-foot syndrome, end organ damage, retinal impairment leading to blindness, priapism, osteoporosis and other associated complications [3]. The *single cure* for the sickle cell disease is **bone marrow transplantation** which is cost-prohibitive in most cases. Only 50% of the patients who are treated with hydroxyurea benefit while blood transfusion remains the only way to provide substantial management in resource-intensive settings. Thus there is a need for a cure or better and less resource-intensive management procedure and/or drug.

2.4 Sickle hemoglobin polymerization: pathogenesis of sickle cell anemia

Sickle cell anemia (SCA) is the first molecular disease to be identified [12]. It was first reported in 1910 [91] and then there were at least four cases documented till 1922 when Mason first introduced the term “Sickle Cell Anemia” [92]. This is an inherited condition where a homozygous mutation is present in the 6^{th} codon that encrypts the β-

chain of the hemoglobin gene. The mutation from GAG to GTG causes the gene to substitute a glutamic acid by a non-polar valine [11, 93]. The transition to nonpolar valine on the surface of HbS does not change the conformation of the protein; in the deoxy state of sickle cell hemoglobin [94] the surface of the ß chain which has nonpolar valine is induced to have hydrophobic contact [95] with other nonpolar residues from other HbS molecules such as with leucine, alanine and phenylalanine [96-99].

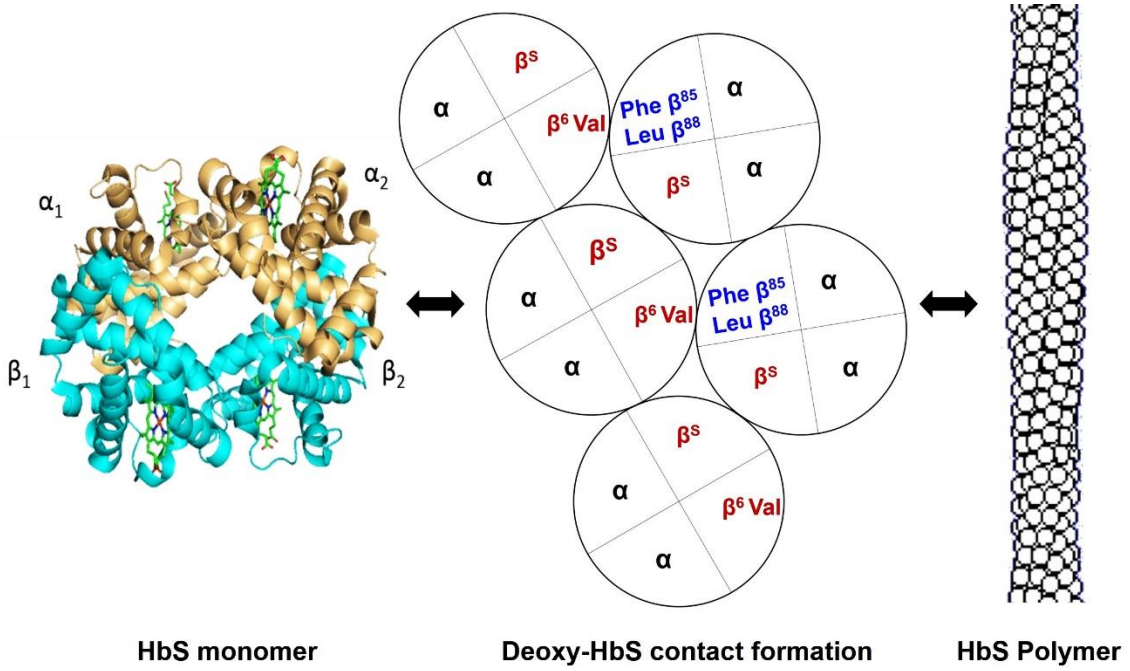

Figure 2.5 HbS polymer formation in deoxy state of hemoglobin through lateral and axial contacts.

This 14-membered polymer [96, 97] deforms the red blood cells and due to increased rigidity of the cells, the flow viscosity also increases [10, 100]. In homozygous individuals with HbS gene, the increased viscosity and induced cell adhesion to the vascular endothelium can lead to episodic obstruction in the blood flow – resulting in pain crises known as vaso-occlusion and other symptoms [101]. This condition can also lead to malaria, bacterial infection (because of aggravation of immune response), stroke,

pulmonary hypertension, splenic sequestration etc. and neonatal and adults die from these conditions [101].

The polymerization of sickle hemoglobin, a first order phase transition owing to the constant density and structure of the polymer [10], has concentration dependence up to 50th order [102]. The **double nucleation mechanism** proposed by Ferrone *et al* in 1980s [103, 104] is characterized by three major steps: *Homogeneous nucleation* of HbS polymers from supersaturated solution (where no polymer is present initially) and under low oxygen environment, their growth and their branching by *Heterogeneous nucleation*, the nuclei from the homogeneous part acting as substrate for the later nucleation and faster growth. The aggregates from heterogeneous nucleation have greater stability than the smaller aggregates formed from homogeneous nucleation.

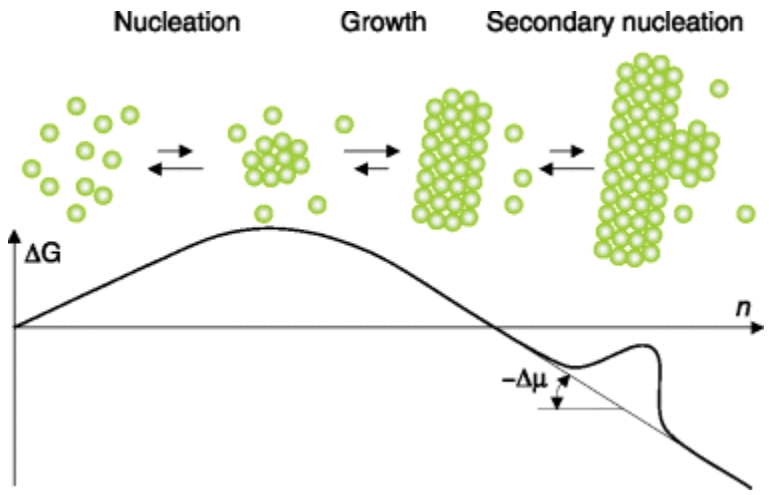

Figure 2.6 Energy landscape of the formation of sickle-cell hemoglobin (HbS) polymers in a supersaturated HbS solution.

The changes in the free energy G of the system [2] are schematically plotted as a function of the number of molecules n, which transfer from the solution to the polymer. The starting point of *G* is its value in the supersaturated HbS solution. The homogeneous

formation of nuclei is accompanied by an increase of G, growth leads to lowering of *G*, the heterogeneous formation of secondary nuclei overcomes a G-barrier lower than the one for homogeneous nucleation. The slope of the decreasing *G* during the growth of a new phase is the solution supersaturation with respect to the polymer.

Delay time of sickle hemoglobin polymerization, treatment strategies and confusions

The double nucleation mechanism actually helped to unravel several features of the sickle cell disease and provided an insight through exploration of the delay time for polymerization [105]. The delay time for polymerization opened a new avenue to look for increasing the delay *in vivo* and thus intervening the disease pathology from a fundamental characteristic phenomena. The mechanism also reinforced explanations for the observations that the polymerization is strongly dependent on the HbS solubility and enhancing HbA (blood transfusion) [106] and HbF [107] (through use of hydroxyurea to enhance HbF gene expression [108-110]) can increase HbS solubility and reduce the severity of the disease. Hydration therapy also increases the water content in the red cells, thus preventing sickling through lowering HbS activity and supersaturation [111, 112].

The molecular-level data on the variant hemoglobin and the fiber structure gave rise to the hope that understanding HbS polymerization, the initiator of the sequence, would lead to cure [113]. A treatment avenue was suggested: to find a hemoglobin ligand, which directly or through allostery would prevent the hydrophobic contact and the formation of the fiber [114-117]. After more than 30 years of research, yielding about 200 molecules which, in vitro, bind to hemoglobin and prevent polymerization, this hope has not been fulfilled [118]. A major obstacle for antisickling drugs has been the high concentration of

hemoglobin inside the erythrocytes, ~5 mmol l^{-1}, which requires unacceptable concentrations of a ligand targeting most HbS molecules [119].

Table 2.1 Treatment strategies for SCD. [120, 121]

	Strategy	**Comments**
Curative	Hematopoietic Stem Cell Transplantation (Only potential cure for SCD)	Related donors – highest engraftments rate Unrelated donors – high risk of immunological rejection
Non-curative	Hydroxyurea	Helps by inducing generation of increased HbF level – thus diluting the HbS, it reduces the severity. Used in pain management. But only 50% of the patients benefit from the treatment.
	Analgesics, NSAIDs, fluids and heating pads	Pain management during mild pain
	Opioids	Severe pain
	Blood transfusion and oxygen therapy	Increase HbA level to dilute the HbS and to ameliorate the symptoms
	Antibiotics	Takes care of infections associated with SCD
	Vasodilators	To increase blood flow during ischemia and pain crises
Novel Therapies	Antisickling actions, increasing HbF, endothelial and platelet adhesion reduction, increasing NO bioavailability, controlling viscosity and vascular tone, reducing inflammation and oxidative injury – these are the currently being pursued to develop new drugs and therapies.	

The clinical features of SCD in patients seem to offer more than just a linear relationship between the polymerization event and disease progression [122, 123]. Observations suggested that people with identical concentrations of HbS do show large variation in the crises scenarios [110, 124-126]. It was found that: more than half of the red cells of sickle cell patients undergo sickling on every passage through the venous circulation, yet sickle cell crises are significantly rarer [127]; the propensity for sickling of different red cells is not correlated to their density [123]; etc. These contradictions indicated that HbS polymerization may be just one of many factors associated with the pathogenesis of SCD and other factors such as red cell adhesion to endothelial walls [128], platelet and neutrophil activation [129], NO dysregulation changing the vascular tone, intravascular hemolysis, membrane damage, chronic inflammation due to ROS generation [1, 123, 130, 131] etc. should be considered as contributing factors.

There is a potent recent finding, which suggests that delaying polymerization may still be a practical venue in the search for clinical therapies. Transgenic mice expressing human HbS were employed [6]. Their HbS was genetically modified: valine at the β6 position was left intact, but the residues, with which it forms hydrophobic contacts in the fiber, were modified. In this way, the sickle cell gene was not touched, and its suspected pleiotropic consequences would not be affected. It was found that the additional mutations inhibit incorporation of the modified HbS into the polymer. The modification was found to delay polymerization, strongly reduce the fraction of sickled red blood cells, and reduce the severity of sickle crises. Significantly, two features of the disease, red cell dehydration and the count of irreversibly sickled cells, sometimes considered as independent factors for the disease, were also reduced [6]. These results provide a cure for sickle cell anemia, at

least in mice, other than bone marrow transplantation. The remarkable fact is that the cure works through delay of HbS polymerization. This delay was achieved through a genetic modification of hemoglobin, and it is likely that such gene therapy in humans will be delayed by many years. Hence, other means to delay polymerization should be sought.

As Table 2.1 summarizes all the treatment strategies that are currently being used and also strategies which are being pursued to develop new therapies, it can be seen that none of them is based on delaying polymerization. In the light of the above discussion and 20 sec circulation time half of which is hypoxic, delaying polymerization can be a viable strategy for reducing the collective jamming of sickled red blood cells i.e., vaso-occlusion.

2.5. Free heme in human physiology

Biosynthesis of heme takes place mostly in red cells in bone marrow and the rest in liver, before being taken up by hemoproteins to perform biological functions which depends on the type of proteins they are associated with [132, 133]. Heme is beneficial and physiologically important in numerous biological processes (in combination with hemoproteins such as hemoglobin, myoglobin, catalases, peroxidases, guanylate cyclase, nitric oxide synthase) including oxygen transport, electron transfer for mitochondrial respiration [16], signal transduction [134], drug and steroid metabolism [135, 136], microRNA processing, NO production for endothelial activation [137], gene expression regulation [138] in cellular environment etc. [139].

Despite all these regulatory and important biophysical functions, free heme is known to be toxic [140, 141] and injurious to cellular function [142, 143]. Heme is toxic by several mechanisms: generation of reactive oxygen species [144, 145], lipid peroxidation [146, 147], neurotoxic effects [148] and activation of cytotoxicity leading to

cell death [149, 150]. Free heme also takes part in inflammation through promotion of vascular and endothelium adhesion molecules [151-153], tissue injuries through activation of innate immune response of neutrophils and macrophages [154-156] and also injuries leading to renal failure [157]. Thus it is very important that we should try to elucidate role of free heme in the prognosis of sickle cell disease.

2.5.1. Free heme in sickle cell disease

Role of free heme in sickle cell vasculopathy has been observed from the point of view of hemolytic free heme pool that is present in the plasma [158, 159]. Free heme contributes to lipid peroxidation of the membranes [160], alteration of cell osmotic pressure [161] and red blood cell cytoskeletal deformation [162] that lead to cell lysis and release of high concentration of heme/hemoglobin high iron loading [159] in vascular environment. The free heme (from vascular hemolysis) plays role in cell signaling [163] for activation of endothelium adhesion molecules [153, 163, 164] and neutrophil, monocyte activation [165-167] in the pathogenesis of sickle cell disease. Plasma free heme/hemoglobin also scavenge nitric oxide (NO) leading to loss of vascular tone. Change in the vascular tone contributes to the constriction of endothelium leading to vaso-occlusion [168]. It has been also found that intravascular heme can deteriorate and trigger acute chest syndrome in transgenic sickle mice [169]. Though there have been ample work on investigating the effect of extracellular free heme on sickle cell disease pathogenesis as in the few references mentioned above, there has been no work except for one *in vitro* work relating sickle cell hemoglobin polymerization to free heme [7].

2.5.2. Free heme and sickle hemoglobin polymerization

It is known that in solution one or more (less likely) heme from hemoglobin can detach from the hemoglobin molecule [170, 171]. The two major consequences of hemoglobin gene mutation in sickle cell patients are: (i) HbS polymerizes in deoxy state stretching and rigidifying the red blood cells and (ii) heme is more readily released from unstable hemoglobin. The rate of heme detachment is higher from HbS [8] due to sickle hemoglobin's higher susceptibility to autoxidation leading to formation of methemoglobin [171] which readily releases heme. The relationship between these two consequences of hemoglobin gene mutation has not been explored and only our group experimented to find out if free heme has any influence on the sickle hemoglobin polymerization [7].

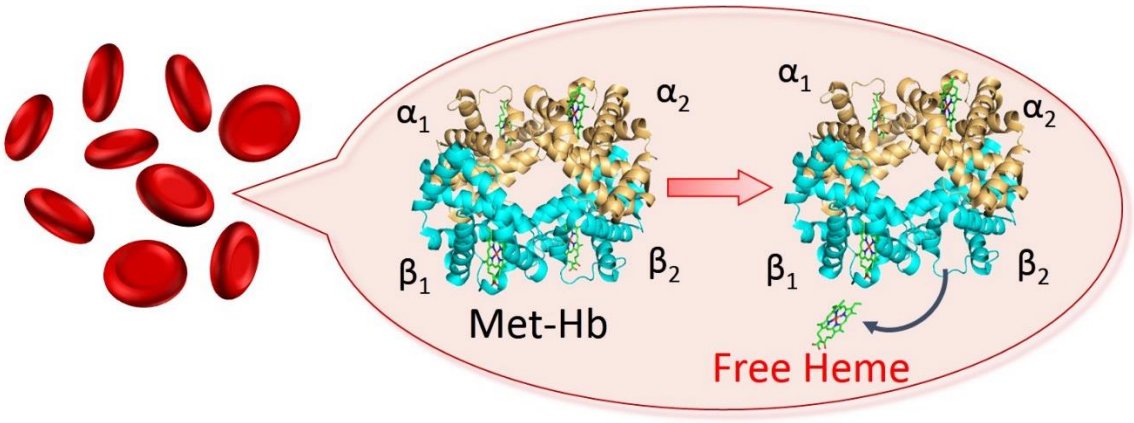

Figure 2.7 Schematic of free heme release in red cell cytosol.

The results in those experiments showed (Fig. 2.8) that complete removal of free heme arrests polymerization and increasing free heme concentration increases the polymerization rate. The dialyzed solution free of heme did not show any evidence of polymerization during longer period of time; and the non-dialyzed and dialyzed solution with added heme showed evidence of polymerization. It can also be seen that upon ageing of HbS solution has higher rate of polymerization. Characterization of the aged HbS

solution by mass spectroscopy reveals that no unexpected species, e.g., pieces of protein chains, appear in the solution upon ageing, and that heme is the only solution component, which appears to increase its concentration. In the dialyzed solution (for removing free heme), no polymers form, even at temperatures as high as 36.5^{o}C (higher concentrations and temperatures lead to faster polymerization); an illustrative example is shown in Fig. 2.8.

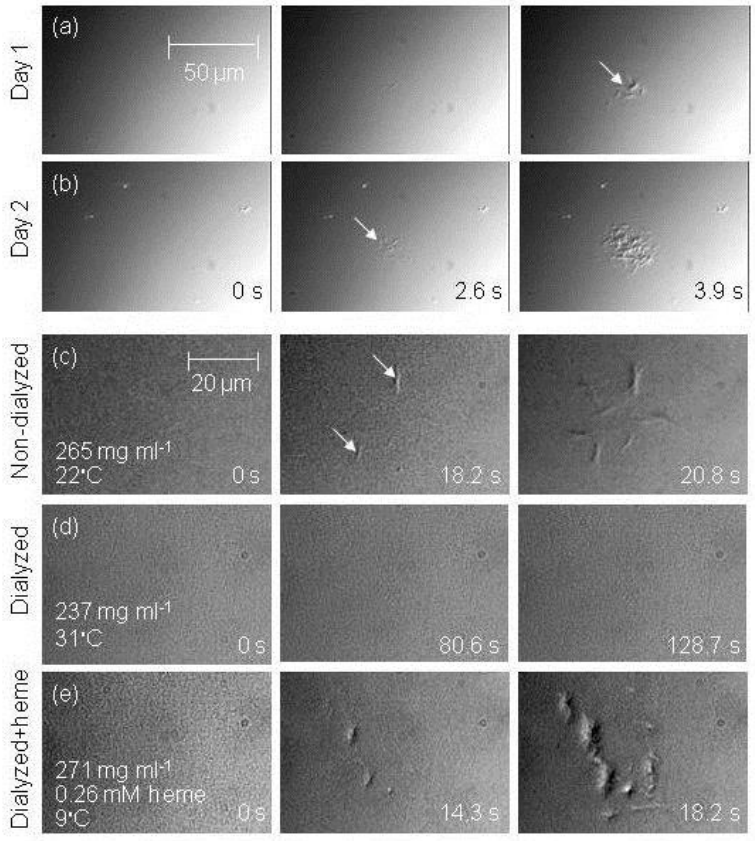

Figure 2.8 Effects of aging and heme on the polymerization of sickle cell hemoglobin.

Quantification of the kinetics of nucleation and growth shows that (i) the rates of nucleation and growth in solutions prepared from one-week old stock are lower and the nucleation delay times are longer than in solutions prepared from one-month old stock in Refs. [172-174]. The faster rates after longer storage may have originated due to release

of heme, likely during thawing and re-freezing. (ii) After dialysis, no HbS polymers nucleate, i.e., nucleation rate is zero and delay time is infinite for nucleation. (iii) The addition of 160 or 260 μM of heme to dialyzed solutions enhances nucleation rate and growth rate of fibers by more than two orders of magnitude and shortens delay time for nucleation by approximately the same factor in comparison with the rates and times prior to heme removal. These observations show that *free heme accelerates HbS polymerization*; in the absence of heme, polymerization is prevented under the conditions tested here and, likely, is significantly delayed in a broader range of conditions. These results suggest that free heme in the erythrocytes of sickle cell anemia patients may be a major factor for the puzzling complexity of the clinical manifestations of sickle cell anemia. But at first we need to be able to answer the following questions:

1. Are the concentrations of heme (100 – 260 μM) used in the polymerization experiments physiologically relevant as single determination [9] of free heme in erythrocytes showed that sickle cell patients may have only up to 0.75 μM?
2. What is the mechanism of heme release in erythrocytes?
3. What are other factors that may be associated with heme release and their effects on sickle hemoglobin polymerization leading to vaso-occlusion?
4. Can free heme concentration contribute to the variability present in clinical manifestations in sickle cell patients?

To find out answer to these relevant queries, we decided to re-measure the heme content that is present in healthy, sickle trait and sickle cell erythrocytes and thus to find out if the physiologic concentrations and heme release mechanism can elucidate more on the pathogenesis of sickle cell disease.

CHAPTER

3

METHOD TO DETERMINE FREE HEME CONCENTRATION IN HUMAN ERYTHROCYTES

The overwhelming difficulty in the determination of free heme in erythrocytes is its separation from hemoglobin without additional heme release or loss of existing free heme; even advanced spectroscopy methods fail to quantify free heme in the presence of hemoglobin [175]. Most of the recent work on heme quantification has focused on the determination of the total heme content, including the one bound to globins, often in the context of forensics [176]. Earlier methods were mostly based on the spectroscopic response of heme and heme-containing proteins [177]; recent studies rely on HPLC [178], capillary electrophoresis[179] and other electrochemical methods [180], and, most recently, mass-spectrometry [176, 181].

In the only previous determination of free heme present in healthy and sickle erythrocyte cytosol [9], Liu *et al* separated free heme from hemoglobin by charge, using ion exchange liquid chromatography. As we proceed to separate heme from hemolysate solutions, we employ a much gentler separation method, dialysis through membrane, in buffer solution and this leads the concentration of heme in the solution to be less than 1 nM which cannot be determined through colorimetric or spectroscopic assays [182].

To quantify such low concentrations of hematin (Fe^{3+} protoporphyrin IX OH), we adopted and modified a recently developed method [183]. The method relies on the reconstitution of apo-horseradish peroxidase (HRP) in the presence of hemin (Fe^{3+}

protoporphyrin IX Cl) or hematin (Fe^{3+} protoporphyrin IX OH), as illustrated in Fig. 3.1. In excess of apoHRP, the concentration of the reconstituted enzyme equals that of the initial hemin or hematin. The reconstituted HRP catalyzes the breakdown of hydrogen peroxide to two hydroxyl radicals, which interact with luminol (3-amino-phthalhydrazide) to produce aminophthalate.

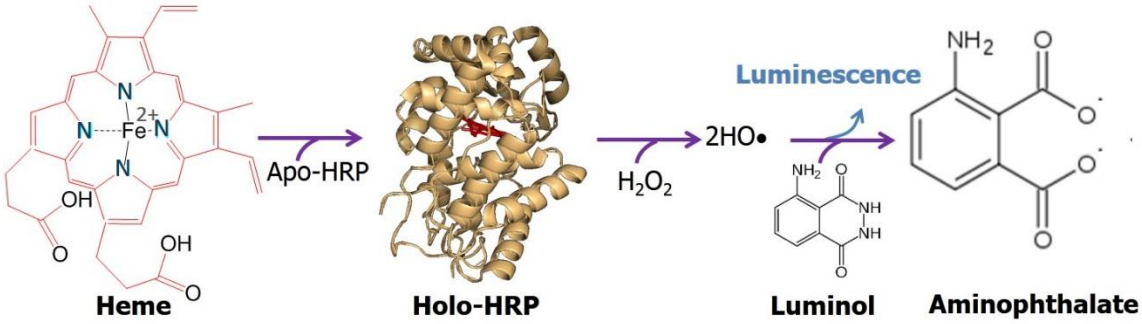

Figure 3.1 Enzymatic determination of free heme concentration using chemiluminescent reaction.

The latter reaction is accompanied by luminescence at 428 nm [184, 185]. The luminescence intensity is proportional to the rate of catalytic decomposition of peroxide. This rate, in turn, is proportional to the concentration of reconstituted peroxidase; hence, the concentration of hemin. This method is highly sensitive to the concentration of hemin because the analyte quantitatively transforms to a catalyst of a reaction with a well detectable product. Furthermore, the luminol reaction is an example of very strong chemiluminescence [184, 185]. The strong amplification of the signal allows detection and quantification of hematin or hemin levels as low as 50 pM making it a viable choice for quantitating putative amount of free heme present in the buffer after dialysis of the ghostless hemolysate.

3.1. Materials and methods

3.1.1. Reagents and solutions

We purchased apo horse radish peroxidase (apo-HRP) from BBI Enzymes, Gwent, UK and used it without further purification. We used two batches of apo-HRP, 109 and 118; as demonstrated below, they showed identical results. We purchased luminol (3-amino-phthalhydrazide) and peroxide as two parts of the binary reagent Immobilon from Millipore. The concentration of luminol in the commercial solution is a trade secret. We spectrophotometrically determined the concentration of peroxide as 4 mM; for this we used an extinction coefficient of 43.6 $M^{-1}cm^{-1}$ at 240 nm [186]. We purchased hematin (Fe^{3+} protoporphyrin IX OH), hemin (Fe^{3+} protoporphyrin IX Cl), and all other reagents from Sigma-Aldrich. 96 well plates (half area flat, white) were purchased from Greiner. We used a multi-channel pipette (Mettler Toledo) for solution deposition into the wells. The consistency of the volumes disposed by the individual channels was found to be within 3%. We used an Infinite 200 PRO microplate reader (Tecan) to monitor the luminescence intensity.

Deionized (DI) water was prepared by Millipore reverse osmosis – ion exchange system RiOs-8 Proguard 2 – MilliQ Q-guard. To prepare standard solutions of hematin and hemin for the analytical procedure we dissolved the respective dry powder in DMSO at concentration ~1 mg mL^{-1}. We diluted these solutions by a mixture of 66.5% ethanol, 17% acetic acid, and 16.5% DI water (v/v). We spectrophotometrically determined the exact concentration of each preparation using the solvent-specific extinction coefficient 144 $mM^{-1}cm^{-1}$ at 398 nm for both reagents [187]. We prepared hematin solutions for the study of its solubility and oligomerization by dissolving dry powder in 0.1 M NaOH at pH

= 13 to a concentration of ~ 2mM. We adjusted the solution pH to the desired value by addition of H_3PO_4 and/or KH_2PO_4.

We prepared a stock solution of apo-HRP in deionized water with approximate concentration 2.5 mM. We spectrophotometrically determined the exact concentration using extinction coefficient of 20 $mM^{-1}cm^{-1}$ at 278 nm[188] We stored the apo-HRP stock in a freezer at -80^oC and thawed samples when needed.

We prepared Tris (tris(hydroxymethyl) aminomethane, $(HOCH_2)_3CNH_2$) buffer by dissolving dry powder in deionized water at concentration 100 mM and adjusting the pH to 8.4 by adding HCl. We prepared 0.15 M phosphate buffer at pH = 7.35 by dissolving K_2HPO_4 and KH_2PO_4 in water and adjusting the pH with KOH or H_3PO_4.

Source of blood

Sickle cell blood was collected at Texas Children Hospital, Baylor College of Medicine (BCM) from sickle cell patients less than 21 years of age, following a protocol approved by the BCM Institutional Review Board. The blood was drawn in green cap Vacutainer tubes, which contain sodium heparin to prevent blood coagulation. After all identifiers of the blood donors were removed, we transferred the blood to the University of Houston. The time between the blood drawing and the start of analysis was approximately two hours. The tubes with blood were held in a Ziploc bag enclosed in a Styrofoam box that maintains a stable temperature of ~23^oC. The protocol for blood transfer and analysis was approved by the University of Houston Committee for Protection of Human Subjects.

Sickle trait blood was collected at the Gulf Coast Regional Blood Center (GCRBC) in Houston. The blood was held in grey cap tubes, which contain fluoride and oxalate to prevent blood coagulation. All identifiers of the blood donors were removed and the blood

was transferred to the University of Houston for analyses. The time between blood drawing and the start of its analysis was up to four hours. During this time, the tubes with blood were held in a Ziploc bag enclosed in a Styrofoam box that maintains a stable temperature of ~23^{o}C.

Blood from healthy adults was collected at the University of Houston Health Center (UHHC) following a protocol approved by the University of Houston Committee for Protection of Human Subjects. The blood was drawn in green cap Vacutainer tubes, which contain sodium heparin to prevent blood coagulation. The time between blood collection and the start of its analysis was approximately 30 min. During the transfer from UHHC to the laboratory, the tubes with blood were held in a Ziploc bag enclosed in a Styrofoam box that holds the temperature of the blood at ~ 23^{o}C. In some cases, to test the effects of the time between blood collection and processing, tubes with blood were held at room temperature, ~23^{o}C, for up to four hours. This length corresponds to the time lag for blood collected at Texas Children's Hospital and to the period for which blood from GCRBC is kept at room temperature. In a few other cases, whole blood was stored in a laboratory refrigerator for up to two days. As shown below, within these parameters of storage, the concentration of free heme in red cells was independent of the time lag between blood drawing and analysis and the temperature at which the blood was stored.

Samples of healthy blood were also provided by GCRBC in Houston in grey cap tubes. This blood was treated identically to the sickle trait blood discussed above. We found no differences in the free heme concentration between blood samples collected at UHHC and GCRBC.

3.1.2. Preparation of hemolysate

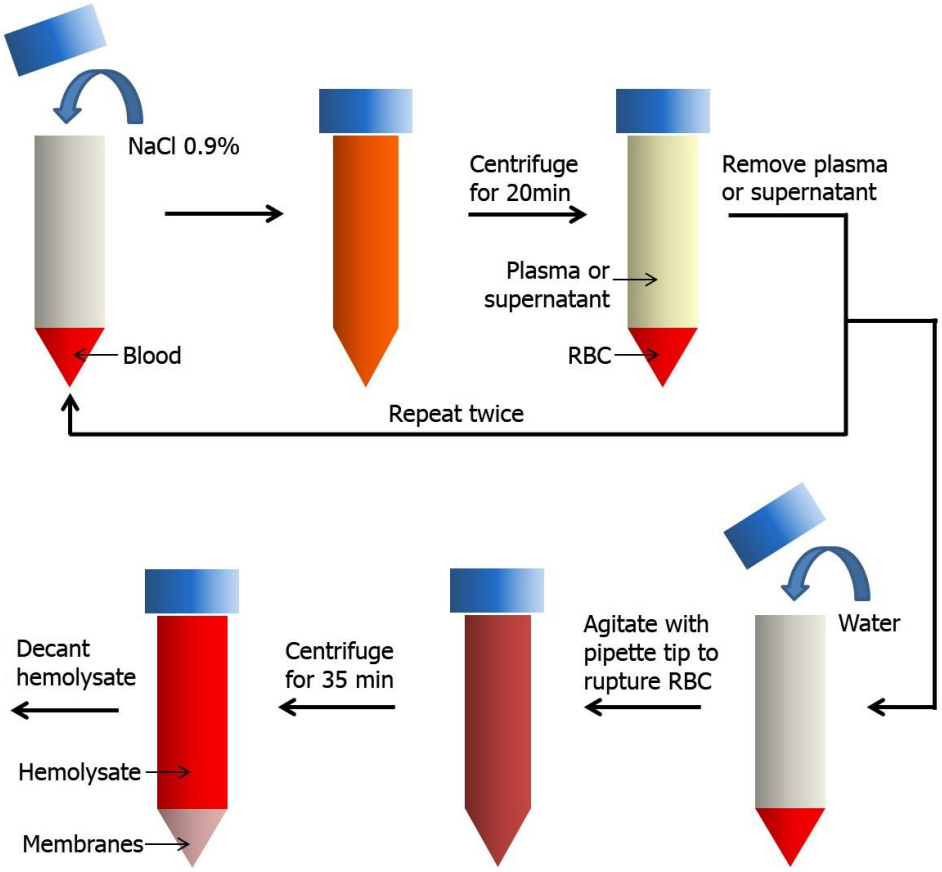

Figure 3.2 Schematic illustration of the hemolysate preparation.

Figure 3.2 illustrates the procedure for preparation of red blood cell hemolysate. We loaded 4 mL of blood in a 50 mL tube. We added 45 mL of isotonic 0.9% NaCl solution in water and gently agitated the tube to homogenize the suspension. We centrifuged the diluted blood for 20 minutes. We decanted and discarded the supernatant containing blood plasma, white blood cells, and other blood components. We repeated the sequence of washing with NaCl solution, centrifugation, and supernatant removal two additional times for a total of three washes. After that we added from 5 to 50 mL of deionized water to the precipitated red blood cells and agitated the solution with a pipette tip to resuspend them in the added water. The red blood cells ruptured under the influence

of the osmotic pressure difference between the cells and the water. We centrifuged this suspension for 35 minutes to compress the cell membranes at the tube bottom, after which we decanted the hemolysate and stored it in a refrigerator at ~5^{o}C in another sterile capped tube.

To evaluate the dilution ratio of the hemolysate compared to the red cell cytosol, we spectrophotometrically determined the hemoglobin concentration in the hemolysate after the addition of Drabkin's reagent using an extinction coefficient for cyanomethemoglobin of 1.512 mL $mg^{-1}cm^{-1}$at 540.0 nm [189]. We assumed that the hemoglobin concentration in the cytosol of healthy red cells is 350 mg mL^{-1} (corresponding to mean corpuscular hemoglobin concentration (MCHC) of 35 g dL^{-1}) and calculated the dilution ratio as the ratio of the two concentrations. While the range of MCHC of sickle cell patients is increased [190], the average cellular hemoglobin concentration is normal [101]. Hence, the same estimate of the dilution ratio was used for sickle hemolysate. Typical hemoglobin concentrations of the hemolysate were between 2 and 20 mg mL^{-1} (~30 and ~300 μM), corresponding to dilution ratios between 175 and 17.5×, respectively.

To test the veracity of this method of evaluation of the hemolysate dilution, we weighed a sample of three-fold washed and centrifuged erythrocytes. We assumed that the measured mass is that of the cytosol only and estimated the volume using a density of 1.00 g mL^{-1}. This estimate yielded a dilution ratio lower by ~ 50% than the one based on the hemoglobin concentration. However, the estimate ignored the mass of the cellular membranes and of the saline solution trapped between the red cells. The latter error is more significant. Since both errors lead to an exaggerated erythrocyte volume and lower

cytosol hemoglobin concentration, the veracity of the concentration-based estimate of the hemolysate dilution ratio is supported.

3.1.3. Separation of free heme from hemoglobin

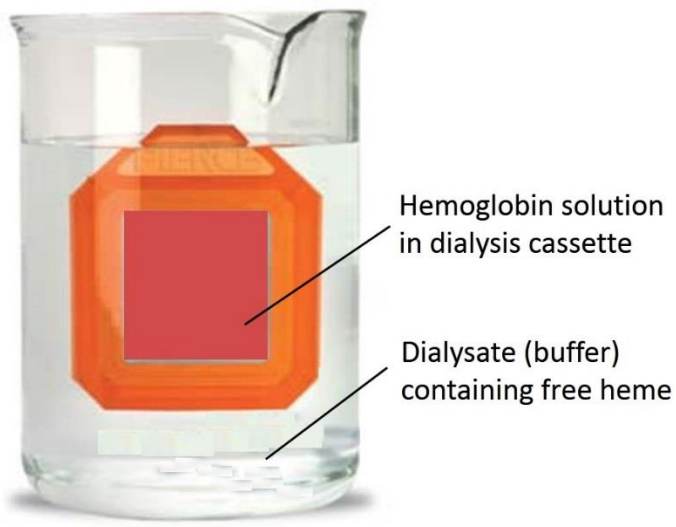

Figure 3.3 Separation of free heme from hemoglobin solution.

We placed 3.00 mL of hemolysate into a dialysis cassette (Slide-A-Lyser from Thermo Scientific with a 2,000 g mol^{-1} molecular weight cut off and 0.5 to 3.0 mL capacity) with a syringe. We inserted another syringe on the other side of the dialysis cassette to remove the compressed air. We suspended the cassette in a beaker containing 1.000 L (measured by a 1L volumetric flask) of 100 mM Tris (tris(hydroxyl-methyl)-aminomethane) buffer at pH = 8.4. We sealed the beaker with parafilm, placed it on a stir plate in the refrigerator at ~5^{o}C, and agitated the outside solution with a 2 inch stirring bar. The duration of the dialysis was from 14 hours (overnight) to 14 days. We took samples of the dialysate and determined the concentration of free heme in them as discussed below. We calculated the concentration of free heme in the hemolysate from the ratio between the

volume of the solutions in the outside beaker and the dialysis cassette. Typically, the latter ratio was 333×, leading to a dilution ratio of 334×.

3.1.4. Measurement of free heme in dialysate solution

For experimental statistics, we used 96 well plates and filled each column of eight wells with identical solutions. Six of the columns were filled with solutions of known hemin concentration in the same buffer as the one used in the dialysis of the hemolysate and the other six—with samples of dialysate produced as discussed above. The six known concentrations were distributed in a range that bracketed the unknown dialysate concentration. The volume of standard solution added to each well varied from 0 to 40 μL so that the final heme concentrations was from 0 to 600 pM. For experimental statistics, a tested solution was divided into two, four, or six samples and each sample subdivided into the wells of one eight-well column. We added 40 μL of the tested solution to each well.

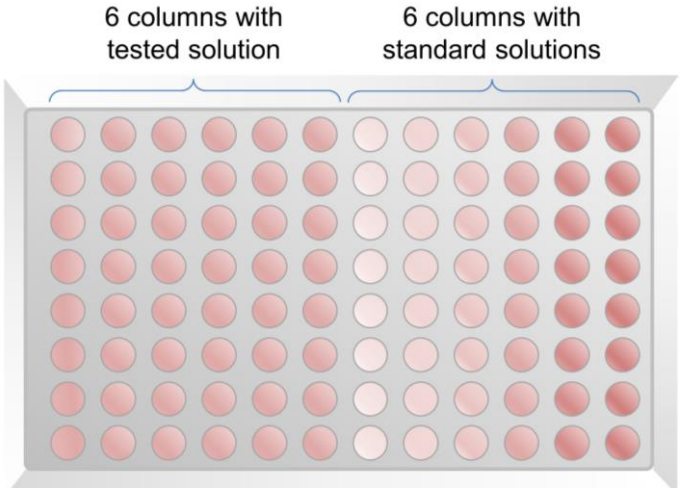

Figure 3.4 Schematic of the loading of the plate with the samples of the tested and six standard solutions.

After we loaded the heme solutions, we added Tris buffer to each well in volume calculated to compensate the different heme solution volumes. Then we added 50 μL of apo-HRP stock solution yielding a concentration of 600 pM in each well. We incubated the microplate for 30 minutes at room temperature, ~23°C, to reconstitute the active HRP [183]. The rate constant for heme binding to 35 different apoglobins is approximately $k_H = 1\times10^8$ $M^{-1}s^{-1}$, regardless of the structure or overall affinity of the apoprotein for iron-porphyrin.[191] We assume that this value applies for apoHRP. With $C_{apoHRP} \approx C_{heme}$ = 200 pM, the half-time for hematin and hemin binding to apo-HRP is, $t_{1/2} \cong (k_H C_{heme})^{-1}$ = 50 s. Thus, 30 min is sufficient time for quantitative binding of free heme to apoHRP. We added 250 μL Luminol + 250 μL H_2O_2 to 3500 μL Tris buffer and after 30 min reconstitution period added this 16 times diluted 20 μL Immobilon to the wells. The final solution volume in each well was 120 μL.

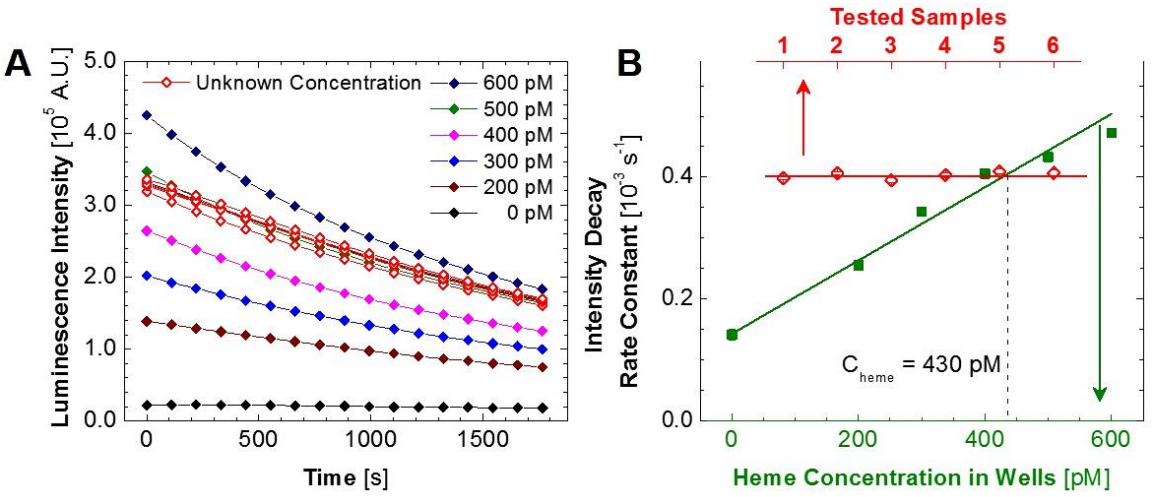

Figure 3.5 A. Luminescence intensity evolution for heme-reconstituted HRP-catalyzed luminol-H2O2 reaction. B. Determination of unknown heme concentration from standard curve using rate constants of the HRP-catalyzed reaction.

We incubated the microplate for 15 minutes at room temperature, ~23°C, and loaded it into the microplate reader. We monitored the evolution of the intensity of the luminescence emitted from each well over 30 min. For each moment of the reaction, the luminescence intensities were averaged over the eight wells with identical composition. An example of the intensity evolution over 30 min for one representative experiment is displayed in Fig. 3.5(A).

3.1.5. Definition of the calibration curve in terms of rate constants

In published implementations of this method, the average luminescence intensity from a tested sample was compared to that from solutions with known concentration at the same reaction time and the unknown concentration was determined by interpolation between the two bracketing values.[183] We developed a new method of determination of the heme concentration based on calibration curves in terms of the rate constant for luminesce intensity decay.

The breakdown of peroxide catalyzed by HRP and other peroxidases is well described by the Michaelis-Menten rate law for the catalytic process [192]

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \overset{k_2}{\rightarrow} E + P \ , \tag{3.1}$$

$$-\frac{dS}{dt} = \frac{k_2 E_0 S}{K_M + S} \qquad \text{where the Michaelis constant} \qquad K_M = \frac{k_{-1} + k_2}{k_1} \ . \tag{3.2}$$

In Eqs. (3.1) and (3.2) *S* denotes the substrate, H_2O_2, and its concentration; *t*, time; *E*, the enzyme, reconstituted HRP; *P*, the product, OH·; *ES*, the enzyme-substrate complex; k_1, k_{-}

$_1$ and k_2 are the respective rate constants, and the subscript 0 indicates initial values. The Michaelis-Menten equation is integrated to

$$\ln\frac{S}{S_0} - \frac{S_0 - S}{K_M} = -\frac{k_2\, E_0}{K_M}\, t \quad , \tag{3.3}$$

Independent determinations of the Michaelis constant K_M for HRP have yielded values from 1.9 to 2.6 mM [193, 194]. The values in this range are significantly higher than the initial concentration of H_2O_2 in the reaction mixture, S_0 = 42 μM, determined from the concentration in the commercial product, see above, and the dilution ratio. We use the inequality $K_M >> (S_0 - S)$ to simplify the integrated form of the Michaelis-Menten expression:

$$S \cong S_0 \exp\left(\frac{-k_2 E_0 t}{K_M}\right) \qquad \text{and} \qquad -\frac{dS}{dt} \cong \frac{k_2 E_0 S_0}{K_M} \exp\left(\frac{-k_2 E_0 t}{K_M}\right) \quad . \tag{3.4}$$

Thus, a pseudo-first order rate law is obtained. Deviations from the exponential decay represented by Eqs. (3.5) are not expected even at long reaction times, when $S \rightarrow 0$ and $(S_0 - S) \rightarrow S_0$: the inequality $K_M >> (S_0 - S)$ is always obeyed.

Since the reaction of OH· with luminol is faster than the preceding kinetic step in Eqs. (3.1), its rate is determined by that of the preceding step. With this, the luminescence intensity I is proportional to the rate of production of OH·, i.e., $-dS/dt$. Thus, the rate constant for the luminescence intensity evolution,

$$k = k_2 E_0 / K_M \quad , \tag{3.5}$$

is proportional to the initial concentration of the enzyme E_0, i.e., to the concentration of heme. Eq. (3.5) demonstrates that k only depends on E_0, the total concentration of

reconstituted HRP. On the other hand, Eq. (3.4) shows that the luminescence intensity also depends on S_0, the initial H_2O_2 concentration. Thus, determinations of the rate constant k are only influenced by inconsistent concentrations of HRP in the reaction mixture, while an additional source of error of the measured intensity is the inconsistent concentration of H_2O_2. Hence we use standard curves in terms of k as a basis for the determination of unknown concentrations of free heme.

The Fig. 3.5(A) shows the decay of luminescence intensity obtained from the plate reader reading. The standard solutions with known concentration (represented with solid symbols) give out luminescence in increasing order of the concentration and the unknown concentrations (represented with open symbols) of the tested samples give out read outs in between the standard solutions. We determine k from the intensity evolution $I(t)$, similar to those plotted in Fig. 3.5(A), employing linear regression analyses of the dependence ln $I(t)$. Using data obtained with known hemin or hematin concentrations, we plot $k(C)$ and use this as a standard curve. For a solution with unknown concentration, we determine k in an identical way and use this standard curve to obtain the concentration of free heme, as illustrated in Fig. 3.5(B).

To calculate the concentration of free heme in the red cells in the analyzed blood sample, we multiply the dilution ratio of the tested dialysate in the plates by the dilution ratios of the dialysate and the hemolysate: $2.5\times334\times350$ mg mL^{-1}/C_{Hb}. With C_{Hb}.= 5.12 mg mL^{-1} for the sample represented 3.5(B), the total dilution ratio is 5.69×10^5 and we obtain that C_{heme} = 24.47 μM in the erythrocytes of the analyzed blood sample. Below, we discuss tests aimed at validation of the method and evaluation of its sensitivity and accuracy.

3.2. Validation of the method

Is heme released during storage of the blood and hemolysate?

The sickle cell blood was kept for about two hours at ~23ºC before analyses. The sickle trait and healthy blood drawn at GCRBC was kept up to four hours at ~23ºC. The healthy blood drawn at UHHC was typically kept at ~23ºC for about 30 min. In a few cases, healthy blood was stored in a laboratory refrigerator for up to four hours. We carried out two tests to judge if significant amount of heme are released in the time between blood collection and analysis, either during storage at room temperature for up to four hours, or in the refrigerator for up to one day. Furthermore, since the dialysis of the hemolysate takes from 10 to 20 hours, we tested if heme is released during this step of the analysis.

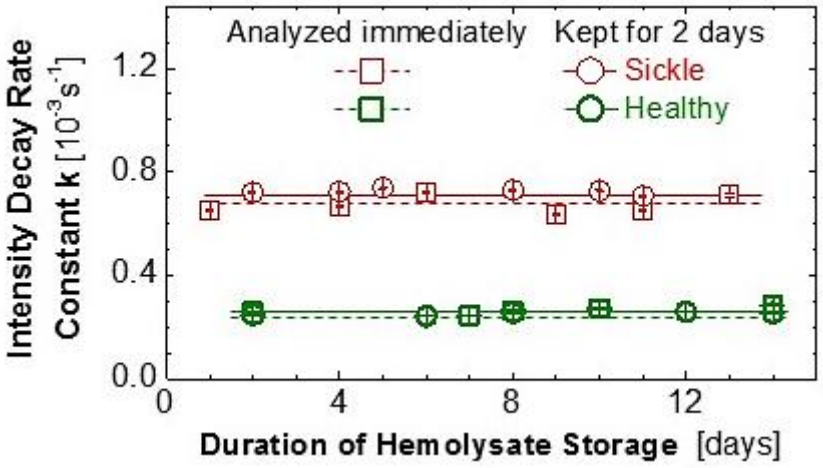

Figure 3.6 Effect of storage on free heme release in sickle and healthy hemolysate.

We carried out three tests with both sickle cell and healthy blood drawn at UHHC. For the first test, we separated the tubes from one drawing into two groups. We analyzed the first group within 30 min of delivery to the laboratory and the second—after four hours. Both groups were kept at room temperature, ~ 23ºC, and were analyzed identically, following the procedure discussed above. We ensured that the dilution ratio of the

hemolysate was similar in both groups. This was verified by measuring the hemoglobin concentration in the hemolysate: it was ~ 2 mg mL^{-1} for both groups. We observed that the decay rate constant *k* of the luminescence intensity was identical in both groups, i.e., storage of the blood at room temperature for up to four hours does not lead to additional amounts of heme released.

We combined the second and third tests. As above, we separated the tubes from one blood drawing into two groups. Blood from the first group of tubes was analyzed immediately upon delivery to the laboratory. The second group of tubes was kept in the laboratory refrigerator at ~5^{o}C for two days. After the red blood cells were lysed, we determined the hemoglobin concentration in the hemolysate. Both hemolysate samples were kept in the refrigerator for 14 days from the respective moments of preparation. At one or two day intervals, we took solution samples, dialyzed them to separate the free heme from the hemoglobin, and then determined the concentration of free heme in the dialysate in terms of the decay rate constant *k* of the luminescence intensity.

The results, displayed in Fig. 3.6, indicate that (i) the rate constant *k* is similar for both groups of tubes, i.e., heme is not released during storage of whole blood at ~5^{o}C for up to two days, and (ii) the rate constant does not change over 14 days of storage of the hemolysate at ~5^{o}C. The results of the three tests demonstrate for both sickle cell and normal blood that heme is not released during storage of blood at ~ 23^{o}C for up to four hours, during storage of blood at ~5^{o}C for up to two days, and during storage of hemolysate at ~5^{o}C for up to 14 days. So storage of whole blood and hemolysate does not affect the free heme determination.

Is heme released during dialysis?

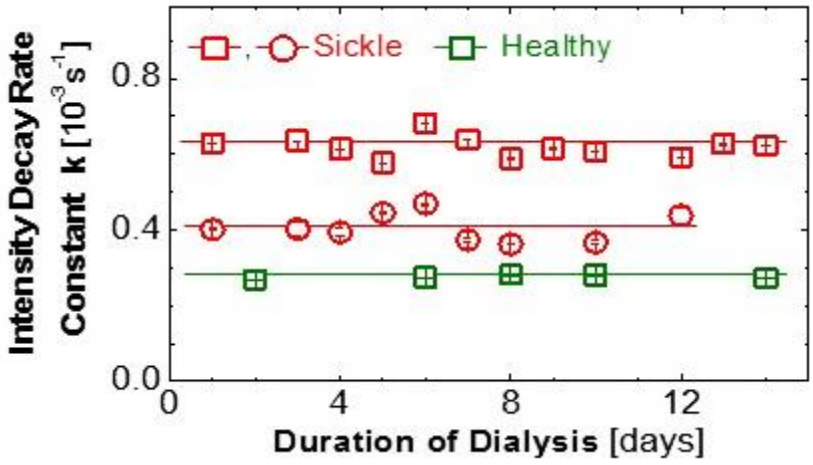

Figure 3.7 Effect of dialysis on free heme release in sickle and healthy hemolysate.

The above tests demonstrate that heme is not released from the hemoglobin in the hemolysate held at ~5°C. However, when we place the hemolysate in a dialysis cassette, the available free heme migrates to the dialysate solution. This lowers the chemical potential of the free heme in the hemolysate and creates a driving force for additional heme release. To test if additional heme is released in the hemolysate held in a dialysis cassette during dialysis, we held sickle hemolysate from two patients and healthy hemolysate from one donor in a dialysis cassette in contact with dialysate for 14 days. At one or two day intervals, we took samples of the dialysate and determined the concentration of heme. Figure 3.7 demonstrates that the concentration of heme in the dialysate was steady over this period, i.e., no new heme is released in the hemolysate during dialysis.

Is free heme present in the red cell cytosol, or is it released upon dilution?

The dissociation of heme depends on the concentration of hemoglobin [195]. The reason for this dependence is that at low concentrations, the native hemoglobin tetramers decay to dimers and the binding of heme to the dimer is weaker [171]. Thus, it is possible that the heme we detect in the hemolysate was not present in the red cell cytosol, but was released after the dilution of the hemoglobin upon cell lysis.

To test if the detected heme is released in the hemolysate, we carried out the following experiment. A freshly prepared hemolysate was kept in a dialysis bag in contact with dialysis buffer for 14 hours. After this time, the dialysate was removed and the concentration was determined using a standard curve measured simultaneously with the sample characterization. A new dialysis buffer was loaded and the bag with the tested hemolysate was placed in contact with that buffer for additional 14 hours. At the end of this period, the concentration of the free heme in the dialysate was determined using a newly measured standard curve (for tests of the reproducibility of the standard curves, see below).

For sickle red cells, we analyzed blood samples from two donors. Three samples of each of the two dialysates were analyzed simultaneously. The results are displayed in Fig. 3.8(B). Using the standard curve measured with the first dialysate, we observe that the free heme concentrations in the dialysate of the two blood samples are unexpectedly close and equal to ~330 pM. Using the second standard curve, we see that the concentrations of the second dialysates are zero, within the uncertainty of the method, for both donors. The results for six samples of a healthy blood dialysate from a single donor

in Fig. 3.8(A) are similar: the concentration of the first dialysate is 278 pM, while that of the second—practically zero.

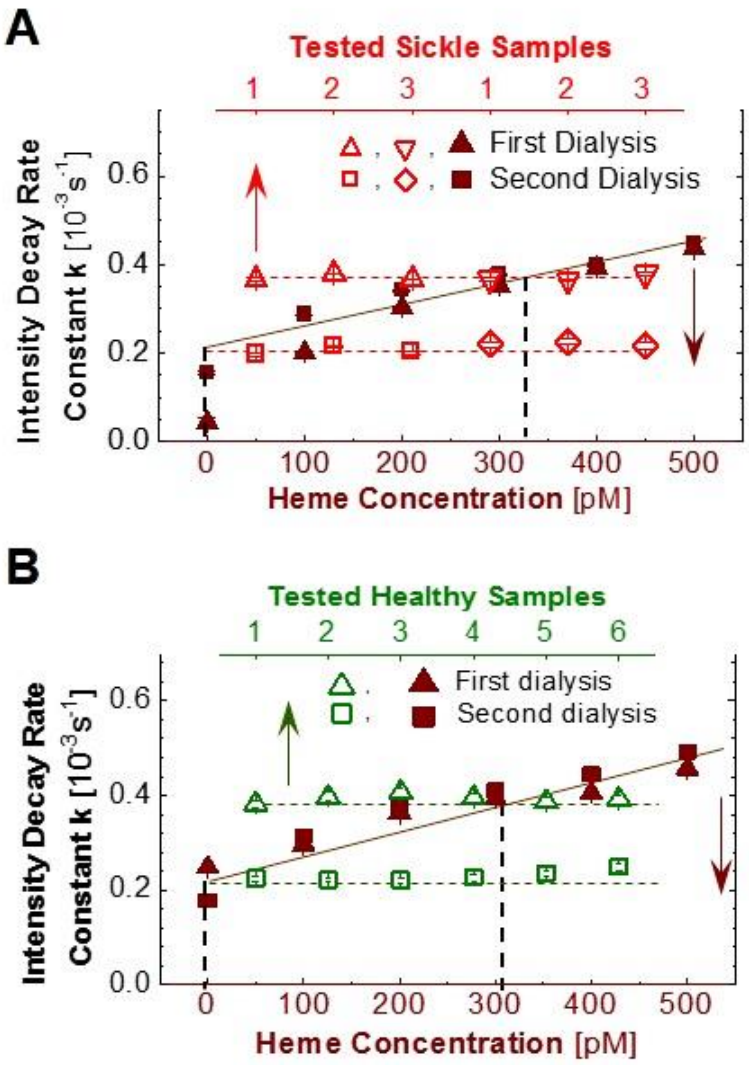

Figure 3.8 Test for increased heme release upon dilution of hemolysate during dialysis.

The lack of free heme in the second dialysates in the three experiments discussed above could either mean that no heme is released in the hemolysate, or that heme is released in the hemolysate, but this release is arrested after the first dialysis. We analyze two processes that may result from the first dialysis and provoke arrest of heme release after it:

(i) dilution of the hemoglobin concentration and (ii) chemical modification of hemoglobin leading to strong heme binding. To exclude (i), we note that we fill the dialysis bag to its capacity with 3 mL of hemolysate. Thus, the hemolysate is not diluted after the first day of dialysis. Option (ii) is also unlikely: as Fig. 3.6 demonstrates, if the hemolysate is stored in a container that preserves the concentration of the free heme present in it, this heme readily transfers to the dialysate even after 14 days of storage. Thus, no changes in the hemoglobin that could lead to stronger heme binding occur. Excluding options (i) and (ii) indicates that a scenario of limited duration heme release is implausible.

We conclude that the release of heme is arrested in the hemolysate and all heme found after dialysis of the hemolysate comes from the erythrocyte cytosol. The reason why heme release is arrested is a separate and very intriguing question. The reason underlies in the mechanism of the heme release which is hypothesized and explained later sections.

Is heme bound to the erythrocyte membranes released in the hemolysate?

Hematin released from hemoglobin has been found bound to the membranes of both sickle and healthy red cells [196]. Even though the detachment of hematin from the membranes for these analyses required the application of saponin [196], it is possible that the heme that we detect does not come from the erythrocyte cytosol, but detaches from the membranes upon cell lysis in a large volume of water. We carried out two tests of this possibility:

(i) We lysed the red blood cells from one donation in two volumes of water: 5 and 50 mL. If hematin detaches from the membranes due to its low concentration in the hemolysate, we would expect higher heme amounts in

the diluted hemolysate. We found that the amount of heme did not depend on the hemolysate dilution.

(ii) We extended from 2 min (the typical length) to 30 min the time between cell lysis and centrifugation applied to remove the red cell membranes. If heme were released from the membranes, we would expect higher heme concentrations in the hemolysate that is kept in contact with the membrane ghosts for an extended period. We found that the concentration of the heme in the two ghostless hemolysates was similar.

The tubes for blood collection

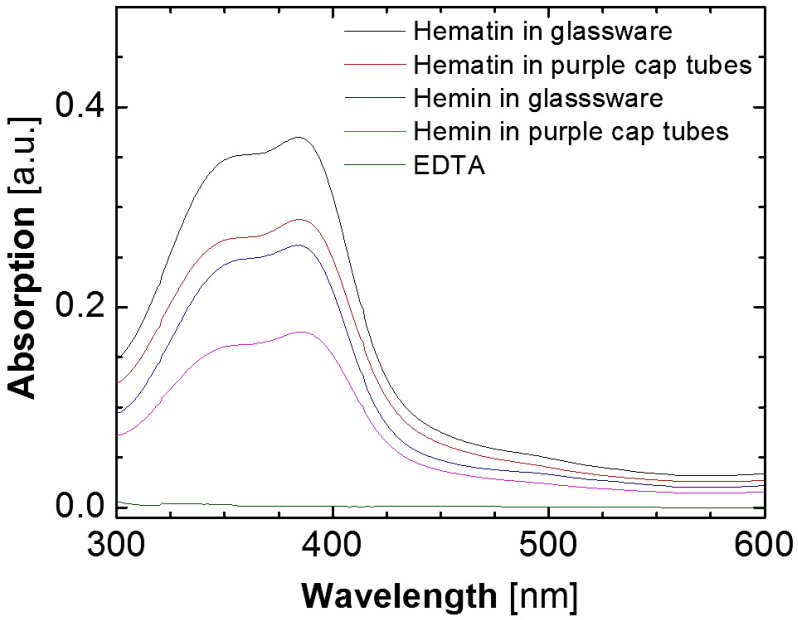

Figure 3.9 Comparison of the UV-Vis-spectra of hematin solutions held in laboratory glassware and purple cap blood collection tubes.

We collect the blood in tubes containing anticoagulants, which allow easy isolation of the erythrocytes. We considered three types of tubes: with green caps, containing sodium heparin, with purple caps, containing ethylenediaminetetraacetic acid (EDTA), and with

grey caps, containing fluoride and oxalate. The other types of anticoagulant tubes contain the same reagents at different concentrations or reagent combinations. To test if these anticoagulants interfere with the determination of free heme, we tested their effects on hematin and hemin dissolved in Tris buffer at pH = 8.4. For this, we filled the tubes with hematin or hemin solutions at concentrations ~ 20 μM, flipped the tubes to dissolve the anticoagulant and kept the solution in them for 30 min. We used Beckman DU-800 spectrophotometer to obtain the spectra from the solutions kept in the tubes.

We compared the spectra of the solution held in the tubes to that of identical solutions kept in the preparation containers. Hematin concentration was 20 μM, that of hemin—14 μM. The spectrum of 8 mM EDTA, similar to the amount in the tubes, 7.2 mg in 4.0 mL, is also shown. We observed that the spectra of hematin and hemin solutions held in green and grey cap tubes were essentially undistinguishable from those of solution kept in laboratory glassware. However, Fig. 3.9 demonstrates that in purple cap tubes, in the presence of EDTA, the absorbance of both hematin and hemin is significantly reduced in the entire wavelength range, indicating that the concentration is reduced. A hypothetical mechanism of this reduction is through the chelation of Fe^{3+} by EDTA. These observations suggest that EDTA may interfere with the determination of free heme in the red cell cytosol; hence, we avoided using purple cap tubes and only used those with green and grey caps.

Accuracy and sensitivity of the method

The accuracy and sensitivity of the enzymatic method of quantification of free heme can be evaluated from the reproducibility of the standard curves, such as those in Fig. 3.5(B). We plot in Fig. 3.10 eight standard curves. We see that for hemin concentrations

≥ 200 pM, the relative error of the standard curves is ± (12 – 15)%. The main source of uncertainty is the inaccurate preparation of the solutions of H_2O_2, luminol, and apoHRP, loaded in the wells: it involves several steps, in which we pipette small volumes. To minimize this uncertainty, we determine a standard curve for every analyzed sample. In this way, the standard and the tested solutions are prepared with identical reagent concentrations. With this, the error of the determination of the concentrations of free heme in the dialysate is estimated as 5 %.

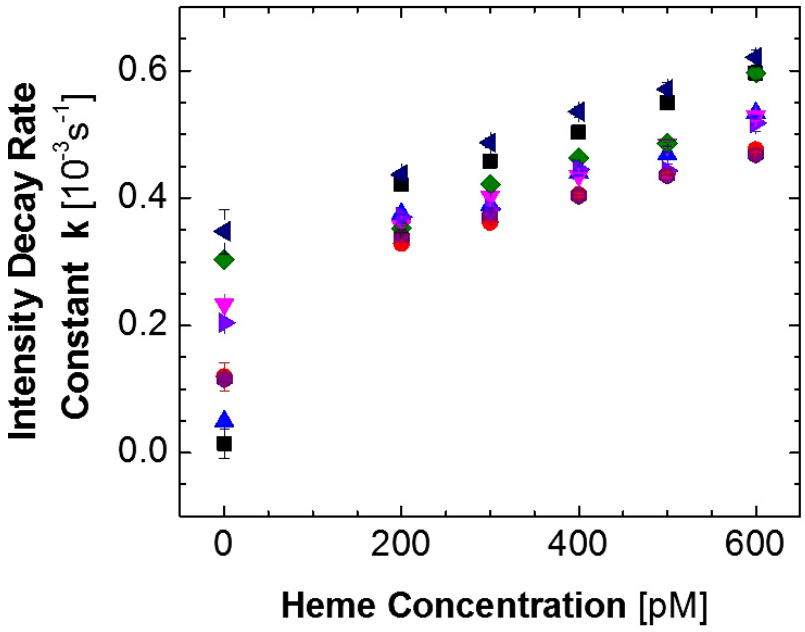

Figure 3.10 Test of accuracy and sensitivity of standard curves.

The sensitivity of the method, i.e., the lowest amount of free heme that can be accurately detected and measured, is determined by the standard value of the intensity decay rate constant *k* at C_{heme} = 0 pM. As Fig. 3.10 demonstrates, this value varies from zero to the point, at which the standard data at non-zero heme concentrations intercept the *k* axis. Values higher than the intercept point were never recorded. If the value of the standard curve at C_{heme} = 0 pM is lower than the intercept point, the lowest value that can

be determined by this method is that of the lowest standard concentration that belongs to the linear $k(C_{heme})$ dependence. If $k(C_{heme} = 0)$ is a part of the linear dependence, the sensitivity is determined by the combination of the uncertainties of the determinations k and C_{heme}. In all cases, in which we measured low hematin concentrations, we used standard curves of the latter type. With this, the sensitivity of the method varies from 20 to 50 pM. Thus the sensitivity and accuracy of this method surpasses any current method that is available for heme determination in solution through spectroscopic measurements.

The apo-HRP preparation

The standard curves, such as those in Fig. 3.10, do not extrapolate to $k = 0$ at $C_{heme} = 0$. Similar dependencies that do not extrapolate to the coordinate origin were reported in other paper on the enzymatic method of heme determination.[183] Two feasible explanations are: (i) the apo-HRP preparation that we use contains residual amounts of holo-HRP that catalyze the decomposition of H_2O_2 even in the absence of added heme, and (ii) H_2O_2 decomposes even in the absence of HRP and the rate of this decay is reflected in the intercept.

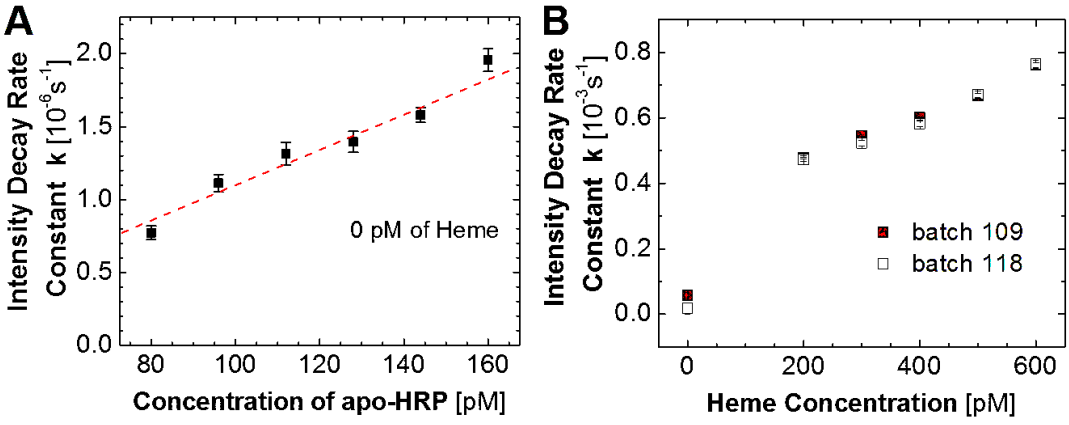

Figure 3.11 Test for (A) residual apoHRP activity and (B) consistency of different apoHRP batches.

To test the combined effect of both processes, we estimated the catalytic activity of apo-HRP in the absence of free heme. We intentionally chose relatively low apoHRP concentrations, from 80 to 160 pM, in which we determined the rate of decay of H_2O_2. The results in Fig. 3.11(A) indicate that apoHRP has a detectable catalytic activity. To evaluate this activity at the concentration used in the heme determination runs, we extrapolate the value of k from Fig. 3.11(A) to 600 pM and obtain $k \approx 7.5\times10^{-6}$. This value of k is (50 – 100)× lower than the value for 600 pM of hemin Fig. 3.10. This ratio suggests that the apoHRP preparation that we use contains about (1 – 2)% of residual holo-enzyme.

Figure 3.11(A) demonstrates that the $k(C_{apoHRP})$ dependence extrapolates to $k = 0$ at $C_{apoHRP} = 0$. This extrapolation suggests that the spontaneous decay of H_2O_2 in the absence of enzyme is an insignificant contributor to the kinetics that we monitor. To test if the quality of apoHRP is consistent, we compare in Fig. 3.11(B) the decay rate constant for the luminescence intensity measured in solutions with known hemin concentrations using apo-HRP from two different production batches, 109 and 118, from the same manufacturer. The two standard curves are very close, indicating that the activities of the apo-HRP preparations from the two batches are similar.

The results in Fig. 3.11 suggest that the standard curves do not extrapolate to zero at low hemin concentrations not because of spontaneous decomposition of H_2O_2 and that the contribution of holo-HRP, present in the apoHRP preparation is less than (1 – 2)%. Comparing the value of k at C_{apoHRP} = 600 pM extrapolated from the low concentration data in Fig. 3.11(A) to the values at the same C_{apoHRP} = 600 pM from the standard curves, e.g., in Fig. 3.5(B), we see that in some cases they are close, while in other cases they differ by orders of magnitude. The inconsistency of the peroxide decay rate recorded in pure

apoHRP suggests a non-linear kinetic response, leading to irreproducible data. However, in the presence of any amount of added hemin or hematin, this response is maximized and consistent and it does not affect the signal from the added heme.

Does heme unbound to apo-HRP affect the determination?

It is possible that a fraction of the free heme in the tested solutions may remain unbound to apo-HRP and independently contribute to the decomposition of H_2O_2 and the luminescence intensity.[185] If the distribution of the heme between the bound and unbound states is inconsistent, equal total heme concentrations might elicit different intensities. Thus, the distribution of free heme between bound and unbound states may lead to an error in the determination of the total hematin concentration by this method. To evaluate the magnitude of this error, we determined the catalytic activity of hematin in H_2O_2 decomposition in the absence of apo-HRP, using the method discussed above.

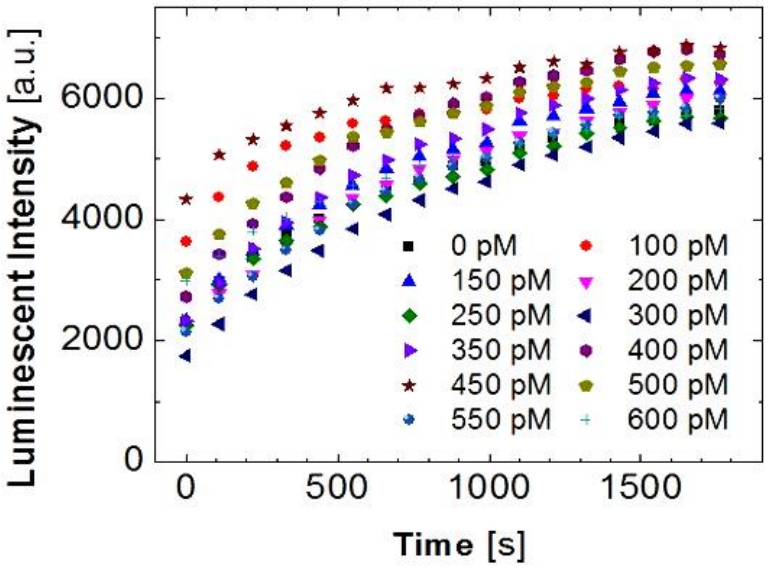

Figure 3.12 Evolution of the luminescence intensity emitted from solutions with hematin concentrations as indicated in the plot in the absence of apo HRP.

Figure 3.12 demonstrates that: (i) the luminescence intensity is approximately two orders of magnitude lower than in the presence of apo-HRP, compare to Fig. 3.5(A); (ii) it does not decrease, as one would expect if the concentration of H_2O_2 decreased as a result of its decay; and (iii) this intensity changes randomly in response to the increasing concentration of hematin, e.g., in Fig. 3.13, the highest intensity is recorded at C_{hematin} = 450 pM and the lowest—at C_{hematin} = 300 pM. We conclude that the data in Fig. 3.13 do not correspond to hematin-catalyzed decay of H_2O_2, but rather to a fluctuating reading within the error range of the intensity measurement that may be due to electronic or other noise. The possible reason for the lack of activity of hematin in these solutions is the relatively low pH = 8.4 in our determinations—hematin activity has been demonstrated at more basic pH's.[184, 185] We conclude that the error in the determination of the hematin concentration by the enzymatic method that may be introduced by its incomplete binding to apoHRP is < 1%.

Does hemoglobin leak into the dialysate?

A disadvantage of the luminol reaction, used for quantification of the heme concentration, is that it has very low selectivity for iron containing compounds.[185] Thus, if hemoglobin leaks through the dialysis membrane due to a defect, it may contribute to the total luminescence intensity and in this way bias the quantification of free heme.

To evaluate the possible error due to leaking of hemoglobin from the dialysis bag, we determined the catalytic activity of healthy hemolysate in which the hemoglobin concentration was 100 pM and compared it to the catalytic activity of 400 pM hematin, in which the total heme concentration is equal to that in the hemoglobin solution. We characterized the activity in terms of the rate constant for decay of the luminescence

intensity. Since our goal was to simulate the leak of hemoglobin into the dialysate, we determined the activity of hemoglobin in the presence of apoHRP.

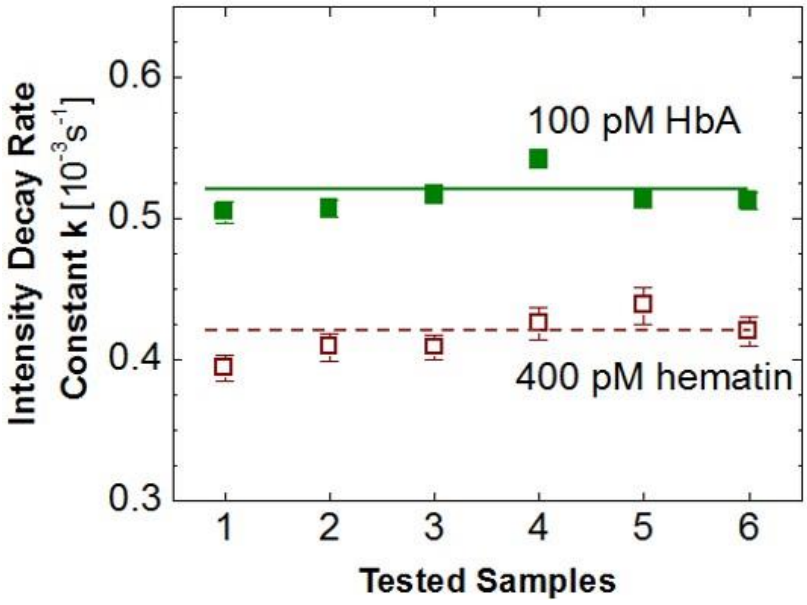

Figure 3.13 Comparison of the activity of normal hemoglobin and hematin at the concentrations indicted in the plot.

Fig. 3.13 demonstrates that the activity of hemoglobin is ~ 25% higher than the activity of an equivalent concentration of hematin. Thus, if hemoglobin leaked it would contribute significantly to the intensity of the luminescence light and to the rate of peroxide decomposition. On the other hand, it is highly unlikely that such leaks in independent dialysis runs would be identical. Hemoglobin leakage would lead to inconsistent values of *k*. Since the data presented above reveal consistent *k* values, we conclude that hemoglobin leakage did not significantly contribute to the values of *k* used for the quantification of free heme concentration.

Standard curves with hematin and hemin

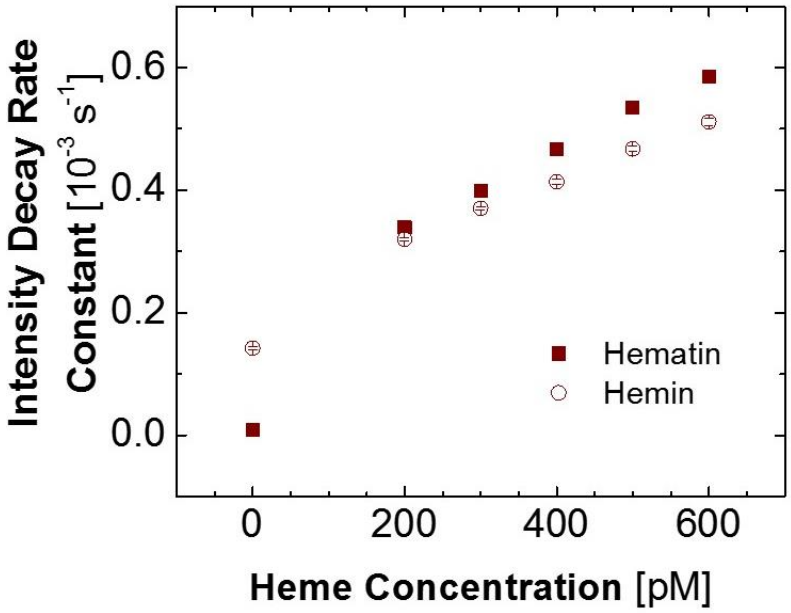

Figure 3.14 Standard curve consistency with both hemin and hematin.

In Fig. 3.14 we compare standard curves determined with hematin and hemin. We see that the difference between the two curves is lower than the variability of the standard curves for hemin. Thus it can safely be assumed that the method is suitable for both the form of heme present in the solution to be determined.

In summary, we successfully validated our method for determination of heme concentration in ways where issues and questions regarding the source of free heme in hemolysate, its time delay related evolution scenario, method sensitivity and reactivity from apo-HRP have been addressed. Looking at the discussion of results of the experiments performed to validate the method, we can say that the measurement procedure is robust and the results measured with this procedure can be trusted with scientific accuracy.

CHAPTER

4

FREE HEME CONCENTRATION IN HEALTHY, SICKLE TRAIT & SICKLE CELL ERYTHROCYTE CYTOSOL

Using the enzymatic method described in Chapter 3, we determined the average concentrations of free heme in the erythrocytes of seven sickle cell patients, three sickle trait donors, and five healthy donors. From the results presented in Fig. 4.1(A), the average of sickle cell patients is 44±9 μM, sickle trait donors, 33±2 μM, and healthy donors, 20±2 μM (to evaluate the dilution of the hemolysate, we used that the average total hemoglobin concentration in the three types of erythrocytes is 350 mg ml^{-1}).

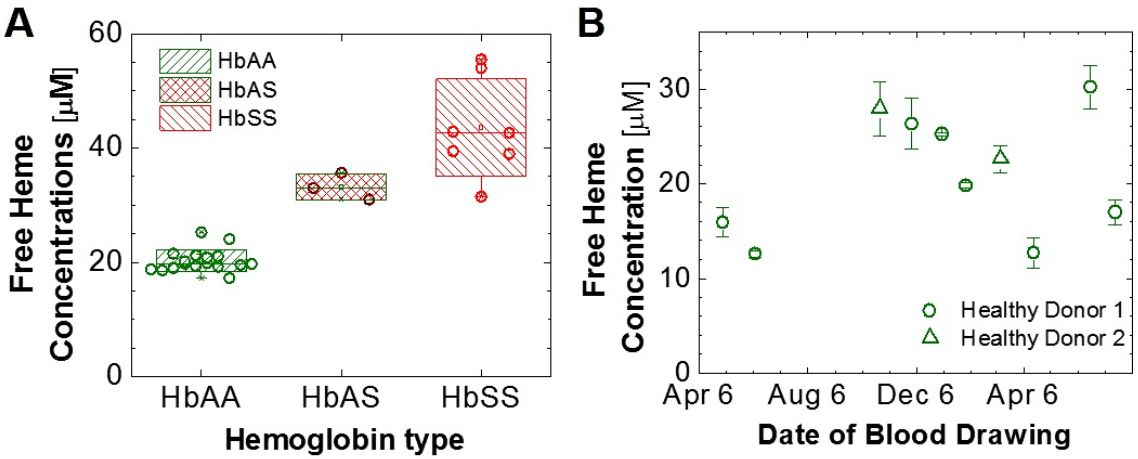

Figure 4.1 A. The average concentrations of free heme in blood samples of sickle cell patients, sickle trait individuals, and healthy donors. B. The variation in time of the average free heme concentration of healthy donors 1 and 2.

In figure 4.1, the bottom and top line of the boxes represent first and third quartile of the samples respectively and the horizontal line in the middle shows the median value for the samples. We performed statistical significance tests using Analysis of Variance

(ANOVA-single factor) in Microsoft Excel on the pairs of groups of heme concentrations: HbAA-HbSS, HbAA-HbAS and HbAS-HbSS and the p-values obtained are 2.52×10^{-10}, 5.89×10^{-9} and 0.079 respectively. The null hypothesis was that the average of the heme concentrations in those pairs would have similar values. The p-values (< 0.05) show that there is significant difference in the heme concentration averages of HbAA (healthy donors) with HbSS (sickle cell patients) and HbAS (sickle cell trait individual), while similar values are expected in HbAS and HbSS individuals (p-value > 0.05). More donors of sickle trait should be part of the study to provide clearer picture of the scenario.

Nonetheless, it is possible the match between HbAS and HbSS heme concentrations can be related to sickle hemoglobin mutations. Though sickle trait individuals do not normally show symptoms of sickle cell disease, high rate of sudden deaths among these group of people under strenuous physical training are questioned [197, 198]. The analysis of such similarity in intraerythrocytic free heme concentration values of HbAS and HbSS individuals may facilitate understanding of pathogenesis of recently identified acute and severe life-threatening pathological conditions like exercise related sudden death, acute chest syndrome, renal dysfunctions, venous thromboembolism and other complications in sickle trait individuals [199-204]. Though sickle trait health complications are intriguing questions that need to be answered, in this work we will limit our discussions only on results comparing healthy donor and sickle cell patients.

The p-values show that there is significant difference in average intraerythrocytic free heme concentrations between healthy donors and sickle cell patients. Based on the higher autoxidation rate of hemoglobin in sickle cell patients [8], it is expected to have higher average free heme concentrations in red cell cytosol. The variation between

individual donors is significantly higher for sickle cell patients than for healthy and sickle trait donors. Figure 4.1(B) demonstrates that the free heme concentration in two healthy donors varies in time from 13 to 30 μM. This variation may be due to environmental and physiological factors. The time average is 21 μM, very close to the average over seventeen donors. The population averages and the individual concentrations in Fig. 4.1 are greater by about 100 × than the values found in Liu *et al* [9]. To resolve the reasons for the discrepancy we used several strategies:

1. We investigated to find out if the heme measurement procedure used in the previous determination had something which may have interfered with correct determination of the concentration and underestimated the values.
2. We tried to find out the aggregation states in which the free heme molecules prevail in the biophysical conditions and also looked at the equilibrium reactions that are supposed to drive the hemoglobin-methemoglobin-heme homeostasis in the physiological conditions;
3. We considered the possibility of a non-equilibrium process that may be involved in the physiological scenario and may provide the answer for the high concentration of free heme found in our determinations.

4.1. Comparison with the previous determination

There is a single previous determination of the concentration of free heme in sickle and healthy erythrocytes,[9] which found concentrations of, respectively, 0.75 and 0.15 μM. The concentration found in healthy adult erythrocytes is more than two orders of magnitude lower than the one in Fig. 4.1(A). Liu *et al*. separated free heme from hemoglobin by charge, using ion exchange liquid chromatography,[9] in contrast to the

separation by size using dialyses, employed by us. To isolate the hematin, Liu *et al*. added 2 M NaCl to the hemolysates. At this electrolyte concentration, the hematin was retained on the column. The captured hematin was then eluted by a solution of sodium dodecyl sulfate (SDS). To validate the method, Liu *et al*. added hematin to the hemolysate and retrieved 75% of the added amount after the SDS elution.

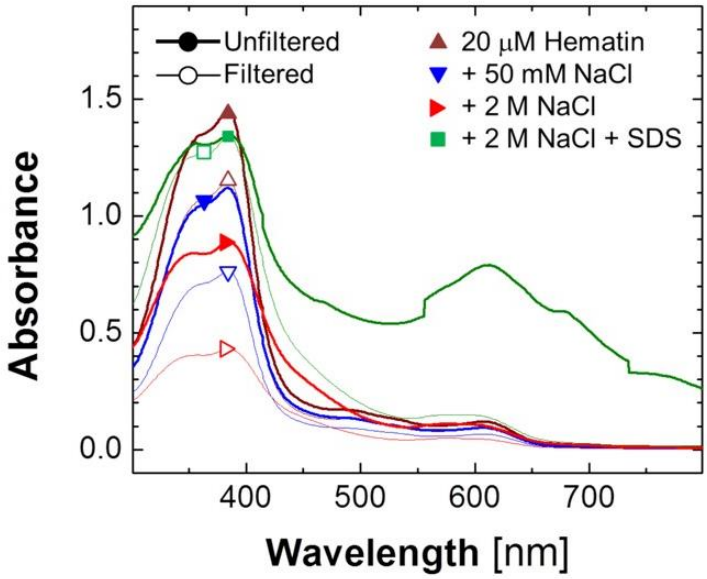

Figure 4.2 The effects of NaCl addition on the free heme in solution – spectral observation.

To understand the effect of NaCl to the state of hematin in solution, we added 50 mM or 2 M NaCl to a 20 μM solution of hematin in 0.15 M phosphate buffer. To test for hematin aggregation or crystallization, we filtered these solutions through a 0.22 μm filter and compared the UV-Vis spectrum of filtered and unfiltered solutions. Since hematin dissolution in aqueous solvents is slow [205], we expect the presence of undissolved aggregates in the initial solution. The spectra in Fig. 4.2 confirm this aggregation: the solution is turbid, judging from the absorbance at wavelengths greater than 700 nm, and

filtration leads to reduction in absorbance. The spectrum of the filtered initial hematin solution corresponds to those in the literature [7].

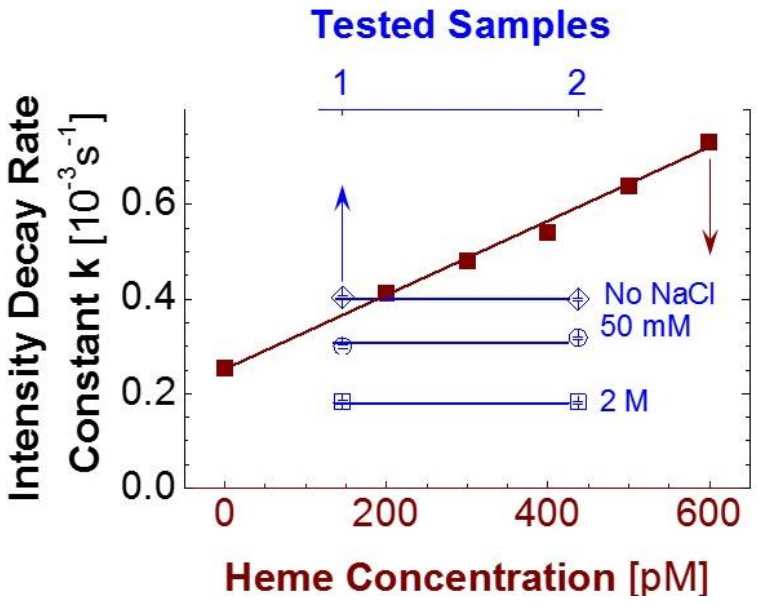

Figure 4.3 The effects of NaCl addition on the free heme determination in hemolysate.

The addition of 50 mM or 2 M NaCl to an unfiltered hematin solution lowers the absorbance at all wavelengths. The turbidity in the respective spectra in Fig. 4.2 is removed by filtration, indicating that it is due to hematin aggregation. Microscopic observation of the unfiltered solutions revealed the presence of brownish clusters that are, likely, precipitated hematin. Comparing the spectra of filtered solution before and after the additions 50 mM and 2 M NaCl reveals that of the additions remove, respectively, ca. 80 % and nearly 100 % of the dissolved hematin.

The addition of SDS to unfiltered solutions holding 2 M NaCl fully solubilizes the precipitate (note the disappearance of turbidity) and likely some undissolved hematin and the optical absorbance increases to levels above those of the initial solution. The dissolved hematin is likely held in SDS micelles. Filtering the SDS containing solution increases its

turbidity, likely due to micelle flocculation. These observations suggest that the hematin added for calibration by Liu *et al*.[9] was precipitated, retained as a solid at the top of the column, and subsequently dissolved and eluted by SDS for nearly complete recovery. This conclusion casts doubt on the method calibration employed by Liu *et al* [9].

To test if the presence of NaCl may affect the release of heme from hemoglobin, we added 50 mM or 2M NaCl to hemolysate samples. In this way, we reproduced the conditions of the determination of Liu *et al* [9]. Figure 4.3 displays the determination of the concentration of free heme in these solutions; to preserve the NaCl concentrations during the determinations, NaCl was added to the dialysis buffer at the same respective concentrations. Fig. 8B demonstrates that the addition of NaCl arrests the release of heme from the hemoglobin. Microscopic observation of the hemolysate extracted from the dialysis cassette revealed the absence of solid residue. Thus, the hematin in stoichiometric ratio to apoglobin was not precipitated by NaCl, but likely driven back to the apoglobin due to its increased chemical potential. The reattached heme cannot be separated from hemoglobin, leading to the low amounts of free heme found by Liu *et al* [9].

In summary: we have effectively shown that the previous determination actually underestimated the concentration of free hematin that is present in human erythrocytes.

4.2. Aggregation states of hematin in red cell cytosol

The hematin present in the erythrocytes can be in the form of monomers, dimers, higher oligomers, and crystalline or amorphous aggregates. The solubility of hematin with respect to crystals has not been measured, but is expected to be low, probably in the micromolar range [206], i.e., lower than the concentrations measured in Figs. 2a and b. To test if hematin concentrations higher than the solubility lead to crystal formation and

growth, we dissolved hematin at 1.5 mM at pH = 13, at which it is monomeric and its solubility is several mM [207], and slowly, over two hours, added phosphoric acid to pH = 7.4; this procedure mimics the release of monomeric hematin from hemoglobin. One aliquot of this solution was placed in contact with hematin crystals from the commercial preparation; another one was stored as prepared. After removal of the newly formed or exogenous solid phases by filtration, we determined the hematin concentrations and found that they are steady for 78 days, Fig. 2c, implying that crystallization does not proceed in the seeded and unseeded solutions. These observations indicate that both nucleation and growth hematin crystals are extremely slow and hematin solutions retain steady concentrations even if they are higher than the solubility.

Hematin is known to form dimers bound by π–π stacking interactions [207]. In solutions similar to the red cell cytosol, other types of dimers and higher hematin oligomers are not expected [207]. To judge about the presence hematin oligomers or larger aggregates in hematin solutions of concentration similar to that in the erythrocytes cytosol, we characterized by dynamic light scattering (DLS) a 55 μM solution of hematin at pH 7.4 in 0.15 M phosphate buffer. The detailed procedure and results are discussed in the following section.

The dynamic light scattering and aggregation state of hematin

We filtered each hematin solution through 0.22 μm Millipore filter and loaded it into a cylindrical cuvette with 1 cm internal diameter. We mounted the cuvette in the goniometer of the ALV-5000/EPP Multiple tau Digital Correlator (ALV-GmbH, Germany), which uses a 35 mW He-Ne laser with a wavelength of 632.8 nm. We recorded the intensity of the light scattered at 90°. The intensity correlation function, $g_2(\tau)$, where τ

is the delay time, was recorded every 60 sec for a total of 30 min and the individual correlation functions were averaged. The intensity distribution function $G(\tau)$ was calculated from the average $g_2(\tau)$ using the standard CONTIN algorithm [208]. For further details of the procedure, see the prior study by Pan *et al* [209].

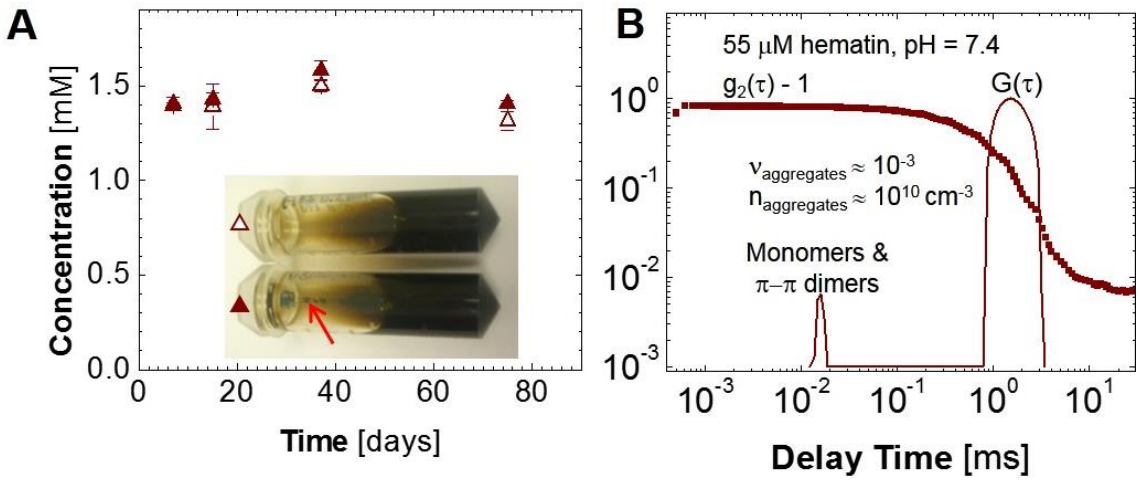

Figure 4.4 Dynamic light scattering (DLS) characterization of aggregation states of hematin in solution of physiological relevance.

The results of DLS analysis in Fig. 4.4(B) reveal the presence of small scatterers diffusing with characteristic time τ_1 from 13 to 16 μs and larger entities diffusing with $\tau_2 \approx 1$ ms. The diffusion coefficient D_1 of the small scatterers is in the range $D_1 = (q^2\tau_1)^{-1} = (2.17 - 1.66)\times10^{-10}$ m^2s^{-1} ($q = 4\pi n/\lambda \sin(\theta/2) = 1.88\times10^7$ m^{-1} is the scattering vector: $n = 1.33$ is the refractive index of the buffer; $\lambda = 628$ nm is the wavelength of the used laser; and $\theta = 90^o$ is the scattering angle). The diffusion coefficients of the hematin monomer and π–π dimer, determined in solutions with viscosity $\eta = 0.99\times10^{-3}$ Pa s, are 1.9×10^{-10} m^2s^{-1} and 1.2×10^{-10} m^2s^{-1}, respectively [207]. Correcting for the viscosity of the buffer used in the determinations in Fig. 4.4, $\eta = 1.05\times10^{-3}$ Pa s [209], the diffusion coefficient range of the small scatterers in Fig. 4.4(B) corresponds to a mixture of monomers and

dimers. Since no aprotic co-solvents, needed for the formation of other hematin dimer structures [210], see discussion below, are present in this solution, we conclude that the dimers contributing to the light scattering signal are π–π dimers. Concurrently with De Villiers *et al.* [207], the DLS data contain no signal from oligomers larger than that π–π dimers.

The size of the two scatterers was determined using the Einstein-Stokes relation modified with $\tau_i^{-1} = D_i q^2$, which yields for the hydrodynamic radius R_i (i = 1,2 denotes small and large scatterers, respectively)

$$R_i = \frac{k_B T\, q^2}{6\pi\eta}\tau_i \qquad , \tag{4.1}$$

where k_B is the Boltzmann constant, and T is the absolute temperature. The resulting average radius R_2 of the large scatterers is ~80 nm: these entities are likely hematin aggregates. In view of the finding of Fig. 4.4(A), of slow crystallization in this solution, they are likely unstructured; hematin is known to form amorphous aggregates [211]. The average size of the monomer/dimer mixture is R_1 = 1.1 nm.

To evaluate the fraction of hematin occluded in the aggregates, we compare the total intensity scattered by the aggregates to that scattered by the monomers and dimers. We evaluate the ratio of the intensities scattered by the 80 nm aggregates and the monomers from the ratio of the areas of the respective peaks A_2/A_1 in Fig. 4.4(B), 600:1. The fraction of hematin in the aggregates $\nu_{aggregates}$ is approximately equal to the ratio of the volume fractions of the aggregates and monomers, φ_2/φ_1 [212]. Assuming that aggregates exhibit similar density and refractive index as the monomer and that the monomers do not interact, from the relation for φ_2/φ_1 [209], we get

$$\nu_{aggregates} \approx \frac{\varphi_2}{\varphi_1} = \frac{A_2}{A_1}\left(\frac{R_1}{R_2}\right)^3 \quad , \tag{4.2}$$

where the ratio $(R_1/R_2)^3$ stems from the Rayleigh's law, according to which the scattered intensity is proportional to the sixth power of the particle radius. We obtain that $\nu_{aggregates}$ ≈ 0.1%. From the ratios φ_2/φ_1 and R_2/R_1, we compute the number concentration of large aggregates $n_{aggregates} \approx 10^{10}$ cm^{-3} [209]. If the aggregation state of hematin in the red cells is similar to the one in the solution analyzed in Fig. 4.4(B), with the volume of a red blood cell ~90×10^{-12} cm^3, there should be approximately one such aggregate per erythrocyte. The low values of $\nu_{aggregates}$ and $n_{aggregates}$ suggest that presence of the aggregates may be of insignificant consequence. Therefore, we conclude that physiological environment the major form of heme is not in form of crystals or amorphous aggregates, rather they are present possibly in the form of dimers.

4.3. The equilibrium amount of free heme

To understand the factors that may have resulted in the high concentration of free heme in Fig. 4.1(A), we first evaluate the equilibrium concentration of free heme in a solution similar to the erythrocyte cytosol. The mechanism and the relevant kinetic and thermodynamic parameters of dissociation of heme from normal hemoglobin and the reverse reaction, the association of heme to apoglobin, are well studied.[170, 171, 191] The main conclusions of these and other works are:

(i) The dissociation of heme from ferrous hemoglobin (Hb(Fe^{+2})) is extremely slow; heme mostly dissociates from methemoglobin (oxidized hemoglobin) (Hb(Fe^{+3})) [8, 170].

(ii) The dissociation of heme is ~10× faster from the $\alpha_1\beta_1$ dimers, prevalent at low concentrations, than from the native $\alpha_2\beta_2$ tetramers [171].

(iii) The dissociation of heme from the β-subunits of the dimer and tetramer is 25 to 60× faster than from the respective α-subunits [171]. Thus, it is safe to assume that most of the dissociation is from β-subunits.

(iv) The dissociation is reversible and the rate constant of association of hematin to apoglobin is independent of the globin and the oligomer state, tetramer or dimer [191].

With these observations, the observed first order rate constant k_{-H}(obs) of the reaction of dissociation of hematin (H) from methemoglobin (Hb^+) producing apoglobin (apo-Hb)

$$Hb^+ \underset{k_H}{\overset{k_{-H}}{\rightleftarrows}} apo\text{-}Hb + H \ , \tag{4.3}$$

is a weighted sum of the rate constants for dissociation from dimers k_{-H}(dimer) and tetramers k_{-H}(tetramer)

$$k_{-H}(\text{obs}) = \gamma\, k_{-H}(\text{dimer}) + (1 - \gamma)\, k_{-H}(\text{tetramer}) \ , \tag{4.4}$$

where γ is the fraction of hemoglobin dimer.[171] The values of k_{-H}(dimer) and k_{-H}(tetramer) for hematin dissociation from the respective β-subunits of normal adult hemoglobin were determined at pH = 7.0 in 0.15 M potassium or sodium phosphate buffer in the presence of 0.45 M sucrose and at 37°C: $k_{-H}(\text{dimer}) = 4.2\times10^{-3}\ s^{-1}$ and $k_{-H}(\text{tetramer}) = 4.2\times10^{-4}\ s^{-1}$ [171, 213]. The dimer fraction γ is higher at low hemoglobin concentrations according to the relation

$$\gamma = -\frac{K_{4,2}}{2H_0} + \frac{1}{2}\sqrt{\left(\frac{K_{4,2}}{H_0}\right)^2 + 4\frac{K_{4,2}}{H_0}} \quad , \tag{4.5}$$

where $K_{4,2}$ = 1.5 μM [195] is the equilibrium constant of dissociation of methemoglobin tetramers into dimers and H_0 is the total heme concentration, $H_0 = 4\,C_{Hb+}$ [171].

The second-order rate constant for the association of hematin to apoglobin, the reverse reaction in Eq. (4.3), was determined at pH = 8 in 50 mM Tris buffer and 50 mM NaCl at room temperature as $k_H = 1\times10^8$ $M^{-1}s^{-1}$ for both dimers and tetramers.[171, 191] The equilibrium constant for Eq. (4.3) is $K_{-H} = k_{-H}(\text{obs})/k_H$. Following Hargrove *et al*. [171], we can calculate formal values for K_{-H} using the above values of k_{-H}(obs) and k_H.

Since we expect a low concentration of released heme, corresponding to a leftward shift of the equilibrium in Eq. (4.3), it is safe to assume that the equilibrium concentration of methemoglobin [Hb^+] is equal to its total concentration C_{Hb+}. With this assumption, from the mass balance of Eq. (4.3), the concentration of free heme in equilibrium with apoglobin and methemoglobin is:

$$C_{heme} = \sqrt{K_{-H}[\mathrm{Hb}^+]} \cong \sqrt{K_{-H}C_{Hb+}} \quad , \tag{4.6}$$

In Table 4.1, we list the values of γ, k_{-H}(obs), K_{-H}, and the equilibrium free heme concentration C_{heme} for three values of C_{Hb+} computed using the above relations and values of the relevant constants for normal hemoglobin.

In Table 4.1, we have the values of the fraction of hemoglobin dimer γ, defined by Eq. (4.5); the observed rate constant of dissociation of hematin from methemoglobin $k_{-H(obs)}$, defined by Eq. (4.4); the equilibrium constant for reaction (4.3) $K_{-H} = k_{-H(obs)}/k_H$ computed using $k_H = 1\times10^8$ $M^{-1}s^{-1}$ for both dimers and tetramers [171, 191]. The value of

C_{Hb+} = 3.5 mg mL^{-1} in the top row of Table 4.1 corresponds to the average fraction of methemoglobin in healthy erythrocytes of 1% [214]. C_{Hb+} = 11.5 mg mL^{1} corresponds to 3% methemoglobin content, which is the upper limit of non-pathological methemoglobin concentration. C_{Hb+} = 100 mg mL^{-1} corresponds to a case of severe methemoglobinemia, in which 28% of the total hemoglobin is oxidized to methemoglobin [215].

Table 4.1 The equilibrium concentration of free heme C_{heme}, calculated using Eq. (10) for three values of C_{Hb+}.

C_{Hb+}		γ	k_{-H}(obs)	K_{-H}	C_{heme},
mg mL^{-1}	mM		s^{-1}	M	nM
3.5	0.054	0.077	7.10×10^{-4}	7.10×10^{-12}	20
11.5	0.18	0.045	5.93×10^{-4}	5.93×10^{-12}	33
100	1.55	0.015	4.73×10^{-4}	4.73×10^{-12}	85

The values of C_{heme} in the last column of Table 4.1 are lower by two or three orders of magnitude than those measured in healthy, sickle trait and sickle individuals in Fig. 4.1. The pH, temperature, and solution composition, at which the rate constants for hematin association to apoglobin were determined, are different from those for dissociation of heme from methemoglobin. Moreover, some of these settings are also different from those in the erythrocytes. Still, it is unlikely that the discrepancy between the data in Fig. 4.1 and the predictions in Table 4.1 is entirely due to the disparate conditions of determination. The differences in temperature, pH, ionicity, and osmolality could lead to at most a 5× higher amount of released heme.

There may be two types of processes that lead to a deviation of the measured erythrocyte concentration of the free heme from the predictions based on equilibrium assumption: (i) the release of heme is faster than predicted by the value of k_{-H} in Eq. (4.4), and (ii) the association of hematin to apoglobin is slower than predicted by the value of k_H. Furthermore, the deviation of the ratio of C_{heme} in sickle and normal erythrocytes from the equilibrium prediction suggests that at least one of these processes is non-equilibrium. An example of a concurrent equilibrium process is the formation of dimers or higher aggregates of hematin that may delay the association of hematin to apoglobin, the reverse reaction in Eq. (4.3). We discuss this mechanism below and demonstrate that its effects are too weak for the observed high heme release.

4.4. The dimerization of hematin

In aqueous solutions, at any pH, hematin forms stable dimers. Two dimer chemical structures have been put forth and are illustrated in Fig. 4.5: π–π dimers, in which the Fe^{3+} ions are located on the outer sides of two parallel monomers bound by overlapping π electron density (Fig. 4.5(A)), and μ-oxo dimers, in which the Fe^{3+} ions of the constituent monomers face each other and are bound to a shared oxygen atom (Fig. 4.5(B)) [216]. Careful recent work indicates that in aqueous solvents or in the presence of protic co-solvents, at neutral and moderately basic pH's the π–π dimer is the only hematin oligomer [207, 210]. The presence of aprotic co-solvents, such as pyridine and DMSO, or ionic compounds at high concentration, induces the formation of μ-oxo dimers [210].

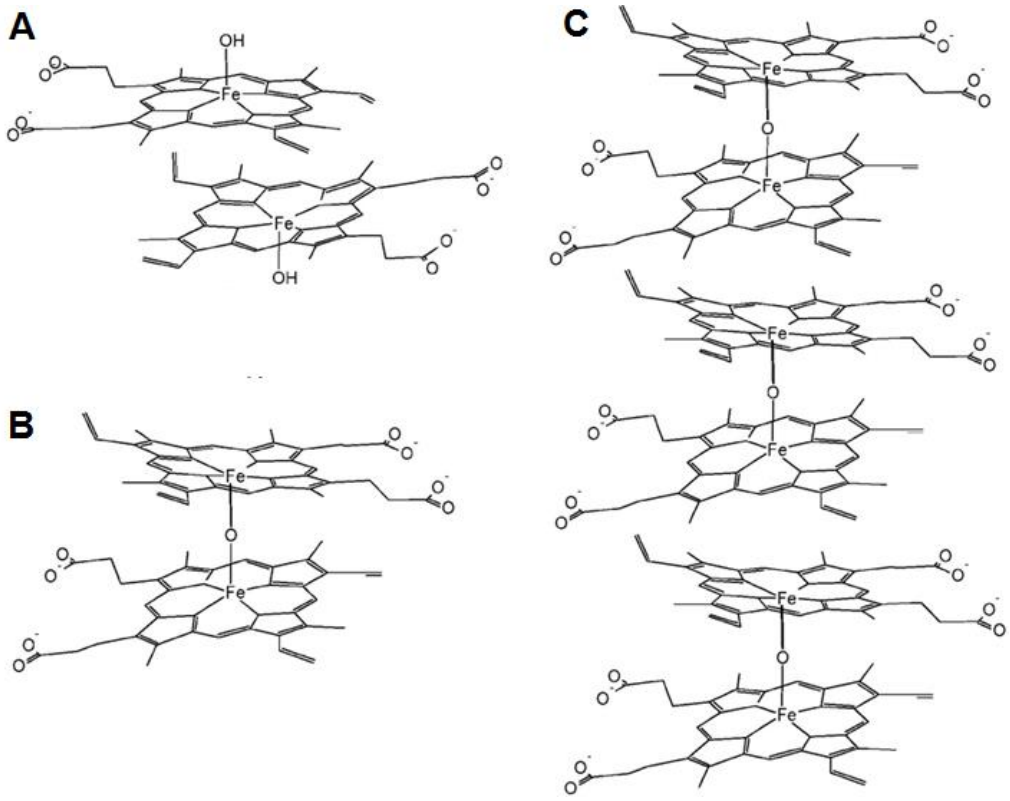

Figure 4.5. The structures of A. π–π dimer, B. μ-oxo dimer, and C. oligomers consisting of stacks of μ-oxo dimers.

Hemoglobin contains numerous polar hydrogen-containing groups on its surface and is thus a protic compound. Hence, mostly π–π hematin dimers are expected in the red cell cytosol. Because of their small size, the π–π hematin dimers easily pass through the dialysis membrane; since they are weakly bound,[210] they easily dissociate to enable hematin binding to apo-HRP. Thus, the presence of the π–π dimers does not interfere with the quantification of the free heme.

The equilibrium constant of formation of hematin dimers at pH = 7.38 has been determined as $K_D = 1.05\times10^8$ M^{-1} [207, 217]. Straightforward mass balance calculations using the hematin dimerization constant reveal that the fraction of monomeric hematin in

equilibrium with the dimers is 0.018 in solutions with C_{heme} = 44 μM, as in the sickle erythrocytes, and 0.030 in solutions with C_{heme} = 20 μM, as in the healthy erythrocytes.

To account for the effect of hematin dimerization on its release from hemoglobin, we solve a system of equations consisting of the expressions for the equilibrium constants for heme release K_{-H}, and hematin dimerization K_D. We use two additional mass balance constraints: that the total free heme concentration is equal to that of the apoglobin, and that the initial methemoglobin concentration is equal to the sum of its equilibrium value and the apoglobin concentration. A system of four equations with four unknown emerges:

$$\begin{gathered} K_{-H} = \frac{[H_{monomer}][apoglobin]}{[methemoglobin]} \\ K_D = \frac{[H_{dimer}]}{[H_{monomer}]^2} \\ C_{heme} = [apoglobin] = 2\,[H_{dimer}] + [H_{monomer}] \\ C_{Hb+} = [apoglobin] + [methemoglobin] \quad . \end{gathered} \tag{4.7}$$

We apply two reasonable assumptions: that the apoglobin concentration is significantly lower than that of methemoglobin, and that the equilibrium concentration of monomeric hematin is much less than its total concentration and obtain total heme concentration:

$$C_{heme} = (2K_D K_{-H}^2 C_{Hb+}^2)^{1/3} \quad . \tag{4.8}$$

If the second assumption is relaxed, i.e., if both $H_{monomer}$ and H_{dimer} are present in significant amounts, then C_{heme} is the solution of a cubic equation

$$C_{heme}^3 - K_{-H} C_{Hb+} C_{heme} - 2K_D K_{-H}^2 C_{Hb+}^2 = 0 \quad . \tag{4.9}$$

Equation (4.9) has a single real root: for C_{heme} = 0, the cubic polynomial on the left hand side is negative, while for sufficiently high values of C_{heme}, it is positive. It can be solved numerically. For the lowest methemoglobin concentration in Table 1, the difference

between the values of C_{heme} returned by Eqs. (4.8) and (4.9), 35 and 31 nm, respectively, is about 13%. This difference decreases as C_{Hb+} increases since greater C_{Hb+} leads to greater C_{heme} and higher fraction of dimers.

Table 4.2 The total concentration of free heme C_{heme} computed using Eq. (4.9).

C_{Hb+}		K_{-H}	C_{heme}
mg mL^{-1}	**mM**	**M**	**nM**
3.5	0.054	7.10×10^{-12}	35
11.5	0.18	5.93×10^{-12}	68
100	1.55	4.73×10^{-12}	232

In Table 4.2 we list values of the total concentration of free heme C_{heme} computed with account for hematin dimerization using Eq. (4.9) for the same methemoglobin concentrations as in Table 4.1. While the values of C_{heme} in Table 2 are greater than those in Table 4.1, they are still significantly lower than the values in the erythrocytes of normal individuals Fig. 4.1. Accounting for the disparity in temperature, pH, ionicity, and osmolality, at which the constants involved in Eq. (4.7) were measured, may increase C_{heme} to about 0.1 μM, at best, in methemoglobinemia-free individuals.

In summary in this chapter: we have shown that the process of dimerization acting as an equilibrium process reducing the rate of association of hematin to apoglobin do not account completely for the high concentration of hematin that we have found in the red cell cytosol. Thus, a non-equilibrium process resulting in heme release faster than the one assumed in the calculation of K_{-H} is needed to understand the found concentrations of free heme in healthy and sickle erythrocytes.

CHAPTER

5

AUTOCATALYTIC RELEASE OF HEME IN HEALTHY & SICKLE RED CELL CYTOSOL

The discrepancies between the results in Fig. 4.1(A) and predictions assuming (i) equilibrium and (ii) dimerization of hematin in solution as a process to slowdown the association of hematin with apoglobin suggest that a fast process contributes to heme release and pushes the system to a steady state with a free heme concentration exceeding that at equilibrium. The arrested heme release in the hemolysate, at low hematin concentration, Figs. 3.6-3.8 and the high hematin concentration in sickle and healthy erythrocytes, seen in Fig. 4.1, suggest that the rate of heme release depends on the concentration of free heme in the solution, i.e., *heme release is autocatalytic*. Autocatalytic processes govern the behaviour of self-organizing spatiotemporal structures in chemistry, biology, economics, and sociology [218]. Hematin can catalyse its own release by oxidizing hemoglobin to methemoglobin. Hematin is known to partake in several oxidation reactions using its Fe^{3+} ion [219] and its numerous deleterious physiological effects are mostly due to its oxidizing potential. Thus it is important to decipher the possible mechanism that underlies the autocatalytic release of hematin in physiologic condition and to see if the mechanism can elucidate some features of sickle cell disease pathophysiology.

5.1. Proposed mechanism for autocatalytic release of heme

We propose the following mechanism of autocatalytic release of heme

$$\begin{gathered} \mathrm{Hb} + n\,\mathrm{H} \xrightarrow{k_{auto}} \mathrm{Hb^+} + \mathrm{H^-} + (n-1)\,\mathrm{H} \\ \mathrm{Hb^+} \xrightarrow{k_{redox}} \mathrm{Hb} \\ \mathrm{Hb^+} \underset{k_H}{\overset{k_{-H}}{\rightleftarrows}} \mathrm{apoHb} + \mathrm{H} \\ \mathrm{H^-} \xrightarrow{O_2} \mathrm{H} \end{gathered} \quad , \qquad (5.1)$$

where k_{auto} is the rate constant for the oxidation of hemoglobin by hematin catalyzed by previously release heme, k_{redox} depends on the concentration of cytochrome b_5 reductase in solution; for more on cytochrome b_5 reductase – a NADH dependent enzyme that catalyzes methemoglobin reduction back to oxyhemoglobin in red cell cytosol, see Refs. [220, 221]; and H^- is Fe^{2+} protoporphyrin IX or heme, which is immediately oxidized to hematin by any available oxidizing agent, e.g., dissolved O_2. With this, the rate of oxidation of hemoglobin $v_1 = k_{auto} C_{Hb} C_{heme}^n$.

The main assumption of this mechanism is the top reaction in Eq. 5.1, which describes the oxidation of hemoglobin by hematin. In support of this assumption, we note that hematin is known to partake in several oxidation reactions using its Fe^{3+} ion [219]. The presence of hematin in the blood plasma and the red blood cells is associated with numerous deleterious effects and most of them are due to its oxidant potential: hematin causes lysis of mouse [222, 223] and human erythrocytes [224], the malaria agents *Plasmodium berghei* [225] and *Plasmodium falciparum* [226], neurons and neuron-like cell cultures [227, 228]. Hematin induces oxidation of low-density-lipoprotein and is

implicated in the development of atherosclerosis [229, 230]. The presence of hematin in membranes disrupts their function increasing disorder and permeability [231].

The mechanism expressed in Eqs. 5.1 ignores several processes. The first one is the presence of equilibrium amount of methemoglobin. Including this methemoglobin concentration in the kinetic scheme does not introduce mathematical complications or significantly different kinetics response [218]. The effect of this methemoglobin can also be accounted by assuming a nonzero initial hematin concentration $C_{heme,0}$. A second ignored reaction is the dimerization of hematin. This process effectively lowers the concentration of the autocatalyst and can be viewed as an inhibition reaction. Inclusion of inhibition into the autocatalysis mechanism leads to higher order non-linear effects discussed below. A third reaction that may lead to complex kinetics laws is the removal of heme from the reaction mixture, for instance by glutathione-catalyzed degradation [232]. The glutathione process releases the iron ion from the organic moiety and precipitates it as insoluble residue [232]; these processes extinguish the oxidizing potential of hematin. Glutathione degradation may also act as effective inhibition of hematin catalysis of its release.

Clearly, the understanding of the processes that regulate the concentration of free heme and their interplay requires significant experimental and theoretical efforts. However, with certain assumptions, we can simplify the mechanism discussed above and provide meaningful insights into autocatalytic heme release.

5.2. Reaction order and autocatalytic behaviour of heme

When we ignore heme dimerization and degradation and limit our considerations to early reaction times or low Hb concentrations, at which the concentrations of Hb^+ and apoHb are low, and the rates of the two reverse reactions with constants k_{redox} and k_H are slow; we sum the three forward reactions in Eqs. (5.1) and obtain a possible net reaction of heme release from hemoglobin

$$\mathrm{Hb} + n\,\mathrm{H} \rightarrow \mathrm{apoHb} + (n+1)\mathrm{H} \quad , \tag{5.2}$$

where (Hb—hemoglobin, H—heme;). Equation (5.2) has the form of a typical autocatalysis reaction where hematin itself is working as a catalyst for its own release, thus providing the possibility of nonlinear autocatalytic rate law behavior. Similar rate laws govern processes and lead to oscillations and other self-organizing spatiotemporal structures in chemistry, biology, economics, and sociology [218].

If the stoichiometric coefficient $n = 1$, the autocatalytic reaction is classified as quadratic; for $n \geq 2$, we have cubic or higher order autocatalysis [218]. If the release of heme complies with a quadratic autocatalysis law, i.e., $n = 1$, and the first step in the mechanism expressed by Eqs. (5.1) is rate limiting, than the rate of release of heme $\frac{d[H]}{dt}$ according to reaction (5.2) will be equal to v_1, so that

$$\frac{d[H]}{dt} = k_{auto} C_{Hb} C_{heme} \quad , \tag{5.3}$$

Eq. (5.3) can be integrated [233, P. 915] to obtain an expression for concentration of heme.

If the initial concentration of hemoglobin $C_{Hb,0}$ is greater than that of hematin $C_{heme,0,}$ the time dependence of the hematin concentration attains a simple form:

$$C_{heme} = C_{heme,0} \exp[(C_{Hb,0} + C_{heme,0})k_{auto}t] \approx C_{heme,0} \exp[C_{Hb,0}\, k_{auto}t], \quad (5.4)$$

In quadratic autocatalysis C_{heme} increases as $C_{heme,0}\cdot\exp[(C_{Hb,0} + C_{heme,0})k_{auto}t]$, where $C_{Hb,0}$ and $C_{heme,0}$ are the initial concentrations of hemoglobin and hematin, respectively, k_{auto} is the rate constant of the above autocatalytic reaction and t is time [218, 233, p. 915]. Cubic and higher order autocatalytic schemes cannot be integrated; however, numerous features of the resulting kinetics have been identified: the rate increases exponentially with time, the pre-exponential expression is a function not only of $C_{heme,0}$ but also of $C_{Hb,0}$, and $C_{Hb,0}$ enters the exponential increment at a high power [218]. Thus we decided to test if the heme release in the red cell cytosol shows autocatalytic behaviour and if the reaction order obtained from such experiment can elucidate the discrepancy in the hematin concentration we found in red cell cytosol.

5.2.1. Test of order of reaction

As a first test of autocatalytic heme release, we monitor the evolution of the concentration of free heme in one sickle and four healthy hemolysate samples after the addition of 0.1 or 0.2 μM of hematin. The five evolutions of free heme concentration in Fig. 5.1 fall into three kinetics curves, which have several features expected for both types of autocatalytic rate laws [218]:

(i) The increase of free heme concentration C_{heme} is exponential.

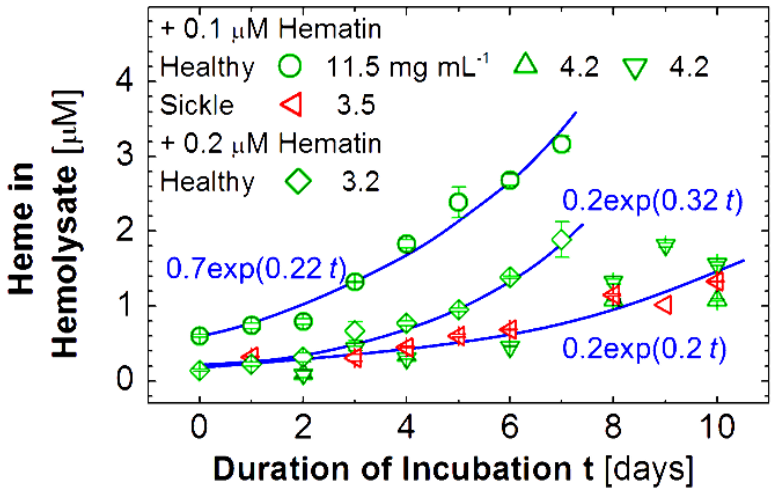

Figure 5.1 The evolution of the concentration of free heme in sickle (one experiment) and healthy (four experiments) hemolysate.

(ii) The best fit $C_{heme,0}$ (0.7, 0.2, and 0.2 μM for the respective curves) approximately equals the measured $C_{heme,0}$ and the sum of endogenous, C_0, evaluated from Fig. 3.5(A), and added, C_{added}, hematin concentrations.

(iii) In sickle hemolysate, in which faster autoxidation should lead to a higher k_{auto}, the rate of release is approximately equal to that in one of the healthy hemolysates even though $C_{Hb,0}$ is lower.

(iv) Increasing $C_{Hb,0}$ increases the rate of heme release.

However, one additional feature of the data diverges from expectations for quadratic autocatalysis: the rate of heme release depends strongly on the initial hematin concentration, as seen by comparing the two lower kinetic curves in Fig. 5.1. Since the lowest hemoglobin concentration, 3.2 mg mL^{-1} ≅ 50 μM, is significantly higher than the highest $C_0 + C_{added}$ ≅ 0.7 μM, in the case of quadratic autocatalysis C_{heme} should not depend on C_{added}. Thus, the dependence of C_{heme} on C_{added} indicates that the participation of

solution hematin in the release of additional heme follows a cubic or higher order autocatalytic rate law [218].

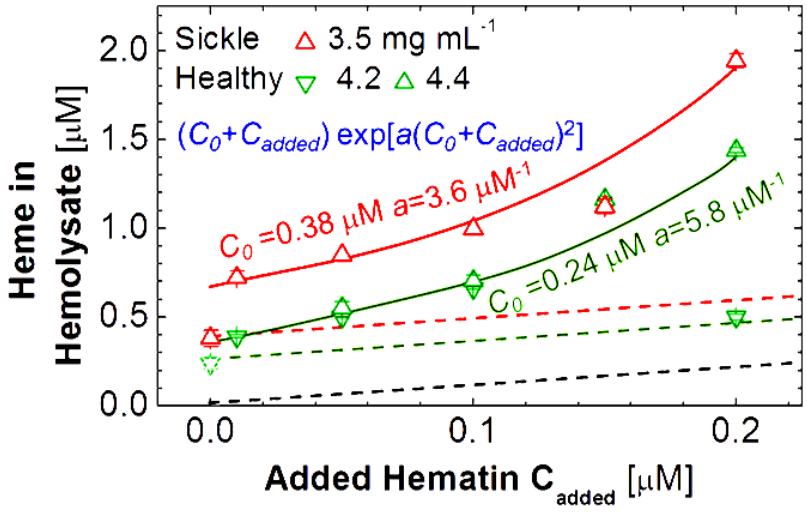

Figure 5.2. The concentration of free heme in hemolysate incubated for 14 days at ~5ºC with hematin concentrations ranging from 0.01 to 0.2 μM.

For additional tests of higher order autocatalytic heme release, we determined the amount of heme released in sickle and normal hemolysate 14 days after the addition of hematin concentrations from 0.01 to 0.2 μM and display the results in Fig. 5.2. In Fig. 5.2, all measured hematin concentrations C_{heme} are higher than (C_0 + C_{added}) and increase as an exponential function of the square of the total initial hematin concentration, $(C_0 + C_{added})^2$. This observation strongly reinforces the conclusion of cubic or higher order autocatalytic heme release.

5.2.2. Importance of cubic or higher order autocatalysis

The features of cubic or higher order autocatalysis we need to consider here are that the rate increases exponentially with time, as in the case of quadratic autocatalysis, the pre-exponential expression is a function not only of $C_{heme,0}$ but also of $C_{Hb,0}$, and $C_{Hb,0}$ enters

the exponential increment at a high power. Typically, higher order autocatalysis is seen when the autocatalyst is inhibited by a parallel process [218]. It is feasible that the dimerization and glutathione degradation of hematin, which significantly reduce its concentration, could have kinetic effects similar to inhibition. Processes with mechanisms involving autocatalytic steps of cubic or higher order exhibit multiple steady states even in closed systems [218]. The equilibrium is only one of the steady states and may never be reached [218]. Non-equilibrium steady states are reached if the initial concentrations of the reagents are strongly dissimilar. Clearly, the release of heme from hemoglobin in the red cell cytosol complies with the latter condition: $C_{Hb,0}$ = 5.4 mM, while the equilibrium concentration of free heme, evaluated to be of order 0.1 μM. With this, it is feasible that the concentration of released heme reaches a steady value higher by orders of magnitude than the equilibrium concentration.

In summary in this chapter: we have put forth autocatalytic release of heme in physiological condition as a faster non-equilibrium process which is the probable explanation for high values of intraerythrocytic free heme observed in our determinations. Autocatalytic processes are source of non-linear kinetics and instability, at the same time it is a process with multiple possible steady states. Autocatalytic release of heme coupled with heme effects on sickle hemoglobin polymerization may be a key factor in the pathogenesis of sickle cell disease. It is imperative that mechanism of such action in physiologic relevant settings be sought and explained.

CHAPTER

6

AUTOCATALYTIC RELEASE OF HEME, METHEMOGLOBIN & OXIDATIVE STRESS IN SICKLE CELL DISEASE

To explore heme release at concentrations closer to those in the erythrocytes and to highlight additional factors affecting heme release, we concentrated sickle and healthy hemolysates by (10 – 60)× and monitored the increase in free heme concentration over time. Hemoglobin concentration increase was achieved by ultracentrifugation, which removed all molecules with molecular mass less than 10,000 g mol^{-1}. Thus, the *starting free heme concentration* was zero.

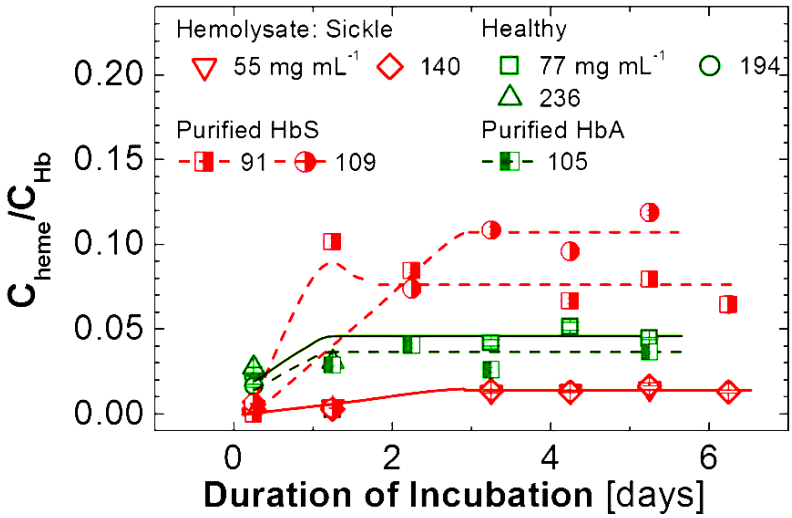

Figure 6.1 The evolution of the concentration of free heme in concentrated sickle and healthy hemolysate and in purified sickle hemoglobin, HbS, and normal hemoglobin, HbA.

Figure 6.1 reveals that a steady and high free heme concentration is reached within several days in both sickle and healthy hemolysates. The steady ratio C_{heme}/C_{Hb} = 0.014

for sickle hemolysate and 0.046—for healthy. These ratios are, respectively, 1.7 and 12× higher than the ratios stemming from Fug. 4.1(A), indicating that heme release in the concentrated hemolysate is stronger than in the red cell cytosol. This enhancement may be due to the removal of glutathione, which catalyses the degradation of hematin [232] and has molecular mass of 307 g mol^{-1}, by ultracentrifugation.

To understand the reason for the higher concentration of free heme in concentrated healthy hemolysate, we purified sickle and normal hemoglobins from the respective hemolysates by ion exchange HPLC [173]. We monitored the release of heme from solutions of the purified hemoglobins. The result presented in Fig. 6.1 reveals that for HbS the steady ratio C_{heme}/C_{Hb} increases from that found in concentrated sickle hemolysate (0.014) to 0.075 or 0.11, depending on C_{Hb}; for HbA it is practically identical to that in the concentrated hemolysate at 0.038. Purification removes all non-hemoglobin proteins from the solution, including cytochrome b_5 and NADH reductase enzyme which reduce methemoglobin to hemoglobin [220, 221]. Methemoglobin reduction is one of the reverse reactions in the autocatalytic mechanism and the removal of its catalyst from the sickle hemolysate leads to significantly higher released heme amount. The variations of the hematin concentration in purified HbS and HbA are greater than in the respective hemolysates and an overshoot above the steady values is observed for the 91 mg mL^{-1} HbS trace. These effects are signatures of kinetic instability inherent to high-order autocatalytic reactions.

Thus it can be assumed that autoxidation of oxyhemoglobin to methemoglobin by hematin and its reverse reaction plays very important role in the autocatalytic release of hematin. To validate the proposed autocatalysis mechanism in Eq. 5.1, we tested if:

(1) hematin really oxidizes hemoglobin

(2) the reduction of methemoglobin is crucial for autocatalysis mechanism.

6.1. Autoxidation of oxyhemoglobin in presence of heme

To test for autoxidation of oxyhemoglobin by hematin as part of reaction mechanism in Eq. (5.1), we filtered oxyhemoglobin solution by centrifuge (to start from zero heme presence in solution), treated the filtered solution with reducing agents (to convert all the methemoglobin back to oxyhemoglobin) and then added hematin in the solution. We observed spectrophotometrically the evolution of methemoglobin in the hematin added and control solutions (no hematin). The detailed procedure and results are discussed below.

6.1.1. Experimental method & data analysis

We performed the experiments with both the hemolysate and pure hemoglobin solutions from healthy adult and sickle cell patient blood. After preparation (as described in Chapter 3) we concentrated the hemolysate and purified solutions and aliquots of each were treated with 2-fold concentrated solutions of ascorbic acid and sodium dithionite (Sigma Aldrich) (both dissolved in DI water) separately. This treatment would ensure the reduction of methemoglobin present in the solution back to oxyhemoglobin [234, 235]. After this treatment for 4 hours, we dialyzed the solutions against 0.15M (pH =7.35) phosphate buffer solution overnight to remove the excess reductants from the solution and

then the solutions were concentrated. The concentrated hemolysate and purified solution were diluted to 100 mg/ml and 10 mg/ml (for two sets of experiments) as both controls and samples with hemin added at 100:1 (hemoglobin : hemin) ratio. We determined fractions of oxyhemoglobin and methemoglobin present in these samples using Beckman DU-800 spectrophotometer and performing a multi wavelength analysis using python programming. We kept the samples for at least 12 days and determined the change in the methemoglobin concentration from the spectrophotometer data analyzed every day.

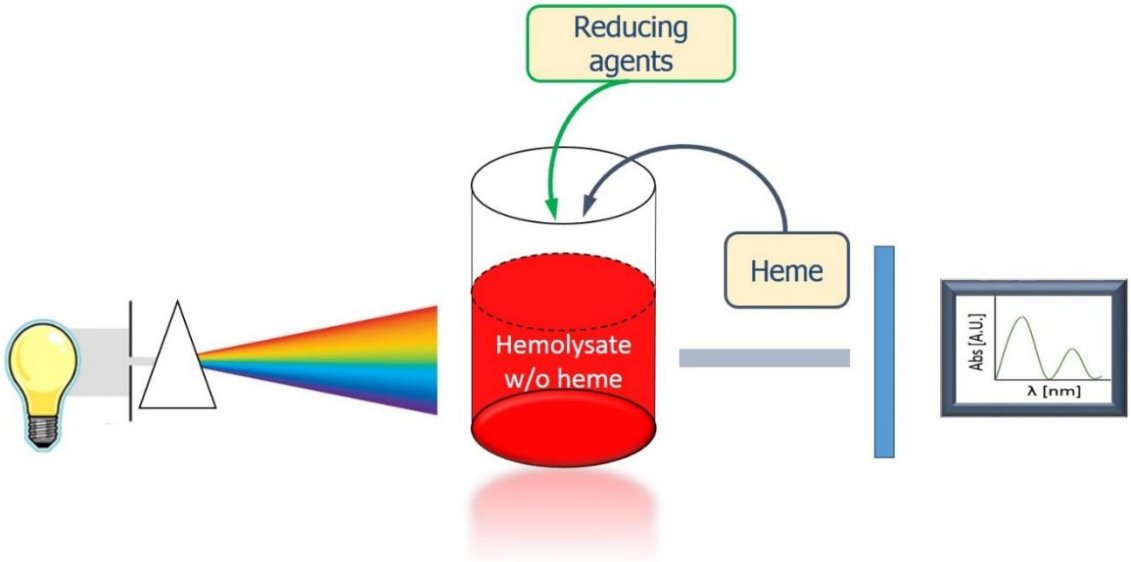

Figure 6.2 Schematic of spectral observation of autoxidation of oxyhemoglobin by heme.

Multi-wavelength fit

The principle of data processing from the spectrophotometer analysis on the samples was the absorption at any wavelength is the sum of the absorption by the mixture components at that component (at a dilution where it follows Beer's law) [236].

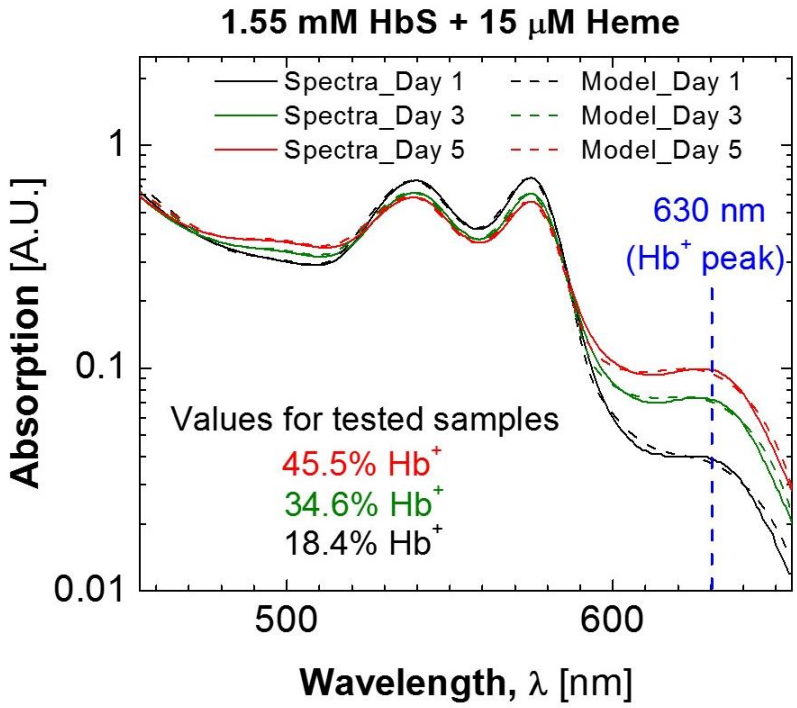

Figure 6.3 Multi-wavelength fit of experimental spectrometry data to determine metHb concentration.

The extinction coefficients for both oxyhemoglobin and methemoglobin at 20 different wavelengths were obtained from ref [237]. Then using python programming, the experimental data was fitted. We read the absorption spectra (A) as a function of wavelength (W). Then we have a matrix consisting of the extinction coefficients which also varies according to the wavelengths. So we can arrange the matrices in the following form:

$$\begin{bmatrix} E11 & E21 \\ E12 & E22 \\ & \cdot \\ & \cdot \\ E1n & E2n \end{bmatrix} \begin{bmatrix} C1 \\ C2 \end{bmatrix} = \begin{bmatrix} A1 \\ A2 \\ \cdot \\ \cdot \\ An \end{bmatrix} \tag{6.1}$$

$$\Rightarrow \bar{\bar{E}}\,\bar{C} = \bar{A}$$

$$\Rightarrow \bar{\bar{E}}^T\,\bar{\bar{E}}\,\bar{C} = \bar{\bar{E}}^T\bar{A} \quad .$$

Now if we consider the minimum sum squared error with respect to $\bar{C}$, i.e., $\sum(\bar{\bar{E}}\ \bar{C} - \ \bar{A})^2_{\text{min}}$, we can find the following:

$$\bar{C} = \ (\bar{\bar{E}}^T\ \bar{\bar{E}})^{-1}\ \bar{\bar{E}}^T \bar{A}\ . \tag{6.2}$$

So from this fit, we obtain the fractions of individual hemoglobin derivative that is present in the solutions. The variance for each concentration was also calculated based on the fit as follows [238]:

$$variance = \ \sigma\ (\bar{\bar{E}}^T\ \bar{\bar{E}})^{-1}_{ii} \text{ of } \bar{C}\ , \tag{6.3}$$

where, $\sigma = \frac{1}{n-p}$ (sum squared error from theoretical and experimental absorption values); n = number of points and p = number of parameters. The variance found for each concentration was on the order of 10^{-6} to 10^{-7} which is very negligible and shows the accuracy of the fit. The standard deviation would be just the square root of variance which is pretty low (~10^{-3}). The assumption during these fits is that there are no hemoglobin derivatives other than oxyhemoglobin and methemoglobin present in the solution. So we only used the extinction coefficients of those derivatives.

6.1.2. Heme induces methemoglobin formation

Previously investigators observed higher levels of methemoglobin in sickle blood than normal blood incubated in media [239]. In our experiment, we treated the solutions with reducing agents to make sure that there is only oxyhemoglobin present initially in the solutions. We concentrated the solutions using ultracentrifuge membrane (10000 MWCO) so that no heme is present as well. Then hemin was added to the solution and the evolution of methemoglobin was observed by spectrophotometry as described in methods section. In

control solutions, we did not add any hemin. We studied both hemolysate and purified solutions of healthy adult and sickle hemoglobin.

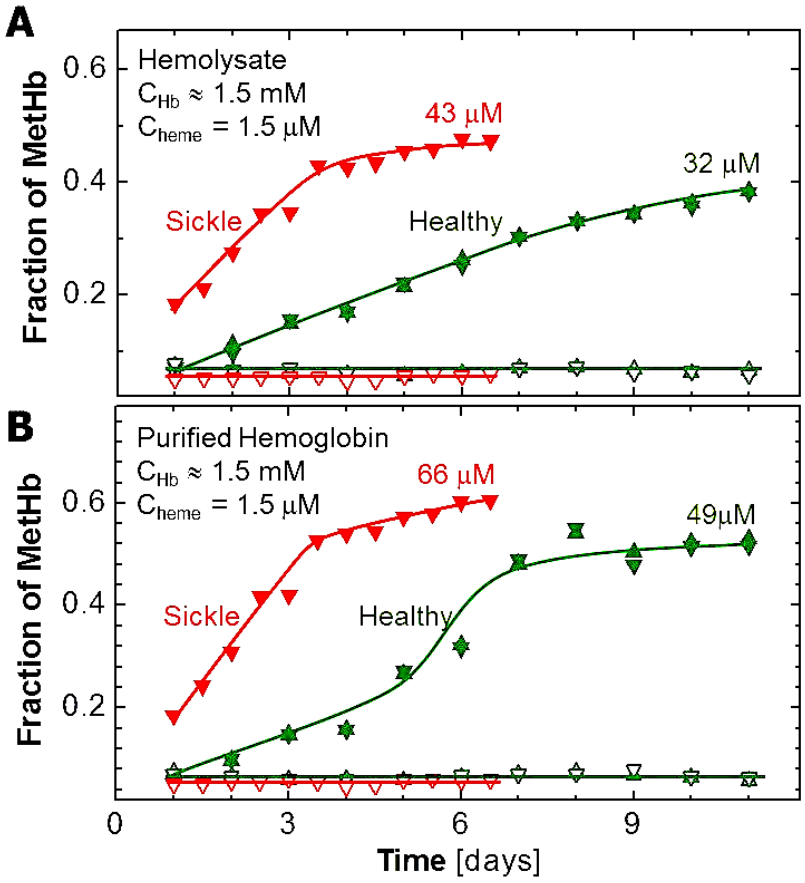

Figure 6.4 The oxidation of hemoglobin to met-hemoglobin by free heme at 1.5 mM concentration of hemoglobin.

The result presented in Fig. 6.4 shows that hematin added to the solutions causes oxidation of oxyhemoglobin to form methemoglobin as expected from the first reaction in Eq. 5.1 and the evolution is stronger in sickle hemolysate than normal hemolysate. This strongly supports the mechanism which we propose for the autocatalytic behaviour of free heme.

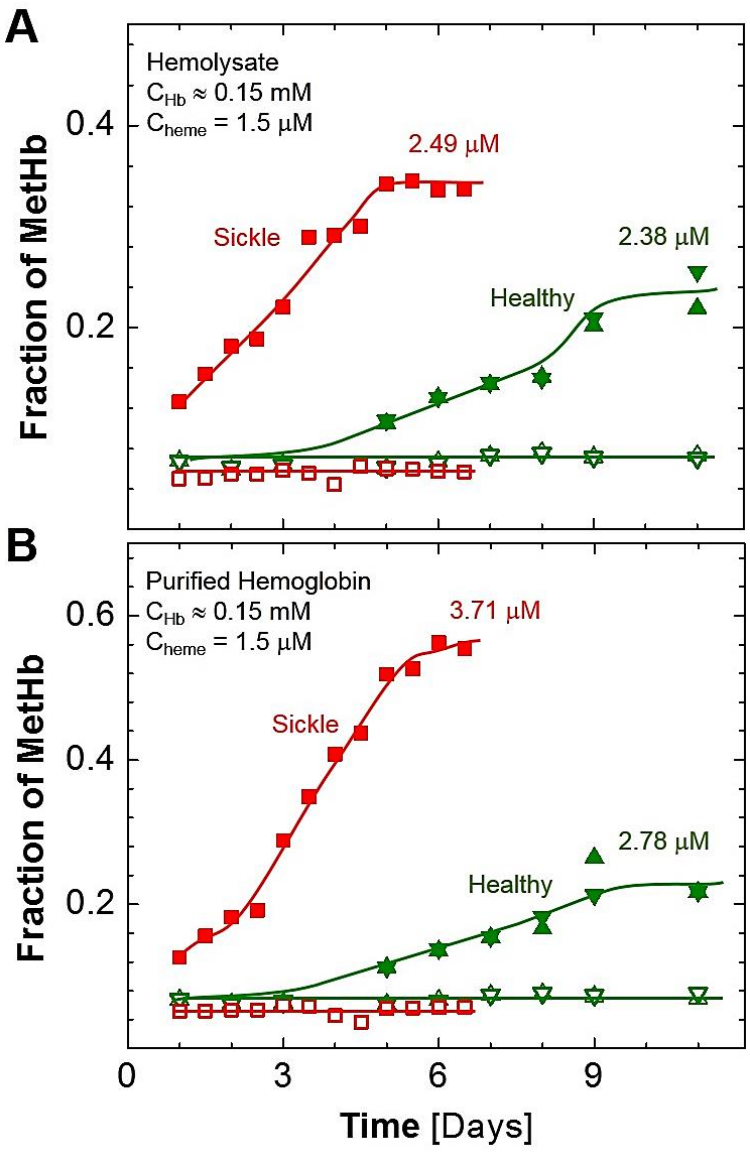

Figure 6.5 The oxidation of hemoglobin to met-hemoglobin by free heme at 0.15 mM concentration of hemoglobin.

If we compare the data of Fig. 6.4(A) and 6.4(B) we see that using reductants (with no hematin) reduces the free heme release in high concentration HbS hemolysate solutions leading to C_{heme}/C_{Hb} = 0.00032; but while we use heme in the solution of $C_{heme,0}/C_{Hb,0}$ = 0.01 – this leads to very high ratio of heme release, C_{heme}/C_{Hb} = 0.028. This manifests the *heme action to be part of the autocatalysis mechanism*. The comparison of Fig. 6.4 and 6.5

reveals that higher levels of heme release occurs from hemoglobin with higher concentrations as expected in autocatalytic reaction scenario. At the same time higher C_{heme}/C_{Hb} ratio in purified samples reveals higher susceptibility of purified oxyhemoglobin solution to autoxidation indicating *significant role of methemoglobin reductase enzymes combined with heme degradation product* to avoid autoxidation of oxyhemoglobin in biophysical scenario.

6.2. Oxidative stress-hemoglobin/methemoglobin homeostasis in sickle cell anemia and possible connection with heme induced sickle hemoglobin polymerization: a proposed mechanism

Being the transporter of oxygen and going through continuous oxygenation and deoxygenation red blood cells basically work as the "chief oxidative sink" of the body [240]. The regulation of reactive oxygen species (ROS) such as hydrogen peroxides, oxygen, peroxide ions, superoxide anoins etc. in red blood cells is very complex [241] and the matured cells without genetic material cannot produce new proteins to regulate the oxidative stress [240]. Thus in red blood cell the ROS control has to be very self-sustainable [242] and it requires steady homeostatic regulation of ROS scavenging enzymes such as superoxide dimustase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), NADPH reductase and NADH-cytochrome b_5; and low-molecular-weight antioxidants such as glutathione (GSH), Vitamin C and Vitamin E [243]. The sickle erythrocytes have been shown to go through more oxidative stress and low level of antioxidant biomarkers are observed due to high metabolic cost from loss of deformability and from the HbS polymerization [244, 245]. The effect of oxidative stress due to free radical formation in SCD erythrocytes [128, 246] and perturbation in the levels of oxidative

stress controlling redox enzymes and low-molecular-weight antioxidants in sickle cell disease pathology have been well documented [128, 243] – which can be associated with increased levels of ROS generation in sickle erythrocytes [245, 247, 248]. Also mediation through NADPH activity and cell signalling molecules present in the plasma have also shown to contribute towards increasing the oxidative stress in sickle erythrocytes [249].

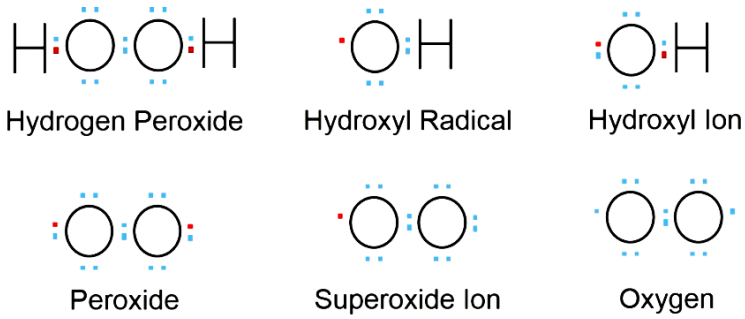

Figure 6.5. Examples of reactive oxygen species active in the biophysical conditions

High level of autoxidation of mutated hemoglobin is inherent to sickle cell disease pathophysiology [8]. The autoxidation of hemoglobin to form methemoglobin involves several reactive oxygen species in healthy erythrocytes; on average only around one percent methemoglobin is expected [243, 250]. Higher than usual methemolgobin content has been found to be associated with sickle cell disease [251, 252]. The major stabilizing enzyme responsible for methemoglobin reduction to have steady methemoglobin (metHb) level in the red cell is NADH-dependent-Cytochrome b_5-reductase. About five (5%) percent of the metHb reduction is taken care by a Flavin protein NADPH [241], and the rest is taken care of by small molecules such as glutathione and ascorbate [253]. Close to 40 mutated forms of NADH enzyme have been found so far that represents high level of methemoglobin formations [250] and this fact actually depicts the essential role of NADH in regulation the autoxidation of hemoglobin *in vivo.* Reduced level of NADH content has

been found in sickle erythrocytes [254, 255] and this links together high levels of methemoglobin formation and oxidative damage in sickle cell disease [251]. Glutathione (GSH) also helps to degrade excess free heme in red cell cytosol [256] and is found in lower amounts (sometimes 50%) in HbS sample compared to HbA sample [128, 257].

Thus it is evident that the increased methemoglobin formation under high oxidative stress and substantial failure to counteract this phenomena by the reduced levels of responsible redox enzymes and heme degradation molecules accentuate a pivotal role of autoxidation of inherently unstable sickle hemoglobin in the disease pathophysiology. Our results depicted in Fig. 6.4 indicate a possible role of free heme acting as an oxidizing agent for the production of methemoglobin from oxyhemoglobin in both healthy and sickle cell hemolysate and pure hemoglobin solutions. In view of the above discussion, we propose a mechanism for the overall scenario in a sequential manner:

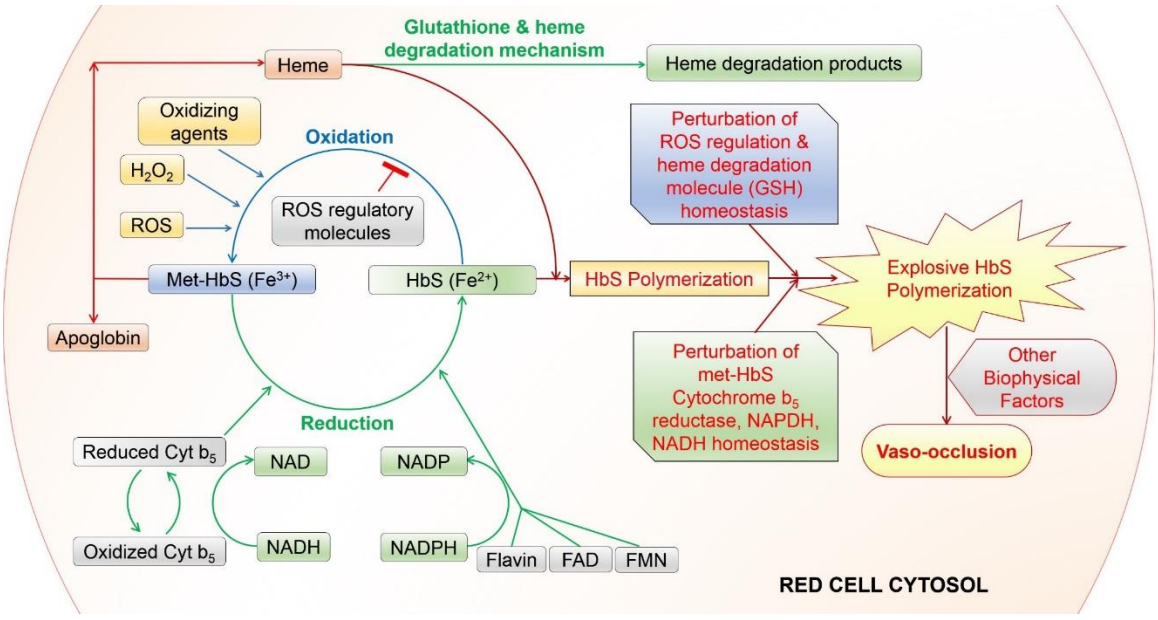

Figure 6.6. Proposed mechanism of sickle cell disease pathophysiology involving oxidative stress, autoxidation of HbS, autocatalytic release of free heme and HbS polymerization.

1. The inherent instability of hemoglobin is the primary reason for autoxidation and formation of methemoglobin in sickle erythrocytes. Though in normal erythrocytes redox couple mechanism of NADH-Cytochrome b_5-metHb reductase, NADPH dependent reduction mechanism, glutathione and ascorbate controlled reduction keep the methemoglobin level at a very low steady state (1%) value, in sickle erythrocytes, under high oxidative stress, level of these methemoglobin reduction mechanisms are altered and autoxidation is accelerated.

2. Due to increased autoxidation, increased levels of methemoglobin is present in sickle red cell cytosol and as methemoglobin is known to release at least one heme molecule in solution [170, 171], that increase in heme level in red cell cytosol is expected and is evident from the comparison in our data on free heme concentrations in sickle and healthy erythrocytes in Fig. 3.5(A). There has been evidence of fluorescent heme degradation products (HDPs) from hemoglobin autoxidation associated with the red cell membrane [242] – indicating the increased level of autoxidation with hemoglobinopathies including sickle cell disease [258]. Strong correlation between high level of heme degradation products and increased methemoglobin content has been proposed to be indicative of higher degradation of intact hemoglobin and/or increased degradation of free heme present in pathologic red cell cytosol of transgenic mice [258].

3. The increased levels of heme and methemoglobin should put a consumption pressure on the reductive enzymes and heme degradation molecules. In case

of severe oxidative stress and associated autoxidation, cytosolic heme content may increase in such level that the heme degrading molecules may saturate and accumulation of free heme is inevitable. This free heme pool due to high oxidation potential can act as a catalytic agent (alongside ROS) for further oxidation of methemoglobin as evident from our data. It will perpetuate the accumulation and release of free heme in red cell cytosol.

4. Thus we propose that under circumstances of high oxidative stress and perturbed homeostatic regulation of reduction enzymes and heme scavenging molecules, triggering autocatalytic release of heme is possible in physiological conditions. Triggering may induce high degree of HbS polymerization [7] and consequent aggressive erythrocyte deformation [2]. This combined with exogenous factors such as erythrocyte adhesion to endothelial cells [259], intravascular hemolysis [260], bio-inavailability of nitric oxide substances for vasodilation [261, 262] etc. may contribute to the clinical manifestation of the disease and its variability present among sickle cell patients [123, 125].

*The proposed mechanism for the **first time ever** relates the two major consequences of the mutation (HbS polymerization and accelerated heme release) with the physiological scenario of high oxidative stress in sickle cell disease pathology.*

CHAPTER

7

SUMMARY OF ANALYSIS OF FREE HEME IN HUMAN ERYTHROCYTES: A NEW PARADIGM IN SCD RESEARCH

The determination and analysis of free heme present in human red cell cytosol has presented us with a new perspective in SCD research. In the course of finding out free heme levels, we developed a new method of determination, analysed the possible mechanisms, proposed a new non-equilibrium process as possible explanation and coupled known pathological scenarios to the new mechanism. We claim the followings to be the key outcomes of the research:

1. We developed a novel enzymatic/chemiluminescence method to quantify picomolar concentrations of free heme present in red cell cytosol.
2. We found average free heme concentrations sickle cell patients is 44±9 μM, sickle trait donors, 33±2 μM, and healthy donors, 20±2 μM. The p-values from statistical analysis indicate towards a significant difference of average heme concentrations in sickle cell patients and healthy donors. Average values in sickle trait individuals and healthy donors are also statistically different. These are expected as sickle hemoglobin has higher propensity of autoxidation and heme release.
3. The average values of HbAS and HbSS are statistically similar. This result may provide insight about clinical complications seen in sickle trait individuals which are not associated with sickle cell disease.

4. Free heme is present as π–π dimers in red cell cytosol, not in crystalline form and dimerization has futile effect on slower association of heme.
5. The release of heme is autocatalytic and follows a high order rate law.
6. HbS oxidation to met-HbS is part of mechanism for autocatalysis. Cellular oxidative stress (inherent to SCD) coupled with perturbation in met-HbS reductase and heme degradation molecule homeostasis may trigger autocatalysis. Subsequent autocatalytic release of heme may induce catastrophic HbS polymerization and thus vaso-occlusion. Thus autocatalysis, a source of non-linearity, may explain extensive variability in clinical manifestations among SCD patients.
7. Two major consequences of hemoglobin mutation in SCD patients – a) HbS polymerizes and b) HbS autoxidizes and readily releases heme. For the first time, a possible physiological link coupling the two major consequences with known SCD associated oxidative stress has been established through autocatalytic release of intraerythrocytic free heme.
8. We propose **intraerythrocytic free heme as new target** for sickle cell therapies.

Thus our work paves a new avenue for alternative therapeutic strategies for sickle cell anemia patients integrating physico-chemical parameters with molecular mechanisms of the disease pathophysiology.

CHAPTER

8

MICROFLUIDICS & SICKLE CELL RESEARCH

The modification of environment into micro space and volume representing similar physical, chemical and kinetic behavior of the system is the basis of microfluidic manipulation of any kind. The necessity arising from low volume generation by various systems & their low detection level and the requirement of handling as less of the reagents as possible for experimental works are the very bases of microfluidic devices fabrication [263, 264]. The evolution of micro electrical mechanical systems (MEMS) technology also induced the search for ways to manipulate the applicability of such systems within the experimental setups which has enabled the scientific community to come up with means to manipulate systems with low volume characteristics and also to help with the analysis and detection in these systems. The devices which actually play with the volume of fluid of around 10^{-9} to 10^{-18} liters need to be of dimension in micrometer range in one direction [264]. This validates the naming of these devices or systems as microfluidics [263]. So the microfluidics is the science of fluid motion behaving differently from traditional fluid mechanics associated with the small length scale [264].

The microfluidic system can be viewed as a combination of miniature unit operation modules which are integrated in one platform to ensure feasible handling and compactness of a process rather using multiple conventional units to process and analyze data. The potential of microfluidic devices as parallel processing capable, high throughput-low cost system [265, 266] for analysis of biological, chemical and physical processes and

well established development technology of MEMS have contributed to its rapid development and usage during last three decades [263]. The advancement of microfluidic devices with integrated analytical systems are known as μTAS (micro total analytical system) [266] or lab-on-chip (LOC) [265].

Though LOC platforms are being developed extensively in many aspects of scientific research and commercial products, majority of the platforms are helping biotechnology and biological development [267] such as molecular and cell biology [268], synthetic biology [269, 270], systems biology & medicine [271], bioanalytical systems [272-274], point-of-care diagnostics [275, 276], drug delivery [277], single cell manipulation [278, 279], regenerative medicine [280], cancer research [281]. Here we accumulated examples of review articles published in the field of microfluidics during 2013-2015. These articles were found by performing a search in Web of Science for review articles tagged "microfluidics" from 2013 till March 12 2015. More than 50% of the articles that the search returned were about the biological, biomedical and biotechnological application of LOC platforms. We tried to categorize a few of the examples in the following table to show the extent and variation of the research that are enabled by microfluidics LOC platforms. The list provided in Table 8.1 is just a glimpse of the extensive research efforts being pursued in the integrated field of biotechnology and microfluidics.

Table 8.1 Examples of review articles published between January 2013 and March 12, 2015 which encompass microfluidic advancement and usage in broad sectors of biomedical, biological and biotechnology research.

Omics research	Metabolomics [282, 283]; Single cell genomics [284, 285]; Transcriptomics [286]; Proteomics – integrated with mass-spectroscopy [274, 287, 288]
Bioanalytical systems	Membrane proteins [289]; Biomarker analysis [290-292]; Single cell protein analysis [293], Microbioreactors [294]; Single molecule detection [295]; Enzyme kinetics [296]; Immune response [297, 298]
Biosensors, detection and point of care diagnostics	Infectious diseases diagnosis [292, 299-303]; Paper based biosensors [304-308]; Noninvasive prenatal diagnostics [309, 310]; Different sensor technologies for diagnostics [311-315]; Single Cell diagnostics [316]
Synthesis & engineering of particles and biological macromolecules	Barcode particles [317]; Drug delivery particles [318, 319]; Functional materials for biomedical & biochemical applications [320-323]; Protein Crystallization & nucleation [324, 325]; PET tracer [326, 327]
Stem Cell Applications	Differentiation, mechanobiology & analysis platform [328-332]; Stem cell derived therapies [333, 334]
Animal models	Neurodevelopment studies with *C. elegens*, Zebra fish and Fruit flies [335, 336]
Biomimetic Physiologic Environment	Organs on chip [337-339]; 3D cellular & Tumor microenvironment [335, 340-342]; Respiratory & vascular tissue models [343-348]
Nucleic Acid Analysis	Extraction & analysis [349-352]
Single cell analysis and Cell-Cell interaction	Single cell biophysics [353, 354]; High throughput analysis [355-358]; Systems immunology [359]; Cancer Research [333]; Plant cell analysis [360]; Microbial Ecology [361]; Chemotaxis Studies [362]; Cell manipulation [278, 279]
Drug delivery, discovery and therapeutics	Crystallization [363]; Droplet-based production [364]; Personalized medicine [365]; Stem cell derived therapeutics [334]; Cancer targeted drug delivery [366]; Cell encapsulation & emulsion formation for drug delivery [318, 367]; *In vitro* drug screening [368]; Nanomedicine [369]; Dose-response study [370]
Cancer Research	3D cancer model [281, 371-373]; Diagnostics [291, 333]; Therapy [333, 366]
Cell separation and cell sorting	Electrophoresis based separations [374, 375]; Methods & challenges [376]; Blood cell sorting and function analysis [377, 378]

Sickle cell disease and microfluidics

Like all the brunches of biological and biomedical research, sickle cell research has also been facilitated by microfluidics development. The current approaches to use LOC platforms sickle cell research are diverse. They include LOC based genotyping [379, 380], microfluidic model of sickle cell vaso-occlusion [381-387], diagnostics [388, 389], study of cell-cell mechanics, physics and pathogenesis of sickle cell disease [390-394] and assessment of drug efficacy [395]. Thus microfluidics is playing a vital role in deciphering questions regarding sickle cell pathophysiology.

Microfluidic cell separation and sickle cell research

In venous low oxygen pressure in sickle cell patients, the variation in the percentage of sickled cells is observed [396]. In 100% deoxygenated condition, theoretically it is expected that 100% homozygous sickle erythrocyte should deform; but investigators found that at most 80% of the cells are sickled [personal communication with Dr. J.M. Higgins, 2013]. Thus, separation of sickled blood cells from unsickled cells is crucial for investigating the biophysical and molecular factors and to find out relevant biomarkers that demonstrate propensity of sickling. We were able to find only two investigations from separation of sickled cells from unsickled and one of them is based on neutral density separation [397] and other one is based on deformability using gel filtration [398].

Microfluidics has become important tool in separation of small particles and cells which rely on different separation techniques [399] such as using microfluidic filters [400], partitioning flow using hydrodynamics at low Reynolds number, size-based deterministic lateral displacement [401], flow-field fractionation using parabolic velocity profile [402], vector chromatography using in flow lateral obstruction [403], inertial microfluidic

separation [404], gravitation and sedimentation based separation [405], biomimetic separation [402], microfluidic magnetophoresis, aqueous two phase system (ATPS), separation using acoustic radiation force, dielectrophoretic cell separation etc. [406, 407].

Though there have been several efforts using microfluidics to separate blood cells, components and parasites present in whole blood [400, 407-413], there has been only one example of separation of red blood cells depending on difference in deformability for malaria infected cells [402].

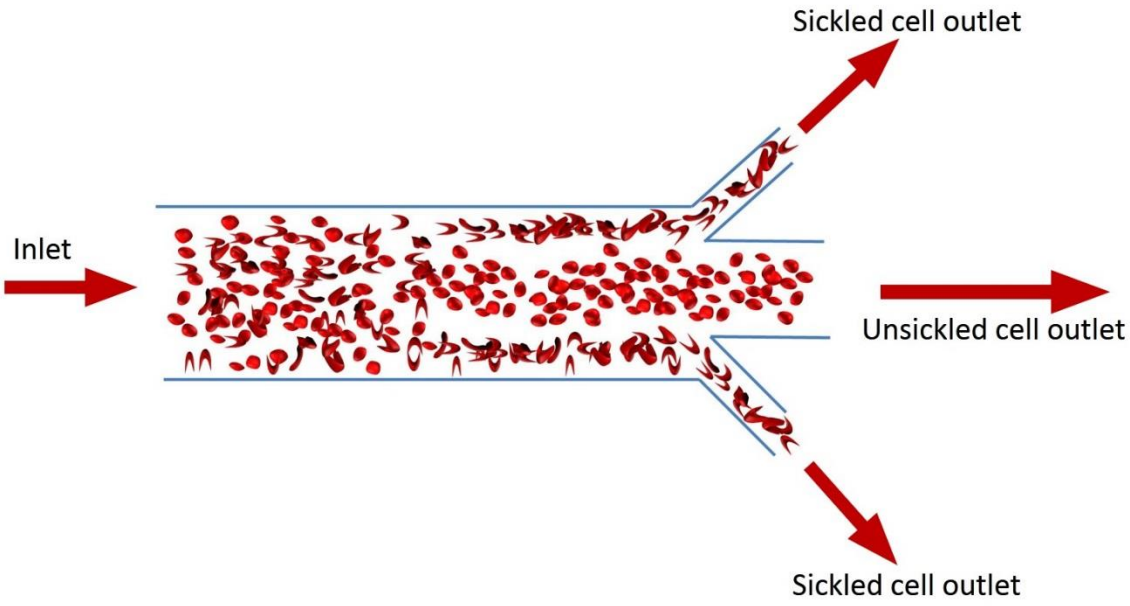

Figure 8.1 Schematic of cell separation principle using margination flow.

Based on parabolic flow profile and biomimetic flow margination [414-416], infected malaria cells were separated with 80-90% purity [417]. So we intend to use the same principle in a microfluidic device (Fig. 13) where stiff and deformed sickle red blood cells (under deoxygenation) are expected to merge to the side walls as they experience less force in flow than the deformable unsickled cells. Once the cells are separate, different biophysical parameters could be assessed.

CHAPTER

9

MICROFLUIDIC DEVICE FOR SEPARATION OF SICKLED & UNSICKLED ERYTHROCYTES

We set out to design and build a microfluidic device for separation of sickled and unsickled cells from whole blood using previously described flow margination technique. To establish the parabolic velocity profile and hydrodynamic separation using induced Fahraeus-Vejlens effect [414] and difference in deformability of sickled and unsickled red cells (under hypoxic conditions), we used the following design considerations:

1. Our design of channel contains one single entrance (width ~ 100 μm) for the buffered red cell solution (devoid of plasma) which narrows down to a 30 μm wide 3-cm long stretch of channel. The long stretch would provide sufficient time for the cells to be subject to margination and be separated – more deformable unsickled cell going into the central flow and less deformable sickled cells marginating towards the wall.
2. The height of channel is kept to 10 μm to eliminate the possibility of margination along the top and bottom wall of the microfluidic chip. At the end of the 3-cm stretch wider stretch (~100 μm) for visualization of separation.
3. The 100 μm end stretch will then be divided into three diverging outlet channel – the middle one being ~60 μm wide and the two others being ~20

μm wide each. The outlets will be connected to microtubes to collect the samples.

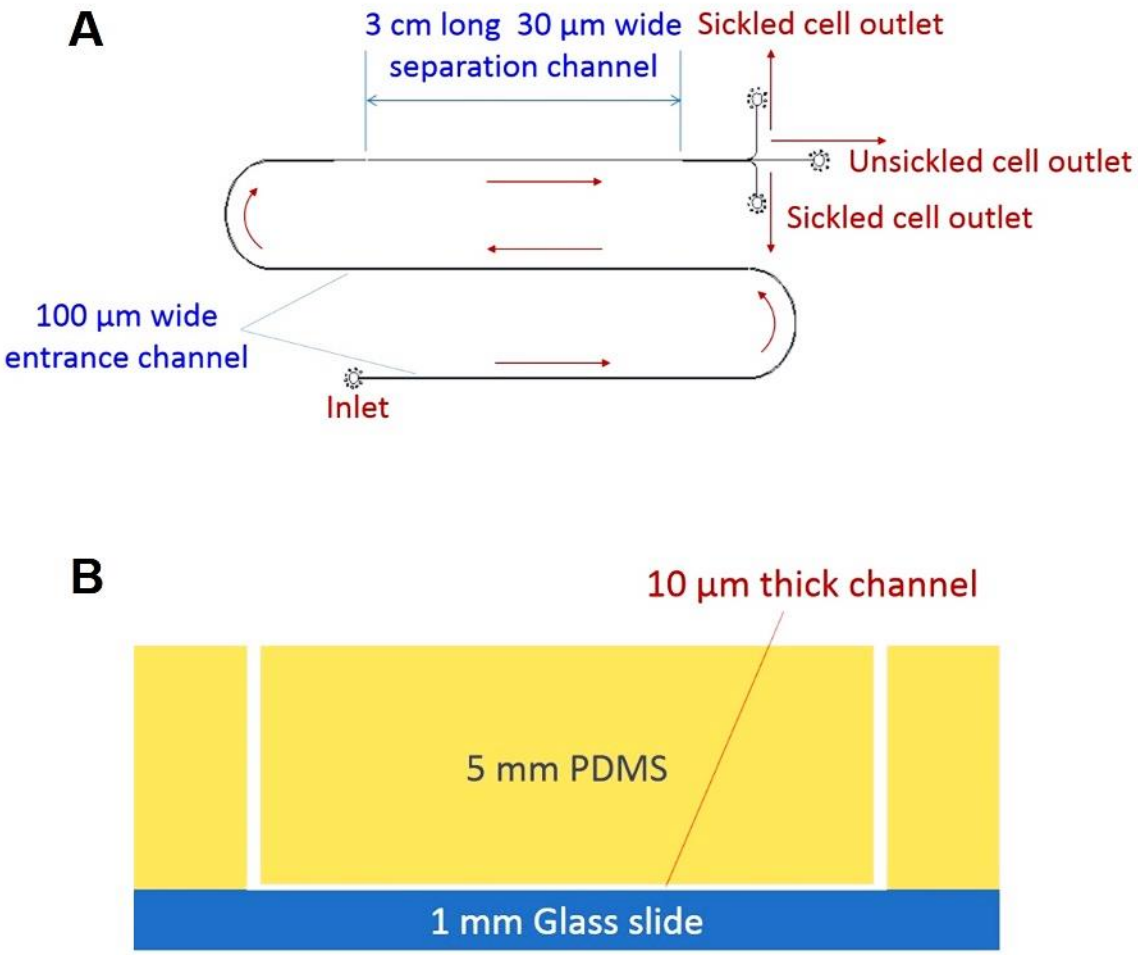

Figure 9.2 A. Microchannel design for the margination flow-based cell separation device. B. Schematic of PDMS-Glass microfluidic device thicknesses.

4. The microfluidic device needs to be biocompatible and transparent; thus we will build the device from thick (~5 mm) PDMS (polydimethylsiloxane) [418, 419].
5. Deoxygenation of cells will be carried out beforehand and deoxygenated buffered red cell solution will be used for the separation. Due to high flow velocity and very low oxygen diffusion time in the thick PDMS layer, we expect to maintain deoxygenated condition in the device during the separation.

As we chose to use PDMS as the structure material for our microfluidic device, we needed to use soft lithography [420, 421]. Soft lithography is a process of fabricating micro and nano structure by replica molding using a curable elastomeric material on a negative master generated by photolithography or other lithography techniques [422]. The process of rapid prototyping of PDMS devices requires three major processes to be involved in our case:

1) Preparation of SU-8 master mold on Si substrate using photolithography
2) PDMS casting and curing on SU-8 master mold – soft lithography
3) Pealing PDMS mold after curing and bonding to glass slide

These three processes lead to manufacturing of a PDMS-glass microfluidics device and adding ports to the inlets and outlets provides a working device.

9.1. SU-8 master mold preparation

9.1.1. Photolithography

Patterning a design (geometry defined on masks) using UV radiation on photosensitive material is known as photolithography – the most used technique for pattern transfer in MEMS and microfluidic industry. The photosensitive polymer material which is exposed to radiation [423] for pattern formation is known as photoresist. Photoresists go under chemical reaction upon UV irradiation. Following the chemical reaction – when submerged in developer solution – the exposed part on the resist can either dissolve away (Positive photoresist) or the rest can dissolve (Negative photoresist). The name "Photolithography" is derived from the resemblance of the process with photography [263].

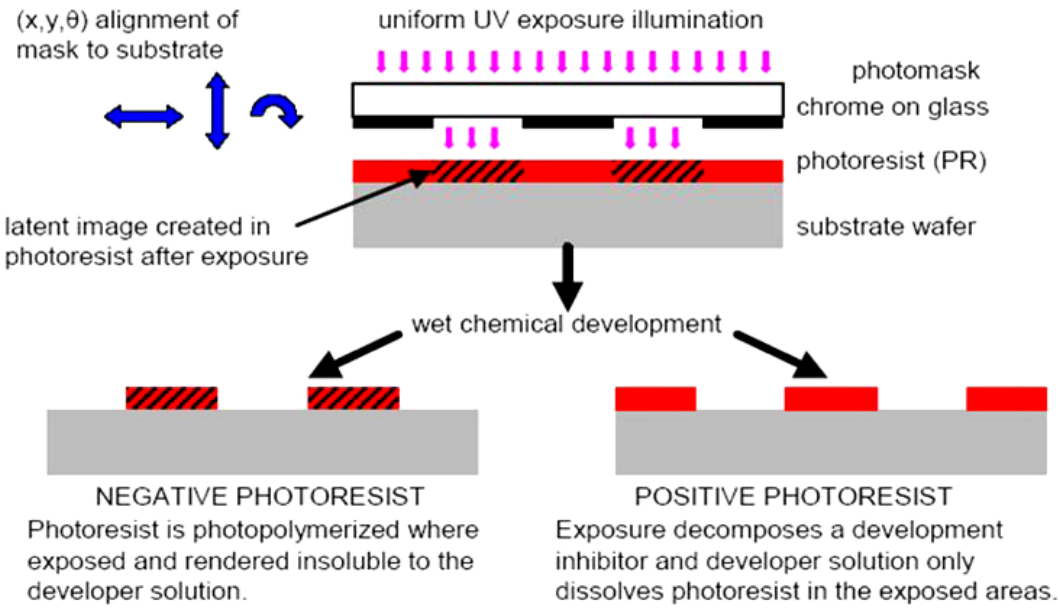

Figure 9.3 Distinction between positive and negative photoresist pattern formation.

9.1.2. SU-8 chemistry and recipe for channel fabrication

SU-8 photoresist (introduced by IBM) [424], a widely used class of negative photoresists [425], is an epoxy-based photoresist which has low molecular weight component dissolved in a highly concentrated solvent [426, 427]. This makes SU-8 a very good candidate for attaining high aspect ratio structures while having enough mechanical stability. Upon UV exposure the photo initiator, triarylsulfonium hexafluoroantimonate salt [426, 428] dissolved in the organic solvent gamma-butyrolacton (GBL) in high concentration around 70-80%, starts strong crosslinking among the eight epoxy functional groups of Bisphenol A Novolak epoxy oligomer (Fig. 9.4) [429], named EPON resin [430], within itself and also with neighboring molecules.

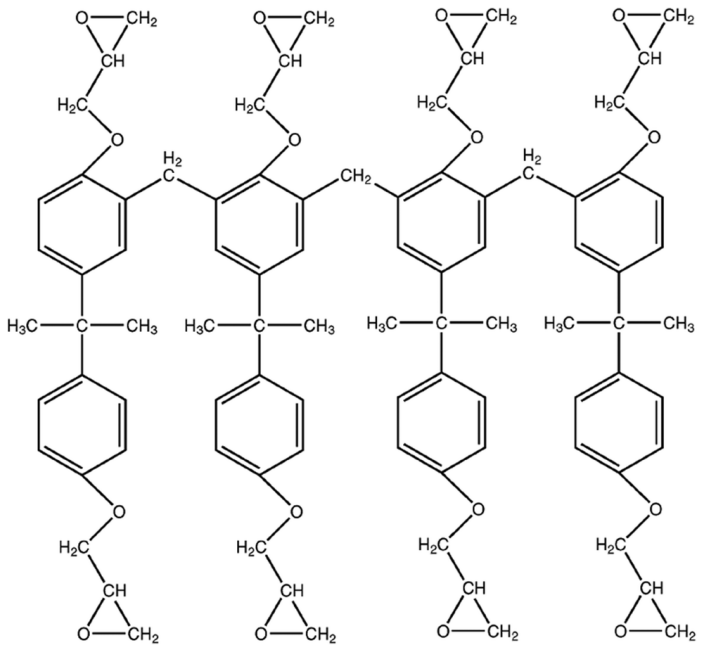

Figure 9.4 Chemical structure of SU-8 monomer.

The processing of SU-8 3005 (from MicroChem Corp.) on 100 mm Si wafer (purchased from University Wafers) substrate is as follows:

A. Substrate preparation: Substrate preparation is the first crucial step because dust and contaminants usually interfere with adhesion of the photoresist to the substrate. In the UH Nanofabrication facility we rinse the Si wafers with acetone and DI water just before using. A crucial parameter for optimizing the adhesion property of SU-8 layer to substrate is dryness of the substrate [431]. The moisture present in the substrate may reduce adhesion between the substrate and SU-8 layer. Thus we heat the substrates at 150°C for 10 minutes as per literature [431].

B. Photoresist Coating: We use Brewer Cee 200 spin coater at UH Nanofabrication facility to deposit ~10 μm thin film of SU-8 layer on the Si substrate following recipe: a. 500 rpm @ 100 rpm/s (10 sec), b. 750 rpm @ 500 rpm/s (30 sec).

Figure 9.5 Brewer Cee 200 spin coater in UH nanofabrication facility.

C. Soft bake: Soft bake is performed to densify the SU-8 layer i.e., to remove the solvent from the SU-8 layer. This is a very important step which has important influence in the resolution and structure of the features on the layer [432]. Soft baking step allows the SU-8 to reflow as T_g of uncrosslinked SU-8 is 55°C and self planarize the layer and for this reason the flatness of the hot plate is desired. We used two step soft bake: 65°C/1min and 95°C/5 min.

D. Exposure: Exposure to near UV spectrum of 365-436 nm is the key to initialization of the cross linking on the polymer layer and pattern formation according to the mask prepared. As soon as the layer is exposed the photo initiator decomposes to produce a photoacid [430] which in turn protonates the epoxides on the oligomer. The crosslinking reaction is initiated at this step which is enhanced further in the post exposure bake step. The exposure enhances the adhesion between the substrate and SU-8 layer.

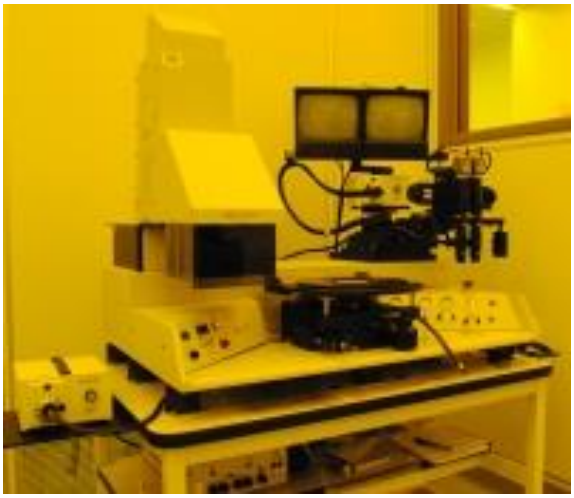

Figure 9.6 Kasper mask aligner in UH nanofabrication facility.

Overexposure may lead to backscatter which will expose unintended region; on the other hand underexposure may lead to peel off during development [432, 433]. These defects can be reduced by using a filter [434] to limit the band width of UV-light that is used for exposing the film. The filter only passes the UV light above 350nm and hence reduces exposure non-uniformity inside the film. In our case, we are using the filter and exposing the SU-8 layer for 75 s which is much longer than the exposure time 15 s when we did not use the filter.

E. Post exposure bake: As the kinetics of the crosslinking is very slow at room temperature, during post exposure bake the increased mobility of the SU-8 molecules (at higher temperature) helps to crosslink themselves better. The crosslinking is catalyzed by the photoacid which helps the SU-8 molecules to form epoxide bonds among themselves and with neighboring molecules [435]. Longer PEB enhances crosslinking; on the other hand contributes to the residual stress if not controlled carefully [436]. To avoid adhesion problem and reduce cracked area [431], primary heating at 65°C near T_g of SU-8 is

conducted. In our experiment, we do PEB at 65°C for 1 minute and at 95°C for 10 minutes. Optimizing this step is the key to have good working samples.

Table 9.1 Recipe for SU-8 master mold preparation.

Process	**Recipe**
Cleaning	1. Acetone and DI water rinsing 2. Drying with air
Dehydrate	20 min (150°C)
Spin Coating	500 rpm @ 100 rpm/s (10 sec) 750 rpm @ 500 rpm/s (30 sec)
Soft bake	65°C/1 min 95°C/5 min
Exposure	75 s (@ 33 mJ/cm^2-s)
Post Exposure Bake	65°C/1 min 95°C/10 min
Development	Keep in SU-8 developer 4 min
Rinse and dry	IPA followed by air drying

F. Development: The development of the image is done by washing with PGMEA (propyleneglycol monomethylether acetate**)** [437]**.** Developer solution diffusion depends on the time spent by the structure within the solution and also on the temperature of the solution [430]. We dip the wafer in developer solution for about 4 minutes and then take it out and rinse with Iso-Propanol.

The prepared SU-8 master depicts negative image of the channel design. Once the SU-8 master mold is prepared, PDMS can be cast on the mold and cured to get the desired channel structure.

9.2. PDMS Soft lithography

Polydimethylsiloxane (PDMS) is a widely used elastomer combined with a crosslinker and is popular as structure material of microfluidic devices. Since its first use in 1993 [438] for elastomeric stamp-pattern transfer (now known as "softlithography"), it has just become the popular choice for microfluidic device fabrication and other softlithography applications due to its rapid prototyping capability, transparency, biological and chemical compatibility [419]. Because PDMS pre-polymer is liquid in room temperature and can freely flow, it can reach around and across every possible micro and nano-scale features and replicate features of those length-scales upon crosslinking.

PDMS is a thermoset polymer which has a silicon-based organic repeating unit $(CH_3)_2SiO$. The pre-polymer is viscous liquid in room temperature and undergoes a crosslinking reaction at elevated temperature. To enable the crosslinking reaction, some of the methyl ($-CH_3$) groups attached to the silicon atom are replaced with hydrogen (H) atoms or vinyl groups ($-CH_2=CH_3$). Dow corning's Sylgard 184 comes as a two part pre-polymer: a) the base containing vinyl terminated groups and b) the curing agent containing hydrogen terminated groups and platinum catalyst. In the presence of a platinum based catalyst through an exothermic reaction, the hydrogen atoms and vinyl functional groups combine and crosslink while the methyl groups remain inert. Once the prepolymers are mixed together in proper ratio (suggested 10:1 by Dow corning) and heated to an elevated temperature ($\sim 65 - 75$℃), PDMS goes under fast crosslinking reaction and the cross linking usually takes less than three hours. Based on the ratio of the base and curing agent, the structural property of PDMS layer changes. High ratio of base and curing agent leads to soft layer and the hardness increases with increasing amount of curing agent.

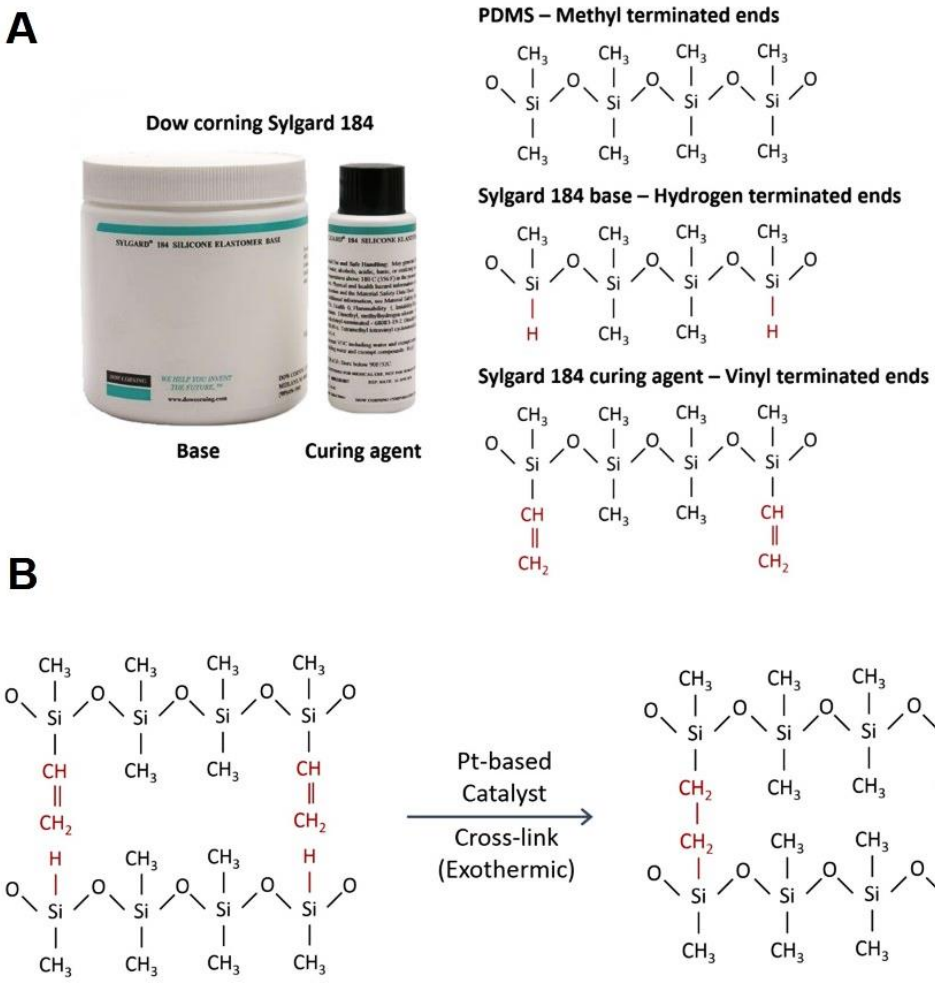

Figure 9.7 A. PDMS monomer and pre-polymers in Dow Corning Sylgard 184. B. Crosslinking reaction of Sylgard 184 base and curing agent.

9.2.1. PDMS processing

1. We start the process of casting PDMS on mold in the nanofabrication facility to avoid any types of dust particles that may be found in principal lab conditions. We use a small weigh boat on a weighing scale to put 30 g of Dow Corning Sylgard 184 PDMS base polymer and 3 g curing agent in the boat. Then we mix the PDMS base and curing agent vigorously using a stirrer.

2. After mixing of the PDMS-curing agent, we put the weigh boat in a vacuum chamber under high vacuum to get rid of the bubbles. We leave the vacuum pump running for about 30-40 minutes. It is important that there is no bubble on the surface of the PDMS layer in the weigh boat when we remove it from the vacuum chamber.
3. We place aluminum foil in a high petridish and put the SU-8 master mold Si wafer in there having the channel structure facing up. Now we pour the mixed and degassed PDMS mixture on top of the wafer. We also try to make sure, there is no bubble formation when we pour the PDMS on the wafer.
4. Next we put the PDMS casted wafer in a preheated convection oven at 75℃ and cure it for at least 2 hours. We keep the petridish covered with aluminum foil to avoid dust in the convection oven. Thermal curing takes place through formation of crosslinks and the PDMS layer solidifies. Higher curing time can also be tried, but we have it optimized it for two hours.
5. After crosslinking, demolding of PDMS layer is carried out manually. We cut the piece of PDMS with a utility knife and debond from the mold. Thick PDMS layers (~ 1 – 5 mm) can be demolded easily and the structures do not under any change because PDMS can take large strains. In case of very thin (~ 200 – 600 μm) layers, the demolding is very difficult due to high demolding stress and it can lead to tearing of the membranes. This is the last step before the bonding process to form the closed channel microfluidic device.

9.3. Bonding of PDMS to Glass and microfluidic assembly

Bonding of PDMS surface containing the channel to glass provides a complete seal to fabricate the final microfluidic device. The bonding is usually performed by PDMS and glass surface treatment with oxygen or air plasma. PDMS surface is hydrophobic in nature due to presence repeating units of -O-Si(CH_3)$_2$- groups; so to bond it to glass, it is required to eliminate the hydrophobic groups and air/oxygen plasma can produce silanol (Si-OH) groups to induce hydrophilicity on the PDMS surface.

1. We wash the glass slide to be bonded with acetone and rinse with DI water to remove dust particles. Then we put the slide on a hot plate to dehydrate it for 10 minutes at 120℃.
2. Next we clean the Oxford's RIE 80 plasma machine with oxygen cleaning recipe to make sure that the chamber do not contain any contaminant that can interfere with the bonding of the PDMS and glass.
3. During cleaning, we punch holes with biopsy punches for inserting tubings the inlet and outlet positions in the PDMS layer.
4. After cleaning of the chamber, we take the demolded PDMS membrane and glass slide and put them in the RIE chamber – the sides to be bonded facing up. We run the optimized recipe for plasma activation of the PDMS and glass surface – O_2 process flow – 36 SCCM, pressure – 700 mTorr, RF power – 20 W, time – 30 sec.
5. After the end of the cycle and venting of the chamber, we immediately align the surfaces and press to bind them together. There should not be any wait

time for the bonding as the in the ambient environment the PDMS losses the hydrophilicity extremely fast.

6. After attaching the PDMS and glass manually, to strengthen the bonding, we put the assembled device in a convection oven at 95℃ for about five (5) minutes.

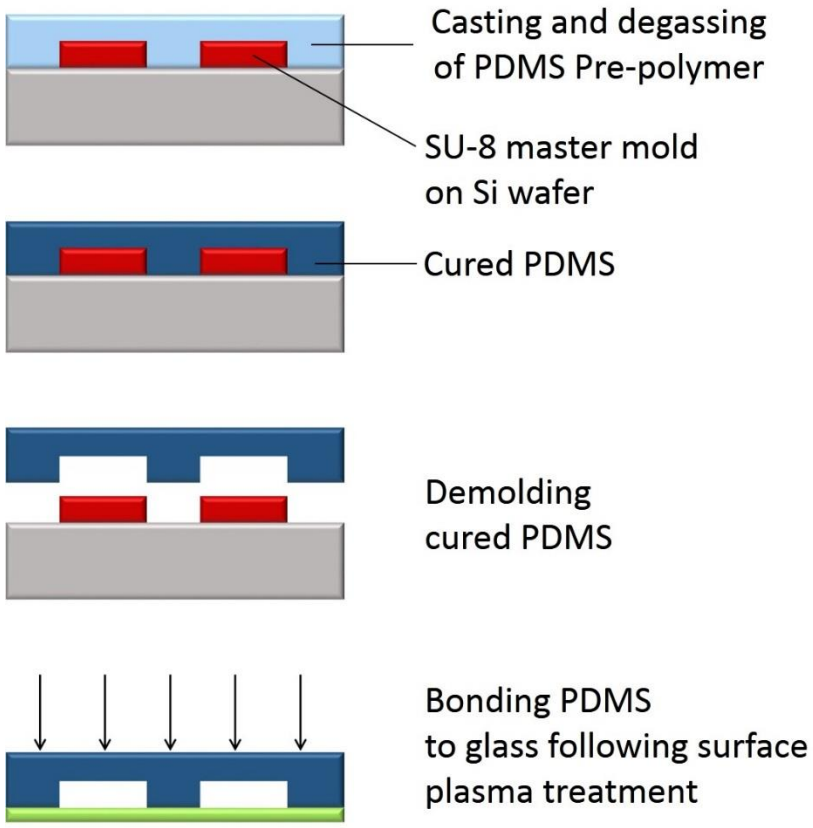

Figure 9.8 PDMS processing and bonding of cured PDMS with channel features to glass.

7. After we bring out the assembled device from oven, we put a stainless steel tubing that fits the hole in the inlet port. To strengthen the inlet port (so that it can sustain high pressure flow) we cut out a cured PDMS piece and make a hole with diameter at least 50 mm larger than the diameter of the tubing.

Next we plasma activate the PDMS cut-piece and the microfluidic device and attach the PDMS piece with the device around the inlet. The hole in the PDMS piece is filled with uncured PDMS pre-polymer and then the device is placed in an oven to cure. The PDMS cures and provides strong support for the inlet port.

Thus bonding of PDMS and glass and subsequent assembly of the ports lead to construction of final working device. Then we put the device in refrigerator in laboratory and keep till we are ready to use it.

9.4. Testing of the device

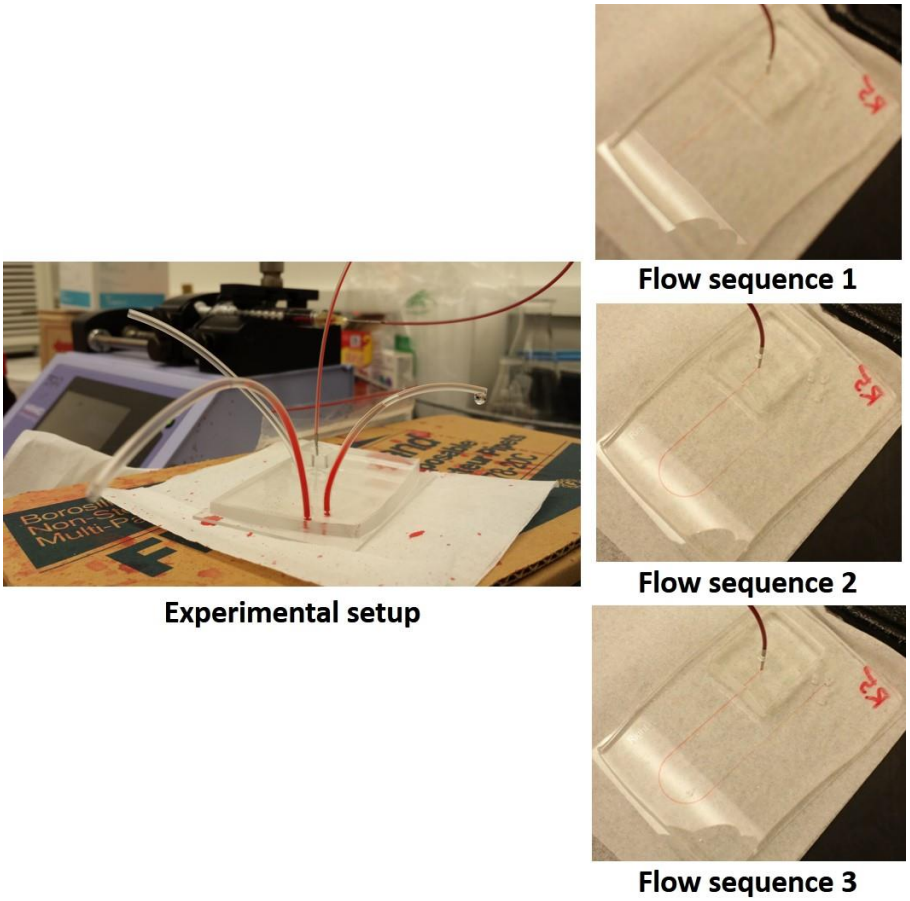

Figure 9.9 The left figure shows the experimental setup consisting of a pump and the microfluidic device. The right panel shows sequence of food dye flow in the microfluidic device.

We tested the assembled microfluidic device only to check the flowability – but not for the actual function of separating the cells based on deformability. The flow is induced by attaching a micro-volume dispensing pump and the flow is observed in the device using food dye and whole blood as flow medium as illustrated in Fig. 9.9.

The designed microfluidic device is yet to be tested for its actual purpose which is to separate the cells based on the deformability. The first test would be to try separating a model system where red blood cells will be mixed with fluorescent microspheres having the same sizes as the blood cells. The microspheres will act as less deformable cells and should marginate during the flow to channel walls.

CHAPTER 10

FUTURE WORK

In our work to study free heme concentrations in human erythrocytes, we have identified heme to have an important biophysical effect in the event of sickle hemoglobin polymerization and its associated oxidative stress. The latter work involving microfluidic separation of sickled and unsickled red blood cells is an unfinished one, but the proposed technology provides a promising avenue for identifying biomarkers in sickle cell disease. In light of these insights, here I envision avenues that will provide further elucidation about the mechanism in play and may assist in evaluation of biomarkers in SCD.

10.1 Microfluidic Cell Separation

The preliminary work has been done on the design and fabrication of the microfluidic device. The next step is to first validate the separation principle and then use the device for actual separation experiment:

1. To validate the separation capability of the device, I propose use of fluorescent polystyrene microparticles of similar size (~ 6 μm) as red blood cells in the solution with healthy red blood cells as a model system where the stiff microparticles will be acting as less deformable sickled cells and healthy red cells will act as a model for unsickled cells.
2. In our design by conservative estimate we assumed that the flow velocity of the deoxygenated blood will be sufficient to avoid oxygenation. We also

propose incorporation of an oxygen sensing layer in the microfluidic device design [439] so that oxygen saturation can be properly monitored.

3. If the oxygen saturation of deoxygenated solution cannot be kept at zero level during flow in the microdevice, we propose a modification of the device by introducing a two layer system. The new design will incorporate a deoxygenation channel above the microfluidic channel through which inert gas will be flown and the design will further contain a 150 μm PDMS membrane in between the microfluidic and gas channels to facilitate the deoxygenation. The long approach path in the entrance in current microchannel design will act as deoxygenation zone.

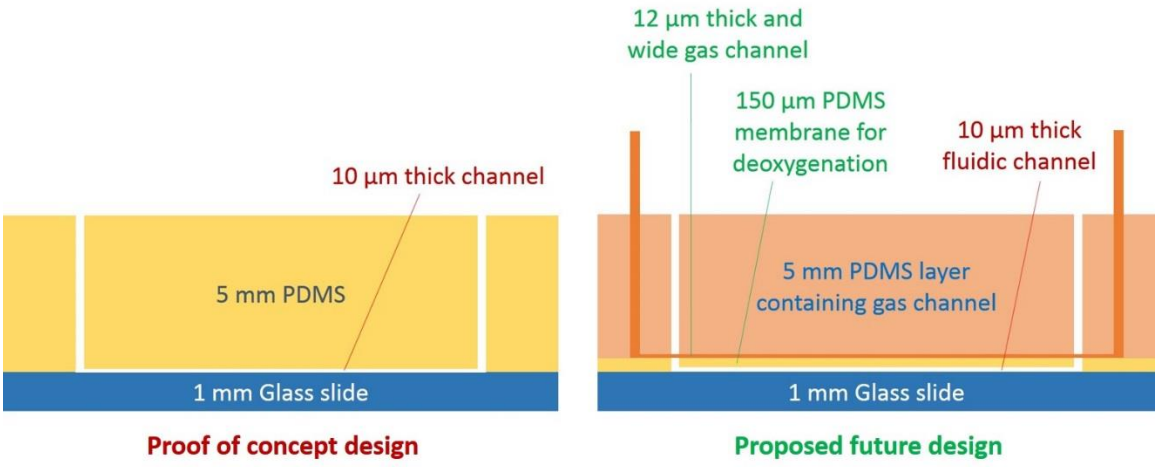

Figure 10.1 Side-by-side schematic of current proof of concept design and proposed design of deformability based margination flow induced microfluidic cell separation device.

Incorporation of such improvements may be necessary following test runs with sickle blood. They will allow more control over the experimental parameters. The completed microfluidic cell separation will facilitate possible characterization of

differences between sickled and unsickled cells during 100% deoxygenation and can lead to possible biomarker detection relevant to SCD pathogenesis.

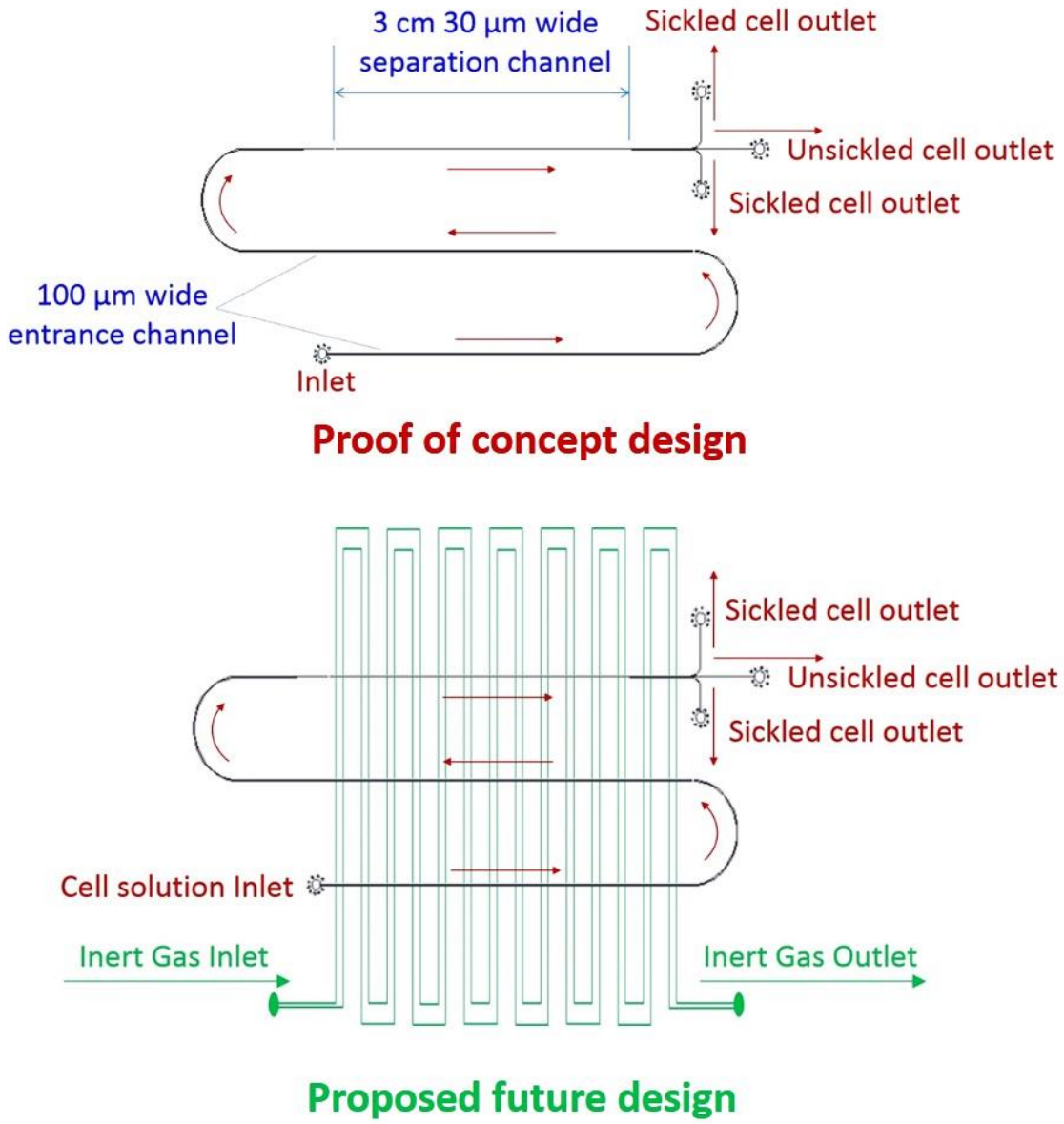

Figure 10.2 Comparison of current and proposed design of microchannel in deformability based margination flow induced microfluidic cell separation device.

10.2. Biomarkers in SCD and Free Intraerythrocytic Heme

"*A joint venture on chemical safety, the International Programme on Chemical Safety, led by the World Health Organization (WHO) and in coordination with the United Nations and the International Labor Organization, has defined a* ***biomarker*** *as "any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease*"" [440]. Thus biomarkers are important to provide information about state and/or progression of disease pathology in a patient. More than 100 biomarkers have been identified for sickle cell disease – a few examples of which are shown in Table 10.1.

Table 10.1 Examples of biomarkers in sickle cell disease.

Pathophysiology of SCD	**Related Biomarker**	**Comment (ref.)**
HbS Polymerization	Rate of polymerization	Surprisingly NO direct biomarker has been found
	HbF levels (Y), MCH levels (~)	[1]
Red Cell Dehydration	Density based separation (Y), Irreversibly Sickled Cells (ISC) on blood film (N)	Visual diagnostics by separation in aqueous multiphase solution (GW group) [441]
Increased Red Cell Rigidity	Red cell filterability (~), Whole blood viscosity (Y), Red cell deformability (Y) – Ektacytometry, Optical Tweezers, Visoelasticity	[442-444]
Induced Adhesion	Red cells (Y), White Cells (Y), Soluble endothelial adhesion molecules (Y)	[445-450]
Many more biomarkers have been identified relating features of SCD pathology: inflammation, hemolysis, anemia, hypoxia, oxidative stress, reperfusion injury, hypercoagulability, NO metabolism, vasculopathy, endothelial dysfunction, damages to specific organs etc.		

Y = significant, N = insignificant and ~ = inconclusive. [5]

As can be seen from Table 10.1, it is quite surprising that even after identifying more than 100 biomarkers, there is **NOT** a single biomarker that can be correlated directly with rate of HbS polymerization (primary pathogenic event of sickle cell disease). In the view of our investigation regarding autocatalytic release of intraerythrocytic heme and its possible direct effect on HbS polymerization, we can propose that levels of free in red cell cytosol in sickle cell patient can probably be used as biomarker for disease progression and severity in relation to rate of HbS polymerization. It is important that intraerythrocytic free heme concentrations in patients treated and untreated with different therapeutic interventions be assessed for any relevant correlation. If the levels correlate directly with pathological conditions of patients, a new avenue for assessing disease progression in SCD can be obtained. It is imperative that we understand the promising aspect of such correlation in clinical diagnostics scenario and thus a lot of groundwork to assert or decline substantial role of intraerythrocytic free heme as a biomarker is necessary. ***Three possible avenues*** to confirm such role of free heme are discussed below.

10.2.1. Free heme induced ROS activity in SCD erythrocytes

Oxidative stress due to inherent high level of reactive oxygen species (ROS) generation is a primary feature of sickle cell disease [128, 245, 451] and we have analyzed and proposed a possible link between the oxidative stress and autocatalytic release of free heme. There are numerous ways to evaluate ROS generation in cell cultures [452-454] and myriad of biomarkers related to oxidative stress in different disease pathology have been identified [455]. There have been some studies for assessing biomarkers linked to oxidative stress in SCD which relates lipid peroxidation products [456, 457], glutathione system [458] and NADPH oxidase [249]. Thus it is important that a cellular ROS generation assay

is performed on sickle and healthy red blood cells in presence and absence of heme in solution in controlled environment (hypoxic and oxygenated conditions). The information gained from such experimentation will provide insight to possible role of free heme as biomarkers for oxidative stress in sickle cell disease.

10.2.2. Effect of antioxidants on autocatalytic release of heme

Though the oxidative stress associated with sickle cell anemia has been a target [128, 246, 451, 459] for remedy since 1986 [460] and several antioxidant treatment strategies originating from nutritional approach [459, 461-465] have been proposed so far, the complex interdependent mechanism of molecular and enzymatic factors affecting the oxidative stress and antioxidant effects of conventional drugs such as hydroxyurea in sickle cell disease are yet to be understood. The free heme perspective should be added to the list and we intend to carry out experiments on the autocatalytic process in presence of already-in-use anti-oxidants and conventional drugs from the following list: hydroxyurea, vitamin C (ascorbic acid), vitamin E, glutamine supplements, N-acetylcysteine, α-lipoic acid, acetyl-L-carnitine, omega-3 fatty acids and cocktail of these supplements [463-465]. The evolution of free heme in presence of anti-oxidant molecules will provide insight to the current understanding of oxidative stress in sickle cell anemia.

10.2.3. Portable microfluidic device for diagnostics and detection of free heme levels

As we set out to use microfluidic technology for sickle cell separation and assess the biomarker-like characteristics of free heme in SCD, a diagnostics and detection tool based on microfluidic separation coupled with heme detection assay components can

be developed. This project will be elaborate and will require incorporation of several components namely: a) microfluidic separation of red blood cells, b) compartment containing separated cells, c) a membrane for separation of free heme from separated red cell solution, d) a preloaded assay for detection of heme levels dialyzed from red cell solution and e) an actuation system for the operation of the device. The idea of such a device is very preliminary and a more detailed layout and feasibility of such device needs to be assessed before embarking on this technological endeavor. It is possible that manufacturing of such diagnostic device will become viable through industry collaboration only if free heme in red cell cytosol proves to be an efficient biomarker in sickle cell disease pathology.

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