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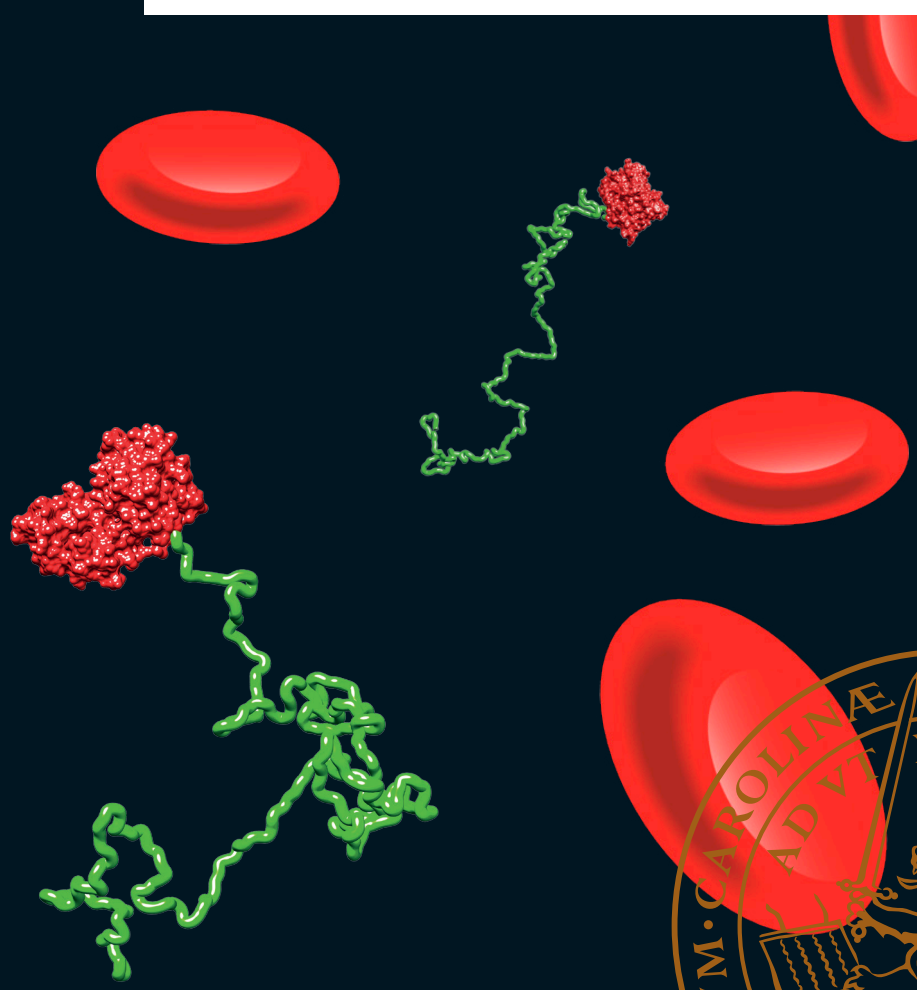

Towards New Generation of Hemoglobin-Based Blood Substitutes

Sandeep Chakane

LUND
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Abstract

Blood transfusion is a clinically significant and crucial process, which saves millions of lives every year. However, shortage of donated blood and the risk of virus transmission through transfusible blood seriously affect the availability of the blood. Hemoglobin (Hb), owing to its oxygen carrying capacity, has been studied as a starting material for the development of artificial blood substitutes/Hb-based oxygen carrier (HBOC). Several kinds of HBOC products have been developed and tested for their safety at different stages of clinical trials with minimal success. The failure of such products is mainly associated with intrinsic toxicity of cell-free Hb which damages lipids, proteins, DNA and surrounding tissues. This thesis describes two approaches aiming to gain further knowledge of potential side effects of Hb molecules on genetic material. Additionally, genetic engineering approach was used as an alternative to chemical modification of Hb molecule, which is essential for the performance of HBOC product in cell-free environment.

Using the comet assay, we have evaluated the genotoxic effect of the penultimate tyrosine residues of the alpha and beta chains. Replacement of a tyrosine residue with phenylalanine, in the alpha chain (α-Y140F) has shown 40% higher DNA damage compared to wildtype HbA. However, a similar mutant on the beta chain had negligible effect on the genotoxicity of Hb molecule.

In a plasmid DNA cleavage assay, we have demonstrated that Hb itself can interact with DNA molecules and initiate their cleavage. Conversion of supercoiled plasmid DNA (sc pDNA) into open circular (ocDNA) or linear DNA (LDNA) was used to determine the DNA cleavage activity of Hb. Our investigation revealed that fetal hemoglobin (HbF) was three-fold less active than adult hemoglobin (HbA). Thus, we have proposed HbF as a potential starting material for creation of safe HBOC product.

In a second approach, we have demonstrated beneficial effects of a polypeptide tag (dubbed XTEN) genetically attached to fusion fetal hemoglobin (fHbF), forming XTEN-HbF. The main purpose of this XTEN polymer is to avoid the chemical processing such as PEGylation, which often increases the production cost. Additionally, PEGylation also impair the structural and functional properties of Hb molecule. Using XTEN polypeptide, the functional properties of a fHbF remains largely unchanged, reflected by identical oxygen affinity and absorption spectra. XTEN-HbF was produced as a homogenous mixture of product and increased the molecular size of fHbF by a factor of 2.2 folds.

In addition, we have produced fluorescent Hb, referred as GFP-HbF. It is composed of green fluorescent protein (GFP) linked to fHbF at the DNA level. The primary results suggest that the purified protein is fully functional, as reflected by spectral properties of fHbF and characteristic fluorescence of the GFP molecule. Furthermore, the adsorption properties of the molecularly imprinted polymers (MIP) have been estimated using fHbF, with or without GFP. These MIPs have a capacity to facilitate the separation and purification of Hb molecules.

Key words: Hemoglobin, adult hemoglobin, fetal hemoglobin, comet assay, DNA cleavage, protein-based radicals, XTEN, green fluorescent protein, hemoglobin-based oxygen carrier

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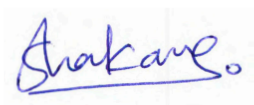
Signature  Date 28th April 2017

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Cover photo by: Sandeep Chakane, Red blood cells and genetically modified hemoglobin (XTEN-HbF).

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Dream is not what you see in sleep,
it is the thing which does not let you sleep.
Dr. APJ Abdul Kalam

Dedicated to my family and friends

Contents

Popular science summary

Why do we need blood substitutes?

Many hospitals are today often lacking a secure and constant supply of fresh blood. The most common reasons are the decreasing numbers of donors and the short shelf-life of donated blood. An increasing and aging population often requires blood transfusions in advanced surgical settings. This issue is critical in several developing nations and will most likely even worsen. Moreover, the risks of blood borne pathogens such as human immunodeficiency virus (HIV), hepatitis etc. resulting from blood transfusions, are other matters that need to be handled. Natural disasters, including earthquakes and tsunamis, always raise the requirement of clinical access to blood. In general, it is almost impossible in a catastrophic scenario to secure a rapid and safe blood supply. For all these reasons, we need a blood substitute that can perform some of the roles normally fulfilled by blood in vivo.

During the past decades, extensive research has therefore been carried out to design a suitable and alternative source of blood. Most of these approaches have been centered on the development of cell-free hemoglobin (Hb) as blood substitutes, often referred to as Hb-based oxygen carriers (HBOCs). The main goal of these agents is to restore the oxygen supply under emergency conditions or in other clinical applications when whole blood is not readily available. Such artificial oxygen carriers could greatly improve the established blood transfusion system in terms of long-term storage, lack of pathogens and availability. Moreover, this is a product that ideally can be used without prior blood typing.

Hb is an essential component of the red blood cells (RBCs). It is tetrameric protein composed of two α- and two β-subunits. Each subunit contains a prosthetic group called heme, which accommodates the iron atom in the ferrous state and gives a characteristic red color to the RBCs. The main function of the Hb is to transport the oxygen from lungs to respiring tissues. Therefore, Hb becomes the target of choice for development of HBOCs due to its oxygen carrying capacity.

The Hb molecules are well protected inside the RBC environment by an array of enzymes, which maintain the protein in a reduced state, necessary for its functionality. However, outside the RBCs, cell-free Hb can induce a cascade of oxidative reactions to surrounding cells and tissues. These oxidative reactions are

mainly linked to the iron moiety that is present in the central cavity of Hb, which also can be responsible for the generation of protein-based radicals. Therefore, previously developed HBOC products have shown negative side-effects on patients in clinical trials. However, these side-effects can be greatly reduced with different protein engineering strategies. Mutagenesis of Hb through site-directed mutagenesis may thereby improve the performance of Hb in cell-free environments. For instance, amino acid residues responsible for unwanted reactions can be replaced to form a more stable Hb molecule. Moreover, optimization of functional properties, e.g. oxygen binding, nitric oxide (NO) scavenging etc. of Hb is possible. Generally, under oxidative conditions, cell-free Hb reacts with proteins and tissues in its surrounding environment. The reactions of Hb with nucleic acids, however, have not been studied in detail. In this study, we have shown that Hb interacts with double stranded DNA and initiates its cleavage. Importantly, we observed that fetal hemoglobin (HbF) is less reactive on DNA compared to adult hemoglobin (HbA), which makes HbF a suitable alternative starting material for HBOC development.

To address the negative side-effects often encountered with HBOCs, new Hb variants have been evaluated. These designs have incorporated chemical or genetic modifications to reduce especially dimer formation of Hb. These strategies have eliminated the rapid clearance of dimeric Hb from the circulatory system and reduced toxic effects on kidneys. In addition, increased retention times (in the blood stream) have been achieved through chemical processing which also can limit the extravasation of Hb. Thus, chemically modified Hb mitigates the nitric oxide binding associated with the frequently observed vasoconstriction. We have developed a new molecular design for HBOC development through genetic modifications. An unstructured protein polypeptide tag (XTEN) was linked to fetal Hb (HbF). This strategy has enabled us to obtain large quantities of pure protein without additional chemical modifications. The structural and functional properties of HbF remained largely unchanged when linked to the XTEN polymer. This approach may be useful to develop a new generation of HBOC product by obviating negative effects related to chemical processing of the protein.

Genetic or chemical modifications may alter the performance of Hb in cell-free environments. Although various kinds of HBOC products have been developed and evaluated in certain animal models, it is important to understand the operative mechanism of such a product *in vivo*. To meet this issue, we have developed a fluorescent Hb. The designed fusion protein is composed of a green fluorescent protein linked to fusion HbF at the DNA level. The expressed protein exhibits dual properties, i.e. green and red color under UV-irradiation and normal light exposure, respectively. Purification of Hb is often a major bottleneck in the production of HBOC, and more efficient downstream processing options need to be generated. In this study, molecularly imprinted polymers (MIPs) have been evaluated for binding of fluorescent Hb to examine their binding capacity.

Populärvetenskaplig sammanfattning

Varför behöver vi blodersättningsmedel?

I dagsläget är det många sjukhus som saknar tillräcklig och trygg tillgång till donerat blod. Det sjunkande antalet donatorer och den begränsade lagringstiden för donerat blod är bland de främsta orsakerna till denna utveckling. Samtidigt krävs en tilltagande mängd blodtransfusioner för alltmer avancerade kirurgiska ingrepp i takt med en åldrande befolkning. Detta problem är än mer kritiskt i utvecklingsländer och kan på sikt förvärras om rådande omständigheter fortgår. Ytterligare problematik som behöver behandlas kring blodtransfusioner är risken för blodburen smitta från smittämnen som humant immunbrist-virus (HIV), olika typer av hepatitvirus, m.fl. Vid naturkatastrofer, som till exempel jordbävningar och tsunamis, höjs alltid kravet på tillgång till blod, och det är nästintill omöjligt under katastrofala omständigheter att garantera snabb och säker blodtillförsel. Med dessa skäl i åtanke står det klart att vi skulle behöva ett blodsubstitut som kan tillgodose livsuppehållande funktioner som normalt utförs av blod *in vivo*.

Under de senaste årtiondena har det bedrivits omfattande forskning för att komma fram till ett alternativ till donerat blod. De flesta ansträngningarna har fokuserat på utvecklingen av blodsubstitut med cell-fritt hemoglobin (Hb), ofta kallat Hb-baserade syrebärare (Hb based oxygen carriers, HBOCs). Huvudsyftet med dessa preparat är att återställa syretillförsel i akuta situationer eller i andra kliniska tillämpningar när donerat blod inte är tillgängligt. Artificiella syrebärare skulle avsevärt kunna förbättra etablerade blodtransfusionssystem med avseende på smittorisker, lagringsmöjligheter och tillgänglighet. Dessutom skulle även en sådan produkt kunna användas utan att behöva göra blodtypsmatchning.

Hemoglobin är en grundläggande komponent i de röda blodkropparna. Det är ett tetrameriskt protein som består av två alfa- och två beta-subenheter. Varje subenhet innehåller en prostetisk hemgrupp med en central järnatom som i funktionell tvåvärd (Fe^{2+}) form ger hemoglobin och den röda blodkroppen dess karaktäristiska röda färg. Huvudfunktionen för hemoglobin är att transportera syre från lungorna till kroppens vävnader, och

det är denna syrebärande egenskap som ligger till grund för valet av detta protein som startmaterial för att utveckla blodersättningsmedel.

Hemoglobinmolekylerna är väl skyddade inuti de röda blodkropparna genom en uppsättning av enzymer som ser till att proteinet med dess järnatomer bevaras i ett reducerat tillstånd. Detta är nödvändighet för att bibehålla proteinets funktionalitet. Om proteinet därmot befinner sig utanför den röda blodkroppen kan hemoglobin inducera en kaskad av oxidativa reaktioner i de kringliggande vävnaderna. Dessa oxidativa reaktioner är huvudsakligen kopplade till järnet i syrebindningsfickan, vilket vars reaktioner också kan ge upphov till proteinbaserade radikaler. På grund av detta har föregående hemglobinprodukter påvisat negativa bieffekter hos patienter i kliniska försökstudier. Sådana bieffekter kan emellertid motverkas och reduceras avsevärt genom olika proteintekniker. Mutation av hemoglobin genom lägesspecifik mutagenes kan förbättra prestationsförmågan hos proteinet även i extracellulär miljö. Exempelvis kan aminosyror som är ansvariga för icke-önskvärda reaktioner bytas ut för att skapa en mer stabil hemoglobinmolekyl. Dessutom finns möjligheten att optimera funktionella egenskaper som till exempel syrebindning, kvävemonooxidbindning, m.m.

I allmänhet kan hemoglobin reagera med proteiner och vävnader under oxidativa förhållanden, men hur proteinet reagerar med nukleinsyror har inte förut studerats i detalj. I detta arbete har vi påvisat att hemoglobin interagerar med dubbelsträngat DNA och initierar klyvning av DNA-molekylen. En viktig upptäckt är att fosterhemoglobinet (HbF) uppvisar signifikant mindre DNA-klyvningsaktivitet i jämförelse med hemoglobin från vuxna människor (HbA). Det styrker slutsatsen att HbF är ett lämpligt alternativ som startmaterial för HBOC-utveckling.

För att hantera de negativa bieffekterna som ofta påträffas med HBOCs har nya Hb-varianter blivit utvärderade. Dessa varianter innefattar kemiska eller genetiska modifikationer som särskilt syftar till att reducera dimer-formering av hemoglobin. Dessa strategier har eliminerat snabb clearance av dimeriskt hemoglobin från blodomloppet och minskat njurtoxicitet. Därtill har förlängd cirkulationstid genom ökad retention uppnåtts med kemisk behandling av proteinet och det har också medfört minskad extravasation av Hb. På samma gång mildrar kemiskt modifierat hemoglobin kväveoxidbindning, som påvisats bidra till kärlsammandragning och ökat blodtryck i samband med administration av HBOCs.

Vi har utvecklat en ny molekylär design för HBOCs genom genetisk modifikation. En ostrukturerad polypeptidkedja, XTEN, har sammanlänkats

med fuserat HbF. Denna strategi har möjliggjort utvinning av större mängder rent protein utan ytterligare kemiska modifieringar. De strukturella och funktionella egenskaperna hos HbF är mestadels bevarade vid sammanlänkning med XTEN och denna strategi kan vara användbar för utveckling av en ny generation HBOC-produkter eftersom den undanröjer negativa effekter relaterade till kemisk behandling av proteinet.

Som tidigare nämnts kan genetiska och kemiska modifikationer ändra prestationsförmågan av Hb i extracellulära miljöer. Även om flera olika typer av HBOC-produkter har utvecklats och blivit utvärderade i djurmodeller, är det viktigt att förstå den operativa mekanismen hos dessa *in vivo*. För att ta itu med den frågan har vi utvecklat ett fluorescerande hemoglobin. Det designade fusionsproteinet består av grönt fluorescerande protein (GFP) sammanlänkat på gennivå med fuserat HbF. Proteinet uppvisar egenskaper som härrör från båda proteinerna, det vill säga grön fluorescens under UV-ljus och röd färg i synligt ljus. De fluorescerande egenskaperna hos detta protein kan underlätta observationer av cellulära och oxidativa skador som associeras med cell-fritt hemoglobin.

Upprening av hemoglobin är oftast en betydande flaskhals vid HBOC-produktion och alltmer effektiva nedströmsprocesser behöver utvecklas. I detta arbete har polymerer med molekylära avtryck (molecularly imprinted polymers, MIPs) blivit utvärderade med avseende på bindning av fluorescerande hemoglobin för att undersöka materialets bindningskapacitet.

List of Papers

This thesis is based on the following papers, which will be referred in the text by their Roman numerals. The publications and manuscripts are appended to the thesis.

Paper I **The penultimate tyrosine residues of human hemoglobin are critical for its genotoxic effects.**
Sandeep Chakane, Vijay Markad, Kisan Kodam, Leif Bülow
Advances in Experimental Medicine and Biology (In press, 2017).

Paper II **Fetal hemoglobin is much less prone to DNA cleavage compared to adult protein.**
Sandeep Chakane, Tiago Matos, Karin Kettisen and Leif Bülow
Redox Biology, Vol. 12, Pages 114-120, 2017.

Paper III **Enhanced hydrodynamic volume of fetal hemoglobin carrying a genetically linked polypeptide tail.**
Sandeep Chakane, Khuanpiroon Ratanasopa, Cedric Dicko, Tommy Cedervall, Abdu Alayash and Leif Bülow
Submitted

Paper IV **Genetic conjugation of green fluorescent protein to fetal hemoglobin: Functional properties and binding to hemoglobin imprinted polymers.**
Sandeep Chakane**,** Ka Zhang and Leif Bülow
Submitted

My contribution to the papers

Paper I	During the work on this article, I performed site-directed mutagenesis, expression and purification of the hemoglobin mutants examined. The comet assay was performed by V.M. I drafted the manuscript.
Paper II	I planned and performed all the experiments. K.K. prepared and purified the HbF mutants. T.M. took part in the discussion. I wrote the manuscript together with L.B.
Paper III	I cloned, expressed and purified the protein. K.R. and I performed some of the experiments together. C.D. and I performed the SAXS experiment, and C.D. wrote parts of the manuscript. I wrote the manuscript together with L.B.
Paper IV	I planned and performed the cloning, expression and purification of the protein. K.Z. performed MIPs experiments. I wrote the entire manuscript.

I have also contributed to the following articles and manuscripts:

1. **Trapping of human hemoglobin by haptoglobin: molecular mechanisms and clinical applications.**
   Khuanpiroon Ratanasopa, **Sandeep Chakane**, Muhammad Ilyas, Chanin Nantasenamat, Leif Bülow
   Antioxidants & Redox Signaling, Vol. 18, Issue 17, Page 2364-77; 2012

2. **Characterization of a Haptoglobin Mimic Prepared by Molecular Imprinting - Recognition and Protection against Lipid Peroxidation**
   Ka Zhang, Tongchang Zhou, **Sandeep Chakane**, Lei Ye, Leif Bülow
   (Submitted)

3. **Expression of fusion fetal hemoglobin in *Nicotiana benthamiana***
   Selvaraju Kalagarajan, **Sandeep Chakane**, Magnus Karlsson, Khuanpiroon Ratanasopa, Li-hua Zhu and Leif Bülow (Manuscript)

Abbreviations

2,3-DPG	2,3-diphosphoglycerate
A	Alanine
A1M	Alpha-1-microglobulin
ALA	δ-aminolevulinic acid
AOC	Artificial oxygen carrier
ATP	Adenosine triphosphate
CD	Circular dichroism
CO	Carbon monoxide
cRBC	Cultured red blood cell
Cys	Cysteine
DAPI	4',6-diamidino-2-phenylindole
DCLHb	Diaspirin-crossed linked Hb
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
E	Glutamic acid
E. coli	*Escherichia coli*
EPR	Electron paramagnetic resonance
FDA	Food and drug administration
fHbF	Fusion fetal hemoglobin
G	Glycine
GFP	Green fluorescent protein
GSH	Glutathione
H_2O_2	Hydrogen peroxide

Hb	Hemoglobin
HbA	Adult hemoglobin
HbF	Fetal hemoglobin
HBOC	Hemoglobin-based oxygen carrier
HDL	High-density lipoprotein
HIV	Human immunodeficiency virus
Hp	Haptoglobin
IPTG	Isopropyl-β-D-thiogalactopyranoside
LDNA	Linear plasmid DNA
Mb	Myoglobin
MG	Methyl green
MIP	Molecularly Imprinted Polymer
NO	Nitric oxide
ocDNA	Open circular plasmid DNA
P	Proline
P_{50}	Partial oxygen pressure where Hb is half loaded with oxygen
pDNA	Plasmid DNA
PEG	Polyethylene glycol
PFC	Perfluorocarbon
PFCOC	Perfluorocarbon-based oxygen carrier
RBC	Red blood cell
ROS	Reactive oxygen species
S	Serine
SAXS	Small angle X-ray scattering
sc pDNA	Supercoiled plasmid DNA
SOD	Superoxide dismutase
T	Threonine
Tyr	Tyrosine

Introduction and Aims

"Blood connects us all[a] and we bleed from similar veins[b]".

Blood or blood product transfusion helps to save millions of lives every year. Blood transfusion plays a crucial role in patients suffering from life-threatening conditions during surgical procedures. It also has an essential, life-saving role in maternal childcare, surgery and in the wake of natural disasters. In 2015, 108 million units of blood were collected worldwide, however, the need of transfusible blood is continuously increasing compared to the actual supply. This situation is often more troublesome in developing countries [1, 2]. In India, the total blood requirement is 9-9.5 million units of blood per year, but blood banks in the country are able to collect only 5-5.5 million units [3]. In addition, the overall risk of death after blood transfusion has been estimated to 1 in 1 million. Emerging pathogens such as hepatitis, human immunodeficiency virus (HIV), Creutzfeldt-Jakob disease or west Nile virus are also of major concern. Furthermore, a blood product has a short life span of only 42 days, because over time, red blood cell (RBC) membranes lose their flexibility, and the cell leaks potassium, and becomes more prone to hemolysis [4, 5]. To overcome this issue, there is a growing interest for production of an alternative source of blood. These alternatives are often synthetic solutions with the ability to bind, transport, and unload oxygen in the body, but do not perform other normal blood functions, such as transport of nutrients, immune response, and coagulation [6].

In the past decades, significant research efforts have been directed towards the production and application of functional hemoglobin-based oxygen carrier (HBOC), that have potential to be transfused in place of the RBCs. In the human body, Hb is present inside the RBCs, and is responsible for transportation of oxygen from lungs to tissues. However, outside the RBCs, the tetrameric Hb molecule quickly dissociates into dimers, which cannot carry oxygen. A cell-free Hb, lacking the antioxidant network of RBCs, participates in several deteriorating reactions and scenarios, i.e. physiological stress and organ damage [7, 8].

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[a] World health organization (WHO) encourages people around the world for blood donation. This slogan was used on the world blood donor day in 2016.

[b] Quote by the American rap artist, Tupac Shakur.

Heme mediated toxicity, radical reactions and reactions with nitric oxide (NO), are often regarded as the major complications associated with cell-free Hb [9].

The reactions of Hb with nucleic acids have not been studied in detail. In this thesis, we have determined the potential toxic effects of Hb variants on a genetic material. In **paper I**, we have developed a traditionally used comet assay to characterize the Hb mutants based on their genotoxic effects. In this approach, nuclear DNA cleavage can occur due to the mitochondrial or membrane damage. Furthermore, in **paper II**, we have developed the DNA cleavage assay to demonstrate that the interaction of Hb with plasmid DNA (pDNA), eventually leads to cleavage of DNA. Several fetal hemoglobin (HbF) variants were examined to understand the mechanism of Hb induced DNA cleavage.

Chemical modification (PEGylation) is often regarded as an essential manufacturing process in the development of HBOCs, but this may alter the structural properties and final yield of the product. In **paper III**, we have used the XTEN technology to modify Hb genetically, obviating chemical modification steps. XTEN is a recombinant polymer composed of negatively charged amino acid residues. This XTEN polymer was genetically linked to fusion fetal hemoglobin (fHbF), and used as an alternative to PEG moiety. In **paper IV**, we have generated fluorescent Hb by genetic linkage of fusion fetal hemoglobin (fHbF) with the green fluorescent protein (GFP), referred as GFP-HbF. The purified GFP-HbF protein was used to evaluate the efficiency of molecularly imprinted polymers (MIP), which can be used as a simple purification alternative for Hb molecules.

1. Past and present status of HBOCs

Currently, there are two types of blood substitute/artificial oxygen carriers (AOCs) under development, based on the mechanism of oxygen transport: perfluorocarbon-based oxygen carriers (PFCOCs) where the oxygen is physically dissolved in perfluorocarbons (PFCs) and hemoglobin (Hb)-based oxygen carriers (HBOCs), where oxygen is bound to the iron moieties of the Hb molecule [10]. These two types of AOCs have differences in molecular structures, uptake and release profiles of oxygen and carbon dioxide, toxicity effects, transport mechanisms, nature of the manufacturing process and costs. PFCs are inert, liquid hydrocarbon compounds, which can be linear or cyclic, and can carry dissolved molecular oxygen without binding to it [11, 12]. However, HBOCs have attracted most attention due to similarities with the function of RBCs, such as oxygen transport ability and less toxicity. Therefore, HBOCs have been extensively studied, developed and characterized.

1.1 Early blood substitutes

In 1616, William Harvey described the circulation of blood. Later many medical experts examined several substances including milk, wine, or sheep blood as alternatives for blood. The first successful human blood transfusion was done in 1667, however, subsequent transfusion therapy trials led to the death of patients due to incompatibility reactions. After that, many blood transfusion experiments were performed using albumin, different Hb solutions etc. with minimal success [13, 14]. In 1957, Chang reported the first artificial red blood cell, which was composed of microencapsulated Hb [15]. The development of such blood substitutes was soon thereafter terminated due to reticuloendothelial stimulation, sterility and high production costs.

In the 1980s, artificial blood was highlighted again after the almost epidemic explosion of HIV infections, which imposed a major risk of its transmission by blood transfusion. The sensitive status of regular transfusion became obvious and safety concerns stimulated initiatives addressing the production of alternative and safe blood substitutes [16]. The first approved blood substitute by FDA was Fluosol-

DA, a PFCOCs, which was later withdrawn due to insufficient clinical benefits [17]. After that, research was focused on manufacturing HBOCs. However, the first generation HBOCs failed due to serious side effects including hypertension, renal toxicity, vasoconstriction and oxidative stress (Table 1) [18]. Nevertheless, Hemopure was the first product of its kind to be FDA approved for human use in South Africa, and for veterinary use elsewhere [19].

Table 1. Current status of early developed oxygen carriers. They are divided into three main categories on the basis of chemical modifications employed. Cross-linked Hbs are made by crosslinking of Hb subunits. Whereas Hb molecules, polymerized using chemical entities are called polymerized Hb. In conjugated Hb, chemical modifications are carried on Hb surfaces. Table contents adapted from a review article by Tao and Ghoroghchian [19].

Product type	Product name	Technology	Present status
Cross-linked Hb	Heme assist	αα cross-linked human Hb	terminated
	Optro (rHb)	Recombinant Hb	terminated
Polymerized Hb	PolyHeme	Glutaraldehyde, pyridoxal human Hb	terminated
	Hemopure	Glutaraldehyde bovine Hb	approved*
	Hemolink	o-raffinose cross-linked human Hb	terminated
Conjugated Hb	Hemospan	Maleimide PEG-human Hb	terminated
	PEG-Hb	PEG conjugated bovine Hb	terminated
	PHP	Polyoxyethylene-conjugated human Hb	terminated

* approved in South Africa for the treatment of anemia, and for veterinary use in USA and Europe

1.2 Current developmental status of HBOCs

The first generation of stroma free or unmodified cell-free Hb molecules failed miserably in clinical trials [20]. Further analysis indicated the presence of Hb dimers in the blood stream, due to rapid dissociation of tetrameric Hb. Since then, a variety of chemical methods have therefore been employed to suppress the unwanted properties of Hb. Currently, a variety of products have been developed, and some of them have reached clinical trials. Most of them are based on chemical conjugation of Hb with polyethylene glycol (PEG) or on crosslinking of the globin chains with glutaraldehyde solution [21].

PEGylated bovine carboxyHb (SANGUINATE) is an oxygen transporter as well as a carbon monoxide (CO) releasing molecule. It is composed of bovine Hb, which is modified with PEG at lysine residues of the Hb molecule. It was found to be safe in phase I clinical trials and has now entered further clinical trial phases [22, 23]. MP4 is another HBOC product, developed by Sangart Inc. (USA), which is also based on PEGylation of bovine Hb. It has a very high oxygen affinity, and passed through

early clinical trials in Sweden. However, financial issues have terminated further development of this product [8]. Another example is MP4CO, also developed by Sangart Inc., which is a PEGylated human Hb saturated with CO. Adult sickle cell patients have been administrated with MP4CO in clinical trials (phase 1b) and this product has shown minimal adverse effects unlike other early generation HBOCs [24].

Hb vesicle (HbV) is human Hb encapsulated in phospholipid vesicles, which are further surface modified with PEG. Approximately 30,000 Hb molecules are entrapped in each HbV. Extensive research by among others Sakai et al. has shown the efficiency of HbV as resuscitative fluid for hemorrhagic shock, oxygenation of ischemic tissues and improved fetal development in a rat preeclampsia model [25-27]. However, short blood circulation life and prompt degradation have limited further clinical use.

Arenicola marina is a polychaeta annelid, which produces hexagonal bilayer Hbs (AmHb), composed of ~3.6 MDa globins, linked with non-globin linker chains. This annelid Hb-based blood substitute is called HEMO2Life and can be isolated from the native host or produced by expression in *E. coli* [28, 29]. In addition, it has been tested in a mouse model and shown wide spread bio-distribution. It has also shown beneficial properties for organ preservation [30]. Similarly, cross-linked HBOCs have been developed, including Hb intramolecularly cross-linked with ATP and GSH (HemoTech) [31]. More complex carriers have also been generated carrying Hb crosslinked with SOD and catalase using glutaraldehyde (PolyHb). Hb molecules covalently wrapped with albumin, designated as “Hemoact”, have also been investigated in preclinical studies [32]. OxyVita Hb is a new generation HBOCs developed through a zero-linked polymerization process [33]. Despite the advancement in the modern HBOC design, none of these latter products are approved for clinical applications yet.

1.3 Red blood cells from hematopoietic stem cells

In search of alternatives to blood, significant research is pursued for production of artificial RBCs. The human body produces millions of RBCs every second to maintain a steady-level of red cell mass. Hematopoietic stem cells undergo numerous steps of differentiation to form matured RBCs in a process known as erythropoiesis (Figure 1.1) [34]. A low oxygen levels stimulates the kidneys to produce the hormone erythropoietin into the blood. This hormone subsequently initiates the production of RBCs. An RBC has the shape of a biconcave disc which

carries oxygen from lungs to tissue. Each RBC is composed to approximately 95% of Hb molecules, which give the characteristic red color to the cells. In recent years, stem cells have proved to be a potential source for cell and tissue replacement therapies. Extensive research has been carried out to permit *in vitro* production of RBCs using cord blood, yolk sac, fetal liver and peripheral blood [35, 36].

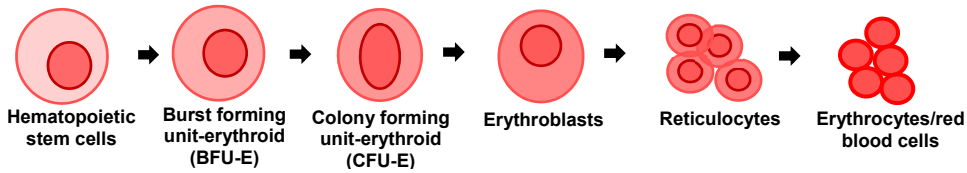

Figure 1.1 Schematic representation of red blood cell (RBC) formation. The principal factor that stimulates RBCs prodcution is erythropoietin, which stimulates hematopoetic stem cells (HSCs) to diffrentiate into matured RBCs. A complete process involves a maturation of nucleated cells into nucleus-free and Hb filled erythrocytes through several steps. The complete process requires 7-8 days.

In many cases, CD34-positive cells from different development stages have been used as a primary source of cultured RBCs (cRBCs). Murine embryonic stem cells and pluripotent stem cells have also been evaluated as an initial source for RBCs production. In addition, recent studies have shown that murine and human fibroblast can be converted into erythroid precursor cells by stimulation of specific transcription factors [37]. cRBCs generated from peripheral blood hematopoietic cells have been tested in healthy volunteers, and have shown a potential for clinical applications [38].

Unfortunately, the production of stem cell based RBCs has only been achieved at laboratory scale and mass production is not yet feasible. Several other challenges also need to be considered for clinical applications. The Hb content of cRBCs is an important feature, which can alter the oxygen transport capacity [39]. Besides the issue of scaling up, transfusion related acute lung injury is among other complications of these RBCs [40].

1.4 Recombinant Hb as starting material

In many developed HBOC products the Hb used as a starting material originated from bovine, outdated whole blood (human) or was obtained by heterologous expression in *E. coli.* Major issues in developing HBOCs are the cost, safety and availability of the Hb supply. There are several advantages of using recombinant Hb as starting material including lack of pathogens [41]. Recent advances in the recombinant DNA technology thus allow the production of human Hb in *E. coli* and other heterologous host cells. Site-directed mutagenesis has been utilized for

expression and production of Hb variants, providing the possibility to achieve optimized biochemical properties in terms of reduced oxidative toxicities, globin stability, adjusted oxygen affinity etc. [2]. The current cost of donated blood to hospitals ranges from $ 150/U or roughly $5/gram of Hb. Thus, the recombinant Hb needs to produce for the cost of $5 - $10/gram, which is challenging goal for any recombinant Hb. To reduce the production cost, more robust and stable Hb molecules must be prepared to improve yields.

2. Human hemoglobin (Hb)

Every molecule in our body has a story to tell. It is believed that the Hb oxygenation and oxidation have evolved to fulfill the environmental and physiological demands of respiring organisms. Globins have evolved during the Earth's long early period of hypoxia/anoxia. The α- and β-globins are synthesized at progressive stages of development and are closely related. Moreover, the amino acids sequences are related, indicating the presence of a common ancestor in early vertebrates approximately 1,000 million years ago (Figure 2.1).

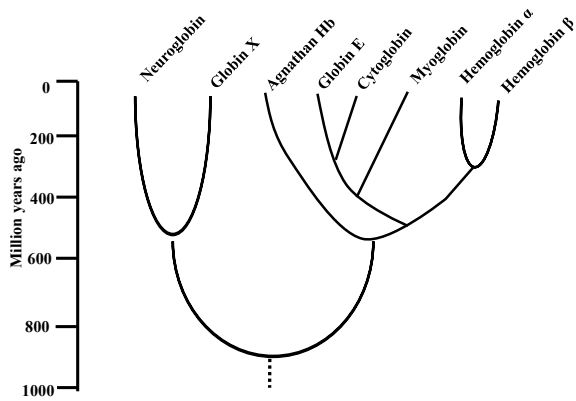

Figure 2.1 Phylogenetic history of the evolutionary appearance of the vertebrate globins. The vertical scale represents the time in millions of years when the heme proteins were evolved. An ancestral globins appeared on earth approximately 1,000 million years ago. The globins synthesized at early stages of evolution had a common amino acid sequences. The image is adapted from Brunori et al. [42].

In animals, Hb is an abundant protein that gives the red color to blood cells which carry oxygen from lungs to tissues. Hemoglobin (Hb) and myoglobin (Mb) are often considered as the only globin proteins involved in oxygen transport [43]. The first globin, (Hb), was discovered in 1840 by Hunefeld and about 60 years later the second (Mb) was discovered by Mörner [44]. A wide range of animals, plants, vertebrates and invertebrates, depends on Hb for transportation of the oxygen.

In vertebrates, there are about six different globin types that have been identified apart from Hb and Mb: 1) Neuroglobin (Ngb) is a monomer composed of 151 amino acids. This globin protein is predominately present in neurons [45]. 2) Cytoglobin (Cyg) is homodimeric, composed of 190 amino acids and present in various organs including liver, heart, brain, lung, retina, gut, esophagus and many others [46]. 3) Androglobin (Adgb) is expressed in testis and composed of around 1,600 amino acids. Recent studies suggest that this protein has a role in reproduction [47]. 4) The eye specific globin (GbE) is found in chicken and is highly expressed in chicken eye [48]. 5) The globin Y (GbY) has been recently found in Xenopus, however, the function of this protein has not been fully elucidated [49]. 6) Globin X (GbX) is membrane bound globin that has been identified in many metazoan animals. This protein may be involved in membrane related cellular signaling processes, but the exact role is still unknown [50].

2.1 The globin structure

The main proteins in the globins family are Mb and Hb. Each globin is 16 kDa in size and the globin polypeptide chains are helical, made up of 8 helices. The 8 helices are denoted as A through H (Figure 2.2), each helix at its turn is connected to short helices called AB, BC, CD etc. which sometimes are referred to as a corner. However, the D helix in the α-chain is replaced by a long coiled segment named as CE corner.

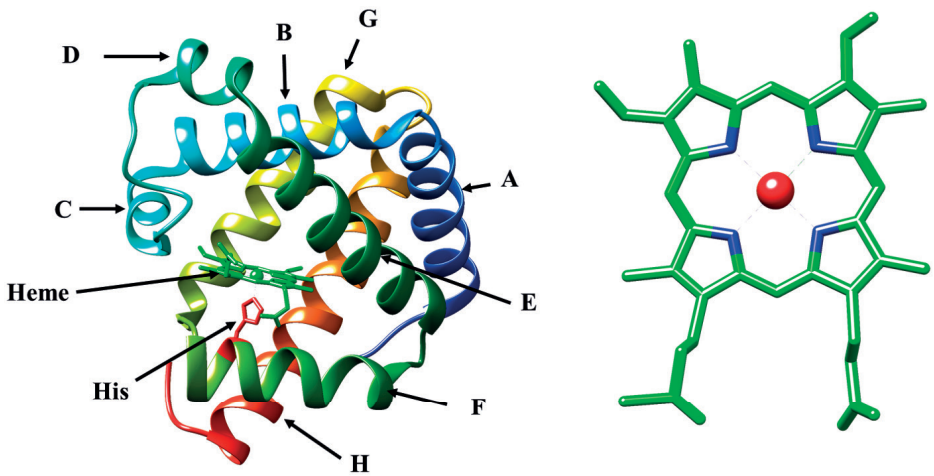

Figure 2.2 Tertiary structure of the globin. Left panel shows a representation of the typical helical fold present in globins illustrated by myoglobin (PDB code 2W6X). Right panel shows a structure of the heme group *(Fe-protopporphyrin IX)*. The iron is coordinated by four nitrogen atoms (blue). Each globin possesses the heme group that facilitates oxygen binding through its iron atom (red).

The helices are amphipathic and the protein interior has non-polar residues. The heme is surrounded by hydrophobic patches along with proximal and distal histidine residues. The heme is bound in a deep pocket formed by the E and F helices, and the iron is pentacoordinated by nitrogen atoms present in the heme group (Figure 2.2). The distal sites of the heme is composed of HisE7 and ValE11 residue, while the proximal site is constituted by α-His87 in the α-chain and β-His92 in the β-chain. The ligand (oxygen, carbon monoxide etc.) binds between the iron atom and distal histidine residue [51]. Heme has a dome shaped configuration in the deoxy form/tense state (T-state), as high spin iron cannot fit into the porphyrin plane and is therefore pulled out of the plane approximately 0.5 Å by the proximal histidine residue. However, binding of a ligand at the distal side pulls the iron into the plane [52].

2.2 Human adult hemoglobin (HbA)

Human adult hemoglobin (HbA) consists of two α (141 residues) and two β (146 residues) chains forming a homodimer of $\alpha_2\beta_2$ (Figure 2.3). Tetrameric Hb has a molecular weight of 64 kDa in size. The α-chain is somewhat smaller than the β-chain due to the missing D helix. Each chain has the heme group at the center, which is attached to the iron atom in the central cavity and is responsible for reversal binding of various ligands reversibly.

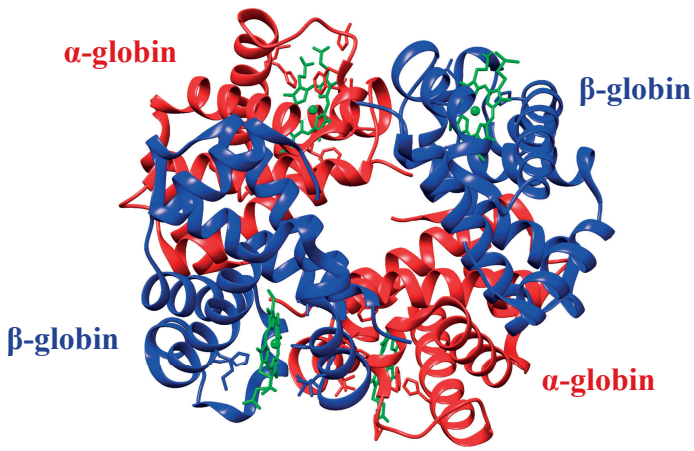

Figure 2.3 Tertiary structure of deoxy HbA (PDB code 2DN2). It is composed of two α-globins (red) and two β-globins (blue). Each globin carries the heme (green) group which is responsible for the binding of oxygen.

Conventionally, the two α-chains are referred to as α1 and α2, similarly, the β-chains are described as β1 and β2. There are only minor contacts between the two

α-chains, whereas the β-chains lack interaction points. Each α-chain connects with the β-chains in α1β1/ α1β2 and α2β1/α2β2 contacts. Helices B, G and H of the α- and β-chains form an α1β1 interface. The α1β2 interface is generated by helix C, F and G corner of each chain. The α1β2 interface is changed during the oxy and deoxy conformation of Hb, forming an allosteric character of the protein [51]. In diluted solution, Hb easily dissociates into dimers, where α1β1 is the main population. Inside the red blood cell, dissociation of tetrameric Hb is rare, because the concentration of Hb is high (millimolar).

The deoxy state is called the T (tense) state whereas the oxy bound state is often referred as the R (relaxed) state. Crystallographic studies by Perutz [53] have revealed that the quaternary structure of the deoxy form differs from liganded Hb (R-state). Several allosteric molecules can bind to the Hb molecule in other places than the oxygen binding sites, which can modulate the T or R state of Hb. The effect of protons on oxygen affinity of Hb is called the Bohr effect, by which the oxygen affinity increases at below pH values 6.0, whereas more alkaline conditions (pH 6.0-9.0) enhances the oxygen unloading. This has an important physiological consequence for Hb. The protons produced in the metabolic formation of carbonic acid, stimulates the unloading of oxygen in the venous blood. Carbon dioxide (CO_2) binds at the N-terminus of the α-chain, which destabilizes the R-state, thereby contributing to the release of oxygen from Hb. Another natural allosteric effector is 2,3-diphosphoglycerate (2,3-DPG), which binds at the β-cleft and which ties the two β-subunits together. As a result of the structural constraint induced by 2,3-DPG, oxygen affinity decreases significantly. Another allosteric effector, inositol hexaphosphate (IHP), has also been studied extensively due to its ability to form more stable complex with Hb than for 2,3-DPG [54].

2.3 Human fetal hemoglobin (HbF)

Besides HbA, human blood also contains very low levels of HbF (<2%) [55]. During the first developmental stages, there is expression of an embryonic form of a β-like globin known as ε-globin, expressed within the yolk sac-derived primitive lineage of erythrocytes. Soon after, the ε-globin expression is exchanged to γ-chain production. This globin is encoded by two separate genes found within the β-globin gene cluster. The Gγ gene carries a GGA codon (codon for glycine), while this is exchanged to GCA (encoding alanine) in the Aγ gene. HbF has Gγ and Aγ chains in the ratio of 70:30. The γ-chains combines with the α-chains forming a tetrameric HbF. After gestation, the predominate HbF switches to HbA [56].

HbF is generally more stable and resistant to alkaline conditions compared to HbA. There are four critical differences in the α1γ1 interfaces that can be associated to the increased stability and resistance to dissociation into dimers or monomers. Especially differences at the N-terminal end is a probable reason for the observed enhanced tetrameric stability of HbF. HbF also has a higher oxygen affinity than HbA owing to the replacement of a histidine in the β- chain to a serine in the γ-chain at position 143. This reduces 2,3-DPG binding site to the γ-chain [57] .

2.4 HbF as a starting material for HBOCs

An elevated level of HbF confers important clinical benefits to patients with sickle cell anemia (SCA) and β-thalassemia. Both diseases are related to a genetic mutation in the β-chain of Hb, which dramatically reduces the oxygen carrying capacity of the affected patients [58]. Several chemical agents have been evaluated for induction of the γ-globin gene, for example, hydroxyurea therapy has shown beneficial clinical improvement for β-thalassemia patients.

HbF shares the same α-chain as HbA, but the γ-chain differs from the β-chain by 39 amino acids. Some of these amino acids play an important role in the physical and chemical properties of HbF. Oxidation of Hb with H_2O_2 is responsible for extensive and irreversible modifications of amino acid residues in the protein, particularly β-Cys93 and β-Cys112 [59]. Oxidative modifications of cysteine residues may in turn lead to heme degradation and α-globin crosslinking, which eventually collapse the structure and function of Hb. The γ-globin lacks the second cysteine residue, which is replaced by the threonine residue at position 112. This amino acid substitution suppresses the further destructive reactions caused by oxidative modification associated with cysteine residues [60].

The redox reactions of Hb often result in formation of ferryl Hb, which is well known as one of the most harmful Hb species. Ferryl HbF shows faster auto-reduction rate in presence of ascorbate. Additionally, HbF also exhibits approximately 70-fold higher tetramer stability than HbA. The alkaline stability of HbF relies on amino acids γ-112T and γ-130W [61]. The replacement of β-His116 with Ile in the γ-subunits also enhances the stability of the α1γ1 interface [62]. The increased oxygen affinity of HbF compared to HbA related to amino acids at γ-43Asp and γ-143Ser [63]. Figure 2.4 depicts the tertiary structure of HbF and the specific residues that are responsible for rendering this Hb a suitable starting material for HBOC development.

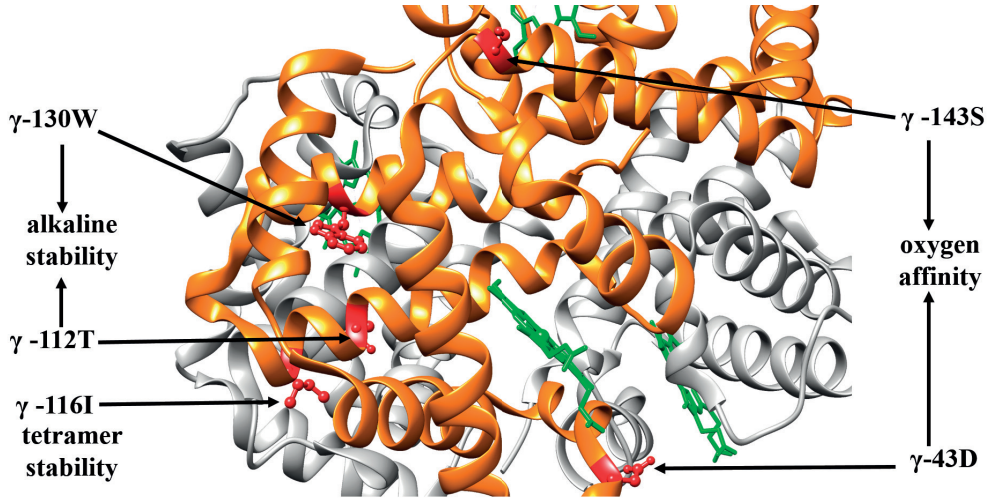

Figure 2.4 Tertiary structure of HbF (PDB code 4MQJ), the gamma chain (orange) differs by 39 amino acids from the beta chain. Highlighted amino acids (red) provides a beneficial properties of HbF.

One of the critical aspects of recombinant Hb production is to obtain the protein in an active and functional form. The production of recombinant HbF can be achieved more efficiently than HbA in *E. coli*. Recombinant HbF is expressed as intact tetramers with higher yields compared to HbA [64]. This also becomes evident when analyzing the different protein variants during chromatographic separations. Recombinant HbA hence tends to produce a non-functional protein fraction, which can be observed as second peak during anion exchange chromatography. This peak is possibly the hemichrome formed due to oxidative processes during Hb production [64].

In **paper II**, we evaluated the DNA damaging effects of HbF and some mutant variants. It was observed that native HbF was almost three-fold less reactive on supercoiled plasmid DNA (sc pDNA) compared to HbA. Since this is one of the critical parameters when identifying safe Hb-based products, we propose to further examine the use of HbF as a blood substitute candidate. In **paper III** and **IV**, we have therefore utilized the designed fetal hemoglobin (fHbF) for additional genetic modifications using negatively charged polypeptide tail (dubbed XTEN) and green fluorescent protein (GFP) forming XTEN-HbF and GFP-HbF, respectively.

3. Oxidative reactions of Hb

3.1 Heme-derived reactions

A range of HBOCs have been extensively characterized and evaluated over the years as alternative blood substitutes. However, cell-free Hb has also been linked to organ dysfunction and exert inflammatory and toxic effects, arising from several alternative oxidative reactions by the reactive heme group. This in turn limits its application as a safe blood substitute [65]. Hemolysis or free circulating Hb-based therapeutics, when administrated at varying quantities, may therefore induce severe radical generating reactions. Inside the RBCs, oxidative reactions produced by Hb are almost completely inhibited by an extensive antioxidant network composed of among others, SOD and catalase [66]. Besides its intrinsic red color, the optical properties of Hb facilitate studies of its redox properties. The failures of especially the first generation HBOCs have been scrutinized in details. Mainly *in vitro* studies have been carried out to understand the performance of Hb molecules in a cell-free environment [67]. Several methods are available and have been developed during the last decades to characterize the properties of cell-free Hb, some of them are listed below.

3.1.1 Autoxidation

The iron of the heme group is responsible for oxygen binding, where it is present in the ferrous (Fe^{2+}) state. However, Hb continuously undergoes autoxidation to produce superoxide radicals ($O_2^{\bullet -}$) [Equation 1], converting the ferrous to a non-functional ferric state (Fe^{3+}), also referred as metHb, which is unable to bind oxygen. Moreover, conformational changes occur during this process allowing water and small anion interactions with the heme. This may thereby lead to oxidation of the iron to form metHb [68].

$$HbFe^{2+}{=}O_2 \longrightarrow HbFe^{3+} + O_2^{\bullet -} \qquad \text{[Equation 1]}$$

Furthermore, autoxidation of Hb produces hydrogen peroxide (H_2O_2) through spontaneous dismutation of superoxide [Equation 2].

$$2O_2^{\bullet -} + 2H^+ \longrightarrow H_2O_2 + O_2 \qquad \text{[Equation 2]}$$

Inside the RBCs, metHb can easily be reduced back to the ferrous form in presence of the enzyme metHb reductase [69]. In addition, an extensive network of RBCs antioxidants neutralizes the formed reactive oxygen species (ROS), superoxide and hydrogen peroxide. In case of cell-free Hb, the ferric Hb produced through autoxidation, has a higher tendency to heme loss and globin denaturation. Thus, application of an advanced protein -engineering strategy could be useful to generate Hb molecules that are more resistant to autoxidation. One simple method to analyze the autoxidation process is to incubate Hb (or Hb variants) in an air-equilibrated buffer and monitor the spectral changes linked to the different oxidative forms of iron. The human oxyhemoglobin (HbO_2) shows a biphasic autoxidation curve. The calculation of autoxidation rate provides a valuable information about the oxidative stability of Hb [2]. This information is crucial for creation of a new molecular design of an HBOC product. In **paper III,** autoxidation rates of genetically modified Hbs were determined and proved to be identical for fHbF and XTEN-HbF. Figure 3.1 depicts typical absorbance changes associated with the autoxidation of Hb. A decrease in the absorbance at 576 nm is generally used to calculate the autoxidation rates.

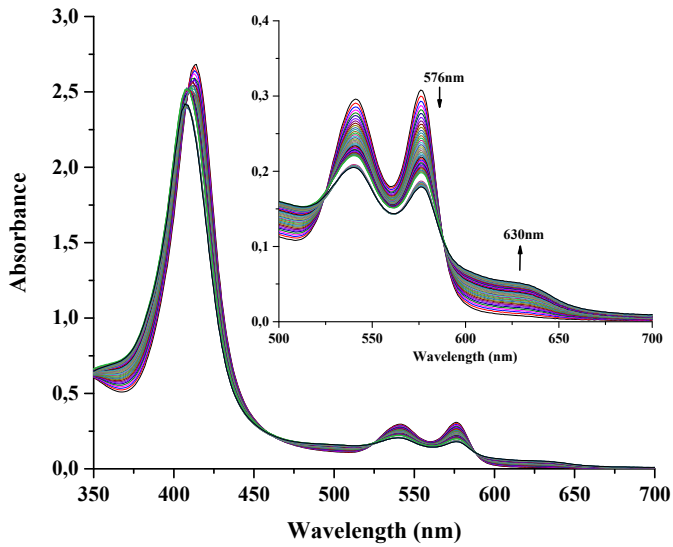

Figure 3.1 Absorbance changes during the autoxidation of XTEN-HbF (20 µM) when placed in 0.1 M sodium phophate buffer pH 7.4 at 37^{o}C (paper III). Cell-free Hb tends to lose oxygen in the absence of an antioxidant network system of red blood cells (RBCs), which eventually leads to formation of non-functional ferric Hb. An increase in the absorbance peak at 630nm indicates the formation of ferric Hb.

3.1.2 Peroxidase activity

The reaction between H_2O_2 and Hb has been studied and characterized via a two-electron oxidation process. Firstly, H_2O_2 reacts with ferrous or ferric Hb; when the reaction occurs with ferric Hb, a potent oxidant is formed in which heme is in the ferryl (Fe^{4+}) state along with the formation of protein-based radicals The ferryl state is highly reactive and may induce oxidative damage to a variety of biological molecules, e.g. DNA, lipids and amino acid residues [70]. This activity has been detected in human and animal whole blood, particularly by electro paramagnetic resonance (EPR) spectroscopy [71]. The reaction between Hb and H_2O_2 is responsible for the structural changes that occur due to the heme derived radical reaction with globin amino acids [72]. In vitro, this reaction leads to heme release and globin oxidation within Hb molecule (Figure 3.2). Fe^{2+} can decompose H_2O_2 into hydroxyl radical (OH**.**) and hydroxyl ion (OH^-), which ultimately produce a non-functional metHb.

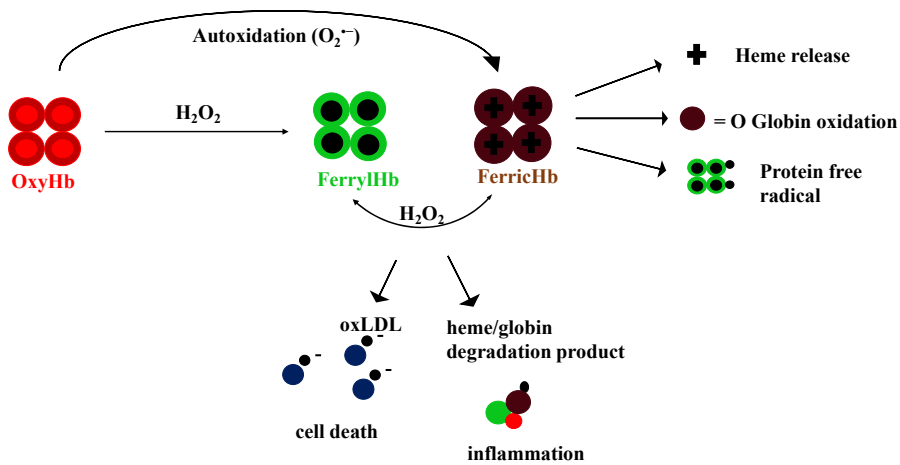

Figure 3.2 Reactions of Hb with hydrogen peroxide. Ferrous Hb (red) produces ferric Hb (brown) through autoxidation process, followed by formation of ferryl Hb (green). The reaction of ferric Hb and H_2O_2, also forms a ferryl Hb. Overall, these reactions leads to heme release and oxidation of amino acid residues within Hb molecule. The image adapted from Schaer et al. [73] with copyright to cold spring harbor laboratory press.

The natural antioxidant system that is present in RBCs can easily reduce the ferric or ferryl state of Hb into the desirable ferrous form. Superoxide dismutase catalyzes the removal of superoxide radicals that are generated during autoxidation reaction. The H_2O_2 is then normally decomposed by its reaction with catalase [74]. Apart from the enzymatic antioxidant system, there are various reductants present in vivo e.g. ascorbate, glutathione etc. Thus, ferric or ferryl Hb are easily reduced back to functional Hb (Fe^{2+}). In **paper III**, the reaction between Hb and H_2O_2 has been

determined under pseudo first order conditions. A biphasic reaction was observed with fast and slow phases. The observed rates could be fitted to a double exponential equation to obtain rate constants (fast and slow) [60, 75]. The oxidation rates of genetically modified fHbF and XTEN-HbF were found identical in the presence of H_2O_2.

3.1.3 Nitric oxide and Hb

Nitric oxide (NO) is a gaseous radical molecule, important for signaling during cardiovascular hemostasis. The most widely accepted interpretation of blood pressure effects caused by Hb is faster scavenging of NO, resulting in prevention of smooth muscle relaxation [76]. NO is synthesized by enzymatic and non-enzymatic reactions in mammalian cells. Enzymatic reactions involve NO synthase, which uses L-arginine as substrate, while non-enzymatic reactions occur in presence of deoxy Hb. NO has a dual function, i.e. it acts both as a toxin and signaling molecule [77].

Hb induced hypertension has been linked to the interaction of NO with both oxy and deoxy Hb. Nitric oxide reacts extreme rapidly with Hb. Reaction of Hb and NO with oxy and deoxy Hb produces metHb, iron-nitrosyl Hb and nitrate ions (Figure 3.3). NO depletion through this reaction causes vasoconstriction and platelet aggregation. NO is also responsible for the production of reactive oxygen species (peroxynitrite), which damages DNA and proteins [6, 73, 78]. Notably, vacant heme in the R state reduces NO at a faster rate compared to the T state of Hb.

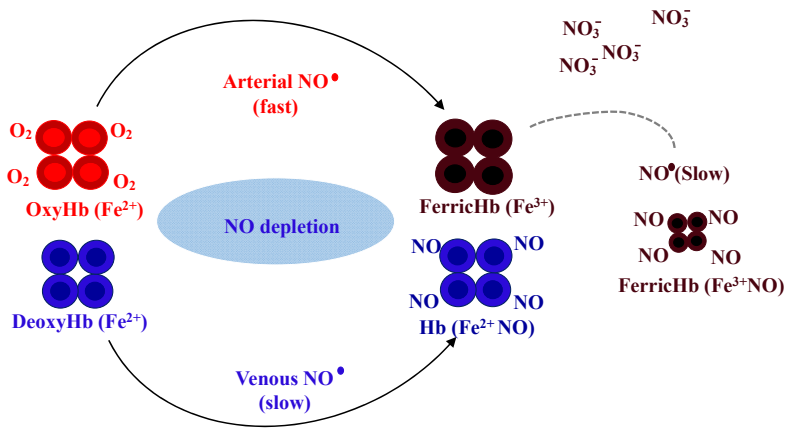

Figure 3.3 Reactions involved between Hb and nitric oxide (NO). When Hb molecule releases from red blood cell (RBC), it can rapidly reacts with nitric oxide (NO) forming ferric Hb (brown) and nitrate (NO_3). If the reaction involves a deoxyHb (blue) then iron nitrosyl hemoglobin is generated. The Image adapted from Schaer et al. [73] with copyright to cold spring harbor laboratory Press.

3.2 Antioxidant mechanism against cell-free Hb

A network of scavenger proteins and enzymes accomplishes clearance of cell-free Hb to avoid a cascade of further downstream oxidative reactions [79]. Generally, the primary Hb scavenger in plasma is haptoglobin (Hp). Hp is a tetrameric protein composed of two α- and two β-chains of 9 and 33 kDa, respectively. Humans possess three main phenotypes of Hp, designated Hp 1-1, Hp 2-1, and Hp 2-2 [80]. The Hp binds to Hb dimers, which are released from RBCs during hemolysis, in a noncovalent manner. It thereby prevents the oxidative damage mediated by free Hb, and facilitates its rapid clearance through CD163-receptor mediated endocytosis. However, the redox properties of Hb, e.g. rates of autoxidation, formation of protein-based radicals, NO binding etc. have been found to be identical in the Hb:Hp complex [81, 82]. Mollan et al. have examined the redox properties of Hb when complexed with polymeric human haptoglobin. Their studies demonstrated that irrespective of the type of Hp phenotype, redox properties of Hb were identical with or without the Hp molecule.

Hemopexin (Hpx) is another plasma glycoprotein that forms a second line of defense against cell-free Hb. This protein binds to a free heme groups, released from Hb, and forms a tight complex ($Kd < 10^{-13}$ M). It protects the lipoproteins from the heme-mediated oxidative damage and limits the interaction of the hemin, the reactive ferric protoporphyrin-IX group released from a cell-free Hb, with cell surface receptors. In intravascular hemolysis, depleted levels of Hpx have been found in patients. Multiple other proteins are present in plasma that bind hemin including albumin, LDL and HDL with low Kd values [79, 83]. Heme oxygenase (HO) is another heme degrading protein, converting free heme to iron, biliverdin and CO. Alpha-1-microglobulin (A1M) is a 27kDa glycoprotein, and is another example of a heme and radical scavenger protein, that acts against cell-free Hb [84].

3.3 Protein-based radicals

The protein-based radicals are formed via reaction between Hb and H_2O_2. As mention in section 3.1.2, EPR and mass spectroscopic data has been used to identify specific amino acid residues that are prone to oxidation within the Hb molecule. Previous studies revealed that tyrosine and cysteine residues of Hb are the most sensitive to oxidation. For example, α-Tyr42, β-Tyr145, β-Tyr35, β-Tyr130 and β-Cys93 are the primary radical formation sites within the HbA molecule. Moreover, these amino acids also promote the migration of free radicals between the α- and β-globin chains of Hb [59, 71, 85, 86]. The short-lived radicals can be determined in

presence of the spin-trap 5,5-dimethyl-1-pyrroline N-oxide(DMPO), which is used for detecting O-, N-, S-, and C-centered free radicals. Recently, Vallelian et al. have identified DMPO-adducted amino acids residues, within Hb molecule, that were sensitive to oxidation (Figure 3.4) [87].

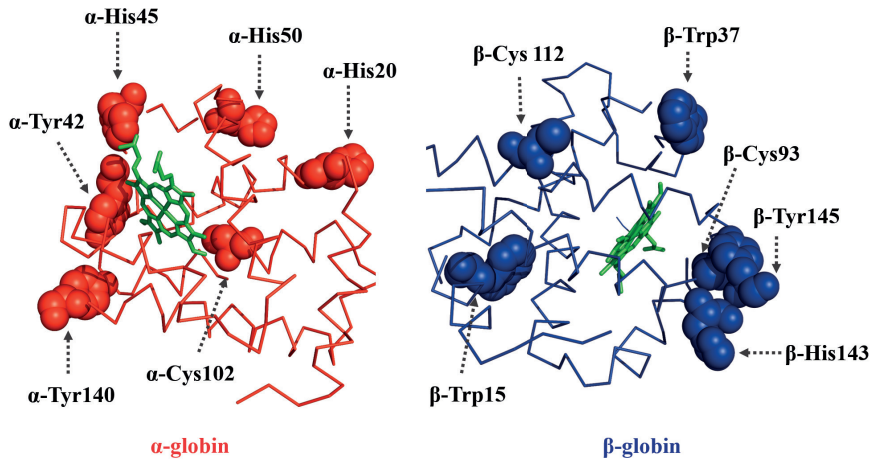

Figure 3.4 Protein-based radicals are generated during the oxidative reactions of Hb and reside on specific amino acid residues (PDB code 2DN3).The α-globin (red) showed six and β-globin (blue) have six amino acids that are prone to oxidation. The figure is adapted from Vallelian et al. [87] with permission.

3.3.1 Penultimate tyrosine residues of the alpha and beta chains

Tyrosine is a key target residue within the protein molecule for oxidation under oxidative stress [88]. Each subunit of Hb carries three tyrosine residues, and α-Tyr140 and β-Tyr145 are at penultimate positions of the α- and β-chains, respectively. Carbonyl groups of α-V93 and β-V98 forms hydrogen bonds with α-Tyr140 and β-Tyr145, respectively, which stabilizes the structure of Hb in the T-state [89, 90]. Mutations at these positions markedly altered oxygen affinity of Hb, particularly at α-Tyr140 [89]. Moreover, there are several natural mutants at this position on the β-chain, Hb Osler (β-Tyr145Asp), Hb Bethesda (β-Tyr145His), Hb Rainier (β-Tyr145Cys) and Hb McKees Rocks (β-Tyr 145 terminated) with high oxygen affinity and lack of T state conformation [91]. There is only one natural mutant for the penultimate tyrosine of the α-subunit in which α-Tyr140 has been replaced by histidine, and which shows high oxygen affinity [91]. Previously, Kneipp et al. [89] showed that replacement of penultimate tyrosine residues with phenylalanine eliminate the Bohr effect. Moreover, the oxygen affinity is increased by five and eight folds for α-Y140F and α-Y140/β-Y145F respectively, compared to wildtype HbA when evaluated at pH 6.0. It has been shown that these tyrosine residues are radical forming site within the Hb molecule [85]. In **paper I,** we have

chosen to replace tyrosine with phenylalanine, which is similar aromatic amino acid lacking a hydroxyl group. We have evaluated the genotoxicity of penultimate tyrosine residues using a comet assay (section 4.1.2). Moreover, the observed effects were verified by DNA cleavage assay.

4. Evaluation of DNA damage caused by Hb

DNA is a molecule that carries the instructions needed for development, survival and reproduction of an organisms. It has been shown that the cell-free Hb, both ferryl Hb and protein-based radicals, are capable of initiation of the oxidation of a wide variety of substrates, including DNA. The hydrophobic heme, released from Hb molecule, has been linked to DNA damaging activity of Hb [67, 79]. Recombinant Hb molecules can be designed with suitable biochemical properties to produce safe and effective HBOC products. Several methods are available to evaluate the toxic effects of newly developed Hb variants. However, detection of DNA damage can serve as the basis of rapid screening of Hb variants. In **paper, I and II**, we have developed existing screening methods for characterization of Hb mutants with respect to their DNA cleavage activity.

4.1 Comet assay

The comet assay is known as a simple and sensitive method for detection of single and double stranded breaks in DNA. This single cell gel electrophoresis (SCGE) or comet assay was first developed by Ostling and Johanson in 1984 [92]. A few years later, Singh et al. [93] developed this assay for use under alkaline conditions. Notably, Ostling et al. described the "comet like" image after the assay, in which the head of the comet contains high molecular weight DNA and the comet tail shows fragmented DNA. Detergent and high salt concentrations are used to remove the membrane and cytoplasm of the cells embedded in agarose gel. Negatively charged DNA migrates towards the anode in electrophoresis. Thus, if DNA is degraded by strand breaks, it will be visualized as a comet tail under fluorescence microscopy (Figure 4.1). The comet assay has been used for analysis of DNA damage in various fields related to genetic, biotechnological and environmental sciences. A range of human cells, tissue samples of mouse, sheep, and bacteria have been tested. The method has been widely explored worldwide for examining genotoxic effects of plant extracts, chemical compounds, nanoparticles, waste water etc. and for clinical applications [94-96].

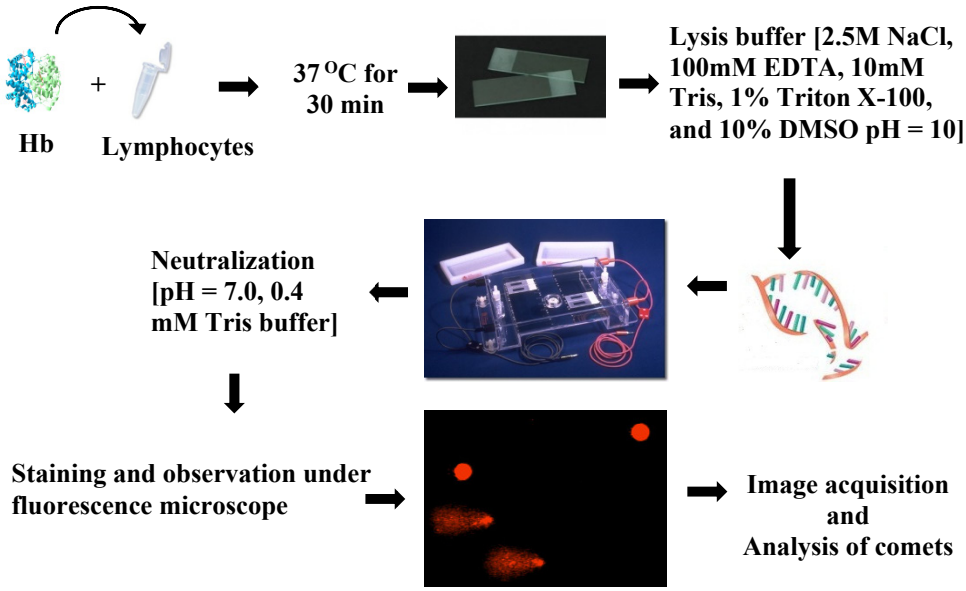

Figure 4.1 Schematic representation of the comet assay. Hb mutants were incubated with lymphocytes and were analyzed using the comet assay. Samples were coated inside the agarose gel and electrophoresis was carried out at alkaline conditions. Fluoresecne microscopy was used to analyze the comet images.

In 2006, Glei et al. first reported the use of the comet assay for detecting DNA damages caused by the iron moiety present in the Hb molecule. Their studies described the DNA damaging effects of Hb on human colon tumor cells [97]. In **paper I**, we have evaluated the radical reactions and subsequent DNA damage generated by Hb variants using the comet assay.

4.1.1 Comet assay of HbA mutants

The tyrosine residues at the penultimate positions of the α (Tyr-140) and β (Tyr-145) chains were replaced by phenylalanine residues. The genotoxic effects of wild type (HbA) and Hb mutants were examined on lymphocytes (**paper I**). Lymphocytes are diploid cells and because they circulate through the whole body, were chosen for this study. Single cell gel electrophoresis or comet assay was carried out to investigate the ability of Hb to induce breaks in the DNA strands. Lymphocytes (1×10^4 cells/ml) were incubated with various concentrations of

mutant metHb (2.5, 5 and 10μM, 30 min, 37^{O}C). After each treatment, cells were washed with cold PBS. The alkaline comet assay was carried out as described by Singh et al. [93]. The parameter selected for quantification of DNA damage was percent tail DNA (% tail DNA). It is calculated as the ratio between the total intensity of the tail and the total intensity of the comet (head and tail together). As shown in Figure 4.2, the mutant α-Y140F induced significantly more (40%) DNA damage ($P < 0.05$) when compared with HbA. The β-Y145F and double (αY140/β145F) mutants showed the same levels of DNA damage as HbA.

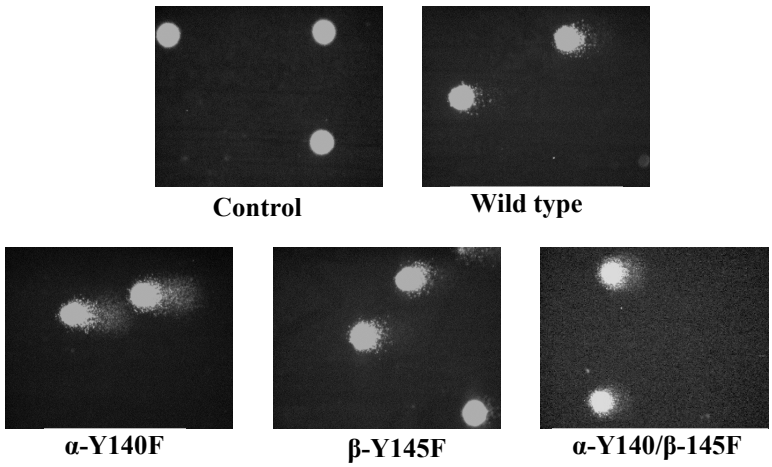

Figure 4.2 Lymphocytes were treated with different Hb variants and images were captured using fluorescence microscope after the comet assay. Wild type Hb and β-Y145F showed identical and negligible genotoxic effects. While, α-Y140F showed the highest level of damage as calculated by a computer-assisted image analysis system.

4.2 DNA cleavage assay

Oxidative DNA damage induced by heme or iron-driven processes has been studied in detail [9]. Recent studies have also demonstrated that polymerized bovine Hb, when infused into a guinea pig model, suppresses the transcription of antioxidant enzymes (SOD, catalase) [98]. The impact of Hb and/or HBOCs on genetic material could provide important information about safety of such a product. Therefore, it is important to develop a simple and rapid technique for the determination of Hb induced DNA damage. In **paper III**, we have implemented a simple method, for analysis of the Hb toxicity to DNA molecules using plasmid DNA (pDNA).

The pDNAs are extrachromosomal, double stranded DNA molecules, which are used for expression of proteins in bacteria by recombinant DNA technology. The pDNA is a double stranded DNA molecule. If the DNA is fully intact with both strands uncut, and with an integral twist, it will have a compact form called

supercoiled plasmid DNA (sc pDNA). However, oxidative reactions can induce conversion of sc pDNA into open circular DNA (ocDNA) by nicking one of the strands. If this reaction continues, a linear DNA (LDNA) is formed where both DNA strands are broken (Figure 4.3) [99, 100].

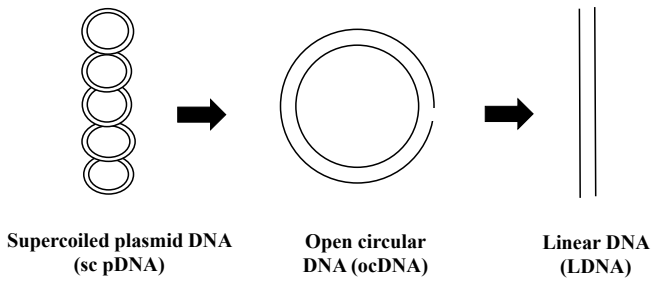

Figure 4.3 Schematic representation of plasmid DNA (pDNA) structure. Under oxidative conditions, supercoiled plasmid DNA is converted into open circular DNA. Linear DNA is formed when both strands of DNA are broken.

An endonuclease-like activity and binding capability of Hb with DNA or pDNA has been reported in earlier studies [101]. Moreover, myoglobin (Mb) also induces pDNA cleavage in the presence of H_2O_2. Mb mutants (recombinant), especially if the mutation occurs near the heme pocket, play an important role in pDNA cleavage [102, 103]. In **paper II**, we have evaluated the pDNA cleavage activity of Hb molecules when incubated with pDNA. Recombinantly produced HbA, HbF and Hb mutants were examined using our implemented DNA cleavage assay. Samples were analyzed using agarose gel electrophoresis, and the decreased intensity of DNA bands were determined using the 'Quantity One' software (BioRad). DNA cleavage was calculated based on the decreased percentage of sc pDNA. It was observed that the Hb molecule itself without addition of H_2O_2, can initiate the unwinding of sc pDNA. The pDNA cleavage activity was proportional to the Hb concentrations applied (Figure 4.4A). When the remaining sc pDNA concentration was plotted against time, the degradation could be fitted to an exponential decay equation to obtain the reaction rate constant. The decay constant was found to be 3,300 $M^{-1}\,h^{-1}$, with an R^2 value of 0.99. Figure 4.4B shows an agarose gel electrophoresis images of pDNA taken after every 2 h in the presence of various Hb concentrations.

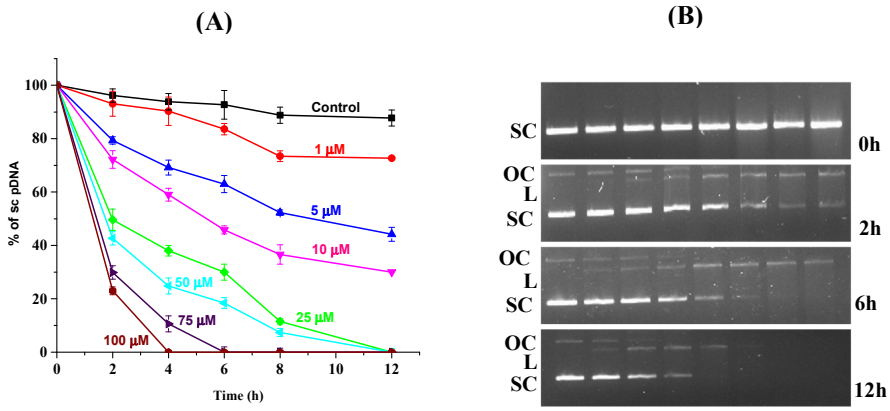

Figure 4.4 Plasmid DNA cleavage kinetics and agarose gel electrophoresis (Paper II) (A) The pDNA cleavage was in direct propotion to the applied Hb concentrations. B) Agarose electrophoresis gel pictures were taken at 0, 2, 6 and 12 h. Complete degradation of pDNA was observed in 12 h at the higher concentrations of Hb (25-100 µM).

Firstly, we eliminated the possibility that Hb may retard the movement of DNA on an agarose gel. For this purpose, we analyzed the migration of pDNA samples, in presence of Hb, on an agarose gel. The same gel was stained using coomassie blue dye and destained overnight. It was observed that Hb did not alter the migration of pDNA on agarose gel. Hb was observed very close to the wells of the gel, whereas pDNA migrated more far away (Figure 4.5).

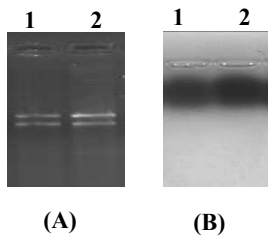

Figure 4.5 Agarose gel electrophoresis of pDNA in presence of Hb (A) The image of agarose gel, lane 1, Hb (25 µM) + pDNA; lane 2, Hb (50 µM) + pDNA. (B) The same gel was stained with coomassie blue dye and destained overnight. Hb samples were observed near to the wells of the agarose gel.

4.2.1 Factors influencing DNA cleavage

It has been reported that the DNA cleavage activity of myoglobin is influenced by many factors. A high NaCl concentration inhibits the DNA cleavage activity of Mb [103]. Similar results were obtained when Hb and pDNA were incubated in presence of NaCl (**paper II**). It indicates that electrostatic interactions exist between Hb and pDNA. Previous study has shown that Mb, bound to oxygen or present in ferric

form, exhibit the DNA cleavage activity [103]. In our studies, we have also analyzed Hb molecules bound to oxygen or carbon monoxide (CO). It was observed that HbO_2 induced the pDNA cleavage. The oxidation (autoxidation) of Hb releases the oxygen molecule, forming ferric Hb along with the superoxide radicals, which may accelerate the DNA cleavage [104]. CO binds to the iron moiety of the heme, almost blocking any further redox activities of Hb [105]. However, it was observed that the CO-Hb also induced 40% pDNA cleavage (**paper II**).

In **paper II**, the DNA cleavage activity of Hb was investigated in presence of different sodium phosphate buffer concentrations (20-100 mM). Similarly, the reaction was carried out at different temperatures (10-40^{O}C). It was observed that the pDNA cleavage activity of Hb was significantly elevated at higher temperature and in presence of sodium phosphate buffer. Similar findings were observed for the Fe ion in presence of sodium phosphate buffer when incubated with pBR322 plasmid [106]. KCN has higher affinity for heme, which does not allow ligands (CO, oxygen) to bind at the heme site [105]. Thus, pDNA cleavage was not observed in presence of KCN. The pDNA cleavage activity was evaluated in presence of DNA binding dyes. As shown in Figure 4.6A, the minor groove binding dye, 4',6-diamidino-2-phenylindole (DAPI), did not inhibits the pDNA cleavage activity of Hb. However, Methyl green (MG), major groove binding dye, significantly decreased the pDNA cleavage. Note that, at the higher concentrations of Hb, pDNA cleavage activity was not altered in presence of DNA binding dyes.

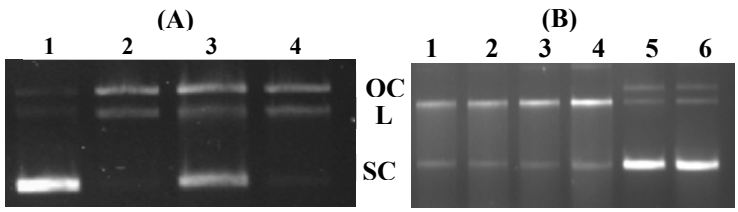

Figure 4.6 The plasmid DNA cleavage activity of Hb in presence of DNA binding dyes and Hp (paper II). (A) lane 1, pDNA; lane 2 Hb +pDNA; Lane 3,4 in presence of MG and DAPI, respectively (B) Agarose gel electrophoresis of Hb in presence of haptoglobin. lane 1-4 represent Hb + Hp in ratio 1:0.25, 1:0.5, 1:1 and 1:2; lane 5-6 respresent the Hp alone (0.25 and 0.5 µM).

As a scavenger of a cell-free Hb, Hp is known to reduce the toxic effects generated by ferryl Hb or protein-based radicals [82, 85, 107]. As shown in Figure 4.6B, our studies suggest that pDNA cleavage was not inhibited in presence of Hp (**paper II**). The ferryl state of Hb can be monitored by absorption spectra at region (400-700 nm), and it has different spectral properties compared to metHb [84]. The absorption spectra of the metHb was monitored for 12 h in presence of pDNA, and scanned every 30 mins. After 12 h, no shift in the soret region was observed. This data clearly indicated that the ferryl Hb was not discovered in the reaction, and thus was not responsible for the DNA cleavage (**paper II**).

4.2.2 DNA cleavage caused by Hb variants

In **paper I**, the comet assay revealed that α-Y140F had a significant influence on the genotoxicity of Hb. These mutants were also analyzed using the DNA cleavage assay. It was found that the 30% of the sc pDNA remained in presence of α-Y140F, while 63% and 55% sc pDNA remained in β-Y145F and α-Y140/β145F, respectively. These results are consistent with the findings from the comet assay. To further evaluate the plausible mechanism of DNA damage, several HbF mutants were examined (**paper II**). HbF showed three-fold less pDNA damage compared to HbA, suggests that HbF is more stable, and this finding is consistent with previous findings [60]. An introduction of a cysteine residue in the α-chain (α-A19C) and (γ-C93A/ α-A19C) had a significant role in the pDNA cleavage activity of Hb (Figure 4.7). These data clearly suggest the role of redox active residues on the protein surface in the pDNA cleavage activity of Hb.

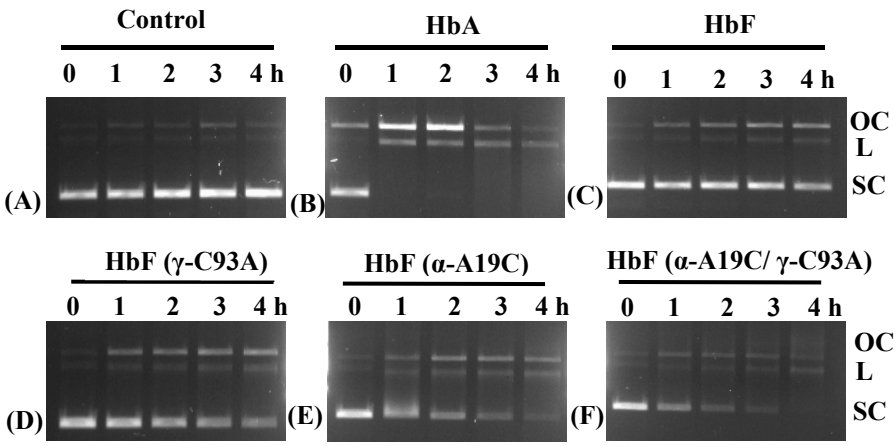

Figure 4.7 The plasmid DNA (pDNA) cleavage activity of HbF mutants (Paper II). (A) control pDNA (B) HbA (C) HbF (D) HbF (γ-C93A) (E) HbF (α-A19C) (F) HbF (γ-C93A/ α-A19C). HbF was more stable compared to HbA. On the other hand, insertion of a Cys residue in the α- chain escalated the pDNA cleavage.

5. Genetic modifications of Hb

During the past decades, various kinds of molecular designs have been incorporated into HBOC products. As mentioned earlier, dissociation of Hb into dimers was responsible for the failure of earlier generations of HBOC products. Thus, the Hb surface of several HBOC products has been chemically or genetically modified to stabilize Hb in its tetrameric conformation. Based on the modifications, present HBOCs belong to three categories including cross-linked HBOCs, conjugated HBOCs and polymerized HBOCs (Figure 5.1) [12, 108]. The cross-linked HBOCs are developed by the formation of intermolecular covalent bonds through the globin chains of Hb molecules, using a chemical reagent or by recombinant technology. For example, Diaspirin-crossed linked Hb (DCLHb), was developed by Baxter and the US army, by utilizing (3,5-dibromo-salicyl)-fumarate to crosslink the two α-subunits. Somatogen, the USA based company, developed a low oxygen affinity Hb mutant, consisting of two α-subunits that are linked by glycine residue (di-α-gly-α).

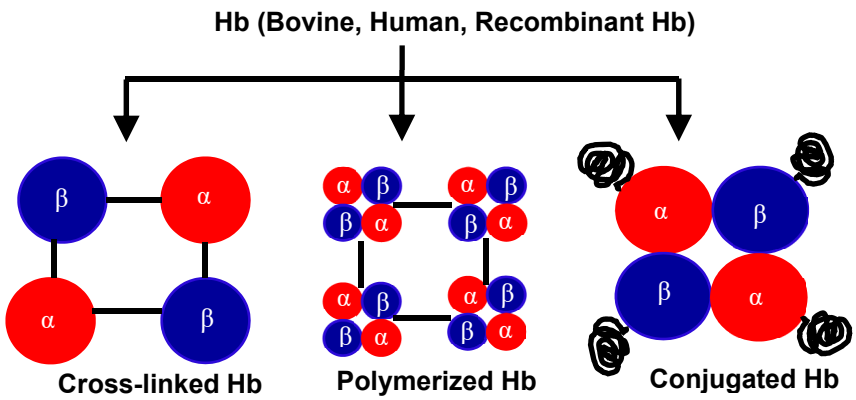

Figure 5.1 Schematic representation of the three main categories of HBOC products. In cross-linked Hb, Hb chains are linked by intermolecular covalent bond. Polymerized Hbs are preapared by crosslinking 4-5 Hb molecules. In the conjugated Hb, a polymer is bound to the surface of Hb molecule.

Conjugated HBOCs were developed by surface modification of the Hb molecule using a PEG moiety. For example, MP4, composed of PEGylated bovine Hb. Polymerized HBOCs is another example of HBOC product in which 4-5 Hb molecules are crosslinked. For instance, glutaraldehyde solution was used to polymerize bovine Hb (Hemopure) [6, 8].

Overall, these chemical modifications were employed to increase the molecular size and intravascular retention time of the Hb molecule. However, an in vitro modification of a cell-free Hb often alters the structural and functional properties of Hb. For example, PEGylation at β-Cys93 residue causes structural disturbances of Hb, which in turn leads to tetrameric instability and exposure of the heme moiety [109]. Besides that, in many cases, it has been reported that PEGylation also alters the oxygen affinity of Hb as reflected by either low or high P_{50} value. Furthermore, these chemical processes on purified products, increase the production cost and often result in a heterogeneous product. The administration of a heterogenic HBOC product may lead to adverse events in itself [110]. Several alternative approaches have been suggested to overcome the drawbacks of PEGylation. For instance, trapping of Hb molecule in clusters of albumins [32], or crosslinking of SOD and catalase together with the Hb molecule [111]. However, each method has its own limitations that mainly interferes with the essential properties of Hb.
In **paper III**, we employed the XTEN technology, to improve the biophysical properties of Hb molecules, while avoiding chemical modifications and their setbacks. For this purpose, we have used a designed fetal hemoglobin, developed in our laboratory which is obtained through genetic linkage of the α- and γ-chains.

5.1 Design of fusion HbF (fHbF)

The most important problem in recombinant production of Hb is linked to its low expression yield. In recent years, many strategies have been employed to obtain higher productivity [2]. For example, co-expression of the human α-subunit and bovine β-subunit [112]. Hb mutants, α-Gly15 to Ala and β-Gly16 to Ala (single or in combination), have been proposed to increase the production yield [41]. The instability of the α-subunit is one of the limitations for recombinant expression of Hb in *E. coli*. One of the strategies described has been to co-express α-hemoglobin stabilizing protein (AHSP), which stabilizes the free α-subunit [112].

In our laboratory, a fusion HbF has been developed in which the C- terminus of the α-chain is genetically linked with the N- terminus of the γ-chain by 12 amino acid residues $(GGS)_4$. The designed protein is expressed as a single polypeptide chain composed of 300 amino acid residues, and named as fusion fetal hemoglobin (fHbF). Notably, the production yield of the purified protein has been significantly higher than for recombinant HbA or HbF. The absorption spectra of fHbF (CO, deoxy, oxy) were identical to the corresponding HbF. Moreover, it can bind to gas ligands such as carbon monoxide and oxygen with a complex mechanism (Ratanasopa et al.). As shown in Figure 5.2, fHbF exists in a partial tetrameric conformation, as determined by small angle X-ray scattering (SAXS) studies. The

molecular size of the fHbF was estimated to be 32 kDa, which in turn may contribute to its rapid kidney clearance. Therefore, it was genetically modified to determine the beneficial impact of the XTEN polymer over the PEGylation (**paper III**).

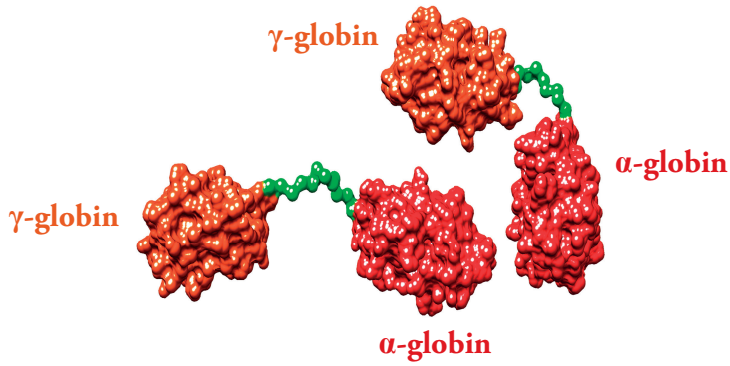

Figure 5.2 Small angle X-ray scattering (SAXS) was employed for evaluation of in solution structure of fHbF. The dimeric protein was found to be in partial tetrameric state due to an interaction between the α-(red) and γ-(orange) globin, linked by 12 amino acids linker (green).

5.2 XTEN as an alternative strategy for PEGylation

XTEN polymers are recombinant PEG mimetics designed to improve the performance of therapeutic proteins. This polymer, an unstructured protein polypeptide of hydrophilic amino acids, avoids several disadvantages associated with the PEG moiety. The XTEN polymer is biodegradable and non-immunogenic, composed of chemically stable amino acids, which include A, E, G, P, S and T. To create a chemically and genetically stable protein polymer, other amino acids were excluded from XTEN. For example, hydrophobic amino acids (F, I, L, M, V, W and Y), which may cause protein aggregation [113], and the positively charged amino acids (H, K and R) can be responsible for protein binding to cell membranes. Almost 1500 unique protein segments were initially screened, to assure that XTEN lack any secondary structures, and to optimize maximum expression in *E. coli*. The XTEN can be genetically linked either to the C- or N-terminal of the protein of interest [114].

In 2009, an initial construct of XTEN containing 864 amino acid residues, was linked to protein Exenatide (glucagon like peptide 1). The N-terminally linked protein was called E-XTEN, and showed improved plasma half-life of exenatide by 71, 65 and 121-folds in mouse, rat and monkey, respectively. This E-XTEN protein was analyzed by several analytical techniques including mass spectrometry (MS), size exclusion chromatography (SEC), RP-HPLC etc. It was observed that XTEN not only increased the molecular size of exenatide, but also appeared as homogenous mixture in solution [113].

Since then, a variety of therapeutics drugs and proteins have been genetically linked to XTEN and expressed successfully. GLP-2G and antiretroviral T-20 drugs were chemically conjugated to the XTEN polymer, and showed improved biophysical properties in terms of long circulation life and solubility [115]. The length (number of amino acid residues) of the XTEN polymer can be adjusted using a genetic engineering approach. Regardless of length, the XTEN polymer behaved as a homogenous class of random coil polymer, and proved to be essential to control plasma half-life. For example, Annexin A5 is a human protein, used to detect apoptosis and cell death, with only 288 amino acids long XTEN tail, showed increased half-life in blood up to 63 min compared with 7 min for wild type Annexin A5 [116]. VRS-859 (XTENylated exenatide) and VRS-317 (XTENylated hGH) have also been extensively studied and are presently in clinical trials phase III [114, 117].

5.2.1 XTENylated fetal Hb (XTEN-HbF)

We genetically fused the XTEN polymer, composed of 313 amino acids, to the N-terminal of fHbF (**paper III**). The purified protein was designated as XTEN-HbF. The SDS- PAGE is a common method to determine the purity of proteins. However, XTEN migrates slowly in SDS -PAGE, which is typical XTEN fusion protein due to large hydration shell of XTEN polymer [116]. Thus, the XTEN-HbF protein showed an apparent size of ~ 110 kDa on SDS-PAGE. However, the theoretical molecular mass of 63 kDa was confirmed with MALDI-TOF (**paper III**).

The low isoelectric point (pI) of Hb molecule has been proposed as a key factor to increase the intravascular retention time [118]. Negatively charged Hb may obstruct the leakage from vasculature walls due to electrostatic repulsion between the cell surface of glomerular basement membrane around the endothelial cells [32]. The XTEN-HbF has lowered the pI (5.0) compared with the fused fHbF (pI: 6.9). Moreover, the XTEN polymer itself does not have any protease sites, which may attribute to increased intravascular retention time of the target protein [119].

Although, it is believed that Hb produced through recombinant technology provides a limitless supply of starting material, the production cost is the main question. Recombinant Hb production may also produce some unwanted protein fractions, such as protein with reverse heme orientation, which disrupts the functional properties of Hb. Circular dichroism (CD) studies have been employed for detection of the heme orientation of the Hb molecules. In **paper III**, the XTEN-HbF was analyzed by CD-spectroscopy, and showed unique CD spectra in the soret region, reflected by the positive peak at 260 nm and a prominent peak at 422 nm. This data suggests correct insertion of the heme moiety in the XTEN-HbF, and is consistent with previous findings [119]. The XTEN-HbF was also analyzed in the UV-CD

region ranging from 200-250 nm. Typical α-helical CD spectra was observed for fHbF protein. However, XTEN-HbF showed a rise in a negative peak at 205 nm, mainly due to the presence of a random coil XTEN polymer (Figure 5.3).

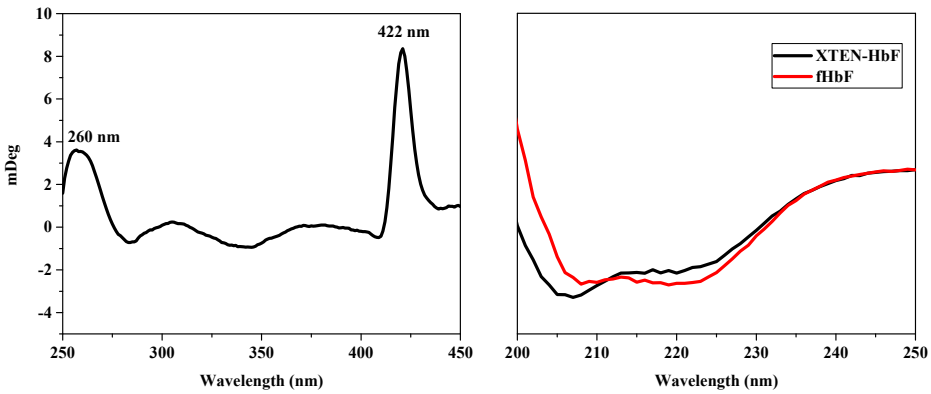

Figure 5.3 CD spectra of the XTEN-HbF and fHbF were analyzed in the soret and far-UV regions. Left panel shows a CD spectra of XTEN-HbF in soret region, prominent positive peaks at 260 nm and 422 nm suggest correct heme insertion, consitent with previous finding by Nagai et al. [120]. Right panel shows far-UV region CD spectra, XTEN-HbF shows a negative peak at 205 nm, presumbly due to random coil character of XTEN polymer.

Moreover, the absorption spectra of XTEN-HbF were identical to fHbF. For example, in the soret region, XTEN-HbF bound to CO and oxygen, showed a maximum peak at 419 and 415 nm, respectively. XTEN polymer displayed monodispersity in aqueous solution, as determined by size exclusion chromatography (SEC) and SAXS studies. The hydrodynamic radius of XTEN-HbF was measured to be 6.2 nm, which was increased by 2.2 folds compared to fHbF (2.8 nm) (**paper III**).

5.2.2 Oxygen binding properties

The oxygen binding curve of Hb is sigmoidal in shape. The partial pressure of oxygen at which 50% of Hb is saturated known as the P_{50} value. It is conventionally measure of Hb affinity for oxygen. Typically, it is 26 and 19 mmHg for native HbA and HbF, respectively. Recombinant technology also make it possible to modulate the oxygen binding properties of the Hb molecule. This can be engineered by substitution of amino acids at both allosteric and active site i.e. α-H58Q and α-V96W [2, 121]. PEGylation often yield a Hb with altered oxygen affinity, which may induce a distinct physiological response [122]. In **paper III**, the oxygen binding curves of XTEN-HbF and fHbF were evaluated using a Hemox analyzer. The P_{50} value for both constructs was found to be ~ 4.20 mm/Hg and Hill coefficient

was > 1 (Figure 5.4). This data indicates that genetically modified Hb can rapidly take up the oxygen, but cooperativity was disturbed due to the design of the α- and γ-chain separated by the flexible linker. Notably, the XTEN polymer has a negligible influence on the oxygen binding properties of fHbF.

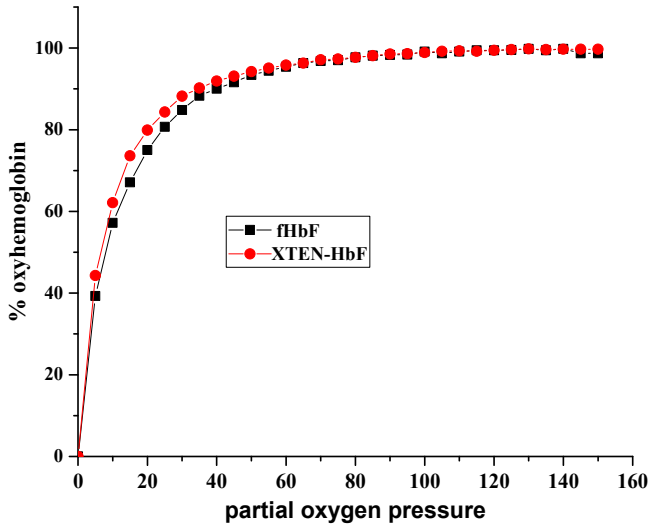

Figure 5.4 P_{50} values of fHbF and XTEN-HbF. The oxygen binding data were determined by hemox analyzer and were found to be identical for both proteins.

5.2.3 In-solution structure

SAXS is a powerful tool, and routinely used method to characterize the biological molecules in solution. SAXS provides low resolution information on shape, conformation and assembly state of proteins. In 1930, French physicist Andre Gunier observed the correlation between scattered light beams and the grain size of the material. Since then, it has become a widely-used technique for low resolution structural characterization of macromolecules [123, 124]. Different Hb molecules have been evaluated using SAXS studies. For example, the size, shape and temperature stability of the Hb molecule originating from *Rhinodrilus alatus* (HbRa), was evaluated by SAXS [125]. Moreover, *Glossoscolex paulistus* hemoglobin (HbGp) was evaluated at different oxidation states [126].
We have also determined the in-solution structure of the XTEN-HbF (paper III). It was observed that XTEN-HbF appears in monomeric form, in contrast to fHbF, which exists in a partial tetrameric state. As depicted in Figure 5.5, XTEN polymer does not interact with the fHbF.

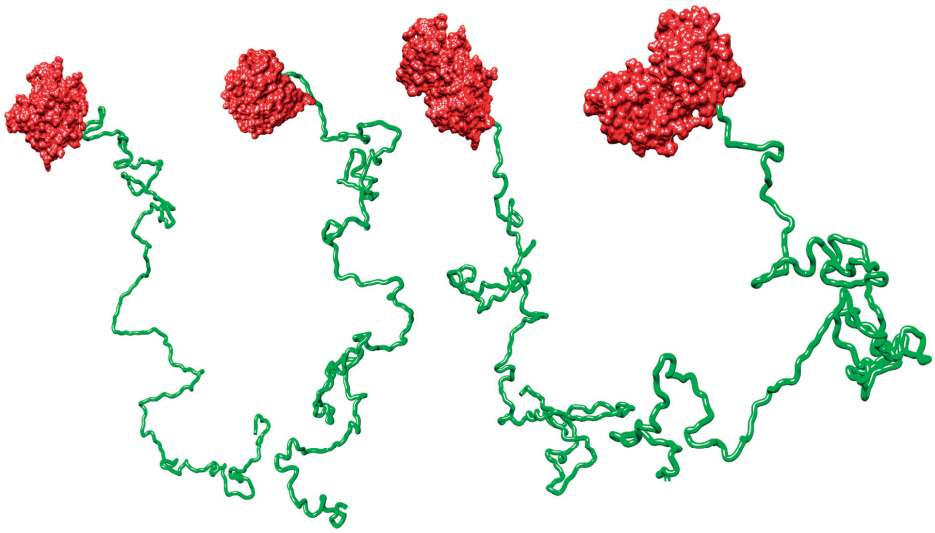

Figure 5.5 PDB models of XTEN-HbF obtained by the small angle X-ray scattering studies (paper III). The red globular units are fHbF and the elongated tails are XTEN (green).

5.3 Green Fluorescent Protein

Green Fluorescent protein (GFP) is a well-known chemiluminescent protein from Aequorea jellyfish. In 1961, Shimomura et al. discovered it as a byproduct of extraction of aequorin from the *Aequorea victoria* jellyfish [127-129]. The discovery of this chromophore led to cloning and expression of GFP, which enabled the widespread usage of GFP in measuring gene expression and cell tracking [130]. GFP is composed of 238 amino acids (27 kDa), but its intrinsic chromophore self-generates from three amino acids, Ser65, Tyr66 and Gly67. Its tertiary structure is composed of eleven anti-parallel β-sheets, known as a 'β-can', and the chromophore is well protected in the central α-helix [131]. The formation of a functional chromophore involves three steps: protein folding, cyclization of protein motif and finally oxidation of cyclized motif. Wild-type GFP has an excitation peak at 390 nm and a primary emission peak at 508 nm [132, 133]. In the past decades, a range of GFP mutant have been created to accommodate different properties in terms of improved fluorescence properties, solubility and folding efficiency [134]. Nowadays, wild type GFP and its variants have been used in numerous applications to study mammalian and plant systems.

5.3.1 Characterization of GFP-HbF

GFP has been genetically linked with plant hemoglobin to facilitate the visualization of subcellular location of Hb in plants [135]. In **paper IV**, a GFP molecule was genetically linked at the N-terminus of the fHbF, and expressed in *E. coli*. The purified protein, designated as GFP-HbF, showed red color in normal light, but turned green in presence of UV-light. Moreover, bacterial cells were collected during the expression of GFP-HbF, and observed under fluorescence microscope (Figure 5.6). Green color of the cells demonstrates that the fluorescence property of the GFP was retained after fusing with fHbF.

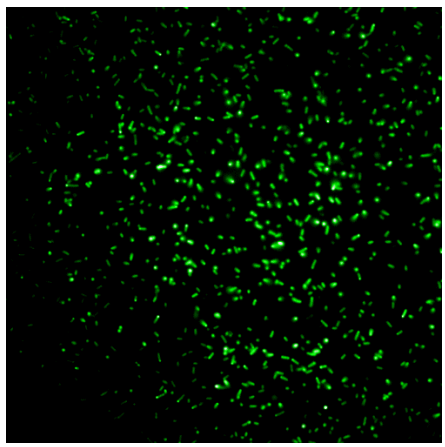

Figure 5.6 The image of a bacterial cells expressing GFP-HbF. Fluorescent property of GFP shows a green colored cells when observed under the fluoresence microscope.

The functional properties of GFP-HbF were also evaluated using the absorption spectra. It was observed that the maximum absorption peaks for GFP-HbF, when bound to CO and oxygen, largely remained unchanged. During the cultivation of GFP-HbF protein in bacterial cells, aliquots of 1ml were collected at different time intervals. These samples were examined using the fluorescence spectroscopy, and showed a gradual increase in peak intensity at 508 nm (**paper IV**). GFP-HbF was applied onto a gel-filtration column to determine the molecular size of the protein. As shown in Figure 5.7, the known molecular weight markers: ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), carbonic anhydrase (29 kDa) and ribonuclease (13.7 kDa), were used to calculate the molecular size of unknown protein. GFP tagged fHbF was eluted earlier than aldolase, showing the size 118 kDa (calculated theoretical value is 58 kDa).

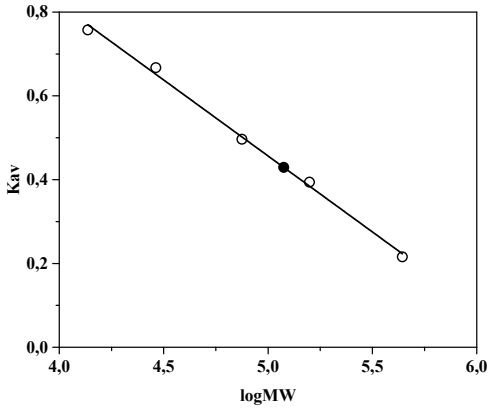

Figure 5.7 Calibration curve (open circle) for the determination of molecular weight. GFP-HbF showed a size of 118 kDa (closed circle), which is two-folds higher than calculated molecular weight.

5.3.2 Efficiency of Molecularly Imprinted Polymers (MIPs)

Molecularly imprinted polymers (MIPs) are highly crosslinked microporous materials with recognition properties similar to biological systems such as enzymes and antibodies. The highly specific binding sites of MIPs are formed in such a way that they have a "memory" of the original template molecules. The imprinting is like that you leave your footprints after you walk on a wet beach. The footprints fit with your feet in terms of both their size and shape. The MIPs have tailor-made binding cavities complementary to the template molecules in shape, size and functional groups. These imprinted cavities can specifically bind and interact the original template through specific molecular recognition. To date, molecular imprinting has been successful mainly for small molecules. It has been more challenging for biological macromolecules [136-138].

Zhou et al. [139] synthesized the Hb imprinted polymers in Pickering emulsion. The obtained particles showed a high selectivity to HbA and the maximum Hb binding capacity was estimated to be 25 mg/g. The binding capacity of the imprinted polymers largely depends on the Isoelectric point (pI) of the protein. Human HbA (pI:6.9) adsorbed to MIPs at pH 6.0, mainly through ionic interaction, and elution was achieved at pH 8.0.

In **paper IV**, GFP-HbF and fHbF were tested in terms of their binding capacity to MIP particles. A solution of GFP-HbF (1-25 μM) was mixed with MIP particles (5 mg), settled, and free Hb in the supernatant was analyzed using UV-Vis spectrophotometer. It was observed that the GFP-HbF exhibited slightly lower binding onto MIP particles, due to the hydrophobic surface of the GFP molecule.

The calculated binding capacity was estimated to be 3.1 μmol g^{-1} and 2.8 μmol g^{-1} for fHbF and GFP-HbF, respectively.

The Phyre2, online tool [140] was employed for the prediction of GFP-HbF structure (Figure 5.8). It suggests that GFP molecule does not interact with the fHbF, although experimental studies suggested that GFP-HbF appears as a dimer in solution, as determined by SEC (section 5.3.1).

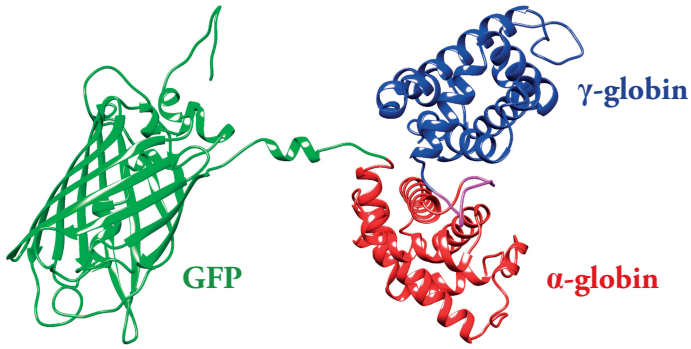

Figure 5.8 Tertiary structure of GFP-HbF. Online tool (Phyre 2) was employed for determination of the structure [140]. GFP (green) molecule was genetically linked to the α-globin (red), which was linked to γ-globin (blue).

6. Production of Hb in *E. coli.*

Production of Hb can be achieved by heterologous expression in bacteria. In 1984, Nagel et al. first developed an expression system for the α- and β-globins in *E. coli*. The expressed protein was obtained in an insoluble form, but it could be solubilized to form a tetrameric Hb when incubated with subunit partners in presence of hemin to form tetrameric Hb [141]. Since then, a variety of other protein engineering techniques have been employed for producing Hb. Nagai and coworkers developed an expression system where both genes were inserted into a single plasmid, followed by addition of heme externally during cell growth. This allowed expression of a fully functional and tetrameric Hb in *E. coli* [41].

In this thesis, we have used different constructs for expression of HbA and HbF. Moreover, the genetically modified Hb molecules have been expressed and purified using different expression and purification conditions.

6.1 Expression and purification of HbA and HbF

The genes encoding HbA and HbF were synthesized by a commercial vendor and cloned in a pETDuet -1 vector, and expressed in *E. coli* strain BL21 (DE3). The genes expressing α-globin and β/γ globin were controlled by the T7 promotor. Similarly, fusion HbF (fHbF) was cloned in a pETDuet vector (Figure 6.1).

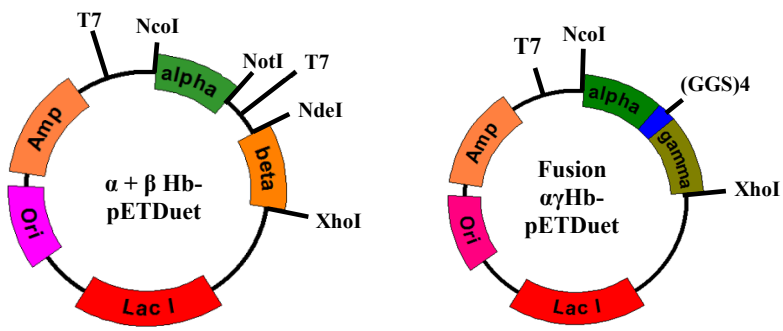

Figure 6.1 Schematic representation of the pETDuet vector harbouring the alpha and beta/gamma chains of Hb. Left plasmid shows a plasmid expressing HbA, both genes were coexpressed under the control of the T7 promotor. Right panel shows a plasmid expressing fHbF, which contains the alpha and gamma chains, linked by a 12 amino acids linker.

All Hb variants were produced by site-directed mutagenesis, and were expressed and purified using an established method [142]. In brief, an overnight starter culture (5 ml) was inoculated into 500 ml Terrific Broth (TB) medium, and allowed to grow at 37^{O}C until $OD_{620} \geq 2$. The culture was induced with 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG) and δ-aminolevulinic acid (ALA, 0.3 mM) was added as the heme precursor. The induced culture was grown at 22^{O}C with modest shaking (60 rpm) [53]. Addition of ALA and IPTG, proved to be a critical step in Hb production. ALA is a primary source for heme production, as it is essential precursor [143, 144]. In addition, the culture was bubbled with CO gas for 10 seconds at each step of cultivation and purification. A slightly modified strategy was used for expression of HbF. An overnight culture was added to 500 ml of TB media and induced with IPTG and ALA directly. This culture was incubated overnight with more vigorous shaking (150 rpm) at 30^{O}C [64].

The HbA and HbF proteins could be purified using two steps of chromatography. Firstly, cells were suspended and sonicated in 10 mM sodium phosphate buffer pH 6.0. This lysate was centrifuged and the supernatant was applied to a cation exchange column (CM Sepharose) connected to an ÄKTA Explorer. The elution of sample was monitored at 419 nm and 280 nm, and the peak eluted at 419 nm was collected and the buffer was exchanged to 20 mM Tris-HCl pH 8.3. The anion exchange chromatography (Q-HP) was employed as a second step of purification. Purified sample was concentrated and kept at -80 ^{O}C until further use. A similar protocol was used for the purification of fHbF. The expression and purification Hb can be observed by the naked eyes due to presence of its red color, as shown in Figure 6.2. Optimization of expression conditions for HbF resulted in significantly higher final yields of protein compared to HbA. The HbF protein induced from the beginning showed a yield of 6-8 mg/L, while in case of HbA, due to presence of an unwanted protein peak, the calculated yield was only 3-6 mg/L. The genetically modified fusion hemoglobin (fHbF) increased the production yield by almost three folds (20-24 mg/L) compared to wildtype HbF.

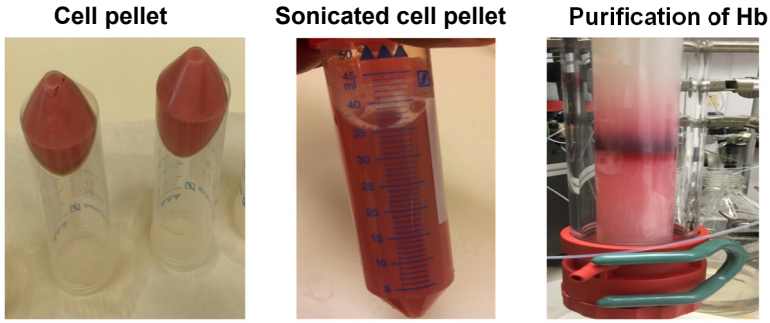

Figure 6.2 Expression and purification of HbA in *E. coli.* The overnight cell culture was harvested and the red colored cell pelletes were sonicated. The Hb was purified by two steps of chromatography. The right image shows elution of concentrated Hb during the first purification step (CM-Sepharose).

6.2 Design and production of XTEN-HbF

In **paper III**, the gene encoding XTEN-HbF was inserted into the pET-28b vector (Figure 6.3) and expressed in *E. coli* strain BL21 (DE3). Earlier studies have demonstrated that the expression of HbA should be carried out with gentle shaking (60 rpm), since aeration affected the final yield of protein [64, 142]. The expression conditions for XTEN-HbF were slightly different from fHbF production. The cells were induced with 0.2 mM IPTG, and allowed to grow with vigorous shaking (150 rpm) at 28^{O}C, resulted in maximum expression. The genetically attached XTEN moiety also acts as purification tag, which facilitated the purification of Hb using anion exchange chromatography [116]. As shown in Figure 6.3, during the second step of purification (Q-HP), negatively charged XTEN-HbF eluted at 250 mM NaCl, while less charged fHbF eluted earlier. Notably, the yield of the proteins was identical when expressed with or without the XTEN polymer, and measured to be 20-24 mg/L.

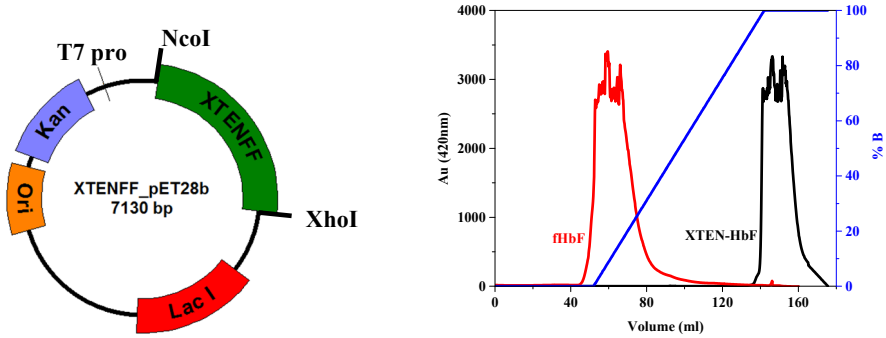

Figure 6.3 Cloning and purification of XTEN-HbF (A) Schematic drawing of the pET-28b vector expressing XTEN-HbF (B) Chromatogram of anion exchange chromatography (Q- HP), which was employed for purification of XTEN-HbF and fHbF, respectively, as a second step of purification. The negatively charged XTEN moiety increased the hydrophilicity of fHbF.

6.3 Genetic conjugation of GFP to fHbF

Enhanced expression of oxygen dependent GFP has been observed, when co-expressed with *Vitreoscilla* hemoglobin (VHb) [145]. Fusion of GFP and VHb was used to analyze the culture during expression, allowing efficient product optimization. Similarly, we have produced a fusion protein, designated as GFP-HbF. A codon optimized gene encoding GFP and fHbF were inserted into the pET28b vector at restriction sites NcoI and XhoI (Figure 6.4).

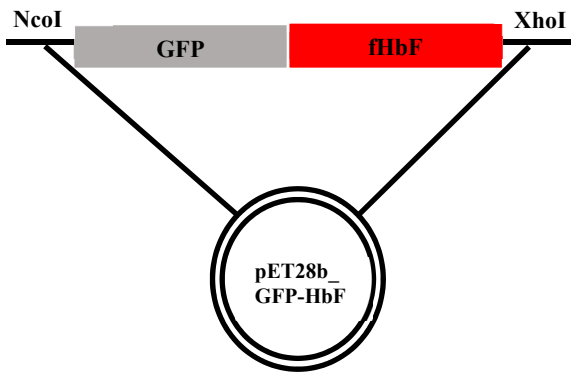

Figure 6.4 Schematic drawing of the pET28b vector expressing GFP-HbF. The C- terminal end of the GFP was linked to the N-terminal of fHbF, and cloned in the pET28b vector.

The pET28b_GFP-HbF vector was transformed into *E. coli* strain BL21 (DE3). GFP requires oxygen to form its chromophore structure. Co-expression of VHb

significantly enhances GFP expression, as reflected by fluorescence intensity, and was found to be dependent on aeration conditions during cultivation [145]. Therefore, GFP-HbF construct was expressed under vigorous shaking (150 rpm) conditions after induction. The expressed protein was purified by two steps of anion exchange chromatography. The final yield of the GFP-HbF was lower (6-8 mg/L) compared to fHbF (20-24 mg/L).

Conclusions and future prospects

The need for an alternative blood substitute is significant in the modern era of transfusion therapy. In the past decades, several strategies have been explored to produce safe and reliable alternatives. Hb-based oxygen carriers have a potential to be used as blood substitutes; however, the intrinsic toxicity of Hb is a major concern, and this needs to be addressed accordingly. The redox active iron of the heme group can be involved in oxidative reactions that can damage the surrounding cells and tissues.

In **paper I**, we have developed a widely-used comet assay for the characterization of tyrosine mutants of Hb. These tyrosine residues are known to be the primary radical formation sites within Hb molecules, therefore, we replaced them with the similar, aromatic amino acid (phenylalanine). The genotoxic effects determined by the comet assay, demonstrated that α-Tyr140 play an important role in maintaining the structure of Hb. However, β-Tyr145, also known as the radical stabilizing site [85], showed a negligible effect on genotoxicity. These data clearly indicate that β-Tyr145 is the leading radical formation site in the Hb samples used in this study. To conclude, the comet assay can be used as a simple test to estimate the initial safety of HBOCs. However, it would be an interesting study to evaluate the genotoxicity of other Hb variants, particularly β-Cys93, α-Tyr42 etc.

In **paper II**, we demonstrated that Hb can also interact with the DNA and initiate its cleavage by conversion of sc pDNA into ocDNA or LDNA. The rate of reaction was directly proportional to the applied concentration of Hb. The underlying mechanism is not completely understood. However, examination of HbF mutants clearly indicates that the protein-based radicals are involved in the DNA cleavage activity. We have also demonstrated that HbF is less reactive on DNA than HbA, which makes HbF attractive as alternative starting material for HBOCs development.

An ideal HBOC should possess a long circulation life and optimal size, to prevent its renal clearance. Therefore, it is essential to design a HBOC product that can be produced in large quantities and in a few steps. In **paper III**, we have used the XTEN technology, originally developed by Schellenberger et al. [113] with slight modifications. The expressed XTEN-HbF maintain the structural properties of fHbF. Notably, XTEN-HbF was found to be as a monomer in solution with an elongated tail as determined by SAXS studies. In conclusion, the XTEN polymer

could be used as an alternative strategy to PEG modification, to produce HBOCs with high yields and with beneficial biophysical properties. It would be interesting to link tetrameric HbF onto the XTEN polymer. However, further *in vivo* studies using mouse or guinea pig models will be needed to assess the efficiency of the recombinant Hb, linked to the XTEN polymer.

Finally, in **paper IV,** fHbF was tagged with GFP at the DNA level, and first results suggest that the functional properties of GFP-HbF are identical to fHbF. The GFP-fHbF construct was used to evaluate the binding capacity of Hb to MIP particles. The imprinted polymers could be simple alternatives for purification of Hb. The low protein yields obtained in *E. coli* can most likely be improved by modifying the expression and purification strategies. GFP will enable us to visualize the function of cell-free Hb molecules in animal models, and may be beneficial to design and improve the safety and efficacy of the HBOC product.

Taken together, our studies suggest that new generation of HBOC products must be tested for their DNA cleavage/damage activity. These types of qualitative and quantitative data on the DNA damage impact of Hb molecules are needed to determine risk potentials. Additionally, the XTEN polymer could facilitate the modifications of a new generation HBOC products. GFP-HbF/fluorescent Hb, may be useful as a parallel/dummy molecule for the incorporation of new molecular design into HBOC products.

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Paper I

The Penultimate Tyrosine Residues are Critical for the Genotoxic Effect of Human Hemoglobin

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Abstract

Hemoglobin (Hb) is a potent oxidant outside the erythrocyte. The tyrosines α140 and β145 play an important role in the structure and function of Hb by forming switch and hinge contacts. These carboxy-terminal residues of the alpha and beta chains, respectively, were replaced to phenylalanine and several different methods were used to characterize the obtained mutants including a comet and plasmid DNA cleavage assay. It was observed that the genotoxic effect was 40% higher for αY140F compared with the wildtype, the βY145F and the double (αY140/β145F) mutants as determined by the comet assay. Cleavage of purified plasmid DNA after Hb application also revealed that the αY140F mutant showed 2-fold higher activity, while the βY145F and αY140/β145F mutants reduced the activity compared to wildtype Hb. This study clearly indicates that the penultimate tyrosines are involved in the genotoxicity of Hb.

Keywords

Hemoglobin, Genotoxicity, Comet assay, Plasmid DNA, Supercoiled DNA

1. Introduction

Free or acellular Hb in the circulatory system is responsible for several serious and potentially life threatening conditions [1]. The protein is involved in a plethora of different reactions, e.g. various redox reactions and binding of nitric oxide (NO). Particularly its oxidative reactions together with hydrogen peroxide have been

dissected in detail [2]. Cooper et al. have demonstrated that βY145F is one of the critical radical formation sites in Hb [3].

The tyrosines adjacent to the C-terminals of the alpha and beta chains of Hb play important roles in stabilizing the T state. The residues αY140 and βY145 thus form hydrogen bonds with the carbonyls groups of αV93 and βV98, respectively [4,5]. The replacement of these penultimate Tyr residues with aromatic amino acids such as Phe has been shown to have a major impact on the oxygen affinity of Hb, especially for the αY140F mutant [5]. Earlier studies have largely focused on the influence of Hb reactivity on lipid and protein modifications. Here, we demonstrate that simple assays can also be utilized to characterize toxicity effects on nucleic acids caused by Hb. In the present study, we have employed the comet and a plasmid DNA (pDNA) cleavage assay for characterizing the genotoxic effects of wildtype (wt) and mutant Hb proteins. It was observed that αY140F escalated DNA cleavage activity of Hb, while the hydrolytic effects were much less pronounced for β145F.

2. Methods

The wt and mutant Hbs were constructed, expressed and purified as described earlier [6]. Primers were purchased from MWG Biotec (Ebersberg, Germany). Enzyme Phusion (Finnzymes Oy, Espoo, Finland) was used for site-directed mutagenesis. The methylated DNA was digested by DpnI (Fermentas, Helsingborg, Sweden) and mutated plasmids were transformed into E. coli strain BL21 DE3. The expressed protein was purified by CM Sepharose followed by Q Sepharose chromatography [7]. To prevent oxidative side reactions during handling, the protein samples were kept in their CO forms by bubbling with carbon monoxide (CO) at every step of expression and purification. The protein concentration was calculated on the basis of molar heme equivalent. To prepare metHb, the CO forms were incubated with potassium ferricyanide (10 mM) at 4ºC in presence of strong light. The excess ferri/ferrocyanide was removed from the ferric proteins by filtration through a size exclusion Sephadex G-25 column (approx. 5 x 0.5 cm). The concentration of Hb was determined by reduction of an aliquot of the ferric protein to the deoxy form using sodium dithionite, and using the extinction coefficient of 133 mM^{-1} cm^{-1} at 430 nm.

Single cell gel electrophoresis or comet assay was carried out to investigate the ability of the Hb samples to induce DNA strand breaks. Lymphocytes were isolated from peripheral blood (collected from healthy male volunteers) using lymphocyte separation medium (HiSepTM LSM 1077, Himedia, India). Cells were washed with phosphate buffered saline (PBS) and finally resuspended in RPMI 1640 medium (Hyclone, Thermo Scientific). Lymphocytes (1×10^4 cells/ml) were incubated with various concentrations of mutant metHb (2.5, 5 and 10μM, 30 min, 37ºC). Hemin

(10 μM), the reactive ferric protoporphyrin-IX group released from a cell-free Hb, was used as control. Cells incubated in RPMI 1640 (30 min, 37°C) and H_2O_2 (100μM, 5 min, 4°C) were used as the negative and positive control, respectively. After each treatment, the cells were washed with cold PBS and processed for the comet assay.

The alkaline comet assay was carried out as described by Singh et al. [8]. The cells (10 μl) were mixed with 90μL of 0.55% low-melting agarose, and layered on slides (precoated with 1.0% normal-melting agarose) and kept on ice for 5 min. After solidification of the agarose, slides were covered with another layer of 90μL of low melting agarose (0.50%) and then immersed in lysis solution (2.5M NaCl, 100mM EDTA, 10mM Tris, 1% Triton X-100, and 10% DMSO) for 2 h at 4°C. After lysis, DNA was allowed to unwind in alkaline buffer (300mM NaOH, 10mM Na_2EDTA, pH> 13.0) for 25 min before electrophoresis at 25 V and 300 mA for 25 min at 4°C. To neutralize the excess alkali, slides were washed thrice with neutralizing buffer (0.4 M Tris, pH 7.5) for 5 min at 4°C. Slides were then stained with 75μL of ethidium bromide and observed under the fluorescence microscope (Olympus CX41, Japan) equipped with a CCD camera. Comet images (50 cells from each of two replicated slides, three independent experiments and 300 images per sample) were analyzed by a computer-assisted image analysis system (CASP software). Percent tail DNA (% tail DNA) was determined for quantification of DNA damage.

The DNA cleavage assay was also carried out by incubating each ferric Hb (metHb) variant with purified pUC18 plasmid (25 ng/μl) at 37°C. DNA degradation was monitored by analyzing samples every 2 hours. The percentage of remaining supercoiled plasmid DNA (sc DNA) was determined by agarose gel electrophoresis as estimated by densitometry analysis with the help of the 'Quantity one' software from Bio-Rad. Calculations of statistical significance were made by one way Anova followed by Tukey's multiple comparison test.

3. Results

Lymphocytes were incubated with several concentrations (2.5-10 μM) of each mutant metHb. Any possible interference from intrinsic haptoglobin [9] in the assay was minimized by avoiding the use of calf serum in this assay. These experiments demonstrated that Hb induced severe DNA damage in lymphocytes already after 30 min incubation at 37°C. The mutant αY140F induced significantly more (40%) DNA damage ($P < 0.05$) compared with wt Hb. In the range tested, DNA damage effects were proportionally dependent on the Hb concentration used. The βY145F and double (αY140/β145F) mutants showed the same DNA damages as wt Hb (Fig. 1, Table 1). Hemin was used as a control to examine if the molecular mechanisms behind the DNA cleavage observed could be directly linked to the heme groups or

associated with radicals on the globin chains. It resulted in similar cleavage results as control cells, indicating that formation of radicals on the globin polypeptide chains is involved in the reaction. Generally, the Tyr residues are the primary radical formation sites within the Hb molecule, under the oxidative stress [10]. Therefore, another Phe mutant was examined, αY24F, but it showed identical damage patterns as wt Hb further strengthening the proposed roles of αY140 and βY145 in modulating DNA damage effects.

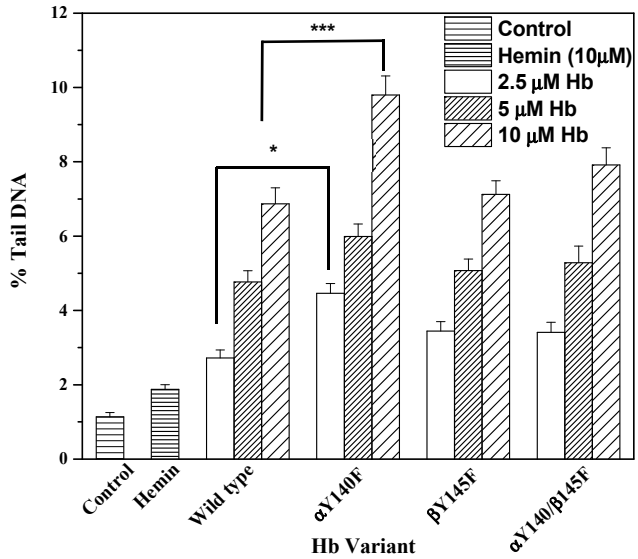

Fig. 1. DNA damage by different Hb variants as determined by the comet assay. αY140F showed significantly more DNA damage than wildtype Hb. Comet images from three independent experiments were performed and 50 comet images were analyzed for each experiment in duplicate. Data were analyzed by one-way ANOVA followed by Tukey's multiple comparison test. (* P <0.05; *** P <0.001)

Table 1. Comet assay of different Hb samples. The % tail DNA at different protein concentrations were determined.

Sample	% tail DNA		
	2.5 μM Hb	5 μM Hb	10 μM Hb
Wild type	2.72	4.77	6.89
αY140F	4.46*	5.99^{ns}	9.80***
βY145F	3.45^{ns}	5.07	7.29^{ns}
αY140/β145F	3.41^{ns}	5.28	8.00^{ns}

Control sample and hemin did not cause DNA damage. An average of 300 images/sample were analyzed and statistical calculations were performed by one-way ANOVA followed by Tukey's multiple comparison test (* P <0.05; *** P <0.001; ns- not significant).

To further characterize the DNA cleavage activity of Hb, the protein samples were incubated together with purified supercoiled plasmid DNA (scDNA). The cleavage activity proved to be directly related to the concentration of Hb applied. Under the conditions used, wt showed 40% of remaining sc DNA, whereas only 30% remained after 12 hours of incubation using the αY140 mutant. However, when examining the βY145F and the αY140/β145F mutants, 63% and 55% scDNA remained, respectively (Fig. 2).

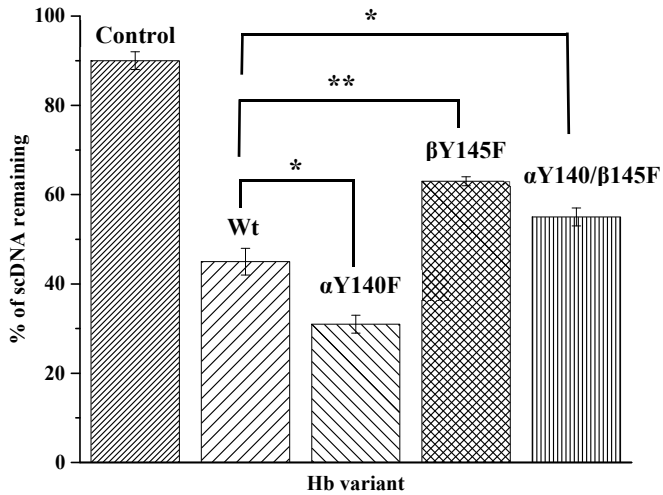

Fig. 2. Effects of different Hb variants on plasmid DNA cleavage as analyzed by the reduced level of supercoiled DNA after incubation for 30 min at 37°C (n=6, * P <0.05; ** P <0.01).

4. Discussion

Many enzymes use Tyr residues in catalysis or long-range electron transfer. This study focused on the penultimate Tyr residues of Hb. There are several natural mutants at these position: Hb Osler (Tyr β145Asp), Hb Bethesda (Tyr β145His), Hb Rainier (Tyrβ145Cys) and Hb McKees Rocks (Tyr β145 terminated) with a high oxygen affinity and lack of T state conformation. However, only one natural mutant for αY140 has been reported where the Tyr has been replaced with His (HbVar database) [11].

Hb is a redox active protein that easily can modify proteins, lipids and nucleic acids. Two different assays were developed to characterize the damaging effects of Hb on DNA. In the comet assay, lymphocytes were used to characterize the genotoxic effects of different Hb mutants. In this approach, Hb molecules penetrate the cell membranes and are then allowed to react with available nucleic acids. The effects of Hb on cells are rapid, within minutes, and take place even at very low

concentrations, in the μM range. Both the oxy and met forms of Hb generate similar DNA cleavages. Glei et al. have shown that cell-free Hb can cause DNA damage in primary colon cells as well in a colon cancer cell line. Moreover, hemin proved to be more cytotoxic than genotoxic by interacting strongly with cell membranes [12].

The comet assay was complemented with a more direct plasmid DNA based assay. Two levels of DNA degradation caused by Hb were monitored, the conversion of sc to open circular (oc) DNA, which requires a single nick on the DNA molecules, as well as the more extensive cuts needed for formation of linear DNA. Both the comet and plasmid assays generated similar results. It was observed that αY140F showed higher DNA cleavage activity than the βY145F and αY140/β145F. Recent studies by Zhao et al. [13] and Deshpande et al. [14] using myoglobin have also confirmed the role of reactive oxygen species in pDNA cleavage as well as the involvement of catalytically active amino acids for DNA cleavage. Lipid peroxidation was examined for comparative purposes and it was observed that αY140F produced higher levels of conjugated diene than wt (unpublished data). Thus, similar data were obtained from the lipid peroxidation and comet assay, but this is not absolute since they occur with different mechanisms. In comet assay, DNA damage can occur due to the mitochondrial or membrane damage [15]. However, peroxyl and alkoxyl radicals are produced from lipid hydroperoxides by the redox active heme [16]. Interestingly, βY145F and αY140/β145F generated less marked DNA cleavage compared with wt, indicates the penultimate Tyr of the beta chain is a leading radical formation site and is consistent with previous findings [3]. This can be valuable for the further development of hemoglobin-based oxygen carriers (HBOCs) with reduced genotoxicities.

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Paper II

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Research paper

Fetal hemoglobin is much less prone to DNA cleavage compared to the adult protein

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ABSTRACT

Hemoglobin (Hb) is well protected inside the red blood cells (RBCs). Upon hemolysis and when free in circulation, Hb can be involved in a range of radical generating reactions and may thereby attack several different biomolecules. In this study, we have examined the potential damaging effects of cell-free Hb on plasmid DNA (pDNA). Hb induced cleavage of supercoiled pDNA (sc pDNA) which was proportional to the concentration of Hb applied. Almost 70% of sc pDNA was converted to open circular or linear DNA using 10 μM of Hb in 12 h. Hb can be present in several different forms. The oxy (HbO_2) and met forms are most reactive, while the carboxy-protein shows only low hydrolytic activity. Hemoglobin A (HbA) could easily induce complete pDNA cleavage while fetal hemoglobin (HbF) was three-fold less reactive. By inserting, a redox active cysteine residue on the surface of the alpha chain of HbF by site-directed mutagenesis, the DNA cleavage reaction was enhanced by 82%. Reactive oxygen species were not directly involved in the reaction since addition of superoxide dismutase and catalase did not prevent pDNA cleavage. The reactivity of Hb with pDNA can rather be associated with the formation of protein based radicals.

1. Introduction

Hemoglobin (Hb) is a tetrameric protein, composed of two alpha (141 residues) and two beta (146 residues) chains, forming an $\alpha_2\beta_2$ heterotetramer of 64 kDa. Each subunit harbors a heme in the center pocket responsible for its oxygen binding capability [1]. Although often viewed solely as an oxygen transporting protein, Hb has a very rich chemistry related to the reactivity of the iron atom in the heme groups. Hb is thus involved in several redox reactions and shows e.g. peroxidase-like activity, in which ferric or metHb (Fe3+) can react with hydrogen peroxide (H_2O_2) to form a potent oxidant ferryl Hb (Fe4+) along with protein-based radicals. These oxidized forms of Hb are in turn highly reactive and are involved in oxidative damages to a variety of biological molecules [2,3]. Modifications of particularly lipids and protein side chains caused by Hb have been examined in detail. Moreover, Hb can bind and react with nitric oxide (NO) to form nitrate and ferric heme. NO depletion through these reactions leads to vasoconstriction and platelet aggregation [4]. Hb is normally encapsulated in the protective environment of the erythrocytes, but when released upon hemolysis its redox reactions may cause serious damages to surrounding cells and tissues. This becomes especially pronounced at various hematological disorders or at blood transfusion events [5–7], when substantial amounts of cell-free or acellular Hb are released into the circulatory system. To prevent these harmful reactions, several defence proteins have evolved. The plasma protein haptoglobin (Hp), binds cell-free Hb rapidly and almost irreversibly, and transports the complex Hp-Hb to the CD163 receptors mainly located on the Kuppfer cells of the liver for enzymatic degradation and reuse [8,9]. Hemopexin and alpha-1-microglobulin are also involved in the protective system against toxic acellular Hb [10,11]. Cell-free Hb may thus insert a substantial burden to our body at a range of conditions and the use of Hb protective and degradative proteins have been proposed to soon also find clinical practice [12].

Despite its clinical manifestations, only limited knowledge of the interactions between Hb and nucleic acids are available. Hb can bind to calf thymus DNA and the protein also carries an endonuclease-like activity which has been examined by the conversion of supercoiled plasmid DNA (sc pDNA) to nicked circular DNA in the presence of H_2O_2 [13]. Similarly, myoglobin (Mb) showed a distinct pathway for cleavage of DNA upon chemically induced reductions in presence of oxygen or when Mb was present in its met form [14,15]. Hb can also induce DNA damages directly at the cellular level by rapid uptake and

Abbreviations: Hb, hemoglobin; metHb, ferric hemoglobin (Fe3+), HbA, adult hemoglobin; pDNA, plasmid DNA; sc pDNA, supercoiled plasmid DNA; ocDNA, open circular plasmid DNA; LDNA, linear plasmid DNA, CO, carbon monoxide; KCN, potassium cyanide; HbF, fetal hemoglobin; Hp, haptoglobin; SOD, superoxide dismutase; DMSO, dimethyl sulfoxide

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cleavage of available nucleic acids. This has been demonstrated for primary colon cells [16], leukocytes [17] and lymphocytes [18]. The use of the comet assay has been instrumental for quantifying the genotoxic effects of Hb, which occur rapidly and already at low concentrations.

In the human population several different Hb variants have been identified [19]. In this study, wild-type and mutant Hb molecules have therefore been examined to characterize their reactivity to nucleic acids. For this we have studied plasmid DNA (pDNA) cleavage by Hb. Purified pUC18 plasmid was incubated with various Hb samples, adult and fetal Hb, i.e. HbA and HbF, respectively, at different experimental conditions. Hemin (free heme in the met form) was included in the study to evaluate the effects of globin polypeptides and heme separately. It was observed that metHb interacts with pDNA and exhibit high endonuclease activity, responsible for conversion of sc pDNA into open circular DNA (ocDNA) or linear DNA (LDNA). Interestingly, HbF, which shares the same alpha chains as HbA, but has unique gamma chains, exhibited no or only low measurable reactivity against nucleic acids. This may have several clinical implications for treatment of a range of hematological conditions, like sickle cell anemia (SCA) and different forms of thalassemia.

2. Material and methods

2.1. Plasmid purification

Plasmid was prepared and isolated as described in our previous work [20]. The *E. coli* strain TG1 (GE Healthcare, Uppsala, Sweden) was used as host in all experiments for production of plasmid pUC18 of 2686 base pairs (GE Healthcare, Uppsala, Sweden). Cells were grown in Luria-Bertani (LB) media at 37 °C overnight, harvested by centrifugation and plasmid DNA was isolated using a dedicated purification kit (NucleoSpin Plasmid, MACHEREY-NAGEL, Germany). The DNA was eluted with 20 mM sodium phosphate buffer pH 7.2 and purified plasmid was stored at −80 °C until further use. The concentration of pDNA was determined by an Implen Nanophotometer (Labvision AB, Sweden).

2.2. Hb preparation

Recombinant HbA was constructed, expressed and purified as described earlier [3]. In brief, the alpha and beta chains of Hb were cloned in the pETDuet-1 vector and transformed into *E. coli* strain BL21 (DE3). Transformed cells were grown in Terrific Broth (TB) media with vigorous shaking (150 rpm) in baffled 2 L flasks at 37 °C until $OD_{620} \geq 2$. The cultures were then induced with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG, Saveen Werner AB, Sweden) and supplemented with 0.3 mM δ-aminolevulinic acid (Sigma Aldrich), and allowed to grow overnight at 22 °C with reduced aeration (60 rpm). Cells were collected and sonicated in 10 mM sodium phosphate buffer pH 6.0. Protein was purified using a weak (CM Sepharose, GE Healthcare, Sweden) followed by strong (Q Sepharose HP, GE Healthcare, Sweden) ion exchange chromatography. The purified recombinant Hb samples were identical with the native protein and stored at −80 °C until further use. To keep the Hb samples in a stable form, carbon monoxide (CO) gas was bubbled through all solutions used at induction and each step of the purification.

The ferric form of Hb (metHb) was obtained by incubating Hb with 10 mM excess of potassium ferricyanide (Sigma Aldrich). This solution was passed through a gel filtration column Sephadex G-25 (5×0.5 cm, GE Healthcare, Sweden) to remove excess of ferricyanide. The concentration of Hb was determined by reducing an aliquot of the ferric Hb with sodium dithionite (Sigma Aldrich) to the deoxy form (430 nm=133 mM^{-1} cm^{-1}). The Hb concentration used in all experiments is given on the basis of molar heme. To obtain the oxy form of Hb, the CO-Hb was exposed to a continuous stream of oxygen gas in presence of light. All the experiments were carried out in 20 mM sodium phosphate buffer pH 7.2 at 37 °C using the ferric form of Hb or as otherwise specified.

2.3. DNA cleavage assay

The pDNA (25 μg/ml) was incubated with different concentrations (1–100 μM) of the ferric Hb at 37 °C using PCR tubes (total reaction volume of 20 μl). Sample was removed after every 2 h and analysed by agarose gel (1%) electrophoresis carried out for 60 min at 100 V using TAE buffer (Tris-acetate-EDTA, pH 8.0). A control sample was included in all experiments containing only pDNA. The different forms of DNA (supercoiled, open circular and linear) were quantified by densitometric analysis using the 'Quantity one' software from Biorad. To evaluate the temperature dependent degradation of pDNA, Hb (20 μM) was incubated with pDNA at different temperatures (10–40 °C). The DNA damage effects were also evaluated in different concentrations of sodium phosphate buffer (20, 50 and 100 mM, pH 7.2). Similarly, oxy and CO adducts of Hb were examined. The absorption spectra of Hb (5 μM) with or without pDNA was taken every 30 min at 37 °C for 12 h using a Cary60 UV–Vis spectrophotometer (Agilent Technologies). All data were analysed from minimum three independent experiments.

2.4. Preparation of HbF mutants

Site-directed mutagenesis was employed for removing a cysteine residue in the gamma chain (γ-C93A) close to the heme group. Similarly, an alanine residue located on the surface of the alpha chain was substituted to cysteine (α-A19C). Primers used were purchased from integrated DNA technologies (IDT, Germany). Forward sequence of primers employed are as follows (reverse primers were complementary to forward primers): bold sequence represents mutation, 5′-GGGGTAAAGTTGGT**TGC**CATGCCGGTGAA-3′ (α-A19C) and 5′-GAGTGAACTGCAC**GCC**GATAAACTGCAC −3′ (γ-C93A). A double mutant was prepared using similar primers. After PCR, the methylated DNA was removed using DpnI (Thermo Scientific) digestion. These mixtures were transformed into *E. coli* strain BL21 (DE3). Sequences of the transformed clones were confirmed (GATC Biotech, Germany). The expression conditions were slightly different than for HbA. In brief, the starter culture was inoculated into 500 ml TB media and was induced immediately. This culture was allowed to grow overnight at 30 °C and 150 rpm. These mutants and wildtype HbF variants were purified as described elsewhere [21]. HbF and HbF mutants (25 μM) were incubated with supercoiled plasmid pUC 18 (10 μg/ml) at 37 °C. Sample were removed after 1, 2 and 3 h respectively, and analysed by agarose gel electrophoresis.

2.5. Inhibition studies of Hb reactivity

A potassium cyanide (KCN) solution (100 μM, Sigma Aldrich) was mixed with Hb (50 μM) prior to the experiment for 1 h to allow complete complexation of the heme iron to prevent any oxidation reaction. The complex was subsequently incubated with pDNA (25 μg/ml) at 37 °C for 12 h. In another set of experiment, Hb samples were incubated together with pDNA containing sodium chloride (100 mM and 500 mM). Similarly, metHb (20 μM) and pDNA was incubated with superoxide dismutase (SOD, 20 units, Sigma Aldrich), catalase (20 units, Roche), ascorbate (20 μM, Sigma Aldrich) and dimethyl sulfoxide (DMSO, 100 μM).

2.6. Protection by haptoglobin

A haptoglobin (Hp) sample containing primarily dimers (Hp 1-1) and to a lesser extent polymer (Hp 2-1, and Hp 2-2) was kindly provided by Bio Products Laboratory (BPL, Hertfordshire, UK). Hp: Hb in ratios (1:1.25, 1:0.625, 1:0.25, 1:0.125) were incubated for 10 min.

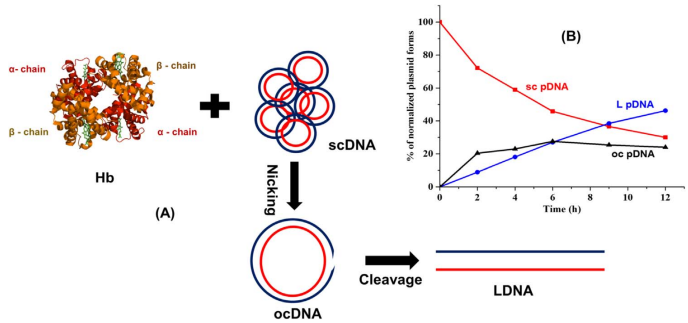

Fig. 1. Schematic representation of pDNA cleavage activity by metHb. A) Hb tetramers (alpha and beta chains in red and orange color, respectively) interact with sc pDNA (red and blue strands) and convert it to open circular (oc) DNA by nicking. The ocDNA may be further cut into linear (L) DNA. B) Different isoforms of pDNA were observed after incubation with Hb (10 μM). The relative fraction of each isoform, as determined by agarose gel electrophoresis, was plotted against time and the gradual increase of ocDNA and LDNA , respectively, was monitored. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

The Hb-Hp complex formed was subsequently incubated with pDNA (25 μg/ml) at 37 °C and analysed as described earlier.

3. Results

Hemoglobins have in general a rich redox chemistry, which results in oxidation or reduction of the heme iron frequently followed by formation of protein radicals. Several alternative approaches have been used to monitor these reactions, both *in vitro* and *in vivo*.

3.1. DNA cleavage activity of HbA and factors responsible

In this study, we have used sc pDNA as screening nucleic acid molecule to characterize the DNA cutting activity of different Hb samples. Upon cleavage of one of the strands, the sc pDNA form is converted into ocDNA. If such a cleavage reaction is allowed to proceed, a linear DNA molecule is generated (Fig. 1A). These modifications of pDNA in the presence of Hb, can easily be monitored over time using agarose gel electrophoresis. Different concentrations of ferric Hb were first incubated with pDNA at 37 °C in 20 mM sodium phosphate buffer pH 7.2. Hb (10 μM) induced significant cleavage of pDNA and the reaction could be quantified over time. As shown in Fig. 1B, under the conditions used, only 30% sc pDNA remained after 12 h, while ocDNA and LDNA increased to 46% and 23%, respectively, of the total DNA composition. These data thus suggest that sc pDNA can be rapidly degraded into ocDNA and/or LDNA in the presence of ferric Hb alone.

Agarose gel electrophoresis could be used to quantitatively follow the progressive cleavage of sc pDNA in the presence of ferric Hb. It was observed that even low Hb concentrations could initiate the unwinding of sc pDNA. After 12 h, 5 and 10 μM of Hb showed 50% and 70% conversion of sc pDNA, respectively. The degree of pDNA cleavage proved to be directly correlated with the concentration of Hb used in the experiment (Fig. 2A). When using higher concentration of Hb (75 μM and 100 μM) complete degradation of pDNA was observed over the time (Fig. 2B). When the remaining sc pDNA concentration was plotted against time, the degradation could be fitted to an exponential decay equation to obtain the reaction rate constant. The decay constant was found to be 3300 M^{-1} h^{-1}, with R^2 value of 0.99.

Most of the experiments were carried out at physiological temperature, i.e. at 37 °C. The GC content of pUC 18 plasmid is 50.6% and the melting temp (Tm) is approximately 75 °C (online tool), while for the human hemoglobin it is 64 °C. In order to analyse if any small structural rearrangements in the pDNA or Hb molecules could influence the degradation, the reaction was also monitored in the temperature range (10–40 °C). There was a clear temperature dependence and pDNA cleavage was more rapid at the higher temperature analysed. The sc pDNA was completely cleaved at 40 °C in 12 h. However, at the lower temperature (10 °C), 50% of sc pDNA remained after 12 h. Similarly, at 20 °C and 30 °C, almost 70% and 90% of sc pDNA were damaged after 12 h, respectively (data not shown). These data clearly suggest that increasing temperature also influence the pDNA cleavage action of Hb. The buffer component also influences the DNA cleavage activity of iron, Saran et al. [22] explained 'crypto OH radicals', as reactive species responsible for interaction of phosphate buffer and iron (Fe2+). In our studies, low buffer concentrations (20 mM) initiate the pDNA cleavage activity at slower rate. Notably, at 100 mM buffer concentration showed the highest pDNA cleavage, almost complete degradation of sc pDNA was observed. However, 43% and 9% of sc pDNA remained using 20 mM and 50 mM buffer concentration, respectively .

Besides the met form, Hb can also be present in several other different redox states. In addition, different ligands may be bound to the heme iron, including oxygen and CO. As shown in Fig. 3, the carboxy-Hb form (CO-Hb) reduced the pDNA cleavage activity compared to the oxy and ferric forms of Hb. CO binds strongly to the ferrous form of Hb and block further redox activities.

The CO-Hb molecule is also stable and was therefore used during purification of the protein [23]. CO-Hb thus showed 40% damage to sc pDNA compared to 70% damage by metHb. Autoxidation of oxy-Hb (HbO_2) easily produces metHb and superoxide radicals ($O_2^{\bullet-}$) which can dismutate to O_2 and H_2O_2, followed by formation of ferryl (Fe4+) species. This can in turn oxidize surrounding biological molecules i.e. DNA, lipids and proteins [24]. This could also be observed in our pDNA assay showing that oxyHb was six-fold faster compared to CO-Hb.

The physical interactions between DNA and Hb have been studied earlier, and it has been demonstrated that protein conformational changes increase the absorbance. Since HbA and HbF differ in their DNA degradation activities, absorption spectra were recorded for Hb in presence of pDNA for 12 h and scanned every 30 min at 37 °C. After 12 h, no shift in the soret region peak (405 nm) was observed. However, the absorbance intensity of HbA alone decreased by 15% (Fig. 4A). In the case of HbF, no such changes were detected. Interestingly, an addition of DNA increased the stability of HbA, while HbF showed gradual increase in peak intensity at soret region (Fig. 4B). In another set of experiment, protein unfolding was monitored at increasing temperatures, intrinsic tryptophan and tyrosine fluorescence was analysed to evaluate the Tm of the Hb variants examined. It was observed that the Tm values of the proteins were

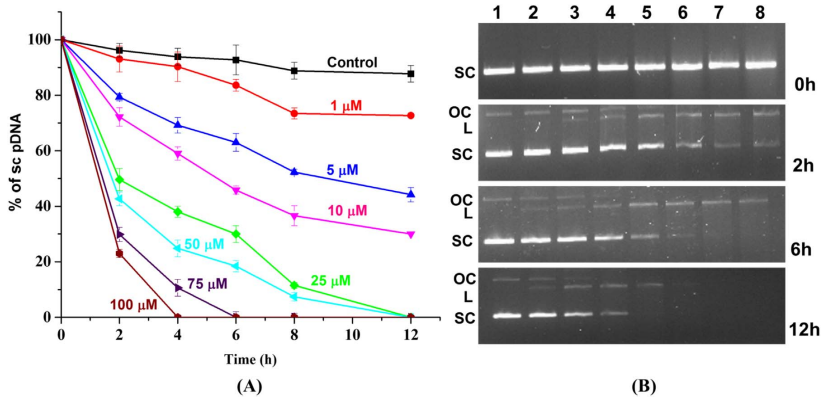

Fig. 2. pDNA cleavage kinetics by ferric Hb as determined by agarose gel electrophoresis. (A) Characterization of the dose dependent cleavage of sc pDNA by Hb in the concentration range 0–100 μM at 37 °C (B) Agarose gel electrophoresis of DNA samples isolated at 0, 2, 6 and 12 h using pUC18 pDNA (25 μg/ml). Lane 1, pUC18 DNA without Hb addition; lane 2, 1 μM Hb+pDNA; lane 3, 5 μM Hb+pDNA; lane 4, 10 μM Hb+pDNA; lane 5, 25 μM Hb+pDNA; lane 6, 50 μM Hb+pDNA; lane 7, 75 μM Hb+pDNA; lane 8, 100 μM Hb +pDNA. Data present mean ± S.D., n=3.

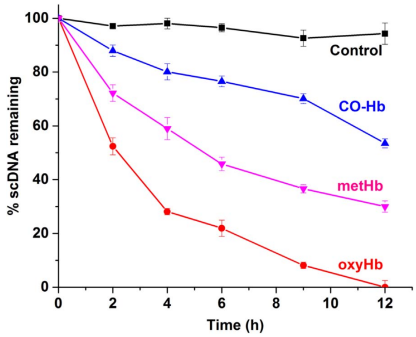

Fig. 3. Dependence of Hb redox state and ligand binding on pDNA degradation. The different forms of Hb were incubated in presence of pDNA at 37 °C for 12 h and the percentage of remaining sc pDNA was determined. Control (black), CO (blue), oxy (red) and ferric (pink) form of Hb (10 μM) was incubated with pDNA and samples were taken at intervals of 2 h. CO-Hb was less reactive compared to oxy and metHb. Data present mean ± S.D., n=3. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

decreased by 2–3 °C in presence of pDNA. This suggests that physical interactions between pDNA and Hb occur before the cleavage activity. The pDNA alone did not show any significant differences in its spectral properties (data not shown).

3.2. Protective nature of HbF and mutants

Several different Hb variants may be present in the human body. Of particular interest is the fetal Hb (HbF) which has been shown to harbor a different redox activity compared with HbA. This has been shown to be due to the absence of a redox active amino acid network in HbF [25]. It was found that HbF cleaved pDNA at a much slower rate. For instance, while HbA under the same conditions initiated pDNA cleavage after 1 h incubation and complete degradation of sc pDNA was observed in 2 h, HbF initiated unwinding of sc pDNA first after 3 h and 76% of sc pDNA was retained (Fig. 5A). Besides the wildtype, different HbF mutants carrying redox active side chains were examined. The cysteine residue present on the gamma chain of HbF, γ-C93, is the first primary location of protein radical formation, and is known to be redox active in Hb introduced on the protein surface [25,26]. When this cysteine residue was replaced with alanine (γ-C93A), prominent difference in DNA cleavage was not observed, 36% of sc pDNA was cleaved compared to 24% for the native protein. However, introduction of cysteine on the surface of the alpha chain (α-A19C) promoted the reaction substantially, resulting in conversion of 55% of the sc pDNA after 3 h, an 82% escalation of the reaction rate. The double mutant (αA19C/γC93A) showed similar pDNA activity as α-A19C (Fig. 5B). These data clearly suggest the role of redox active residues on the protein surface in the pDNA cleavage activity of Hb.

3.3. Inhibition of DNA cleavage induced by Hb

KCN has very high affinity for heme iron and effectively displaces other ligands from the protein and prevents further redox activity [23]. When the pDNA cleavage activity was monitored in the presence of KCN treated Hb, no reactivity was observed (Fig. 6A, lane 1). It suggests that involvement of the central heme, which initiates oxidative or radical formation reactions, is critical for pDNA cleavage. The absorption spectra also suggest physical interactions of Hb and pDNA, which can be based on both hydrophobic and ionic interactions. When the reaction was followed in the presence of high sodium chloride concentrations (500 mM), the pDNA cleavage activity of ferric Hb could be inhibited (Fig. 6B, lane 1). Also at lower (100 mM) NaCl levels (Fig. 6B, lane 3), the DNA cleavage was reduced, but did not completely inhibit the reaction. These results suggest that electrostatic interactions are needed between Hb and pDNA to generate DNA cleavage. Free hemin (10 μM), the reactive ferric protoporphyrin-IX group potentially released from cell-free Hb, was also examined. When added alone to the reaction mixture and incubated for 6 h, it was observed that hemin showed no pDNA cleavage activity (Fig. 6C). It clearly further supports that, not heme alone, but rather amino acid residues oxidized by the heme groups are responsible for pDNA cleavage.

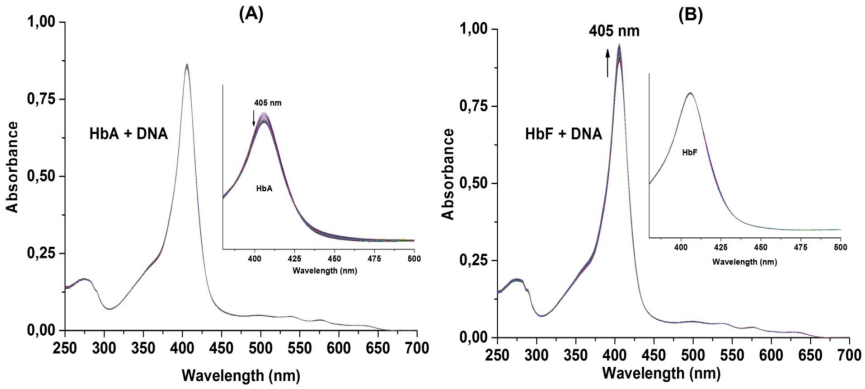

Fig. 4. The absorption spectras of metHb (5 μM) and pDNA (25 μg/ml) were recorded every 30 min for 12 h at 37 °C. A) The absorption spectra of HbA was not altered in presence of pDNA, however, gradual decrease in peak intensity of HbA (without pDNA) was observed (A, inset). B) The peak intensity of HbF, in presence of pDNA was increased over the time. HbF alone did not showed any change in absorbance during an incubation period (B, inset).

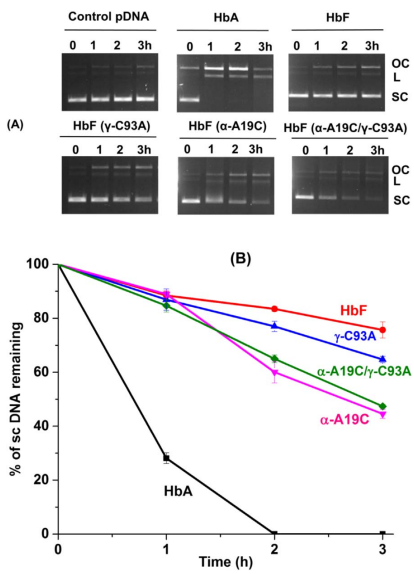

Fig. 5. HbF mutants (25 μM) were incubated with pUC18 (10 μg/ml), and agarose gel electrophoresis was carried out to determine the levels of remaining sc pDNA. A) Agarose gel electrophoresis of pDNA after treatment with HbF mutants. HbF did not induce pDNA cleavage in 3 h, while complete cleavage was observed in presence of HbA. An insertion of Cys residue in the alpha chain escalate the pDNA cleavage. OC, L and SC represent open circular, linear and supercoiled DNA, respectively. B) The percentage of remaining sc pDNA was plotted against the time interval. Data present mean ± S.D., n=3.

Hp is a plasma protein that binds and captures Hb released from red blood cells (RBCs), thereby minimizing the toxic effects of cell-free Hb [24,27]. The Hb (10 μM) and pDNA were incubated in presence of Hp in different ratios (Hp: Hb-1:1.25, 1:0.625, 1:0.25, 1:0.125). It was observed that Hp could not inhibit the pDNA cleavage activity of Hb (Fig. 7).

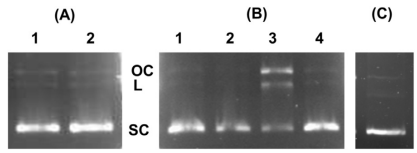

Fig. 6. Inhibition of the Hb cleavage activity by KCN and NaCl, evaluated by agarose gel electrophoresis of pDNA (25 μg/ml) and Hb (50 μM) after 12 h at 37 °C. (A) Inhibition was studied by using KCN (100 μM), which abolished the pDNA cleavage activity; Lane1, Hb and pDNA in presence of KCN; lane 2, pDNA incubated with KCN (B) Hb and pDNA were incubated with NaCl: lane1, Hb and pDNA with 500 mM NaCl; lane 2, pDNA with 500 mM NaCl; Lane 3, Hb and pDNA with 100 mM NaCl; lane 4, pDNA with 100 mM NaCl. (C) Effect of hemin (10 μM) was analysed and it did not induce any pDNA cleavage when observed on agarose gel electrophoresis after 6 h.

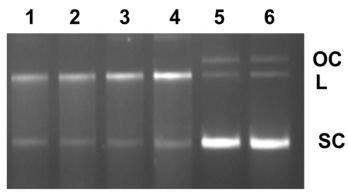

Fig. 7. Inhibition studies of DNA cleavage in presence of haptoglobin (Hp) as determined by agarose gel electrophoresis. The HbA was incubated with Hp, followed by addition of pDNA, agarose gel electrophoresis of different ratios of Hp:Hb. lane 1, Hb+pDNA+Hp (0.5 μM, 1:1.25); lane 2, Hb+pDNA+Hp (1 μM, 1: 0.625); lane 3, Hb+pDNA+Hp (2.5 μM, 1:0.25); lane 4, Hb+pDNA+Hp (5 μM, 1: 0.125); lane 5, pDNA +Hp (2.5 μM), lane 6, pDNA+Hp (5 μM).

4. Discussion

Hb is one of our most abundant proteins, normally densely packed inside the erythrocytes. The protein is well protected by an antioxidant network in the RBCs [28], but when released during hemolysis, acellular Hb can promote a cascade of deteriorating reactions [29]. Early studies have thus shown that stroma free Hb in circulation can induce a number of unwanted side effects including inflammation and hypertension [30,31]. Several reactions regarding Hb toxicity have been elucidated in detail, including effects of heme loss, quenching of

nitric oxide and redox activity of the protein. Particularly oxidative Hb reactions have been identified as a key factor contributing to the known pathophysiology of some Hb inherited disorders [4,29,31]. When bound to the globin chains, the redox active heme iron shows reduced oxidation of the ferrous iron. Nevertheless, it is well known that Hb undergoes continuous autoxidation in presence of oxygen with formation of the met form and production of superoxide anion as in Eq. (1) [5,24].

$$Hb\ (Fe2{+}O_2)_4 \rightarrow metHb\ (Fe3{+}) + 2O_2^{\bullet -} \quad (1)$$

$$2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (2)$$

$$Hb\ (Fe3{+}) + H_2O_2 \rightarrow {\bullet}Hb\ (Fe4{+}) + H_2O \quad (3)$$

Superoxide dismutase acts on the superoxide ($O_2^{\bullet -}$) anions, forming molecular oxygen and H_2O_2 (Eq. (2)). Hydrogen peroxide can in turn be decomposed into water in the presence of catalase but can also further oxidize metHb to the ferryl form (Eq. (3)). The protein based radicals are generated within the Hb molecule by the redox active heme molecule when the ferryl protein is produced. The radicals located within the Hb molecules, can migrate to individual amino acid residues within the protein and/or to surrounding biological molecules. Previous studies have demonstrated that the protein based radicals are unstable and easily can migrate far away from the heme moiety [32]. Tyrosine residues have been proposed to be one of the initial target locations [33]. Particularly β–Y145 is known to be one of the radical sites in the Hb molecule. Natural mutations have also been identified at this location [34]. In addition, site-directed mutagenesis to β-Y145F has been made to characterize the redox property of the mutant. Replacement of this active tyrosine reduced the DNA cleavage activity [18]. Cysteine residues are also highly susceptible to redox modifications and particularly the irreversible oxidation of β-Cys93 have been emphasised [25,26]. This cysteine residue is conserved amongst all vertebrates and was evolved during transition of vertebrates from aquatic to terrestrial life. The antioxidant function of β-C93 has also been examined in a mouse model expressing human Hb [28]. The γ-C93A mutant slightly enhanced DNA cleavage compare to the native Hb, most likely due to migration of the protein radicals to other residues on the proteins. However, insertion of a cysteine (α-A19C) residue on the surface of the alpha chain had a major impact on the pDNA damage, which was almost 82% higher than the native HbF. As shown in Fig. 8, a plausible pathway for protein based radicals is the transfer of electrons from the α-heme iron to α-Tyr24 (13.6 Å), and subsequently to α-Cys19 (7.5 Å). Additionally, α-Tyr42 (5.3 Å) can receive electrons from the heme iron, and transfer them to α-Cys19 via α-Tyr24 (16.9 Å).

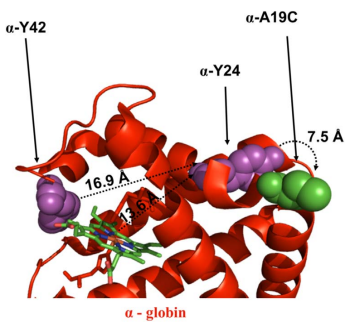

Fig. 8. Schematic model of a primary radical formation sites in the alpha chain of HbF. The alpha (red) chain (PDB code 4MQJ), amino acids prone to oxidation are shown (magenta) and cysteine mutant (green) as a spheres. The generated radical transfer from α-Tyr42/α-Tyr24 to an engineered α-Cys19 residue, consequently damages the pDNA. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

The involvement of protein radicals is further supported by the fact that superoxide radicals were not responsible for DNA cleavage. The pDNA cleavage activity thus remained largely unchanged in the presence of SOD and catalase. In addition, formation of CO-Hb or CN-Hb blocks the oxidative conversion of iron in the heme pocket [23] and thereby slows down the radical reactions. Furthermore, DMSO, did not inhibit the pDNA cleavage activity, which strengthens the hypothesis that Hb produces protein based radicals which are responsible for pDNA cleavage.

The binding reaction between DNA and Hb has been studied previously, resulting in a high affinity constant ($10^{-5}\ M^{-1}$) [13]. The binding of Hb to DNA can be followed by a small change in the spectroscopic absorbance of the protein. This has been observed for Hb in presence of salmon [35] and calf thymus DNA [13]. When using pDNA, this interaction did not alter the heme environment since no change in the location of the soret peak was observed. However, there was a small increase in peak absorbance. This elevation of absorbance in the presence of pDNA further supports that one or more Hb molecules bind to pDNA. Taken together, this suggests that Hb firstly interacts with DNA, and electrons from the heme iron distributed to different protein locations subsequently contribute to the pDNA cleavage activity of Hb.

Erythrocytes do not normally contain a nucleus and are unable to proliferate. Enucleated RBCs are present in the blood of all mammals, suggesting that nuclear removal provides an evolutionary advantage. One of the reasons for the absence of nucleus can be related to the aggressive character of HbA on DNA as demonstrated here. This is also supported by the fact that Hb easily can penetrate the nuclear membrane and thereby generate fragmentation of the chromosomes [36,37]. The precursor cells of RBCs, the pro-erythroblasts, pass a series of cell divisions where Hb is gradually accumulated while the size of the nuclease is reduced [38]. During the gestational period (fetal life), HbF is produced which is followed by a switch to HbA only after birth. The tetrameric structure of these Hbs consists of two alpha chains and either two beta chains in HbA or two gamma chains in HbF [39]. The 39 amino acids difference in the gamma compared to the beta subunit has modified the properties of HbF in terms of enhanced solubility and oxygen affinity. Recent studies have also shown that HbF is oxidatively more stable compared to HbA [25], A lack of a cysteine residue at position 112 in the gamma chain, has been proposed to be responsible for this enhanced oxidative stability. The present investigation also indicates that HbF is less prone to DNA cleavage activity compared to HbA which may be an efficient way of protecting the nucleus in the first development phases of the embryo.

Acellular Hb molecules are normally captured by the plasma protein Hp, which binds almost irreversibly to Hb dimers. This Hb-Hp complex is cleared by CD163 present on the macrophages [8,9]. Hp not only removes acellular Hb from circulation, but is also responsible for attenuation of the oxidative reactions raised by Hb [34,40]. However, in this study it was shown that the Hb-Hp complex did not mitigate the pDNA cleavage activity. It may be explained by the large freely accessible surface of Hb which can interact with DNA even after Hp complex formation [41].

Besides, developing a basic understanding of the interactions between Hb and nucleic acids, it is important to stress that such knowledge is highly relevant also in clinical settings. Since the 1980s, hemoglobin based oxygen carriers (HBOCs) have been extensively evaluated as a blood substitutes. Initial safety tests for HBOCs development include peroxidase [27,42], mass spectrometry [26,31] and electron paramagnetic resonance measurements [24,32]. The present approach may be explored as a simple and rapid technique for quantitative and safety characterization of HBOCs requiring minimal quantities of samples. It is also supports the use of HbF instead of HbA for such applications [21].

5. Conclusion

To conclude, it was observed for the first time that Hb can initiate DNA cleavage in the absence of hydrogen peroxide. The reaction mechanism has not been elucidated but certainly involves a redox reaction initiated by Hb. Importantly, DNA cleavage was not observed for HbF.

Acknowledgement

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Paper III

Enhanced hydrodynamic volume of fetal hemoglobin carrying a genetically linked polypeptide tail

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Abstract

Blood shortage is increasing in our hospitals. To address this global challenge several alternative strategies have been pursued, such as the development of various blood substitutes. Chemical modification of hemoglobin (Hb) is crucial to convert the native protein into a safe and functional hemoglobin-based oxygen carrier (HBOC). Such processes, including chemical cross-linking and PEGylation, often reduce yields and modify the intrinsic properties of the protein. This paper reports the synthesis, structure and properties of an HBOC candidate expressed in *E. coli*. A recombinant unstructured polypeptide composed of hydrophilic amino acids was genetically linked to a fusion fetal hemoglobin. The entire construct could be produced as a single polypeptide in high yields. Small angle X-ray scattering studies demonstrated the presence of a homogeneous protein in aqueous solution. The polypeptide tail increased the hydration shell of Hb from 2.8 to 6.2 nm. The isoelectric point (pI) was also lowered, from 6.9 to 5.0, for the native and polypeptide tagged Hb, respectively. The biophysical properties of the modified Hb were identical to the untagged protein as reflected by absorption spectra and oxygen affinities (p50 = 4.20 mm Hg). The construct is anticipated for use as an alternative material for present PEGylated products.

Keywords

Fetal hemoglobin, fusion protein, oxygen affinity, dynamic light scattering, circular dichroism, small angle X-ray scattering

1. Introduction

Blood transfusion helps to save millions of lives every year and plays an important role in patients suffering from life-threatening conditions during surgical procedures (Clinat et al., 1999; Pape et al., 2009). However, blood transfusion is not risk-free and has limitations due to the potential risks of blood-borne pathogens. Besides that, donated blood has a short storage life of 42 days and must be kept refrigerated. Decreasing numbers of donors often lead to shortage of blood (Chamberland et al., 2001; Dodd et al., 2004). Consequently, a blood substitute is required to fulfil the increasing demands of blood in modern medicine. In the past decades, several alternative strategies have been pursued for generating functional blood substitutes (Alayash, 2017; Sakai, 2017). Among others, hemoglobin (Hb)-based oxygen carriers (HBOCs) have been studied extensively due to the intrinsic oxygen carrying capacity of the Hb molecule (Sakai, 2017). Hb is a tetrameric protein composed of two alpha and two beta subunits forming a 64 kDa protein (Mozzarelli et al., 2013). Earlier studies demonstrated that Hb itself is not stable when administered in a cell-free environment, since unmodified Hb easily dissociates into dimers. This may result in several side-effects including renal toxicity and organ damage (Alayash, 2014). Additionally, cell-free Hb also contributes to vasoconstriction effects by reacting with nitric oxide (NO), which is one of the main reasons for the failure of many HBOC products (Olson et al., 2004). Nevertheless, several chemical or genetic modification strategies have been explored to avoid such negative effects (Alayash, 2017). The main goal of these efforts is to increase the molecular size of the Hb molecule, thus limiting the extravasation of Hb through the endothelium layer. Moreover, most of these approaches are aimed to stabilize the natural tetrameric conformation of the Hb molecule (Coppola et al., 2011; Simoni, 2017). Most of the HBOC products that are manufactured for clinical trials consist of chemical conjugation of polyethylene glycol (PEG) to the Hb surface (Winslow, 2008).

Recent setbacks suffered by chemically modified HBOCs have directed the research towards the use of recombinant hemoglobins as starting material for a new generation of products (Varnado et al., 2013). Advances in recombinant DNA technology have made it possible to create a more stable Hb molecule in terms of less degree of dimer dissociation, higher oxygen affinity and reduced NO scavenging. Various mutagenesis strategies have also been employed to develop safer Hb molecules (Graves et al., 2008; Olson et al., 2006). However, the low

production yields have been a major issue associated with production of Hb recombinantly. The unequal expression of alpha and beta subunits leads to formation of undesired protein side-products, particularly in the case of adult Hb (HbA), which affects the final productivity. However, fetal Hb (HbF) is less sensitive and can be expressed with higher yields, 40-55% lower compared to HbA (Ratanasopa et al., 2016). Therefore, we have addressed the issue related to yields by designing a fusion Hb molecule. It is composed of the alpha and gamma subunits which are linked together by a flexible linker consisting of twelve amino acids residues, $(GGS)_4$, forming a fusion fetal Hb (fHbF). This genetically modified construct has 3-fold higher production yields compared to native/wildtype HbF. Moreover, this fusion protein can bind to gaseous ligands such as carbon monoxide (CO) and oxygen in a similar manner to native HbF (Ratanasopa et al. submitted manuscript).

PEG-decorated Hb molecules obtained from bovine or human blood, have been evaluated in different stages of clinical trials (Weiskopf, 2014). However, one of the main disadvantages associated with PEGylation is the formation of a heterogenous final product that needs an extra purification step to obtain the desirable HBOC. Moreover, perturbation of the structural and functional properties of the Hb molecule through chemical processing may limit its usefulness in human subjects (Coppola et al., 2011; Ronda et al., 2011; Weiskopf, 2014). In recent years, alternative approaches have been employed to avoid chemical processing of therapeutic proteins including conjugation to albumin (Kratz, 2008), using PASylation (Schlapschy et al., 2013) and the XTEN technology (Schellenberger et al., 2009). XTEN has been developed as a recombinant PEG mimetic and which is an unstructured protein polypeptide comprised of repeated hydrophilic amino acid residues. The protein of interest can be genetically fused to this biodegradable XTEN polypeptide enabling its direct recombinant expression (Ding et al., 2014). It provides a bulking effect in the same manner as PEG to the corresponding protein with improved half-lives and stability of the fused protein. The genetically fused XTEN is chemically defined and is expressed as a single polypeptide, thereby avoiding subsequent synthetic chemical steps. Notably, XTEN shows several advantages over traditional methods of chemical conjugation, for example, it can serve as a purification tag or can be visualized when combined with a fluorescent protein. Several pharmaceutical proteins have been investigated when expressed together with the XTEN polymer either at the N- or C-terminal ends of the protein (Haeckel et al., 2014; Podust et al., 2016).

In the present report, we describe the fusion of XTEN to HbF. The construct was expressed as a single polypeptide carrying the XTEN polypeptide (313 residues) followed by the alpha and gamma chains. The construct was expressed in *E. coli* forming a fusion protein XTEN-HbF. A comparative study was carried out

between purified preparations of XTEN-HbF and fHbF, a fusion protein carrying a polypeptide with linked alpha and gamma chains. Biophysical properties such as conformation, stability and oxygen binding were determined.

2. Materials and Methods

2.1. Molecular Cloning

Fusion fetal hemoglobin (fHbF) is composed of the alpha chain linked to the gamma chain at the genetic level by a flexible twelve amino acid long linker composed of glycine and serine residues in the format of $(GGS)_4$. A gene encoding XTEN (Schellenberger et al., 2009) of 313 amino acids was designed and synthesized by Epoch Life Sciences (USA). The DNA sequence was codon optimized to maximize the translation efficiency in *E. coli*. The XTEN sequence was fused to 5′end of the fHbF using a HindIII restriction site, forming a 1902 bp gene. The construct was inserted into a pET28b vector (Novagen) at NcoI and XhoI restriction sites (Fig. S1). The final vector was named pET28b-XTEN-HbF and the correct inserted sequence was confirmed by DNA sequencing by a commercial vendor (GATC Biotech, Germany). Fig. S1 shows the amino acid sequences of XTEN-HbF and fHbF.

2.2. Protein expression and purification

The expression and purification of fHbF was carried out as described elsewhere (Ratanasopa, et al., submitted manuscript). The plasmid pET28b-XTEN-HbF harbouring the T7 promotor was transformed and expressed in *E. coli* strain BL21 (DE3). A 5-ml starter culture was grown overnight by vigorous shaking at 37^{O}C in Luria-Bertani (LB) medium containing kanamycin (50 µg/ml, Duchefa Biochemie). This inoculum was added to fresh 500 ml Terrific Broth (TB) medium supplemented with kanamycin and incubated at 37^{O}C until $OD_{620} \sim >2$. The cells were induced by addition of 0.2 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG, Saveen Werner AB) and 0.3 mM δ-aminolevulinic acid (ALA, Sigma Aldrich). The cultivation was then continued overnight at 28^{O}C in an orbital shaker set at 150 rpm. The cells were collected by centrifugation and resuspended in 20 mM sodium phosphate buffer pH 8.0, followed by sonication for 15 min with amplitude 30%, 3 min pulse and 2 min pause on ice bath. The obtained crude extract was centrifuged at 12,000 rpm for 45 min and the supernatant was filtered using a 0.45-µm Minisart filter (Sartorius). The XTEN-HbF protein was purified using two chromatographic steps. Firstly, the crude extract was passed through a DEAE column (XK26/40, packed to 32 cm bed height) previously equilibrated with 20 mM sodium phosphate buffer pH 8.0. Bound XTEN-HbF was eluted with a step gradient of 40%, followed by a linear gradient (40-100%) using the equilibration buffer containing 250 mM NaCl. Flow

rate was maintained at 5 ml/min. In the second step of purification, samples were first dialyzed against 50 mM Tris-HCl buffer pH 8.0. The collected samples were loaded on a Q-Sepharose High Performance prepacked column (5 ml, GE Healthcare) at a flow rate of 3 ml/min and an elution was achieved by 20 mM sodium phosphate buffer pH 8.0 containing 250 mM NaCl. The eluted protein was further concentrated using a viva spin column (30 kDa molecular mass cut off, Sartorius). Carbon monoxide (CO) gas was bubbled after each step of purification for a few sec. The concentered sample was stored in the CO bound form at -80^{O}C until further use. Protein purity was analyzed on precasted 4-12% SDS-PAGE gels (BioRad). The absorption spectra of the CO, oxy, ferric and deoxy forms of Hb were obtained by spectral scanning (350-700nm) using an Agilent Cary 60 spectrophotometer. Native recombinant fetal hemoglobin (rHbF) was expressed and purified as described elsewhere (Ratanasopa et al., 2016), and compared with fHbF and XTEN-HbF.

2.3. Determination of molecular size

Size exclusion chromatography (SEC) was performed using Superdex Hi-Load 16/60, HR (GE Healthcare, 121 ml) equilibrated with 50 mM sodium phosphate buffer pH 7.2, containing 150 mM NaCl and connected to an ÄKTA Explorer (GE Healthcare, Sweden). Samples (50-100 µM, heme equivalent) were applied on a column and elution was monitored at 280 nm, 405 nm and 420 nm. The molecular weight was determined using known molecular weight markers.

Molecular size of a protein was also determined using MALDI-TOF (MALDImicroMX, Micromass MS Technologies, Manchester, UK) mass spectrometer, calibrated with bovine serum albumin and trypsinogen in a linear mode. Sample concentration of 10 pmol/µl was used for the experiment.

2.4. Hydrodynamic radius measurement using dynamic light scattering (DLS)

The molecular sizes were also ascertained by dynamic light scattering (DLS) using a Dynapro Plate Reader II (Wyatt Technology, USA) at 25^{O}C. Samples were prepared in 50 mM sodium phosphate buffer pH 7.2 at various concentrations ranging from 0.1 to 1.5 mg/ml. The hydrodynamic radius was determined from the particle size distribution.

2.5. Isoelectric focusing (IEF)

2-Dimensional electrophoresis (2-DE) was performed at the SCIBLU centre, Lund University. The Ettan IPGphor isoelectric focusing (IEF) system was used in the first dimension and the Criterion cell system for the second dimension SDS-PAGE. IEF was performed according to the protocol recommended for IPG-phor (Amersham Biosciences, Sunnyvale, CA). Proteins resolved in 8 M urea, 2% CHAPS/IPG buffer 3–10 NL (5 µL/mL)/DTT (2.8 mg/mL) were equilibrated with IPG strips (3–10 NL/11 cm) overnight at 20^{O}C, and first dimension was run on

IPGphor (pre running conditions, 1 h at 500 V, 2 h at 4000 V, and 1 h at 8000 V at 20^{O}C, followed by 8000 V for 7 h). Second dimension was run on precasted polyacrylamide gels at 200 V for 50-55 min and stained with commassie brilliant blue dye.

2.6. Circular dichroism (CD) spectroscopic studies

Hb spectra were recorded on a Jasco J-815 spectrometer at 25^{O}C using 1 mm light path cuvette. The Hb concentration was 60 µM in 50 mM sodium phosphate buffer pH 7.2 and monitored at 500-250 nm at a speed of 100 nm/ min. The thermostability of Hb (10 µM) was determined at the far UV-CD ranging from 200-250 nm.

2.7. Scattering experiments and data analysis

2.7.1. Small Angle X-ray Scattering (SAXS)

The purified and refolded fHbF and XTEN-HbF in 50 mM sodium phosphate buffer pH 7.2 containing 50 mM NaCl was measured at the bio-SAXS beamline (911-SAXS) at the Max IV Laboratory, Lund, Sweden (Labrador et al., 2013). The data were collected at a fixed sample detector distance of 1945 cm and wavelength 0.91Å. Two-dimensional SAXS images were recorded using a PILATUS 1 M detector (Dectris) with an exposure time of 60 sec. Control measurements were performed to ensure that the results were not compromised by radiation damage. The scattering vector q range was calibrated with a silver behenate sample. Data were reduced and transformed to absolute scale using BioXTAS RAW software (Nielsen et al., 2009). Reported scattering profiles I(q) were obtained as the difference of the azimuthally averaged SAXS 2D images from the sample and solvent. To account for the interparticle interactions, solutions of fHbF and XTEN-HbF at three protein concentrations in the range of 0.25-1 mg/ml were measured. The Guinier analysis was conducted using PRIMUS (Konarev et al., 2003) and BayesApp (Hansen, 2012) web interface. The obtained molecular weight values were confirmed by using BSA as a calibration standard and from the concentration-independent excluded Porod volume (MMPorod).

2.7.2. Ab-initio modelling (DAMMIF)

Pre-processing of the data for ab-initio modeling was done in GNOM, the standard settings were chosen for the pair distribution function p(r) and qmax of 0.15 Å^{-1}. The Dmax was estimated visually and confirmed using BayesApp (Hansen, 2012). DAMMIF simulations were run 20 times using the ATSAS online web server (http://www.embl-hamburg.de/biosaxs/atsas-online/); the final models were clustered, averaged and filtered. GASBOR simulations were run three times

in slow mode using the ATSAS online web server, the final models presented were the best fit models.

2.7.3. Combined rigid body modelling and ab-initio (CORAL) of fHbF

CORAL simulations were run at least three times using the ATSAS online web server. In the modelling of fHbF, the two subunits were linked by a 12 amino acid linker defined by a string of bead. The α- and γ-subunits were taken from 4MQJ PDB. Guided by the DAMMIF simulations, a dimer constraint was imposed on the model.

2.7.4. Ensemble optimization modelling of XTEN-HbF (EOM)

The high flexibility of the XTEN-HbF was modeled using the EOM approach. Briefly a pool of PDB structures (10,000) was generated and their scattering intensity calculated. A genetic algorithm was applied to select from the ensemble a sub-ensemble (a Pool) that fitted the experimental scattering data. The ensemble generation and genetic algorithm was performed on the ATSAS online web server. A model similar to the one used for CORAL modelling was built with the addition of the XTEN tail.

2.8. Determination of oxygen binding curves

Oxygen equilibrium binding studies were carried out using a Hemox Analyzer (TCS Scientific, New Hope, PA) using the Hemox buffer (135 mM NaCl, 5 mM KCl, and 30 mM TES; pH 7.4 at 37^{O}C) provided by the manufacturer. A computer-based analysis of oxygen binding curves of the samples was performed to obtain the P_{50} values.

2.9. Autoxidation studies

The CO-bound Hb was converted to the oxy form by a continuous stream of oxygen in presence of light. Oxy-Hb (20 μM) in 0.1 M sodium phosphate buffer pH 7.4 was incubated at 25^{O}C for 12 h, and the absorption spectra (350-700 nm) were recorded every 30 min. Autoxidation rate constants were obtained by the normalized absorbance change at 576 nm fitted into a first order single exponential equation, $y=y_{max}*e^{-kt}+y_0$, where y is the observed absorbance reading as a function of time.

2.10. Analysis of oxidation reaction with hydrogen peroxide

To obtain ferric Hb, the samples were incubated with potassium ferricyanide (10 mM) and the excess of ferricyanide was removed by size exclusion chromatography using a Sephadex G-25 column (5 x 0.5 cm, GE Healthcare). The reaction of ferric Hb (10 μM) with an excess of H_2O_2 (100 - 500μM) under pseudo first order conditions, was monitored as described previously (Ratanasopa et al., 2015). Time course of reaction was followed at 405 nm using a Cary 60 (Agilent

Technologies) instrument attached to an RX-2000 rapid mixing accessory. Data were fitted to a double exponential equation to obtain rate constants, which were plotted as a function of H_2O_2 concentration.

3. Results

3.1. Purification and yield of XTEN-HbF

The designed gene sequence of XTEN-HbF, encoding a single polypeptide harbouring the XTEN sequence linked to fHbF was successfully cloned and expressed in *E. coli*. XTEN, which is composed of several negatively charged amino acid residues, could act as a purification tag to facilitate the isolation of the fusion protein. In the second ion exchanger step, the hydrophilic XTEN-HbF protein was eluted at 250 mM NaCl (100% gradient), while fHbF eluted much earlier in the salt gradient, at ~ 20% (Figure 1A). As listed in Table 1, both proteins showed identical yields after two steps of purification (20-24 mg/L), demonstrating that expression of XTEN with fHbF had negligible effect on the final yield of the protein. However, the expression vector and conditions used were not identical. Notably, when compared with rHbF, the final yields were three-folds higher for both proteins studied [1]. Figure 1B depicts the purity of XTEN-HbF, which was > 95%, as determined by SDS-PAGE. The calculated molecular weight of XTEN-HbF is 63 kDa, but it migrated slowly on SDS-PAGE, showing an apparent molecular size of ~110 kDa. The XTEN part reduced the binding of SDS to the protein, which is responsible for the modified migration of the protein in the gel (Haeckel et al., 2014).

Table 1. The yields of fHbF and XTEN-HbF were monitored during each step of purification.

Purification step	Yield mg/L	
	fHbF	XTEN-HbF
Crude lysate	55 ± 3	45 ± 2
DEAE-IEX[a]	40 ± 1.7	29 ± 3.1
Q-IEX	22 ± 2	23 ± 1.6

[a] Ion exhange chromatography

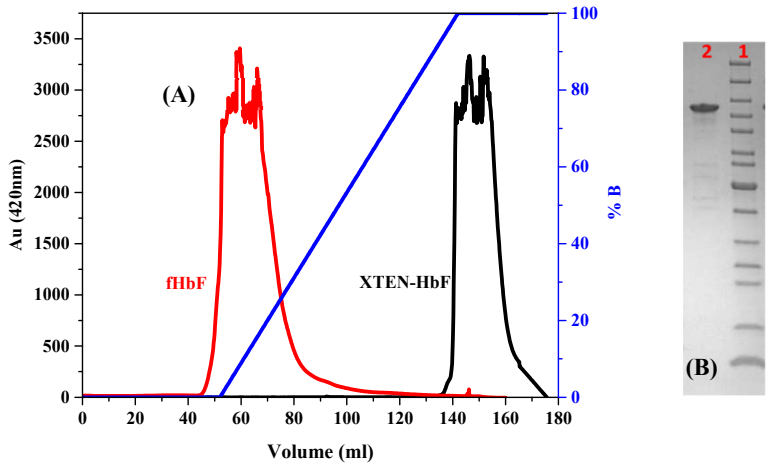

Figure 1. Purification of XTEN-HbF and fHbF by Q-HP ion exchange chromatography A) Comparative chromatographic curves of XTEN-HbF (black) and fHbF (red) on a Q-HP. fHbF is less charged and eluted earlier than XTEN-HbF. B) 4-12% gradient SDS-PAGE gel stained with commassie blue dye, Lane 1: PageRuler unstained protein ladder, Lane 2: XTEN-HbF after two steps of purification.

3.2. Biophysical properties

3.2.1. Molecular weight determination

The molecular sizes were determined by an analytical SEC column. The fHbF (theoretically calculated Mw of 31,908), carrying a linker of 12 amino acids and proteolytically stable, showed a size of 32 kDa on a Superdex Hi-Load. For purified XTEN-HbF, a single homogeneous peak was observed and the elution volume was significantly decreased. The large hydrodynamic radius of XTEN-HbF resulted in an apparent size >320 kDa as determined by SEC. This result is consistent with previous studies (Schellenberger et al., 2009) demonstrating the volume enhancing effect of XTEN on fHbF. Data from MALDI-TOF demonstrated [M+H]1+ ion at m/z= 32,162.15 and 63,167.75 for fHbF and XTEN-HbF, respectively. The XTEN part thus increased the molecular size two-fold, but the hydrodynamic volume was enhanced even further.

3.2.2. Isoelectric point and absorption spectra

IEF was carried out under denaturing conditions, followed by SDS-PAGE in the second dimension. The XTEN moiety of the fusion protein lowered the pI value to 5.0 (theoretical 4.71) in relation to fHbF (pI: 6.9) and native HbF (pI: 6.8). The negatively charged XTEN part thus displayed an extensive and characteristic influence on fHbF. The absorption spectra of different ligands bound to XTEN-HbF are shown in Figure 2. In the visible region of the Hb absorption spectra, the CO-Hb (pink) form showed a characteristic peak at 539 and 569 nm, whereas peaks at 541 and 576 nm were observed for oxy-Hb (black). Moreover, an

absorption spectrum at the soret region resulted in a prominent peak at 415 and 419 nm for oxy and CO-Hb, respectively. The deoxy-Hb (blue) sample generated a single peak at 555 nm in the visible region. The spectrum of oxidized Hb (ferric-Hb, red) showed typical absorption maxima, with peaks at 541, 576 and 630 nm.

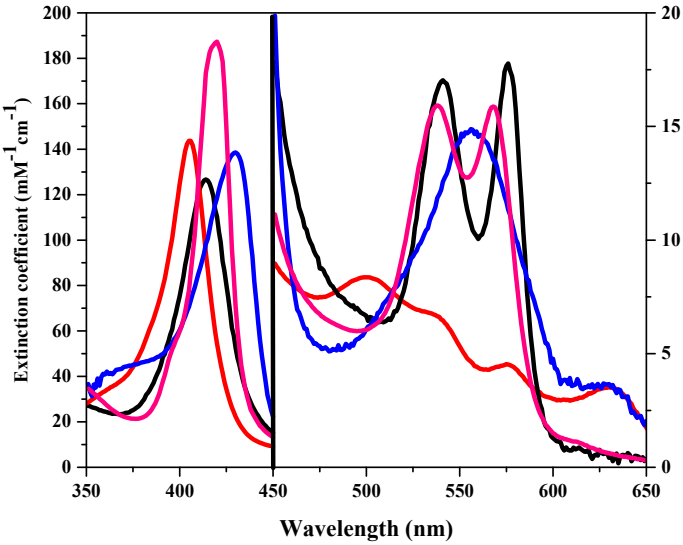

Figure 2. Representative absorption spectra of Hb forms obtained by spectroscopic scans between 350-700 nm. XTEN-HbF showed characteristic peaks in the visible region to the corresponding fHbF. (CO-Hb (pink), 539 and 569 nm; oxy-Hb (black), 541 and 576 nm, ferric Hb (red), 500 and 630 nm and deoxy-Hb (blue), 555 nm).

3.2.3. Secondary structure and thermostability

CD spectroscopy was used to investigate the effect of the XTEN moiety on the secondary structure of fHbF. The fusion Hb showed a unique CD spectrum, for example, a positive band at 260 nm and a prominent band at 422 nm. The CD spectrum in the soret region remained almost unchanged for XTEN-HbF in comparison with fHbF. In the far CD-UV region, the fHbF showed typical α-helical protein spectra. However, the spectra of the corresponding XTEN fusion protein showed significant deviation, a negative peak around 205 nm arose, indicating the effect from the random coil in XTEN. The data from these CD measurements, particularly in the soret region, are consistent with previous studies (Nagai et al., 2008; Ratanasopa et al., 2016) indicating that both proteins show identical CD spectra in relation to rHbF.

The effects of temperature induced unfolding were studied in the far CD-UV region. As shown in Figure 3, increasing temperature led to unfolding of the alpha helical structure of fHbF at 60^{O}C. Interestingly, no such effect was observed for

XTEN-HbF when investigated at the same temperature. Unlike XTEN-HbF, fHbF was completely unfolded at 70^{O}C, a shift in the peak around 200 nm was observed for XTEN-HbF when investigated at 70 and 80^{O}C (Figure 3). Taken together, the microenvironment of the heme was not altered by expressing XTEN at the N-terminal of fHbF. Notably, by adding the XTEN part, a much more thermostable Hb protein can be produced.

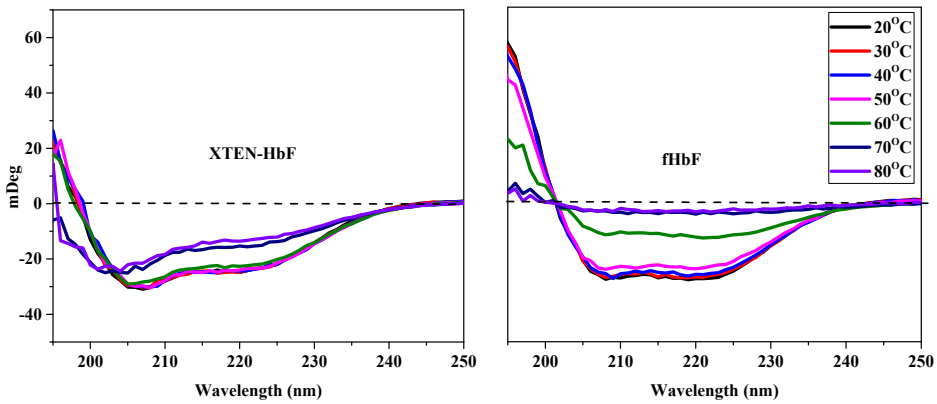

Figure 3. CD spectra of temperature induced unfolding of fHbF and XTEN-HbF, respectively. Left panel shows the temperature induced unfolding of fHbF. The XTEN moiety increases the temperature stability of the corresponding fHbF part (right panel).

3.2.4. Measurement of hydrodynamic radius and small angle X-ray scattering

The molecular radii of the proteins were also determined by DLS. Chemical modifications (i.e. PEGylation or crosslinking) can be viewed as common practice to increase the hydrodynamic radius of cell-free Hb. As listed in Table 2, XTEN of 313 amino acids attached at the N-terminal of fHbF, showed a larger molecular radius (6.2 nm) compared with 2.8 nm for fHbF.

Additional characterization of fHbF and XTEN-HbF was performed using small angle X-ray scattering (SAXS). Figure 4 (Left panel) shows the scattering intensities of the two proteins, where the right panel illustrates the pair distribution function (P(r)).

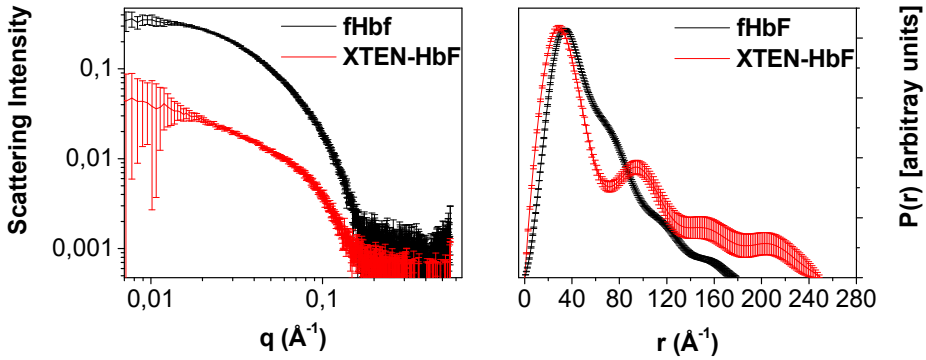

Figure 4. **Small angle X-ray scattering**. Left panel shows the typical scattering intensity of fHbF and XTEN-HbF on absolute scale. Right panel shows the pair distribution function (P(r)) obtained using Gnom inverse transfor algorithm. Both P(r) shapes indicated that the proteins were elongated. The XTEN-HbF P(r) was re-scaled for convenience.

From the analysis of the scattering intensity, we extracted the radius of gyration (Rg), the total size (Dmax) and the molecular weight. Table 2 summarizes the findings.

Table 2. DLS and Small angle X-ray scattering results

	fHbF		**XTEN-HbF**	
Hydrodynamic Radius Rh (nm)	2.8 ± 0.09		6.2 ± 0.08	
Polydispersity index	15-24%		18-23%	
	Guinier [a]	P(r)[b]	Guinier	P(r)
Radius of gyration Rg (Å)	43.2 ± 7.3	48.82	50 ± 12.5	68.25
Dmax (Å)	107		250	
MW (Da) [c]	85149.4		83444.1	
Oligomeric state [d]	2.5		1.3	
Rg / Rh [e]	1.2	1.39	0.8	1.13

[a] Guinier analysis
[b] Size analysis from pair distribution function P(r)
[c] molecular weight determined from Porod volume
[d] ratio of SAXS molecular weight to nominal molecular weight
[e] typically Rg/Rh for a sphere is 0.774, for a coil 0.816 and for a rod 1.732

The shape and level of flexibility called for caution during data analysis (Receveur-Bréchot et al., 2012). The P(r), in Figure 4 (Right panel), showed the

typical signature of an elongated and flexible system. To ensure that the Rg and Dmax are unbiased, a direct determination using the Guinier approximation and an indirect method with the P(r) were employed. Table 2 indicates that fHbF Rg is consistent with both methods. On the other hand, XTEN-HbF showed a bigger discrepancy. When computing the ratio Rg/Rh (SAXS and DLS size parameters ratio) fHbF ratio was between 1.2 and 1.4, an indication of elongation. The XTEN-HbF ratio was 0.8 (a coil) or 1.13 (elongated) when considering the more correct Rg. Estimation of the molecular weights and comparison to the nominal values suggested that fHbF is a dimer and XTEN-HbF is monomeric.

One advantage of SAXS is the ability to model the scattering intensities back into a shape envelope (DAMMIF) and eventually build a 3D model based on the protein crystal structures. For fHbF, a system with less flexibility, we modelled the shape using DAMMIF approach. Figure 5 shows the fit and bead model plus envelope of the fHbF system. Calculation of the bead volume confirmed the dimeric nature of the protein.

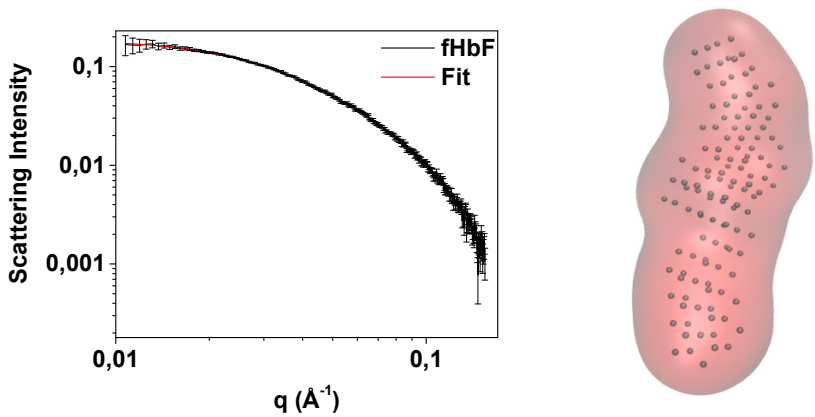

Figure 5. 3D ab-initio reconstruction using DAMMIF (Atsas online). Left panel shows the fit from the consensus and refined model ($\chi 2 = 0.746$). The right panel shows the envelop and beads for the refined model. The bead models are consensus models after clustering, averaging and filtering 20 independent models.

To gain more insight on the relative position of the fHbF subunits within the scattering envelope, we used the CORAL software on the Atsas online application. The approach allowed us to construct the fHbF using known PDB structures and add the linker between the α- and γ-subunits. Figure 6 shows the fit and resulting 3D model.

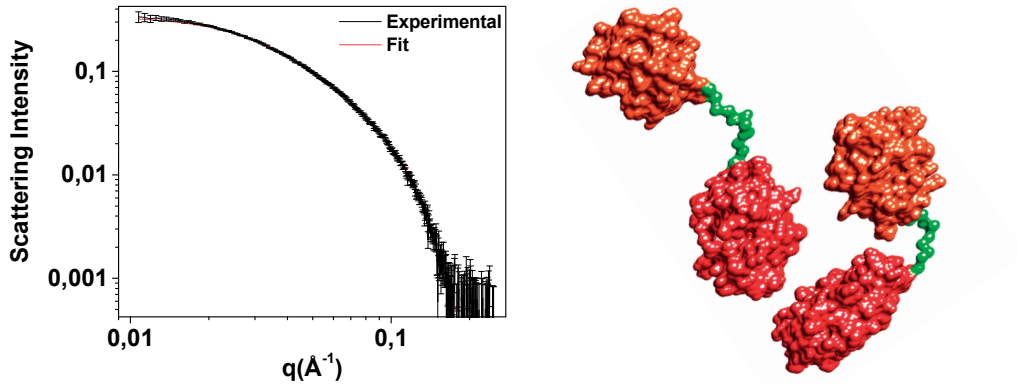

Figure 6. **Combined Rigid body and *ab-initio* modelling (CORAL) of fHbF protein**. Left panel shows the fit from the model (χ2 = 1.283). Right panel shows the resulting model appearing as a dimer.

Inspection of the 3D model revealed that the dimer of fHbF was formed by the interaction of the α- and γ-subunits. The absence of conformational changes during modelling was most likely favouring the contact between the two subunits, since they formed a complementary pair from the dimeric crystal structure they originate from.

3D modelling of the XTEN-HbF was done using the ensemble optimization method (EOM), which helps dealing with highly flexible systems by generating a large number of structures and averaging them to obtain the scattering intensity. An initial pool of 10,000 PDB was generated and designed to be representative of the XTEN-HbF conformational space. A genetic algorithm selects and refines the type and number of structures to form an ensemble that fitted the experimental scattering curve. Figure 7 summarises the fit and Rg distribution of the initial pool and final ensemble.

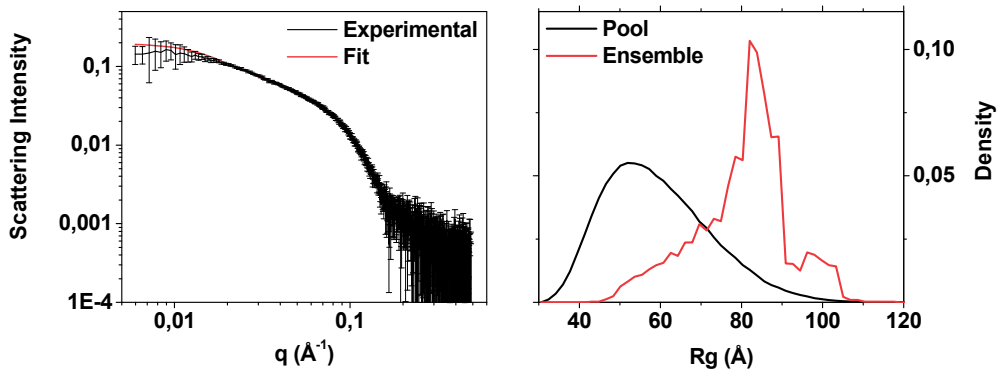

Figure 7. Ensemble optimization method of XTEN-HbF. Left panel, experimental and fitted data (χ2 = 0.982). Right panel, radius of gyration of the initial pool and final ensemble. The Rg of the ensemble was 80Å. R_{flex} / Rσ: ~ 80.00% / 0.88. For the pool R_{flex} is 84.41% and Rσ is 1.

Measures of flexibility are estimated using R_{flex} and R_{σ} parameters. Using R_{flex}, the selected ensemble distribution can be numerically compared to that of the pool, the latter representing a reference for flexibility. For convenience, R_{flex} can be reported as a percentage in the range 0 to 100%, with R_{flex} = 100% indicating maximum flexibility. Here the pool R_{flex} had a calculated value of 84.41%. Rσ indicates the variance of the ensemble distribution with respect to the original pool, yielding values close to 1.0 when the ensemble distribution describes a fully flexible system and largely reproduces the conformational space of the pool. We found for the ensemble Rσ of 0.88. The ensemble R_{flex} and Rσ were in the pool values, hence confirming the validity of the results.

We found in the ensemble four representative structures that could explain the experimental scattering intensity. Figure 8 summarizes the four PDB structures.

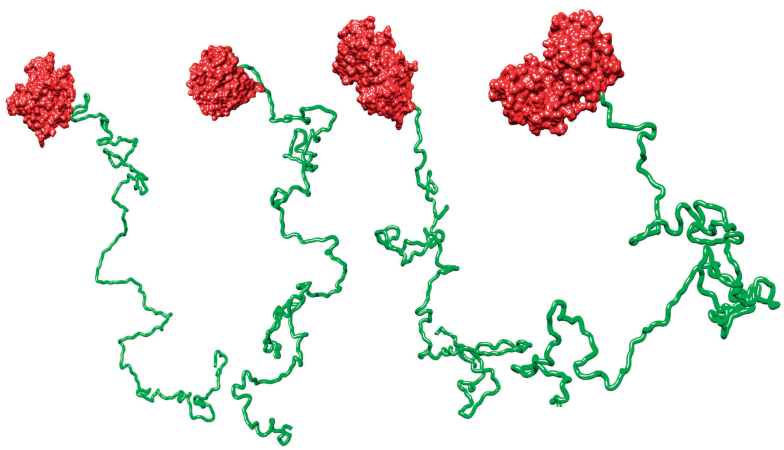

Figure 8. **PDB models of XTEN-HbF from EOM final ensemble**. The red globular units are the fHbF and the elongated tail are the XTEN (green).

3.3. Oxygen affinity

The oxygen affinity (P_{50}, oxygen partial pressure at which Hb is half saturated with oxygen) for both Hb proteins were determined. It was observed that the P_{50} value for fHbF and XTEN-HbF were 4.20 and 4.18 mm Hg, respectively. The designed fHbF lowers the P_{50} value compared with rHbF, resulting in the higher oxygen affinity. The Hill coefficients for both proteins (1.13 and 1.08) were lower than native HbF, indicating a decrease in cooperativity caused by the genetic modification. However, the addition of XTEN in itself had a negligible effect on the oxygen binding properties of fHbF.

3.4. Autoxidation

The ferrous iron (Fe^{2+}) of Hb can easily be converted into ferric Hb (Fe^{3+}) with production of superoxide anion ($O_2^{\bullet-}$), a process known as autoxidation. The decrease in absorbance at 576 nm was used to calculate the rate of this reaction. It was observed that the autoxidation rate for fHbF, with or without XTEN, was largely unaffected. The autoxidation rate constants showed identical values of $0.117\ h^{-1} \pm 0.016$ and $0.119\ h^{-1} \pm 0.011$. These data suggest that fusion of XTEN to fHbF, did not cause any structural perturbations, but the calculated autoxidation rates were lower than for rHbF ($0.132\ h^{-1}$).

3.5. Oxidative reaction

The pseudoperoxidase activity of ferric Hb with H_2O_2 was examined by UV-Vis spectroscopy. This reaction often generates a most oxidizing ferryl Hb, known for generating damages to tissue or organs. Hb (10 μM) was mixed with various concentrations of H_2O_2, and the reaction was immediately monitored at 405 nm. Second order rate constants were calculated by a slope from the plot of first order rate against the H_2O_2 concentrations. The obtained values were identical for both proteins as represented in Figure 9 and Table 3.

Table 3. The second order rate constant of XTEN-HbF and fHbF were calculated using double exponential fit. Both protein show identical oxidation reaction with H_2O_2.

Hb	Fast reaction ($\mu M^{-1}S^{-1}$)	Slow reaction ($\mu M^{-1}S^{-1}$)
fHbF	$3.88 \pm 0.08\ X10^{-4}$	$7.03 \pm 0.05\ X10^{-5}$
XTEN-HbF	$3.96 \pm 0.03\ X10^{-4}$	$6.99 \pm 0.03\ X10^{-5}$

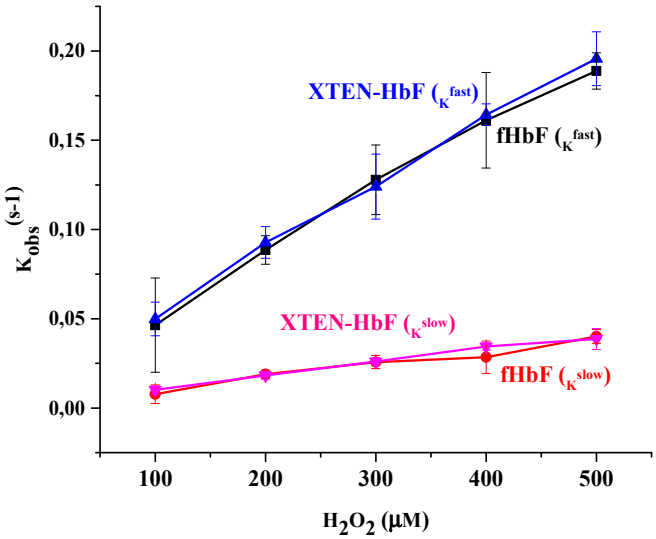

Figure 9. Oxidation of XTEN-HbF and fHbF proteins in presence of hydrogen peroxide (100-500 µM). The absorbance changes (405 nm) were fitted to a double exponential equation and obtained rates were plotted against H_2O_2 concentrations. Data were obtained from three independent experiments and data shown are mean ± standard error.

4. Discussion

In the search of universal blood substitutes, several studies have been undertaken focusing on the development of safe and efficient HBOC products. HBOCs have been widely investigated due to several inherent potential advantages compared to whole blood, including long storage life, universal blood typing and lack of blood-borne pathogens (Weiskopf, 2014). Earlier studies, however, demonstrated that cell-free Hb rapidly can dissociate into αβ dimers, and additionally, Hb is also responsible for scavenging of available nitric oxide (NO) (Alayash, 2014, Buehler et al., 2010). Historically, most HBOCs are manufactured based on the chemical modifications of bovine or outdated human Hb using PEG or glutaraldehyde solutions. Such chemical modifications, can at least to some extent, provide beneficial effects including reduced extravasation when administrated into certain animal models (Manjula et al., 2003). PEGylation is most commonly used, and considered as an essential step to improve the performance of Hb in cell-free environments. However, a major concern associated with the PEGylation method is the formation of a heterogeneous product mixture, which may result in the distribution of different Hb species in the body. Such heterogeneity may therefore generate adverse side-effects, which significantly can influence the biochemical and pharmacokinetic properties of an HBOC product (Ronda et al., 2011). Previously, several drawbacks related to a PEGylated compound in animal models

where PEG antibodies were detected, which thereby enhanced blood clearance (Armstrong et al., 2007). Since large quantities of an HBOC product need to be infused in humans, generation of PEG antibodies can therefore be anticipated, especially if the treatment is to be repeated. Alternative approaches have therefore been evaluated in order to obviate chemical modifications of Hb. The Hb-albumin cluster designated as "Hemoact" was developed and found to be a safe alternative in preclinical studies (Haruki et al., 2015). Here, we have developed a new molecular design of an HBOC product utilizing a polypeptide tail (XTEN). The XTEN protein polymer was first developed by Schellenberger et al. to increase the molecular size of therapeutic proteins (Schellenberger et al., 2009). Several beneficial properties of XTEN over the PEGylation have been reported, such as genetic and chemical stability, formation of a homogeneous product as well as increased molecular size and hydrodynamic radius (Podust et al., 2016). When the XTEN polymer was linked to HbF at the DNA level, biophysical evidence especially by DLS studies, clearly indicated that the diameter of XTEN-HbF was two-fold greater than that for fHbF.

XTEN mimics the overall advantageous properties of PEG. Notably, the length (number of amino acid residues) of the XTEN polymer can be varied to achieve the targeted half-life. Regardless of length, it exerts a significant hydrodynamic volume to the fusion protein. XTEN composed of 144 up to 1728 residues elutes earlier than large globular proteins on size exclusion chromatography (SEC) (Podust et al., 2016). SAXS studies provided additional important pieces of information about protein aggregation (Kikhney et al., 2015). When XTEN was attached to fHbF, the random coil polymer produced a homogeneous preparation of the fHbF protein. These data were also consistent with SEC, further supporting the presence of a monodisperse XTEN-HbF preparation in solution. To the best of our knowledge, this is the first time the "in solution" structure of XTEN polymer has been determined. This data clearly indicates that the XTEN moiety does not interact with the attached protein, and the structural and functional properties of the native HbF molecule are maintained.

The low pI of the Hb molecule may play a vital role in achieving a long blood circulation time (Simoni et al., 2009). The negatively charged XTEN-HbF may thus obstruct the leakage from the vasculature walls due to electrostatic repulsion between the cell surface of glomerular basement membrane around endothelial cells and the fusion protein. Moreover, the XTEN polymer is designed in such a manner that it does not contain any plasma protease cutting site. Overall, this effect may also contribute to a long circulation life of the Hb molecule *in vivo*.

Earlier developed HBOC products obtained via chemical conjugations modulate the oxygen affinity (P_{50} value) of the Hb molecule after the modifications. For instance, MP4 and Euro-PEG-Hb are PEGylated HbA products which displayed

reduced P_{50} values (higher oxygen affinity), compared to the starting Hb molecule (Hu et al., 2005; Wang et al., 2017). The site of PEGylation plays a crucial role in oxygen affinity. For instance, Cys-93 (β) is a commonly used residue for this purpose, and which is located close to the adjacent His-92 (β) that coordinates the iron atom. Ultimately, PEGylation at this site can alter the microenvironment of the Fe-His residue and thereby significantly alter the oxygen affinity (Wang et al., 2014). It has been suggested that an increased P_{50} value (decreased O_2 affinity) elevates the O_2 unloading (Sakai et al., 2009). Generally, dimeric Hb binds oxygen tightly and releases it only at very low oxygen tension. Since, our study involves a dimeric Hb, the P_{50} value was reduced to ~4 torr compared to native HbF (19 mm Hg). Interestingly, the XTEN polymer does not induce any structural alteration to the Hb molecule, and the oxygen affinity can thereby be maintained. For comparison, the P_{50} value of recombinant HbF (tetrameric) was also measured, and it was found to be ~9 mm Hg. This data suggests that the oxygen binding properties of fHbF partly differ from native HbF. The distance between the C- and N- terminus of the alpha and gamma chains, respectively, is ~37Å. A 12-amino acid long linker spans this distance, but may still result in partial perturbation of the interface normally existing in tetrameric HbF. Thus, the reduced cooperativity could be a direct consequence of tension in the fusion protein.

The cell pellets as well as the crude extracts obtained after sonication were red in colour indicating the authenticity of the normal Hb character. However, recombinant expression of Hb can affect the folding of globin chains, and particularly, a reverse heme orientation has been observed when adult Hb was expressed in bacteria (Nagai et al., 2008). However, the absorption and CD spectra supported that the expressed XTEN-HbF protein was correctly folded with proper insertion of the endogenous heme co-factor. Moreover, genetic conjugation of the XTEN polymer to fHbF had very limited influence on the overall structure of Hb, as confirmed by CD. Interestingly, the temperature stability of XTEN-HbF was elevated by 10^{O}C. This effect may be particularly useful during the initial stages of purification. For instance, crude bacterial extracts can be heated to 65^{O}C to denature unwanted host cell proteins, which may eliminate further purification steps. A mechanistic interpretation of the obtained enhanced thermostability will require a more detailed investigation.

The oxidation of ferrous Hb into the non-functional metHb reflects one of the aspects behind the *in vivo* toxicity of HBOC products. This process may also produce a highly oxidizing ferrylHb (Fe^{4+}) species, which can undergo heme loss and globin precipitation (Schaer et al., 2013). Generally, Hb is densely packed inside the RBCs, and protected by an antioxidant network system, including e.g. SOD and catalase (Rifkind et al., 2014). Previous reports demonstrated that all modified Hbs increased the autoxidation rate of cell-free Hb, which has been suggested to be inversely proportional to the oxygen affinity of Hb (Jia et al.,

2004). On the contrary, our study demonstrates that the autoxidation rate of modified fHbF largely remains identical when observed at 25^{O}C. This data further supports the finding that the XTEN polymer does not perturb the heme environment of Hb. The formation of ferryl species is known to damage a range of biological materials (Alayash, 2017). This was tested using a rapid-mixing approach with an excess of H_2O_2. Unlike chemically modified Hbs, XTEN-HbF appeared to exhibit a similar transformation rate from ferric to ferryl Hb as fHbF. The iron atom of XTEN-HbF thus has a similar redox cycling in presence of H_2O_2 as fHbF.

Large quantities of Hb, lacking mammalian infectious agents, can be produced in bacteria. In addition, the physiological properties of Hb, i.e. oxygen binding, NO scavenging etc., can be modulated using site-directed mutagenesis to obtain an ideal blood substitute (Olson et al., 2006; Varnado et al., 2013). However, the expression of HbA in *E. coli* has often turned out to produce a complex mixture of protein products. Notably the formation of hemichromes during production has lowered the final yields. On the contrary, HbF showed higher productivity (40-50%) than HbA, with more well defined secondary structure and heme orientation (Ratanasopa et al., 2016). To be used as blood substitute, large quantities are needed for the desired applications in the clinical usages. Therefore, we designed the fusion fetal hemoglobin, fHbF, which showed a higher yield (20-24 mg/L) compared to both native recombinant HbA and HbF. However, the obtained genetically linked dimers are 32 kDa, which is small enough to be cleared from renal tubules. Thus, the utility of the XTEN polymer was tested by linking it genetically to fHbF. As listed in Table 1, the production yield of the XTENylated Hb was identical to native unmodified fHbF. However, the differences observed in the initial phase may be a direct consequence of different expression vectors and cultivation conditions.

Conclusion

The goal of the present study was to demonstrate that the production of a recombinant Hb molecule, with increased molecular size and hydrodynamic volume, can be achieved without additional chemical processing. For this purpose, HbF was genetically linked to XTEN as an alternative to PEGylation. The attached XTEN polymer did not significantly alter the structural and functional properties of the linked Hb molecule. The use of this gene fusion technology represents a one-step method to produce a ready to use HBOC without the need of additional chemical modifications.

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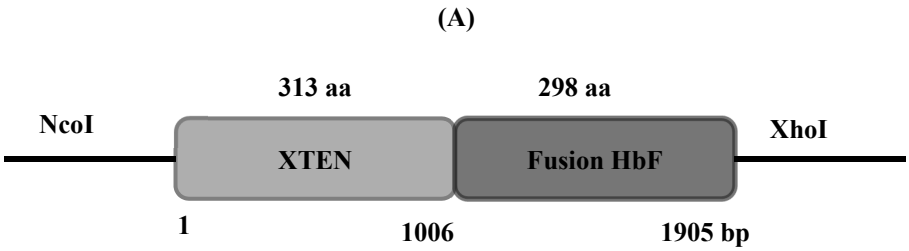
supplementary material



(B) Amino acid sequence of XTEN

MGSSHHHHHH SSGLVPRGSH MTSESATPES GPGTSTEPSE GSAPGTSESA
TESGPGSEPA TSGSETPGTS ESATPESGPG SEPATSGSET PGTSESATPE
SGPGTSTEPS EGSAPGSPAG SPTSTEEGTS ESATPESGPG SEPATSGSET
PGTSESATPE SGPGSPAGSP TSTEEGSPAG SPTSTEEGTS TEPSEGSAPG
TSESATPESG PGTSESATPE SGPGTSESAT PESGPGSEPA TSGSETPGSE
PATSGSETPG SPAGSPTSTE EGTSTEPSEG SAPGTSTEPS EGSAPGSEPA
TSGSETPGTS ESATPESGPG TSTEPSEGSA PGKL

(C) Amino acid sequence of fHbF

VLSPADKTNV KAAWGKVGAH AGEYGAEALE RMFLSFPTTK TYFPHFDLSH
GSAQVKGHGK KVADALTNAV AHVDDMPNAL SALSDLHAHK LRVDPVNFKL
LSHCLLVTLA AHLPAEFTPA VHASLDKFLA SVSTVLTSKY RGGSGGSGGS
GGSGHFTEED KATITSLWGK VNVEDAGGET LGRLLVVYPW TQRFFDSFGN
LSSAAIMGNP KVKAHGKKVL TSLGDAIKHL DDLKGTFAQL SELHCDKLHV
DPENFKLLGN VLVTVLAIHF GKEFTPEVQA SWQKMVTGVA SALSSRYH

Fig. S1. Amino acid sequences of XTEN and fHbF were cloned and expressed as a single polypeptide chain A) Schematic representation of DNA sequence for XTEN-HbF, composed of 1902 base pairs. NcoI and XhoI restrictions sites were used to insert XTEN-HbF in pET28b vector. B) XTEN, expressing 313 amino acid residues, was linked to N terminal of the fHbF. C) Amino acid sequence of fHbF (299 amino acids), the alpha (red) and gamma (blue) chains were genetically linked using a 12 amino acids (black and underlined).

Paper IV

Genetic conjugation of green fluorescent protein to fetal hemoglobin: Functional properties and binding to hemoglobin imprinted polymers.

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Abstract

Hemoglobin (Hb) is a tetrameric protein responsible for the transportation of oxygen molecule in the body. Due to the frequently occurring clinical situations of blood shortages, several efforts have been undertaken to generate alternatives to regular blood. A range of different Hb molecules have therefore been evaluated as starting materials for the development of artificial oxygen carriers to meet the needs of blood transfusion. The preparation of such blood substitutes is complex and requires an extensive characterization followed by biological and clinical testing. In order to facilitate this work, we have developed a fluorescent Hb fusion protein composed of green fluorescent protein (GFP), which has been linked to fetal hemoglobin (HbF) at the DNA level. The intrinsic fluorescent property of the GFP moiety has been used for facilitating the evaluation of the biophysical properties HbF. The fusion protein, GFP-HbF, was expressed in *E. coli* and maximum expression (6-8 mg/L cell culture) was achieved 14 h after induction. The molecular size of native GFP-HbF was estimated to 118 kDa, which corresponds to a dimeric form of the protein. Fusion of GFP to HbF had negligible effects on the functional properties of the individual proteins as reflected by identical absorption spectra of both proteins. Previously, molecularly imprinted polymers (MIPs) have been prepared to allow molecular recognition of different Hb variants. Here it was shown that the fusion protein GFP-HbF exhibited almost identical binding capacity (2.8 μmole/mg MIP particles) compared with HbF. The prepared fluorescent Hb may be used to evaluate the action and performance of Hb molecules both in cell-free environments as well as in biological test systems.

Keywords

Hemoglobin, green fluorescent protein, fusion protein, molecularly imprinted polymer

Abbreviations

ALA, aminolevulinic acid; CO, carbon monoxide; fHbF, fusion fetal hemoglobin; GFP, green fluorescent protein; GFP-HbF, green fluorescent tagged with fusion fetal hemoglobin; Hb, hemoglobin; HBOC, hemoglobin-based oxygen carrier; IPTG, isopropyl β-D-thiogalactopyranoside; metHb; ferric hemoglobin (Fe^{3+}); MIP, molecularly imprinted polymer; OD, optical density; oxy-Hb, oxygen bound hemoglobin; PAS, protein polymers; RBC, red blood cell; SEC, size exclusion chromatography; XTEN, protein polymers.

1. Introduction

Fusion proteins obtained by genetically conjugating two structural genes have been explored in both basic and applied research fields. For instance, fusion proteins with dual or multifunctional properties have been used to facilitate protein purification, modify cell metabolism, stabilize a target protein or monitor cellular and subcellular expression levels. In addition, several therapeutic proteins have been fused to the Fc domain of antibodies or to human serum albumin (HSA) to extend their plasma half-lives [1, 2]. Long polypeptide tails (XTEN, PAS) have been attached to various biopharmaceutical proteins to enhance their therapeutic effects [3, 4]. Green fluorescent protein (GFP) is a widely used marker protein to determine the functional or subcellular location of the target protein [5]. Additionally, GFP has been used to optimize the overexpression and purification of eukaryotic proteins owing to its intrinsic fluorescence [6].

During the last decades, different hemoglobin (Hb) variants have been evaluated extensively as starting material for developing blood substitutes, also referred as Hb-based oxygen carriers (HBOCs) [7, 8]. In this study, we have used fetal hemoglobin (HbF) as our Hb source, since this is a more robust protein compared with the conventionally used adult Hb (HbA). Low expression yields have often been a challenge for producing large quantities of recombinant Hb. This is often due to uneven expression levels of the two globin chains. We have overcome this issue by designing a dimeric Hb molecule composed of the alpha and gamma chain linked together by a short flexible linker of 12 amino acids.

A highly purified Hb preparation is one of the prerequisites needed for generating a functional HBOC. For this purpose, various methods including ion exchange, hydrophobic interaction and immobilized metal affinity chromatography, have been developed to isolate Hb from different crude cellular starting materials [9, 10]. When starting with red blood cells (RBCs) various separation materials have been prepared and tested to achieve a high capacity in the purification step. The Hb content is high and a highly purified preparation can often be achieved simply by mixing with organic solvents, followed by centrifugation/precipitation [11]. In a recombinant context, host cell proteins may interfere with the purification and it is essential to utilize an efficient and robust technique for isolating Hb from microbial lysates [12, 13]. Several recent dedicated separation materials, such as microparticles synthesized by polymerization of negatively charged acrylic acid, have shown promising results in terms of binding capacity [14]. In addition, adsorption of Hb to porous materials (cryogels) has proved to be useful for Hb isolation [15]. Previous studies have also identified the unique recognition ability of molecularly imprinted polymers (MIPs). These are artificial receptors with the ability to specifically recognize template molecules. The highly specific recognition sites are formed by using a molecular template to control the distribution of functional monomers during a cross-linking polymerization. The stability and low cost of MIPs have led to their effective application in several fields, including chemical sensing, separation, catalysis and drug delivery [16, 17]. Recently, we have developed a new synthetic strategy for molecular imprinting of Hb via combining the surface imprinting and the Pickering emulsion techniques. MIP beads obtained by this approach exhibited fast adsorption kinetics and significant selectivity for Hb [16].

In this study, we have genetically fused GFP to the N-terminus of the alpha chain of HbF. To further simplify the production of this construct, the alpha and gamma chains were also linked to generate a single polypeptide chain harboring GFP and the two globin chains. The obtained fusion protein, GFP-HbF, was thus expressed as a single polypeptide chain. Its functional properties, i.e. absorption and emission spectra, ligand binding, stability and autoxidation were examined. The surface properties of GFP-HbF were studied by comparing the molecular recognition of the fusion protein with fHbF (HbF with linked alpha and gamma chains) to Hb-MIP particles.

2. Materials and Methods

2.1. Molecular cloning, expression and purification

A DNA fragment (744 bp) encoding the GFP protein was synthesized by a commercial vendor (Epoch Life Science, USA). The designed fusion fetal hemoglobin (fHbF) harbours the α- and γ-chains genetically linked by twelve amino acid residues $(GGS)_4$. Both genes were codon optimized for maximum translation efficiency in *E. coli*. The C-terminus of the GFP was fused to the N-terminus of fHbF protein using HindIII and BamHI restriction sites, forming GFP-HbF. This gene was inserted into a PET28b vector between the NcoI and XhoI restriction sites and transformed to competent *E. coli* strain BL21 (DE3) cells. The final vector was named as pET28b-GFP-HbF and the inserted sequence was confirmed by DNA sequencing from commercial vendor (GATC Biotech, Germany). A single colony was picked up to produce 5 mL of starter culture grown at $37^{O}C$ in an orbital shaker set at 150 rpm. An overnight culture was then used to inoculate 500 ml TB (Terrific Broth) medium supplemented with kanamycin. The shake flasks (2L) were kept at $37^{O}C$ in an orbital shaker at 150 rpm until $OD_{620} \geq 2$, followed by induction with 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG) and 0.3 mM δ-aminolevulinic acid (ALA) to support heme biosynthesis. The induced culture was further grown at $28^{O}C$ for 14 h. The bacteria were then collected by centrifugation at 10,000 rpm in a cold environment ($4^{O}C$) and resuspended in 20 mM sodium phosphate buffer pH 8.0. The cells were opened by sonication and a clarified bacterial extract was obtained by centrifugation at 12,000 rpm for 40 min at $4^{O}C$ and finally filtered using a 0.45 µM Minisart filter (Sartorius). The fusion protein was purified using two chromatographic steps. Firstly, the protein samples were loaded onto Q-Sepharose Fast Flow (GE Healthcare, Sweden) column equilibrated with same buffer, and elution was achieved using 50 mM sodium phosphate buffer pH 7.2, containing 100 mM NaCl. Collected samples were subsequently buffer exchanged to 20 mM Tris-HCl buffer pH 8.0. In the second step, samples were purified using strong anion exchange chromatography (Q-HP, GE Healthcare), and bound protein was eluted using same elution buffer used in the first step. To stabilize the Hb protein and to avoid oxidation, carbon monoxide (CO) gas was bubbled (few sec) through all solutions at all steps starting from the induction of the culture to each step of purification. The purity of the samples was analyzed by precasted SDS-PAGE gels (4-12%) from Biorad. Fusion HbF without the GFP moiety was produced using an established protocol (submitted article) and used as a control in the subsequent characterization studies.

2.2. Emission spectra

During cultivation, bacterial cell growth was monitored at OD_{620}. Additionally, fluorescence spectra of the cells were measured using a PTI Quanta master 40

spectrofluorometer (Photon Technology International, Lawrenceville, NJ). The excitation and emission wavelengths were set at 390 and 508 nm, respectively.

2.3. Absorption spectra

The purified Hb samples were buffer exchanged to 0.1 M sodium phosphate buffer pH 7.4. To obtain oxy-Hb, the samples were continuously flashed with a stream of oxygen gas in the presence of strong light. To prepare metHb, oxy-Hb was incubated with a 10-fold excess of potassium ferricyanide. To remove the excess of ferricyanide, the solution was passed through a gel filtration column (5 X 0.5 cm, GE Healthcare). Deoxy-Hb was obtained by adding a few grains of sodium dithionate to the sample. UV-visible spectra were recorded in the wavelength range 350-700 nm using a Cary 60 spectrophotometer (Agilent technologies).

2.4. Size exclusion chromatography

The molecular weight of GFP-HbF was determined by size exclusion chromatography (SEC). A sample in 50 mM sodium phosphate buffer pH 7.4 supplemented with 150 mM NaCl, was loaded to a Superdex Hi-Load 16/60, HR (GE Healthcare, 121 ml) column, pre-equilibrated with a same buffer. The column was connected to an ÄKTA Explorer (GE Healthcare, Sweden), and elution of the sample was monitored at 419 and 280 nm. The molecular weight was calculated using a calibration curve obtained from established markers [ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), carbonic anhydrase (29 kDa) and ribonuclease (13.7 kDa)].

2.5. Autoxidation

Samples (20 μM) in 20 mM sodium phosphate buffer pH 7.4 were placed in a 1 mL cuvette. Absorption spectra were recorded every 30 min at a wavelength range 350-700 nm at 37^{O}C for 12 h. Normalized absorbance changes at 576 nm was fitted to a single exponential function (equation 1), where y_0 is the absorbance at t =6 h.

$$y = Ae^{(-xt)} + y_0 \qquad \text{(Equation 1)}$$

2.6. Preparation of MIP particles and assay

Hb imprinted particles were synthesized on the surface of silica via the Pickering emulsion method as described previously (15). To investigate the adsorption capacities (Q) of imprinted polymers, 5 mg of MIPs were added into 1 mL of Hb-CO solution (1-45 μM, heme equivalent) in 20 mM sodium phosphate buffer pH 6.0. The mixture was stirred at 4^{O}C for 5 min, and then centrifuged at 13,000 rpm for 2 min. The amount of free Hb in the supernatant was measured spectrophotometrically at 419 nm. The adsorption capacities of MIPs were calculated by the following equation:

$$Q = \frac{(Co - Ct) \times V}{m}$$

In the above equation, C_0 (μM) is the initial concentration of protein solution, Ct (μM) is the final protein concentration of the supernatant solution, V (mL) is the volume of the initial solution and m (mg) is the mass of MIPs. To determine the kinetics of adsorption, experiments were performed with an initial Hb concentration of 5 μM while the other parameters were the same as in equilibrium binding test. Samples were taken at regular time intervals (10, 20, 30, 60, 120, 300 and 900 sec) to determine the unbound Hb concentration.

3. Results

The fusion and expression resulted in a chimeric protein harboring GFP linked to the N-terminal of fHbF. The protein was designated as GFP-HbF. When cultivated in shake flasks, cells appeared red in color, and yields were measured to 6-8 g cells/L. GFP-HbF could be purified by two chromatographic steps from *E. coli* crude extracts. The final yield of the purified protein was 6-8 mg/L with > 95% purity as determined by SDS-PAGE. GFP-HbF was found to possess dual properties. It appeared strongly red in color under normal light exposure while turned green under UV irradiation (Fig. 1). Moreover, fluorometric scanning generated an emission maximum at 508 nm, which was identical with native GFP. These results provide a first indication that the fusion event did not interfere with the intrinsic functional properties of both proteins.

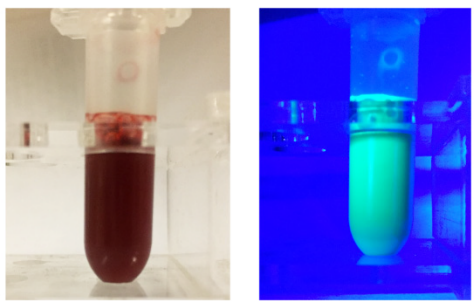

Fig. 1 Visualization of the expressed GFP-tagged fetal hemoglobin (GFP-HbF). Left panel shows the red color solution of the GFP-HbF under normal light. Right panel shows a green fluorescence under UV illumination due to the GFP molecule.

The *E. coli* cells harboring the GFP-HbF expression plasmid were induced at OD_{620} ≥ 2 using 0.1 mM IPTG. In order to monitor cell growth and protein production, samples from the cultures were removed every 60 minutes for SDS-PAGE. Similar

samples were analyzed using spectrofluorometer, and emission fluorescence was measured at wavelength between 400-600 nm. As shown in Fig. 2, the SDS-PAGE gel image shows a band at ~58 kDa, present in all the samples (expected theoretical molecular size is 60 kDa), corresponding to the GFP-HbF fusion protein. The same samples were analyzed by spectrofluorometer to observe the GFP peak at 508 nm. We found absence of fluorescence emission at 508 nm in a sample before induction. Notably, peak intensity at 508 nm was increased gradually over the time, and the highest peak intensity was observed after 14 h of cultivation. Samples were also analyzed simultaneously by SDS-PAGE and the band intensity of the gel was calculated (Quantity One software, BioRad). The obtained data indicated a gradual increase in band intensity after induction until 4 h. The band intensity was slightly decreased after overnight cultivation. This data demonstrated that induced culture initiate the expression of GFP-HbF after 4 h itself. However, complete folding of the protein occurs only after overnight cultivation.

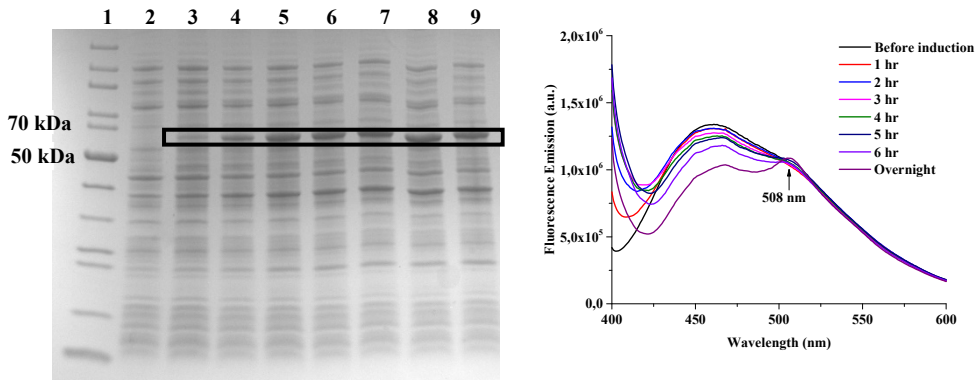

Fig. 2 The expression of GFP-HbF was monitored during the cell cultivation. Left image shows the SDS-PAGE gel image of crude *E. coli* cell extracts. Lane 1: Molecular protein markers. Lane 2: Before induction. Lane 3-9: 1 h, 2 h, 3 h, 4 h, 5 h, 6 h and 14 h after induction. Right panel shows the fluorescence emission of cells before and after induction, characteristic peak intensity for GFP at 508 nm was increased during cultivation.

3.1. Functional properties

Generally, Hb shows unique spectral properties in wavelength ranges from 400 to 650 nm. The spectral properties of GFP-HbF bound to different ligands was evaluated using UV-visible spectrophotometer.

GFP-HbF bound to CO and Oxy showed a soret peak at 419 and 415 nm, respectively (Fig. 3). The Q bands were observed at 539 and 569 nm for CO form, and oxy form showed peaks at 541 and 576 nm (Fig. 3, inset). This confirms that GFP tagged fHbF can bind to oxygen and CO. Moreover, the deoxy form was obtained by addition of few grains of sodium dithionite, showed an identical spectral property to fHbF. Ferric form also showed a single peak at 555 nm and prominent

peak at the soret region (405 nm). This data suggests that the spectral properties of fHbF were not disrupted by genetically linked GFP molecule.

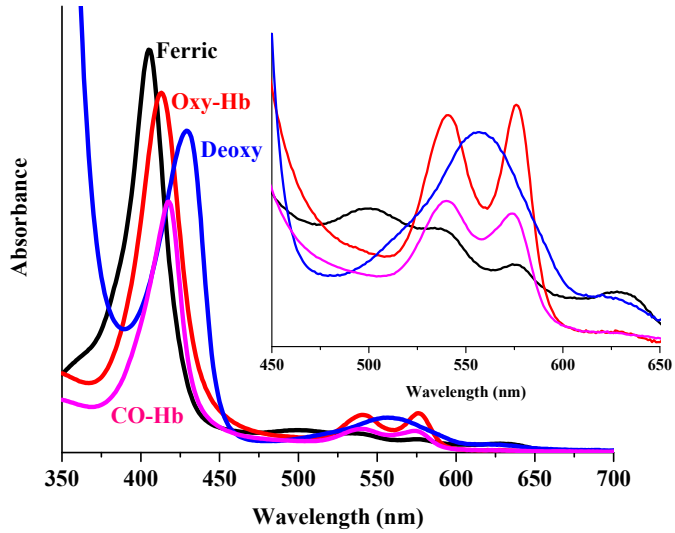

Fig. 3 Absorption spectra of GFP-HbF when bound to different ligands. CO and oxy bound GFP-HbF showed typical spectral properties as a native Hb molecule. Deoxy and ferric form were also identical to the corresponding protein.

3.2. Molecular size of GFP-HbF

The theoretical molecular size of the GFP-HbF was estimated to be 60 kDa, which agrees well with the results from SDS-PAGE. The molecular size of the GFP-HbF as determined by SEC was 118 kDa indicating that the fusion protein is present as a dimer. The Phyre2, online tool [18], was employed for the prediction of GFP-HbF structure (Fig. 4).

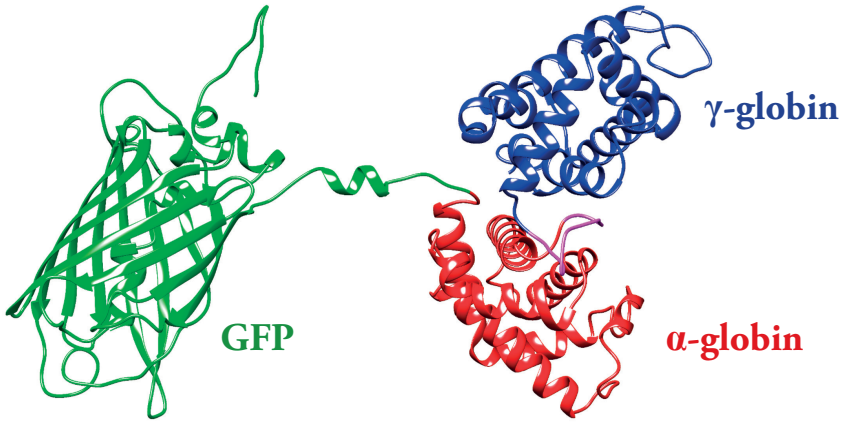

Fig. 4 Structure of GFP-HbF was determined by using online tool (Phyre2). Green fluorescent protein (green) was linked to the alpha globin (red). The alpha (red) and gamma (blue) globins are connected together by flexible twelve amino acids (magenta) linker $(GGS)_4$.

3.3. Oxidative properties of GFP-HbF

Outside the protective environment of RBCs, Hb continuously undergoes an autoxidation. In this reaction, Hb (O_2) produces superoxide radicals (O_2•−) and is converted into non-functional ferric Hb (Fe3+). This reaction can be monitored in vitro to calculate autoxidation rates by determining the redox state of the iron moiety. Autoxidation rates were calculated form absorption spectras recorded every 30 min for 6 hours. As shown in Fig. 5, the absorbance changes (576 nm) were normalized to calculate the autoxidation rates. The calculated autoxidation rates for fHbF and GFP-HbF were determined to be 0.20 ± 0.022 h^{-1} and 0.14 ± 0.05 h^{-1}, respectively. This data clearly indicates that genetically linked GFP lower the autoxidation rate by 1.5 folds.

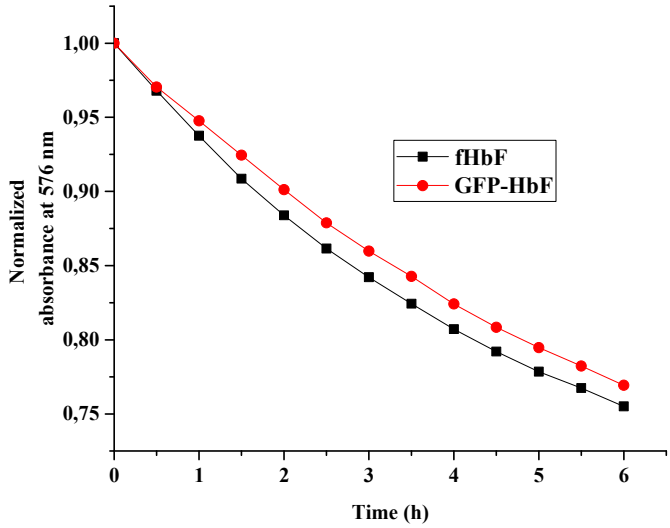

Fig. 5 The autoxidation rates of GFP-HbF (red) and fHbF (black). Normalized absorbance changes at 576 nm were fitted into single exponential function.

3.4. Adsorption Studies

The designed MIPs have cavities for the functional groups on the surface of the Hb molecule. When protein samples were mixed with MIP particles, the Hb molecules were rapidly adsorbed by the imprinted polymers. The binding isotherm (Fig. 6) showed that MIPs displayed slightly higher binding capacity (3.1 μmol g^{-1}) for fHbF than for GFP-HbF (2.8 μmol g^{-1}), most likely due to the increased size of the fusion protein.

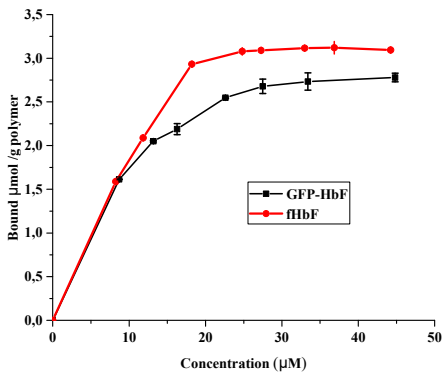

Fig. 6 Binding capacity of GFP-HbF and fHbF for imprinted polymers at different Hb concentrations. The concentration of MIPs used in each test was 5 mg ml^{-1}. The binding capacity was calculated based on bound and unbound protein present in the solution after incubation for 5 min at 4^{O}C in 20 mM phosphate buffer pH 7.4

The kinetic sorption data were fitted to pseudo-second-order kinetic model (equation 2):

$$\frac{t}{q_t} = \frac{1}{k_2 q_e^{\,2}} + \frac{1}{q_e} t \qquad (2)$$

in which k_2 is the rate constant of sorption, q_t and q_e are the amounts adsorbed at time t and at equilibrium. If the initial sorption rate $v = k_2 q_e^{\,2}$, equation (2) may be modified to equation (3).

$$\frac{t}{q_t} = \frac{1}{v} + \frac{1}{q_e} t \qquad (3)$$

By plotting of t/q_t against t, we can get the values of v and q_e. As listed in Table 1, the initial sorption rate for fHbF was higher than GFP-HbF.

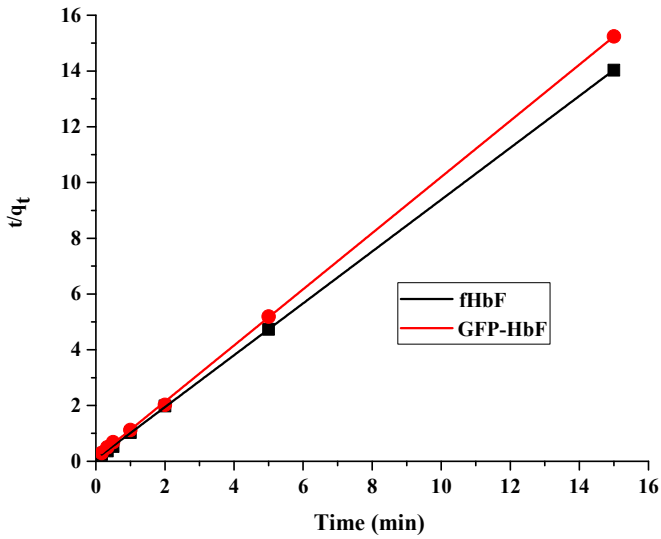

Fig. 7. Analysis of binding kinetics of GFP-HbF and fusion HbF using a pseudo-second-order kinetic model.

Table 1. The initial sorption rates and maximum binding capacity of fHbF and GFP-HbF on Hb-MIP at 4^{o}C in 20 mM sodium phophate buffer pH 6.0.

Sample	v (umol g^{-1} min^{-1})	q_e (μmol g^{-1})
fHbF	11.93	1.08
GFP-HbF	7.97	0.99

4. Discussion

Immunological assays and cellular screening methods often require access to conjugates between a target protein, often an antigen, and a marker enzyme. These approaches include the necessity of separately isolating the antigen and marker, as well as chemical conjugating them without disrupting the active site of the enzyme or the antigen-antibody binding site [19, 20]. Furthermore, these conjugates tend to be heterogeneous mixtures as conjugation often occurs at various and multiple residues. An attractive alternative to the above mentioned chemical cross-linking method would be the preparation of protein-marker complexes by gene fusion [1, 2]. Previously, bifunctional proteins prepared by gene fusion have found applications for facilitating protein purifications and modifying cell metabolism [21]. GFP has been used to enhance the solubility of fusion proteins. The use of GFP in these instances is highly advantageous. GFP is a small dimeric and robust enzyme with an intrinsic fluorescence [22, 23]. When GFP was fused to fHbF it could easily be tracked by fluorescence measurements. It requires no co-factor for monitoring of the fluorescence [24]. However, after its initial translation GFP requires an autocatalytic oxidative modification to create a functional chromophore. Intracellular access to oxygen is therefore critical for formation of fluorescence [25]. Another Hb, Vitreoscilla hemoglobin (VHb) has previously been used to enhance protein production under aerobic conditions. Fusion of GFP to VHb showed a significant impact on the growth of different *E. coli* strains [26]. Increased aeration during the cultivation of the cells resulted in a higher expression levels of GFP due to the activity of VHb for transferring oxygen to GFP or for providing a microenvironment with a high local oxygen level [27]. In our studies, we have also used vigorous shaking conditions (150 rpm) after induction of the culture. However, the HbF has a much higher oxygen affinity compared to VHb. The final yield of the purified GFP-HbF was also reduced (6-8 mg/L) compared to fHbF (20-24 mg/L). The recovery of the GFP-VHb was reduced when using *E. coli* strain BL21 [26]. Thus, the lower yield of the GFP-HbF may be directly linked to *E. coli* strain used for expression or the conditions used for aerating the bacterial culture.

The structure of native GFP molecule is composed of 11-stranded β-barrel that wraps around the central α-helix [28]. Data obtained from SEC suggest that GFP-

HbF is present as a dimer. The fusion partner (fHbF) also exists in a partially dimeric state as reflected by small angle X-ray scattering studies (SAXS). Overall, the aggregation of the GFP-HbF may thereby responsible for the poor yields. Heme precursor, ALA, play an important role in formation of stable Hb molecule by incorporating into readily formed apo-globin [29]. After its translation, GFP requires oxidation for the formation of functional chromophore. Fluorescence is completely lost in denatured GFP [24]. We observed that fluorescence peak at 508 nm was significantly elevated after 14 h of induction. Moreover, functional properties of Hb were determined by the absorption spectras. Taken together, it clearly indicates that formation of active GFP is in direct connection with the proper folding of Hb molecule.

Hb is known to undergoes continuous oxidation (autoxidation), in which released oxygen is reduced to superoxide, and the iron is oxidized to ferric/metHb (Fe^{3+}). Further reactions may lead to formation of ferryl Hb (Fe^{4+}), heme loss and globin denaturation [30, 31]. Herein, fusion of GFP to fHbF reduced the autoxidation rates. Moreover, GFP-HbF, may facilitates the interaction of two GFP molecule together, and/or electrostatic interaction between two fHbF molecule is another possibility, thus could be linked to reduced autoxidation rates.

MIPs display high selectivity for a molecule used as a template for it synthesis. For example, imprinted polymers demonstrated high selectivity for Hb molecule compared to other proteins differ by molecular size or isoelectric point [16]. It is well known that protein possess negative or positive charge at pH values above or lower its pI value. The adsorption capacity of the MIPs largely depends on the isoelectric point (pI) of the protein [32]. In our studies, imprinted polymer used were synthesized using adult hemoglobin as a template in its tetrameric form. Thus, Hb (pI: 6.9), has an overall negative charge at pH 6.0, which favours the binding to cavity of the functional groups present on imprinted polymers. The alpha chain of fHbF may thereby facilitate the adsorption of protein onto MIPs. On the contrary, additional GFP present on the N-terminus of the fHbF has decrease the pI of the protein (calculated pI:6.7), and thus decreased the binding capacity of a MIPs. Moreover, GFP increase the size of protein by 3.6 folds, which may also have contributed in lower binding.

Rudolph et al. [33] studied the bio distribution of liposome encapsulated Hb (LEH), radiolabelled with technetium-99m (99mTc). The pharmacokinetics of similar product developed as artificial oxygen carrier, designated as HbV, was studied using 125I [34]. All these studies, pharmacokinetic or pharma dynamics, would ultimately leads to development of a safe Hb molecule. However, Hb molecule cannot be used directly as a blood substitute due to its natural ability to dissociate into dimers [35]. Chemical or genetic modification onto Hb surfaces has shown some beneficial effects. Theses chemical entities often alter the structural and functional properties

of Hb molecule [36, 37]. Thus, the behaviour of decorated Hb molecule in cell-free environment remains a major question before its applications. Although, significant research has been undertaken for development of safe and effective HBOCs, no such a product available in the market for human usages [38]. Overall failure of the HBOC product may linked to intrinsic toxicity of the Hb molecule. In our previous report, we have demonstrated that Hb can also interact with plasmid DNA and initiate its cleavage [39]. However, the underlying mechanism is not completely understood. Therefore, GFP-HbF owing to its fluorescent property may facilitate the study to explore the action of cell-free Hb on genetic material.

Conclusions

In this report, we have shown that it is possible to prepare a fluorescent hemoglobin using protein-engineering approach. The expressed protein was functional as reflected by the fluorescence properties of GFP and spectroscopic properties of fHbF. Additionally, we have shown that MIPs may be useful to isolate pure Hb molecule and are also specific for Hb molecule.

Acknowledgement

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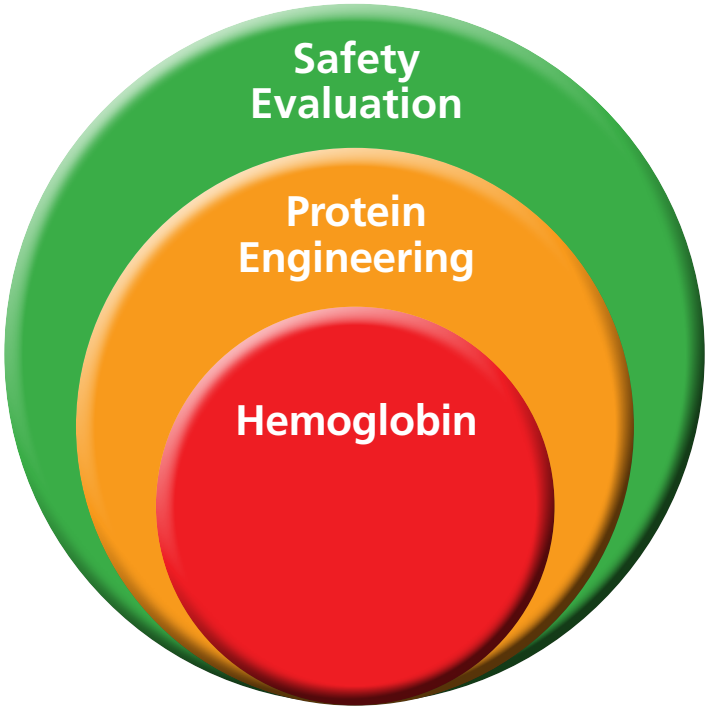

Schematic illustration of the research efforts undertaken for developing a safe and functional blood substitute.

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