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Kettisen, Karin

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LUND UNIVERSITY

PO Box 117
221 00 Lund
+46 46-222 00 00

Engineering of Human Fetal Hemoglobin

KARIN KETTISEN
FACULTY OF ENGINEERING | DEPARTMENT OF CHEMISTRY | LUND UNIVERSITY

Engineering of Human Fetal Hemoglobin

Karin Kettisen

LUND UNIVERSITY

DOCTORAL DISSERTATION

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Faculty opponent
Prof. Angela Fago
Department of Biology, Aarhus University, Denmark

Organization
LUND UNIVERSITY
Division of Pure and Applied Biochemistry
P.O. Box 124
SE-221 00 Lund, Sweden
Author: Karin Kettisen

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Abstract

In blood, the oxygen-transporting protein hemoglobin (Hb) governs the oxygenation of cells and tissues. Naturally, this protein has claimed a place in the center of the research field of artificial oxygen therapeutics. Such Hb-based products have used cell-free Hb purified from human, bovine, invertebrate, or recombinant sources to create hemoglobin-based oxygen carriers (HBOCs). However, administration of these cell-free Hb products into the bloodstream initiates several unwanted adverse events. The inherent toxic reactivity of Hb related to heme-mediated oxidation, nitric oxide scavenging, and heme release give rise to serious side effects. These issues have persisted despite attempts at finding a formulation strategy to tame native Hb outside the red blood cell. This dissertation describes work regarding the engineering of human fetal Hb (HbF) for screening of beneficial protein design strategies on the protein itself, both in terms of retaining oxidative stability and from a production perspective.

Oxidative side effects are central to the extracellular toxicity exhibited by Hb. We examined the effect of modifying redox active cysteine residues in HbF by removing and/or adding cysteine at the conserved hotspot γCys93 and a surface-located site on the α-subunit. The conserved cysteine was important for the oxidative stability of the protein and removal produced a more unstable Hb molecule. In contrast, the addition of cysteine on the surface of the α-subunit alleviated damaging reactions during oxidative conditions by providing an alternative oxidation hotspot.

As the surface of the α-subunit appeared to be promising as a target area for mutagenesis, we explored a set of mutants where alanine residues were substituted into negatively charged aspartic acid. This lowered the pI of HbF and reduced the DNA cleavage rate without affecting the overall structural integrity. We also observed an extended half-life *in vivo* as well as unchanged oxidation and heme loss rates, indicating that improved functions could be attained with modification of the net surface charge without adversely affecting key functions and stability.

We continued to focus on the surface of HbF and created mutants with more dramatically changed net surface charge by replacing positive surface residues with negatively charged residues. We improved Hb yields in the crude extracts during recombinant expression in *E. coli* with this strategy, but non-target Hb fractions were present in significant quantities in two of the three mutant samples. This indicated an unbalanced assembly of subunits in the HbF variants carrying the γ-subunit mutations, leading to the formation of homotetramers. In addition, the chosen γ-subunit mutations contributed to a more oxidatively unstable Hb molecule, as seen by increased autoxidation rates.

The best-performing negatively charged mutant was subjected to a more in-depth characterization study. The crystal structure of this mutant was solved and thus confirmed the surface-exposed locations of the mutations. The mutant showed no significant differences in oxidation rate reactions but differed in reduction and heme loss rates from wild-type HbF in reactions governed by the α-subunit. In contrast to a previously studied HbF mutant, this mutant did not show any increased effect on retention time *in vivo*. However, a dramatic decrease in DNA cleavage rate was seen, indicating a much less damaging behavior towards important cellular components.

We conclude that there are strategies for modifying HbF itself for the improvement of protein properties towards a better, and more easily produced Hb for HBOC development. The suggested modifications to the HbF protein presented in this work could be used in combination with other protein engineering strategies for Hb development.

Key words: fetal hemoglobin, protein engineering, recombinant protein production, protein purification, chromatography, oxygen carrier, redox reactions, net surface charge, DNA cleavage

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Engineering of Human Fetal Hemoglobin

Karin Kettisen

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Division of Pure and Applied Biochemistry

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Generation of figures for displaying 3D molecular graphics and analysis of protein structures were performed with the UCSF Chimera package [1, 2]

Popular summary

Towards ”blood” without donors.

Modern medicine is dependent on the supply of donated blood. A shortage or stop in blood donations could have devastating effects on healthcare. In troubled times such as right now, during a pandemic caused by SARS-CoV-2, the limitations of relying exclusively on donated blood for maintaining/restoring oxygen-transport capacity in routine procedures and medical applications are even more tangible. The infrastructure around blood donations and blood banks vary across the globe, as well as the main uses of the collected blood donations. Three-quarters of the blood transfusion performed in high-income countries are given to patients over 60 years of age. The most common uses are cardiovascular and transplant surgery, cancer therapy, and massive trauma. In low-income countries, on the other hand, over half of the transfusions are given to children under 5 years of age, and the most common uses involve treating severe childhood anemia and managing pregnancy-related complications. Donated blood also has drawbacks that dictate the safety of its use. Blood-borne pathogens, blood-type matching, secure supply of donors, and the perishability and short shelf-life of oxygen-carrying red blood cells, are all disadvantages that must be carefully managed by the blood banking systems. The potential benefits of a safe alternative to donated red cells for providing oxygen transport capacity in a clinical setting would greatly support modern medical care as well as alleviate shortages in circumstances where infrastructure is unable to sustain a sufficient influx of blood donations.

Without oxygen, the human body will cease to function. The essential component responsible for distributing oxygen is the protein hemoglobin (Hb), which is contained in the red blood cells at high concentrations. Hb is the component that gives these cells their characteristic red color. The idea to base an artificial oxygen-carrier product on Hb has been around since the 1930s and has sparked extensive research on hemoglobin-based oxygen carriers. However, despite several attempts, no artificial oxygen-carrier product has been able to get approval for human use by key government agencies, and this has kept these products from widespread use on a global scale. Two products – one Hb-based and one perfluorocarbon-based – have been accepted in a limited number of countries where they are allowed to be used in certain situations, despite safety concerns regarding toxic side-effects. Thorough investigations of the Hb-based therapeutics developed so far have pinpointed several crucial reactions/features of Hb that contribute to the adverse toxicity seen with these products. The studies in the wake of the unsuccessful attempts at realizing a globally accepted artificial oxygen carrier have contributed to a better understanding of the Hb protein and its many roles and side reactions in the body.

Hb is made up of four parts. Each separate part is folded into a specific structure that shelters a heme group, which can bind and release oxygen with its central iron atom. The heme group also enables the redox reactions which are key in the toxic effects exhibited by free Hb outside the red cell. The red blood cell contains several types of protective enzymes and molecules that maintain the iron in a reduced and functional state. However, if the protein is released into plasma, concentration-dependent dissociation, heme release, and cascading oxidative damage become prevalent. Thus, purified, unmodified human Hb is not safe to use despite its exceptionally suitable oxygen transport abilities. The previously developed Hb-based products successfully eliminated the dissociation problems of cell-free Hb, but the oxidative side-effects remain. Additional formulation strategies and/or protein engineering designs of Hb are required to tackle these issues.

Biotechnological advances over the past decades have allowed the production of wild-type and genetically engineered protein molecules in heterologous host organisms. Instead of relying on Hb from human and animal sources, products can now be based on specifically designed Hb produced in bacteria, yeast, or plants, which can be cultivated under controlled and pathogen-free conditions. This enables researchers to freely explore protein engineering strategies to tame Hb's toxic side effects. Such engineering studies have discovered ways to custom-tailor the oxygen-binding characteristics and managed to uncover site-specific mutations to reduce Hb's effects on vascular function. The remaining issue now, besides controlling the oxidative side reactions, lies with increasing the yield and production scale of these designed Hb molecules to enable practical and sustainable sources of artificial Hb.

To that end, the work presented in this dissertation has been conducted on the fetal version of human Hb (HbF). Rationally designed mutants have been investigated based on their redox reactions, cultivation yields in bacterial host cells, and other desirable traits such as stability and DNA cleavage ability. This work can be added to the large host of research efforts onto which eventually a safe, sustainable, artificially produced oxygen carrier may be based.

Populärvetenskaplig sammanfattning

Mot ”blod” utan blodgivare.

Den moderna sjukvården är beroende av bloddonationer från blodgivare. Blodbrist eller uppehåll i tillförseln av donerat blod kan ha förödande effekter på sjukvården och i samhället i stort. I oroliga tider som nu, mitt i en pandemi orsakad av SARS-CoV-2, blir det uppenbart hur sårbar den nuvarande situationen är med endast bloddonationer som källa till syretransporterande medel. Blodtransfusioner är avgörande för att återställa/bibehålla syresättning i kroppen i akuta situationer, rutinmässiga medicinska ingrepp och under allt mer avancerade tillämpningar.

Infrastrukturen bakom bloddonationer och blodbanker är omfattande och möjliggör den medicinska välfärd som man i flera delar av världen är van vid idag. Man får inte glömma att detta välstånd varierar över världen, liksom även hur användningen av de insamlade bloddonationerna fördelas. Tre fjärdedelar av alla blodtransfusioner i höginkomstländer ges till patienter över 60 år. De vanligaste användningarna innefattar hjärt- och transplantationskirurgi, cancerterapi och traumavård. I låginkomstländer å andra sidan ges över hälften av transfusionerna till barn under 5 år, och där är de vanligaste användningsområdena behandling av anemi hos barn och komplikationer i samband med graviditet. Donerat blod kommer även med en rad nackdelar som påverkar säkerheten av blodtransfusioner. Blodburen smitta, blodtypsmatchning, tillgång på blodgivare och den förhållandevis korta hållbarheten hos de syrebärande röda blodkropparna är risker som måste hanteras löpande för att säkerställa tillgång av blodprodukter för sjukhusens dagliga bruk. Ett konstgjort alternativ till röda blodkroppar som kan syresätta vävnader med liknande kapacitet i kliniska sammanhang skulle vara ett välkommet komplement och stöd till den alltmer avancerade sjukvården. Samt förhoppningsvis även förbättra sjukvård i länder där infrastrukturen kring bloddonationer är underutvecklad eller satt ur spel.

Utan syre slutar kroppen att fungera. Transporten av syre från lungorna till kroppens celler och vävnader hanteras av proteinet hemoglobin (Hb), vilket också är den komponent som ger de röda blodkropparna sin karaktäristiskt röda färg. Redan på 1930-talet beskrevs idén om att framställa en konstgjord syrebärarprodukt baserad på Hb. Denna idé har genom åren gett upphov till omfattande forskning och utveckling av Hb-baserade syrebärarprodukter. Trots flera försök att få dessa produkter godkända för användning som läkemedel för människor världen har man ännu inte lyckats. Två produkter, en Hb-baserad och en perfluorerad kolväte-baserad, har nått en liten framgång hos beslutsfattande myndigheter i ett begränsat antal länder. I dessa områden har de tillåtits trots rapportering om allvarliga

bieffekter, främst för att lindra konsekvenserna då kritisk blodbrist råder. De föregående produkternas avslag från de mest inflytelserika läkemedelsmyndigheterna har lett till mer ingående forskning och genom detta har man fastställt funktioner som ligger bakom de skadliga bieffekterna som observerats med de tidigare Hb-baserade syrebärar-produkterna. Detta har bidragit till en ökad förståelse kring detta livsviktiga protein.

Mänskligt Hb består av fyra delar och var och en av dessa fyra delar/subenheter innehåller en hemgrupp med en järnatom som kan binda syre. Denna hemgrupp möjliggör också de redoxreaktioner som har visat sig vara centrala för de bieffekter som uppvisats med extracellulärt Hb, d.v.s. fritt Hb utanför den röda blodkroppen. Inuti de röda blodkropparna finns det flera typer av skyddande enzymer och molekyler som förebygger oxidation och ser till att järnet befinner sig i ett reducerat och funktionellt tillstånd. Miljön inuti de röda blodkropparna är anpassad för att kontrollera och minimera Hbs oxidativa sidoreaktioner. När Hb befinner sig utanför denna skyddande miljö, fri i plasman, uppvisar proteinet skadliga effekter som grundar sig i koncentrationsberoende dissociation av subenheterna, oxidationsreaktioner som påverkar omgivande vävnader och frigörande av hemgruppen. Således är fritt Hb inte lämpligt att använda som syrebärande komponent i ett läkemedel för syretillförsel trots dess förträffligt lämpliga syretransportförmåga. De föregående Hb-baserade produkterna formulerades så att de framgångsrikt eliminerade dissociation av Hb i plasman, men de oxidativa bieffekterna har dessvärre kvarstått och är de som i grunden ligger bakom biverkningarna som lett till avslag för kliniskt bruk. Därför krävs ytterligare formuleringsstrategier och proteindesign av Hb för att eliminera och kontrollera Hbs toxiska sidoreaktioner.

Framsteg inom bioteknikområdet under de senaste decennierna har möjliggjort produktion av främmande proteinmolekyler i värdorganismer – så kallade rekombinanta proteiner. Detta gör det möjligt att basera nästa generations syrebärarprodukter på Hb-molekyler som producerats under patogenfria odlingsförhållanden i t.ex. bakterieceller, jästceller eller växter, istället för att som tidigare samla in Hb från humana och animaliska källor. Det medför också möjligheter för forskare att genetiskt designa nya Hb varianter och undersöka innovativa proteindesignstrategier för att tämja Hbs skadliga bieffekter.

Forskning på proteindesign av Hb har fastställt strategier för att finjustera proteinets syrebindande egenskaper, samt funnit mutationer som kan tillämpas för att minska Hbs effekt på blodkärlsutvidgande processer. De återstående problemen inom Hb-design ligger nu i att finna något sätt att kontrollera de oxidativa sidoreaktionerna, samt att öka odlingsutbyte och produktionsvolym för att möjliggöra hållbar storskalig produktion. Detta kommer att kräva optimering av

rekombinanta produktionssystem som sedan kan användas som tillförlitliga källor för utvinning av specialdesignat Hb.

För detta ändamål har studierna som ligger till grund för denna avhandling utförts på rekombinant producerat humant foster-Hb (HbF). Rationellt designade HbF-mutanter har undersökts och karaktäriserats med fokus på redoxreaktioner, odlingsutbyte i bakteriella värdceller och andra önskvärda egenskaper såsom stabilitet och minskad DNA-klyvningsaktivitet. Forskningsresultaten som presenteras i denna avhandling kan läggas till den redan omfattande forskningen på Hb, och förhoppningsvis så småningom bidra till förverkligandet av en säker och hållbart producerad syrebärare.

List of papers

The dissertation is based on the following scientific research articles and manuscripts, which are appended after the introductory chapter (kappa). The papers will be referred to in the text by Roman numerals.

Paper I ***Site-directed Mutagenesis of Cysteine Residues Alters Oxidative Stability of Fetal Hemoglobin***

Karin Kettisen, Michael Brad Strader, Francine Wood, Abdu Alayash, & Leif Bülow.
Redox Biology. 2018, 19: 218-225.

Paper II ***Site-Specific Introduction of Negative Charges on the Protein Surface for Improving Global Functions of Recombinant Fetal Hemoglobin***

Karin Kettisen, Cedric Dicko, Emanuel Smeds, & Leif Bülow.
Frontiers in Molecular Biosciences. 2021, 8:649007

Paper III ***Introducing negatively charged residues on the surface of recombinant fetal hemoglobin improves yields in* Escherichia coli**

Karin Kettisen & Leif Bülow.
Submitted

Paper IV ***Structural and Oxidative Characterization of a Recombinant High-Yield Fetal Hemoglobin Mutant***

Karin Kettisen, Maria Gourdon, Emanuel Smeds & Leif Bülow.
Manuscript

My contribution to the papers

Paper I ***Site-directed mutagenesis of cysteine residues alters oxidative stability of fetal hemoglobin***

I planned and performed the experimental work except for the quantitative MS (M.B.S.) and the oxygen-binding assay (F.W.). I drafted the manuscript and finalized it with revision from the co-authors.

Paper II ***Site-Specific Introduction of Negative Charges on the Protein Surface for Improving Global Functions of Recombinant Fetal Hemoglobin***

I planned and performed the experimental work except for the SAXS experiment and analysis (C.D), and the animal handling (E.S.). I drafted the manuscript and finalized it with revision from the co-authors.

Paper III ***Introducing negatively charged residues on the surface of fetal hemoglobin improves yields in* Escherichia coli**

I planned and performed the experimental work and drafted the manuscript. I finalized the current version of the manuscript with revision from L.B.

Paper IV ***Structural and Oxidative Characterization of a Recombinant High-Yield Fetal Hemoglobin Mutant***

I planned and performed the experimental work except for the X-ray crystallography (M.G) and the animal handling (E.S.) I drafted the manuscript and finalized the current version of the manuscript with revision from L.B.

Papers not included in the dissertation

Potential electron mediators to extract electron energies of RBC glycolysis for prolonged in vivo functional lifetime of hemoglobin vesicles

Karin Kettisen, Leif Bülow & Hiromi Sakai.
Bioconjugate Chemistry. 2015. 26, 4, p. 746-754.

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

Sandeep Chakane, Tiago Matos, **Karin Kettisen**, & Leif Bülow.
Redox Biology. 2017, 12, p. 114-120.

Composite imprinted macroporous hydrogels for haemoglobin purification from cell homogenate

Solmaz Hajizadeh, **Karin Kettisen**, Magnus Gram, Leif Bülow, & Lei Ye.
Journal of Chromatography A. 2018, 1534, p. 22-31.

Chromatographic separation of hemoglobin variants using robust molecularly imprinted polymers

Ka Zhang, Tongchang Zhou, **Karin Kettisen**, Lei Ye & Leif Bülow.
Talanta. 2019, 199, p. 27-31.

Rapid separation of human hemoglobin on a large scale from non-clarified bacterial cell homogenates using molecularly imprinted composite cryogels

Solmaz Hajizadeh, **Karin Kettisen**, Leif Bülow, & Lei Ye.
Frontiers in Bioengineering and Biotechnology. 671229

Production of human fetal hemoglobin in Nicotiana benthamiana leaves by transient expression – a first step towards plant produced hemoglobin-based oxygen carriers

Selvaraju Kanagarajan, Magnus Carlsson, Sandeep Chakane, **Karin Kettisen**, Emanuel Smeds, Ranjeet Kumar, Magnus Gram, Leif Bülow, & Li-Hua Zhu.
Submitted

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

Sandeep Chakane, Khuanpiroon Ratanasopa, Cedric Dicko, **Karin Kettisen**, Emanuel Smeds, Tommy Cedervall, Abdu Alayash, & Leif Bülow.
Manuscript

Abbreviations

2,3-BPG	2,3-bisphosphoglycerate
AHSP	α-helix stabilizing protein
ALA	δ-aminolevulinic acid
AOC	artificial oxygen carrier
Cygb	cytoglobin
Da	Dalton (1.6605×10^{-24} g)
DSF	differential scanning fluorimetry
DTT	1,4-dithiothreitol
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
EMA	European Medicines Agency
FDA	US Food and Drug Administration
Fe^{2+}	ferrous iron
Fe^{3+}	ferric iron
Fe^{4+}	ferryl iron
GFP	green fluorescent protein
gMb	green myoglobin (recombinant apomyoglobin mutant H64Y/V67F)
Hb	hemoglobin
HbA	adult hemoglobin
HbF	fetal hemoglobin
HbS	sickle cell hemoglobin
HBOC	hemoglobin-based oxygen carrier
HbV	hemoglobin vesicles
HbVar	database of Human Hemoglobin Variants and Thalassemias
HCP	host cell protein

HO-1	heme oxygenase-1
Hp	haptoglobin
Hpx	hemopexin
HSA	human serum albumin
IEX	ion exchange chromatography
IEF	isoelectric focusing
IPTG	isopropyl β-D-1-thiogalactopyranoside
MAP	methionine aminopeptidase
Mb	myoglobin
metHb	ferric hemoglobin (Fe^{3+})
NADH	nicotinamide adenine dinucleotide
Ngb	neuroglobin
P_{50}	partial oxygen pressure when hemoglobin is at 50% capacity
PDB	Protein Data Bank
pDNA	plasmid DNA
PEG	polyethylene glycol
PFC	perfluorocarbon
pI	isoelectric point
POE	polyoxyethylene
R-state	relaxed state, fully oxygenated hemoglobin
RBC	red blood cell
ROS	reactive oxygen species
SOD	superoxide dismutase
T-state	tense state, fully deoxygenated hemoglobin
SAXS	small-angle X-ray scattering
WHO	World Health Organization
wt	wild type

1 Introduction

A sought-after dream in medical research is to be able to provide a safe, universally compatible, artificially produced, *oxygen therapeutic*, or *artificial oxygen carrier* (AOC). Such a therapeutic could enable innovative healthcare applications and be applied as a complement to the existing blood banking systems to alleviate blood shortages and improve healthcare in acute situations or at remote locations.

Severe blood loss due to injury or surgical procedure has two consequences, 1) loss of liquid volume in the intravascular system, and 2) loss of oxygen supply to the tissues. Intravascular liquid volume can be restored with isotonic solutions with suitable colloidal properties, but if the loss of blood volume results in a critical shortage of the oxygen-carrying red blood cells (RBCs), transfusion of RBCs is necessary to maintain oxygen homeostasis for life-supporting cellular metabolism. Through many years of clinical practice, transfusion treatment with donated RBCs has been proven to be effective, but unfortunately, it also comes with several risks. Blood-type matching is critical to avoid fatal consequences, and although the discovery and application of primarily the AB0- and Rh-systems have resolved many of the early blood-matching issues, many more blood type antigens have since been discovered, and substantial resources and efforts go into managing the matching between different blood groups. Another risk with donated blood is the blood-transmitted infectious agents such as viruses and bacteria. Outbreaks such as seen in the 1980s with the HIV crisis highlight the danger of new pathogens appearing and being transmitted through blood transfusions. Testing for both known and potential new infectious agents in donated blood is, therefore, another critical task for blood banking organizations. Another shortcoming is that the supply of blood and blood components for transfusion is hugely dependent on the altruistic individuals taking part in repeated blood donations. With an aging world population and the corresponding need for more advanced surgical procedures, the demand for donated blood is increasing. Furthermore, RBCs derived from donated blood are not very stable and have a fairly short lifespan. The oxygen-carrying RBCs can be stored in a refrigerated environment for up to 42 days, depending on the local regulations [3]. Although, there is currently also an ongoing debate on the subject of “fresher” or “older” stored blood, as well as the possible implications of donor age, sex, etc., in short – the “quality” of the transfused RBCs – in regards to the safety of donated blood [4-6].

In summary, blood type-matching, pathogen testing, donor availability, altruism, as well as and demanding storage criteria and short shelf-life, are serious and costly disadvantages of relying solely on donated blood to alleviate the loss of oxygenation

capacity in patients and other medical applications. For these reasons, AOCs are pursued to support modern medicine and the blood banking systems.

For several decades, researchers, companies, and military institutions have been working towards the realization of a functional, safe, and globally accepted AOC [7]. One approach to achieve this is through the research on hemoglobin-based oxygen carriers (HBOCs) [8, 9]. In this field, the oxygen-transporting protein hemoglobin (Hb) is used as the principal component of the oxygen-carrying therapeutic, aiming to take advantage of Hb's well-suited and compatible oxygen-distributing capabilities. The purpose of an AOC is primarily to mediate oxygen transport, and therefore "blood substitute" is not an appropriate term since it implies other blood-related functions such as distribution of immune system functions or nutrient transportation. An ideal AOC should preferably be readily available, lack antigenicity, be free of pathogens, have a long shelf-life, be stable at room temperature, have compatible colloidal properties, stay in circulation for a significant amount of time, transport CO_2 for removal from tissues, decompose into non-hazardous components, and possess suitable oxygen transport and delivery capabilities under physiological conditions. This is a long list of desirable properties, and despite that several products have successfully achieved many of them, persistent safety concerns in clinical trials have stopped the AOC products developed so far to get approval for human use by key major influential governmental agencies [10]. In 2008, a criticized meta-analysis of the clinical trials suggested that the HBOC products were associated with a higher risk of myocardial infarction and death [11]. Clinical side-effects that are commonly seen with the preceding generation of HBOC products are inflammatory reactions, renal toxicity, neurotoxicity, hypertension, and myocardial infarction [7, 12]. The one HBOC product to have some success is only available in South Africa and Russia, where it was accepted despite the safety concerns preventing it from approval in other countries [12].

Extensive research has been dedicated to addressing the safety concerns raised with previously developed AOC products. Through biotechnological advances, recombinant techniques have enabled complex protein molecules to be produced in suitable heterologous hosts, such as bacteria, yeast, or plants. This allows researchers to independently produce Hb for HBOC development, but it also provides the option to freely explore different genetic variants and constructs of Hb in search of a Hb molecule with tailor-made properties for the intended applications [13]. The adult Hb (HbA) has been one of the main Hb used and investigated for HBOC development, due to the compatible oxygen-carrying properties and availability of this protein. It has been established that the persistent toxicity issues of previous generations of HBOCs are related to oxidative reactivity [14], heme

release causing activation of immunological responses [15], and NO scavenging activity causing vascular dysfunction [16]. Protein engineering studies of Hb to clarify the biochemistry behind these events have elucidated some of the key reactions contributing to the toxic issues [17]. Besides HbA, another variant of human Hb with suitable oxygen transport properties is the fetal Hb (HbF), which is set apart from the adult Hb physiologically by an increased oxygen affinity in the presence of 2,3-BPG, and also by exhibiting greater tetrameric stability and enhanced alkaline stability. HbF has also been shown to exhibit a lower DNA cleavage activity [18], and an enhanced pseudo-peroxidase activity [19], as well as shown promising quantitative and qualitative results during recombinant bacterial production [20]. Although the oxidative reactivity, heme release, and nitric oxide scavenging activity similarly need to be addressed by protein engineering in HbF, the advantage of the greater stability of HbF calls for further examination of engineering strategies with this variant as a template for HBOC development.

For the studies presented in this dissertation, the focus has thus been on protein engineering of HbF. In **paper I** a study on fetal Hb variants with cysteine-related mutations and their oxidative reactions was presented. The redox reactions of Hb are critical to the function and the potential toxicity of Hb. Another critical aspect of Hb is the interactions with surrounding biomolecules, and in **paper II** another set of mutants was investigated in regards to net surface charge modification. To further explore the impact of Hb variants with an increased negative net charge on *E. coli* expression, three mutants with surface-located substitutions were created and the findings are presented in **paper III**. Finally, **paper IV** addresses a more detailed characterization of one particular negative mutant with promising qualities such as increased yield and significantly lower DNA cleavage activity.

2 Hemoglobin

A turning point in evolution was when oxygen started to be utilized in metabolic processes for the extraction of energy. The cellular energy output from the metabolism of glucose is increased fifteen times in the presence of oxygen, and the efficient energy extraction provided support towards the development of more and more complex organisms. For vertebrates, sufficient oxygen supply to all tissues in the organism has been solved by developing a circulatory system that transports oxygen from the lungs to all tissues in the body. The molecule capable of transporting oxygen and delivering it to the cells is the protein known as *hemoglobin* (Hb). This protein is closely bound to the scientific history of biochemistry and is one of the most studied proteins of all time [21]. In the 19th century, researchers started to uncover traits of Hb, such as the oxygen-carrying capabilities, the molecular size, the protein-to-iron ratio (1825), as well as Hb's ability to crystallize (1840) [22, 23]. The name 'Hämoglobin' was coined by F. Hoppe-Seyler in 1864 [24], from Greek's αἷμα (*haima*), literally meaning "blood" and Latin's *globus*, meaning "ball/sphere". Almost 100 years later, in 1960, the crystal 3D structure of Hb was solved as part of the pioneering work by M. Perutz [25]. Through the numerous studies of this life-enabling protein, along with its variants and mutants, Hb has become a model protein in the field of biochemistry [26].

2.1 Structure

The structure of human Hb is made up of four subunits, with two identical pairs of polypeptide chains, α- and β-type, that each folds into seven or eight α-helices commonly denoted from A to H. The assembly of the subunits forms a globular protein with a central cavity. Each subunit forms a characteristic protein structure called the *globin fold* (Figure 1A), with a sheltered pocket that houses the hydrophobic prosthetic group *heme*, an iron protoporphyrin IX molecule (Figure 1B). In human Hb, the iron atom is held in place in the center of heme by four pyrrole nitrogen atoms, and the fifth binding position is coordinated with the imidazole nitrogen atom provided by the histidine residue at position 8 of helix F, commonly referred to as the *proximal histidine*, on the polypeptide chain of Hb. This setup enables the iron atom to reversibly bind the oxygen (O_2) in the empty sixth position. In summary, human Hb is a tetrameric protein, consisting of two identical pairs of polypeptide chains (heterodimers), and contains four heme groups (Figure 1C). As a result, each Hb molecule can carry up to four oxygen molecules, binding 1.39 ml oxygen per gram Hb. The presence of normal concentrations of Hb in the blood increases the oxygen-carrying capacity by 70 times and enables

sufficient oxygen transportation from the lungs to meet the oxygen demand of the tissues [25, 27].

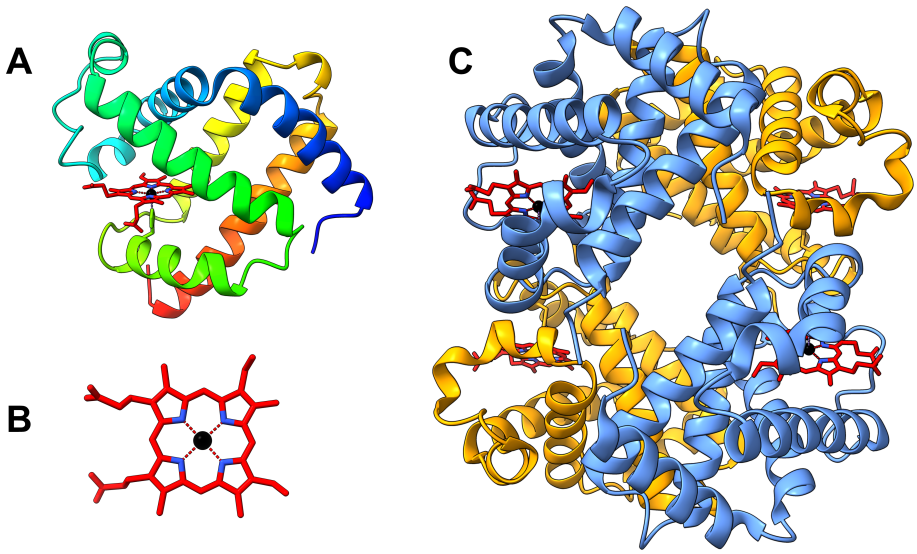


Figure 1: A) Protein structure of sperm whale deoxy myoglobin (PDB entry 1A6N), illustrating the globin fold with the eight α-helices forming a sheltered pocket for the heme (red). B) In heme, the iron is coordinated in the center of the protoporphyrin IX molecule by nitrogen, shown in blue in the top-down view of heme and the central iron atom is black. C) Protein structure of the T-state configuration of deoxy HbA (PDB entry 2DN2). The two α-subunits are shown in blue and the β-subunits in yellow.

The globin fold is conserved in the superfamily of the so-called globins/heme proteins/hemoproteins, and these proteins can be found in all kingdoms of life with varying and sometimes unknown functions [28]. Four examples of globin proteins found in humans besides Hb are: myoglobin (Mb), present in cardiac and skeletal muscle tissues and mediates diffusion/buffering/release of oxygen and nitric oxide management [29, 30]; cytoglobin (Cygb), involved in nitric oxide metabolism [31]; neuroglobin (Ngb), exhibiting a protective role relating to hypoxia, neural tissues, and cancer cells [32]; and androglobin, which contain an embedded globin chain, and recently shown to have a role in ciliogenesis [33, 34]. It is common to classify heme proteins based on their coordination to the heme, which is either penta- or hexacoordinated. The oxygen-carrying proteins Mb and Hb are penta-coordinated and bind ligands in the sixth position as described above.

Oxygen binding of respiratory globins

The accuracy of Hb to bind and release oxygen at the appropriate times derives from cooperative binding mechanisms enabled by heme-interaction and the assembly of the heterodimers. When an oxygen molecule binds in one of the subunits of Hb, the structure slightly changes and facilitates the binding of oxygen in the other subunits in an allosteric manner. Inversely, when the subunit releases an oxygen molecule, the structural changes promote oxygen release from the other subunits. This is called cooperative binding and the oxygen binding curve of Hb, therefore, shows a sigmoid shape (Figure 2). The two endpoints of the cooperative binding are called the deoxygenated T-state (tense) and the oxygenated R-state (relaxed), with a structural difference resulting in a 15-degree rotation in respect of one heterodimer to the other, and an accompanying contraction of the central cavity [27]. In comparison, Mb, the oxygen-carrying protein found in muscle tissue, is a monomer consisting of only one polypeptide chain. Mb and Hb are closely related as oxygen carriers by divergent evolution, but play different roles for oxygen delivery in vertebrates. While Hb is used for the overall transport of oxygen, Mb binds oxygen with higher affinity and functions as a storage molecule that can release its bound oxygen when partial pressures decrease in the muscle tissue. Being made up of a sole polypeptide chain, Mb has a much simpler oxygen binding curve due to the lack of cooperative stereochemistry [21]. Figure 2 displays the difference in oxygen binding curves between Mb and Hb.

Several factors have tuned Hb to provide suitable oxygen transport. An important molecule for the regulation of oxygen delivery is the compound 2,3-bisphosphoglycerate (2,3-BPG), which is present within the red blood cells (RBCs). This compound functions as an allosteric effector that binds in the center of the Hb tetramer in the T-state, and stabilizes this otherwise relatively unstable configuration. This facilitates a dramatic increase in the release of oxygen at physiological conditions, enabling the unloading of 66 % of the carried oxygen to tissues, compared to only 8 % without 2,3-BPG. Another factor that has an impact on how oxygen is released is the Bohr effect, described in 1904 [35], which refers to how pH affects the oxygen affinity of Hb. Lower pH stimulates the increased release of oxygen (shifting the oxygen binding curve to the right) and promotes more oxygen to be released in tissues with a high metabolism. The Bohr effect is divided into the acid Bohr effect, where oxygen uptake promotes the uptake of protons, and the alkaline Bohr effect, where oxygen uptake promotes the release of protons. The alkaline Bohr effect is also influenced by the presence of chloride ions. Thus, 2,3-BPG, CO_2, H^+, and Cl^- are all allosteric effectors of Hb providing fine-tuning of oxygen delivery [21, 36, 27].

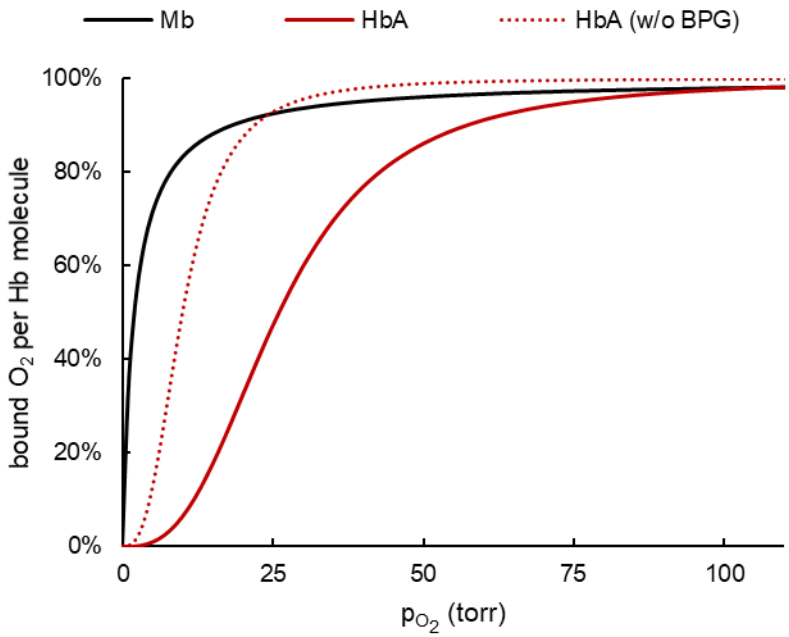


Figure 2: Oxygen binding curves of Mb (black) and Hb (red). The dotted line represents Hb's binding curve in the absence of 2,3-BPG. The cooperative binding of oxygen by Hb is visualized by the sigmoidal shape of the oxygen equilibrium curve.

Conserved structure and invariant residues

The globin fold is a highly conserved structure that prevails even among greatly varied amino acid residue sequences. There are only two categorically invariant amino acid residues in Hb and Mb – proximal histidine in helix F (position F8) and phenylalanine in the corner between helix C and helix D/E (position CD/CE1). These two residues have important heme interactions and mutations at these sites significantly destabilize the protein-heme-binding. Another highly conserved residue is histidine at position 7 in helix E, called *distal histidine*, which is involved in the stabilization of the ligand-bound state. In Hb, two distinct contact surfaces make up the tetrameric assembly of the subunits, $\alpha_1\beta_1$ or $\alpha_2\beta_2$ (interface between the subunits of the heterodimer) and $\alpha_1\beta_2$ or $\alpha_2\beta_1$ (interface between the two heterodimers in the tetramer). The two α-subunits only have a few contact points between one another, while the β-subunits hardly have any contact at all. The largest structural changes going from unliganded to liganded form happen in the $\alpha_1\beta_2/\alpha_2\beta_1$ interface, and the residues present here are also well-conserved in vertebrate Hbs [37-39]. A recent paper by Pillai et al. provides insight into the evolution of Hb from the ancestral globin genes, in which they investigate how cooperativity and allosteric regulation evolved in vertebrae Hbs [40].

Stability of hemoglobin

The building blocks and structural elements determine the stability of a protein. According to Antonini and Brunori, Hb is structurally stable for less than an hour at 40 °C (depending on ligand state) and within the pH interval of 5-10 [29]. When carbon monoxide is bound to Hb it is more stable and temperature resistant [41]. As a non-covalently linked multimeric protein, concentration-dependent tetramer-dimer-monomer dissociation constants are also important to describe the stability of Hb. For example, the deoxy form of Hb has higher tetrameric stability than the liganded form, supposedly due to six extra salt bridges present in the T-state [39]. There is also a backside of the handling of oxygen, since oxygen and derived reactive oxygen species (ROS) readily initiate oxidative side reactions. Hb spontaneously autoxidizes to ferric Hb (metHb) and if oxidation reactions are allowed to proceed without protective reducing activities from the surrounding environment, protein radicals will inflict structural damage and lead to the release of heme and collapse of the globin structure. The oxidative side reactions of Hb also affect biomolecules in the surrounding environment [42, 43, 15]. Therefore, the oxidative stability of Hb is a very important aspect in HBOC development and will be discussed further later on.

2.2 Human hemoglobin variants

The predominant variant of Hb changes during ontogeny at defined time points for different development stages. This is governed by genes for α-type globins on chromosome 16 [44] and β-type globins on chromosome 11 [45]. The human Hbs can be divided into three stages where they dominate the expression: embryonic, fetal, and adult. The embryonic Hbs are called Hb Gower 1 ($\zeta_2\varepsilon_2$), Hb Gower 2 ($\alpha_2\varepsilon_2$), and Hb Portland ($\zeta_2\gamma_2$). These are downregulated some weeks into the fetus development in favor of the expression of fetal Hb (HbF, $\alpha_2\gamma_2$). After birth, the α-gene persists but the γ-gene is gradually downregulated and replaced by β- and δ-genes, and at around 6 months of age, the adult Hb (HbA, $\alpha_2\beta_2$) emerge as the major form for the remainder of the human life span. In a normal human adult, the total circulating Hb content is around 97 % HbA, 2 % HbA_2 ($\alpha_2\delta_2$), and 1 % HbF [26, 46].

Fetal hemoglobin

The presence of HbF was first noticed by E. Körber in 1866, who reported increased resistance to alkali denaturation of Hb from fetal blood [47]. Later, in 1927, it was shown in experiments on goats that the oxygen dissociation curves of the maternal and fetal blood were different [48]. In 1963, Schröeder et al. published the amino acid sequence of the purified subunits of HbF [49, 50]. Like the HbA, HbF is a globular protein that consists of four subunits. The two α-subunits in HbF are the same as in HbA, but in HbF they are paired with γ-subunits instead of β-subunits.

Although HbA and HbF are very similar in size and structure, the differences between the β- and the γ-subunits are determined by the amino acid residues that differ between them. The difference is either 39 or 40 residues as the γ-globin gene comes in two functionally identical variants, γ^G and γ^A, which only diverges by one amino acid residue at position 136 in the polypeptide chain (glycine or alanine). 22 of the residues that differ between the β- and the γ-subunit are located on the surface of the protein. Four residues differ in the $\alpha_1\gamma_1$ contact interface, and one residue differs in the $\alpha_1\gamma_2$ interface. Figure 3 shows the unliganded and liganded structures of HbF.

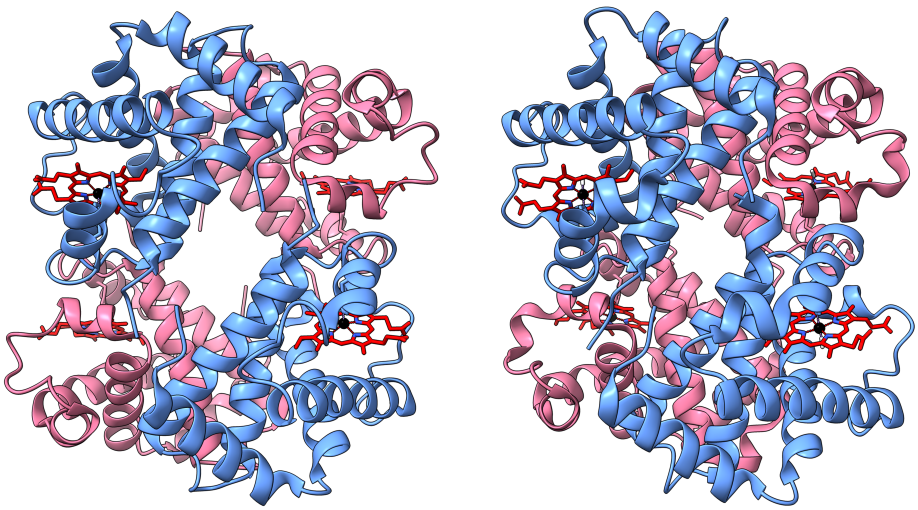


Figure 3: Protein structures of HbF, unliganded structure to the right and liganded structure to the left, displaying the noticeable tightening of the central cavity (PDB entries 1FDH and 4MQJ, respectively). The α-subunits are colored blue and the γ-subunits are shown in pink, while the heme groups are red with the iron atom colored black.

Below is the amino acid residue sequence alignment between the β-subunit and the ^{G}γ-subunit, made in Clustal Omega at ebi.ac.uk/Tools/msa/clustalo/ [51]:

```
  0  MVHLTPEEKS AVTALWGKVN VDEVGGEALG RLLVVYPWTQ RFFESFGDLS  49
     |.|.|.|:|: .:|:|||||| |::.|||.|| |||||||||| |||:|||:||
  0  MGHFTEEDKA TITSLWGKVN VEDAGGETLG RLLVVYPWTQ RFFDSFGNLS  49

 50  TPDAVMGNPK VKAHGKKVLG AFSDGLAHLD NLKGTFATLS ELHCDKLHVD  99
     :..|:||||| |||||||||. :..|.:.||| :||||||.|| ||||||||||
 50  SASAIMGNPK VKAHGKKVLT SLGDAIKHLD DLKGTFAQLS ELHCDKLHVD  99

100  PENFRLLGNV LVCVLAHHFG KEFTPPVQAA YQKVVAGVAN ALAHKYH     146
     ||||:||||| ||.|||.||| |||||.|||: :||:|.|||: ||:.:||
100  PENFKLLGNV LVTVLAIHFG KEFTPEVQAS WQKMVTGVAS ALSSRYH     146
```

In the above alignment, the lines (|) represent the residues that are the same, the colons (:) denote residues that are exchanged but have similar properties, and the dots (.) show residue changes with divergent properties.

The substituted residues that differ between the β-subunit and the γ-subunit give rise to some distinct biophysical dissimilarities, for example, a higher affinity for oxygen in the presence of 2,3-BPG [52, 53]. The oxygen binding curves of HbA and HbF are shown in Figure 4. From several studies, this property has been suggested to be determined by the residues γGly1, γGlu5, γAsp43, and γSer143 [52, 54, 55]. These residue substitutions are located at binding sites for 2,3-BPG, effectively lowering the affinity for the allosteric regulator in the T-state, compared to HbA. This difference in oxygen affinity is biologically crucial to ensure oxygen transfer from the maternal blood to the fetus.

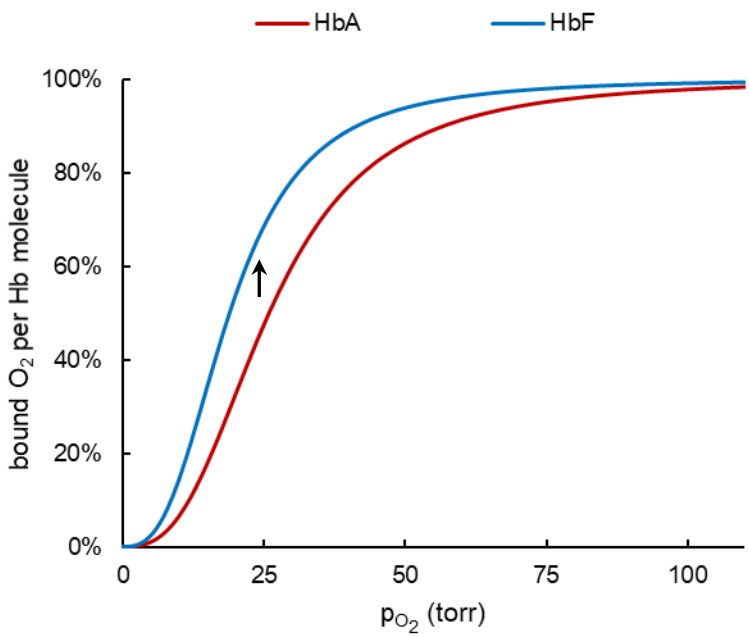


Figure 4: Comparison of the oxygen-binding curves of HbA and HbF in presence of 2,3-BPG. The difference enables oxygen to be transferred from the mother to the fetus.

Deoxy HbF also has higher solubility compared to deoxy HbA. The proposed substitution responsible for this difference is position γAsp22 with the aspartate instead of glutamate as seen in the β-subunit, possibly weakening an intermolecular contact usually seen between neighboring Hb molecules in Hb crystals [56].

The greater alkali denaturation resistance of HbF is useful in clinical settings for quantifying the presence of HbF [57], but the exact structural mechanism behind this resistance has not yet been fully elucidated. It was proposed to be due to substitutions of weakly acidic internal residues βCys112 and βTyr130, which can be ionized at pH ~12 [58]. Their counterparts in the γ-subunit are γThr112 and γTrp130 respectively, and the anticipated effect would be reduced destabilization at the $\alpha_1\gamma_1$ contacts by ionization and attraction of water into the non-polar region at higher pH values. From the analysis of the natural mutant Hb Kenya – a γβ fusion hybrid where the sequence up to at least residue 81 is γ, and the latter part is β – it appears that the alkali denaturation property indeed is mediated by the residues located after the 86th position as this mutant displayed an alkali denaturation resistance similar to HbA [59]. However, a recombinant mutant of HbF with point mutations at the anticipated responsible residues, γThr112 → Cys and γTrp130 → Tyr, did not show a decrease in alkali denaturation resistance and remained equally

resistant as the unmodified HbF [60], indicating that these two specific residues are not responsible for this difference as initially proposed. Another recombinant mutant HbF was created, this time for examining the role of the 18 residues long N-terminal sequence containing 8 substitutions between the β-subunit and the γ-subunit. It was found that this mutant had a slightly lower alkali resistance compared to HbF, indicating that the N-terminal and A-helix contribute to some extent to the alkali resistance of HbF [61]. Overall, the mechanism appears thus to be governed by complex interactions involving several regions of the γ-subunit.

Another difference between HbA and HbF is the reported 70-fold lower tetramer-dimer dissociation constant of HbF. Five residues differ in the α/β or the α/γ interfaces between the subunits, at positions 43, 51, 112, 116, and 125. A recombinant HbA β-subunit mutant with the γ-subunit equivalent residues in these five positions did show a lower dissociation constant, but not as low as HbF, indicating that other residues are involved to fully account for this property [62]. Another recombinant HbA mutant where first 18 residues of the N-terminal sequence for the A-helix was exchanged for the γ-subunit sequence showed even more decreased dissociation constant, close to that of HbF [63], and it was suggested that γGly1, γGlu5, and γAsp7 were the major contributing residues [64]. However, the inverse corresponding recombinant HbF mutant mentioned earlier (with 8 β-subunit substitutions in the N-terminal γ-subunit) showed that substituting the resides in the N-terminal region into the β-subunit equivalent residues did not increase the dissociation constant markedly in HbF [61]. It does however seem that γGly1 is quite important for the tetramer-dimer dissociation constant since it was found that acetylation of this residue causes HbF to have a similar dissociation constant as HbA [65]. Complex interactions between different regions appear to contribute to this favorable attribute of HbF as well.

It can be concluded that the 39 residue dissimilarities between the β-subunit and the γ-subunit give rise to some interesting property differences that are not clear-cut to explain. The level of HbF present is significant in the pathophysiology of several hematological diseases and can be used for diagnosis, prognosis, and treatment. For some diseases, medical therapy includes inducing upregulation of the γ-gene to promote the production of HbF in patients and has shown potential to treat some severe hemoglobinopathies, such as sickle cell anemia and β-thalassemia [66, 67].

Hemoglobin mutations

Numerous mutations in human Hb have been found during the years of Hb research. Some of these mutations have been shown to give rise to severe hematological diseases, while some go by unnoticed without clinical implications.

One of the most well-known mutations is the single point mutation causing sickle cell disease, and this mutation causes the negatively charged glutamic acid at position 6 in the polypeptide chain of the β-subunit to be replaced by a hydrophobic valine residue (βE6V). This mutant Hb is called HbS. The critical effect of this mutation is that the valine provides a surface-exposed hydrophobic patch in the T-state configuration that enables interaction with neighboring Hb molecules. When HbS associates with the surrounding Hb molecules it will initiate aggregation and create fibers. As these Hb fibers form in the RBC, the flexibility of the RBC is lost and the cell will transform into irregular shapes, such as a sickle-like shape, which has given the name of the disease. The misshapen RBCs clog capillaries and disrupt blood flow, causing poor circulation, swelling, and risk of stroke. These irregular RBCs also have a shorter life span in circulation which contributes to an anemic state of the patient.

Another Hb-related disease is thalassemia. Thalassemia is the name for a set of diseases, caused by irregular expression of the hemoglobin chains. α- and β-thalassemia refers to the reduced or absent expression of the α-subunit or the β-subunit, respectively. Many different genetic variations have been associated with thalassemia, and the severity of the disease varies with what type of mutation is causing it and which subunit is affected. Poor expression of the α-subunit will for example cause increased formation of β_4-tetramers and dysfunctional oxygen unloading due to loss of cooperativity and increased oxygen affinity, while reduced production of the β-subunit will cause deficient maturation and loss of RBCs due to aggregation of α-subunits, causing anemia.

When discovering a new natural mutation of Hb, the standard procedure for naming the mutant Hb is by the city or region where the mutant Hb was found. Some examples are Hb J Paris I (αA12D), Hb Kenya (γβ fusion hybrid), Hb Stockholm (βE7D), and Hb Malmö (βH97Q). Some of the most clinically relevant variants are named with just a letter, e.g. HbS, HbC (βE6K), HbE (βE26K). The reported mutations are collected in a database called HbVar – A Database of Human Hemoglobin Variants and Thalassemias [68]. At present, 1834 entries are collected in the database (Table 1). Far from every mutation in the Hb genes cause a negative consequence that leads to a diseased state of the person carrying the mutation, as is evident by the number of registered mutations found in this important database. Some mutation sites are more critical than others to the Hb functionality, and from a protein engineering perspective, this database provides a way to evaluate potential mutations for protein engineering strategies to an extent that is not yet possible for any other human protein. The Hb analysis performed in hospitals and laboratories all over the world for screening purposes, as well as the efforts of the scientific community to publish and collect information, enable this great resource.

Table 1: Entries in the HbVar database as of April 2021 (https://globin.bx.psu.edu/cgi-bin/hbvar/counter).

Category	Number of entries
Total entries in database	1834
Total hemoglobin variant entries	1396
Total thalassemia entries	534
Total entries in both variant and thalassemia categories	69
Variants with compound variants	312
Entries involving the alpha1 gene	363
Entries involving the alpha2 gene	456
Entries involving the beta gene	942
Entries involving the delta gene	138
Entries involving the Agamma gene	63
Entries involving the Ggamma gene	77
Entries with an insertion mutation	87
Entries with a deletion mutation	239
Entries with a fusion gene mutation	11
Entries with a substitution mutation	1481
Hemoglobins with high oxygen affinity	101
Hemoglobins with low oxygen affinity	48
Unstable hemoglobins	153
Methemoglobins	13

3 Protein engineering of hemoglobin

Protein engineering has emerged as an exceptional tool to investigate the properties of Hb and protein functions in general. Since the first successful recombinant production of tetrameric Hb in microorganisms [69, 70], several research groups and companies have explored genetic modification of Hb.

The first genetically modified Hb by recombinant means was achieved by Looker et al., who constructed a plasmid vector harboring two copies of the α-globin gene fused by a one glycine linker, creating an αα complex of 283 amino acid residues, and expressed in tandem with one copy of the β-globin gene carrying the β108 Asn→Lys mutation (Hb Presbyterian) [71]. Since then, several research groups have been exploring genetically modified Hbs to find mutations to enhance the suitability of Hb for HBOC products. Advances such as tailor-made oxygen affinity, modified NO scavenging, additions of reactive groups to attach other molecules or crosslinking subunits, etc., are all appealing examples of the power of rational protein engineering. A review article by Varnado et al. summarizes the development of recombinant HBOCs up until 2013 [13], and the recent paper by Olson presents a summary of 50 years of protein engineering work on Mb and Hb [17].

Hemoglobin building blocks – amino acids and their side-chains

Proteins are made up of 20 building blocks called amino acids. As proteins are translated, the amino acids are linked by peptide bonds and form polypeptide chains that fold into specific structures that make up the functional protein. At the beginning of a polypeptide chain there is a terminal amino group and at the end a terminal carboxyl group, both of which are ionizable with pKa 7.7 ± 0.5 and 3.3 ± 0.8 [72], respectively. The 20 different side-chains vary in size, charge, polarity, reactivity, and shape, and provide the proteins with their vast versatility [73].

3.1 Introducing mutations in hemoglobin

Mutations of human Hb happens spontaneously and randomly through DNA replication processes, as seen in the great number of entries in the HbVar database (http://globin.bx.psu.edu/hbvar). The probability of erroneous DNA replication is generally very low, but this small plasticity in the DNA replication process enables evolution. The mutations sometimes lead to genetic diseases, and sometimes to the enhancement of favorable traits. Cracking the code of DNA, genes, and phenotypes have led to great biomedical and technological advances in many fields. The discovery of the single adenine to thymine nucleotide base change in the sixth codon of the DNA sequence of the β-subunit gene [74] causing the amino acid substitution from glutamic acid to valine at β6 in HbS [75], resulting in patients suffering from

sickle cell disease, provides an exemplary illustration of the relationship between genotype and phenotype. For researchers, specific mutations of interest can be performed with recombinant techniques in the laboratory by straightforward means. The gene coding for Hb can easily be modified to a coding sequence with the desired site-specific mutations. The modified gene can then be combined into host organisms through transformation and be expressed under controlled conditions (Figure 5).

Bacteria have, aside from the genetic DNA, small circular DNA fragments called plasmids. These plasmids are completely separate from the genetic DNA and can be transferred between different bacterial cells. This has been shown to provide bacteria with quick adaptation by sharing environmental survival genes such as antibiotic resistance encoded by plasmids. This has become a growing problem in hospitals as antibiotics steadily are rendered ineffectual to treat bacterial infections [76]. However, these plasmids are also an efficient tool for recombinant expression technology. They are essentially transferrable coding elements that can be easily modified and inserted into bacteria for the expression of any gene from any species. The incorporation of antibiotic resistance genes applies selective pressure to ensure the survival of the bacterial cells harboring the plasmid during cultivation and expression in a controlled environment.

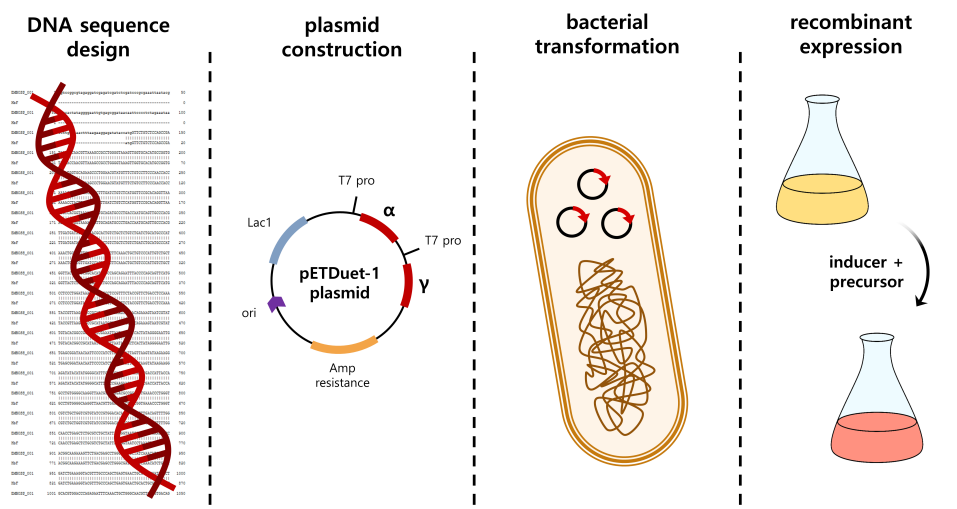


Figure 5: Simplified scheme of cloning procedures and expression of HbF in E. coli *cells for recombinant production.*

Protein surface of hemoglobin

Hb has been evolved to perform its task sequestered inside the RBCs and is adapted for this particular intracellular environment. Viewing the surface of Hb (Figure 6), it is clear that it presents a mosaic-like property map, neither particularly hydrophobic/hydrophilic nor dominantly positively/negatively charged. However, the γ-subunit does appear to have more negatively charged surface-exposed residues than the α-subunit, as visualized by the red coloring. Calculation of the theoretic isoelectric point (pI) by the Expasy Compute pI/MW-tool [77, 78] for the different subunits also suggests that the pI of the γ-subunit (6.64) is lower than the α-subunit (8.72).

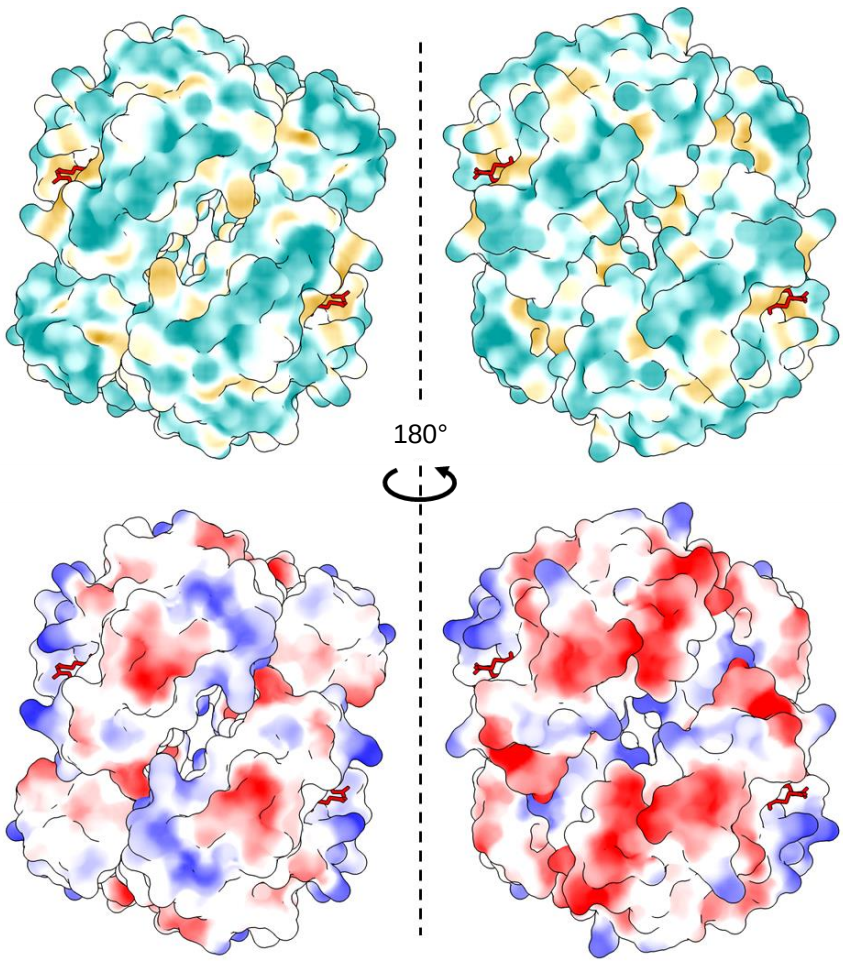


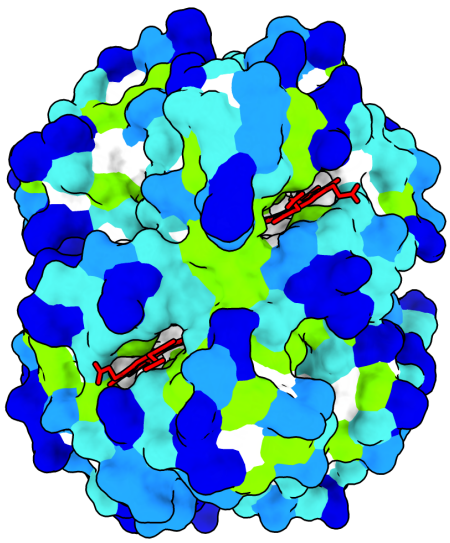
Figure 6: Hydrophobic and electrostatic surface maps of HbF using PDB entry 1FDH as 3D model (hydrophobic/hydrophilic – dark gold/dark cyan, positive/negative – blue/red). The structures on the left side are turned to show the α-subunits, while the structures on the right side are turned 180° to show the γ-subunits.

Considering the possibilities of engineering the Hb surface, examples of supercharging proteins towards extensive positive or negative net surface charge have demonstrated generous plasticity of protein surfaces while still retaining protein function [79, 80]. Important biomolecules such as nucleic acids and cellular membranes display a negative net charge, and proteins, peptides, and particles with a positive net surface charge or positively charged patches promote the association to cell surfaces and cellular uptake [81]. Additionally, the soluble expression of proteins appears to correlate with the lack of positively charged surfaces [82]. General strategies of improving recombinant protein production in *E. coli* have shown that adaptation of the protein surface to fit the cytosolic electrostatic environment of the host cell could significantly improve mobility of the expressed proteins inside the cells, indicating less unwanted interactions [83]. Genetic modification of the protein surface charge has been explored in various enzymes for biocatalytic purposes [84], but this strategy has not been pursued in recombinant Hb adaptation towards HBOC development. Taking into account the correlation between increased net surface charge and concentration of myoglobin in tissues of deep-diving mammals [85], and the stability of these myoglobin variants in correlation to expression yields in *E. coli* [86], it could be worth exploring the mutational plasticity of HbF's exposed surface residues. Engineering surface-exposed residues on the subunits of Hb could potentially produce a more stable protein capable of better yields in recombinant hosts and may be able to alter interactions both within the host cell and in the vascular compartment. As the subunits still must be able to readily combine into tetramers to retain Hb functionality as a cooperative oxygen carrier, the choice of mutation sites for such a strategy should avoid key organizational and functional residues.

An inspection of the surface-exposed residues in HbF using the molecular graphics program UCSF ChimeraX [2] reveals that the α- and γ-subunits have 53 residues each with > 50 $Å^2$ area exposed on the surface of the tetramer. Table 2 lists the number of residues with the largest calculated surface-exposed area. From this inspection, it can be seen that the majority of the charged residues have positively charged side-chains at physiological pH.

Table 2: The number of surface-exposed residues of HbF as calculated by the ChimeraX program [2] using PDB entry 4MQJ. The color-coded structure show range of surface exposure: Area >25 Å² (green) >50 Å² (turquoise) >75 Å² (blue) >100 Å² (dark blue).

	Solvent accessible surface area									
	>25 Å²		**>50 Å²**		**>75 Å²**		**>100 Å²**		**Residues with >100 Å² (dark blue)**	
	α	**γ**	**α**	**γ**	**α**	**γ**	**α**	**γ**	**α**	**γ**
Total residues	74	71	53	53	30	33	16	21	1 Val	2 His
Positive	19	19	17	17	14	13	10	12	4 Pro	5 Glu
Negative	10	13	7	13	3	12	1	6	11 Lys	6 Glu
									16 Lys	17 Lys
									19 Ala	22 Asp
									44 Pro	43 Asp
									45 His	49 Ser
									50 His	58 Pro
									56 Lys	59 Lys
									60 Lys	61 Lys
									61 Lys	66 Lys
									74 Asp	76 Lys
									78 Asn	77 His
									89 His	79 Asp
									90 Lys	82 Lys
									141 Arg	87 Gln
										95 Lys
										117 His
										120 Lys
										121 Glu
										144 Arg



In this work, **papers II**, **III**, and **IV** focus on genetic modification of surface-exposed residues to add negatively charged side-chains on HbF. We confirmed the impact of the mutations on pI by isoelectric focusing (IEF) and observed corresponding changes during production that will be addressed further, e.g. the adaptation of the liquid chromatography purification protocols.

3.2 Interactions and reactions of hemoglobin

Although Hb's primary function is oxygen transport, this protein has the potential to bind to and react with several other physiologically relevant biomolecules. Heme-derived reactions such as autoxidation, NO binding, and peroxidase activity, are some examples of reactions that are highly relevant to the physiological presence of Hb.

Unliganded and liganded Hb states produce specific spectra, as shown in Figure 7. Depending on the oxidation state of the iron and the bound ligand, the Hb protein displays distinct and characteristic absorbance peaks. The planar ring structure of conjugated π-electrons in the porphyrin ring structure of heme absorbs strongly in the violet region around 400 nm (Soret band), as well as in the visible spectrum with extinction coefficients around ten-times lower (α/β bands) [87].

Ligands of hemoglobin

Oxygen (O_2) is the primary ligand for Hb for respiratory purposes and the cooperative binding abilities have been discussed previously. Other gases such as carbon monoxide (CO) or nitric oxide (NO) can also bind to the central iron atom in heme. The details of the molecular mechanisms behind the binding of diatomic gases in mammalian Mbs and Hbs have been studied by J. Olson with collaborators and consist of at least four distinct phases: 1) hydrogen-bonded H_2O molecules being displaced from the heme pocket on the distal side; 2) gas molecule moving into the space left by H_2O in the distal pocket, close to the sixth position of the heme iron; 3) the iron atom in heme forms a covalent bond with the gas molecule; and 4) electrostatic stabilization of the bound ligand-heme complex by the distal histidine residue, by donation of a hydrogen bond [17]. The third step, the covalent bond formation, is the driving force behind the whole process and the chemistry of this bond is the main determinant of the ligand affinity.

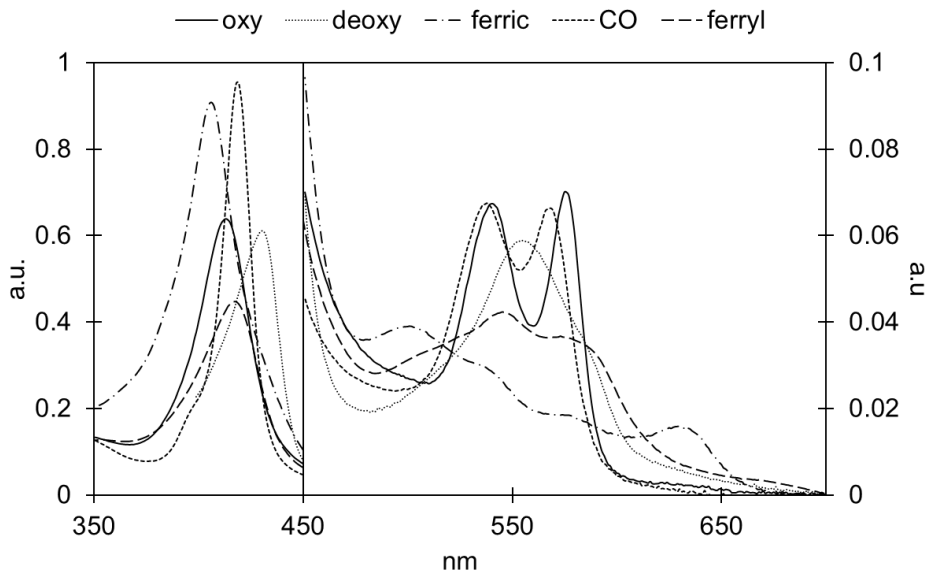


Figure 7: Optical spectra of HbF showing the unliganded (deoxy), liganded (oxy and CO), and oxidized, ferric (Fe^{3+}) and ferryl (Fe^{4+}), states. The absorbance peaks show characteristic differences between the different heme states.

Carbon monoxide (CO) is extremely toxic to respiratory organisms relying on oxygen delivery by Hb. Human Hb binds CO with about 250-fold higher affinity than O_2, which results in CO competitively displacing O_2 from heme if inhaled, and exposure to this gas will effectively impair oxygen delivery altogether and have fatal consequences. Treatment of CO poisoning is tricky because of the high affinity of Hb with CO compared to O_2, and medical efforts mostly involve treating the patient with pure O_2 gas and hope that CO is released from Hb in time. Another, more efficient way to remove CO from Hb is by exposure to strong light. Photodissociation will more easily break the bond between heme-CO than heme-O_2, and this would potentially be efficient to use in a critical setting. It is however complicated to standardize this type of treatment and quickly expose the Hb in circulation to strong light. Efforts on developing a so-called “CO photo-remover”-device are nonetheless underway and could potentially be of use in the future medical treatment of CO intoxication [29, 88]. On the other hand, in terms of stabilizing Hb, CO gas is convenient to use in the recombinant preparation of human Hb solutions. After producing Hb in a recombinant host the protein typically needs to be extracted through several purification processes. As Hb is prone to spontaneous autoxidation causing loss of function and subsequent deterioration, using CO gas to stabilize Hb during downstream processing efficiently halts heme-

associated oxidative processes. CO-binding to Hb also increase the protein's resistance to thermal denaturation, as can be seen in Figure 8.

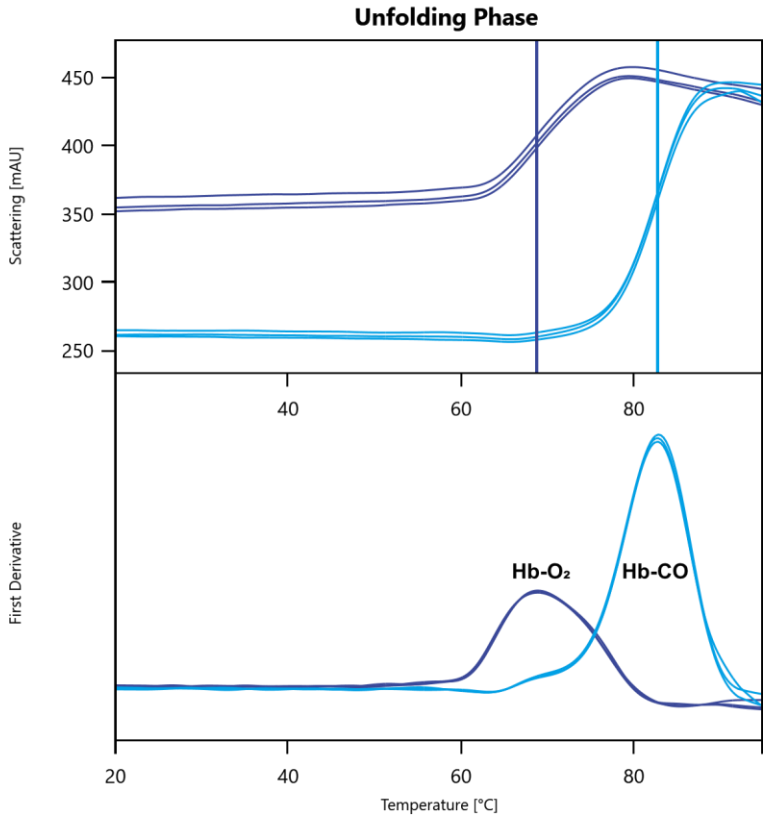


Figure 8: Thermal denaturation of HbF bound to O_2 (dark blue) or CO (light blue) measured in a Prometheus NT.48 instrument (Nano Temper Technologies) using a temperature gradient from 20-95 °C at 7 °C/min. The upper curves show the scattering data and the lower curves are the first derivatives of the collected data.

Nitric oxide (NO) also binds to heme in Hb with very high affinity, 1000-fold greater than CO. In contrast to O_2 and CO, NO can bind to Hb in both the ferrous and ferric forms. NO is a free radical endogenously synthesized in the body and is essential as a signaling molecule and regulator of vascular tension, acting as a vasodilator. Because of the high affinity for NO, free Hb can scavenge NO from endothelial cells and disrupt the NO-derived regulation, directly contributing to vascular dysfunction [89, 29]. The strength of affinity amongst the three above-mentioned ligands, NO > CO > O_2, is found to be consistent in many penta-coordinated heme proteins [90]. There is a chance of NO binding to the cysteine at β93, forming an SNO-Hb. This is only possible if either NO^+ or a thiol radical is present [91].

Redox reactions of hemoglobin

Autoxidation and hydrogen peroxide

Through the interactions of Hb with oxygen, the iron in the heme spontaneously oxidizes from the ferrous Fe^{2+} to the ferric Fe^{3+}, forming methemoglobin (metHb). When this happens, the heme can no longer bind O_2 and Hb becomes non-functional as an oxygen carrier. Inside the RBCs, there are antioxidant systems in place to maintain Hb in the useful ferrous form, such as methemoglobin reductase, which is an NADH-dependent enzyme that reduces metHb back to functional ferrous Hb [92]. A byproduct of the spontaneous autoxidation of heme is the formation of a superoxide radical ($O^{\bullet}_2{}^{-}$) [93]. This radical can be neutralized by the enzyme superoxide dismutase (SOD) by forming hydrogen peroxide, H_2O_2. H_2O_2 is also a highly reactive compound but can be converted into H_2O and O_2 by the enzymes catalase, glutathione peroxidases, and peroxiredoxin [15]. It has been observed that autoxidation rates are lowered in the presence of SOD and catalase, as these work together to remove superoxide and H_2O_2 [94]. Other ROS scavengers in the RBCs are glutathione, ascorbic acid, and vitamin E [15]. If either $O^{\bullet}_2{}^{-}$ or H_2O_2 are not neutralized through antioxidant actions, they will go on and react with Hb or surrounding biomolecules to form other harmful reactive species. If H_2O_2 is allowed to react with ferrous Hb or metHb, unstable ferryl Hb (Fe^{4+}) or oxoferryl Hb will form. These ferryl species of Hb are highly oxidizing and have been shown to cause cellular and tissue damage [15]. The ferryl Hb species can convert back to ferric Hb by reacting with H_2O_2, acting as a pseudoperoxidase. As all these highly reactive species are produced through the redox reactions of Hb, unless kept under control by a reducing environment, the protein will break down from radicals forming in the heme pocket and propagating through the protein [7, 95, 96]. Figure 9 depicts a scheme illustrating the redox reactions of Hb and H_2O_2. When the heme is released from Hb in the degradation process, the subunits of Hb will collapse. The characteristic spectra of the oxidized states of HbF are displayed in Figure 7 together with ligand-bound spectra.

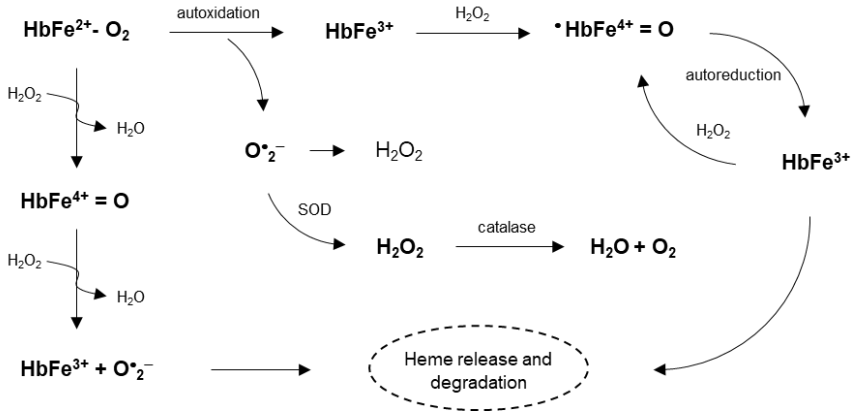


Figure 9: Scheme of the redox reactions of human Hb and hydrogen peroxide. Adapted from Mollan & Alayash [14]. Ferrous O2-bound Hb spontaneously autoxidizes to ferric Hb and generates a superoxide radical as a byproduct. In presence of enzymes SOD and catalase, the reactive radical is neutralized into water and O_2. If hydrogen peroxide is present or formed as a by-product both ferrous and ferric Hb species can oxidize into reactive ferryl forms of Hb, leading to damage to the protein and surrounding biomolecules and tissues.

There are a few primary radical formation sites in Hb, also known as *oxidative hotspots*, as a result of the reaction between H_2O_2 and Hb. Tyrosine, cysteine, histidine, tryptophan, and methionine residues are prone to harbor radicals and/or oxidize during such conditions, as shown by EPR and mass spectrometric data [97, 98]. Examples of residues susceptible to radicals and modifications induced by oxidation within the HbA molecules are βCys93, βTrp15, βTyr35, βMet55, βTyr130, βTyr145, and αHis20, αTyr24, and αTyr42 [99]. βC93 and βC112 are extensively oxidized during H_2O_2 oxidation and appear to be key residues participating in a programmed oxidative process of the β-subunit [99]. βCys93 has also been shown to be important in the role as a superoxide scavenger by capturing the superoxide radical formed in the heme pocket during oxidation from $HbFe^{2+}$-O_2 to ferric $HbFe^{3+}$ by combining and forming a thiyl radical [100]. This ability of βCys93 to act as a protective scavenger is important for the oxidative stability of Hb. Another interesting feature of the redox active residues is that they permit radicals/electrons to migrate through the protein in so-called electron pathways, which can protect the heme from oxidative damage [101, 102], and also provide enhanced susceptibility to reducing agents [103]. Reeder et al. demonstrated the capacity of tyrosine residues to enable electron flow pathways in a model Mb [104], and the potential advantage of this ability was examined in a recombinant Hb for HBOC development in a paper by Cooper et al. [103]. The protective trait of electron pathways leading radicals away from the active site through redox active residues has also been shown in other heme-containing proteins such as cytochrome c peroxidase [102].

In **paper I**, oxidative reactions in recombinant HbF (rHbF) were investigated in three mutants harboring cysteine-related substitutions and compared to wild-type (wt) rHbF. Two single mutants and the corresponding double mutant were created to examine the impact of adding a cysteine residue on the surface of the α-subunit (αA19C) and removing the conserved cysteine in the γ-subunit (γC93A). As this cysteine was replaced by alanine we observed increased autoxidation rates, increased H_2O_2-induced rates, and increased heme release from the γ-subunit. The results indicated that the conserved γCys93 had a similar role as the βCys93 in HbA, probably as a scavenger of superoxide formed in the heme pocket during oxidation. On the other hand, adding a cysteine in place of an alanine residue on the surface of the protein at position α19 showed a decreased rate of autoxidation, decreased H_2O_2-induced oxidation rate, and reduced heme release from the γ-subunit. Quantitative mass spectrometry was used to examine the irreversible oxidation of hotspot residues after extended exposure to excess H_2O_2, and the results showed that the added cysteine on the α-subunit could act as a new alternative hotspot, lowering irreversible oxidation at γCys93 when present together on the protein, or displaying significantly increased oxidation when γCys93 was mutated to alanine and no longer could act as the primary oxidation hotspot target. Similar to the results obtained by Silkstone et al. [101], the addition of a new electron pathway mediated by a reactive side-chain on the surface of the Hb molecule significantly affected the oxidation pattern of Hb.

An aspect of using cysteine instead of for example tyrosine to provide reactive side-chains is cysteine's ability to form disulfide bridges through its thiol group. During liquid chromatography purification of the mutants in **paper I**, it became evident that the mutants harboring the αA19C mutation sometimes exhibited an extra elution peak (Figure 10). This peak disappeared after the addition of 1,4-dithiothreitol (DTT), indicating that the peak was formed by Hb bound together by disulfide bonds that could be broken with DTT. Thus, the αA19C mutation appeared to enable a fraction of the mutant Hbs to spontaneously form disulfide bridges. This could potentially be utilized for specific design involving surface-exposed cysteine residues, as has been demonstrated by Brillet et al., based on a reported natural variant β83 Gly→Cys, which spontaneously induces a stable octamer formation by two inter-tetramer disulfide bonds [105]. Another example is a recombinant Hb variant with human α-subunit in combination with bovine β-subunit carrying the Hb Porto Alegre mutation (βS9C) [106], which have been used to formulate a potential HBOC called Hb Polytaur [107, 108]. Interestingly, reptilian Hbs often contain a high number of cysteine residues, and it was demonstrated by Petersen et al. that these reptilian HbA molecules are prone to polymerize without affecting the normal functionality of the protein and appear to contribute to antioxidant capabilities

[109]. Moreover, the presence of surface-exposed cysteine was examined in a study comparing two variants of mouse Hb – one with two cysteine residues in the β-chain, βCys13 and βCys93, and the other with only the single cysteine at βCys93 – and this study reported that the presence of the extra βCys13 lowered metHb formation during H_2O_2 exposure, demonstrating the protective effect during oxidative conditions [110].

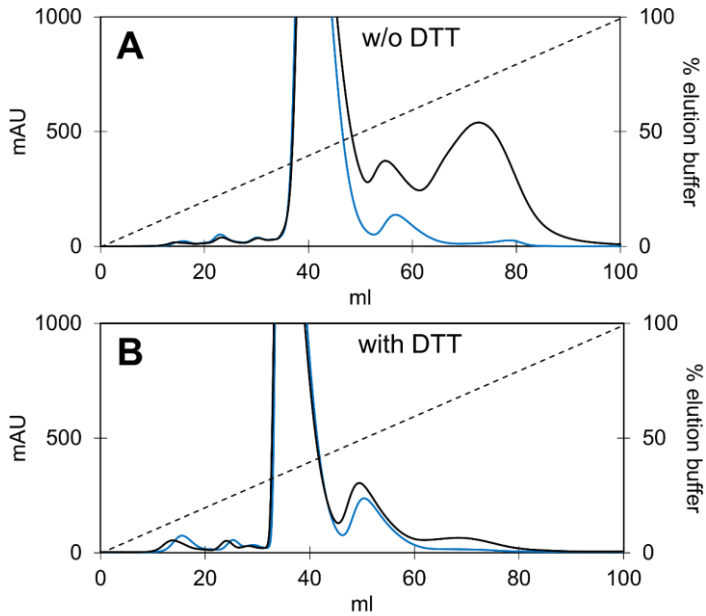


Figure 10: Example of the rHbF αA19C mutant elution step chromatograms when applied to a 5 ml Q HP column without (A) or with (B) addition of DTT. Black line: 419 nm UV trace, blue line: 280 nm UV trace, and dashed line: elution buffer concentration. An additional Hb peak is seen when DTT is not added to the mutant Hb sample before applying to the column.

Another feature of placing cysteine residues on the surface of the Hb molecule is enabling a surface-exposed handle that could be used to link other molecules to Hb, given that the placement of the cysteine ensures minimal structural perturbation. A recent paper by Cooper et al. [111] describes mutants of HbA using the αA19C mutation, or a similarly placed β-subunit substitution βA13C, both in combination with βC93A substitution, to specifically couple a PEG-molecule in these locations. Their results show no effect on neither oxygen affinity nor cooperativity, indicating negligible structural disruption. Additionally, when the mutants were PEGylated at these sites, autoxidation and heme loss rates were decreased. Other biomolecules apart from PEG could be considered to be coupled to Hb at this type of surface-exposed binding site, for example, other proteins (albumin, enzymes), lipids, or functional peptides.

DNA cleavage

An interesting feature of the mammalian RBC is the erythroid enucleation. During the RBC maturation process, the Hb concentration gradually increases and DNA and organelles are excluded from the cells [112]. The evolutionary advantage of this complicated process is not completely clear, but it may be beneficial for rheological purposes and enable the necessary flexibility of RBC to adapt maximum surface exposure in narrow capillaries for increased oxygen delivery. In contrast, the circulating RBCs in birds, reptiles, amphibians, and fish still contain the nucleus and organelles excluded in the mammalian RBC maturation process [113]. Out of these non-mammalian vertebrates, only birds are endothermic, like mammals, but they have evolved a smaller RBC size in combination with a higher Hb content, which contributes to the oxygen delivery capacity for sustaining constant body temperature [113]. Although, a few species of fish, like tuna and shark, do have regional endothermy [114], and recently the opah (*Lampris guttatus*) was the first species of fish shown to maintain endothermy in the whole body, with an average of five degrees higher body temperature compared to the surroundings [115]. One aspect to contemplate when considering the intracellular environment of the RBC or other host cells is Hb's ability to damage DNA. Experiments with purified DNA in solution with Hb show that DNA will readily be cleaved by Hb [18]. The exact mechanism of DNA cleavage by Hb is not fully known, but the damaging activity appears to be associated with ROS, and Hb easily forms oxygen and hydroxyl radicals that are very reactive to nucleic acids [116]. Although, physical interaction between the DNA and Hb appears to be important as well, and interestingly, HbF appears to cleave DNA at a slower rate than HbA, suggesting a difference between these two proteins [18]. Studies have also shown that the presence of surface-exposed residues with the ability to carry protein-based radicals, e.g. βTyr145 [117] and αCys19 [18], increases the rate of DNA cleavage.

Docking DNA to HbF using the HDock server [118] suggested that DNA preferentially docks in the vicinity of the heme pockets (Figure 11). Positively charged lysine residues are located close to the heme pocket entrance and may contribute to electrostatic attraction of the negatively charged phosphate backbone of DNA. The importance of electrostatic interaction to mediate the nicking damage was supported by the finding that the presence of high salt concentration inhibits the DNA cleavage activity of Hb [18]. In the study by Lawrence et al. [79], describing supercharged proteins, the positively charged green fluorescent protein (GFP) variant was reported to co-precipitate with RNA and DNA, while the addition of NaCl reversed the interactions; in contrast, the negatively charged GFP variant did not precipitate in the presence of nucleic acids. Thus, increasing the negative net charge on protein surfaces appear to reduce interactions with DNA due to the

repelling electrostatic forces. Besides the electrostatic attraction, the shape at the sites of interaction may be important as well, as the DNA molecule docking changes the angle between the T-state (Figure 11 panels A-C) and R-state of the HbF (Figure 11 panels D-F). There are several known and characterized DNA-binding motifs in proteins [119], and in the case of Hb, the prevalence of α-helical segments may present favorable protruding shapes which might fit into the major groove of a DNA helix and enable close interactions.

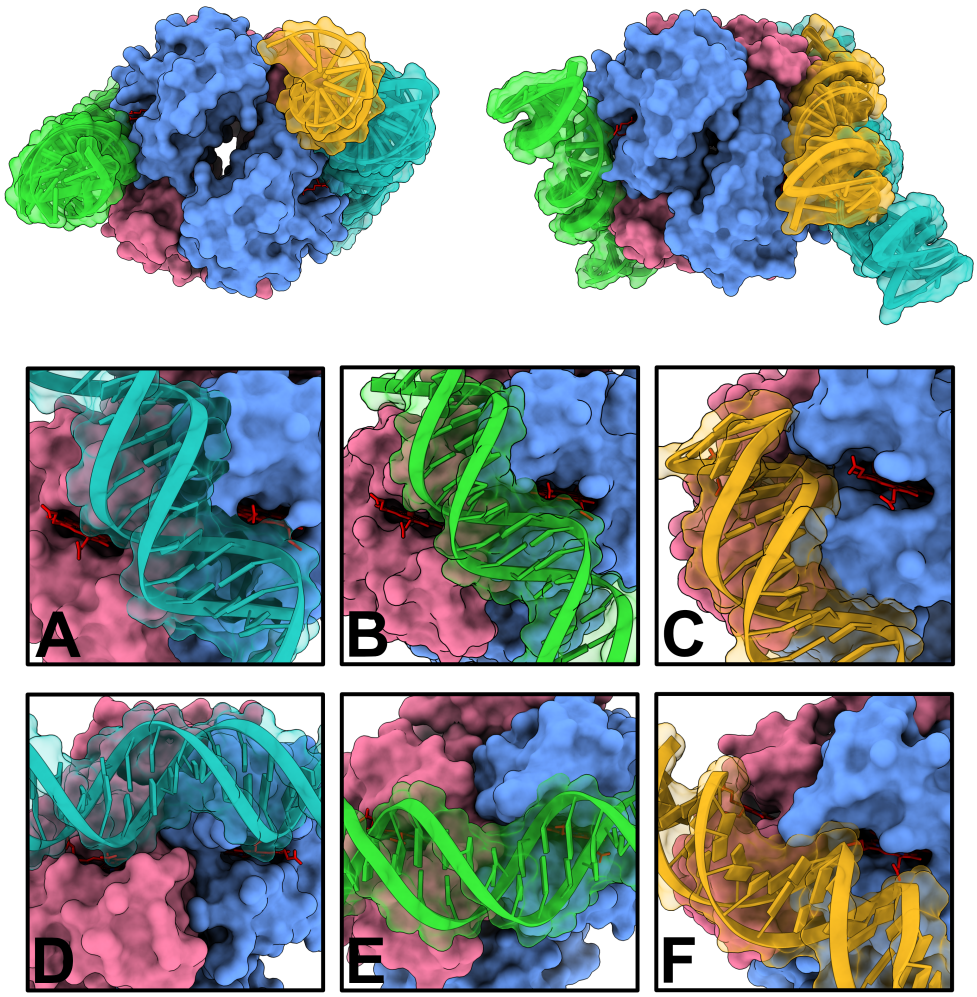


Figure 11:DNA-HbF docking with the HDock server [118]. The HbF crystal structures (left: PDB entry 1FDH, right: PDB entry 4MQJ) are shown with blue α-subunits and pink γ-subunits, and the DNA is colored differently for the distinction of the modeled docking sites (turquoise - best score, green - second, yellow - third). The A-F panels show close-up figures of the docking, where A-C is with 1FDH and D-F is with 4MQJ. The 20 highest scoring docking results all modeled DNA in close vicinity of the heme pockets.

Considering the prokaryote *E. coli* as the recombinant host for Hb production, the genomic DNA is not separately stored within a nucleus as it is in eukaryotic cells. Speculatively, achieving high concentrations of human Hb within *E. coli* cells during recombinant production may result in DNA interaction and/or damage that could affect expression. In **papers II** and **IV** the DNA cleavage activity was assessed by incubating HbF variants with purified pUC18 plasmid, which enable monitoring of single-stranded cuts in a simple and sensitive way. Figure 12 shows the main plasmid DNA (pDNA) structures that can be separated by agarose gel electrophoresis – supercoiled (sc) pDNA, open circular (oc) pDNA, and linear pDNA. In **paper II**, mutants with alanine replaced for aspartate residues in the α-subunit that provide additional negative charges on the surface of the HbF molecule were examined. It was observed that the plasmid DNA cleavage rate decreased with an increasing number of negative charges, and this was again observed in **paper IV**, with another mutant of HbF that had four extra surface-exposed negative charges. The rHbFα4 mutant in **paper IV** displayed a 4-times lower DNA decay constant ($\mu M^{-1}h^{-1}$) as determined by plotting the supercoiled pUC18 plasmid signal over time, in increasing concentrations of HbF (Figure 13). One of the four mutations on the mutant in **paper IV** is αK90E, which changes a positively charged lysine residue for a negatively charged glutamate in the docking sites found by the DNA-HbF modeling, and may be directly influential in the reduced cleavage rates found in this study. The mutants in **paper II** do not carry their negative mutations in the vicinity of the heme pockets but still influence the DNA cleavage, although to a smaller extent than the mutant in **paper IV**. It appears thus that the increased negative net charge of these mutants contributes to reducing the association to DNA and subsequently a lower rate of nicking the negatively charged phosphate backbone of DNA. The effect may be increased further if a specific site of interaction on the Hb surface could be more accurately identified and the local residues are mutated accordingly, or possibly by designing a Hb molecule with an even greater negative net surface charge.

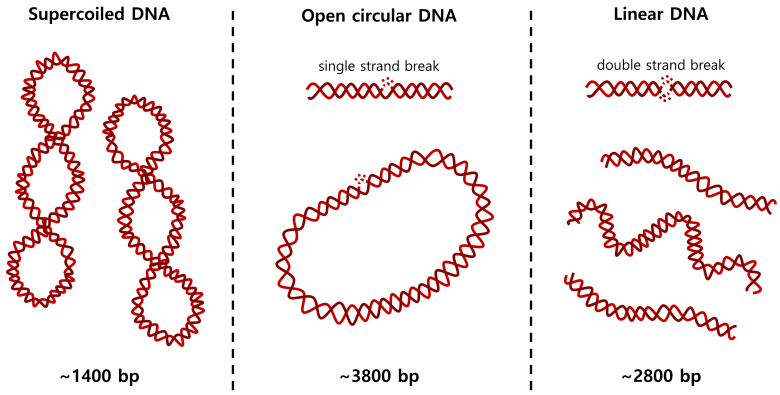


Figure 12: Schematic figure of the pDNA cleavage process. When separated by electrophoresis in an agarose gel, sc pDNA appears smaller than expected due to the tightly wound coil structure. Nicking of one of the DNA strands in the sc pDNA leads to open circular conformation that migrates as a larger than expected fragment. Base pair (bp) size examples correspond to the apparent sizes of the different pDNA conformations of the pUC18 plasmid when separated next to a linear DNA ladder.

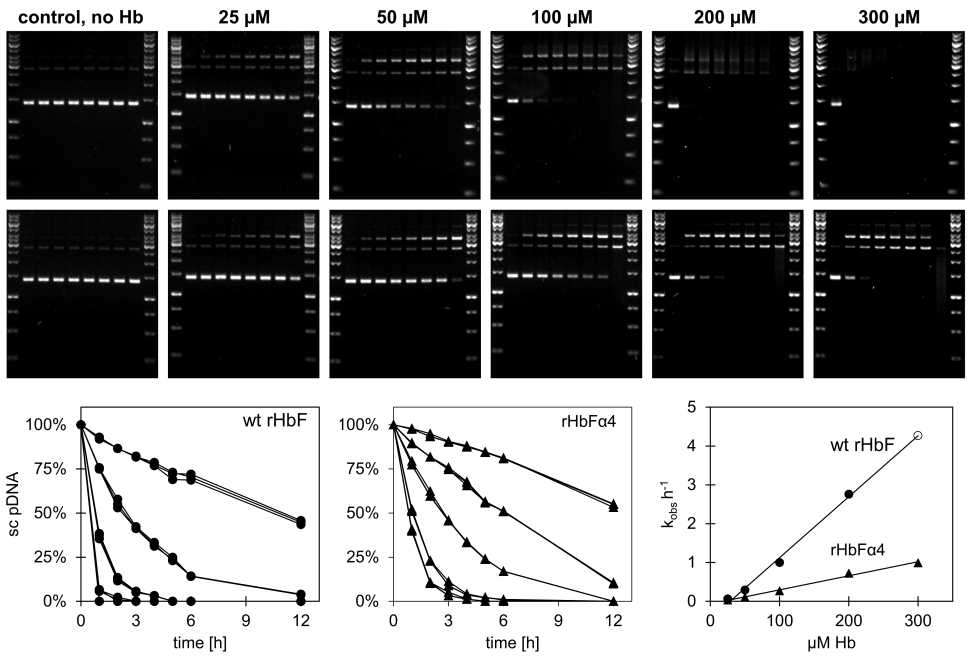


Figure 13: Data from DNA cleavage activity experiment of wt rHbF and the rHbFα4 mutant. The gel pictures above the graphs show wt rHbF (upper row) and rHbFα4 mutant (lower row). In the control samples, no DNA cleavage was detected over time. In the Hb-containing samples, the decay of sc pDNA is noticeable and the oc and linear pDNA bands emerge and disappear as they are also cleaved within the experiment timespan at higher Hb concentrations. The graphs show the signal of the supercoiled band over time up to 12 h. Single exponential decay rates were calculated from the gel data and plotted against Hb concentration. A linear relationship was obtained and used for determining the decay constant of the DNA cleavage activity in the presence of Hb.

Mechanisms of clearance and control of hemoglobin

Human red blood cells have an approximate lifespan of 120 days in circulation before they are destroyed and phagocytized by macrophages present in sinusoids. Sometimes, the RBCs lyse in the intravascular compartment in an event called hemolysis, which leads to leakage of cell-free Hb into the bloodstream. Several different events will unfold when Hb is released in the plasma environment, and the severity of the effect depends on the extent of hemolysis [15]. Extracellular Hb in plasma mediates several oxidative reactions, release heme, and contributes to NO depletion in endothelial cells causing vascular dysfunction as mentioned previously. Clearance pathways have developed to protect the surrounding tissues and control the harmful side effects of decompartmentalization of Hb [120].

Hemolysis is promoted by several pathological conditions, e.g. sickle cell disease, resulting in elevated levels of extracellular Hb. Such elevated levels and the ensuing reactions of extracellular Hb have received more and more interest in the field of HBOC research since many formulations are based on the administration of extracellular Hb solutions [7]. As Hb clearing pathways become overloaded upon administration of HBOC products, the toxic side-effects of Hb in an HBOC product must be carefully controlled [121].

Haptoglobin and plasma heme scavengers

Haptoglobin (Hp) is an abundant plasma protein that binds Hb αβ dimers with high affinity. This Hb scavenger protects from Hb-mediated toxicity at three levels, by 1) seizing the small 32 kDa dimers in a larger protein complex, preventing renal and vascular injury caused by the diffusion of dimers into the surrounding tissues, 2) reducing heme release and iron loss, and 3) altering/controlling oxidative side-reactions by protecting key oxidation-prone residues in Hb, preventing radical transfer to other molecules, and stabilizing the ferryl state. Hp is an acute-phase glycoprotein made up of two polypeptide chains – an α-chain and a β-chain – where the α-chain exists in two variants, resulting in three primary phenotypes – Hp1-1, Hp2-1, and Hp2-2. Hp binds to Hb dimer in the tetramer interface, and the first 3D structure was determined by Andersen et al. with the porcine Hp-Hb complex [122]. Recent reviews summarize the current accumulated knowledge of Hp and its role in Hb/heme metabolism and as a moderator of Hb toxicity [123, 124]. Figure 14 shows size-exclusion chromatograms of HbF and Hp. It can be seen how the 419 nm Hb peak shifts towards a larger molecular complex when mixed with Hp, clearly the capture of both wt rHbF and mutant rHbFα4 by Hp – indicating that the mutations do not affect the binding of this important plasma protein.

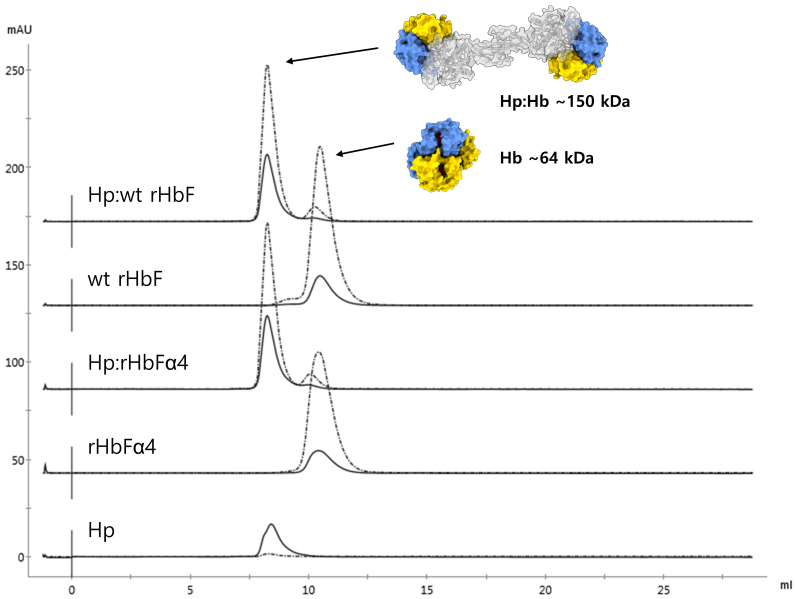


Figure 14: Chromatographic separation of Hp, Hb, and Hp:Hb complexes on a Superdex® 75 10/300 GL column (GE Healthcare) in 50 mM sodium phosphate buffer pH 7.2, supplemented with 150 mM NaCl, at a flow rate of 0.5 ml/min. Solid lines show the 280 nm (general protein indicator) and the dotted lines represent the 419 nm (Hb protein indicator) UV trace. Both wt rHbF and mutant rHbFα4 readily bind to Hp and elute as high molecular complexes after mixing, as seen by the shift of the 419 nm lines. As expected, the chosen mutation sites did not affect the binding of HbF to Hp. The inset PDB 4F4O structure shows the porcine Hp:Hb complex as reported by Andersen et al. [122]. Hp is colored gray while Hb α-subunits are shown in blue and β-subunits in yellow.

After capturing Hb, the Hp-Hb complex associates to the CD163 receptor on macrophages for endocytosis, leading to lysosomal proteolysis of Hb, and heme recycling. This process involves heme oxygenase-1 (HO-1) and produces biliverdin/bilirubin, carbon monoxide, and iron. Hemopexin (Hpx) is another plasma glycoprotein essential to protection against hemolysis due to its role in binding free heme. Other proteins capable of binding heme are albumin, alpha-1-microglobulin, and lipoprotein LDL/HDL. Hpx is the major heme scavenger and the Hpx-heme complex binds to the macrophage receptor CD91 for heme metabolism/recycling [125]. Figure 15 displays a simplified scheme of the most important processes of Hb metabolism processes following RBC lysis.

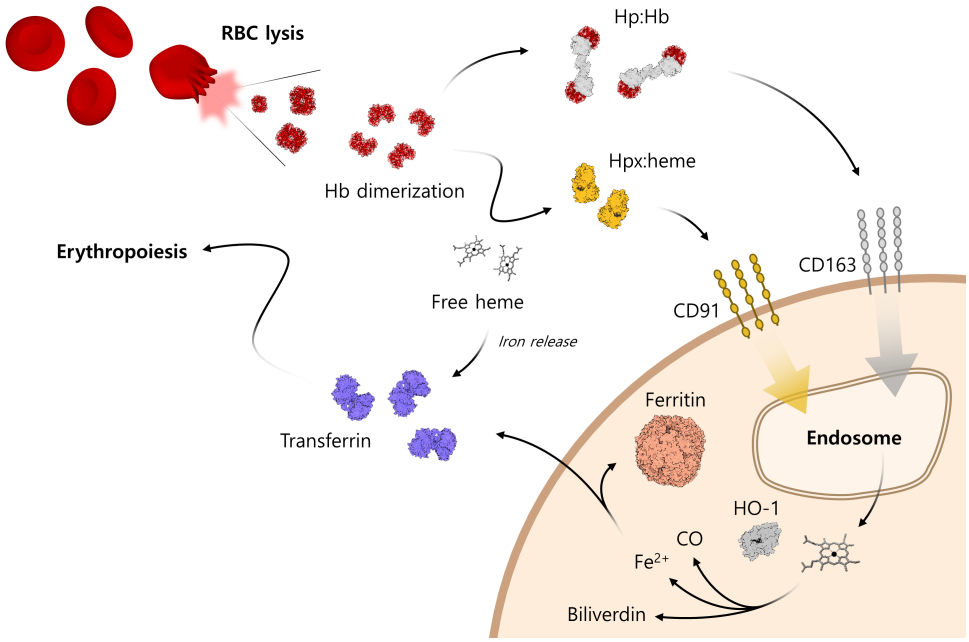


Figure 15: Schematic figure of RBC lysis in plasma and the clearance pathways of Hb and heme. Haptoglobin (Hp) and hemopexin (Hpx) are the main plasma scavengers handling the release of Hb by hemolysis. After capture, the components are transferred into macrophages through CD163 and CD91, and heme and the iron are metabolized/recycled.

If the clearance pathways of cell-free Hb are overwhelmed, for example in the instance of disease- or drug-induced hemolysis, free Hb will be removed from circulation primarily by passing through the renal glomeruli. i.e. by filtration in the kidneys, and excreted in the urine [126]. The dissociation of Hb greatly contributes to a more rapid clearance as the 32 kDa dimer is small enough to more easily pass through the glomerular filters [126]. However, as Hb is filtered through the glomerular barrier it will also associate with the cells and exhibit oxidative side-effects that effectively induce renal damage and leads to proteinuria [127]. Even polymerized high-molecular Hb complexes, designed to prevent dimerization and prolong circulation times of native Hb, induce glomerular dysfunction [128]. This indicates that not only size is determinant of the Hb-induced observed effects on the glomerular filtration barrier. The barrier may be described as a negatively charged fine mesh that besides molecular size also selectively filters molecules based on negative net charge and steric configuration, and thus albumin is selectively retained in circulation while tetrameric Hb, despite having a similar size, is not [129, 126].

Papers II and **IV** report animal experiments using a mouse model to evaluate the plasma half-life of select HbF variants. In the graphs presented in Figure 16, the

results of the different HbF constructs are compiled for comparison. The fusion rHbF [130, 131] and fusion rHbF-XTEN [132, 133] variants are shown to provide additional HbF samples for comparison. The fusion rHbF construct is prone to exist in dimer form and as the calculated half-life of the fusion rHbF, wt rHbF, and rHbFα4 are similar, ~36-38 min, it may be assumed that dimerization greatly contributes to the rapid clearance of these variants, as expected. The double mutant rHbFαA12D/A19D was also cleared rapidly but showed an increased calculated half-life of 47 min, notably, the plasma samples at 6 h and 24 h had higher concentrations of Hb remaining than wt rHbF. In contrast, the fusion rHbF-XTEN construct, carrying a genetically attached unstructured negatively charged polypeptide tail [134], had a half-life of 5.5 h, clearly showing the effects of negative charge and steric configuration on retention time in plasma. The graph displaying albumin concentration in the urine shows that the glomerular filtration was affected and led to higher concentrations of albumin in the urine when Hb was simultaneously detected at significant concentrations in plasma.

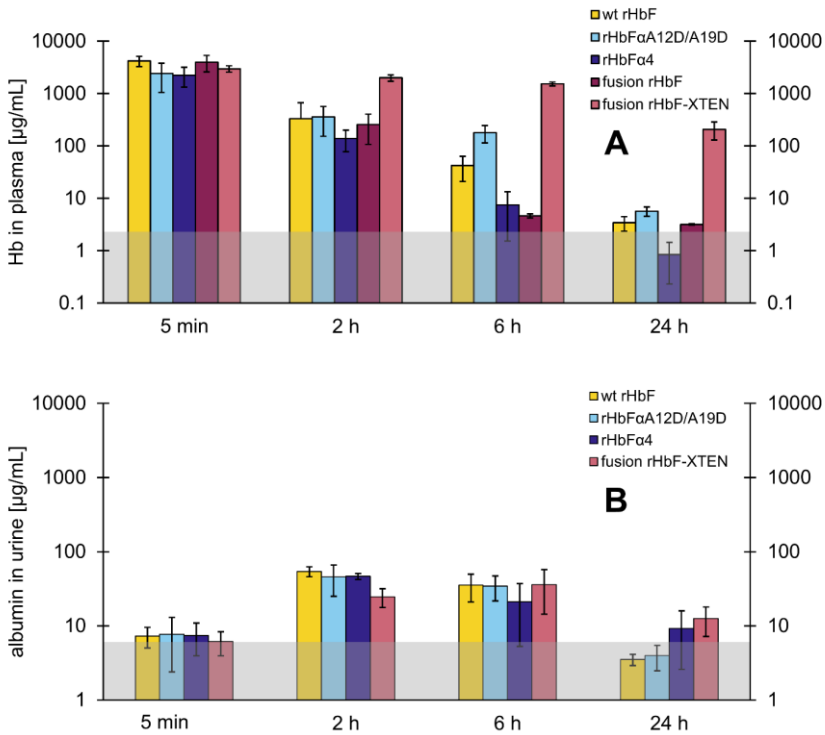


Figure 16: A) The pharmacokinetics of wtHbF, rHbFαA12D/A19D, rHbFα4, fusion rHbF, and fusion rHbF-XTEN were analyzed in plasma samples at four different time-points (5 min, 2 h, 6 h, and 24 h, n = 5 per time point and Hb variant). The Hb concentration was assessed by ELISA with HbF specific antibodies. B) The albumin assay showed that the concentration of albumin in the urine increased after challenge with Hb, and was later reversed as plasma Hb concentration decreased. The grey area in the graphs shows the signal of the non-injected control (n = 5).

4 Production of hemoglobin

4.1 Recombinant production of hemoglobin

Microbial expression of Hb in bacteria and yeast make up the bulk of current recombinant Hb biosynthesis [135], and while shake flask cultivations generally serve to produce a sufficient amount of protein for exploratory lab-scale testing, large-scale production trials of potential recombinant Hb for commercial use will require large-scale bioreactors. Whichever host is chosen for recombinant protein production the cDNA must be optimized according to the codon usage of that organism, both for efficient translation of the recombinant protein and for the cellular economy of translation [136]. In the case of Hb, both subunits must also be expressed at suitable rates to avoid precipitation by unbalanced expression.

In all papers presented in this dissertation, *E. coli* BL21 (DE3) has been used as the recombinant production host cell. Tandem expression of the two genes encoding the α- and γ-subunit was enabled with the pETDuet-1 plasmid from Novagen as described previously by Ratanasopa et al. [20]. The rHbF proteins were produced in shake flask cultures in Terrific Broth medium supplemented with an antibiotic agent, the heme precursor δ-aminolevulinic acid (ALA), and the inducing agent isopropyl β-D-1-thiogalactopyranoside (IPTG). In **papers I** and **II**, expression yields of different HbF mutants ranged from 6-15 mg/L purified Hb protein in lab-scale shake flask cultures. In **paper III**, two rounds of optimization of the cultivation procedure were performed to investigate if the yield in shaking flask cultures could be improved. By improving the protocol in regards to OD at induction, temperature, shaking speed (rpm), precursor concentration, and inducer concentration, the yield of purified rHbF was enhanced. Table 3 shows the screening parameters and the results after overnight expression. The first screening round revealed that the best expression was achieved with the high-level values of all the selected parameters. The second round was thus performed with fixed high temperature and induction OD while altering agitation (rpm), precursor, and inducer concentrations. A new cultivation protocol was based on the results of the second round and used for Hb production in **papers III and IV**.

Table 3: Optimization screens of shake flask cultures of E. coli *producing recombinant HbF.*

1st screening

	Parameters:					*Results:*				
exp no.	induc. OD	temp [°C]	rpm	ALA [mM]	IPTG [mM]	final OD	cell w. [g]	crude vol. [mL]	Hb [µM]	Hb [mg]
1	0.1	37	150	0.1	0.05	14.9	2.1	5.4	21.9	1.9
2	0.1	23	50	0.1	0.2	1.5	0.4	4.3	8.8	0.6
3	4	37	50	0.1	0.05	4.3	0.9	4.3	19.8	1.4
4	4	23	150	0.1	0.2	10.4	3.6	9.1	9.5	1.4
5	0.1	23	150	0.5	0.05	6.2	1.7	4.5	56.7	4.1
6	0.1	37	50	0.5	0.2	1.8	0.4	5.2	2.9	0.2
7	4	23	50	0.5	0.05	3.9	1.0	4.5	18.4	1.3
8	4	37	150	0.5	0.2	9.2	1.6	4.1	129.6	8.5
9	2	30	100	0.3	0.125	5.2	1.3	4.1	61.1	4.0
10	2	30	100	0.3	0.125	5.0	1.3	4.3	59.5	4.1
11	2	30	100	0.3	0.125	5.5	1.3	4.4	45.6	3.2

2nd screening (35 °C, induction OD 4)

	Parameters:			*Results:*				
exp no.	rpm	ALA [mM]	IPTG [mM]	final OD	cell w. [g]	crude vol. [mL]	Hb [µM]	Hb [mg]
1	100	0.4	0.1	5.0	1.4	4.1	58.0	3.8
2	180	0.4	0.5	7.5	1.9	5.3	88.7	7.5
3	180	0.4	0.1	6.9	2.0	5.4	171.2	14.7
4	100	0.4	0.5	6.3	1.9	5.1	84.0	6.8
5	180	1	0.1	7.4	1.9	4.9	185.1	14.6
6	100	1	0.5	5.2	1.3	4.3	95.5	6.6
7	100	1	0.1	5.6	1.7	4.8	140.6	10.8
8	180	1	0.5	7.2	1.8	4.8	186.3	14.4
9	140	0.7	0.3	7.0	1.9	5.0	222.4	17.9
10	140	0.7	0.3	8.4	1.9	5.0	227.4	18.2
11	140	0.7	0.3	6.0	1.7	4.7	182.1	13.7

Escherichia coli as a hemoglobin production platform

A commonly used host for recombinant protein production is the bacteria *E. coli* due to quick protein expression, fast growth, and relatively cheap media for cultivation. There are however various drawbacks of using this type of host cell. Since *E. coli* is a prokaryotic organism it lacks some cellular structures and functions found in higher-order organisms such as yeast and plant cells. A relevant difference is the lack of compartmentalization of the genetic material, the DNA, in *E. coli*, which may influence the ability to achieve high yields of intracellular recombinant

Hb. As mentioned earlier in this dissertation, human Hbs have been shown to efficiently cleave DNA over time, and this could become a bottleneck for efficient production, considering that high concentrations of Hb may become harmful to the host cell DNA and/or the expression plasmid itself.

Fortunately, Hb protein does not need any advanced post-translational modifications to be functional. However, the methionine aminopeptidase (MAP) effectively cleaves off the N-terminal Met residue in native Hb production. Even though *E. coli* also have this ubiquitous enzyme, the cleavage rate is not as efficient as in human cells. In general, for heterologously produced proteins it could become a problem if crucial functions are dependent on the removal of the N-terminal Met. During recombinant production of HbA in *E. coli*, the processing of the N-terminal Met is not complete, and both subunits contain the initiating Met residue [69]. This extra Met appears to affect the Bohr effect and play a role in the dynamics of the transition between the R- and T-state. This was considered to be a problem in the early days of recombinant Hb production. Two ways were developed to address the Met extension in the N-terminal: 1) substitution of the starting valine residue in both the α- and β-subunit to methionine, (V1M) [71]; and 2) co-expressing *E. coli* MAP to improve methionine removal [137, 138]. When the V1M mutation strategy was developed, the effect on the oxidation of Hb was not considered as important as it has become to be realized in the current HBOC development, and now it has been shown that the V1M mutant strategy contributes to a less oxidatively stable Hb that autoxidize about three times faster than Met-processed native Hb [139]. The co-expression of MAP on the other hand yields Hb identical to native Hb produced in RBCs [138, 140], and ensures a homogenous protein population.

Another drawback is the supply of heme to form functional Hb subunits. In bacteria such as *E. coli* the heme supply must be boosted by either 1) adding hemin or heme precursor ALA in the cultivation media, preferably in combination with a heme transport system for improved uptake into the cells [141], or 2) enhance the heme biosynthesis pathways by metabolic engineering to keep up efficient ALA and heme production [142, 143]. In the studies incorporated in this dissertation, ALA has been supplemented in the media for enhancement of heme supply during HbF production. In **paper III** it was demonstrated that with increasing concentration of ALA, combined with higher temperature and shaking speed, the yield was significantly improved in lab-scale shake flask culture (Table 3, Figure 17). However, the cost of purchasing purified ALA could become a burden further on in large-scale production, and metabolic engineering of the heme biosynthesis pathways may be key to sustain heme supply for commercially viable *E. coli* Hb production [142].

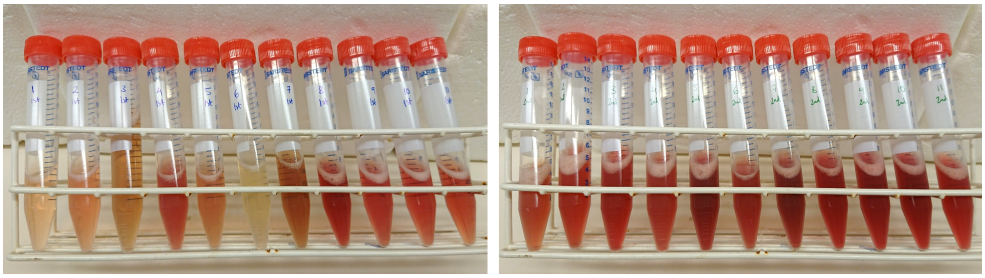


Figure 17: Sonicated, centrifuged, and filtered crude extracts of two optimization rounds of flask culture expression of HbF. Successful expression of Hb is visible by the red color of crude extracts provided by the presence of Hb.

Another important disadvantage of using *E. coli* as the expression host is the bacterial endotoxin (lipopolysaccharide) that comes with bacterial cultivation. Heterologous proteins are often produced intracellularly in *E. coli* and thus require expensive downstream processes involving cell lysis and target protein purification from contaminating host cell protein (HCP) and other components. In the case of endotoxins from *E. coli*, the human body reacts even at quite low concentrations compared to for example mice [144], and this becomes an obstacle to be considered, especially bearing in mind the amounts of Hb protein that would be needed in a potential HBOC application. For larger scale cultivations one might consider exploring alternatives for secretory expression into the periplasmic space or outside the cells [145] to reduce endotoxin levels in downstream processing. Although, this must be considered while keeping in mind the effect on Hb if removed from the reducing intracellular environment, and also addressing the concentration-dependent dissociation.

Yet another problem to be solved is the aggregation-prone Hb α-subunit. As mentioned earlier in this dissertation the α-subunit precipitates without sufficient β-type globin to combine with. Co-expression of the α-subunit chaperone found in maturing RBCs, α-hemoglobin-stabilizing protein (AHSP), has successfully been applied in *E. coli*, first in combination with only α-subunits [146], and later together with both α- and β-subunits [108]. The yield was impaired when both subunits and AHSP were expressed in equimolar ratios [108, 13], suggesting competition between the AHSP and the β-subunit. Managing the tandem co-expression of both α- and β/γ-subunits to ensure similar expression rate is therefore imperative so neither the α-subunit aggregates by the lack of β/γ-subunits nor the β/γ-subunits form β/γ_4-tetramers by lack of sufficient α-subunits to associate with. During the work presented in **paper III,** it became evident that the rHbFγ2 and rHbFγ2/α4 mutant cultivations resulted in reduced yields due to the partial formation of unwanted Hb fractions. The presence of a non-target Hb species was revealed by

chromatographic separation on ion exchange (IEX) resins. Spectral analysis during incubation with the oxidizing reagent potassium ferricyanide $K_3[Fe(CN)_6]$ presents a quick way to evaluate different Hb fractions. Homotetrameric Hb is prone to form hemichrome when challenged with $K_3[Fe(CN)_6]$ as opposed to heterotetrameric Hb which will form the conventional ferric spectrum [147]. Additional analysis with IEF in denaturing conditions further suggested that the non-target Hb fractions formed during rHbFγ2 and rHbFγ2/α4 expression only contained γ-subunits.

During the work incorporated in this dissertation, the top-performing mutant showing the highest yield of purified Hb was the rHbFα4 variant, with 70 mg/L pure protein from production in shake flasks, which is roughly double the amount of the wt rHbF yield. As opposed to the other two mutants in **paper III**, rHbFα4 expression resulted in only one Hb fraction, which also was confirmed as functional by the $K_3[Fe(CN)_6]$ evaluation. In **paper IV** the correct heterotetrameric structure of rHbFα4 was decisively confirmed by the solved X-ray crystal structure. In this study it was also found that rHbFα4 exhibited a significantly reduced DNA cleavage activity (Figure 13), which might contribute to the better yields seen with this mutant, assuming that the host cell was less negatively affected by increasing concentrations of this mutant. To investigate this we also applied the expression protocol established in **paper III** during the expression of the less negatively charged mutants in **paper II**, since these mutants also showed a lower DNA cleavage activity than wt rHbF. We found that these mutants also displayed a higher yield than wt rHbF with the modified cultivation protocol (40-50 mg/L compared to 30 mg/L of the wt rHbF). However, it is difficult to draw conclusions about the complex intracellular environment from simple *in vitro* incubation experiments, and it might be that apoglobin stability and/or affinity to γ-subunits may similarly be affected by the mutations and could be contributing to the observed increased yields. Further studies are required to examine which processes are most influential in terms of improving the recombinant Hb yields, but from the studies here on rHbF production, it appears that this type of surface modification on HbF can have a positive effect on production yield in *E. coli*.

In summary, there are quite a few drawbacks of using *E. coli* as the expression host for large-scale production of recombinant Hb. However, co-expression of MAP and AHSP, as well as metabolic engineering and/or supply of exogenous heme/heme transport systems, alleviate some of the drawbacks, and the advantages of cheap cost and speed of production are appealing. In tandem with protein engineering strategies to improve the Hb protein's suitability for host cell expression, *E. coli* could become an even better candidate for large-scale Hb production.

Yeast and plant production platforms

Yeast has also been used to produce recombinant Hb. *Saccharomyces cerevisiae* was one of the first organisms to produce fully formed Hb, with the additional advantage of showcasing complete processing of the initiating Met residue [70]. Thus, *S. cerevisiae* might be advantageous by producing a more homogeneous Hb product than bacterial host cells. Similar to bacterial alternatives, yeast cultivation media is also relatively inexpensive. For large-scale production, heme biosynthesis must be enhanced with *S. cerevisiae* as well. The heme production pathway can be metabolically engineered for overexpression, enabling better Hb yield to circumvent the need for heme precursor added to the cultivation media [148, 149]. Another yeast host is *Pichia pastoris*, which has been shown to produce fully functional Hb for HBOC development [150]. *P. pastoris* has also been metabolically engineered and used to produce soybean Hb at high yields, to be used as a food additive [151].

Myoglobin has recently been expressed in the tobacco plant, *Nicotiana benthamiana* [152], and a modified HbF construct with a Gly-Gly-Ser linker between the C-terminal of the α-subunit and the γ-subunit N-terminal [130] have also been expressed in this system [131]. N-terminal Met cleavage was reported to be effective in this system, and although the yield so far is low compared to what has been achieved in microorganism host cell systems, efficient expression in plants could enable a straightforward scalable production.

4.2 Purification of hemoglobin

Production of Hb by biosynthesis in host cells requires efficient extraction and purification with minimal loss of target protein and Hb functionality. Unless the production platform enables secretion of the produced Hb – which may or may not be desirable depending on the Hb construct's resistance to dissociation, stability, or other factors – release of Hb from inside the host cell at the end of cultivation and subsequent downstream processing for isolation of Hb from the contaminating HCPs have to be robust and reliable. Liquid chromatography provides versatile separation means at gentle processing conditions suitable for proteins.

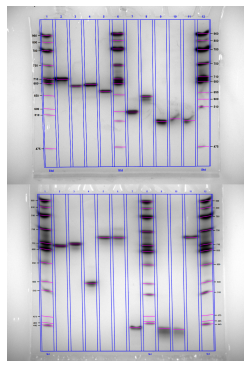
Ion exchange chromatography

IEX is based on the principle of using ionic interactions for separating compounds [153]. The amino acid residue composition of proteins determines their ionic net charge, mainly by arginine, lysine, and histidine providing positively charged side-chains, and glutamic and aspartic acid providing negatively charged side-chains, at physiological pH. The pI of a protein is determined at the pH where the net charge is zero, i.e. where the oppositely charged side-chains cancel each other out. The pIs of the Hb proteins created in this work were determined by IEF gel electrophoresis (**paper II** and **III**), and the values are listed in Table 4. At pH values above or below

the pI value, the protein will exhibit negative or positive net charge, respectively. Through ionic interactions with the stationary phase of the ion-exchange chromatography resin, proteins will be separated according to their net charge, surface charge distribution, and protein conformation, based on the chosen buffer conditions.

*Table 4: Isoelectric points of recombinant Hb (**papers II-IV**). Examples of IEF gels are shown to the right.*

Hb	Isoelectric point
wt rHbA	7.0
wt rHbF	7.1
rHbFαA12D	6.7
rHbFαA19D	6.8
rHbFαA12D/A19D	6.6
rHbFγ2	6.5
rHbFα4	5.8
rHbFγ2/α4	5.0



The HbF proteins produced in this work were purified from *E. coli* lysates by two steps of liquid chromatography with an ÄKTA Avant 25 system. After harvesting and washing the *E. coli* cells were re-suspended in the binding buffer of the following chromatography step, kept on ice, and burst by sonication. The sonicated extract was centrifuged and filtered to remove cell debris. The crude extracts of wt rHbF, rHbFαA12D, rHbFαA19C, rHbFαA19D, rHbFγC93A, rHbFαA12D/A19D, and rHbFαA19C/γC93A were applied on the strong cation exchange resin Capto S, equilibrated with 10 mM sodium phosphate buffer pH 6.0 in the first step to capture the Hbs with ionic interaction. After washing the bulk of the HCPs out, the Hb was eluted by 70 mM sodium phosphate buffer at pH 7.2. It was observed in **paper II** that especially the double mutant rHbFαA12D/A19D did not bind as strongly to the column as the wt rHbF, indicating that the negative charges added to the protein through site-directed mutagenesis were capable of affecting the behavior on the cation exchange resin. For rHbFα4, rHbFγ2, and rHbFα4/γ2 in **papers III** and **IV**, the multimodal anion exchange resin TREN was used instead as the first capture step. The TREN resin was equilibrated with Tris-HCl buffer (pH range 7.4-8.5) to bind the HbF variants, and then a Tris-HCl buffer salt wash, followed by a buffer shift to MES buffer pH 5.6, were used as additional washing steps until elution was achieved in the MES buffer with a NaCl gradient.

The second step, the polishing step, was performed with the strong anion exchange resin Q HP for all HbF proteins described, equilibrated with Tris-HCl

buffer for binding Hb to the column resin, and after washing a salt gradient was used to elute the Hb containing fraction. The strong red color enabled easy detection of Hb on the columns during the chromatographic purification and the eluted samples were collected with Hb peak fractionation by monitoring the 419 nm peak in the chromatogram. Figure 18 shows a collection of pictures from the production and purification procedures used.

Figure 18: Pictures of recombinant production and purification of rHbF. Shake flask E. coli *cultures visibly change color after overnight expression of Hb, and the pelleted cells display a strong red color for well-expressed Hb protein cultivations. There is a clear color difference between resuspended unbroken* E. coli *cells and the released extracellular Hb in the sonicated crude extracts. The lower row of pictures shows dialysis of crude extracts and progressing purification with two steps of liquid chromatography. Finally, the purified Hb protein was concentrated before freezing in liquid nitrogen for storage at -80 °C.*

The cation exchange step with Capto S is very useful to remove a significant amount of *E. coli* HCPs, as seen in Figure 19 and Figure 20. The negatively charged surface of the resin efficiently captures HbF and mutants with pI close to wt rHbF, while the majority of the HCPs do not interact with the column and are washed away at pH 6.0. However, as more negatively charged mutations were added as described in **paper III**, the mutants were repelled by the cation resin and did not bind on the column, and another resin had to be used instead. For IEX the rule of thumb is to use buffers with a pH more than 0.5 pH unit away from the pI of the target protein [154], and the pIs of the rHbFα4, rHbFγ2, and rHbFα4/γ2 mutants were too low to permit the use of the cation exchange purification protocol. The TREN resin proved to be an excellent substitute for the first capture step but required a chromatographic method with additional washing steps to achieve efficient purification before moving on to the second and final polishing step with the established Q HP resin.

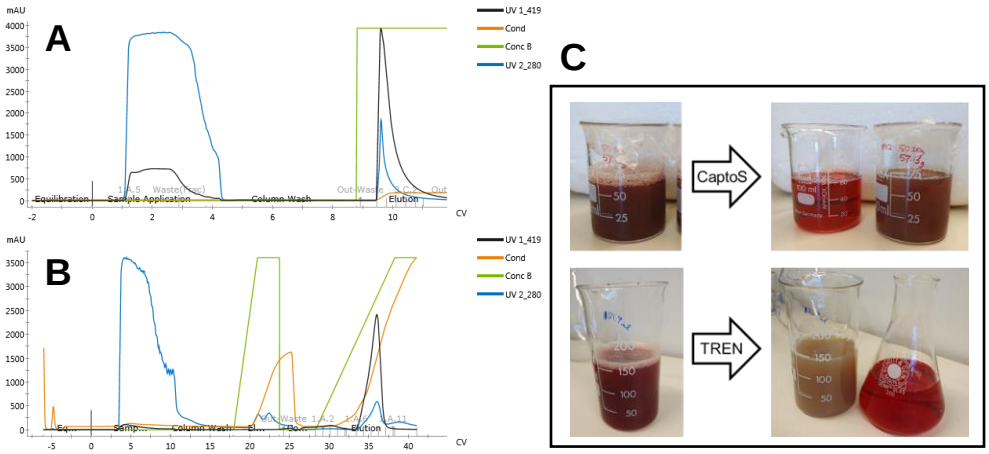


Figure 19: Chromatograms of CaptoS (A) and TREN (B) purification. The inset pictures (C) show the crude extracts before and after application to the columns, with the collected flow through and the purified Hb fractions after collection.

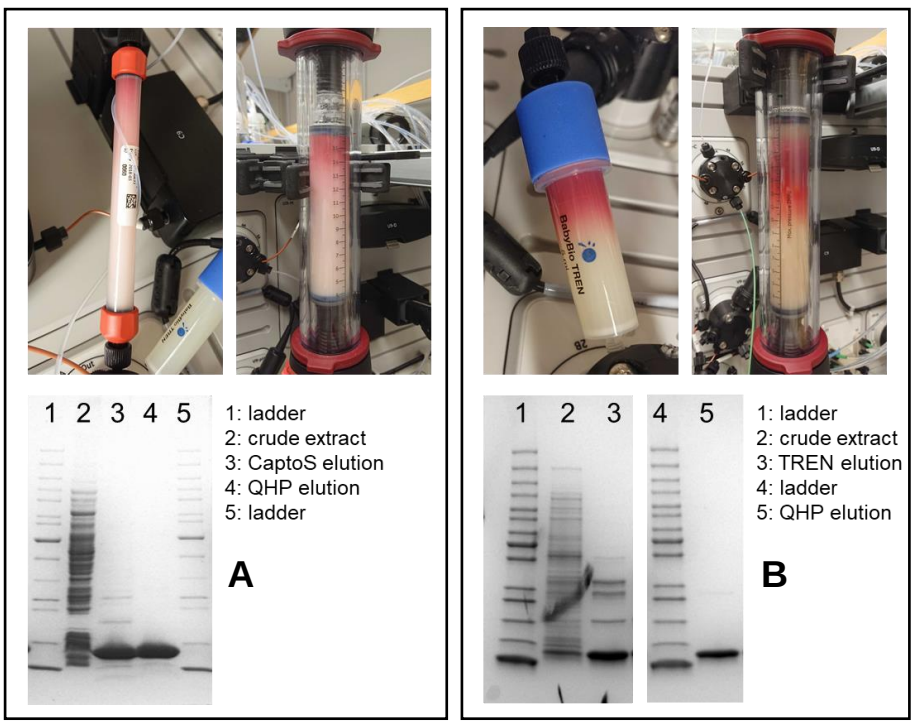
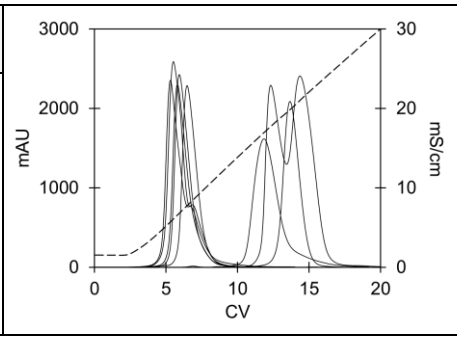


Figure 20: Pictures of first step liquid chromatography purification of HbF, using CaptoS (A) and TREN (B) resins, small- and larger-scale. Corresponding SDS-PAGE gels are shown below. The left gel shows the crude, CaptoS, and QHP samples of wt rHbF purification (A). Right gel show crude, TREN, and QHP samples of rHbFα4 purification (B).

The Q HP resin used as the final step of purification has previously been shown to be useful in separating unwanted Hb fractions such as β_4-tetramers formed during recombinant expression of wild-type HbA in *E. coli* cultures [20], and this trait was observed in the work presented here as well. This resin also provided the possibility of characterizing electrostatically altered mutations by applying appropriate buffer pH conditions and a sufficient salt gradient. During purification of the HbF variants created for the papers included in this dissertation, there were significant differences in peak elution conductivity for the variants harboring negatively charged mutations (**papers II**, **III**, and **IV**), clearly displaying the surface exposure of the chosen mutation sites as intended. Table 5 display the conductivity values for the different rHbF variants.

Table 5: Elution peak conductivity values of different rHbF variants as determined by a NaCl gradient on a Q HP resin in 20 mM Tris-HCl pH 7.4, and the chromatograms are shown to the right.

Hb	Elution peak conductivity [mS/cm]
wt rHbF	6.1 ± 0.2
rHbFαA12D	6.7 ± 0.1
rHbFαA19D	7.1 ± 0.2
rHbFαA12D/A19D	7.7 ± 0.1
rHbFγ2	16.3 ± 0.6
rHbFα4	18.9 ± 0.8
rHbFγ2/α4	21.6 ± 0.5



The scope of the work included the production of Hb in shake flask cultures, but for comparison purposes, wt rHbF and rHbFαA12D/A19D were produced in a pilot trial in a 5 L bioreactor (BIOSTAT® A Plus, Sartorius Stedim Systems GmbH). It was observed that Hb yield per gram cell weight was significantly less in the bioreactor cultivation than in the shake flask cultures. The concentration of Hb is crucial for the tetramer-dimer equilibrium, and the tetrameric form is more stable than dimers. Diluted solutions of Hb will promote dimerization into heterodimers and the dimers are more prone to oxidize and are less stable than the tetramers [155]. If the yield of Hb inside the host cells is less than optimal for ensured tetramer stability in the immediate subsequent downstream processing steps the protein quality will more likely deteriorate. Higher intracellular yields will therefore in extension also lead to better recovery and better quality of the final purified sample. To overcome this, genetic cross-linking of the subunits is a strategy that effectively prevents dissociation, and the dimer formation problem is minimized.

5 Hemoglobin and artificial oxygen carriers

5.1 Blood Substitutes and Oxygen Therapeutics

The history of blood transfusion dates back to the 17th century. In 1667 the first blood transfusion between humans was accomplished, but further trials revealed that incompatibility issues often led to fatal outcomes. Besides human blood, many other fluids were tried as potential blood substitutes, for example, animal blood, wine, milk, and saline solutions, but without success. Later, albumin solutions and purified Hb solutions were also examined, but these attempts were not successful either [156]. With the understanding of donor compatibility and blood type matching, modern blood bank facilities have enabled an organized supply of donated blood in areas where the infrastructure can support the required facilities. According to the World Health Organization (WHO), 118.5 million blood donations are collected globally [3]. High-income countries are responsible for 40 % of the collected blood, while only housing 16 % of the world's population. Availability of donated blood and the use thereof varies across the globe. In high-income countries, the majority (75 %) of the transfusions are given to patients over 60 years of age, and the most common uses are for cardiovascular and transplant surgery, cancer therapy, and massive trauma. In contrast, in low-income countries, the majority (54 %) of transfusions are given to children under 5 years of age, battling severe childhood anemia, and managing pregnancy-related complications [3]. Despite the many uses and modern medicine's dependence on blood-derived products and blood transfusion, there are several drawbacks of relying solely on donated blood. Stability, compatibility, blood-borne diseases, and availability are serious issues that also need to be considered.

Already in 1957, T. Chang reported the accomplishment of creating the first "artificial red blood cell" [157], by encapsulating hemoglobin and RBCs enzymes within a polymeric membrane. However, complications such as *in vivo* safety issues and expensive production stalled further development. In the 1980s, the blood donation systems were put to a halt due to the HIV crisis and sparked a renewed interest in the pursuit of an AOC that could complement the supply of donated blood. Development of blood substitute candidates followed, and two main types of products emerged: perfluorocarbon-based and Hb-based. In later years, an alternative approach has been the development of hematopoietic stem cells for the artificial production of RBCs. Throughout the years, several AOC candidates have been entered into clinical trials, but only one perfluorocarbon (PFC)-based and one Hb-based product managed to get some success, and only in a small number of countries.

Perfluorocarbons

PFCs were originally developed during the Manhattan project as inert storage liquids for uranium and plutonium, but following the discovery that PFCs also were able to dissolve significant amounts of gases, Clark and Gollan published results in 1966 showing how mice could survive while “breathing” oxygen-saturated liquid PFC [158]. Gases like oxygen and carbon dioxide are highly soluble in PFC solutions, and their solubility depends linearly on partial pressure. This enables PFC to “take up” oxygen in areas with high partial pressure like the lungs, and release it in tissues where the partial pressure is lower. Due to their chemical makeup, the PFCs are immiscible in water-based solutions and must be emulsified to be compatible with the intravascular system to be used as AOCs. The emulsions are typically formed by adding different types of surfactants (e.g. egg-yolk phospholipids, Pluronic, etc.), creating PFC-containing spheres around 100-300 nm in size. Several PFC-based products have been in clinical trials [159], but Perftoran is the only PFC-based AOC currently approved for human use, and only in Russia, Ukraine, Kazakhstan, Kyrgyzstan, and Mexico. Under the brand name Vidaphor this product is also aiming to enter the United States and European markets [12].

HBOCs

The first HBOC candidate was simply a solution of free, purified Hb, thought to have the advantage of sidestepping the need for blood-type matching and storage limitations seen with intact RBCs, while at the same time taking advantage of the already optimized intrinsic oxygen-carrying capabilities of Hb. As discussed in previous chapters, it was however quickly realized that free Hb was easily converted into non-functional metHb and exhibited toxic effects initially believed to be caused by contaminating stroma. It was also clear that extracellular Hb is rapidly excluded from the intravascular space by renal clearance (elimination through the kidneys), while at the same time causing kidney damage [126]. After the initial Hb solution trials, four types of modifications of Hb developed to formulate less toxic HBOCs with prolonged plasma retention: cross-linked Hb, polymerized Hb, conjugated Hb, and encapsulated Hb (Figure 21).

Cross-linked Hb was first achieved by H.F. Bunn in 1968 by using bis-N-maleimidomethyl ether, and the stabilized tetramer stayed in circulation for a longer time as dimerization of Hb was no longer possible [160, 156]. Later, another cross-linking agent (bis-3,5-dibromosalicyl fumarate) was used to create one of the first HBOCs called diaspirin cross-linked Hb (DCLHb, HemAssist), in which the two α-subunits are linked together at αLys99 [161]. With recombinant technology, genetic cross-linking was enabled, and a cross-linked Hb was created by fusing the C-terminal and N-terminal of two α-subunits with one glycine residue as linker [71]. Another genetic linker has been proposed using a 12 residue long linker (3x Gly-

Gly-Ser) is inserted between the α-subunit N-terminal and β/γ-subunit C-terminal [162, 20]. Other mutational strategies have also been tried, for example by adding cysteine residues at surface-located positions to initiate specific linkage between Hb molecules, creating octameric Hb [105].

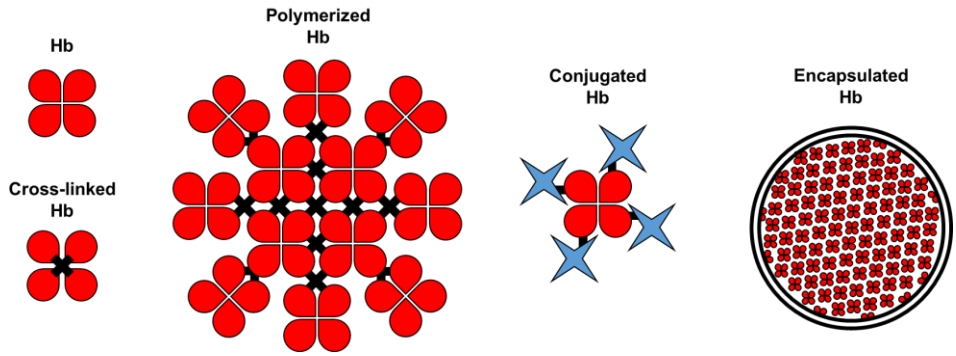


Figure 21: Schematic figure of different modifications of Hb for HBOC formulation.

Polymerized Hb can be achieved by reaction with raffinose or glutaraldehyde. After this process was patented in the 1970s, several HBOC products were developed based on this method. The most successful product is Hemopure, which is polymerized bovine Hb with an average size of 250 kDa and a p_{50} of 38 torr. Hemopure is currently approved for human use, although only in South Africa (since 2001) and Russia (since 2012), but is accepted for veterinary use in other countries.

Conjugated Hb is made by attaching other molecules to surface-exposed residues on Hb. Polyethylene glycol (PEG) is a common molecule used for conjugation to Hb. Hemospan, nowadays called MP4OX, is an HBOC based on human HbA conjugated with maleimide-PEG [163]. The average size is 96 kDa with a p_{50} of 6 torr. Originally MP4OX was developed as a “blood substitute”, but currently it is directed towards an oxygen therapeutic approach instead, for example, treatment of sickle cell anemia [164]. A problem seen with these types of products is unspecific conjugation procedures leading to a product containing a wide range of sizes. A recent article by Meng et al. [165] highlights this issue. Another study by Cooper et al. demonstrates that this may be resolved by designing mutants with specifically placed sites for conjugation using cysteine residues [111]. HemoAct is another conjugated type of HBOC, made up of human Hb conjugated to three human serum albumin (HSA) molecules [166]. There are two products in the market in Japan for veterinary use, directed towards cats and dogs, using feline albumin canine or albumin, respectively.

Encapsulated Hb is achieved by containing Hb solutions within polymeric membranes or liposomes. T. Chang was the first researcher to report a successful artificial cell containing Hb [157, 167]. Several different membrane materials were examined to increase circulation time [160, 167]. Hemoglobin vesicles (HbV) is another HBOC where a highly concentrated human HbA solution is encapsulated in a biocompatible liposome through extrusion. The liposome is made up of four different lipids and one of the lipids is conjugated to a PEG molecule, thus when the vesicles are assembled the HbV is decorated with PEG. These HbV are 250-280 nm in diameter with an internal concentration of Hb at around 38 g/dl. The HbV have been extensively studied in several animal trials [168].

Although many HBOC products successfully addressed the clearance issues of unmodified Hb, the persistence of hypertension, inflammatory reactions, and higher risk of myocardial infarction and death have stalled approval from the most influential government agencies [8]. Table 6 summarizes select HBOC products that have entered clinical trials.

Table 6: Summary of HBOCs entered into clinical trials. The table is adapted from several review articles: Tao and Ghoroghchian [169], Alayash [42], Ferenz and Steinbicker [12], Benitez Cardenas et al. [121], and Jahr et al. [10].

HBOC product		**Source of Hb**	**Modification**	**Mol. size [kDa]**	**P_{50} [torr]**
Discontinued	HemAssist (DCLHb, ααHb)	HbA (outdated blood)	Cross-linked αα	64	30
	Optro	HbA (recombinant)	Cross-linked	64	33
	HemoLink	HbA (outdated blood)	Cross-linked + polymerized	32 - >500	34
	PolyHeme	Bovine Hb	Polymerized	130-250	28-30
	Hemospan/MP4OX	HbA (outdated blood)	Conjugated PEG	95	4-8
	PHP	Bovine Hb	Conjugated POE	100	20
In progress	Sanguinate	Bovine Hb	Conjugated PEG	120	7-16
	$HemO_2Life$	Invertebrate Hb		~3600	7
Approved*	Hemopure	Bovine Hb	Polymerized	130-500	38

* in South Africa and Russia

The results of the clinical trials of preceding HBOC products have discouraged much of the research and funding that previously went into AOC development, and this has slowed down progress from moving as rapidly forward. However, Hb research has focused on providing a better understanding of the underlying reactions of Hb that are causing the persistent toxic side-effects of HBOCs, and progress and development are still ongoing in the pursuit of a safe and functional AOC.

Some examples of HBOCs currently under development/preclinical/clinical phase are HbV [168, 170], human Hb encapsulated in a biocompatible artificial vesicle; HemoAct [171, 172], the protein cluster with a central Hb molecule covalently linked to three HSA molecules; ErythroMer [173], nanoparticle-containing human Hb, leukomethylene blue and 2,3-BPG; OxyVita Hb [174], polymerized bovine Hb using a polymerization technique without linker molecules; and $HemO_2Life$ [175, 176], based on a large natural extracellular Hb complex of 156 globin molecules from the marine worm *Arenicola marina*.

Alternative technologies

An alternative strategy to utilize donated blood is to remove A and B antigens from collected RBCs to create "O-type RBCs" [177]. This would create a pool of "universal" donated blood with no need for blood-type matching, and at least in settings where donated blood is constantly available, this would alleviate specific blood type shortages.

Another alternative route to providing an artificially produced oxygen carrier is through the development of artificial RBC production from stem cells [178]. Compared to the relatively simple approach of producing one target protein such as Hb, the manufacturing of complete and mature RBCs from stem cells require vastly more complicated processes. The main advantages of this approach would be the manufacturing of fully functional RBCs with the correct oxygen transport capabilities, and the possibility to create patient-specific blood products. Laboratory-scale RBC production methods have been able to develop through clinical infrastructure providing collected cord blood and peripheral blood. Currently, RBCs have been produced from hematopoietic stem cells, embryonic stem cells, induced pluripotent stem cells, and mesenchymal stem cells, but the expensive culture conditions involving cytokines needed for differentiation, and complicated multi-step cultivation procedures, results in extremely high cost for the large-scale production needed. Significant advancements in biological and technological aspects of artificial RBC production are needed to make this approach a viable option [179, 180]. A side note worth mentioning regarding AOC uses is that the HBOC product based on the invertebrate Hb from marine worm has found an application in stem cell differentiation by providing oxygenation in hypoxic cultures and engineered tissue [181, 182].

6 Conclusions and future prospects

Successful engineering of a Hb molecule for HBOC development should result in a product with suitable oxygen transport capabilities, reduced oxidative degradation processes, low NO scavenging abilities, and efficient biosynthesis aimed at achieving high yields for commercially viable production. Up to this point, protein engineering work on Hb has demonstrated that oxygen-binding properties, as well as NO scavenging, can be controlled by appropriate mutational strategies. The bottleneck for the realization of an AOC based on recombinant Hb now lies in managing the oxidative side reactions of Hb and solving efficient biosynthesis for large-scale production platforms.

HbF possesses desirable qualities in terms of stability that provide some advantages, especially during upstream production procedures. In this work, the HbF protein was used as a template Hb for the studies presented in this dissertation. The work addressed both aspects of the bottleneck, by employing rational protein design and investigating oxidative behavior and production strategies. We confirmed that substitutions involving redox active cysteine residues in **paper I** had a significant impact on the oxidative stability of HbF, and further studies into possible favorable sites to place/replace these types of electron-pathway altering residues appear to be interesting as a strategy to alleviate oxidative damage in key parts of the protein.

The other strategy, involving modification of the net surface charge of HbF (**papers II-IV**), showed promise especially for protein production yields and in decreasing DNA damage activity. The reduction of damaging activities on important cellular structures and molecules may become increasingly important as higher concentrations of Hb could affect biomolecules and structures sensitive to the side-effects of Hb reactions during expression. Successfully decreasing adverse interactions by adding repelling forces on the surface of the protein could be beneficial in several conditions. Two main types of environments should be kept in mind when considering this engineering strategy: 1) host cell for recombinant production; and 2) intended administration target environment, e.g. the vascular compartment. It was found in **paper III** that negatively charged surface-exposed residues affected expression in *E. coli*, both in favorable and some unfavorable aspects. Specifically, the unbalanced expression of subunits was more prevalent in some mutants than in others, leading to a significant loss of final functional Hb yield. Additional engineering strategies, such as genetic fusion approaches, should diminish such undesirable effects and should be investigated in combination with successful mutations which have been shown to not affect oxidative stability of the

Hb molecule, for example with negatively charged mutations on the α-subunit. In the end, the mutations investigated in this work should be considered to be used in combination with other engineering strategies to achieve a more suitable Hb molecule for use in targeted applications.

Artificial oxygen carriers may have at first been pursued primarily as supplements or alternatives to RBC transfusion for clinical use, but despite having this particular application eluding realization on a global scale up to this point, several other uses of these products have been developed in the wake of AOC research, and new potential applications may emerge as the field is progressing forward.

The specifically optimized oxygen transport that an engineered Hb provides could be used for oxygenating organs in transit for organ donation. In this way, the tissues could be kept in a less damaging environment and avoid the side effects of cold storage, which potentially could decrease damaging effects to the organ in transit. Another potential use could be in cancer therapy, where AOCs could be applied to increase oxygenation in tumor tissue. Designing an AOC with high oxygen affinity and small size could lead to a therapeutic capable of permeating more easily into the affected tissue compared to RBCs. This could contribute to increased oxygenation of the therapy-resistant hypoxic regions, and increase the effect of for example radiation therapy. One might also speculate that a designed AOC could become useful as a therapeutic during clinical conditions where hypoxia is a side-effect, such as when lung capacity is impaired and patients need to be supplied with oxygen gas. A designed AOC could in such situations potentially be used as an oxygenation enhancing agent to alleviate hypoxic stress.

One may also consider potential uses where it is becoming apparent that donated RBCs are associated with risk factors, for example in neonatal care for extremely preterm infants. The use of transfusions with adult red blood cells results in higher oxygen levels, which leads to oxidative stress by an oversupply of oxygen to the tissues in these patients. Many studies regarding RBC production from stem cells report that HbF is the dominant Hb produced with the developed protocols, and could potentially be a source of a more suitable alternative in various applications.

Another, different, use of Hb-based products would be to provide nutrition and/or treat anemia arising from iron deficiency. The body has specialized pathways to capture and recycle iron and heme, and using an artificially produced Hb-product for this purpose may be advantageous and a more effective iron supplement that is easily taken up and metabolized in the body.

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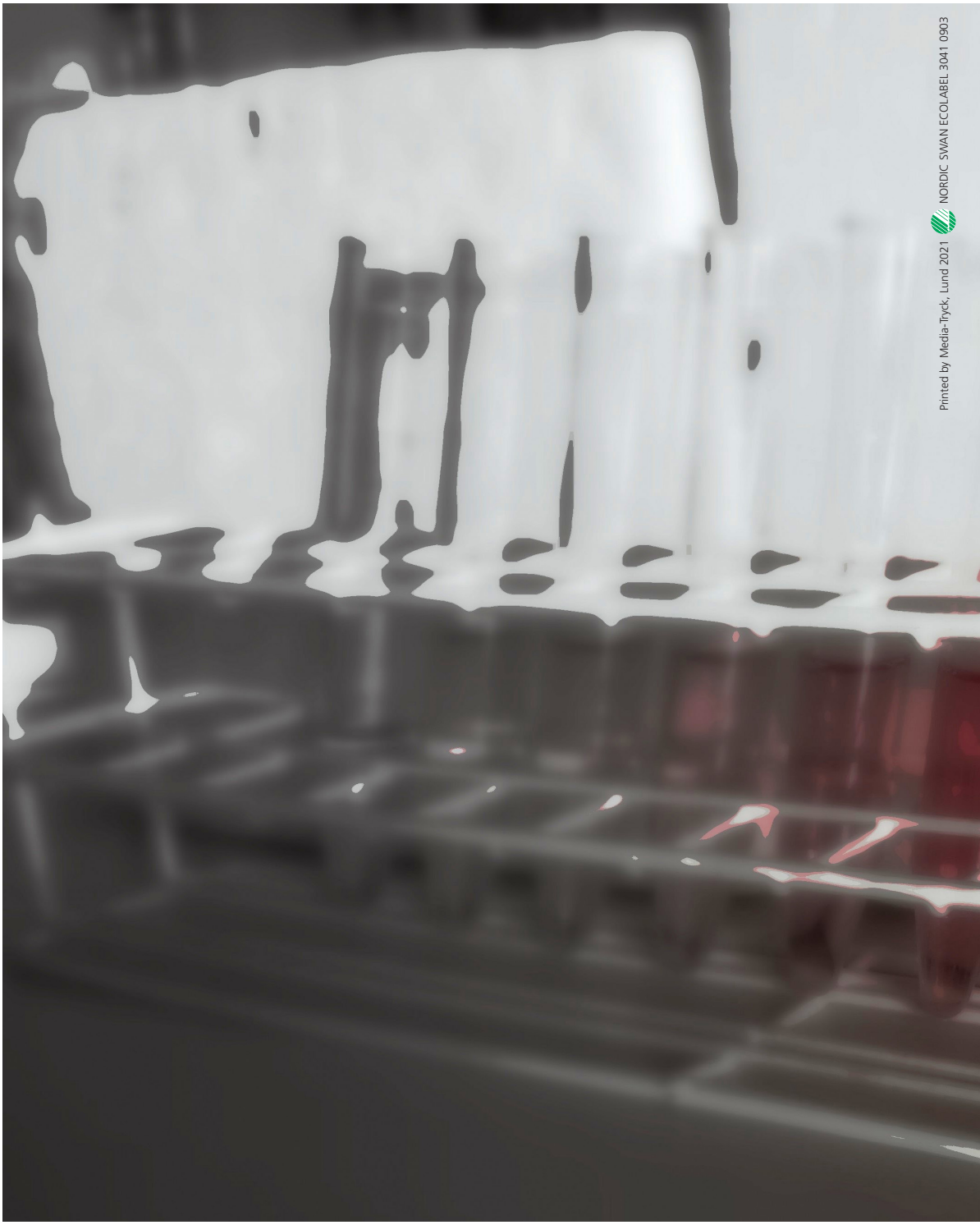

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