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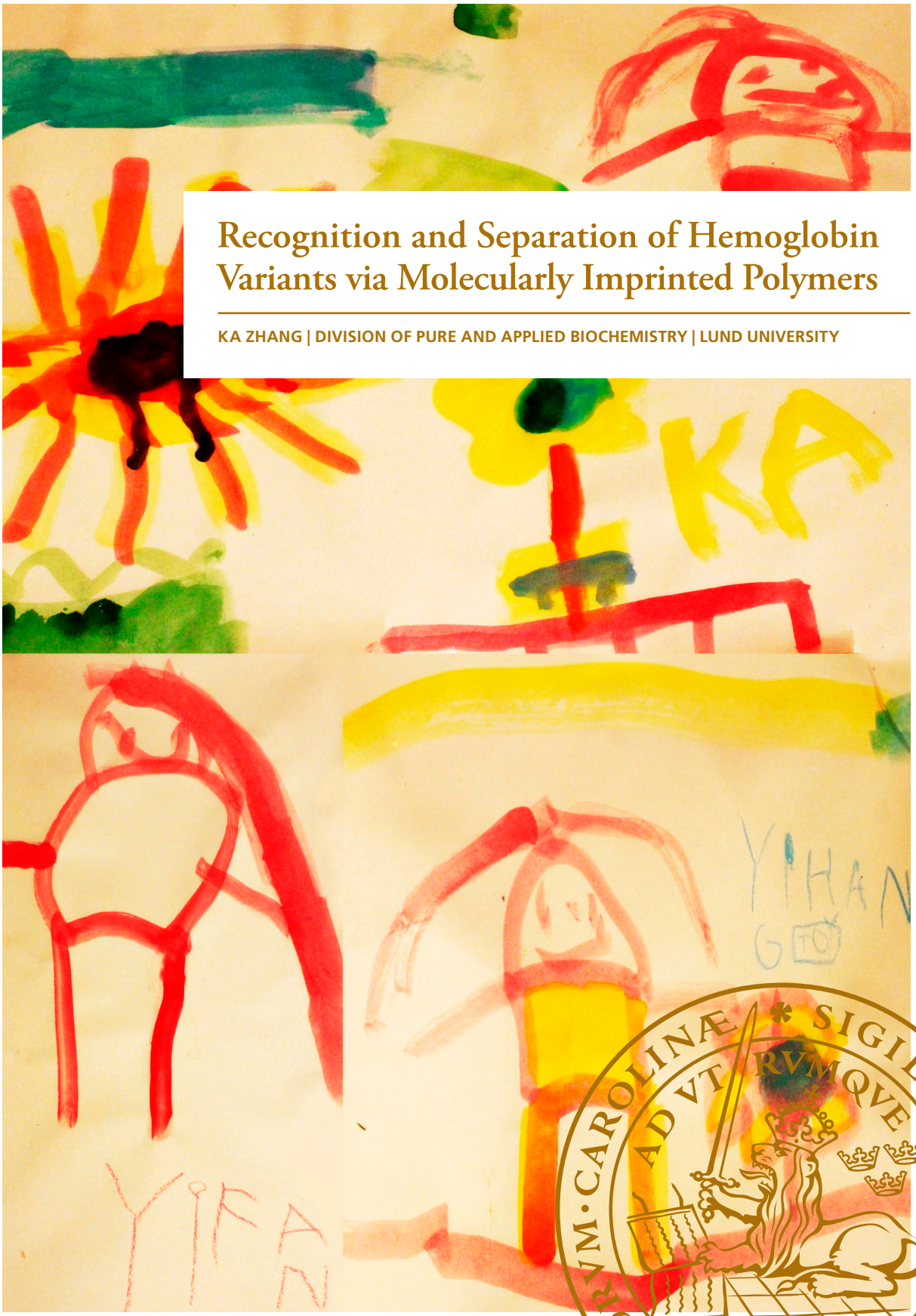


# Recognition and Separation of Hemoglobin Variants via Molecularly Imprinted Polymers

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# Recognition and Separation of Hemoglobin Variants via Molecularly Imprinted Polymers

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LUND
UNIVERSITY

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Abstract

Hemoglobin (Hb) is the predominant protein in red blood cells with allosteric regulation mechanisms for delivering oxygen between the lungs and tissues. After the 1980s, concentrated research on modified Hb emerged because of the necessity for the development of a blood substitute (oxygen carrier). Several purification procedures and genetic/chemical modifications of Hb have been undertaken by both industry and academia to produce viable Hb-based oxygen carriers (HBOCs). In this thesis, molecular imprinting technique has been applied to characterize, recognize and purify different Hb variants. Molecular imprinting is one of the most efficient methods to prepare polymer materials bearing pre-designed molecular recognition sites, which are complementary to the template in their size, shape and spatial arrangement of the functional groups.

By combining Pickering emulsion polymerization and surface imprinting, highly cross-linked Hb-imprinted polymers with recognition sites on the surface were synthesized. The obtained molecularly imprinted polymers (MIPs) possessed high selectivity and could be applied as an efficient chromatography resin to selectively recognize and purify different Hb variants, such as adult Hb (HbA), fetal Hb (HbF), fusion HbF (fHbF) and Hb mutants. From MIP column, we also identified that *E. coli* glyceraldehyde 3-phosphate dehydrogenase (GAPDH) could especially interact with HbF that will further interfere with the regular *E. coli* metabolism and indicated the importance to carefully control cell metabolism when optimizing Hb production in heterologous systems.

The possibility of using MIPs as Haptoglobin (Hp) mimics *in vitro* was also studied. Hb is safe and inert within the confinement of the RBCs but becomes reactive and toxic upon intravascular hemolysis. When red cells are lysed in vivo, Hp binds to free Hb instantly and prevent free Hb-induced vascular dysfunction or injury. We demonstrated that MIPs, as a Hp mimic, could reduce the intrinsic oxidative toxicity associated with Hb.

Furthermore, to facilitate the evaluation of the biophysical properties of HbF both in cell-free environments as well as in biological test systems, we have developed fluorescent Hb (GFP-fHbF) by genetic linkage of fHbF with the green florescent protein (GFP) at the DNA level. It was also applied to evaluate the efficiency of both HbA-imprinted and HbF-imprinted polymer beads. Unlike physical absorption of HbA on silica surface, the preparation of the HbF-imprinted polymers is achieved by covalently immobilizing HbF to silica nanoparticles as templates. Overall, our work opens up new possibilities of carefully designed MIPs for tailored protein purification, separation and analysis.



Key words : Hemoglobin, molecularly imprinted polymers, haptoglobin, protein purification, chromatography glyceraldehyde 3-phosphate dehydrogenase, Pickering emulsion

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Cover image drawn by Yihang Zhou and Yifan Zhou (five-and-a-half-year-old twin boys) – portraits of their mother.



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# *DEDICATED*

*To*

*my wonderful parents and parents-in-law,*
*who took care of my kids when they were small and made it possible*
*for me to complete this work,*

*my lovely husband*
*who has been a constant source of support and encouragement during*
*the challenges of my PhD study and life,*

*my two boys, Yihang and Yifan,*
*who make me keen to learn new things*

*It is not beauty that endears, it's love that makes us see beauty.*

*—— Leo Tolstoy*

*人并不是因为美丽才可爱，而是因为可爱才美丽。*

*——列夫.托尔斯泰*

# Table of Contents

# Popular science summary

What is hemoglobin?

There are thousands of different proteins in the human body. Hemoglobin (Hb) is a protein found in red blood cells (RBCs). RBCs are an essential part of our blood, and they are the most common type of blood cells in the human body. Hb gives blood the red color and constitutes approximately 96% of the RBCs' dry content and approximately 35% of the total content (including water). Hb can deliver oxygen from the lungs to the rest of the body and then carries carbon dioxide from body tissues and transports it to the lungs. The human body can survive three weeks without eating and three days without drinking, but it can't survive even for three minutes without oxygen. Oxygen provides the energy the body needs for all of its normal activities and only Hb is capable of carrying oxygen to the cells. Each RBC contains around 200–300 million molecules of Hb that bind oxygen.

A blood transfusion is a routine medical procedure in which the donated blood is supplied through a narrow tube placed within a vein entering the circulation intravenously. This can help replace the blood lost due to surgery or injury, as well as help if an illness prevents the body from producing blood or a blood component accurately. The 21$^{st}$ century has been medically challenging for human beings – an increasing aging population, generation of new infectious agents and natural disasters are some of the factors. Several scientists believe that one of the future evolutionary innovations could be artificial blood – a substance used to mimic some functions of biological blood, especially the oxygen-carrying capacity of Hb. The research has focused on cell-free Hb-based products (HBOCs). However, recently, HBOCs are still at an early stage of development. To fulfill this vision, we still have to improve our knowledge and be persistent. The major source of Hb of previous developed products is outdated human blood or bovine blood. These available sources have their limits. Therefore, other Hb alternative sources are required for the development of HBOCs. New technologies also need to be developed to increase the yield of the Hb production, purify and characterize these Hb variants.

Molecular imprinting is a synthetic technique to create template-shaped cavities in polymer matrices. It is often described as a method of making a molecular lock to match a molecular key. The imprinting process is like the footprints you leave on a wet beach – the footprints fit your feet in terms of both their size and shape. The

obtained molecularly imprinted polymers (MIPs), like the footprints, hold a memory of the original template molecules complementary to the template molecules in terms of shape, size and functional groups. The MIPs possess several advantages and have been successfully employed in several applications as artificial receptors. They exhibit high selectivity and affinity to the target molecules. They can be used repeatedly and be stored for years without loss of activity for the target analyte. They have high physical robustness, strength and resistance to harsh chemical media, heat and pressure. Additionally, their synthesis is also less expensive. The number of researchers in this technology has increased rapidly due to its potential application in various fields.

In this thesis, a new molecular imprinting method called Pickering emulsion polymerization is employed to prepare Hb-imprinted polymer beads. We demonstrated the possibility of using these MIPs as effective chromatography resins to isolate Hb variants from complex biological solutions. Additionally, the feasibility of these MIPs to characterize protein–protein interaction could be a new application area of MIPs and help to optimize the Hb production in heterologous systems. We believe that molecular imprinting is an effective technology that could be a cost-effective Hb purification strategy and help expand our knowledge regarding Hb and promote its biomedical applications as blood substitutes.

# Populärvetenskaplig sammanfattning

Det kommer ständigt nya larmrapporter om blodbrist på våra sjukhus. I Europa är tillgången relativt säker men i Asien till exempel, finns ofta inget blodbankssystem. Om det händer en olycka måste släktingar ställa upp och hjälpa till. På vissa håll har rädslan för HIV-smitta lett till att människor varken vill ge eller få andras blod. Det finns därför starka drivkrafter för att utveckla ett alternativ till blod - ett konstgjort blod eller ett blodersättningsmedel. Den vanligaste metoden innebär användning av de röda blodkropparnas protein hemoglobin som utgångsmaterial. Dessa produkter kallas ofta för hemoglobin-baserade syrebärare (HBOC) vilket innebär att hemoglobinet har modifierats för att möjliggöra förbättrad stabilitet och effektivitet utanför sin naturliga miljö i den röda blodkroppen. Hemoglobin är en mycket viktig komponent i blod. Detta protein ger blodet dess röda färg och har som sin primära uppgift i att transportera syre i kroppen. Rekombinant humant hemoglobin kan uttryckas stora mängder från olika transgena *E. coli* produktionssystem. En av de viktigaste utmaningarna är dock att utforma en effektiv och ekonomisk protein-uppreningsstrategi för att säkerställa höggradigt upprenade hemoglobinprodukter.

Molekylär avtryckssteknik (molecular imprinting) är på väg att bli alltmer erkänd som en teknik för framställning av polymerer med specifik igenkänning av en målmolekyl. De framställs genom att polymerisera funktionella och tvärbindande monomerer i närvaro av en printmolekyl (molekylär mall). Efter polymerisering elueras printmolekylen och lämnar komplementära bindningssäten vilka tillåter återbindning av printmolekylen med i många fall mycket hög specificitet, jämförbar med antikroppar. Det på så sätt erhållna artificiella materialet har använts i olika applikationer som kräver specifik ligandbindning, såsom separation av strukturellt nära relaterade föreningar, och i immunoassay-liknande bindningsanalyser. Dessutom kan polymerer med molekylär avtryckssteknik användas i organiska lösningsmedel och p.g.a. deras starka kemiska, termiska och mekaniska stabilitet behåller de sitt molekylära” minne” över långa tidsperioder. De kan därför vara fördelaktiga som igenkänningselement i många biomolekylära tillämpningar. Vi har utvecklat en ny metod för framställning av proteinspecifika polymerkulor baserat på molekylär avtryckssteknik i en speciell lösning som kallas Pickering emulsion. Den höga selektiviteten och stabiliteten hos polymerkulorna visar att de är attraktiva som en enkel och kostnadseffektiv metod för upprening av hemoglobin.

# List of Papers

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

| | |
|---|---|
| Paper I | Preparation of Protein Imprinted Polymer Beads by Pickering Emulsion Polymerization<br>Tongchang Zhou, **Ka Zhang**, Tripta Kamra, Leif Bülow and Lei Ye<br>Journal of Materials Chemistry B, 2015, 3, 1254-1260. |
| Paper II | Chromatographic Separation of Hemoglobin Variants Using Robust Molecularly Imprinted Polymers<br>**Ka Zhang,** Tongchang Zhou, Karin Kettisen, Lei Ye and Leif Bülow<br>Talanta, in press. |
| Paper III | Characterization of Protein-Protein Interactions in Recombinant Hemoglobin Producing Escherichia coli Cells Using Molecularly Imprinted Polymers<br>**Ka Zhang,** Tongchang Zhou, Lei Ye and Leif Bülow<br>Advances in Experimental Medicine and Biology, 2017, 977, 367-373. |
| Paper IV | Characterization of a Haptoglobin Mimic Prepared by Molecular Imprinting - Recognition and Protection against Lipid Peroxidation<br>**Ka Zhang**, Tongchang Zhou, Sandeep Chakane, Cedric Dicko, Lei Ye and Leif Bülow.<br>Submitted |
| Paper V | Genetic Conjugation of Green Fluorescent Protein to Fetal Hemoglobin: Functional Properties and Binding to Hemoglobin Imprinted Polymers<br>Sandeep Chakane, **Ka Zhang** and Leif Bülow<br>Manuscript |

Paper VI Preparation and Application of a Highly Selective Fetal Hemoglobin - Imprinted Absorbent

**Ka Zhang,** Tongchang Zhou, Cedric Dicko, Lei Ye and Leif Bülow
Submitted

## Papers not included in the thesis

Paper I Ag-Polymer Nanocomposites for Capture, Detection and Destruction of Bacteria

Haiyue Gong, **Ka Zhang**, Cedric Dicko, Leif Bülow, Lei Ye
Submitted

## My contribution to the papers:

**Paper I** I participated in planning the work, performed the SDS-PAGE experiment, participated in the analytical work, and assisted in writing the manuscript.

**Paper II** I participated in planning, performed all the experiments, and wrote the manuscript.

**Paper III** I participated in planning, performed all the experiments, and wrote the manuscript.

**Paper IV** I participated in planning, performed all the experiments, and wrote the manuscript.

**Paper V** I participated in planning the work, prepared the molecularly imprinted polymers, performed all the analytical work regarding the imprinted polymers and wrote the manuscript together with Sandeep Chakane.

**Paper VI** I participated in planning, performed all the experiments, and wrote the manuscript.

# Abbreviations

| | |
|---|---|
| 2, 3-DPG | 2,3-diphosphoglycerate |
| BSA | Bovine serum albumin |
| CD | Circular dichroism |
| CO | Carbon monoxide |
| $CO_2$ | Carbon dioxide |
| CSF | Cerebrospinal fluid |
| DPGM | Diphosphoglycerate mutase |
| *E. coli* | *Escherichia coli* |
| FBOCs | Fluorocarbon-based oxygen carriers |
| fHbF | Fusion fetal hemoglobin |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| GFP | Green fluorescent protein |
| Hb | Hemoglobin |
| HbA | Adult hemoglobin |
| HbC | Hemoglobin C |
| HbE | Hemoglobin E |
| HbF | Fetal hemoglobin |
| HBOCs | Hemoglobin-based oxygen carriers |
| HCP | Host cell protein |
| HIC | Hydrophobic interaction chromatography |
| HIV | Human immunodeficiency virus |
| Hp | Haptoglobin |
| HPLC | High-performance liquid chromatography |
| IEC | Ion-exchange chromatography |
| Ig | Immunoglobin |
| IMAC | Immobilized metal ion affinity chromatography |
| MAA | Methacrylic acid |
| Mb | Myoglobin |
| MIP | Molecularly imprinted polymer |
| MMC | Multimodal chromatography |
| NIP | Non-imprinted polymer |
| RBCs | Red blood cells |

| | |
|---|---|
| SAXS | Small angle X-ray scattering |
| SBSE | Stir bar sorption extraction |
| SDS | Sodium dodecyl sulphate |
| SEM | Scanning electron microscopy |
| SEC | Size exclusion chromatography |
| SERS | Surface-enhanced Raman spectroscopy |
| SOSG | Singlet oxygen sensor green |
| SPE | Solid phase extraction |
| SPME | Solid-phase microextraction |
| WHO | World health organization |

# 1 Introduction

Proteins belong to the most fundamental molecules of biology, found in all living cells, in animals and plants. Except for water, proteins are the most abundant substance in the human body (1). Hemoglobin (Hb), an iron-containing oxygen-transport metalloprotein in the red blood cells of all vertebrates as well as in the tissues of some invertebrates, occupies a significant place in the annals of protein research. Hb has probably been studied more than any other protein. Much of our knowledge of human physiology and of several aspects of pathology has its antecedents in laboratory and clinical studies on Hb. Work on Hb began in the early 1800s, when it was found to be a major component of mammalian blood cells and, importantly, found to contain iron (2). In 1825, J. F. Engelhard discovered that the Fe/protein ratio is identical in the Hb of several different species. He determined the molecular mass of Hb as approximately 64,000 Daltons – the first determination of molecular mass of a protein (3). The oxygen-carrying protein hemoglobin was discovered by Hünefeld in 1840 (4). In 1959, Max Perutz elucidated the molecular structure of Hb through X-ray crystallography (5). For this achievement he was awarded the Nobel Prize for chemistry in 1962.

In the human body, Hb is not homogeneous and usually different variants and derivatives exist. Hb in adult RBCs, adult Hb (HbA), is the most common form of Hb. Human fetal hemoglobin (HbF) is the primary Hb in fetal erythrocytes during the last seven months of development in the uterus and lasts up to six months after birth (6). Hb mutants are also observed in the human population, caused by mutations in the chromosomes. Over the last century, our understanding of the genetics and functions of Hb proteins has greatly expanded. It is likely that current developments in Hb research will have significant clinical implications for understanding and treating the prevalent diseases related to Hb. In the meantime, research in Hb-based oxygen carriers (HBOCs) has become a relevant topic (7-10). The Hb present in the blood is essential to our survival – we would perish without oxygen transported from the respiratory organs to the rest of the body. Blood transfusion is a potentially life-saving procedure that could help replace the blood lost. A blood substitute (also called artificial blood), conceived to provide an alternative to blood transfusion, is a substance used to mimic and fulfill some functions of biological

blood. The main category of "oxygen-carrying" blood substitutes being pursued is the HBOCs.

For the developments of HBOCs, large amounts of different types of highly purified Hb need to be isolated and characterized for the practical application and new technologies need to be initiated to meet the requirements. With the advent of supramolecular chemistry in the 1980s, considerable efforts have been invested in the design of artificial molecular materials that possess defined, predictable recognition properties (11-12). Molecular recognition refers to the specific interactions that occur between two molecules. Special focus has here been placed on molecular imprinting, often described as a method of making a molecular lock to match a molecular key. This technique involves the creation of molecularly imprinted polymers (MIPs) with tailor-made binding sites complementary to the template molecules in shape, size and functional groups (13-17). Nowadays, this approach is considered as a straightforward and versatile method for the generation of artificial receptors for both small organic compounds and large biological molecules. MIP particles, especially those with high selectivity in aqueous solutions, have recently become attractive as they can be potentially used as selective adsorbents for solid-phase extraction as well as for chromatographic separations (18-22). For these purposes, it is desirable to prepare MIP beads with regular sizes and shapes since this is of critical importance to their performance. Furthermore, it is desirable to develop water-compatible MIPs that can be directly used in aqueous solution, while most of the conventional MIPs exhibited recognition properties only under non-aqueous environment. In this thesis, a new molecular imprinting method based on nanoparticle-stabilized emulsion (Pickering emulsion) was applied to prepare Hb-imprinted spherical beads.

The primary objective of this thesis has been to investigate the application of Hb-imprinted polymer beads, focusing on recognition and purification of different Hb variants. In Paper I, a new imprinting method – Pickering emulsion polymerization – was employed to prepare spherical MIPs with high selectivity. The interfacial protein imprinting led to the formation of recognition sites on the surface of highly cross-linked polymer beads. In Papers II and III, the MIP particles were used as a novel and efficient chromatographic resin to selectively recognize and purify different Hb variants. We also identified one *E. coli* host protein, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), which could interact strongly with fetal Hb (HbF). In Paper IV, the possibility of using MIPs as Hp mimics *in vitro* was studied. The MIPs had significant selectivity for recombinant Hb and could decrease the oxidative damage caused by cell-free Hb. In Paper V, we generated fluorescent Hb (GFP-fHbF) by genetic linkage of fusion fetal hemoglobin (fHbF) with the green florescent protein (GFP) and it was applied to evaluate the efficiency of Hb-imprinted polymers. In Paper VI, instead of using adult Hb as a template, we used fetal Hb and prepared the HbF-imprinted polymer beads.

# 2 Proteins

Proteins are present in all living organisms and most proteins consist of linear (not branched and not forming rings) polymers built from series of up to 20 different amino acids. It has been estimated that the average-sized bacteria contains approximately two million proteins per cell (23). The name "protein" is derived from the Greek word *Proteios*, meaning "primary" or "holding the first place" and was first used by the Dutch chemist Gerard Johann Mulder in 1838 (24).

## 2.1 Primary functions of proteins

According to the Central Dogma of Molecular Biology (originally proposed by Francis Crick in 1958), genetic information is transferred from DNA to RNA to proteins (25). Proteins belong to the most versatile macromolecules in the living systems present on the earth today and serve crucial functions in essentially all biological processes that make life possible. Proteins are also one of the most important nutrients required by our body and must be consumed in an adequate quantity and quality in the diet.

Countless processes use proteins and each protein has one or more specific functions. They can act as catalysts and almost all the complex chemical functions of the living cells are performed by protein-based catalysts called enzymes. They transport and store other molecules such as oxygen and mineral ions. Antibodies called immunoglobins (Ig) are large Y-shaped proteins, produced mainly by plasma cells to recognize and destroy foreign pathogens such as bacteria and viruses. They can also afford mechanical support, generate movement, transmit nerve impulses and control growth and differentiation. Examples of protein functions are listed in Table 2-1 (1).

**Table 2-1.** Examples of protein functions

| Function | Description | Example |
|---|---|---|
| Antibody | Antibodies identify and defend against specific foreign particles, such as viruses and bacteria. | Immunoglobulin G |
| Enzyme | Enzymes are proteins that facilitate biochemical reactions. | Trypsin |
| Messenger | Messenger proteins, such as some types of hormones, transmit signals to coordinate certain biological processes. | Insulin |
| Structural component | Structural proteins are the most abundant class of proteins in nature. Structural proteins confer stiffness and rigidity to otherwise fluid biological components. On a larger scale, they also allow the body to move. | Collagen<br>Actin |
| Transport/storage | These proteins bind and carry atoms or small molecules. | Ferritin<br>Hemoglobin |

## 2.2 Protein–protein interactions

Until the late 1990s, characterization of protein functions primarily focused on single proteins. However, proteins rarely act as isolated species while performing their functions *in vivo*. The vast majority of proteins interact with others for proper biological activity and it has been revealed that over 80% of proteins do not operate alone but rather in various weak or strong complexes (26). Understanding protein-protein interaction and identifying biological networks have thus become vital to understanding how proteins function.

Proteins could bind to each other through a combination of hydrophobic interactions, van der Waals forces and salt bridges at specific binding domains on each protein. These domains can be small binding clefts or large surfaces. They can be just a few peptides long or span hundreds of amino acids (27-28). Protein–protein interaction information can represent both transient and stable interactions:

- Stable interactions are formed in proteins that are purified as multi-subunit complexes, and the subunits of these complexes can be identical or different (e.g. ribosome, hemoglobin).
- Transient interactions are temporary in nature and are considered to control the majority of cellular processes (29-30). Transient interactions can be strong or weak and fast or slow. Weak transient complexes show a dynamic

mixture of different oligomeric states, whereas strong transient complexes change their quaternary state only when triggered by ligand binding.

Protein–protein interactions are key biological events in all living cells. Proteins interact with each other to perform certain functions. The functionality of unidentified proteins can be predicted on the evidence of their interaction with a protein, whose function has already been revealed (31-32). Protein–protein interactions can also be used in protein purification. Protein A is a 42 kDa surface protein originally found in the cell wall of the bacteria *Staphylococcus aureus* (33). It can bind with a strong affinity to immunoglobulins (Figure 2-1). Nowadays, chromatographic separation using protein A immobilized on porous substrates is the most widely established method for purifying monoclonal antibodies (mAbs) from cell culture supernatant (34-35). In Paper III, protein–protein interactions in recombinant Hb producing *Escherichia coli* cells were characterized using molecularly imprinted polymers.

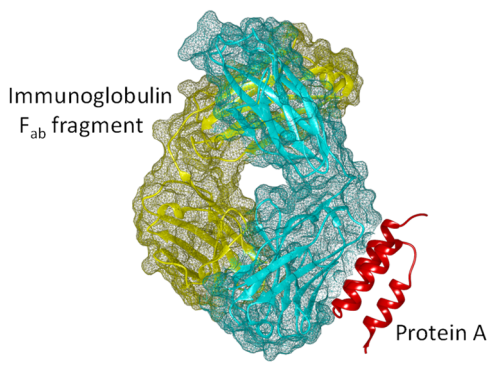


**Figure 2-1.** Structure of the $F_{ab}$ fragment of an antibody bound to Protein A (based on PDB 1DEE). Protein A is folded into a three-helix bundle (red).

## 2.3 Protein purification

Purified proteins are useful as experimental tools, commercial products and therapeutics in the treatment of diseases. With the rapid growth of the protein drug market and recent advances in proteomics, the demand for large quantities of pure, active proteins has been increasing. It is often impossible to obtain satisfactory yields from natural sources; therefore, the challenge for producing proteins at the desired scale and quality has led to the development of a diverse set of methods for heterologous protein production (36-37).

Protein production is referred to as the biotechnological process of generating a specific protein. A protein must be isolated from the host cell and followed by several downstream processes. This process usually begins with cell lysis during which a cell membrane and cell wall are disrupted, releasing its internal contents into a solution and forming a crude extract. The desired protein could then be purified from other cellular components using a variety of techniques exploiting differences in protein size, physicochemical properties, binding affinity and biological activity (38-39). Liquid chromatography is by far the most common method to purify high-value proteins, since this method is reliable and reproducible. Complex strategies are required to obtain a pure protein, including different modalities such as ion exchange, hydrophobic interaction, immobilized metal ion affinity and/or multimodal chromatography. The level of purification can, for instance, be monitored using various types of gel electrophoresis and mass spectroscopy.

### 2.3.1 Ion-exchange chromatography

Ionic interactions are the basis for purification of proteins by ion-exchange chromatography (IEC). The separation of IEC occurs due to the competition between proteins with different surface charges for oppositely charged groups on an ion exchanger adsorbent (40). Proteins usually contain charged amino acid residues. The total net charge at the isoelectric point (pI) of a protein will be zero and the location of this point depends on the amino acid composition. The protein will become charged at pH values lower or higher than its pI due to the acceptance or loss of protons ($H^+$). Positive charges are usually provided by arginine, lysine and histidine, and negative charges are principally provided by aspartate and glutamate residues. Weakly acidic side chains (cysteine, tyrosine and serine) are also able to have a charge if the pH is high enough that they tend to deprotonate.

Anion- and cation exchange chromatography, as the names imply, will bind anionic- and cationic proteins. The interaction between a protein and an ion exchanger depends not only on the net charge and the ionic strength, but also on the surface charge distribution of the protein. The charged amino acid residues could be distributed uniformly on the surface of the protein or they could be clustered such that one region is highly positive while another region is highly negative (41). Additionally, some structural changes can affect the separation by IEC since the chromatographic behavior also depends on protein conformation (42). Figure 2-2 shows an example, a chromatogram of HbA purification from a bacterial extract using cation exchange chromatography.

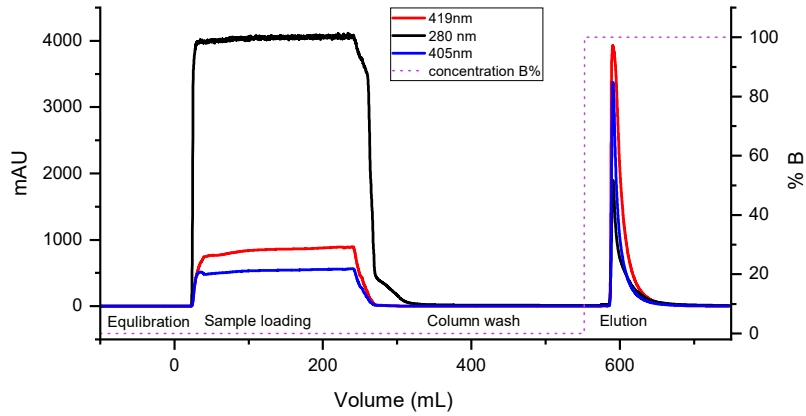


**Figure 2-2.** Chromatogram of purification of recombinant HbA from an *E. coli* crude extract using cation exchange chromatography (HiScale CaptoS, GE Healthcare) including equilibration, sample loading, column wash and elution.

## 2.3.2 Hydrophobic interaction chromatography

Hydrophobic interaction chromatography (HIC) is one of the most widely used methods for separating and purifying proteins in their native state. The term "*hydrophobe*" means "afraid of water" and refers to the physical property of a molecule that is repelled by water. Hydrophobic interaction chromatography is a separation technique that uses the property of hydrophobicity to separate proteins from one another. Generally, hydrophobic amino acids are those with side chains that lack active groups for formation of hydrogen bonds with water. Most hydrophobic amino acid residues are buried on the inside of a protein, but some are also observed on the surface (43). The proteins are separated according to differences in the amount of exposed hydrophobic amino acids. The number and spatial distribution of hydrophobic amino acid residues present on the surface of the protein determine the ability of the protein to bind to hydrophobic column materials. The interaction is dependent on the salt concentration of the solution and the protein mixture is loaded on the column in a buffer with a high concentration of salt to promote hydrophobic interactions. Usually, a decreasing salt gradient is subsequently used to elute proteins from the column. Additionally, elution can also be achieved with mild organic modifiers or detergents (44-45).

### 2.3.3 Affinity chromatography

Affinity chromatography is based on highly specific biological interactions between a protein and other molecules. These interactions could occur with low molecular weight substances but particularly occur with other proteins. A particular ligand is covalently immobilized to an inert chromatographic matrix that favors the specific and reversible binding of the target protein to the ligand. When a complex mixture is passing through the column, only the intended protein is adsorbed from the extract. After other substances are washed away, the bound molecule is stripped from the support by changing the composition of the mobile phase. By affinity chromatography, high-selectivity separation of biomolecules can be achieved through their specific interactions. However, applications of the technique are limited by the availability of immobilized ligands (46-47).

To simplify purification steps, genetic engineering is also used to add chemical features to proteins that make them easier to purify without affecting their structure or activity. For example, a series of histidine residues (a "His-tag") can be genetically attached to the terminus of proteins. When the lysate is passed through a column containing metal ions such as nickel or copper, the histidine residues resultantly become attached to the column while the untagged components of the lysate pass unimpeded. This technique is called immobilized metal ion affinity chromatography (IMAC). IMAC relies on the formation of weak coordinate bonds between immobilized metal ions and some amino acids on proteins, primarily histidine residues (48). It is also referred to as metal chelate chromatography, metal ion interaction chromatography and ligand-exchange chromatography. Figure 2-3 shows an example of an IMAC based chromatography resin.

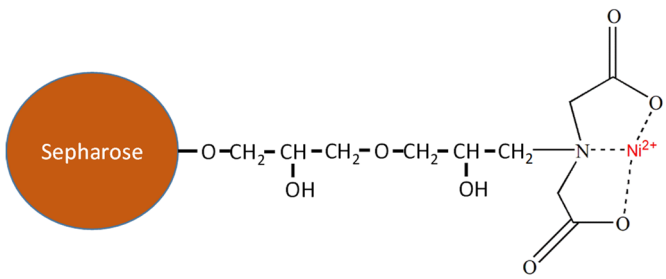


**Figure 2-3.** Schematic illustration of an immobilized metal ion affinity chromatography material using Ni as transition metal ion. A metal chelate forming ligand (iminodiacetic acid) was here coupled to Sepharose via an aliphatic spacer arm.

### 2.3.4 Size-exclusion chromatography

Size exclusion chromatography (SEC) is a method where separation of proteins occurs according to their size and shape in the solution. The principle of SEC is based on how efficiently the proteins penetrate the pores of the stationary phase (Figure 2-4). Unlike other modes of chromatography, it relies on the absence of any interaction between the analyte and the matrices packed in the column. The technique is sometimes also referred to as molecular sieve chromatography. Typically, when an aqueous solution is used as a mobile phase, the technique is known as gel-filtration chromatography, versus the name gel permeation chromatography, which is used when an organic solvent is used. In general, SEC is considered as a low-resolution chromatography form since it does not discern similar species very well and is therefore often reserved for the final step during purification. Size exclusion chromatography is also suitable for quantifying protein mixtures, and is a valuable technique for quality control in recombinant protein manufacture, such as measuring aggregates (dimers, trimers, tetramers, etc.) (49).

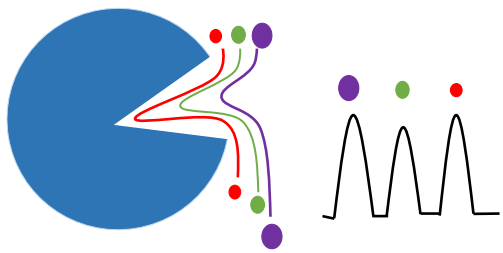

**Figure 2-4.** Molecules permeate the pores of the stationary phase to different extents depending on their size. Small molecules spend longer time in the pores and elute later. Larger molecules spend less time and have less access to the pores and elute sooner.

### 2.3.5 Multimodal chromatography

Multimodal chromatography (MMC) or mixed-mode chromatography, refers to chromatographic methods that combine more than one mechanism of interaction into the design of their ligands. Unlike traditional single-mode chromatography (such as affinity, ion exchange, hydrophobic interaction chromatography, etc.), the secondary interactions in MMC also contribute to the retention of the solutes (50). These different modes of interaction can cooperate or work independently. Electrostatic interactions are commonly involved, and hydrogen bonding and hydrophobic interactions can also be significant. The strength of these individual interactions depends on both the target molecules and the overall process conditions. MMC can be classified into two categories – physical MMC and chemical MMC. In

the physical MMC, the stationary phase is constructed of two or more types of packing materials. In the chemical MMC, just one type of packing material containing two or more functionalities is used. MMC has been explored for purification of a number of different biomolecules, including peptides, proteins, nucleotides and oligosaccharides (51-52).

The requirement for high quality protein products such as pharmaceuticals is expected to increase in the future. Primarily because of final purity demands, the bottleneck in protein production has been shifted from upstream to downstream (53). To meet these demands, new generations of chromatography media are required.

# 3 Human hemoglobin

The primary proteins in the globin family are myoglobin (Mb) and hemoglobin (Hb). The globin family is the most investigated group of proteins in the biological and medical sciences. Mb can reversibly bind oxygen and serves as a local oxygen reservoir that temporarily provides oxygen when blood oxygen delivery is insufficient during periods of intense muscular activity (54). Hb in the red blood cells (RBCs) is the dominant oxygen transport protein in the mammalian circulatory system, and an adult carries almost one kg of the protein (4). Mb was the first protein to have its three-dimensional structure revealed by X-ray crystallography (55). An overall illustration of the tertiary structure is presented in Figure 3-1. The globin is 16 kDa in size and consists of 8 α helices named A through H starting from the N-terminal to the C-terminal (55). Each helix at its turn is connected to a short coil segment named after its location; for example, CD is a coil that separates helix C from helix D. In human Hb, helix D is missing in the α-chain, and is instead replaced by a long coil segment – the CE corner.

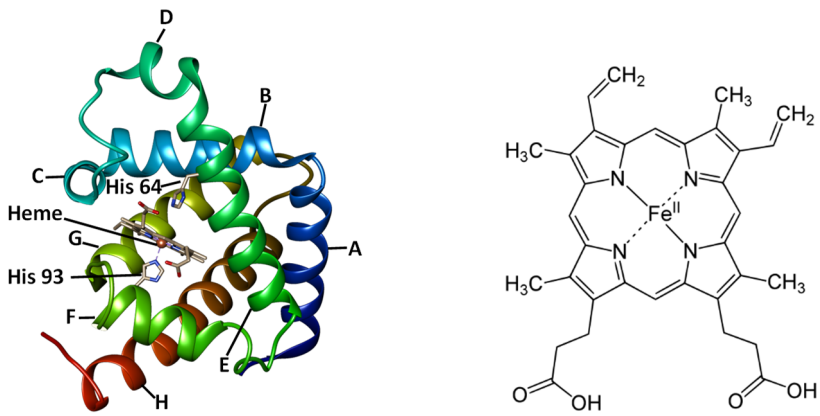


**Figure 3-1.** Left panel shows the tertiary structure of the globin as exemplified by myoglobin (PDB:1MBN). Right panel shows the structure of the heme group that is present in myoglobin and hemoglobin.

The heme group is the chemically reactive unit that is located in the "heme pocket" formed by the E and F helix. The heme group divides the heme pocket in a proximal and distal side. The proximal site (towards the helix F) is closely packed, but the distal site (towards the helix E) is less packed. The heme is surrounded by hydrophobic patches present at the bottom of the heme pocket along with the proximal (His 93) and distal histidine residues (His 64), respectively. An essential part of the heme group is the iron, pentacoordinated by nitrogen atoms present in the heme group and proximal His 93. The iron thus becomes engaged in five of its six coordination sites, while leaving one to coordinate with ligand. The ligands ($O_2$, NO, CO etc.) bind between the iron atom and the distal histidine residue. Globin-fold proteins in combination with heme are of great significance for life.

## 3.1 Human adult hemoglobin (HbA)

Human adult Hb, the most common human Hb, is a tetrameric protein consisting of two identical α (141 residues) and two identical β (146 residues) chains forming a $(\alpha\beta)_2$ homodimer (Figure 3-2).

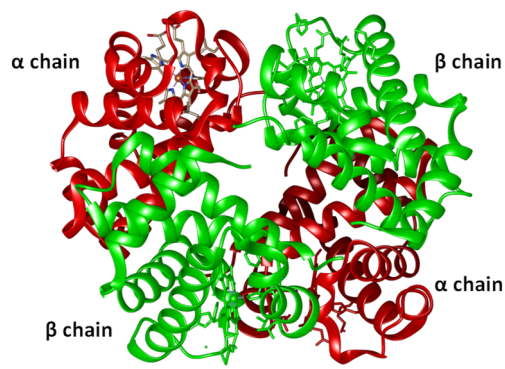


**Figure 3-2.** Structure of adult deoxyhemoglobin (PDB 2DN2). The adult hemoglobin molecule consists of two α (green) and two β (red) chains. Each chain carries a heme group.

The molecular weight is approximately 64 kDa with the monomers weighing approximately 16 kDa. Except the difference in the length of the polypeptide chains, there are also some differences in the composition of the amino acid residues between the α and β subunits. The primary structure of the α and β chains is approximately 50% identical. Almost no contact exists between the two β chains and only minor ones exist between the two α chains. Each α chain is involved with both the β chains

at two contact sites. The αβ interface is changed during the oxy and deoxy conformation, forming an allosteric character of the protein. The deoxyHb is primarily in the T (tense) state while the oxyHb is in the R (relaxed) state. At low concentrations, the tetramer dissociates into dimers consisting of one α and one β chain. However, tetramer-dimer dissociation is negligible inside the red blood cells where Hb concentration is high, i.e. in the millimolar range (9).

Fully loaded Hb can carry four molecules of $O_2$ as each subunit possesses a heme group at the center. The unloading of one $O_2$ molecule facilitates the unloading at other heme groups. This kind of behavior is called cooperativity and is physiologically important to enable efficient $O_2$ transport and delivery (56). The oxygen affinity can also be affected by allosteric effectors such as 2, 3-diphosphoglycerate (2.3-DPG), $CO_2$, $H^+$. Hb can bind CO with 250-fold higher affinity than that of oxygen. Consequently, the replacement of CO leads to a hypoxic state that could cause an acute death.

## 3.2 Human fetal hemoglobin (HbF)

HbF is nearly completely replaced by HbA in new-borns at approximately six months postnatally. In normal adult blood circulation, HbF is present at a low level ( $<$2%) (57). The level of HbF can be used as a clinical marker for the diagnosis and prognosis of diseases (58). HbF production can also be reactivated pharmacologically, which is a promising therapeutic approach in the treatment of diseases such as sickle-cell disease (59-60).

Human fetal Hb (HbF) is composed of two identical α and two identical γ subunits $(\alpha\gamma)_2$. The quaternary structure of HbF is depicted in Figure 3-3 and the amino acid sequence alignment of β chain and γ chain is shown in Figure 3-4. The overall quaternary structure of HbF is very similar to HbA; however, differences in 39 residues between the β and γ chain result in major differences in biophysical properties between HbA and HbF. Compared to HbA, HbF exhibits higher stability and oxygen affinity, enhanced pseudoperoxidase and nitrite reductase activities (61-64). Higher affinity for oxygen enables HbF to take oxygen from maternal blood into cord blood.

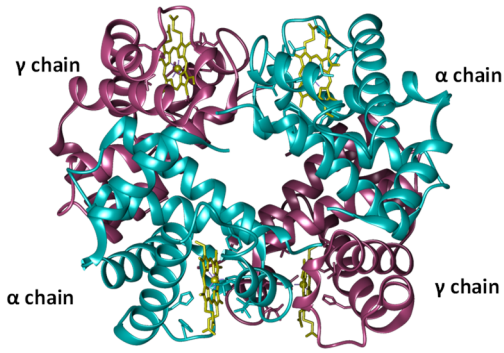


**Figure 3-3.** Structure of fetal deoxyhemoglobin (PDB: 1FDH). Heme groups are shown in yellow color, α chain in cyan and γ chain in hot pink.

```
MVHLTPEEKSAVTALWGKVNVDEVGGEALGRLLVVYPWTQRFFESFGDLS          P68871
MGHFTEEDKATITSLWGKVNVEDAGGETLGRLLVVYPWTQRFFDSFGNLS  50      P69892
TPDAVMGNPKVKAHGKKVLGAFSDGLAHLDNLKGTFATLSELHCDKLHVD          P68871
SASAIMGNPKVKAHGKKVLTSLGDAIKHLDDLKGTFAQLSELHCDKLHVD  100     P69892
PENFRLLGNVLVCVLAHHFGKEFTPPVQAAYQKVVAGVANALAHKYH             P68871
PENFKLLGNVLVTVLAIHFGKEFTPEVQASWQKMVTGVASALSSRYH     147     P69892

HBB Human    Hemoglobin subunit beta                         P68871
HBG2 Human   Hemoglobin subunit gamma 2                      P69892
```

**Figure 3-4.** Alignment of β chain and γ chain of Hb using the CLUSTAL program. The identity between the two chains is 73.5%.

## 3.3 Naturally occurring hemoglobin variants

There are several Hb genes in the human body (65). The gene that encodes the α subunit is located on chromosome 16 and is present in duplicate as HBA1 and HBA2. The β subunit is encoded by the HBB gene on human chromosome 11. The γ subunit is encoded on chromosome 11, and there are two similar copies of the γ subunit gene – HBG1 (γA) and HBG2 (γG). The difference is just one residue at position 136, γG has a glycine and γA carries an alanine.

Mutations in the genes encode the Hb protein result in a range of Hb variants. Several of these mutant forms of Hb are considered silent, which means that they result in no signs or symptoms to the carrier. Some of these mutant forms of Hb, however, are responsible for diseases and are considered as hemoglobinopathies (66).

The most extensively studied hemoglobinopathy is sickle-cell disease, which was the first human disease whose mechanism was understood at the molecular level. This disease is caused by the substitution of a single amino acid (Glu6Val) in the β chain, which is associated with malformation and destabilization of RBCs (67-68). Some other common and medically significant Hb variants include HbC, HbE and thalassemia (69).

In addition to these prevalent mutant proteins, different mutations of the globin chains of the human Hb molecule have been discovered that are rare individually but common collectively. A database of available Hb variants can be found in the globin gene server called *HbVar* (Table 3-1) (70). They are classified according to the type of mutation, the affected globin subunit and by the clinical and hematologic phenotype. Analyses of these variants, which can be considered to be "experiments of nature", have generated valuable insights into structure–function relationships within the Hb molecule.

**Table 3-1.** Summaries of mutation categories in HbVar server

| Query | Count of results |
|---|---|
| Total entries in database | 1760 |
| Total hemoglobin variant entries | 1305 |
| Total thalassemia entries | 493 |
| Total entries in both variant and thalassemia categories | 50 |
| Entries involving the alpha1 gene | 356 |
| Entries involving the alpha2 gene | 439 |
| Entries involving the beta gene | 909 |
| Entries involving the delta gene | 125 |
| Entries involving the gamma gene 1 | 61 |
| Entries involving the gamma gene 2 | 76 |
| Entries with an insertion mutation | 83 |
| Entries with a fusion gene mutation | 11 |
| Entries with a deletion mutation | 225 |
| Entries with a substitution mutation | 1423 |
| Hemoglobins with high oxygen affinity | 101 |
| Hemoglobins with low oxygen affinity | 49 |
| Unstable hemoglobins | 152 |
| Methemoglobins | 12 |

## 3.4 Recombinant hemoglobin

In 1972, Stanford University's Paul Berg first produced recombinant DNA and received the 1980 Nobel Prize in chemistry for it (71). Herbert Boyer from the University of California, San Francisco, transformed *E. coli* cells with recombinant plasmid in 1973 (72) and later founded Genetic Engineering Technology(Genentech, San Francisco, CA, USA). In 1982, Boyer's team developed and later launched the first human recombinant protein–recombinant human insulin (Humulin) (73). After more than three decades of continuous progress, recombinant proteins expand the market, representing the core of the human medical biotechnology industry. There is no doubt that the production of recombinant proteins in microbial systems has revolutionized biochemistry.

### 3.4.1 Fusion HbF

Molecular biotechnology has become an essential tool in Hb research and several strategies have been explored to optimize Hb properties, including fusion globin strategies. The first genetic modification Hb was achieved by Looker *et al.* who fused two α chains together and expressed it as a single polypeptide chain with 283 amino acids, Hb ($\alpha\alpha\beta_2$) (74). The purpose of the work was to develop a Hb macromolecule that would not dissociate into αβ dimers. In our laboratory, a fusion HbF molecule (Figure 3-5) has been designed. The C-terminus of the α chain and the N-terminal of the γ chain has been genetically joined by a linker composed of four repetitions of GGS (glycine-glycine-serine). The designed protein is named fusion fetal hemoglobin (fHbF) and is expressed as a single polypeptide chain. fHbF is a functional protein and behaves similar to a native Hb. In contrast to recombinant HbA and HbF, the expression yield of recombinant fHbF can be enhanced three-fold.

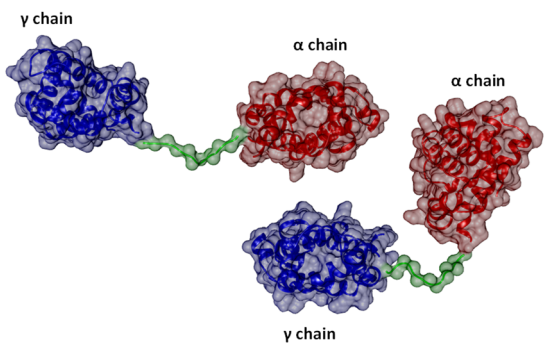


**Figure 3-5.** Small angle X-ray scattering (SAXS) was employed for the evaluation of the in-solution structure of fHbF. The dimeric protein was found to be in partial tetrameric state due to interaction between the α-(red) and γ-(blue) globins, linked by 12 amino acids linker (green).

### 3.4.2 Green fluorescent protein–fHbF

The green fluorescent protein (GFP) exhibits bright green fluorescence when exposed to light in the blue to ultraviolet range. It is composed of 238 amino acid residues (26.9 kDa) and in cell and molecular biology, the GFP gene is frequently used as a reporter of expression (75). Roger Y. Tsien, Osamu Shimomura, and Martin Chalfie were awarded the 2008 Nobel Prize in Chemistry for their discovery and development of GFP.

Hebelstrup *et al.* genetically linked GFP with plant Hb and the GFP-tagged Hb was used for the visualization of subcellular localization of plant Hb under various conditions (76). In Paper Ⅴ, a GFP molecule was genetically linked to the N-terminus of the fHbF (Figure 3-6). The GFP-fHbF displayed red color in normal light but turned green in the presence of UV-light. The expressed GFP–fHbF was functional as reflected by the fluorescence properties of GFP and spectroscopic properties of fHbF. The molecular size of GFP–fHbF as determined by SEC was 118 kDa, indicating that the fusion protein is present as a dimer.

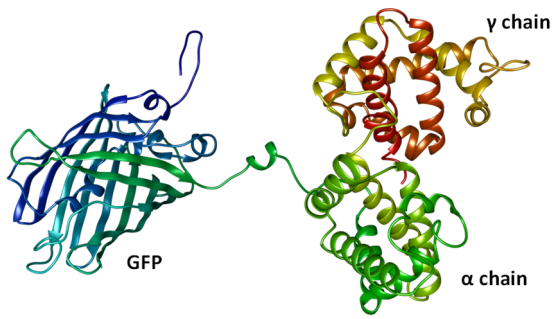


**Figure 3-6.** A Model for the tertiary structure of the GFP-fHbF. Online tool (Phyre 2) was employed for illustrating the structure. GFP (green) molecule was genetically linked to the α-globin (red), which in turn was linked to γ-globin (blue).

## 3.5 Hemoglobin-based oxygen carriers (HBOCs)

Blood is a vital healthcare resource used in a broad range of clinical services to save lives and improve health. According to the World Health Organization (WHO), 1-3% of the world's population needs to be blood donors in order to maintain adequate blood supply. Ensuring sufficient supplies of blood and blood products and prevention of transmission of HIV, hepatitis and other transfusion-transmissible infections have become major public health responsibilities of every national

government. While this may be achievable in the West, it represents a significant challenge for several developing countries. Therefore, searches for blood substitutes – infusible liquids that eliminate the need for refrigeration and cross matching, have a long shelf life and reduce the risk of iatrogenic infection – have been initiated.

Currently there are two types of blood substitutes – hemoglobin-based oxygen carriers (HBOCs) and fluorocarbon-based oxygen carriers (FBOCs). FBOCs are entirely synthetic hydrocarbon compounds capable of carrying and delivering oxygen under physiological conditions (77). HBOCs, however, have attracted most attention and been studied extensively. The main function of HBOCs is merely to mimic the oxygen transport capacity of natural Hb. HBOCs most often consist of concentrated solutions of purified acellular bovine or human Hb, which are either isolated from blood or expressed in different heterologous hosts including transgenic bacteria, mice, swine or yeast (78-83).

Unmodified cell-free Hb is not useful as a blood substitute because of its high oxygen affinity, the short half-life within the intravascular space, the tendency to undergo dissociation into dimers with resultant kidney damage and toxicity and causing vasoconstriction by taking up nitric oxide. Several efforts to overcome these problems have been undertaken, including making genetically engineered versions, cross-linking, polymerization, and encapsulation (Figure 3-7) (84). Several earlier developed HBOCs have entered different levels of clinical trials; however, most of the first generation products are discontinued due to the negative side effects on patients in the later trial phases. Hemopure, developed by Biopure Corp, is the only product approved in South Africa (85-86). It is a chemically stabilized, cross-linked bovine Hb intended for human use. However, some independent medical experts have claimed that Hemopure is not a substitute for blood but simply an effective temporary treatment until safe blood can be procured.

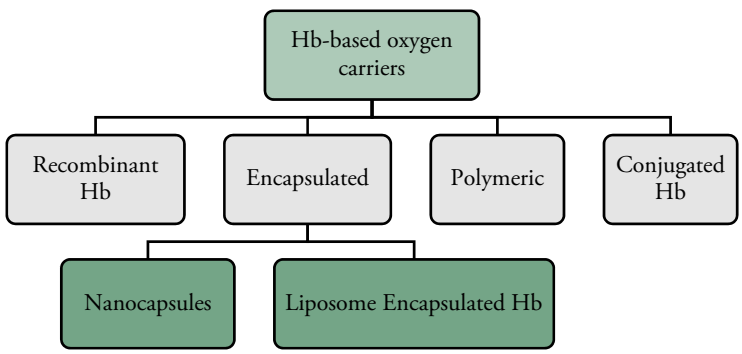


**Figure 3-7** Overview of alternative hemoglobin-based oxygen carriers (HBOCs).

Researchers are still searching for alternative HBOCs. Recent advances in molecular biotechnology have led to the development of increasingly refined and efficient methods for producing recombinant Hb. Some of these methodological improvements have been motivated by the goal of developing recombinant Hb-based blood substitutes (87). The expression yields can be improved by rational mutagenesis, thereby facilitating Hb production on a large scale. By different protein engineering strategies, the physiological suitability of Hb can be improved to address specific efficacy and toxicity issues. Modulating radical transfer via tyrosine residues on Hb is an example of the advantage of using recombinant Hb. By mutating Tyr42 to a non-redox active residue (αY42K) or by inserting tyrosine in an appropriate position (βF41Y), the radical transfer pathway can be modulated (88). Cysteine residues are also highly susceptible to redox modifications (89). By mutation (γC93A), the destructive reactions caused by oxidative modification associated with cysteine residues can be altered. Hb mutant could be a starting point for rational design of the next HBOCs generation.

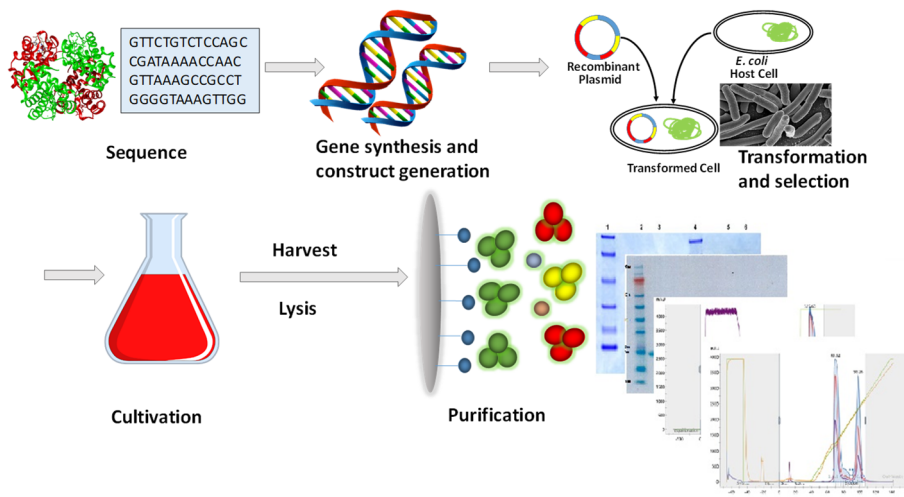


**Figure 3-8.** Recombinant hemoglobin production in an *E. coli* system.

Bacterial expression systems are commonly used for Hb production. These systems provide the most economical route for protein production and the short doubling time of most engineered strains enables rapid evaluation of experimental outcome. *E. coli* is the most popular bacterial system due to the low cost of media, fast growth, easy handling, high yield of target protein and the extensive knowledge available regarding the genetics of *E. coli* (90). Approximately 60% of all recombinant proteins in the literature and nearly 30% of currently approved recombinant therapeutic

proteins are produced this way. As shown in Figure 3-8, isolate the gene of Hb, clone it into expression vector, transform it into *E. coli*, cultivate the cells, induce and then the Hb is ready for purification and characterization.

# 4 Molecular imprinting

## 4.1 Molecularly imprinted polymers (MIPs)

In biological systems, almost all biological functions are performed by various molecular interactions that are based on specific molecular recognition, including enzyme catalysis, ligands and receptors interactions, immune responses, etc. (91-92). An important example of molecular recognition is the interaction between antibodies and antigens, often described in terms of a key in a lock model. Antibodies can normally fit perfectly with their target antigens and are able to recognize them with high selectivity and affinity. It has been a long-term goal of scientists to mimic natural receptors such as antibodies and enzymes. Considerable efforts have been invested in the design of artificial recognition molecular materials that possess defined, predictable recognition properties. Special focus has here been placed on molecularly imprinting, a versatile technique to create template-shaped cavities in polymer matrices with memory of the template molecules to be used for molecular recognition.

Molecular imprinting is based on the co-polymerization of functional and cross-linking monomers in the presence of a molecular template. The functional monomers, which are self-assembled around the template molecule through interaction between functional groups on both the template and monomers, are polymerized to form an imprinted matrix. Subsequent removal of the template molecule leaves cavities with size, shape and chemical functionality complementary to the template. Thus, a molecularly imprinted polymer (MIP) is prepared capable of selectively binding the target with affinities comparable to natural receptors. MIPs have been widely used in the field of separation science, catalysis, sensors and immunoassays and have often been called artificial antibodies (93-96).

## 4.2 Molecular imprinting approaches

The molecular recognition capabilities of MIPs are based on the specific interactions of a template with the functional groups located in the polymer cavities.

Depending on the interactions that occur between the template and functional monomers involved in the imprinting and rebinding steps, three types of molecular imprinting approaches exist for creating these specialized polymers, covalent, non-covalent and semi-covalent approaches (Figure 4-1).

The first approach of molecular imprinting was introduced by Wulff and co-workers in 1972, and is based on a covalent attachment strategy (97). The approach uses a reversible covalent bond to link the molecular template with the functional monomer, while then the covalent bond is broken to free the template from the solid polymer and form an imprinted site. Since the monomer-template conjugates are stable, a wide variety of polymerization conditions (e.g., high temperature, and low or high pH) can be employed. Functional groups responsible for binding are only located in the binding cavities, therefore restricting non-specific binding effects. However, the choice of covalent linkage is crucial and only a few reversible covalent bonds are available for this approach (98-102). Another issue that emerges is that the binding kinetics are often slow due to the necessity to reform the covalent bonds during the rebinding process.

Compared to covalent molecular imprinting, the non-covalent approach has become the most versatile and most widely used. It was first described in the early 1980s by Klaus Mosbach (103). In 1993, the group of Klaus Mosbach published the milestone study in "Nature" where non-covalent molecular imprinting was employed in a competitive binding assay for the detection of theophylline and diazepam in human serum (104). This paper helped popularize the technique of imprinting as means of generating synthetic antibody mimics. In the non-covalent approach, the template-functional monomer complex was stabilized by non-covalent interactions such as hydrogen bonding, van der Waals forces, ionic and hydrophobic interactions. The rebinding of the template to the imprinted sites was also realized by the same non-covalent interactions. The non-covalent approach is preferred due to its simplicity of preparation and the commercial availability of a large number of functional monomers. In addition, imprinted polymers prepared by the non-covalent approach display more rapid rebinding kinetics than those prepared by the covalent approach. However, since all these types of interactions are not strong, excess functional monomers are used in order to maintain the stability of the template-monomer complex under the polymerization conditions. Thus, the binding sites are heterogeneous and not well-defined, resulting in higher non-selective binding.

The semi-covalent approach was developed by Whitcombe and co-workers, where a covalent linkage between template and functional monomer was generated in the imprinting process and the template rebinding occurred through non-covalent interactions (105). The ratio of template molecule to functional monomer normally used in the semi-covalent approach is generally 1:1 or 1:2. The purpose of the semi-covalent approach is to unite the advantages of both covalent and non-covalent

approaches. However, the limited choice of functional monomers and the same limitations as those that arise in the covalent approach restricts the applicability of this technique. Furthermore, the process often requires harsh reaction conditions to cleave the template-functional group bonds for template removal.

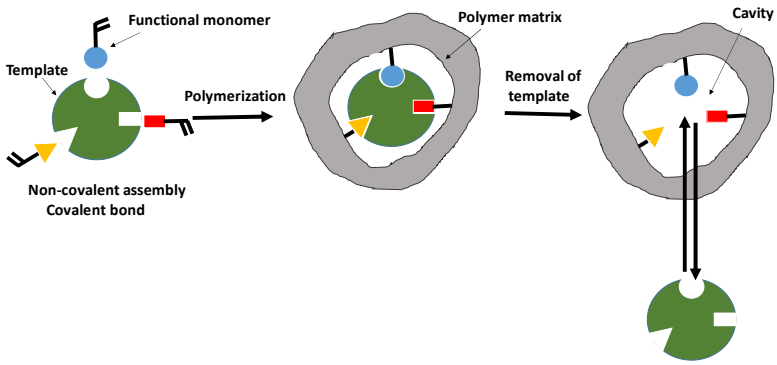


**Figure 4-1.** Schematic representation of the molecular imprinting process. The template and the functional monomers can interact by covalent or non-covalent bonds.

## 4.3 Different physical forms of MIPs

The shape and size of the MIPs are of critical importance for their performance. Different physical forms of MIPs can be obtained by using various synthetic strategies, depending on their final application. Traditionally, MIPs have been prepared as porous monoliths by bulk polymerization (106-107). After fragmentation and sieving, irregularly shaped particles in the desired size ranges required by the specific application are produced. Monolithic MIPs have also been prepared directly within a chromatographic column by *in situ* free radical polymerization process without grinding and column packing (108-109). Although bulk polymerization has been the only method for MIP synthesis for a long time and is still widely used due to its simplicity, some drawbacks such as the poor morphology control, presence of particles with irregular size and shape, imprinted sites being destroyed during grinding and its unsuitability for large-scale production are obvious. Alternative methods to prepare novel MIP forms have been developed in recent years.

One of the important formats especially when they are meant to be used as responsive layers in sensing devices, are MIP membranes. The transport properties across a MIP membrane are controlled through both sieving effects and imprinted cavities. It could overcome the problems associated with the limited accessibility of the imprinted sites of the bulk polymers as well as the lack of selectivity of commercial

membranes. Two types of MIP membranes have been proposed – self-supported MIP membranes and MIP composite membranes (14). Composite membranes have attracted much attention as they combine the mechanical strength of the supporting membrane and the selectivity of the MIPs. Two approaches have been reported to prepare MIP composite membranes – *in situ* MIP synthesis on a supporting membrane and incorporation of pre-synthesized MIP particles into membrane. However, low membrane permeability was still the main obstacle for their application in separation processing.

When used as affinity based chromatographic media or solid-phase extraction media, it is desirable to prepare MIP beads with regular sizes and shapes in sufficient quantity. Since the grinding process is unrealistic, a synthetic approach is required that directly generates beaded MIPs. A variety of procedures have been introduced in the last few years to produce spherical polymers directly, such as seed polymerization, suspension polymerization and precipitation polymerization (110-113). However, most polymerization methods generate organic solvent-compatible MIP beads that lack the selectivity required in aqueous solutions, limiting the application of MIPs in several biotechnological and biomedical systems. Efforts have recently been made to develop water-compatible MIPs that are applicable in aqueous solutions for potential biological and medical applications (Paper I).

## 4.4 Applications of MIPs in analytical chemistry

The peculiar properties of MIPs have made them a highly interesting tool for a wide range of applications in analytical chemistry, as diagrammatically shown in Figure 4-2. MIPs have been widely used in sample pre-treatment, chromatographic separation (SPE, monolithic column chromatography, etc.) and sensing (electrochemical sensing, fluorescence sensing, etc.) (13-15, 17, 94-95, 114).

The choice of appropriate sample pre-treatment techniques plays an important role in qualitative and quantitative determination. Compared to traditional extraction sorbents, MIPs are much better matched to the template molecule, so that they can identify the extract specifically and can be utilized as selective sorbent materials. In recent years, the applications of MIPs in pre-treatment techniques have attracted particular attention and have become a popular area of study among researchers.

MIPs are also used in chromatography as packing materials and monolithic column materials, due to their high affinity and selectivity to the target analytes. MIP columns have been used to carry out analytically relevant separations – the separation of enantiomers from each other and the separation of the template from other substances in a complex sample.

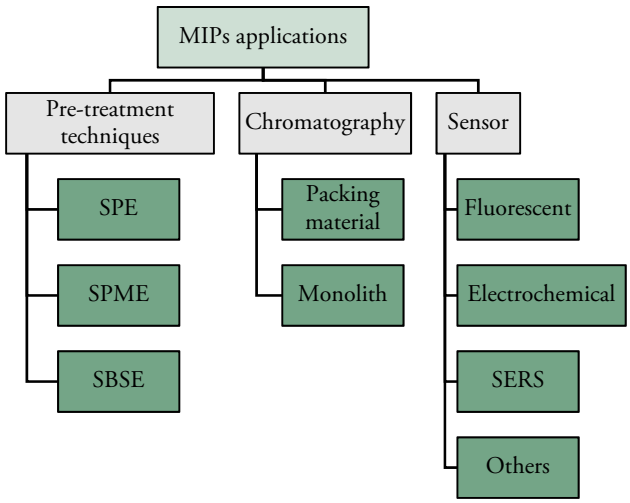


**Figure 4-2.** Structural diagram of the applications of MIPs in pre-treatment techniques, chromatography and sensor. Abbreviations: SPE, solid-phase extraction; SPME, solid-phase microextraction; SBSE, stir bar sorption extraction; SERS, Surface-enhanced Raman spectroscopy.

The employment of MIPs as specific materials in sensors, namely MIP-based sensors, has made considerable progress due to their high selectivity and stability, simplicity, cost-effectiveness, and versatility. It was first proposed by Andersson and Mosbach to monitor the specific binding of vitamin $K_1$ to a ''surface-imprinted'' silicon surface (115). The MIPs have both recognition and transduction properties, that is, as recognition elements can specifically bind target analytes and as transduction elements can generate output signals for detection.

## 4.5 Protein imprinting

### 4.5.1 Challenges in protein imprinting

The high stability and cost-effectiveness of MIPs has led to their application in a range of fields. However, to date, molecular imprinting has been successful primarily for low molecular weight compounds, e.g. drugs, metal ions, steroids and amino acid derivatives (114, 116-118). In the case of biological macromolecules such as proteins, the use of molecular imprinting is still a challenging task. The challenging is largely due to the low binding capacity and slow mass transfer because of the large size of protein, and heterogeneous binding sites. Furthermore, biomacromolecules are often

vulnerable to harsh conditions, such as high/low pH, high temperatures and salt concentrations that are often used to synthesize MIPs (119). However, the study on protein imprinting has shown rapid progress because of the enormous demand for protein-selective materials in the areas of biology and life science. Despite the development of MIPs for proteins had a slow start from 1995, but it exploded in 2005 and currently covers approximately 10% of all publications on MIPs (Figure 4-3)(120).

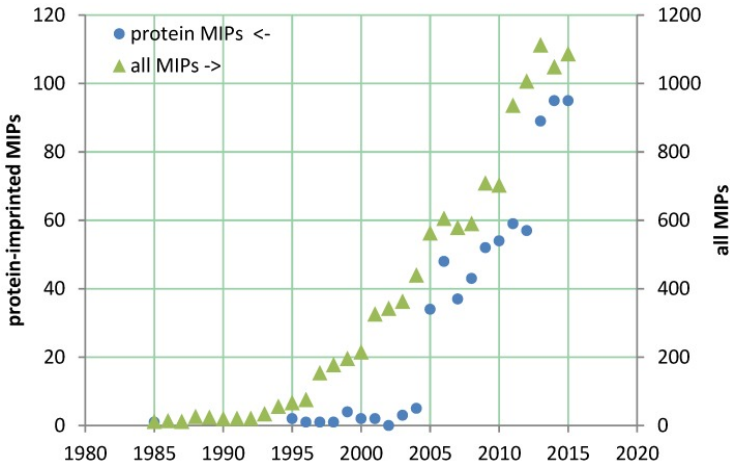


**Figure 4-3.** Number of publications on protein-imprinted (circles) and all MIPs (triangles) until 2015. Reprinted with permission from Menger et al. (2016) (120), Copyright 2016 MDPI.

### 4.5.2 Surface imprinting

Until now, the most convincing examples for protein recognition have been developed by surface imprinting techniques. Ratner and co-workers were among the first to create imprinted sites of proteins in a surface bound polymer film (121). They showed that a bovine serum albumin (BSA) imprinted surface preferentially adsorbed the template from a binary mixture. Surface imprinting improves the performance of MIPs since it solves the issues of limited mass transfer and template removal that is often associated with traditional molecular imprinting. This improvement is especially valuable when imprinting macromolecules, for which diffusion limitation is a major issue. For surface imprinting, the protein is normally absorbed or immobilized on the surface of beads, nanoparticles or flat surfaces. Binding sites generated on the surface often exhibit good accessibilities.

Several common methods are utilized to prepare and optimize MIPs in the spherical form with controllable shape and size distributions. Therefore, the best of both worlds is available – molecularly surface-imprinted beads. It increases the applicability of molecular imprinting for separation application and significantly enhances the possibility of it being used for industrial mass-production. There are various techniques to produce surface-imprinted spherical MIP particles. One of the common strategies is to use preformed support beads for the deposition of imprinted polymers, imparting a core-shell structure to the final imprinted polymer (122). To date, one direct way of creating core-shell MIP particles is through the process of core-shell emulsion polymerization and the common type of material used as support beads is silica due to its stability, favorable physical properties and its ease of derivatization (123-124). This approach involves a two-stage process where core particles are prepared in the first step followed by the creation of an imprinted shell on the seed particles in an emulsion polymerization. A surfactant such as sodium dodecyl sulphate (SDS) is often required to stabilize the emulsion. However, the complexity of the polymerization system and the use of surfactants affect the scope of application of this imprinting methodology.

### 4.5.3 Pickering emulsion polymerization

As discussed above, one challenging task in molecular imprinting is the generation of MIPs that are able to exhibit molecular recognition abilities in aqueous media. Most of the previously developed MIPs are only compatible with organic solvents and often fail to show specific binding in aqueous solutions. Water molecules can disrupt the important interactions required for selective recognition and can cause significant non-specific binding. In recent years, several efforts have been made to prepare water-compatible MIPs that are applicable in aqueous solutions.

In our work, a new strategy based on Pickering emulsion polymerization was developed to synthesize water-compatible molecularly imprinted polymer beads using silica nanoparticles as building blocks (Paper Ⅰ). A Pickering emulsion is an emulsion stabilized by solid particles in place of surfactants, which adsorb onto the interface between the two phases (Figure 4-4). It was named after S.U. Pickering, who described the phenomenon in 1907 (125). Although the first molecular imprinting from Pickering emulsions was reported only a few years ago, the number of publications in this area has increased rapidly and it has shown great promise for the preparation of MIP beads for selective recognition of pre-defined molecular targets (126-128). The surfactant-free character makes them attractive for several applications in various fields, in particular cosmetic and pharmaceutical applications where surfactants often show adverse effects.

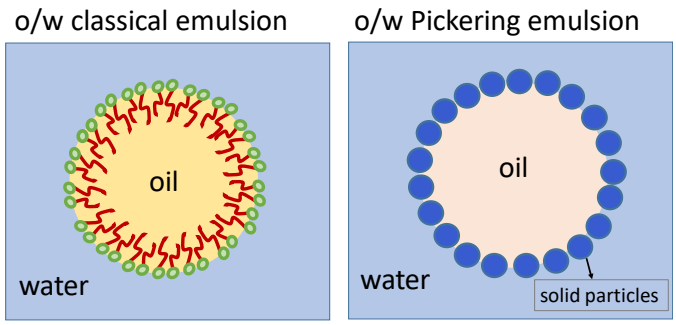


**Figure 4-4.** An oil/water classical emulsion versus an oil/water Pickering emulsion

The basic principle of droplet stabilization is the partitioning of solid particles between the two immiscible liquids. The contact angel θ between the interface and the surface of the particles is an important parameter. When the contact angle is approximately 90°, the energy required to stabilize the system is at its minimum. Depending on the surface tension of the solid particles, Pickering emulsions could be prepared as either oil-in-water or water-in-oil systems. By employing Pickering emulsion polymerization, genuinely surface-exposed and water-compatible MIP polymers composed of large pores decorated with easily accessible molecular binding sites can be prepared.

# 5 Hemoglobin-imprinted polymers

## 5.1 Hb-imprinting in Pickering emulsion

In our recent studies, we have demonstrated that Pickering emulsion offers a versatile system for preparing MIPs. In Paper I, HbA-imprinted polymer beads were prepared by combining the surface imprinting with Pickering emulsion polymerization (Figure 5-1). In the first step, HbA was adsorbed on the silica nanoparticles. The Hb-coated silica particles were subsequently used to stabilize an oil-in-water Pickering emulsion. Following free radical polymerization, the HbA-silica particles were removed and protein recognition sites on the surface of highly cross-linked polymer beads were formed.

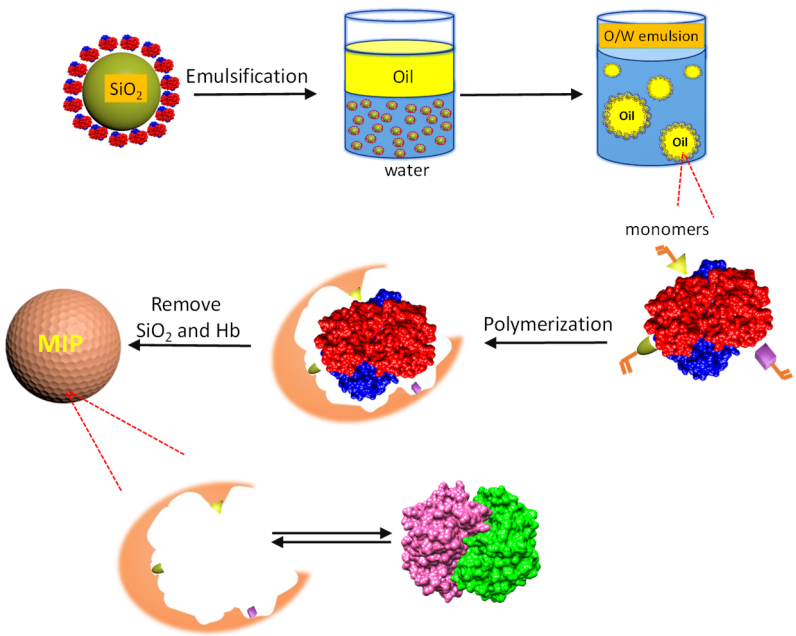


**Figure 5-1.** Preparation of HbA-imprinted polymer using Pickering emulsion polymerization.

As observed from the images by SEM (Figure 5-2), the size of the MIP beads was 25±8 μm and apparent protein aggregates were present on the surface of the beads after protein binding, which may be explained by Hb-Hb interactions. A typical human RBC has a disk diameter of approximately 6.2-8.2 μm and an average volume of about 90 femtolitres. Adult humans have roughly 20-30 trillion RBCs at any given time, comprising approximately 70% of all cells by number. Each human RBC contains approximately 300 million Hb molecules. Inside the RBCs, these Hb molecules are densely packed and some organizations of all Hb molecules could exist. Through such interactions, the apparent protein binding capacity could hence be higher. The maximum Hb binding capacity of MIPs was 32 mg Hb $g^{-1}$ polymer.

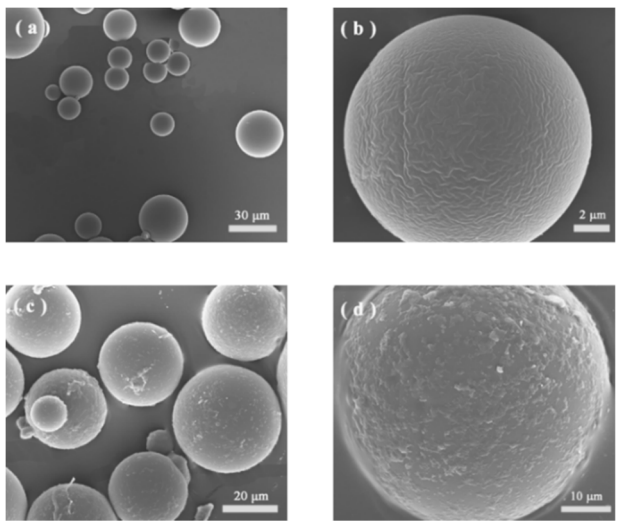


**Figure 5-2.** SEM images of MIP beads before (a), (b) and after (c), (d) binding experiment.

The obtained MIP particles with the binding sites located on the surface exhibited fast adsorption kinetics and significant selectivity for Hb. The optimal binding was observed at pH value around 6.0. By merely changing the pH from pH 6.0 to pH 8.0, the bound Hb could be eluted from the MIP particles (Figure 5-3). At pH 6.0, histidine (pKa=6.10), lysine (pKa=10.79) and arginine (pKa=12.48) residues possess positive charges. Since the MIP particles are based on a polyacrylic acid polymer, they will be negatively charged at pH values above four. At pH 8.0, the ionic interactions between the particles and protein are disrupted. This mild elution condition is attractive for a number of applications especially in bioseparation processes.

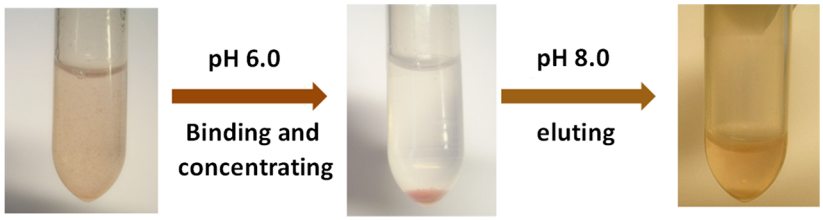


**Figure 5-3.** Capture of HbA at pH 6.0 and elute at pH 8.0. Hb in 1 mL 20 mM pH 6.0 phosphate buffer was added into 5 mg MIPs. After 10 minutes of incubation, the solution was concentrated and the supernatant was removed. The bound Hb was then eluted with 200 µL 20 mM pH 8.0 phosphate buffer.

When Hb was physically absorbed on silica surface, the interactions between silica and Hb could be broken by pH change or the adding of chemicals; therefore, the conditions need to be well controlled in order to produce a stable oil-in-water emulsion. To avoid this, in Paper VI, HbF is first covalently bound through its surface-exposed amino-groups to silica nanoparticles. The HbF immobilized silica nanoparticles then acted as the stabilizing particles to establish the oil-in-water Pickering emulsion (Figure 5-4).

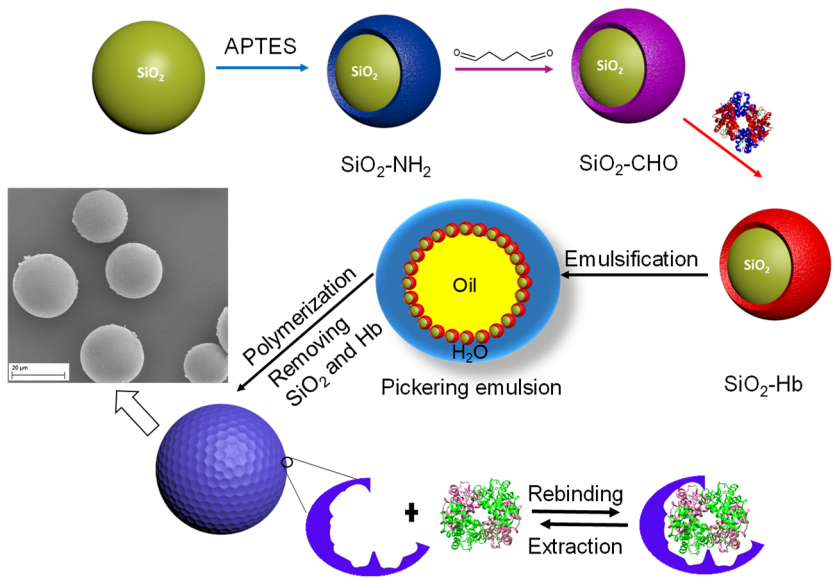


**Figure 5-4.** Schematic of preparation of HbF-imprinted polymers. HbF was firstly immobilized on silica surface.

Instead of using HbA, here HbF was selected as the template molecule. Figure 5-5 shows that compared to HbA, HbF is more effective in competing with GFP-fHbF to interact with MIP particles. This assay demonstrated that the HbF-imprinted polymers displayed better selectivity for HbF than HbA; therefore, HbF had a higher level of inhibition for the GFP-fHbF binding to the specific sites.

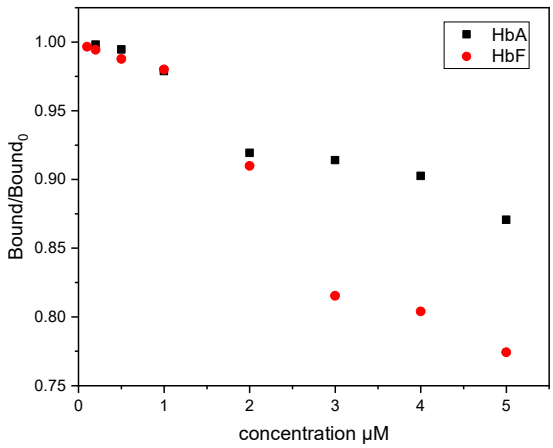


**Figure 5-5.** Displacement of GFP-fHbF from 5 mg of MIP particles by wildtype HbA and HbF. Bound and $Bound_0$ are the amount of the bound GFP-fHbF measured in the presence and absence of competing HbA /HbF, respectively.

## 5.2 Capture of Hb from complex biological solutions

In Paper II, Hb-imprinted polymer beads were used as a resin to selectively recognize and capture different Hb variants from either crude *E. coli* extracts or human body fluids, such as blood plasma and cerebrospinal fluid (CSF). Bacterial production of Hb is complex and several different misfolded and aggregated polypeptides are often observed. In addition, large amounts of *E. coli* host cell proteins (HCP), modified heme, and free porphyrin need to be removed (87, 129).

Figure 5-6 shows the elution profiles of recombinant HbA, HbF and fHbF *E. coli* crude cell extracts from the Hb-MIP column. The bound Hb molecules could be eluted from the HbA-MIP column by merely changing the pH from 6.0 to 8.0. When 0.1 M salt was added into the eluting buffer, Hb samples emerged earlier with sharper peaks. Although the overall quaternary structure of HbF is very similar to HbA, the separation between HbA and HbF illustrated the highly selective effect of molecular imprinting. As described initially, the fHbF protein exists both as a monomer and

dimer. This is further proved by the Hb-MIP column as fHbF appeared as two peaks in the chromatogram. The first peak of fHbF had the same elution volume when compared with HbF. We concluded that the first peak contained the dimer, and the second contained the monomeric form of fHbF.

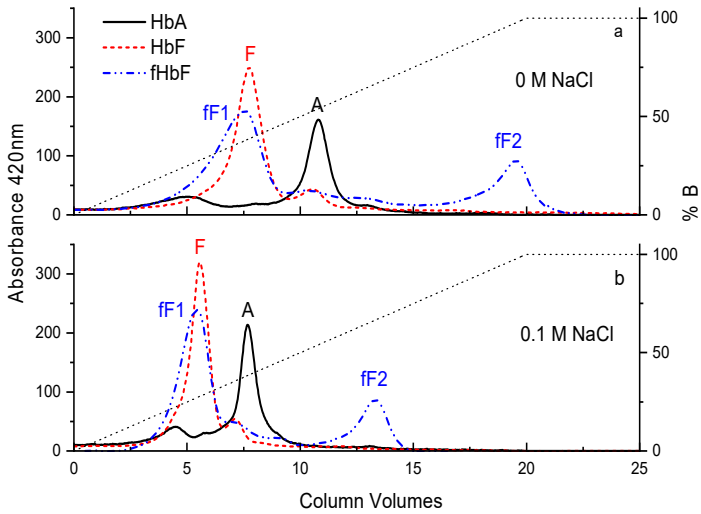


**Figure 5-6.** Elution profiles of recombinant HbA, HbF and fHbF *E. coli* crude cell extracts from the Hb-MIP column using a linear gradient of buffer A (20 mM sodium phosphate buffer pH 6.0) mixed with buffer B (20 mM sodium phosphate buffer pH 8.0 with/without 0.1 M NaCl).

When RBCs are lysed within the intravascular space, hemoglobin enters the plasma. Plasma hemoglobin is now recognized as a biologically active and toxic compound, primarily due to its iron-mediated oxidizing capabilities. The removal of extracellular Hb via extracorporeal filtration could be an easy and less costly method. For instance, there have been numerous studies in which extracorporeal devices have been used as therapy for sepsis (130). In Paper II, the applicability of the Hb-MIP column for Hb removal from plasma and CSF samples containing various dissolved proteins (i.e. serum albumins, globulins and fibrinogen) was examined. The chromatographic profiles (Figure 5-7) illustrated that the MIP resin proved to be completely functional in crude biological environments and could be used to selectively capture Hb from human plasma and CSF. The obtained results suggest that MIPs can have clinical applications in an extracorporeal shunt system.

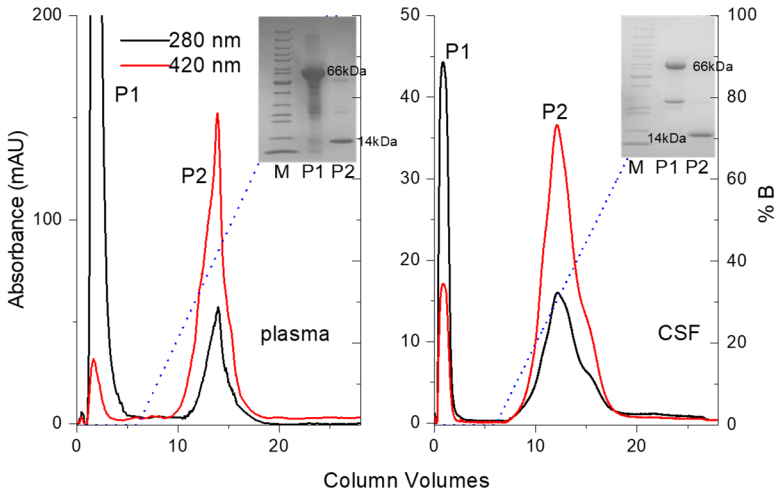


**Figure 5-7.** Chromatographic elution profiles of HbA in plasma and HbF in CSF samples from the Hb-MIP column and the SDS-PAGE of peak fractions (P1, P2). Linear gradient elution with buffer A (20 mM sodium phosphate buffer pH 6.0) mixed with buffer B (20 mM sodium phosphate buffer pH 8.0 supplemented with 0.1 M NaCl).

## 5.3 Hb variants characterization

We also examined the selectivity of the HbA-MIP resin by monitoring the elution behavior of different Hb variants carrying single amino acid substitutions (Figure 5-8) (Paper II). When lysine was removed from the surface of HbA (βK66Y), it eluted from the Hb-MIP column earlier than wildtype HbA. On the contrary, when an additional lysine residue was introduced (αY42K), this protein could interact stronger with the MIP particles; and therefore was eluted later. Irrespective of whether cysteine was replaced or added, for HbF mutants the affinity of the protein to the imprinted polymers did not alter. However, when alanine was exchanged to aspartic acid in the 12th or 19th position of the α chain of HbF (αA12D and αA19D), the retention behavior was altered. The aspartic acid residue on position 19 of α-chain is more exposed than αA12D, therefore αA19D HbF was eluted slight earlier than αA12D HbF.

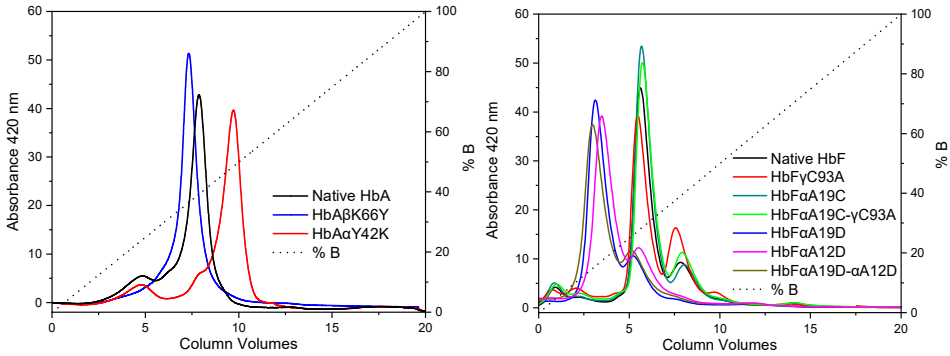


**Figure 5-8.** Chromatographic elution profiles of Hb mutants in *E. coli* crude extracts from the Hb-MIP column. Linear gradient elution with buffer A (20 mM sodium phosphate buffer pH 6.0) mixed with buffer B (20 mM sodium phosphate buffer pH 8.0 supplemented with 0.1 M NaCl).

The molecular recognition of GFP-fHbF to MIP particles was compared with fHbF (Paper V). The binding isotherm (Figure 5-9) showed that MIPs displayed slightly higher binding capacity for fHbF than for GFP-fHbF. Hb (pI: 6.8) has an overall positive charge at pH 6.0, which favors the binding to cavities of the functional groups present on the imprinted polymers. Additional GFP present on the N-terminus of the fHbF has decrease the pI of the protein (calculated pI: 6.7), and thus decreased the binding capacity of MIP particles. Moreover, GFP increases the size of protein by 3.6 folds. This increase could have also contributed in lower binding.

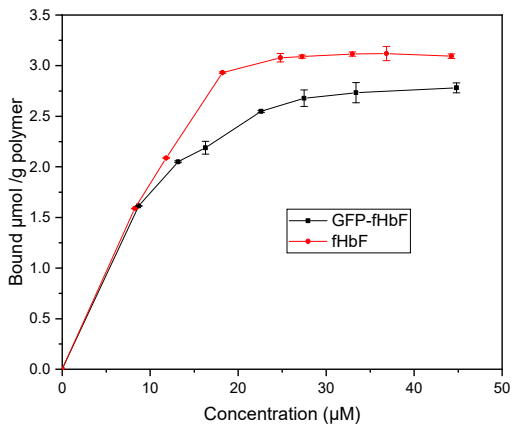


**Figure 5-9.** Binding capacity of GFP-fHbF and fHbF for imprinted polymers at different Hb concentrations. The concentration of MIPs used in each test was 5 mg $ml^{-1}$. The binding capacity was calculated based on bound and unbound protein present in the solution after incubation for five minutes at 4 $^{O}$C in 20 mM phosphate buffer pH 6.0.

## 5.4 Haptoglobin mimic by molecular imprinting

Hb in the red blood cells (RBCs) is the dominant oxygen transport protein in the mammalian circulatory system. Hb is safe and inert within the confinement of the RBCs but becomes reactive and toxic upon intravascular hemolysis, especially under conditions of hematological disorders or blood transfusion (131). Haptoglobin (Hp), an acute phase glycoprotein, counteracts the negative physiological consequences of intravascular hemolysis by binding free Hb released from erythrocytes with high affinity. The interaction between Hp and Hb is actually one of the strongest non-covalent events known in nature. When present in plasma, free Hb is almost instantly captured by Hp to form an Hb-Hp complex. The complex is extraordinarily strong, and it can be considered irreversible once formed. A high resolution structure of the human Hp-HbA complex has been descripted recently (Figure 5-10) (132).

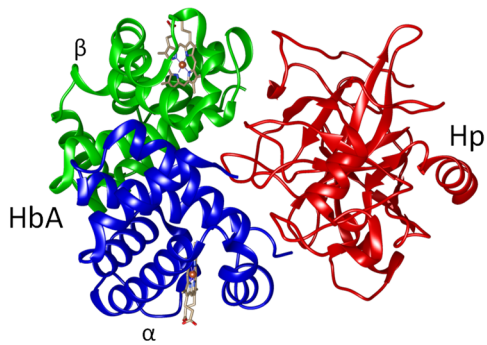


**Figure 5-10.** Structure of the human HbA-Hp complex based on PDB 4X0L.

Hp, in its simplest form, consists of two alpha and two beta chains, connected by disulfide bridges (133). The beta chains of Hp are involved in the binding of Hb. Since there are two possible variants of alpha subunits, α1 and α2, Hp may be present in multiple forms depending on which alpha subunit combines with the beta subunit to form the functional protein. In the human body, Hp occurs in three phenotypes – Hp 1-1, Hp 2-1 and Hp 2-2. Hb is present as dimers in the complex when bound to Hp, and each is located on the ends of the Hp moiety. This binding detoxifies Hb to a large extent and reduces further possible Hb side-reactions. Additionally, the Hb-Hp complex is recognized by CD163, which in turn impairs filtration and clearance of Hb dimers by the kidney (134).

Hp has previously been utilized clinically for treatment of hematological disorders, but limited availability of this protein reduces its clinical applicability (135). Small

amounts of Hp can be isolated and produced from human plasma. However, recombinant Hp production in large scales is not realistic with present technologies, due to the complex structure as well as the functionally important glycosylation. Alternatives are therefore required for allowing both a more extensive clinical management and control of free Hb toxicities in the body.

MIPs with the ability to specifically recognize template molecules offer a valuable alternative source. In Paper IV, we studied the ability of MIPs to protect against radical formation induced by metHb (Figure 5-11) using phosphatidylcholine liposomes as a model. By using the fluorescence sensor SOSG, the formation of singlet oxygen during lipid peroxidation could be detected and conjugated diene formation of liposomes was also measured spectroscopically over time ($A_{234}$ nm).

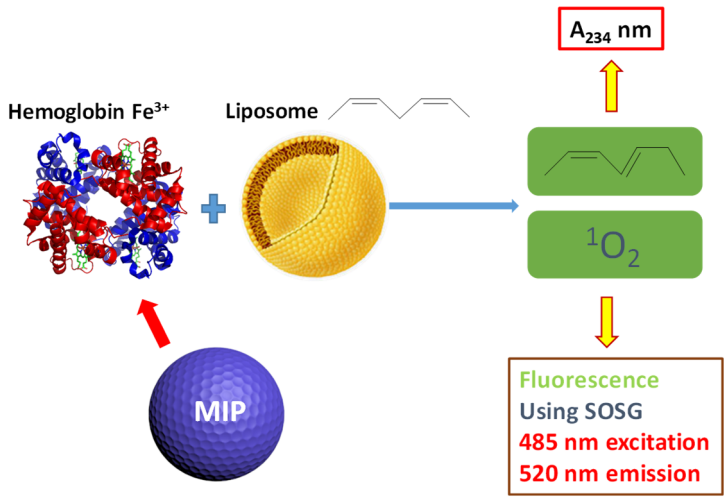


**Figure 5-11.** Schematical illustration of a lipid peroxidation experiment. The effect of MIP particles on the formation of singlet oxygen and conjugated diene were monitored.

In the presence of MIPs, the onset of lipid peroxidation was delayed proportionally to the concentration of the MIPs while the control polymers had no impact (Figure 5-12). Hb molecules are shielded by MIPs implying that lipid peroxides could not so easy get close to the heme of Hb to generate ferryl heme and lipid radical species. The lag period before the cascade event of oxidation was subsequently prolonged, thereby decreasing the oxidative damage by Hb.

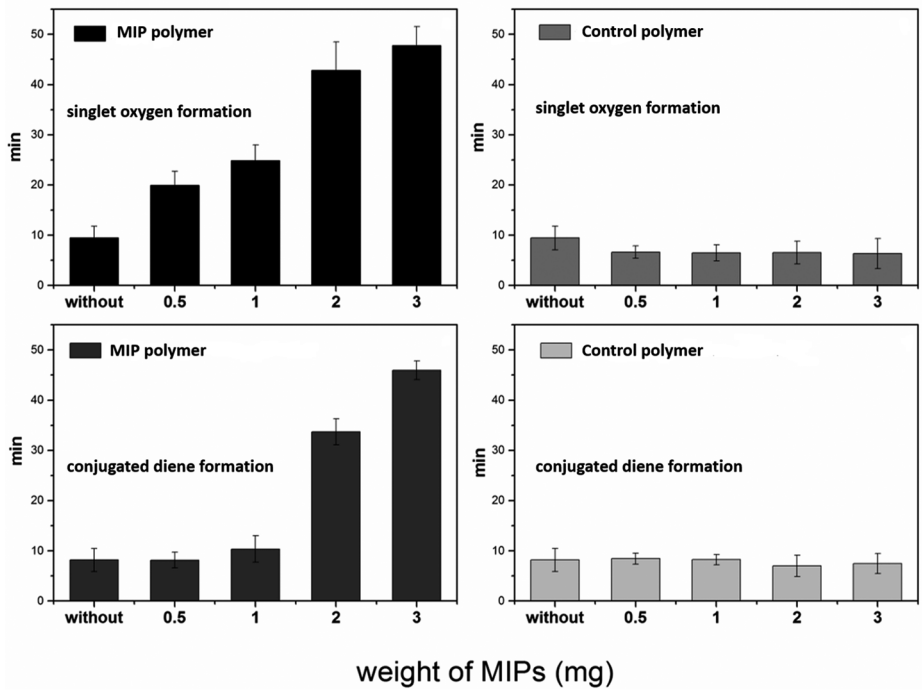


**Figure 5-12.** Lipid peroxidation of liposomes by Hb. Lag phase time of singlet oxygen formation in the presence of MIP (a) and control polymers (b) and conjugated diene formation in the presence of MIP (c) and control polymers (d).

## 5.5 Characterization of protein-protein interactions

In theory, it is straightforward to obtain a recombinant Hb. However, in practice, poor growth of the host, inclusion body formation, Hb inactivity and even not obtaining any Hb at all are some of the problems often encountered. Thus, careful selection of the optimal plasmid, host and the most appropriate growth conditions should be conducted according to the protein characteristics and downstream requirements. For example, Hb could influence the cell metabolism in *E. coli* production systems. Therefore, the identification of modified protein–protein interactions is critical for optimizing Hb production. In this study, MIPs directed against Hb have been utilized to identify host cell proteins that bind to human Hb (Paper III). One *E. coli* host protein, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), was identified to interact with Hb. HbF and its gamma chains especially formed a stable complex with GAPDH that could be broken by high NaCl concentrations (Figure 5-13).

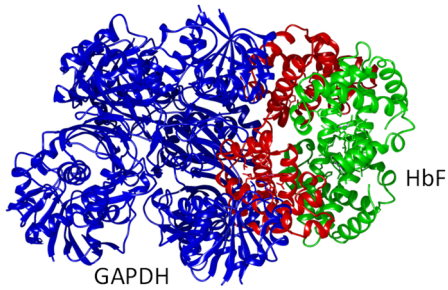


**Figure 5-13.** Proposed structure of for HbF (PDB: 1FDH) and GAPDH (PDB: 1GAD) complex produced with the PATCHDOCK AND FIREDOCK server. The gamma chains of HbF are shown in red and the alpha chains are shown in green.

GAPDH functions as a homotetramer protein with native molecular weight in the range of 140-150 kDa and is composed of four identical subunits (35-37 kDa) (136). GAPDH catalyzes the sixth step of glycolysis, converting glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate (1,3-DPG), and thus serves as an important step to break down glucose for energy and carbon molecules.

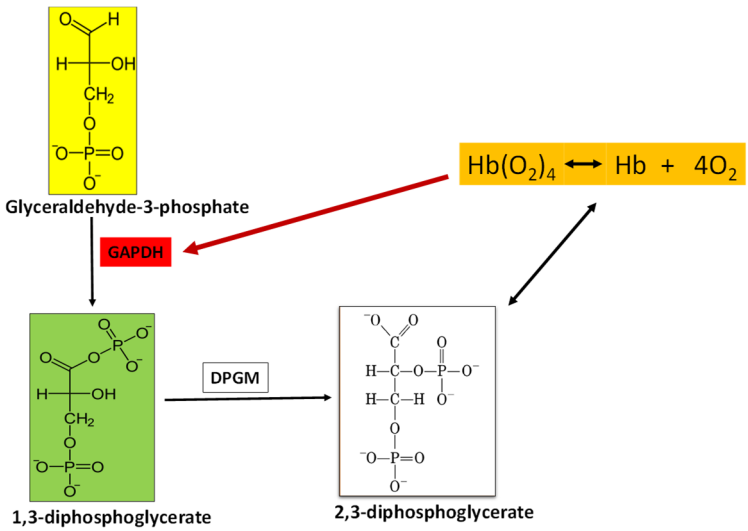


**Figure 5-14.** Schematic representation of a possible physiological function of GAPDH interaction with hemoglobin. GAPDH: glyceraldehyde 3-phosphate dehydrogenase, DPGM: diphosphoglycerate mutase, Hb: deoxyhemoglobin, $Hb(O_2)4$: oxyhemoglobin.

Cultivation of *E.coli* cells producing different Hb variants, for instance, HbA and HbF, differs substantially in terms of growth rates, need of oxygen supply and use of carbon source. Hb could influence the *E.coli* cell metabolism and therefore the identification of modified protein-protein interactions is critical for optimizing Hb production. As shown in Figure 5-14, the product of GAPDH, 1,3-DPG, can be isomerized to 2,3-DPG. Diphosphoglycerate mutase (DPGM) catalyzes the transfer of a phosphoryl group from $C_1$ to $C_2$ of 1,3-DPG, generating 2,3-DPG. 2,3-DPG is an allosteric effector of Hb and it binds with greater affinity to deoxyHb than it does to oxyHb, promoting Hb transition from a state of high oxygen affinity to a state of low oxygen affinity. Therefore, the oxygen-carrying capacity of Hb and the rate of glycolysis are closely linked (137). The expression level of HbF (6-8 mg/L) is significantly higher than that of HbA (3-5 mg/L) although compared to HbA, HbF exhibits a much lower affinity for 2,3-DPG. Hence, glycolysis was stimulated by HbF not by the binding of 2,3-DPG, removing product inhibition of DPGM. This may be explained by the current result – action at the site of GAPDH and not DPGM. The gamma chains of HbF can interact directly with GAPDH and then promotes the activity of this enzyme.

# 6 Conclusion and future remarks

Functional MIP materials have attracted much attention due to their potential applications in practical environments. In this thesis, water-compatible Hb-imprinted polymer beads were synthesized by Pickering emulsion polymerization with the imprinted sites located on the surface. Both HbA and HbF were selected as the template molecules. The high selectivity of the Hb-imprinted beads and their high stability were attractive for a number of applications. For recognition of protein, Pickering emulsion is particularly interesting as various templates and epitope structures can be immobilized on the particles to create surface imprinted binding sites. It is also applicable to other large biological molecules and living cells. We believe that, more functional materials can be expected by combining Pickering emulsion with more controllable synthetic methods.

The high selectivity and protection against lipid peroxidation properties of the Hb-imprinted polymers made it possible to use these sterilizable MIPs as Hp mimics, especially when recombinant Hp production in large scales needed for clinical applications is not realistic with present technologies. However, it would be desirable to mimic the favorable activities of Hp to exploit MIPs intravenously in the form of nanoparticles. In these instances, biocompatible and biodegradable polymers should be examined. It could be beneficial to include further radical protection and stability studies after adding MIPs. Several aspects also have to be further investigated, such as the *in vivo* studies and efficiency.

We have also illustrated that the MIP resin could be used to selectively remove Hb from human plasma and CSF. Hb should be strictly compartmentalized within the red blood cells (RBCs) to maintain it in a reduced and nontoxic state. Cell-free Hb is toxic, damages tissues and results in inflammation and cell death. The removal of extracellular Hb via extracorporeal filtration should be an easy and cost-efficient method. Extracorporeal devices are widely used to treat renal disease patients and support patients undergoing cardiac surgery. The obtained results from our study suggest that MIPs can have clinical applications in an extracorporeal shunt system.

With the development of HBOCs, the vital task is to reduce the production cost. The current cost of donated blood ranges from $130 to $150 /U, which is a challenging goal for any recombinant Hb. To reduce the cost, an effective protein purification strategy is required where various downstream processes account for a large portion of the total cost. In this thesis, we have studied the possibility of using Hb-MIP column to capture Hb molecules from *E. coli* crude extracts. Hopefully, the results can provide a ground for developing more sensitive and specialized chromatography Hb-imprinted media.

Considered together, we think that in the next few years it will be possible to observe unprecedented progress in the preparation of such MIPs and the application of these intelligent structures especially in basic and applied Hb research.

# Acknowledgements

Every time I receive a PhD thesis, I first turn the pages to read the acknowledgements section as it could be the only information I would understand. Therefore, from my point of view, acknowledgements are absolutely a significant part of any thesis (even the most important part ^_^).

I have enjoyed living in Lund these years, the calm and lovely university town. It was an unforgettable and valuable experience, which will stay with me for my whole life. There are so many people I wish to express my gratitude to, without whom this project would never have been possible. I have to apologize in advance if I forget to mention someone's name here.

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**2019-02**

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**2019 年 2 月**

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