Strategies for Engineering Safer, More Efficient and More Stable Recombinant Hemoglobins for Use as O₂ Delivery Pharmaceuticals

Cellular Engineering of bacteria

Protein Engineering of Hb

John S. Olson

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Design of Hemoglobin-Based Oxygen Carriers (HBOCs)

Chemically or genetically cross-linked extracellular Hb tetramers.
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Extracellular Hb is 2 to 3 times more efficient delivering O₂ than red blood cells because the molecules are closer to the vessel walls.
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Chemically or genetically cross-linked extracellular Hb tetramers.

Extracellular Hb is 2 to 3 times more efficient delivering O₂ than red blood cells because the molecules are closer to the vessel walls.
First Hemoglobin-Based Blood Substitute Engineering Challenge: Dimer formation, rapid clearance, kidney damage, and severe hypertension.

Rapid autooxidation, hemin loss, and globin precipitation
Hemoglobin tetramer crosslinking:

1. Non-specific polymerization
2. Specific chemical crosslinking
Hemoglobin tetramer crosslinking:

1. Non-specific polymerization
2. Specific chemical crosslinking
3. Genetic crosslinking
3. Genetic crosslinking

Hemoglobin tetramer crosslinking:

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“Di-alpha” rHb (0.1)
Hemoglobin tetramer crosslinking:

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“Di-alpha” rHb (0.1)

Somatogen/Baxter Optro®
Key Requirements of a Hb-based O$_2$ Carrier (HBOC)

1. Moderate O$_2$ affinity ($P_{50}$) and large dissociation rate constant for efficient transport.

2. Reduced rates of NO scavenging to prevent hypertension.


4. Low rates of heme dissociation to increase shelf-life, reduce oxidative stress, and inhibit bacterial infections.
Engineering $O_2$ affinity by changing the active site structure
Engineering O₂ affinity by changing the active site structure
Engineering $O_2$ affinity by changing the active site structure

Rotate 90°
Human Hb tetramer

Engineering O₂ affinity by changing the active site structure

Rotate 90°
Engineering O₂ affinity by changing the active site structure.
Engineering O$_2$ affinity by changing the active site structure
H-bonding to water and/or bound ligands-discrimination in favor of $O_2$
H-bonding to water and/or bound ligands-discrimination in favor of $O_2$
Non-covalent binding pocket

Steric hindrance

H-bonding to water and/or bound ligands - discrimination in favor of $O_2$
Non-covalent binding pocket

H-bonding to water and/or bound ligands - discrimination in favor of O₂

Steric hindrance

R state high affinity (all ligands)

T state low affinity (all ligands)
Non-covalent binding pocket

H-bonding to water and/or bound ligands-discrimination in favor of O₂

T state
Low affinity (all ligands)

R state
High affinity (all ligands)

Allosteric mutations
Cl⁻, H⁺, DPG binding

Steric hindrance

Allosteric mutations
Cl⁻, H⁺, DPG binding

H-bonding to water and/or bound ligands-discrimination in favor of O₂

Non-covalent binding pocket
Use of Allosteric mutations to adjust $P_{50}$

Chien Ho and coworkers, Carnegie Mellon University

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Use of Allosteric mutations to adjust P50

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**Not Blood Simple**

After decades of setbacks, compounds that act like blood to deliver oxygen are in the final stretch of clinical trials.

Until March 1998, Baxter Healthcare Corp. thought it had a sure-fire winner. The Deerfield, Illinois, company was in phase III trials of HemAssist, an oxygen-carrying solution designed to treat patients in shock from massive bleeding. Analysts were excited because blood substitutes—more accurately known as

Scientists began working on artificial blood in the 1960s, trying to see whether iron-rich hemoglobin molecules extracted from red blood cells would be able to pick up oxygen molecules and deliver them to tissues when transfused into a patient. Those early compounds ran into problems withkid

might cause other problems.” Other experts stress that more needs to be known about how modified hemoglobin delivers oxygen to tissue

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**BLOOD SUBSTITUTES IN ADVANCED CLINICAL TRIALS**

<table>
<thead>
<tr>
<th>Product (Company)</th>
<th>Source</th>
<th>Use</th>
<th>Half-life†</th>
<th>Shelf life</th>
<th>Side effects‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemopure (Biopure)</td>
<td>Modified bovine hemoglobin</td>
<td>Elective surgery</td>
<td>24–36 hours</td>
<td>2 years at room temperature</td>
<td>Vasoconstriction, transient increase in liver and pancreas enzymes</td>
</tr>
<tr>
<td>PolyHeme (Northfield Laboratories)</td>
<td>Modified human hemoglobin</td>
<td>Trauma, urgent blood loss</td>
<td>24 hours</td>
<td>1 year refrigerated</td>
<td>None reported</td>
</tr>
<tr>
<td>Hemolink (Hemosol)</td>
<td>Modified human hemoglobin</td>
<td>Heart surgery</td>
<td>18–20 hours</td>
<td>1 year refrigerated</td>
<td>Vasoconstriction, yellowing of skin</td>
</tr>
<tr>
<td>Oxygent (Alliance)</td>
<td>Perfluorochemical emulsion</td>
<td>General surgery</td>
<td>24–48 hours</td>
<td>2 years refrigerated</td>
<td>Slight rise in body temperature, drop in platelet count</td>
</tr>
</tbody>
</table>

* In the United States. † Dose-dependent. ‡ All transient.
Pressor responses to rHb1.1 and rHb Bethesda: no dependence on O₂ affinity

1. vasoconstriction
2. heart lesions
3. gastro-intestinal problems

Baxter Hemoglobin Therapeutics (Doherty... Olson, and Lemon, Nature Biotechnology, 16, 672-676 (1998).
Detoxification of Nitric Oxide by Hemoglobins and Myoglobins

\[
\text{MbO}_2 \rightarrow \text{Non-covalent ligand pocket} \rightarrow \text{Fe(III)} \rightarrow \text{aquometMb} \rightarrow \text{flavoprotein(FAD) (cyt b5)} \rightarrow \text{O}_2 \rightarrow \frac{1}{2} \text{NAD}^+ + \text{H}^+ \rightarrow \frac{1}{2} \text{NADH}
\]
Detoxification of Nitric Oxide by Hemoglobins and Myoglobins

Highly toxic:
Inhibits aconitase (TCA cycle) and cytochrome c oxidase (electron transport to O₂)

Non-covalent ligand pocket

•N=O

Ile or Leu(G8)

Val(E11)

Leu(B10)

His(E7)

Phe(CD4)

His(F8)

flavoprotein(FAD) (cyt b₅)

MbO₂ → aquometMb

1/2 NAD⁺, H⁺ → 1/2 NADH

O₂

δ(+)  

δ(–)

H₂O

Detoxification of Nitric Oxide by Hemoglobins and Myoglobins
Detoxification of Nitric Oxide by Hemoglobin and Myoglobin

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- Inhibits aconitase (TCA cycle) and cytochrome c oxidase (electron transport to $O_2$)

Non-covalent ligand pocket

- Leu(B10)
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$\text{N}=\text{O}$

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$\frac{1}{2} \text{NAD}^+, H^+ \rightarrow \frac{1}{2} \text{NADH}$

$\text{O}_2$

Flavoprotein (FAD) (cyt b$_5$)
Detoxification of Nitric Oxide by Hemoglobins and Myoglobins

Highly toxic:
Inhibits aconitase (TCA cycle) and cytochrome c oxidase (electron transport to O₂)

Hb or Mb-ethyl isocyanide

Nitrate: non-toxic

O₂

1/2 NAD⁺, H⁺

1/2 NADH
Detoxification of Nitric Oxide by Hemoglobins and Myoglobins

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$Ile$ or $Leu$ (G8)

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$1/2$ NAD$^+$, $H^+$

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**Nitrate:** non-toxic

$H_2$O

flavoprotein (FAD) (cyt b$_5$)
Detoxification of Nitric Oxide by Hemoglobins and Myoglobins

Highly toxic: Inhibits aconitase (TCA cycle) and cytochrome c oxidase (electron transport to $O_2$)

Non-covalent ligand pocket

$Ile$ or $Leu$ (G8)

$O_2$

$MbO_2$

$1/2$ NAD$^+$, $H^+$

$H_2O$

$1/2$ NADH

Peroxynitrite intermediate

Nitrate: non-toxic

Non-covalent ligand pocket

Isomerization is very rapid

flavoprotein (FAD) (cyt b$_5$)

H$\delta$(+)
NO dioxygenase activity for detoxification of \textsuperscript{'}NO

Paul Gardner (Children's Hospital, Cincinnati) and Mike Gustin (Rice): flavoHbs from \textit{E. coli}, \textit{Candida albicans} and \textit{Aspergillus fumigatus} (Gardner et al., 1998, PNAS 95, 10378-10383).

\textbf{Macrophage engulfing bacteria or fungi.}

NO can inactivate aconitase at levels \(\leq 200\) nM (Gardner et al., 1997 J. Biol. Chem. 272, 25071-76)

NO can inhibit cytochrome oxidase at levels \(\leq 1\) \(\mu\)M (Brunori, 2001 TIBS 26, 21-23)
NO dioxygenase activity for detoxification of \( \cdot \)NO

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**FlavoHb**

- **Heme**
- **Globin domain**
- **FAD**
- **Reductase domain**

NO can inactivate aconitase at levels \( \leq 200 \text{ nM} \) (Gardner et al., 1997 J. Biol. Chem. 272, 25071-76)

NO can inhibit cytochrome oxidase at levels \( \leq 1 \text{ µM} \) (Brunori, 2001 TIBS 26, 21-23)

Macrophage engulfing bacteria or fungi.
A secondary function of HbO$_2$ and MbO$_2$ is to remove excess NO.
A secondary function of HbO₂ and MbO₂ is to remove excess NO

**Endothelial Cells with NO Synthase (NOS)**

- NO from NOS activates GC
- Activated GC produces NO₃⁻

**Smooth muscle cells With guanylyl cyclase (GC)**

- NO from NOS activates GC
- Activated GC produces NO₃⁻

**Myoglobin in aerobic skeletal muscle**

- NO from NOS activates GC
- Activated GC produces NO₃⁻
A secondary function of HbO$_2$ and MbO$_2$ is to remove excess NO.

Endothelial cells with NO synthase (NOS)

Smooth muscle cells with guanylyl cyclase (GC)

Myoglobin in aerobic skeletal muscle

Activated GC

Prevents NO inhibition of mitochondrial respiration.
A secondary function of HbO₂ and MbO₂ is to remove excess NO.

**Endothelial Cells with NO Synthase (NOS)**

- NO is produced by NOS.
- NO activates guanylyl cyclase (GC).
- Activated GC leads to the production of cGMP.
- cGMP inhibits smooth muscle cells and prevents NO inhibition of mitochondrial respiration.

**Smooth muscle cells with guanylyl cyclase (GC)**

- Activated GC inhibits smooth muscle cells.

**Myoglobin in aerobic skeletal muscle**

- Myoglobin is involved in the removal of excess NO.

**Red Cells**

- Protection from inhaled NO or excess NO from inflammation.

**Lumen of arteriole**

- Direction of flow with cells in the center of vessel.
Effects of NO inhalation on metHb, HbNO, and nitrate levels
(Gladwin et al., 2000 PNAS 97, 9943-48)

- NO inhalation by healthy human subjects
  - nitrate (~80 µM increase)
  - metHb (~80 µM increase)

Concentration in µM

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO inhalation</td>
<td>80 ppm NO</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HbNO(≈3 µM)</td>
<td>80 ppm NO</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HbNO(≈2 µM)</td>
<td>80 ppm NO</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nitrate (≈0.3 µM)</td>
<td>80 ppm NO</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>metHb (≈0.2 µM)</td>
<td>80 ppm NO</td>
<td></td>
<td></td>
<td></td>
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Alveolar capillaries
Effects of NO inhalation on metHb, HbNO, and nitrate levels

(Gladwin et al., 2000 PNAS 97, 9943-48)

NO inhalation by healthy human subjects

Concentration in µM

Time (hr)

nitrate (~80 µM increase)

metHb (~80 µM increase)

80 ppm NO

HbNO (~3 µM)

HbNO (~2 µM)

0 1 2 3 4 5 6 7 8

Alveolar capillaries

Endothelium

Lumen

Precapillary sphincter

Air

Effects of NO inhalation on metHb, HbNO, and nitrate levels

(Gladwin et al., 2000 PNAS 97, 9943-48)
Myoglobin in aerobic skeletal muscle

Endothelial Cells with NO Synthase (NOS)

Smooth muscle cells with guanylyl cyclase (GC)

Activated GC

Erythrocytes do not interfere with NO signaling due to cell-free layer

Red Cells

Lumen of arteriole

Direction of flow with cells in the center of vessel

Red Cells

Mb prevents NO inhibition of mitochondrial respiration
Myoglobin
in aerobic
skeletal muscle

Endothelial
Cells with NO
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muscle cells
With guanylyl
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Red Cells

Lumen of
arteriole

Direction of flow
with cells in the
center of vessel

Red Cells

Extracellular Hb scavenges NO

Mb prevents NO inhibition of
mitochondrial respiration
Cell-Free Hemoglobin-Based Blood Substitutes and Risk of Myocardial Infarction and Death: A Meta-analysis

Charles Natanson, MD
Steven J. Kern, BS
Peter Lurie, MD, MPH
Steven M. Banks, PhD†
Sidney M. Wolfe, MD

THE DEVELOPMENT OF A BLOOD SUBSTITUTE—an infusible liquid that eliminates the need for refrigeration and crossmatching, has a long shelf life, and reduces the risk of iatrogenic infection—would provide a potentially lifesaving option for surgical patients and trauma patients with hemorrhagic shock, especially in rural areas and military settings. To date, a large proportion of blood substitutes in development have been hemoglobin-based products. Yet randomized controlled trials completed as early as 1996 have raised questions about the safety of these products and have failed to demonstrate clinical benefit. Nonetheless, at least 1 of these products is approved for use outside the United States and new clinical trials are being con...

Context Hemoglobin-based blood substitutes (HBBSs) are infusible oxygen-carrying liquids that have long shelf lives, have no need for refrigeration or crossmatching, and are ideal for treating hemorrhagic shock in remote settings. Some trials of HBBSs during the last decade have reported increased risks without clinical benefit.

Objective To assess the safety of HBBSs in surgical, stroke, and trauma patients.

Data Sources PubMed, EMBASE, and Cochrane Library searches for articles using hemoglobin and blood substitutes from 1980 through March 25, 2008; reviews of Food and Drug Administration (FDA) advisory committee meeting materials; and Internet searches for company press releases.

Study Selection Randomized controlled trials including patients aged 19 years and older receiving HBBSs therapeutically. The database searches yielded 70 trials of which 13 met these criteria; in addition, data from 2 other trials were reported in 2 press releases, and additional data were included in 1 relevant FDA review.

Data Extraction Data on death and myocardial infarction (MI) as outcome variables.

Results Sixteen trials involving 5 different products and 3711 patients in varied patient populations were identified. A test for heterogeneity of the results of these trials was not significant for either mortality or MI (for both, I²=0%, P=.60), and data were combined using a fixed-effects model. Overall, there was a statistically significant increase in the risk of death (164 deaths in the HBBS-treated groups and 123 deaths in the control groups; relative risk [RR], 1.30; 95% confidence interval [CI], 1.05-1.61) and risk of MI (59 MIs in the HBBS-treated groups and 16 MIs in the control groups; RR, 2.71; 95% CI, 1.67-4.40) with these HBBSs. Subgroup analysis of these trials indicated the increased risk was not restricted to a particular HBBS or clinical indication.

Conclusion Based on the available data, use of HBBSs is associated with a significantly increased risk of death and MI.

JAMA. 2008;299(19):2304-2312
Cell-Free Hemoglobin-Based Blood Substitutes and Risk of Myocardial Infarction and Death
A Meta-analysis

Charles Natanson, MD
Steven I. Kern, BS

Context Hemoglobin-based blood substitutes (HBBSs) are infusible oxygen-carrying liquids that have long shelf lives, have no need for refrigeration or cross-
to demonstrate clinical benefit. Nonetheless, at least 1 of these products is approved for use outside the United States and new clinical trials are being conducted or planned worldwide.2-8

Although there are biochemical differences between the products tested to date,9-13 all share the same mechanism of action and apparent mechanism of toxicity.14 Hemoglobin molecules used to manufacture these products are not contained by a red cell membrane, and when released into the vasculature, these molecules rapidly scavenge nitric oxide. This can result in systemic vasoconstriction, decreased blood flow, increased release of proinflammatory mediators and potent vasoconstrictors, and a loss of platelet inactivation,17-20 creating conditions that may lead to vascular thrombosis of the heart or other organs. This mechanism has recently been shown in preclinical models to be responsible for injury during hemolytic states, in which hemoglobin is also released into the circulation.21

Conclusion Based on the available data, use of HBBSs is associated with a significantly increased risk of death and MI.

JAMA. 2008;299(19):2304-2312

For editorial comment see p 2324.

CME available online at www.jamaarchivescme.com and questions on p 2336.

Author Affiliations: Critical Care Medicine Department, Clinical Center, National Institutes of Health, Bethesda, Maryland (Drs Natanson and Banks and Mr Kern); and Health Research Group, Public Citizen, Washington, DC (Drs Lurie and Wolfe).

†Deceased.

Corresponding Author: Charles Natanson, MD, Critical Care Medicine Department, Clinical Center, National Institutes of Health, 10 Center Dr, Bethesda, MD 20892 (c_natanson@cc.nih.gov).
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Extravasation of Hb tetramers

- NO3⁻
- NO

Endothelial Cells

- GC
- Inactive
- NO

Smooth Muscle Cells

- GC
- Inactive
- NO
- Activated

Lumen of Arteriole

- Slower extravasation of polymers and conjugates
- NO3⁻
- NO
Extravasation of Hb tetramers

NO3-

Endothelial Cells

Smooth Muscle Cells

Lumen of Arteriole

GC Inactive

GC Inactive

GC Inactive

GC Inactive

Slower extravasation of polymers and conjugates

NO3-

NO

NO

NO

NO

NO

NO

NO

NO

NO

NO

NOS

Activated

Activated

Activated
Extravasation of Hb tetramers

Lumen of Arteriole

NO₃⁻

NO

NO₃⁻

Endothelial Cells

Inactive

NO

Smooth Muscle Cells

Lumen of Arteriole

GC

Inactive

GC

Activated

These products “work” and are relatively “safe.”

Dependence of the Pressor effect on MW or size

Various polymers (Sakai et al., 2002)

rHb, glutaraldehyde (Doyle et al. 1999)

CL-Hb (Rohlfis et al., 1998)

peg-Hb (Rohlfis et al., 1998)

rHb (Doyle et al., 1998)

(no distal pocket mutants - see Dou et al. (2002 Biophys. Chem. 98, 127-148)

%ΔMAP or %ΔTPR

Approximate MW (kD) or Size

These products “work” and are relatively “safe.”
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Extravasation of Hb tetramers

Lumen of Arteriole

NO

Endothelial Cells

NO

NO

Smooth Muscle Cells

NO

NO

GC

Inactive

GC

Inactive

GC

Inactive

GC

Inactive

Slower extravasation of polymers and conjugates

NOS
1. Re-engineer Hb to lower the rate of NO dioxygenation.

2. Then re-adjust the $O_2$ binding parameters for efficient transport.

3. Use a recombinant Hb expression system.
Side Path / HisE7 Gate / Baseball Glove Model

Side Path / HisE7 Gate / Baseball Glove Model

“Bimolecular ligand capture”

Side Path / HisE7 Gate / Baseball Glove Model

“Unimolecular ligand dissociation”
Inhibition of NO Dioxygenation by “filling” the distal pocket with Phe and Trp mutations without impairing O$_2$ binding and release.
Inhibition of NO Dioxygenation by “filling” the distal pocket with Phe and Trp mutations without impairing O$_2$ binding and release.
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Inhibition of NO Dioxygenation by “filling” the distal pocket with Phe and Trp mutations without impairing O2 binding and release.
Inhibition of NO Dioxygenation by “filling” the distal pocket with Phe and Trp mutations without impairing O$_2$ binding and release.

Correlation between $k^\prime$NOD and $k^\prime$NO,Fe(II)

- 1:1 Apolar His64 mutants
- 0.7:1 (R$^2$=0.69)

Sterically hindered distal pocket mutants:
- L29F/H64Q/V68I
- H64Q/V68W
- L29W
- L29W/H64Q

Leu(B10)29 Phe or Trp

Val(E110)68 Phe or Trp

Gln or allosteric mutants to adjust P$_50$
Elimination of TPR in rHb3011

(TPR = MAP/Cardiac Output)

**Starting rHb for rHb2.0**

- $k'_{NO, \alpha}$
  - $70 \mu M^{-1}s^{-1}$ (rHb1.1)
  - $P_{50} = 32$ mm Hg

- $2 \mu M^{-1}s^{-1}$ (rHb3011)
  - $P_{50} = 43$ mm Hg

350 mg/kg

Top load doses

$n = 6$

Elimination of TPR in rHb3011

(TPR = MAP/Cardiac Output)

\[ k'_{\text{NO, } \alpha} \]

\[ 70 \mu M^{-1}s^{-1} \text{ (rHb1.1)} \]

\[ P_{50} = 32 \text{ mm Hg} \]

\[ \alpha(\text{TrpB10/GlnE7}) \]

\[ \beta(\text{TrpE11}) \]

Starting rHb for rHb2.0

\[ 2 \mu M^{-1}s^{-1} \text{ (rHb3011)} \]

\[ P_{50} = 43 \text{ mm Hg} \]

350 mg/kg

Top load doses

n = 6

Key Requirements of a Hb-based O₂ Carrier (HBOC)

1. Moderate O₂ affinity ($P_{50}$) and large dissociation rate constant for efficient transport.

2. Reduced rates of NO scavenging to prevent hypertension.


4. Low rates of heme dissociation to increase shelf-live, reduce oxidative stress, and inhibit bacterial infections.
Key Requirements of a Hb-based O₂ Carrier (HBOC)

1. Moderate O₂ affinity ($P_{50}$) and large dissociation rate constant for efficient transport.
2. Reduced rates of NO scavenging to prevent hypertension.
4. Low rates of heme dissociation to increase shelf-life, reduce oxidative stress, and inhibit bacterial infections.

All of these properties can be optimized by protein engineering. The problem is production of recombinant hemoglobin in bacteria.
1. Human hemoglobin requires donors, and its availability is limited. Bovine hemoglobin is cheaper and more readily available.

2. Recombinant hemoglobin production is potentially unlimited, but it is costly, and the product is often contaminated with lipopolysaccharide antigens and degradation products.

Need 30-60 g per transfusion unit

Expression of rHb in E. coli is a key limiting factor in production yield.
Hemoglobin Synthesis in E. coli
Hemoglobin Synthesis in E. coli
Hemoglobin Synthesis in E. coli

unfolded α

mRNA

unfolded β

β

α

αβ dimer

apo αβ dimer

unstable at > 15°C

Very rapid precipitation and degradation

Periplasm

Inner Membrane

Outer Membrane
Hemoglobin Synthesis in E. coli
Hemoglobin Synthesis in E. coli
Hemoglobin Synthesis in E. coli

unfolded α

 apo α₁β₂ dimer

 holo α₁β₂ dimer

Hemoglobin tetramer

holoproteins stable for days at 37°C

bacterial heme synthesis

mRNA

protein

Rb

inner membrane

outer membrane

periplasm

Hemoglobin Synthesis in E. coli
\[ \text{Rate}_{\text{holoHb}} = k_{\text{heme}} \frac{K_{UN}}{1 + K_{UN}} - k_{\text{degrade}} \frac{1}{1 + K_{UN}} \]

- Rate of heme synthesis or transport
- Fraction folded
- Rate of precipitation or proteolysis
- Fraction unfolded
Collaborations with:
1. George Phillips, Jr., University of Wisconsin (sperm whale hemoglobin)
2. Mitchell Weiss, Childrens’s Hospital of Philadelphia (alpha hemoglobin stabilizing protein - AHSP)
Collaborations with:
1. George Phillips, Jr., University of Wisconsin (sperm whale hemoglobin)
2. Mitchell Weiss, Childrens’s Hospital of Philadelphia (alpha hemoglobin stabilizing protein - AHSP)
3. Doug Henderson, UT Permian Basin; Neil Varnado and Doug Goodwin, Auburn U. (ChuA and HugA outer membrane heme receptors)
Hemoglobin Synthesis in E. coli

Periplasm

Inner Membrane

bacterial heme synthesis

unfolded \( \beta \)

 apo \( \alpha_1 \beta_2 \) dimer

 holo \( \alpha_1 \beta_2 \) dimer

hemoglobin tetramer
Hemoglobin Synthesis in E. coli

1. Increase globin stability
Hemoglobin Synthesis in E. coli

1. Increase globin stability
   - Increase globin stability
   - Add heme transport genes

2. Add heme transport genes
   - Bacterial heme synthesis
   - Heme transport genes

**Outer Membrane**

**Inner Membrane**

**Periplasm**

**unfolded β**

**unfolded α**

**apo α4β2 dimer**

**holo α4β2 dimer**

**hemoglobin tetramer**
1. Increase globin stability

2. Add heme transport genes

3. Add α subunit chaperone

AHSP

α subunit chaperone

Hemoglobin Synthesis in E. coli

1. Increase globin stability

2. Add heme transport genes

3. Add α subunit chaperone

Folding constant: $K_{UN} = 1/K_{NIKIU}$

Human or wt pig apoMb

$K_{UN} = 1,300$

$K_{UN} = 63,000$

wt SW apoMb and pig multiple mutant

### Normalized CD Change at 222 nm

<table>
<thead>
<tr>
<th>[GdmCl] M</th>
<th>0.0</th>
<th>0.5</th>
<th>1.0</th>
<th>1.5</th>
<th>2.0</th>
<th>2.5</th>
<th>3.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt pig apoMb</td>
<td>K\textsubscript{UN} = 1,300</td>
<td></td>
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</tbody>
</table>

Folding constant: \( K\textsubscript{UN} = 1/K\textsubscript{NIKIU} \)

Pig Mb mutations to the five naturally occurring amino acids in sperm whale Mb.

Normalized CD Change at 222 nm

![Graph showing normalized CD change at 222 nm vs. GdmCl concentration.](image)

- **Folding constant**: $K_{UN} = 1/K_{NIKIU}$
- **Human or wt pig apoMb**: $K_{UN} = 1,300$
- **wt SW apoMb and pig multiple mutant**: $K_{UN} = 63,000$
- **no holoMb expression**
- **constitutive expression**

Pig Mb mutations to the five naturally occurring amino acids in sperm whale Mb

B. Unfolding of SW versus Human apoHb, 5°C
B. Unfolding of SW versus Human apoHb, 5°C

C. Unfolding of wt apoHb: Simulated with 2 step Dimer Model, 5°C
B. Unfolding of SW versus Human apoHb, 5°C

C. Unfolding of wt apoHb: Simulated with 2 step Dimer Model, 5°C

apo $\alpha_1\beta_1$ dimer (D)

molten intermediate (I)

unfolded $\alpha$ (U)

unfolded $\beta$ (U)

$K_{DI} = \frac{[I]}{[D]} \approx 0.01$

$K_{2,1} = \frac{[U]^2}{[D]} \approx 0.05 \mu$M

Unfolded heme pockets
Very stable

\( \alpha_2 \varepsilon_2 \) Hb embryonic (High \( O_2 \) affinity)

\( \alpha_2 \gamma_2 \) HbF (moderate \( O_2 \) affinity)

Very stable

\( \alpha_2 \beta_2 \) HbA (low \( O_2 \) affinity)
Acid, base, and isopropanol tests for hemoglobin stability
Mutations to stabilize the dimer interface based on Hb F (γ chains)

$\alpha_1 \cdots \beta_1$ interface
Mutations to stabilize the dimer interface based on Hb F (γ chains)

$\alpha_1 \cdots \beta_1$ interface

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<td>C112T (G14)</td>
</tr>
<tr>
<td>H116I (G18)</td>
</tr>
<tr>
<td>P125E (H3)</td>
</tr>
<tr>
<td>Y130W (H8)</td>
</tr>
<tr>
<td>V133M (H11)</td>
</tr>
</tbody>
</table>
Mutations to stabilize the dimer interface based on Hb F (γ chains)

α₁⋯β₁ interface

β→γ replacements
- C112T (G14)
- H116I (G18)
- P125E (H3)
- Y130W (H8)
- V133M (H11)
Mutations to stabilize the dimer interface based on Hb F (γ chains)

α₁...β₁ interface

β→γ replacements

C112T (G14)
H116I (G18)
P125E (H3)
Y130W (H8)
V133M (H11)
Mutations to stabilize the dimer interface based on Hb F ($\gamma$ chains)

$\alpha_1 \cdots \beta_1$ interface

$\beta \rightarrow \gamma$ replacements

- C112T (G14)
- H116I (G18)
- P125E (H3)
- Y130W (H8)
- V133M (H11)
Unfolding of Mutant ApoHbs

- $\alpha$(G15A)
- $\beta$(G16A/H116I)
- $\alpha$(G15A)
- $\beta$(G16A)
- WT apoHbA

$[\text{GdmCl}]$ M vs $1-\text{YCD}$
A. Unfolding of Mutant ApoHbs

Higher yield (1.5 to 2.0-fold) of holo-rHb
Hemoglobin Synthesis in E. coli

1. Increase globin stability

Periplasm

Inner Membrane

bacterial heme synthesis

apo $\alpha_1\beta_2$ dimer

holo $\alpha_1\beta_2$ dimer

hemoglobin tetramer

unfolded $\alpha$

unfolded $\beta$

mRNA

Protein

Rb

1. Increase globin stability

Hemoglobin Synthesis in E. coli

1. Increase globin stability
Hemoglobin Synthesis in E. coli

1. Increase globin stability
   - mRNA
   - Protein
   - Apo $\alpha_1\beta_2$ dimer
   - Heme transport genes

2. Add heme transport genes
   - Heme transport
   - Heme synthesis
   - Holo $\alpha_1\beta_2$ dimer
   - Hemoglobin tetramer

Inner Membrane

Periplasm
Heme utilization genes (*hug*)

**Plesiomonas shigelloides**
(Vibrio cholerae, Shigella dysenteriae, Yersinia pestis, Yersinia enterocolitica, and pathogenic E. coli) - heme transport genes under FUR control - Fe\(^{3+}\) binding transcription factor

- HugB
  - slow flipping, high affinity for membrane layers
- HugC
- HugD
- ExbD
- ExbB
- TonB
- Outer Membrane
- Periplasm
- Inner Membrane
Heme utilization genes (hug)

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(Vibrio cholerae, Shigella dysenteriae, Yersinia pestis, Yersinia enterocolitica, and pathogenic E. coli) - heme transport genes under FUR control - Fe$^{3+}$ binding transcription factor

HugA

slow flipping, high affinity for membrane layers

HugB

TonB

ExbD

ExbB

HugC

HugD

Outer Membrane

Periplasm

Inner Membrane
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**Outer Membrane**

**Periplasm**

**Inner Membrane**

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- HugA
- HugB
- HugC
- HugD
- ExbD
- ExbB
- TonB

**Outer Membrane**

**Periplasm**

**Inner Membrane**

- slow flipping, high affinity for membrane layers

H+ "active" uptake?
Heme utilization genes (*hug*)

*Plesiomonas shigelloides* (Vibrio cholerae, Shigella dysenteriae, Yersinia pestis, Yersinia enterocolitica, and pathogenic E. coli) - heme transport genes under FUR control - Fe³⁺ binding transcription factor

- HugA
- HugB
- HugC
- HugD

Slow flipping, high affinity for membrane layers

H+ "active" uptake?

Outer Membrane

Periplasm

Inner Membrane
Heme utilization genes (*hug*)

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- HugA
- HugB
- HugC
- HugD
- HugW, X, Z?

- free Fe$^{3+}$
- heme degradation enzymes

**Outer Membrane**

**Periplasm**

**Inner Membrane**

**ExbB**

**ExbD**

**TonB**

**H+ "active" uptake?**

slow flipping, high affinity for membrane layers
Heme utilization genes *(hug)*

**Plesiomonas shigelloides**
(Vibrio cholerae, Shigella dysenteriae, Yersinia pestis, Yersinia enterocolitica, and pathogenic E. coli) - heme transport genes under FUR control - Fe$^{3+}$ binding transcription factor)

HugA

HugB

HugC

HugD

ExbB

ExbD

TonB

Outer Membrane

Periplasm

Inner Membrane

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slow flipping, high affinity for membrane layers
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HugA → HugB → HugC → HugD

slow flipping, high affinity for membrane layers

H+ "active" uptake?

ExbD → ExbB

H+ incorporation into apoHb
FecA  Ferrous-Citrate complex

Outer Membrane Heme Receptors

HugA - *Pleisomonas shigelloides*

ShuA - *Shigella dysenteriae*

ChuA - *E. coli O157:H7*

± TonB and ABC transporter systems

Collaborations with:

2. Douglas Goodwin, Auburn University (*ChuA*) and Neil Varnado, Rice University

Susan K. Buchanan, “Bacterial Metal Detectors” Molecular Microbiology 58, 1205-1209, 2005
FecA Ferrous-Citrate complex

Outer Membrane Heme Receptors

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Engineering the *E. coli* bacterium

Expression Vectors (ds DNA)

- Tetracycline resistance
- Synthetic hemoglobin Genes + *tac* promoter
- Restriction enzyme site for cloning

Transformation into *Escherichia coli*

- Chromosomal DNA
- Higher copy plasmid
- Chloramphenicol resistance

Fe utilization Regulation (Fur)

Heme Transport genes

- HugABCD/TonB (Henderson)
- ChuA (Goodwin/Varnado)
- ShuA (Henderson)

Chloramphenicol resistance

pACYC vectors with helper genes
Engineering the *E. coli* bacterium

Expression Vectors (ds DNA)

- Restriction enzyme site for cloning
- Synthetic hemoglobin Genes + tac promoter
- Restriction enzyme site for cloning

Transformation into *Escherichia coli*

- Tetracycline resistance
- Mutation

Chromosomal DNA

- Higher copy plasmid
- Low copy plasmid

Chromosomal DNA

Fe utilization Regulation (Fur)

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  - HugABCD/TonB (Henderson)
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Chloramphenicol resistance

pACYC vectors with helper genes
Tetracycline resistance

Synthetic hemoglobin Genes + tac promoter

Expression Vectors (ds DNA)

Restriction enzyme site for cloning

Transformation into *Escherichia coli*

Chromosomal DNA

Grow on tetracycline and chloramphenicol plates to **select for cells** containing both plasmid DNAs and turn Hb on with IPTG and the heme transport genes with iron starvation

1. Colonies that are resistant to tetracycline and chloramphenicol and express both rHb and heme transport genes
2. Red color is due to hemoglobin (HbO₂) expression

Higher copy plasmid

Low copy plasmid

Fe utilization Regulation (Fur)

Heme Transport genes

ChuA (Goodwin/Varnado)
ShuA (Henderson)

HugABCD/TonB (Henderson)

Chloramphenicol resistance

pACYC vectors with helper genes
Tetracycline resistance

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Engineering the E. coli bacterium:

- restriction enzyme site for cloning
- synthetic hemoglobin genes + tac promoter
- expression vectors (ds DNA)
- transformation into Escherichia coli
- higher copy plasmid
- low copy plasmid
- chromosomal DNA
- pACYC vectors with helper genes
- heme transport genes
- chloramphenicol resistance
- Fe utilization regulation (Fur)

HugABCD/TonB (Henderson)
ChuA (Goodwin/Varnado)
ShuA (Henderson)
Tetracycline resistance

Synthetic hemoglobin genes + tac promoter

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rHb0.0 production in BL21 cells with: Co-expression of pHUG21.1 (iron depletion with DIP = 2,2-dipyridine)/pSGE0.0-E4(rHb with IPTG)

A. "Raw" spectra for rHb0.0

![A. "Raw" spectra for rHb0.0](image1)

B. Derivative for rHb0.0

![B. Derivative for rHb0.0](image2)
rHb0.0 production in BL21 cells with: Co-expression of pHUG21.1 (iron depletion with DIP = 2,2-dipyridine)/pSGE0.0-E4 (rHb with IPTG)

Heme transport appears to double the yield of rHb0.0!
Hemoglobin Synthesis in E. coli

1. Increase globin stability

- mRNA
- Protein
- unfolded $\alpha$
- unfolded $\beta$

2. Add heme transport genes

- heme transport genes
- bacterial heme synthesis

- apo $\alpha_4\beta_2$ dimer
- holo $\alpha_4\beta_2$ dimer
- hemoglobin tetramer
Hemoglobin Synthesis in E. coli

1. Increase globin stability
2. Add heme transport genes
3. Add α subunit chaperone

Periplasm

Inner Membrane

bacterial heme synthesis

apo $\alpha_1\beta_2$ dimer

holo $\alpha_1\beta_2$ dimer

hemoglobin tetramer

unfolded $\beta$

unfolded $\alpha$

mRNA → Protein

$\alpha$ subunit chaperone

AHSP
AHSP: $\alpha$ complex

AHSP

Pathways for HbA Assembly

Precipitation

$\alpha$ chain

$\beta$ chain

$\alpha_1 \beta_1$ dimers

HbA tetramer

Precipitation
AHSP: α complex

AHSP

α chain

Precipitation

β chain

Precipitation

Precipitation

α₁β₁ dimers

HbA tetramer
**AHSP:** α complex

**AHSP**

**α chain**

**β chain**

**Precipitation**

**Pathways for HbA Assembly**

**α₁β₁ dimers**

**HbA tetramer**
$\alpha + 1.0 \mu M \text{ AHSP}$

$0.5 \mu M = [\alpha]_{\text{total}}$
\[ \alpha + 1.0 \, \mu M \text{ AHSP} \]

\[ 0.5 \, \mu M = [\alpha]_{\text{total}} \]

A. 

Normalized Signal vs. Time (s)

\[ 10 \, \mu M^{-1}s^{-1} \]
\[ \alpha + 1.0 \mu M + 3.0 \mu M \beta = [\alpha]_{total} \]

Hb dimer assembly is 20 times slower.
\( \alpha + 1.0 \mu M \text{AHSP} \)

\( 0.5 \mu M = [\alpha]_{\text{total}} \)

3.0 \( \mu M \alpha + 3.0 \mu M \beta \)

Hb dimer assembly is 20 times slower.

0.25 \( \mu M \text{AHSP}: \alpha + 9.7 \mu M \beta \)
\[ \alpha + 1.0 \mu M AHSP \]

\[ 0.5 \mu M = [\alpha]_{total} \]

Hb dimer assembly is 20 times slower.

\[ 0.25 \mu M AHSP: \alpha + 9.7 \mu M \beta \]

\[ 10 \mu M^{-1}s^{-1} \]

\[ 0.1 s^{-1} \]

\[ 0.5 \mu M^{-1}s^{-1} \]

\[ \alpha_1\beta_1 \text{ dimer} \]
Pathways for HbA Assembly

AHSP:α complex

AHSP

α chain

β chain

Precipitation

Precipitation

Precipitation

α1β4 dimers HbA tetramer
AHSP: $\alpha$ complex

Pathways for HbA Assembly

Precipitation

$\alpha$ chain

$\beta$ chain

$\alpha_1 \beta_1$ dimers

HbA tetramer

$\beta_4$ tetramer

Precipitation
AHSP: α complex

Pathways for HbA Assembly

Precipitation

Precipitation

Precipitation

α₁β₁ dimers

HbA tetramer
But [AHSP] can't be too high.
$1.5 \, \mu M \alpha, \text{AHSP} + 1.5 \, \mu M \beta$

$[\text{AHSP}] = 0 \, \mu M$
1.5 μM α, AHSP + 1.5 μM β

[AHSP] = 0 μM

Absorbance Change (a.u.)

Time (s)

1.5 μM α, AHSP + 1.5 μM β
1.5 µM $\alpha$, AHSP + 1.5 µM $\beta$

$[\text{AHSP}] = 10$ µM

$[\text{AHSP}] = 5.0$ µM

$[\text{AHSP}] = 2.5$ µM

$[\text{AHSP}] = 0$ µM

$\kappa_{1,2}$

$\kappa'_{2,4}$

$\alpha_1\beta_1$ dimer

HbA tetramer
$1.5 \, \mu M \alpha, \text{AHSP} + 1.5 \, \mu M \beta$

$[\text{AHSP}] = 10 \, \mu M$

$[\text{AHSP}] = 5.0 \, \mu M$

$[\text{AHSP}] = 2.5 \, \mu M$

$[\text{AHSP}] = 0 \, \mu M$

$\alpha: \text{AHSP}$

$\text{AHSP}$

$\beta$

$\alpha \text{HbA tetramer}$

$\alpha_1 \beta_1 \text{dimer}$

$k'_{\text{AHSP}}$

$k_{\text{AHSP}}$

$k'_{1,2}$

$k'_2,4$
1.5 μM α, AHSP + 1.5 μM β

[AHSP] = 10 μM
[AHSP] = 5.0 μM
[AHSP] = 2.5 μM
[AHSP] = 0 μM

Excess AHSP inhibits Hb Assembly
Engineering the *E. coli* bacterium

Expression Vectors (ds DNA)

- Tetracycline resistance
- Synthetic hemoglobin Genes + tac promoter
- Restriction enzyme site for cloning

Transformation into *Escherichia coli*

- Chromosomal DNA
- Mutation

**Mutation**

- Higher copy plasmid
- Low copy plasmid
- pBR322 based vector

**pBAD33-AHSP**

- Chloramphenicol resistance
- pACYC vectors with helper genes

Grow on tetracycline and chloramphenicol plates to select for cells containing both plasmid DNAs and turn Hb on with IPTG and AHSP with arabinose.

1. Colonies that are resistant to tetracycline and chloramphenicol and express both rHb and AHSP
2. Red color is due to hemoglobin (HbO₂) expression

Todd Mollan
Mitch Wiess

**Pbad**

**ahsp gene**
A. Effects of IPTG and Ara Induction

- +IPTG, +Ara
- +IPTG
- +Ara
- nothing
A. Effects of IPTG and Ara Induction

- +IPTG, +Ara
- +IPTG
- +Ara
- nothing

B. Variation of [Ara], no IPTG

- low [AHSP] enhances rHb expression
- high [AHSP] is inhibitory

(6.7 mM Ara)
AHSP does aid assembly, but only at substoichiometric concentrations (i.e. [AHSP] ≤ 0.2 [Hb subunits])
Real Genetic Engineering

Put the helper genes and switches directly into the E. coli genome and create a bacterial cell designed to produce rHb.
Chromosomal DNA

High copy plasmid

Low copy plasmid

Helper genes (heme transport, chaperone, others)

Genes for rHb blood substitute

Helper genes and switches directly into the *E. coli* genome and create a bacterial cell designed to produce rHb.
Recombinant hemoglobin will be the ultimate starting material for all Hb-based Oxygen Carriers.