In Pursuit of Artificial Blood: Developing a Plasmid Expression System for Synthesis of Hemoglobin and Haptoglobin Complex in *Escherichia coli*

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Hemoglobin (Hb) is an essential protein responsible for distributing oxygen to organs and tissues. The demand for this vital molecule is high as blood shortages are noted across the globe. The scarcity of blood supply is further compounded by issues of purity, safety, and storage. Donated blood must be tested for contaminants, and the short shelf-life puts pressure on constantly finding new donors. As a result, the need for artificial blood is greater than ever. Many products are in advanced phases of clinical trials rely on Hb's ability to carry oxygen and are known as the hemoglobin-based oxygen carrier (HBOC). Although many advances have been implemented in the developing HBOCs, oxygen capacity and cell-free Hb toxicity are two major areas that still require further improvements. Using recombinant DNA technology, we designed an *Escherichia coli* plasmid expression system to synthesize recombinant Hb via overexpression of the crocodile Hb genes, which have evolved to bind with byproducts of respiration to improve oxygen capacity and release mechanism. To neutralize the toxicity associated with cell-free Hb, we included a gene for haptoglobin (Hp) which, when expressed, binds to Hb, stabilizes Hb's tetrameric structure, and prevents damaging effects. We hope that our system for recombinant Hb-Hp protein complex production proves efficient, high-yielding, and affordable so that synthetic blood is equitably accessible across the globe.

Keywords: Hemoglobin, haptoglobin, Hemoglobin-based oxygen carrier (HBOC), synthetic blood

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Watch a video introduction by the authors at https://youtu.be/T6cNIIqen6s

Background

While the demand for blood is always high, it often seems to be in short supply. According to the Community Blood Center Organization, around 37% of the US population is able to donate blood, but less than 10% actually do so (Blood Facts, 2022). In the US, someone needs blood every 2 seconds, and 1 in 7 people entering a hospital require blood (WHO, 2022). Blood transfusions save lives and improve health, but many patients do not have access to blood. According to the World Health Organization (WHO), of the 118.5 million blood donated, 40% is collected in high-income countries, which are home to only 16% of the world's population (WHO, 2022). This means that low-income countries have a blood shortage and struggle with recruiting a sufficient voluntary donor base to maintain a steady supply.

Another critical component of donated blood must be considered purity and safety. Efforts must be taken to decrease the risk of transfusion-transmitted infections and treat those who happen to test positive. The donated blood must be examined for viral contaminants such as HIV and hepatitis as well as for bacteria, prions, and parasites (Haldar et al, 2019). Thorough quality checks and assurances are performed in high-income nations but may not be rigorously carried out in low-income countries, putting patients who receive donated blood at risk (Jenny, 2017).In low-income countries, only 34% of blood laboratories are subjected to quality assessment review compared with 81% in high-income countries, resulting in transfusion-transmitted diseases (Jenny, 2017). The paucity of resources results in either inadequate screening methods or tests with low sensitivity.

Furthermore, processing and storing blood is an additional obstacle that must be addressed. Donated blood is spun in centrifuges to separate into transfusable components such as red cells, platelets, and plasma. Each component is packaged as needed per transfusion requirements, tested and stored appropriately (WHO, 2022).The Red Cross recommends storing red cells at 6° C for only 42 days, platelets at room temperature in agitators for up to 5 days, and plasma frozen and stored in freezers at -80° C for up to 1 year (WHO, 2022). The procedures, and equipment associated with processing and storing donated blood are complex and expensive. High-income countries are able to streamline these processes and afford all of the necessary equipment and dedicated personnel. However, low-income countries may not have the necessary resources, funding, expertise, or staff. In addition, time constraints on storing different blood components are challenging and put a constant demand on finding blood donors prior to expiration data.

Due to the aforementioned issues of an insufficient base of volunteers worldwide, difficulty adhering to high standards of purity and safety, and challenges with processing and storing blood, the need for creating artificial blood and blood substitutes is more crucial than ever. Different solutions and various technologies are being explored to replace blood. However, it is difficult to replicate real blood due to its complicated mixture of hormones, proteins, mineral salts, fats, vitamins, platelets, white blood cells, and red blood cells (Moradi *et al*., 2016; Sarkar, 2008; Gupta, 2019). So far, only several blood substitutes that mimic one or two functions of blood have been developed. This task is challenging, but a viable substitute must possess at least the following three characteristics: safe and compatible with the human body, able to transport oxygen throughout the body, and be stored for long periods with no impact on stability (Moradi *et al*., 2016; Sarkar, 2008; Gupta, 2019).

One of the more promising types of blood substitutes in development is hemoglobin-based oxygen carriers (HBOCs) (Gupta, 2019; Perutz *at al.*, 1981). HBOCs transport oxygen throughout the body. An example of an HBOC is Hemopure, a solution made with chemically stabilized bovine hemoglobin that can be stored at room temperature for up to three years. It has been approved in South Africa and Russia for the treatment of anemia. In the US, it is an investigational new drug seeking FDA approval. Hemopure is compatible with all blood types and is administered through standard intravenous lines with immediate release of oxygen to tissues; because of its small molecular diameter, it has the potential to transport oxygen through restricted blood vessels. However, several serious adverse events were noted for this product, such as stomach pain, hypertension, jaundice, and nausea. In the clinical trials, the patients treated with Hempure had outcomes no worse than patients treated with allogeneic red blood cells, supporting the need for further product development and continuation with follow-up clinical trials in humans (Chen, 2009). For example, in one study, 25% of patients given Hemopure experienced at least one serious adverse event compared to 18% of patients receiving red blood cells (Feuerstein, 2002). Even though overall, the study showed Hempure as a promising product, the side effects that were noted the need to be addressed by further optimization

Our research focuses on developing a type of HBOC utilizing recombinant DNA technology. We propose expressing a modified crocodile hemoglobin (Hb) protein which has been shown to have an increased oxygen carrying capacity (Roamcharen *et al.*, 2019; Brunori *et al.*, 1995; Bautista *et al*., 2021; Meegan *et al.*, 2021). Small changes in the crocodile's Hb allow these marsh

predators to hold their breath for longer than an hour. In most animals, the carbon dioxide inhaled with each breath dissolves in the bloodstream to form bicarbonate ions. The crocodile Hb has evolved to contain amino acids that form binding sites to the bicarbonate ions, waste products of respiration, at the interface of the alpha and beta globin chains. When Hb binds to bicarbonate ions, the Hb affinity to the oxygen decreases and thus resulting in more of the oxygen delivered to the crocodile's tissues. Several groups noted that the crocodile's Hb feedback mechanism could be utilized to optimize the oxygen capacity of artificial blood (Roamcharen *et al.*, 2019; Bautista *et al*., 2021; Meegan *et al.*, 2021).

Red blood cells harbor Hb and distribute it to organs and tissues. Outside of red blood cells, the Hb tetramer dissociates into dimer subunits and causes toxicity (Invest, 2012; Buehler *et al.*, 2020). Translocation of Hb dimers initiates Hb toxicity and causes chemical Hb reactions in vulnerable tissue. The causes of Hb toxicity are oxidative reactions and reactions that consume nitric oxide, causing vasoconstriction. Some diseases with Hb toxicity include acute and chronic vascular illnesses, kidney failures, thrombosis, and inflammation (Invest, 2012; Buehler *et al.*, 2020). By the same token, HBOCs have been related to problems in the human body such as transient hypertension, gastrointestinal, pancreatic/ liver enzyme elevation, and cardiac/renal injury. In contrast to the scavenging of vascular endothelial nitric oxide (NO) and heme-mediated oxidative side reactions that have been thought to be major causes of toxicity, oxidative pathways have been claimed by more recent studies to play a major role in the toxicity of HBOCs and free Hb.

Antioxidative strategies are evaluated as potential solutions to reduce adverse reactions to cell-free Hb toxicity (Alayash, 2019). Chemical or genetic engineering has produced several HBOCs with different oxygen-binding characteristics, circulatory half-lives, and oncotic features. The objective was to achieve two goals: first, to stabilize the hemoglobin (Hb) molecule in a tetrameric or polymeric state, and second, to improve Hb oxygen carrying capacity.

Another potential solution to address cell-free Hb toxicity is implementing the haptoglobin (Hp) protective mechanism. Production of Hp is a natural reaction initiated by the immune system to neutralize the toxicity of Hb dimers during hemolysis when red blood cells rapture, and their content is released into the bloodstream (Schaer *et al*., 2013, 2021; Natarajan *et al*., 2011). To counteract the toxic effect, liver cells start producing Hp, which binds to Hb dimers and transports Hb back to the liver for processing. Also, in some cases, the Hp provides structural stability to the Hb tetramer. The Hb and Hp complex is irreversible and is cleared by CD163-positive monocytes and macrophages or recycled (Schaer *et al.*, 2013). The Hp shields oxidative reactions with lipoproteins, so the redox reactivity is stabilized, and Hb is protected against oxidative damage. We intend to incorporate the Hp capacity as a clearance protein and stabilizing agent to neutralize Hb dimer toxicity and protect the Hb structure.

Systems Level

To alleviate the global need for blood donation and challenges associated with the quality and storage of donated blood, we plan to continue the development of synthetic blood substitutes in the category of HBOC. The current HBOC products can be further augmented to improve Hb oxygen capacity and distribution of oxygen throughout the body. In addition, the synthesized Hb molecule must be stabilized to prevent cell-free Hb toxicity. This will be done by expressing crocodile Hb to increase oxygen delivery to the tissues and by expressing Hp to address Hb toxicity and stability.

Device Level

We propose to create an affordable blood substitute of the HBOC type in the form of an intravenous drip bag. Our product name is Hemptoglobin, with an active ingredient Hemoglobin-Haptoglobin protein complex (Hb-Hp) mixed in a soluble solution along with electrolytes (Figure 1).

We plan to use *E. coli* for production of the Hb-Hp recombinant protein complex. Expression of the crocodile Hb protein, known for its high oxygen capacity and efficient oxygen release to the tissue, would greatly increase the therapeutic potential of Hemptoglobin.We plan to express the Hp gene, to address cell-free Hb toxicity and prevent it from dissociating into dimers. The Hp protein is known for neutralizing the toxic effects of cell-free Hb. The Hp has a natural affinity for Hb dimers and will bind upon contact. Hp stabilizes and protects the Hb tetramer (not dissociated) structure (Figure 2).

We selected *E. coli* as the host for transfection and expression of the Hb and Hp genes because its plasmid system is readily available, well-established, and efficient. Due to its popularity in academia and industry, the *E.coli* system allows for easier troubleshooting of any potential issues. In terms of equity, the *E.coli* system can be adapted globally and in low-income countries more easily thanexpensive and complex mammalian systems.

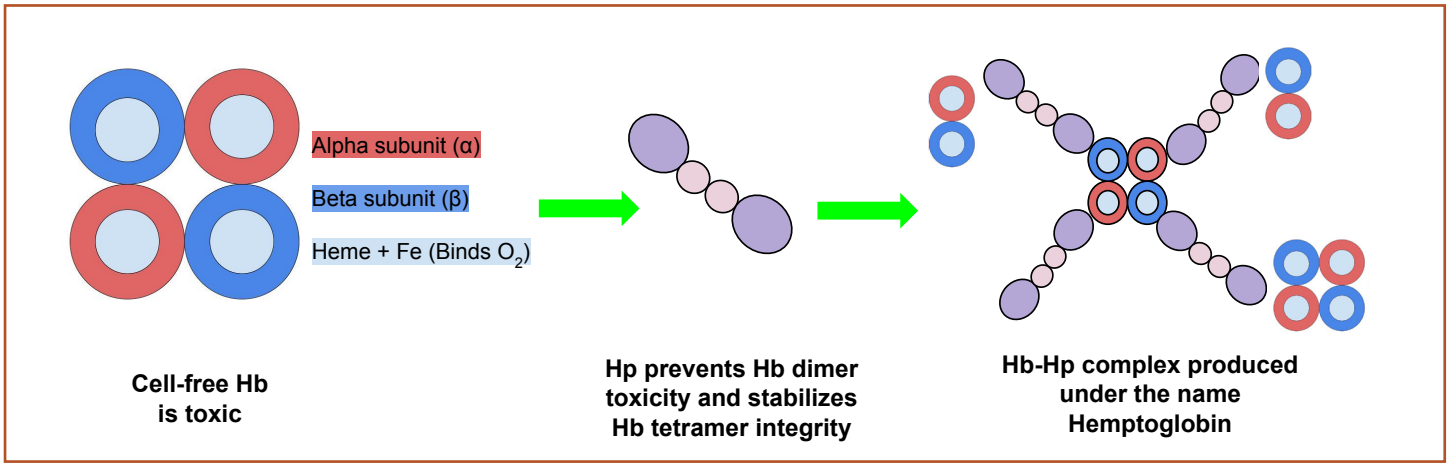

Figure 1: *Design of the Hemoglobin and Haptoglobin Protein Complex: Hemptoglobin. Crocodile Hb evolved to bind with byproducts of respiration, which allows the crocodile to stay underwater for over an hour in one breath. Using the crocodile Hb will result in higher oxygen capacity and a more efficient release of oxygen into the tissue. Genes for Hb: HBA (Hα) and HBB (Hβ). Cell-free Hb dissociates into dimers and causes toxicity. Hp is known to naturally bind to Hb and neutralize the toxic effects. Gene for Hp:Hp.*

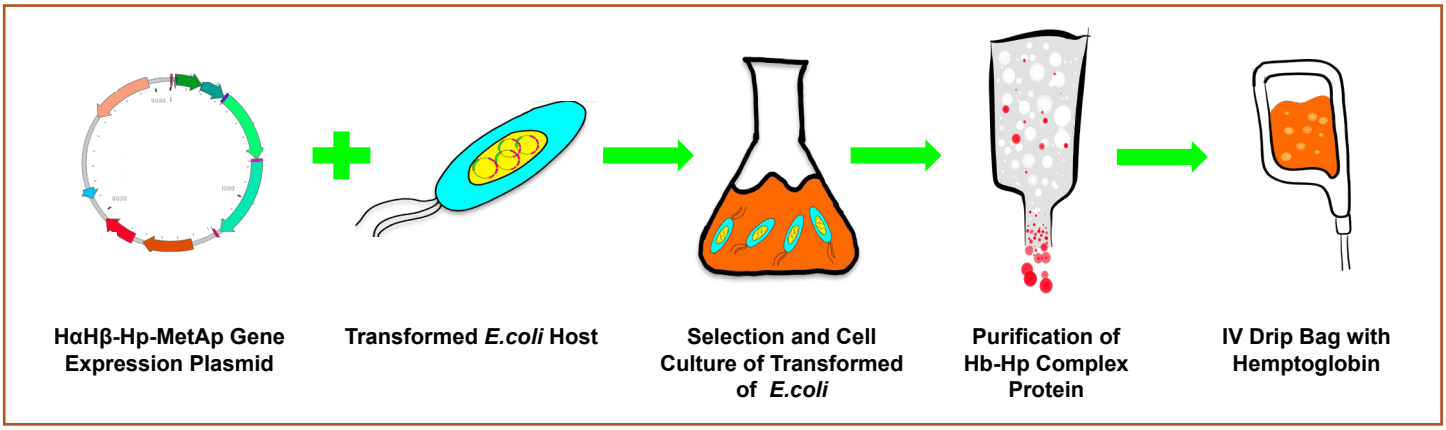

Figure 2: *Development and Production of Novel HBOC: Hemptoglobin. HαHβ-Hp-MetAp Gene Expression Plasmid transformed into E. coli. The transformed cells are cultured in the presence of ampicillin in an appropriate medium to select the population of cells containing the integrated plasmid. The successfully transformed cells grow in media with glucose, IPTG, and hemin to produce the Hb-Hp protein complex. Cell culture is spun down and supernatant subjected to purification to isolate the Hb-Hp protein complex as a drug substance. The drug substance is formulated into the final product: an IV drip bag with Hemptoglobin.*

Since *E. coli* is the host, we included in the plasmid the MetAP gene to cleave the methionine residues, which are an important element of post-translational modification and will enhance Hemptoglobin biocompatibility in humans. In part, we designed our experimental plan based on Natarajan *et al.* research (2011).

Parts Level

We plan to use the *E. coli* pET vector system to express Hb and Hp recombinant proteins along with the MetAP enzyme. We will clone genes of interest into the pET vector under the control of a strong bacteriophage promoter. We optimized all of the gene sequences with respect to *E. coli* codon preferences to maximize translational efficiency (Dataset 1). T7 RNA polymerase activates transcription and translation and is known for high expression. When fully induced, several groups have found that nearly all of the cell's resources are dedicated to making the genes of interest and comprise almost half of the cell's total protein production (Dubendorff *et al.*, 1991; Francis, 2010).

Dataset 1: *Codon Optimized Genes of Interest: Hemoglobin Subunit Alpha, Hemoglobin subunit Beta, Haptoglobin, and MetAP.*

Hemoglobin Subunit Alpha:

ATG GTC CTT TCT CCG GCT GAC AAG ACA AAT GTA AAA GCC GCG TGG GGT AAA GTT GGC GCT CAC GCC GGG GAA TAT GGG GCC GAG
GCT CTT GAG AGA ATG TTC CTG TCA TTC CCA ACG ACT AAA ACT TAT TTC CCC CAC TTC GAC CTT TCT CAC GGG TCC GCC CAG GTT AAG
GGC CAC GGT AAG AAG GTA GCT GAC GCA TTG ACG AAT GCA GTG GCT CAT GTC GAT GAT ATG CCG AAC GCT TTG TCC GCC CTT AGC
GAC CTG CAC GCC CAC AAG TTA AGA GTC GAT CCC GTA AAC TTC AAG CTT CTG AGC CAT TGC TTG TTA GTT ACG CTG GCA GCC CAC TTG
CCG GCT GAA TTT ACT CCG GCA GTT CAT GCC TCA TTG GAT AAG TTC CTT GCC AGT GTG TCC ACC GTA CTT ACG AGT AAA TAT AGA TAA

Hemoglobin Subunit Beta:

ATG GTC CAC CTG ACA CCA GAA GAA AAG TCG GCC GTG ACG GCA TTG TGG GGA AAG GTG AAC GTG GAC GAG GTA GGA GGG GAG GCA
CTG GGG CGG CTG TTA GTT GTT TAC CCC TGG ACC CAA CGG TTT TTT GAA TCT TTT GGT GAC TTA TCA ACT CCA GAC GCG GTT ATG
GGA AAC CCA AAA GTA AAA GCC CAT GGC AAA AAG GTC TTG GGC GCG TTC TCA GAC GGT CTG GCG CAC CTT GAT AAC CTG AAG GGA
ACA TTC GCC ACG CTG TCA GAG CTG CAT TGC GAC AAG TTG CAC GTC GAT CCT GAA AAC TTT AGA TTG TTA GGT AAT GTA CTG GTA
TGT GTC TTG GCT CAT CAC TTC GGA AAG GAA TTC ACT CCT CCA GTG CAA GCA GCG TAT CAA AAA GTG GTA GCA GGA GTA GCG AAT
GCC CTG GCC CAT AAA TAT CAC TAA

Haptoglobin:

ATG TCG GCA CTG GGT GCT GTA ATC GCT TTG TTG TTA TGG GGC CAG CTG TTT GCT GTA GAC TCT GGG AAC GAT GTG ACG GAC ATT
GCA GAT GAT GGT TGT CCT AAA CCG CCA GAA ATC GCT CAC GGC TAT GTA GAG CAT TCT GTT CGG TAT CAA TGC AAG AAC TAT TAC
AAA CTT CGG ACA GAA GGA GAT GGA GTG TAC ACC CTT AAC AAT AAA AAG CAG TGG ATC AAC AAG GCT GTC GGT GAC AAA CTT CCG
GAA TGC GAA GCT GAC GAC GGT TGC CCC AAA CCA CCC GAA ATC GCC CAT GGT TAC GTT GAA CAC AGC GTC CGG TAT CAG TGC AAA
AAC TAT TAC AAG CTG CGT ACA GAG GGT GAT GGA GTT TAT ACA TTG AAC AAT GAG AAG CAG TGG ATC AAT AAG GCA GTT GGC GAT
AAA CTT CCA GAA TGC GAG GCT GTG TGC GGT AAG CCC AAA AAC CCG GCT AAT CCG GTT CAG CGT ATC TTA GGA GGA CAC CTT GAC
GCC AAA GGA TCG TTC CCG TGG CAG GCT AAA ATG GTG TCA CAC CAT AAT TTG ACC ACC GGA GCC ACA CTT ATT AAT GAA CAG TGG
TTA TTG ACG ACA GCC AAA AAC CTT TTT CTT AAT CAT TCT GAG AAT GCG ACG GCT AAG GAC ATC GCA CCC ACA TTA ACG TTA TAT
GTG GGC AAG AAG CAA TTG GTT GAG ATC GAG AAA GTT GTC CTT CAC CCG AAC TAT AGT CAA GTT GAC ATT GGT TTA ATC AAA CTG
AAG CAG AAA GTG AGC GTA AAT GAA CGG GTC ATG CCG ATC TGT TTA CCG TCG AAG GAT TAC GCC GAG GTC GGC CGC GTC GGG TAT
GTT TCC GGT TGG GGG CGT AAC GCA AAC TTC AAG TTC ACA GAC CAC TTA AAG TAT GTT ATG CTT CCG GTG GCG GAC CAG GAC CAG
TGC ATA AGA CAC TAC GAG GGG TCG ACT GTA CCT GAA AAA AAA ACT CCG AAG TCA CCT GTC GGT GTT CAG CCG ATT TTG AAC GAA
CAT ACA TTC TGT GCA GGA ATG AGT AAG TAT CAA GAG GAC ACT TGC TAT GGG GAC GCT GGC TCA GCA TTC GCA GTT CAT GAT CTT
GAG GAG GAT ACC TGG TAC GCC ACC GGG ATA CTG AGT TTC GAC AAA AGT TGC GCT GTC GCG GAA TAT GGT GTG TAC GTT AAG GTG
ACT TCC ATC CAA GAT TGG GTT CAA AAA ACA ATT GCA GAA AAC TAA

MetAP (methionine aminopeptidase):

ATGGCGGGTGTGGAGGAGGTAGCGGCCTCCGGGAGCCACCTGAATGGCGACCTGGATCCAGACGACAGGGAAGAA
GGAGCTGCCTCTACGGCTGAGGAAGCAGCCAAGAAAAAAAAGACGAAAGAAGAAGAAGAGCAAAGGGCCTTCTGCA
GCAGGGGAACAGGAACCTGATAAAGAATCAGGAGCCTCAGTGGATGAAGTAGCAAGACAGTTGGAAAGATCAGCA
TTGGAAGATAAAGAAAGAGATGAAGATGATGAAGATGGAGATGGCGATGGAGATGGAGCAACTGGAAAGAAGAA
GAAAAAGAAGAAGAAGAAGAGAGGACCAAAAGTTCAAACAGACCCTCCCTCAGTTCCAATATGTGACCTGTATC
CTAATGGTGTATTTCCCAAAGGACAAGAATGCGAATACCCACCCACACAAGATGGGCGAACAGCTGCTTGGAGAA
CTACAAGTGAAGAAAAGAAAGCATTAGATCAGGCAAGTGAAGAGATTTGGAATGATTTTCGAGAAGCTGCAGAA
GCACATCGACAAGTTAGAAAATACGTAATGAGCTGGATCAAGCCTGGGATGACAATGATAGAAATCTGTGAAAA
GTTGGAAGACTGTTCACGCAAGTTAATAAAAGAGAATGGATTAAATGCAGGCCTGGCATTTCCTACTGGATGTTC
TCTCAATAATTGTGCTGCCCATTATACTCCCAATGCCGGTGACACAACAGTATTACAGTATGATGACATCTGTAA
AATAGACTTTGGAACACATATAAGTGGTAGGATTATTGACTGTGCTTTTACTGTCACTTTTAATCCCAAATATGA
TACGTTATTAAAAGCTGTAAAAGATGCTACTAACACTGGAATAAAGTGTGCTGGAATTGATGTTCGTCTGTGTG
ATGTTGGTGAGGCCATCCAAGAAGTTATGGAGTCCTATGAAGTTGAAATAGATGGGAAGACATATCAAGTGAAA
CCAATCCGTAATCTAAATGGACATTCAATTGGGCAATA6TAGAATACATGCTGGAAAAACAGTGCCGATTGTGA
AAGGAGGGGAGGCAACAAGAATGGAGGAAGGAGAAGTATATGCAATTGAAACCTTTGGTAGTACAGGAAAAGGT
GTTGTTCATGATGATATGGAATGTTCACATTACATGAAAAATTTTGATGTTGGACATGTGCCAATAAGGCTTCC
AAGAACAAAACACTTGTTAAATGTCATCAATGAAAACTTTGGAACCCTTGCCTTCTGCCGCAGATGGCTGGATC
GCTTGGGAGAAAGTAAATACTTGATGGCTCTGAAGAATCTGTGTGACTTGGGCATTGTAGATCCATATCCACCA
TTATGTGACATTAAAGGATCATATACAGCGCAATTTGAACATACCATCCTGTTGCGTCCAACATGTAAAGAAGT
TGTCAGCAGAGGAGATGACTATTAA

The pET system is typically transferred into a host strain, engineered to carry the T7 RNA polymerase gene under the control of the LacUV5 promoter. We plan to use the *E. coli* strain BL21 (DE3). This bacterial strain contains a chromosomal copy of the gene for T7 RNA polymerase, and transcription is induced by the addition of IPTG to the bacterial culture.

The cell culture will consist of 2xYT medium with ampicillin (100 µg/mL). Ampicillin is used for artificial selection to eliminate the cells which did not uptake the plasmid. The cells will be grown at 37°C in orbital shakers at 200 RPM and induced with 0.2 mM Isopropyl β- d-1-thiogalactopyranoside (IPTG) and then supplemented with hemin (50 µg/mL) and glucose (20 g/L). IPTG is a

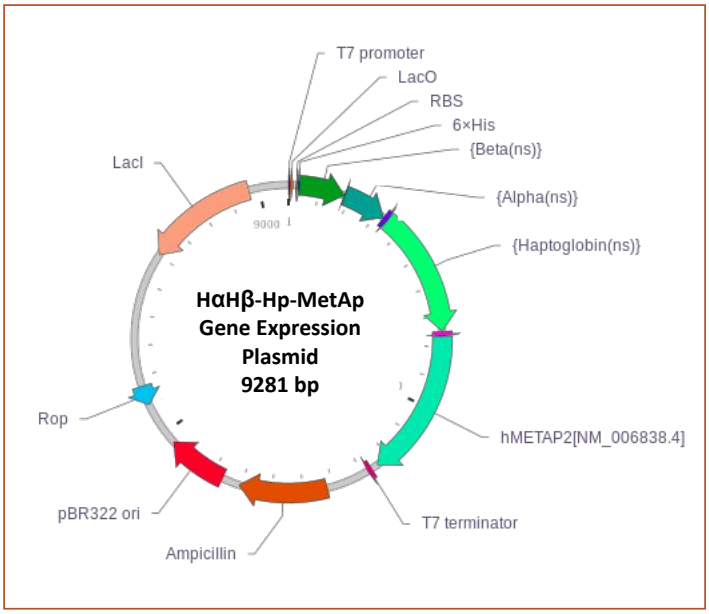

Figure 3. *HαHβ-Hp-MetAp Gene Expression Plasmid*

molecular mimic of allolactose, a lactose metabolite that triggers transcription of the lac operon and is used to induce protein expression where the gene is under the control of the lac operator. The medium is supplemented with hemin to provide a binding site for iron which carries the oxygen. We are also adding glucose to the medium to culture and grow the bacteria under optimal conditions (Studier *et al.*, 1990).

We plan to construct an expression plasmid, HαHβ-Hp-MetAp Gene Expression Plasmid, which contains the Alpha and Beta globin sequences, that are characteristic of the crocodile Hb Alpha and Beta genes (Figure 3 and Table 1). We will fuse the two Hb genes using a 3xGGGGS linker. We plan to coexpress Hp and MetAP genes (stop codon after each gene: UAA), along with the Alpha and Beta globin genes. Hp serves to neutralize the toxic effects of cell-free hemoglobin, and MetAP cleaves the N-terminal methionine residues from the nascent globin chains, a critical post-translational modification of tetrameric Hb, rendering it biocompatible. We will use the ampicillin resistance gene to provide a means of antibiotic selection and T7 promoter to drive a high-level transcription of the genes of interest when T7 RNA polymerase is present. When placed immediately upstream of a lacO element, the entire cassette is known as the T7lac promoter. We also include the LacI gene for induction of the system with IPTG.

Safety

The *E. coli* strain BL21 (DE3) is not hazardous (Catalog Number: C2527 from New England Biolabs, Ipswitch, MA). No special measures are required to safely handle this product. However, we plan to work in a contained laboratory environment to prevent contamination and ensure the safety of our lab area and personnel. The reagents used for the cell culture of *E. coli* are also not hazardous and do not require fume or chemical hoods for handling. The vector is also not hazardous.

We plan to adhere to good laboratory practices and wear personal protective equipment such as gloves, goggles, and lab coats.

Discussions

In the past, many groups have encountered challenges in producing an artificial blood substitute via recombinant DNA technology. One of the major obstacles encountered by others is increasing productivity at a manageable cost. Likely, we will have to optimize cell output to ensure sufficient yields of the Hemptoglobin. Many optimization experiments will be designed to enhance culture conditions and *E. coli* expression output. Temperature, cell culture duration, and media components must be assessed to increase growth and productivity. We will also evaluate different components of the expression vector to increase promoter performance, tweak coding sequences to improve stability and product quality and ensure proper expression.

Developing a purification process to isolate the Hb-Hp protein complex without any impurities is another challenge. There are four basic steps of protein purification: harvest and cell lysis, binding protein to a matrix, washing off undesirable molecules and contaminants, and final elution of the desired protein complex. We will develop and optimize these steps to purify the Hemptoglobin protein substance. Once purified, we will formulate the Hemptoglobin drug product into an intravenous drip bag. Before commercial production and administration to patients, animal and human clinical trials will be conducted to test Hemptoglobin for safety, efficacy, and biocompatibility.

We hope that our research will be applied to other fields. Because our product focuses on utilizing crocodile Hb which is known for higher oxygen release capacity, it could be applied to enhance performance in extreme low-oxygen environments. First-responders during rescue missions caused by fires, earthquakes, and other natural disasters are exposed to low oxygen conditions and would benefit from using an oxygen-enhancing product readily available in stressful and quickly-changing conditions. Hemptoglobin can be administered to survivors as temporary relief to sustain life, while proper hospital help may be far away. Other groups that may benefit from Hemptoglobin are military personnel

Table 1. *Components of the HαHβ-Hp-MetAp Gene Expression Plasmid*

Name	Size (bp)	Type	Description	Application Notes
T7 promoter	17	Promoter	T7 Promoter	Drives high-level transcription of downstream gene in E. coli host cells that express T7 RNA polymerase; can also be used for in vitro transcription.
LacO	25	Miscellaneous	Lac operator	In the absence of IPTG, it is bound by lac repressor (LacI), leading to transcriptional repression of the adjacent gene or shRNA. In the presence of IPTG, LacI can no longer bind to LacO, thus allowing the adjacent gene or shRNA to be transcribed.
RBS	5	RBS	Ribosome Binding site	Recruits ribosome to mRNA and aligns ribosome with start codon to initiate protein synthesis in E. coli.
6xHis	18	Tag	6 tandem histidine tag	Facilitates purification by nickel or cobalt matrix and antibody binding of the tagged protein.
Beta(ns)	441	ORF	*None*	Beta hemoglobin gene
Alpha(ns)	426	ORF	Codon optimized for E. coli	Alpha hemoglobin gene
Haptoglobin(ns)	1218	ORF	Codon Optimized for E. coli	Haptoglobin gene
hMETAP2 [NM_006838.4]	1437	ORF	None	MetAP gene
T7 terminator	47r	Terminator	T7 transcription terminator	Allows transcription termination of RNA transcribed by bacteriophage T7 RNA polymerase.
Ampicillin	861	ORF	Ampicillin resistance gene	Allows E. coli to be resistant to ampiciIIin.
pBR322 ori	589	Replication_ origin	pBR322 origin of replication	Facilitates plasmid replication in E. coli; regulates low-copy plasmid number when Rop protein is the presence (15-20) and medium-copy plasmid number when Rop is absent (100-300).
Rop	192	ORF	Repressor of primer	It encodes a small protein that regulates plasmid copy number in E. coli. The presence of Rop protein, in combination with pBR322 origin of replication on the plasmid results in low copy number of the plasmid.
LacI	1083	ORF	Lac repressor	In the absence of IPTG, it binds to the lac operator (LacO) to repress transcription of downstream gene in E. coli. In the presence of IPTG no longer binds to LacO, thus allowing downstream gene to be transcribed.

or civilians working in high-altitude or deep-water environments. Decreased oxygen causes problems, such as suffocation or inability to complete urgent and critical tasks. Our product has the potential to increase oxygen capacity, enabling emergency first-responders, military personnel, and professionals working under extreme oxygen-depleted conditions to complete their tasks more efficiently while lowering or preventing health risks.

Next Steps

The first set of experiments we plan to perform involves designing the expression plasmid and transforming it into *E. coli*. Transformation experiments must be optimized as the vector we use is unique and carries 4 genes plus the ampicillin resistance gene. Transforming such a large vector maybe a difficult task in a prokaryotic cell line.

After transformation, the cells will be cultured with ampicillin. Since the ampicillin resistance gene is part of the plasmid expression vector, only the cells that contain the plasmid will survive the selection. We will analyze the cultures that survive ampicillin selection, isolate genomic DNA, and use PCR to test for the presence of genes of interest. Also, we will isolate total RNA, reverse-transcrib it to cDNA, and use PCR to test for the presence and level of transcript of a genes of interest.

Once we have verified that the cultures contain the desired genes and make the desired transcripts, we will perform a cloning experiment. The aim is to select a clonal recombinant host cell line that produces the desired protein complex of expected quality and is highly productive.

During the BioBuilder 2022 conference, we met a scientist from Daicel Arbor Biosciences who suggested using a cell-free system for protein expression. We consider using of the myTXTL-cell-free T7 Expression kit as a viable option for initial proof of principle experiments. The myTXTL-cell-free T7 Expression kit has been designed to be used with T7 promoter-driven constructs. Continuous co-expression of T7 RNA polymerase in the *E. coli*-based mix drives protein production for any template with a T7 promoter. In this system, the P70a-T7rnap plasmid delivers continuous production of the T7 RNA polymerase in addition to an *E. coli*-based master mix containing the cellular machinery, energy buffer, and amino acids needed for in vitro protein production in a single tube. Using the cell-free kit is cheaper and will allow us to assess the efficiency of our expression vector and what elements may need to be optimized. Also, initial purification steps may be simplified as we will not need to address host cell proteins and impurities associated with the host.

Purification of recombinant protein from *E.coli* is challenging. For an initial assessment and proof of principle, we will take advantage of the polyhistidine-tag in the expression vector to purify Hb-Hp complex via a widely available purification from Qiagen. For large-scale protein purification, we plan to utilize repeated cycles of freezing and thawing, which have been previously shown to be sufficient in separating highly expressing recombinant protein from the cellular milieu of *E.coli* (Johnson, 1994). The freezing and thawing cycle liberates recombinant proteins from bacterial cytoplasms without releasing the bulk of endogenous host proteins and potential endotoxins. We will further purify the supernatant through HPLC using Q-Sepharose (anion-exchange) followed by SP-Sepharose (cation-exchange) pre-packed columns. We will analyze the purified recombinant complex using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which uses polyacrylamide gel to eliminate the influence of structure and charge, and proteins are separated based on their molecular weight. In addition, we will apply mass spectrometry is a more sensitive method to calculate and assess the exact molecular weight of Hemptoglobin.

Author Contributions

All authors contributed to brainstorming and researching. A.J.L. proposed the idea of developing HBOC and utilizing the crocodile hemoglobin genes. All authors contributed to gathering research articles and developing the idea further, as well as designing figures and tables. A.J.L., I.C., and B.K. drafted the manuscript while A.J.L and X.P. were critical to editing and finalizing the publication. I.C. spear-headed the citation and referencing process.

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