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Engineering Therapeutics to Detoxify Hemoglobin, Heme, and Iron

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Abstract

Hemolysis (i.e., red blood cell lysis) can increase circulatory levels of cell-free hemoglobin (Hb) and its degradation by-products, namely heme (h) and iron (Fe). Under homeostasis, minor increases in these three hemolytic by-products (Hb/h/Fe) are rapidly scavenged and cleared by natural plasma proteins. Under certain pathophysiological conditions, scavenging systems become overwhelmed, leading to the accumulation of Hb/h/Fe in the circulation. Unfortunately, these species cause various side effects such as vasoconstriction, hypertension, and oxidative organ damage. Therefore, various therapeutics strategies are in development, ranging from supplementation with depleted plasma scavenger proteins to engineered biomimetic protein constructs capable of scavenging multiple hemolytic species. In this review, we briefly describe hemolysis and the characteristics of the major plasma-derived protein scavengers of Hb/h/Fe. Finally, we present novel engineering approaches designed to address the toxicity of these hemolytic by-products.

Contents

RED BLOOD CELLS AND HEMOLYSIS

Red blood cells (RBCs) constitute ~45% of the total blood volume and account for ~4% of the total body weight in humans (1). To maintain RBC homeostasis in the circulation, every second, approximately two million RBCs are replaced, each of which contains more than 250 million hemoglobin (Hb) molecules (2, 3). Maintenance of this high RBC turnover rate requires a highly regulated and safeguarded process to ensure proper disposal of senescent RBCs.

Hb is a heterotetrametric protein composed of two alpha and two beta subunits ($\alpha_2\beta_2$). Within each globin subunit (α or β), a hydrophobic porphyrin macrocycle—termed heme (h)—coordinates an iron (Fe) atom (porphyrin + iron = heme), which allows Hb to perform its main function—binding and transport of oxygen (O_2) (**Figure 1**). Without the Hb protein, the hydrophobicity of heme leads to it forming aggregates in aqueous solutions, preventing it from functioning as an oxygen carrier (4). Furthermore, the protein structure of Hb is altered when heme binds to O_2 such that the binding of subsequent O_2 molecules to the neighboring hemes is promoted (a process called cooperativity) (5). The resulting Hb-facilitated O_2 transport enables a more than 60-fold increase in O_2 solubility in blood (~20 mL versus ~0.3 mL of O_2 per 100 mL) and greatly improves exchange of respiratory gases (6). While a remarkable evolutionary feat, O_2 transport and storage via Hb comes with certain caveats. These caveats originate from the toxicity derived from Hb and its major components—heme and the Fe atom coordinated in the porphyrin macrocycle.

Under normal conditions, Hb, heme, and its iron are physically restricted to the cytosol of the RBC, where the reducing environment maintains Hb in the ferrous (Fe^{2+}) state and the high cellular Hb concentration maintains Hb in the tetrameric quaternary state. Although the tetrameric Hb assembly is mainly known for its role in enabling cooperative O_2 binding, tetrameric Hb in the Fe^{2+} state also prevents heme release from the hydrophobic heme-binding pocket of Hb. Therefore, encapsulation of Hb within RBCs ensures proper Hb function, and that heme is bound to the apoprotein of Hb [i.e., apohemoglobin (apoHb)]. However, upon hemolysis (i.e., rupture of the RBC membrane), cell-free Hb is released into the circulation, where the tetrameric Hb concentration drops below its tetramer-dimer equilibrium constant (K_D = 200 nM) (8) and dissociates into αβ dimers. Hb αβ dimers can more readily oxidize into methemoglobin (metHb)

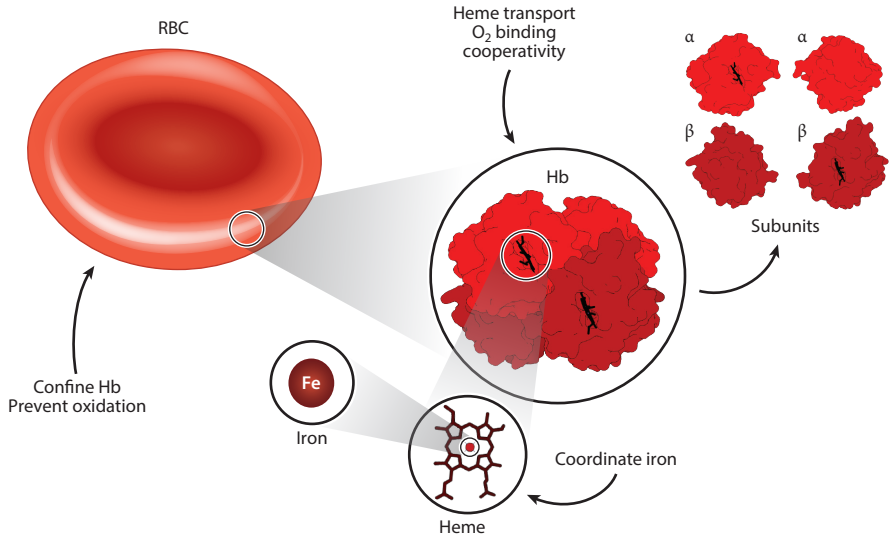

Figure 1

Schematic highlighting red blood cell (RBC) function that enables efficient oxygen (O_2) transport via encapsulation of hemoglobin (Hb). Protein images generated on UCSF Chimera (7) based on Protein Data Bank structure 2HHB.

and unfold to release heme and apoHb. Hb and heme may be further metabolized, leading to the release and buildup of Fe in the bloodstream. These three hemolytic by-products (Hb/h/Fe) are Fe-containing molecules whose Fe must be properly recycled to maintain RBC production given that Fe recycling accounts for 90% of our daily iron requirements, while the remaining 10% are derived via dietary sources (9).

However, regulation of Hb/h/Fe is necessary not only to maintain RBC homeostasis but also to prevent the toxicities associated with each of these components. First, cell-free Hb reacts with and scavenges nitric oxide (NO). Unfortunately, the dimerization of Hb released into the circulation further facilitates its extravasation into the subendothelial space and into the smooth muscle layers of blood vessels. In these tissue compartments, scavenging of NO leads to microvascular, systemic, and pulmonary vasoconstriction, subsequently inducing systemic and pulmonary hypertension (10). The reaction of NO with Hb also oxidizes Hb into metHb, promoting heme release. Second, heme can bind to cellular receptors, transcription factors, and enzymes—disrupting cellular homeostasis (11, 12). For example, heme binds to Toll-like receptor 4 (TLR4), initiating a cascade of inflammatory immune responses (11). Lastly, a significant contribution to the toxicity of Hb/h/Fe originates from their participation in Fenton-type redox reactions mediated by the iron atom, generating free radicals and reactive oxygen species (ROS) (13). These free radicals and ROS can subsequently oxidize and damage lipid membranes, proteins, and nucleic acids. Notably, heme hydrophobicity ensures that most of it circulates bound to either lipoproteins, plasma proteins, or cellular membranes, promoting oxidation of these bound species (11). In the case of cell membranes, while heme rapidly inserts and diffuses through lipid bilayers, its transport across bilayers is slow but is facilitated by transmembrane heme importer or exporter proteins such as the heme responsive gene protein 1 (HRG-1) and the feline leukemia virus subgroup C receptor-related protein 1 (FLVCR1), respectively (14, 15). The increased oxidative stress also induces systemic inflammatory responses, which can lead to tissue injury (12). The major toxic hemolytic components are summarized in **Figure 2**.

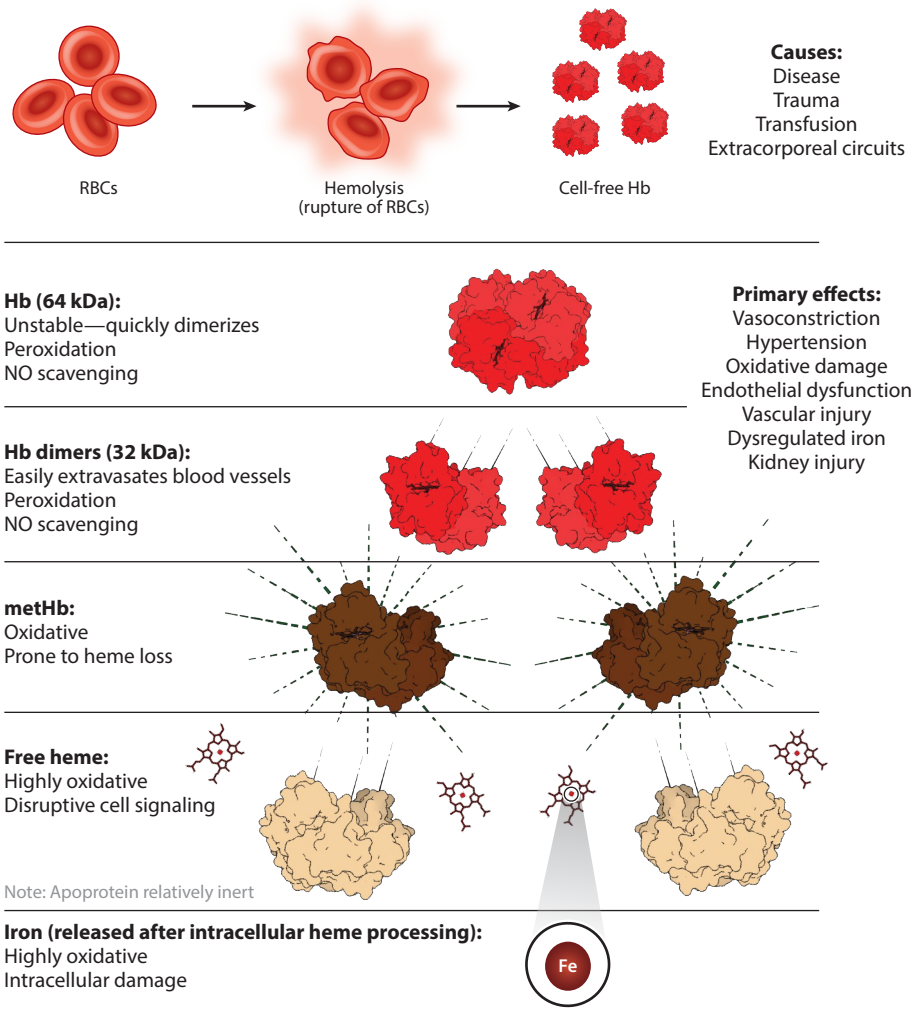

Figure 2

Hemolysis and the major toxic hemolytic species Hb, heme, and iron. Abbreviations: Hb, hemoglobin; metHb, methemoglobin; NO, nitric oxide; RBC, red blood cell. Protein images generated on UCSF Chimera (7) based on Protein Data Bank structure 2HHB.

Common disease states that cause hemolysis include sickle cell anemia, beta thalassemia, and malaria (16, 17). In sickle cell anemia and beta thalassemia, genetic traits lead to RBC hemolysis and subsequent Hb release. In malaria, the parasite (*Plasmodium* spp.) uses RBCs to asexually reproduce until the RBC ruptures, releasing Hb and the sporozoites into the circulation (18). Furthermore, hemolytic conditions have been shown to occur upon massive blood transfusion, fever, sepsis, hemolytic anemia, viral hemorrhage, extracorporeal circulation, burn injury, and radiation exposure (3, 19). Finally, one of the major determinants for the short ex vivo shelf life of stored RBCs is the gradual hemolysis that occurs during storage in the blood bag (20–22).

These toxic and reactive species—Hb/h/Fe—are primarily controlled during mild to moderate hemolysis via the action of three plasma proteins: haptoglobin (Hp), hemopexin (Hpx), and

transferrin (Tf) (23). These serum proteins bind to and neutralize cell-free Hb, free heme, and free iron, respectively. After Hb/h/Fe binding to their protein target, the complexed forms are less reactive and can be safely cleared from the circulation via receptor-mediated endocytosis (23). The toxic effects of Hb, heme, and iron occur when these scavenging proteins are saturated or depleted in the plasma, allowing excess Hb, heme, and iron to freely react with their surroundings. Depletion of scavenger proteins is prominent in hemolytic states such as malaria, beta thalassemia, and sickle cell anemia, where patients have low or undetectable levels of Hp and Hpx in plasma (24, 25). Human serum albumin (HSA) is another serum protein that binds to and transports both heme and iron. Although not as specific nor as strong as the associations present in heme-Hpx or iron-Tf complexes, HSA becomes the main reservoir for heme and iron once the primary scavengers (Hpx and Tf) have been depleted in the plasma.

In addition to direct tissue and organ damage, Hb/h/Fe are also used as substrates for the proliferation of pathogenic microbes (26, 27). Even though some microorganisms can sequester iron from holotransferrin (holoTf), Hb-haptoglobin (Hb-Hp) complex, or heme-bound Hpx, they must compete with the binding affinity of these complexed species for their target cellular receptor and the rapid clearance of these complexes from the circulation (27–29). Therefore, restricting the bioavailability of iron during pathophysiological states could prevent the proliferation of infectious microorganisms (23, 26, 27).

Recently, the scavengers of Hb/h/Fe have been proposed to have an even more vital role in the regulation of hemolysis. Rupture of RBCs has been generally classified into two main categories: intravascular and extravascular hemolysis. Intravascular hemolysis occurs when RBCs rupture within blood vessels, leading to release of Hb/h/Fe into the circulation. On the other hand, extravascular hemolysis is thought to occur when whole RBCs are engulfed (i.e., erythrophagocytosis) by specialized macrophages (mainly, splenic red pulp macrophages or liver Kupffer cells), which directly clears senescent RBCs from the circulation. However, the lack of direct evidence that macrophages perform erythrophagocytosis leads to a new hypothesis: senescent RBCs adhere to the red pulp in the spleen and are lysed under shear flow, releasing their cytosolic contents (mainly Hb) into the circulation such that the RBC ghosts (i.e., the ruptured RBC membrane and underlying cytoskeleton) are captured by the splenic macrophages (30). This potential mechanism for clearance of senescent RBCs greatly increases the importance of scavenger proteins, as hemolysis is an intermediate stage during the RBC turnover process. On the other hand, baseline intravascular hemolysis is estimated to account for 1–10% of hemolytic events (3, 31). Hence, the splenic hemolysis model could provide better clarity on how Hb/h/Fe originates in the circulation—not only random rupture of the RBC membrane and underlying cytoskeleton but deformed or aged RBCs that tend to be more rapidly cleared and increase the rate of release of Hb/h/Fe into the bloodstream.

In this review, we describe the major natural hemolysis scavenging proteins and new engineering approaches for treatment of hemolytic conditions.

HAPTOGLOBIN

Hp is a polymorphic α-2 glycoprotein composed of disulfide-linked αβ dimers with plasma concentration levels ranging from 0.5 to 3 mg/mL (31–33). Each Hp αβ dimer is capable of binding to one Hb αβ dimer and appears to require Hb dimer formation for their association (34). As the primary Hb scavenger molecule, the presence of Hp in plasma gives it an Hb-binding capacity of approximately 1 mg for each milliliter of plasma, with a maximum clearance rate of 0.1–0.2 g Hb/L/h (35, 36). The plasma half-life of Hp ranges from 1.5 to 2 days but is quickly reduced to ~20 min when in the Hp-Hb complex form (37). In humans, the α chain of Hp exists in two allelic

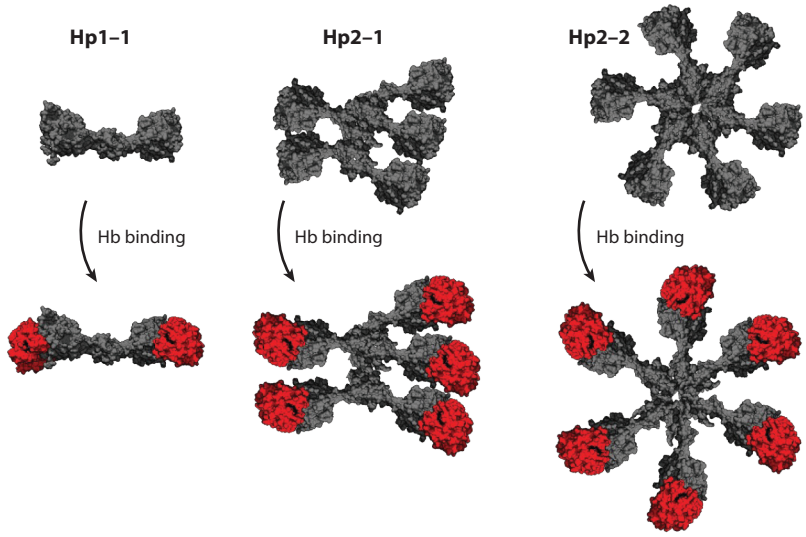

Figure 3

Structure of Hp1–1, Hp2–1, and Hp2–2 (*gray*) based on negative-stain electron micrographs and bound to hemoglobin (Hb) αβ dimers (*red*) (31). Protein images generated on UCSF Chimera (7) based on Protein Data Bank structure 4F4O.

forms—Hp1 and Hp2—which give rise to three phenotypes: Hp1–1, Hp2–1, and Hp2–2, depicted in **Figure 3** (38). In the United States and Europe, the population is composed of 15% Hp1–1, 50% Hp2–1, and 35% Hp2–2 phenotypes (24). While the 36-kDa β chain is the same among all phenotypes, the α-2 chain originated from a partial duplication of the α-1 gene, leading to differing molecular weights (MWs) of 18 and 9 kDa, respectively (39). When assembled, Hp1–1 consists of a single 89-kDa tetrameric protein composed of two αβ dimers linked via disulfide bonds between the α chains. On the other hand, the α-2 chain contains an extra thiol group, which allows for polymerization with other α-2 chains. Accordingly, Hp2–1 is a linear polymeric species with an average MW of 200 kDa, and Hp2–2 is a cyclic polymer with an average MW of 500 kDa (40, 41). While these varying phenotypes exist, all Hp molecules bind to Hb with high affinity (K_D < 1 pM) (42, 43) and have similar effects in attenuating Hb toxicity in animal models (38).

In humans, after binding to cell-free Hb, the Hb-Hp complex is scavenged by $CD163^+$ macrophages and monocytes (44). Cell-free Hb has also been shown to bind to CD163 receptors without Hp, albeit at a lower affinity than the Hb-Hp complex ($K_D \sim 195$ nM versus $K_D \sim 19$ nM, respectively) (3). In mice, murine Hp does not promote binding of the Hp-Hb complex to the CD163 receptor (45). Instead, cell-free murine Hb has a higher CD163-binding affinity compared with human Hb to promote cell-free Hb clearance without Hp binding (45). However, in both mice and humans, when Hb is bound to Hp, the large size of the Hb-Hp complex prevents Hb extravasation into the tissue space, reducing NO scavenging, vasoconstriction, and systemic hypertension (11, 46–48). The increase in size of the Hb-Hp complex also reduces kidney damage by preventing Hb excretion into the urine (hemoglobinuria). Furthermore, Hp binding to Hb prevents heme release from Hb and lowers the ability of Hb to elicit oxidative damage and inflammation (47, 49). On the basis of these benefits, Hp has been approved for clinical use in Japan since 1985, mainly for its role in preventing kidney injury in patients with hemoglobinuria (11). Other more recently discovered properties of Hp are its intrinsic antioxidant potential, chaperone activity, and binding to high-mobility group box protein 1 (HMGB1) (40, 41, 50, 51).

HMGB1 is a damage-associated molecular pattern molecule released in response to infection or injury. Hp binding to HMGB1 may play a role in reducing hemolysis-associated toxicities, given that HMGB1 is released during hemolytic conditions and may synergize with Hb to promote inflammation (52, 53). The heightened inflammatory state during hemolysis not only contributes to tissue damage but also promotes vascular dysfunction (54, 55). In vitro studies suggest that inflammatory responses of lipopolysaccharide-stimulated macrophages may be downregulated by Hp due to its binding to HMGB1 (56).

HEMOPEXIN

Hpx, shown in **Figure 4**, is an ~60-kDa serum glycoprotein (~20% carbohydrate) with the highest affinity for free heme (K_D < 1 pM) and a plasma concentration ranging from 0.5 to 1.5 mg/mL (23, 26, 57). While each Hpx molecule binds tightly to one heme molecule, some studies suggest a secondary heme-binding site in Hpx, which may play a role in heme transport (58). Unlike Hp, which is catabolized after cellular uptake, after intracellular heme release, Hpx may be recycled and released intact back into the circulation (26, 59–61). Yet, the mechanism of Hpx recycling is not well defined, with evidence demonstrating that, during hemolytic states, serum Hpx levels decrease, indicating that Hpx may be degraded upon receptor-mediated uptake (26). On the basis of experimental evidence demonstrating both recycling and degradation, after binding to heme, the Hpx-heme complex has been proposed to be cleared via two mechanisms: a high-affinity, low-capacity system (termed specific) and a high-capacity, low-affinity system (termed selective) (26, 62). Early studies demonstrated in vivo and in isolated rat hepatocytes that the specific system, which is the primary Hpx-heme clearance pathway at low heme concentrations, promoted Hpx recycling. However, the later identification of CD91 receptor LRP1 (low-density lipoprotein receptor-related protein 1) expressed on macrophages, hepatocytes, neurons, and syncytiotrophoblasts as a high-affinity Hpx-heme binding receptor showed Hpx degradation in LRP1 overexpressing cells, contradicting the theory of Hpx recycling via the high-affinity receptor (23, 26, 61, 63). Notably, heme exposure to LRP1 overexpressing cells could have overwhelmed the LRP1 recycling pathway, leading to Hpx degradation in the lysosome (26). Furthermore, while some LRP1 ligands are degraded upon uptake, others such as apolipoprotein E and β1-integrin have been shown to be recycled, demonstrating that LRP1 uptake may mediate ligand recycling

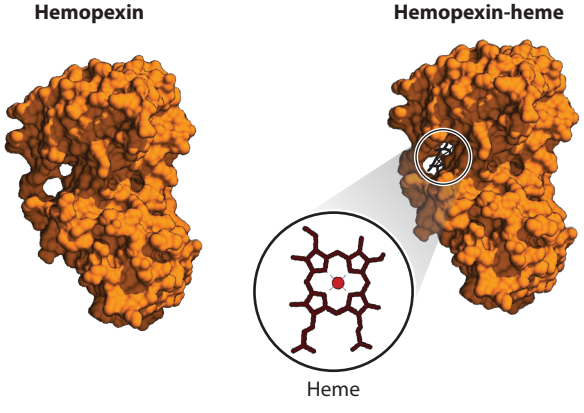

Figure 4

Structure of hemopexin and its heme-binding pocket. Protein images generated on UCSF Chimera (7) based on Protein Data Bank structure 1QJS.

(64). Lastly, both the specific clearance mechanism and LRP1 depend on calcium for uptake, unlike the selective mechanism, which further indicates that LRP1 may be used for Hpx recycling (specific system). Further evidence for Hpx recycling comes from in vitro studies in which uptake of Hpx-heme colocalized with holoTf at various relevant stages of the Tf recycling pathway (26, 65). More importantly, through receptor-mediated heme transport, the rate of heme uptake in cells is reduced such that cells are protected from heme overload that can occur when excess free heme is present (26).

Similar to Hp binding to Hb, Hpx binding to heme prevents the oxidative reactions of heme from occurring (23). Furthermore, Hpx aids Hp in clearance of heme derived from cell-free Hb, cell-free myoglobin, and Hb-based RBC substitutes (3). Recent evidence indicates that the carbohydrate chains of native Hpx confer protection against oxidative heme reactions, which can limit the effectiveness of recombinant nonglycosylated Hpx (26). Hpx also aids during hemolysis by inducing cellular expression of iron regulatory proteins heme-oxygenase-1 (HO-1) and ferritin (57). HO-1 is the primary inducible intracellular enzyme that cleaves heme into bilirubin, carbon monoxide, and ferrous iron, whereas ferritin is an iron storage protein. Both HO-1 and ferritin protect the organism from the oxidative and inflammatory stress induced by heme during hemolysis (57). The importance of HO-1 is evident, as its gradual induction with repeated administration of small heme doses has been shown to improve resistance against heme overload damage (66). Furthermore, HO-1 induction can induce less wound scarring by reducing heme levels in the wound (67).

TRANSFERRIN

Tf is an ~80-kDa serum glycoprotein present at 2–4 mg/mL in plasma (23). Its protein structure, shown in **Figure 5**, is composed of two 40-kDa subunits linked by a short peptide sequence (68). Each subunit (termed the N and C lobes) has one iron-binding site, which tightly binds to ferric iron (Fe^{3+}) with $K_D \sim 10^{-22}$ M (69, 70). Under homeostasis, iron-bound Tf (i.e., holoTf) accounts for 10–50% of total Tf (23). However, during states of hemolysis, Tf saturation with iron increases with a concomitant increase in non-transferrin-bound iron (labile iron), which starts to be detectable upon 70–80% Tf saturation.

While labile iron is toxic, Tf-bound iron is maintained in a nonreactive state (23). Therefore, Tf is vital for metabolism of senescent RBCs and its hemolytic by-products, as their iron component needs to be safely transported without participating in toxic redox reactions. HoloTf binds to the transferrin receptor, which is ubiquitously expressed on cells for iron uptake (68). After endocytosis, iron is released intracellularly and apoTf is quickly recycled back to the cell surface

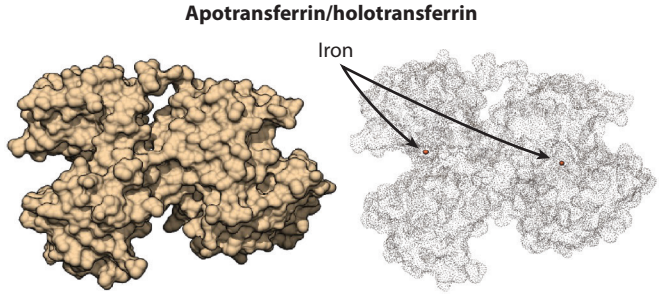

Figure 5

Structure of transferrin and locations of its iron-binding pockets. Protein images generated on UCSF Chimera (7) based on Protein Data Bank structure 3QYT.

(71). This quick rate of recycling is crucial for maintaining the iron balance, as only 1–2 mg of Fe can bind to plasma Tf, but a total of 20–30 mg of Fe are processed per day (9).

Various hemolytic conditions may benefit from Tf treatment. For example, during intracerebral hemorrhage, Hp or Hpx single-agent treatments have been shown in vivo to reduce Hb or heme toxicity, but in vitro studies showed an increase in iron-dependent neural cell damage that may be ameliorated with iron chelation therapy (72, 73). Furthermore, from either chronic blood transfusion or the inherent iron regulatory dysfunction in genetic disorders such as beta thalassemia or sickle cell disease, accumulation of iron in tissue parenchyma leads to hemochromatosis, with studies demonstrating the benefit of Tf administration in vivo (74, 75). Lastly, transfusion with older RBCs has been shown to increase Tf saturation and lead to the presence of labile iron in humans (20). In guinea pig models, the presence of free iron after blood transfusion was shown to increase mortality that could be prevented by either Hp or Tf coinfusion, with free iron accelerating Hb oxidation (76).

HUMAN SERUM ALBUMIN

HSA is a 65-kDa nonglycosylated polypeptide chain (**Figure 6**). Circulating at approximately 35–55 mg/mL in plasma, HSA is the most abundant plasma protein, with a diverse set of functions (57). For example, HSA regulates oncotic pressure, binds to and transports a variety of endogenous and exogenous molecules, and possesses enzymatic and antioxidant properties (77). Clinal applications of albumin include blood volume replacement, emergency shock treatment, treatment of burns, and other cases of hypovolemia (78). Notably, HSA is the major antioxidant in blood, accounting for more than 80% of free thiols in plasma (78).

In the case of hemolytic conditions, HSA can bind to both free heme and free iron (57, 79). Iron binding to HSA prevents oxidative damage from free iron (78, 79). In the case of heme, binding to HSA ($K_D \approx 10$ nM) decreases free heme–mediated oxidative damage (57). However, more than four times excess HSA is required to prevent heme oxidative damage in vitro (80). Furthermore, unlike Hpx, HSA is prone to oxidation due to the bound heme (57). HSA also aids in transport of catabolized heme through its bilirubin-binding properties. Additionally, given the role of bilirubin as a physiological reductant, bilirubin-bound HSA can have enhanced antioxidant properties by preventing lipid peroxidation (78, 81). Interestingly, conditions for which administration of HSA has been recommended may benefit from treatment of hemolytic by-products (Hb/h/Fe). For example, during severe burns, septic shock, organ transplantation, or surgeries, HSA can be

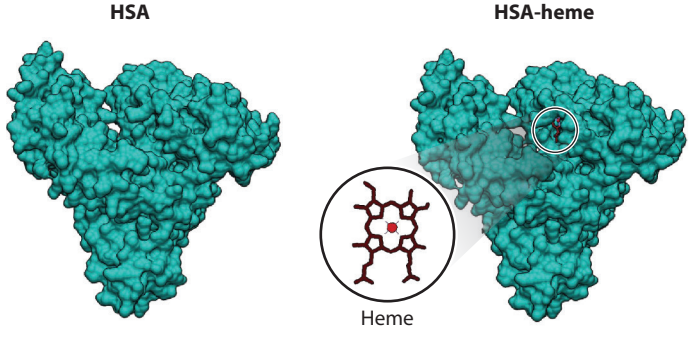

Figure 6

Human serum albumin (HSA) and its heme-bound species (HSA-heme). Protein images generated on UCSF Chimera (7) based on Protein Data Bank structure 1N5U.

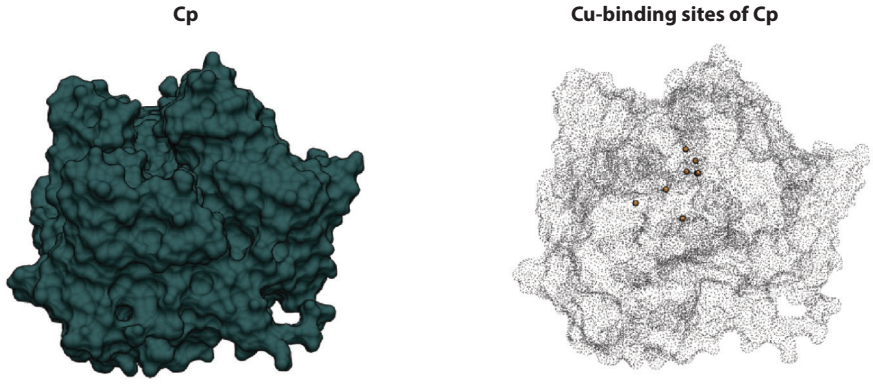

Figure 7

Structure of ceruloplasmin (Cp) and its Cu-binding sites. Protein images generated on UCSF Chimera (7) based on Protein Data Bank structure 1KCW.

administered as a plasma expander (82). Yet, these conditions have also been shown to release hemolytic by-products (Hb/h/Fe) (19, 83–87).

While HSA is generally regarded as an intermediate storage and transport protein for heme and iron until Hpx or Tf can sequester them and deliver them to the appropriate cells, receptor-mediated heme transport via heme-albumin has also been proposed (14, 88–92). Indeed, in vitro studies have identified that the transferrin receptor (CD71) can transport heme-albumin intracellularly (93). Yet, more studies are needed to elucidate the role of albumin beyond being a reservoir for storage of iron and heme until Tf- or Hpx-mediated clearance.

OTHER SERUM PROTEINS INVOLVED IN REGULATING HEMOLYTIC BY-PRODUCTS—CERULOPLASMIN AND Gc GLOBULIN

Ceruloplasmin (Cp) is an ~120 kDa serum protein responsible for binding and transport of copper (**Figure 7**) (94). Cp plays a major role in iron metabolism as a ferroxidase for oxidation of Fe^{2+} into Fe^{3+} and for stabilization of ferroportin (cellular iron exporters) (95, 96). Oxidation of iron to Fe^{3+} is required for iron binding to Tf (transport) or ferritin (storage) (94, 95, 97–99). Thus, Cp is vital for proper iron metabolism, and genetic Cp deficiencies lead to accumulation of iron in organs (100). Moreover, low Cp levels have been associated with patients having a high risk for acute organ failure (101). It has also been shown that Cp activity decreases during hemolytic conditions such as trauma and burn injuries, contributing to inflammation and hypoferremia (100, 102). Studies have also demonstrated that copper may be coendocytosed with heme-Hpx, given that copper binding to Hpx in the endosome may aid in heme release (26). Thus, copper transport may play an important role for controlled transport and metabolism of heme.

There are many other serum proteins that can aid in the reduction of hemolysis-mediated toxicities. Gc globulin, also known as vitamin D binding protein, is a backup actin scavenging protein (**Figure 8**) (103, 104). Serum actin is also a toxic species, and hemolysis has been shown to increase actin levels, which saturate the binding capacity of the natural actin scavenger gelsolin (104–107).

NOVEL THERAPEUTIC PROTEINS

Given the multifaceted by-products of hemolysis, various new therapeutics are being developed to address their toxicities. These include novel protein constructs that scavenge single toxic species, engineered complexes capable of scavenging multiple species, and protein mixtures.

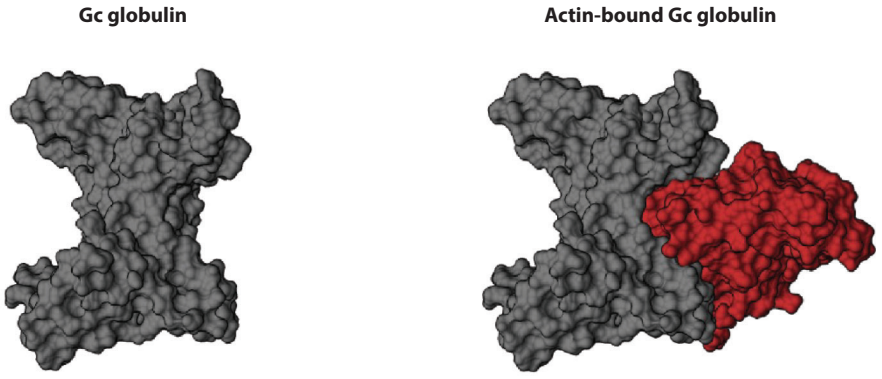

Figure 8

Structure of Gc globulin and actin-bound Gc globulin. Protein images generated on UCSF Chimera (7) based on Protein Data Bank structure 1LOT.

PEGylated Apohemoglobin

Removal of heme groups from Hb yields the apoprotein apoHb, which exists as an αβ dimer (108). The α and β globin subunits in apoHb bind to heme with high affinities (K_D of 1.7 and 42 pM, respectively) (109). On a mass basis, apoHb is capable of binding four times more heme than Hpx (~16 kDa for α/β apoHb globin compared with 60 kDa for Hpx monomer). Recent manufacturing techniques have allowed for facile and scalable synthesis of apoHb from Hb (108). Given that each liter of blood contains more than 100-fold the amount of Hb compared with Hpx, apoHb may be a more readily available blood-derived heme scavenger. However, unlike Hpx, which is stable at physiological temperatures, apoHb precipitates rapidly. Furthermore, as apoHb exists as an ~32-kDa dimer, it is rapidly cleared from the circulation. To address these issues, one strategy relies on the conjugation of polyethylene glycol (PEG) polymers to apoHb, generating PEG-apoHb (**Figure 9**) (110). PEG conjugation has been extensively used to improve pharmacokinetics and pharmacodynamics of therapeutic macromolecule delivery owing to its ability to increase hydrodynamic size, reduce immune recognition, and improve protein stability (111). Prior work has shown that the attachment of approximately 10 5-kDa PEG molecules per apoHb dimer increases its hydrodynamic diameter fivefold and should prevent rapid circulatory clearance of the biomolecule. Moreover, PEG-apoHb was found to have improved protein stability at 37°C in physiological buffer or plasma. In terms of the bioactivity of PEG-apoHb, it was shown that it retained 30–40% of the heme-binding activity of the starting apoHb, maintained high heme-binding affinity on the basis of its capacity to extract heme from HSA, and could bind to Hp (110). Given that PEG-Hb reached phase III clinical trials as an RBC substitute, PEG-apoHb may be a promising safe and effective synthetic heme scavenger.

ApoHb-Hp Complex

On the basis of the ability of Hp to bind to apoHb, the resulting apoHb-Hp complex offers the opportunity to use one biomolecule for the dual scavenging of Hb and heme during hemolytic states (**Figure 10**) (112, 113). Unlike free apoHb, the apoHb-Hp complex is stable at physiological temperatures, and the large molecular size of the complex prevents rapid clearance of the complexed apoHb (114). Furthermore, for apoHb in the apoHb-Hp complex, the reduced heme dissociation rate constant of Hb-Hp may lead to even higher affinity, although quantification is still needed. Importantly, the apoHb-Hp complex exhibits both Hb and heme-binding capacity

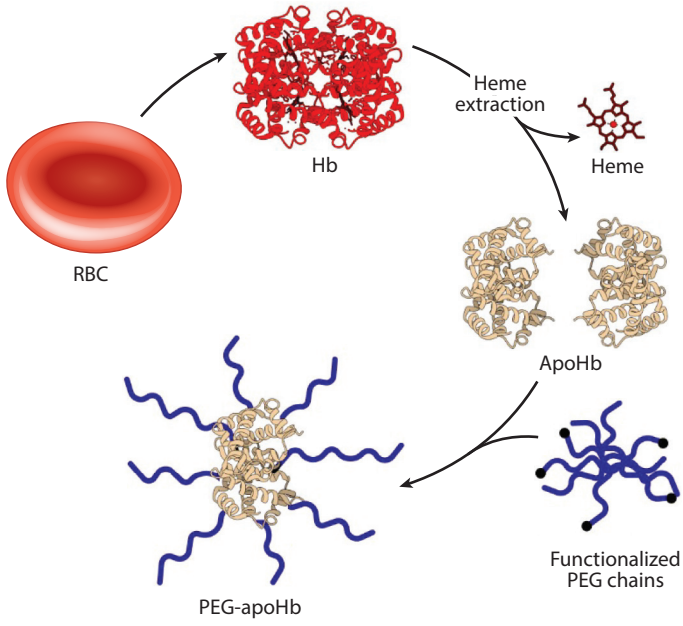

Figure 9

Schematic for the synthesis of PEG-apoHb. Abbreviations: ApoHb, apohemoglobin; Hb, hemoglobin; PEG, polyethylene glycol; RBC, red blood cell. Figure reproduced with permission from Reference 110; copyright 2020, American Chemical Society.

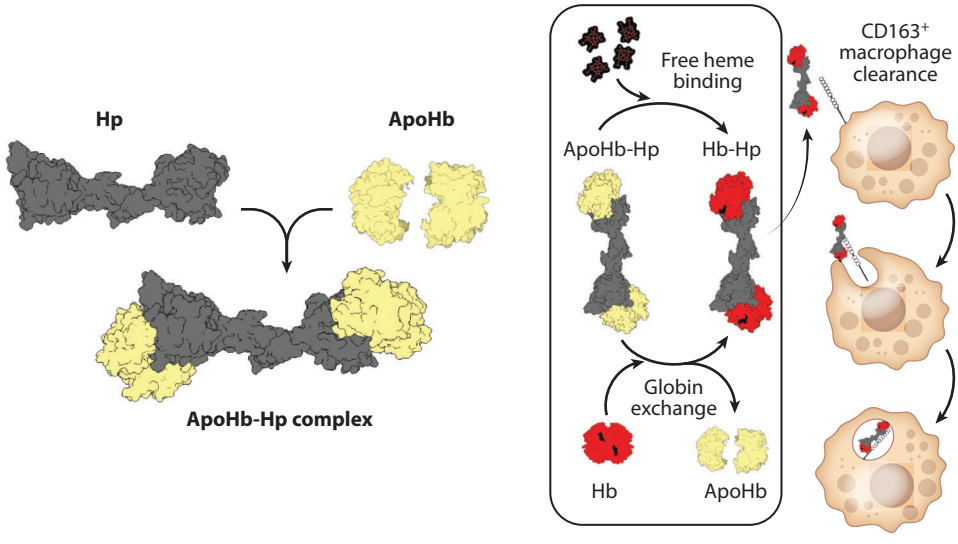

Figure 10

Schematic for the formation of the apoHb-Hp complex from apoHb and Hp and the mechanism of heme and Hb binding to the apoHb-Hp complex. Abbreviations: ApoHb, apohemoglobin; Hb, hemoglobin; Hp, haptoglobin.

without any loss in the bioactivity of each constituent protein (108, 112). Heme binding occurs directly with the apoHb-Hp complex, while Hb binding occurs via apoHb exchange with holoHb, and both were shown to occur both in vitro and in vivo in guinea pig models (**Figure 10**) (112). Moreover, exchange transfusion studies in hamster models challenged with either Hb or heme complexed with albumin demonstrated that the apoHb-Hp complex could prevent the damaging hemodynamic alterations induced by either cell-free Hb or heme and was better than single-agent treatments (i.e., apoHb or Hp) (112). ApoHb-Hp has also been demonstrated to reduce the toxicity of hemoglobin-based oxygen carriers, which have commonly suffered from Hb-mediated damage, and is being explored for its use in treatment of chronic hemolytic conditions such as beta thalassemia (113, 115).

Recombinant Hp

While Hp is an abundant plasma protein, its phenotypic variance in the population may limit clinical translation when sourced from pooled plasma (41). While no differences have been observed in acute hemolysis treatment efficacy among the different Hp phenotypes, prolonged treatment with a mixture of Hp phenotypes could trigger an immune response in parts of the population (38, 41). Furthermore, phenotypic differences have been theorized to result in different pharmacokinetics. Therefore, it is valuable to develop phenotype-specific Hp therapeutics and explore their biophysical properties and therapeutic efficacy. In addition to purifying Hp from donors with a specific Hp phenotype, generation of phenotype-specific Hp products has been achieved through recombinant protein synthesis in mammalian cells (FS293F) (41). Since the primary translation product of Hp mRNA is prohaptoglobin (proHp), production of recombinant Hp required coexpression of the protease complement C1r subcomponent-like protein (C1r-LP) to proteolytically cleave proHp into Hp (41). The final recombinant Hp protein was shown to be fully functional Hp through Hb-binding kinetics, preservation of vascular NO signaling in vitro and in vivo, and prevention of heme release and lipid peroxidation (41). In a later study, selective amino acid mutations enabled the generation of an Hp1–1 protein that was devoid of CD163 binding, which could be useful for certain therapeutic applications such as intracerebral hemorrhage, where capture of Hb-Hp in the brain could lead to toxic iron buildup (116).

Hpx-Hp

Fusion proteins are a promising engineering strategy to modulate the properties and function of biomolecules. With an established system to generate recombinant Hp, its β chain was used as a scaffold to associate with the fusion protein partners, HSA and Hpx (116). While most of the α chain could be omitted from the protein sequence, expression of Hpβ (termed mini-Hp) required a portion from the α chain containing the C1r-LP cleavage site for proper expression. The resulting fusion proteins—HSA-Hpβ and Hpx-Hpβ—maintained Hb-binding properties as Hpβ is the primary chain responsible for capturing Hb. However, Hpβ alone and the Hpx-Hpβ fusion protein showed tenfold and 170-fold lower CD163 binding compared with Hp1–1. The fused Hpx was also found to maintain heme-binding properties similar to those of plasma Hpx, and heme-complexed Hpx-Hpβ bound to CD91 with affinity similar to that of plasma-derived heme-Hpx. These fusion proteins showed similar NO-sparing capacity in an ex vivo vascular function assay and in vitro antioxidant activity in vitro compared with plasma-derived Hp. Interestingly Hpβ alone showed lower NO-sparing capacity than larger fusion protein complexes or Hp2–2 which further highlights the size-restriction model of vascular Hp protection. In this model, the large MW of Hb-Hp complexes prevents Hb extravasation to the NO-sensitive smooth muscle layer of the vascular wall. Therefore, similar to the apoHb-Hp complex, Hpx-Hpβ could bind and scavenge both Hb and heme as depicted in **Figure 11**. Notably, novel mutants

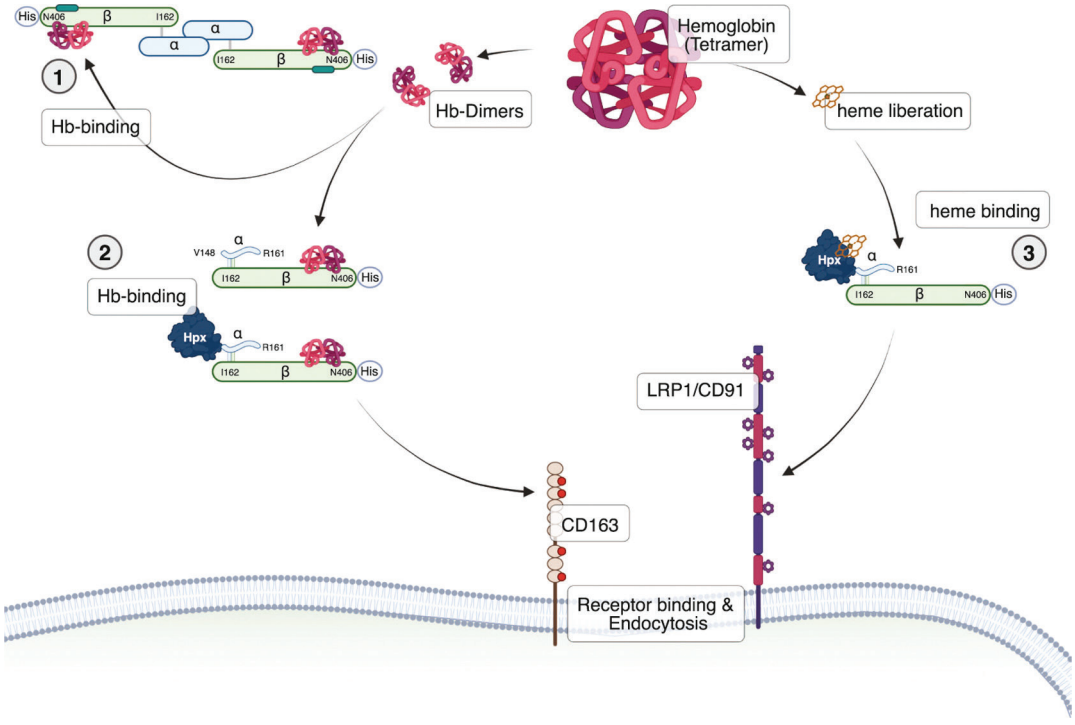

Figure 11

Schematic for the proposed mechanism of action of recombinant mini-Hp (truncated form of Hp), Hpx-Hp, and a Hp mutant with low CD163 affinity. Upon Hb dimerization, the low-CD163-affinity Hp (❶), mini-Hp, or Hpx-Hp (❷) may bind to the dimers. Only mini-Hp and Hpx-Hp are scavenged by CD163. Furthermore, Hpx-Hp can bind to free heme (❸) and be scavenged by LRP1/CD91. Abbreviations: Hb, hemoglobin; Hp, haptoglobin. Figure modified with permission from Reference 116; copyright 2021, American Chemical Society.

of these recombinant heme and Hb scavenging constructs could be engineered to control toxic ligand-binding kinetics, cellular receptor–binding kinetics, or pharmacokinetics. These optimized protein constructs would thus allow for more targeted treatment of the varied hemolytic conditions where plasma-derived scavengers have been shown to function.

Protein Scavenging Cocktail

In addition to engineering molecular systems capable of performing multiple functions such as the apoHb-Hp complex or the Hpx-Hp fusion protein, an alternative strategy is the use of scavenger protein mixtures to scavenge multiple hemolytic by-products. Although this could be accomplished with the use of purified proteins such as Hp, Hpx, and Tf, one approach relies on the purification of a protein cocktail containing these varied therapeutic agents starting from human plasma fraction IV (FIV) (117). FIV is a by-product from the plasma fractionation industry that enables the material to be readily available and sustainably sourced. This purification process uses tangential flow filtration, which is a scalable size-based separation technique, to fractionate the proteins in FIV on the basis of their molecular size. In the fractions with larger protein molecular weights (750 to 100 kDa), Hp with more than 95% purity can be isolated, whereas in the fraction with lower molecular weight proteins (<100 kDa), a protein mixture can be obtained containing HSA, Hp, Hpx, Tf, Cp, and Gc globulin with mass percentages of 40%, 10%, 5%, 35%, 5%, and 5%, respectively (117, 118). This mixture, termed the protein scavenging cocktail, was shown to

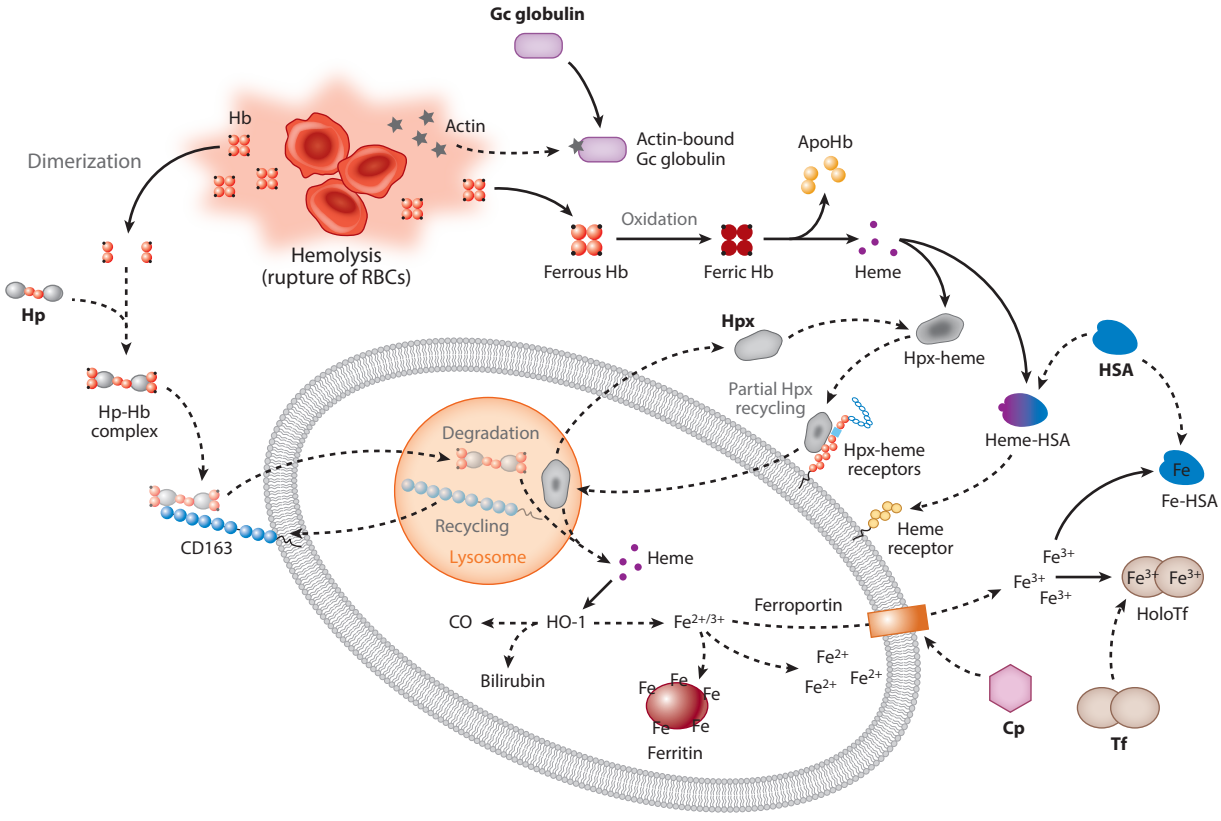

Figure 12

Schematic of the major components in the protein cocktail and their mechanism for detoxification of hemolytic by-products. Primary components of the protein cocktail are identified in bold text. Abbreviations: ApoHb, apohemoglobin; CO, carbon monoxide; Cp, ceruloplasmin; Fe, iron; Fe^{2+}, ferrous iron; Fe^{3+}, ferric iron; Hb, hemoglobin; HO-1, heme-oxygenase-1; HoloTf, holotransferrin; Hp, haptoglobin; Hpx, hemopexin; HSA, human serum albumin; RBC, red blood cell; Tf, transferrin.

possess Hb, heme, and iron-binding activity, which resulted in improved iron distribution and lower organ damage in vivo in hamsters exchange transfused with hemolyzed blood (117). A diagram depicting the components in the protein cocktail and their ability to scavenge Hb/h/Fe is shown in **Figure 12**.

An interesting benefit of HSA as a component in the scavenging protein cocktail beyond the properties mentioned in this review is its extensive ligand binding capacity, making it a flexible drug delivery vehicle (119). For example, as the major store of NO in vivo, HSA in the protein cocktail may be used to deliver NO to the vasculature during states of hemolysis, thus preventing hypertension (120–122). This concept has been demonstrated via nitrate infusions, which have been shown to restrict Hb hypertension during hemolysis (123). Furthermore, HSA may be a carrier for small-molecule drugs that can further enhance the therapeutic effect of hemolysis treatment agents (124).

CONCLUSION

Hemolysis is a damaging multifaceted event that is present under multiple clinical conditions, requiring detoxification of Hb/h/Fe. While there are various natural plasma proteins capable of

neutralizing these toxic hemolytic by-products, these proteins can be rapidly depleted in plasma. Therefore, therapeutic Hb/h/Fe detoxification strategies involve either replacement of depleted proteins or development of newly engineered biomimetic therapeutics. Recent emphasis has been placed on developing more controlled or multifunctional therapeutic products to better treat the multitude of toxic molecules released during hemolysis.

DISCLOSURE STATEMENT

I.S.P. and A.F.P. are listed as inventors on patent applications describing methods of protein purification and strategies to treat hemolysis.

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LITERATURE CITED

1. Am. Soc. Hematol. 2010. Blood basics. *American Society of Hematology*. **https://www.hematology.org/education/patients/blood-basics**
2. Lew VL, Tiffert T. 2017. On the mechanism of human red blood cell longevity: roles of calcium, the sodium pump, PIEZO1, and Gardos channels. *Front. Physiol.* 8:977
3. Smith A, McCulloh RJ. 2015. Hemopexin and haptoglobin: allies against heme toxicity from hemoglobin not contenders. *Front. Physiol.* 6:187
4. Pires IS, Belcher DA, Palmer AF. 2017. Quantification of active apohemoglobin heme-binding sites via dicyanohemin incorporation. *Biochemistry* 56(40):5245–59
5. Bellelli A. 2010. Hemoglobin and cooperativity: experiments and theories. *Curr. Protein Pept. Sci.* 11(1):2–36
6. Pittman RN. 2011. Oxygen transport. In *Regulation of Tissue Oxygenation*. San Rafael: Morgan & Claypool Life Sciences
7. Pettersen EF, Goddard TD, Huang CC, Couch GS, Greenblatt DM, et al. 2004. UCSF Chimera—a visualization system for exploratory research and analysis. *J. Comput. Chem.* 25(13):1605–12
8. Atha DH, Riggs A. 1976. Tetramer-dimer dissociation in hemoglobin and the Bohr effect. *J. Biol. Chem.* 251(18):5537–43
9. Porter JB, Garbowski M. 2014. The pathophysiology of transfusional iron overload. *Hematol. Oncol. Clin. North Am.* 28(4):683–701
10. Buehler PW, Humar R, Schaer DJ. 2020. Haptoglobin therapeutics and compartmentalization of cell-free hemoglobin toxicity. *Trends Mol. Med.* 26(7):683–97
11. Schaer DJ, Buehler PW, Alayash AI, Belcher JD, Vercellotti GM. 2013. Hemolysis and free hemoglobin revisited: exploring hemoglobin and hemin scavengers as a novel class of therapeutic proteins. *Blood* 121(8):1276–84
12. Van Avondt K, Nur E, Zeerleder S. 2019. Mechanisms of haemolysis-induced kidney injury. *Nat. Rev. Nephrol.* 15(11):671–92
13. Hatcher HC, Singh RN, Torti FM, Torti SV. 2009. Synthetic and natural iron chelators: therapeutic potential and clinical use. *Future Med. Chem.* 1(9):1643–70
14. Korolnek T, Hamza I. 2014. Like iron in the blood of the people: the requirement for heme trafficking in iron metabolism. *Front. Pharmacol.* 5:126
15. Light WR, Olson JS. 1990. Transmembrane movement of heme. *J. Biol. Chem.* 265(26):15623–31
16. Morris CR, Kuypers FA, Kato GJ, Lavrisha L, Larkin S, et al. 2005. Hemolysis-associated pulmonary hypertension in thalassemia. *Ann. N. Y. Acad. Sci.* 1054:481–85

17. Rifkind JM, Mohanty JG, Nagababu E. 2015. The pathophysiology of extracellular hemoglobin associated with enhanced oxidative reactions. *Front. Physiol.* 5:500
18. Cowman AF, Healer J, Marapana D, Marsh K. 2016. Malaria: biology and disease. *Cell* 167:610–25
19. Effenberger-Neidnicht K, Hartmann M. 2018. Mechanisms of hemolysis during sepsis. *Inflammation* 41(5):1569–81
20. Hod EA, Brittenham GM, Billote GB, Francis RO, Ginzburg YZ, et al. 2011. Transfusion of human volunteers with older, stored red blood cells produces extravascular hemolysis and circulating non-transferrin-bound iron. *Blood* 118(25):6675–82
21. Rapido F, Bandyopadhyay S, Francis RO, Soffing M, Divgi CR, et al. 2015. Longer duration of red blood cell storage induces progressively increased markers of extravascular hemolysis and hepcidin in autologously transfused healthy volunteers. *Blood* 126(23):657
22. Sawant RB, Jathar SK, Rajadhyaksha SB, Kadam PT. 2007. Red cell hemolysis during processing and storage. *Asian J. Transfus. Sci.* 1(2):47–51
23. Buehler PW, Karnaukhova E. 2018. When might transferrin, hemopexin or haptoglobin administration be of benefit following the transfusion of red blood cells? *Curr. Opin. Hematol.* 25(6):452–58
24. Carter K, Worwood M. 2007. Haptoglobin: a review of the major allele frequencies worldwide and their association with diseases. *Int. J. Lab. Hematol.* 29(2):92–110
25. Santiago RP, Guarda CC, Figueiredo CVB, Fiuza LM, Aleluia MM, et al. 2018. Serum haptoglobin and hemopexin levels are depleted in pediatric sickle cell disease patients. *Blood Cells Mol. Dis.* 72:34–36
26. Smith A. 2013. Protection against heme toxicity: Hemopexin rules, OK? In *Handbook of Porphyrin Science with Applications to Chemistry, Physics, Materials Science, Engineering, Biology and Medicine*, Vol. 30, ed. GC Ferreira, KM Kadish, KM Smith, R Guilard, pp. 311–38. Singapore: World Sci.
27. Cassat JE, Skaar EP. 2013. Iron in infection and immunity. *Cell Host Microbe* 13(5):509–19
28. Pogoutse AK, Moraes TF. 2017. Iron acquisition through the bacterial transferrin receptor. *Crit. Rev. Biochem. Mol. Biol.* 52(3):314–26
29. Hare SA. 2017. Diverse structural approaches to haem appropriation by pathogenic bacteria. *Biochim. Biophys. Acta Proteins Proteom.* 1865(4):422–33
30. Klei TRL, Dalimot JJ, Nota B, Veldthuis M, Mul E, et al. 2020. Hemolysis in the spleen drives erythrocyte turnover. *Blood* 136:1579–89
31. Andersen CBF, Stødkilde K, Sæderup KL, Kuhlee A, Raunser S, et al. 2017. Haptoglobin. *Antioxid. Redox Signal.* 26(14):814–31
32. Muranjan M, Nussenzweig V, Tomlinson S. 1998. Characterization of the human serum trypanosome toxin, haptoglobin-related protein. *J. Biol. Chem.* 273(7):3884–87
33. Yerbury JJ, Rybchyn MS, Easterbrook-Smith SB, Henriques C, Wilson MR. 2005. The acute phase protein haptoglobin is a mammalian extracellular chaperone with an action similar to clusterin. *Biochemistry* 44(32):10914–25
34. Nagel RL, Gibson QH. 1971. The binding of hemoglobin to haptoglobin and its relation to subunit dissociation of hemoglobin. *J. Bioloqical Chem.* 246(1):69–73
35. Laurell C-B, Nyman M. 1957. Studies on the serum haptoglobin level in hemoglobinemia and its influence on renal excretion of hemoglobin. *Blood* 12(6):493–506
36. Faulstick DA, Lowenstein J, Yiengst MJ. 1962. Clearance kinetics of haptoglobin-hemoglobin complex in the human. *Blood* 20(1):65–71
37. Bertolini J, Goss N, Curling J. 2012. *Production of Plasma Proteins for Therapeutic Use*. New York: John Wiley & Sons
38. Lipiski M, Deuel JW, Baek JH, Engelsberger WR, Buehler PW, Schaer DJ. 2013. Human Hp1–1 and Hp2–2 phenotype-specific haptoglobin therapeutics are both effective in vitro and in guinea pigs to attenuate hemoglobin toxicity. *Antioxid. Redox Signal.* 19(14):1619–33
39. Pimenova T, Pereira CP, Schaer DJ, Zenobi R. 2009. Characterization of high molecular weight multimeric states of human haptoglobin and hemoglobin-based oxygen carriers by high-mass MALDI MS. *J. Sep. Sci.* 32(8):1224–30
40. Larsson M, Cheng T-M, Chen C-Y, J S. 2013. Unique assembly structure of human haptoglobin phenotypes 1–1, 2–1, and 2–2 and a predominant Hp 1 allele hypothesis. In *Acute Phase Proteins*, ed. S Janciauskiene, pp. 163–77. Rijeka, Croatia: InTech

41. Schaer CA, Owczarek C, Deuel JW, Schauer S, Baek JH, et al. 2018. Phenotype-specific recombinant haptoglobin polymers co-expressed with C1r-like protein as optimized hemoglobin-binding therapeutics. *BMC Biotechnol*. 18(1):15
42. Hwang PK, Greer J. 1980. Interaction between hemoglobin subunits in the hemoglobin-haptoglobin complex. *J. Biol. Chem.* 255(7):3038–41
43. Polticelli F, Bocedi A, Minervini G, Ascenzi P. 2008. Human haptoglobin structure and function—a molecular modelling study. *FEBS J.* 275(22):5648–56
44. Alayash AI, Andersen CBF, Moestrup SK, Bülow L. 2013. Haptoglobin: the hemoglobin detoxifier in plasma. *Trends Biotechnol*. 31(1):2–3
45. Etzerodt A, Kjolby M, Nielsen MJ, Maniecki M, Svendsen P, Moestrup SK. 2013. Plasma clearance of hemoglobin and haptoglobin in mice and effect of CD163 gene targeting disruption. *Antioxid. Redox Signal.* 18(17):2254–63
46. Boretti FS, Buehler PW, D'Agnillo F, Kluge K, Glaus T, et al. 2009. Sequestration of extracellular hemoglobin within a haptoglobin complex decreases its hypertensive and oxidative effects in dogs and guinea pigs. *J. Clin. Investig.* 119(8):2271–80
47. Belcher JD, Chen C, Nguyen J, Abdulla F, Zhang P, et al. 2018. Haptoglobin and hemopexin inhibit vaso-occlusion and inflammation in murine sickle cell disease: role of heme oxygenase-1 induction. *PLOS ONE* 13(4):e0196455
48. Schaer CA, Deuel JW, Schildknecht D, Mahmoudi L, Garcia-Rubio I, et al. 2016. Haptoglobin preserves vascular nitric oxide signaling during hemolysis. *Am. J. Respir. Crit. Care Med.* 193(10):1111–22
49. Lim S-K, Ferraro B, Moore K, Halliwell B. 2001. Role of haptoglobin in free hemoglobin metabolism. *Redox Rep*. 6(4):219–27
50. Tseng CF, Lin CC, Huang HY, Liu HC, Mao SJT. 2004. Antioxidant role of human haptoglobin. *Proteomics* 4(8):2221–28
51. Sultan A, Raman B, Rao CM, Tangirala R. 2013. The extracellular chaperone haptoglobin prevents serum fatty acid-promoted amyloid fibril formation of β_2-microglobulin, resistance to lysosomal degradation, and cytotoxicity. *J. Biol. Chem.* 288(45):32326–42
52. Gupta K. 2014. HMGB1 takes a "Toll" in sickle cell disease. *Blood* 124(26):3837–38
53. Lin T, Sammy F, Yang H, Thundivalappil S, Hellman J, et al. 2012. Identification of hemopexin as an anti-inflammatory factor that inhibits synergy of hemoglobin with HMGB1 in sterile and infectious inflammation. *J. Immunol.* 189(4):2017–22
54. Mendonça R, Silveira AAA, Conran N. 2016. Red cell DAMPs and inflammation. *Inflamm. Res.* 65(9):665–78
55. Belcher JD, Chen C, Nguyen J, Milbauer L, Abdulla F, et al. 2014. Heme triggers TLR4 signaling leading to endothelial cell activation and vaso-occlusion in murine sickle cell disease. *Blood* 123(3):377–90
56. Krzyszczyk P, Kang HJ, Kumar S, Meng Y, O'Reggio MD, et al. 2020. Anti-inflammatory effects of haptoglobin on lps-stimulated macrophages: role of hmgb1 signaling and implications in chronic wound healing. *Wound Repair Regen*. 28(4):493–505
57. Tolosano E, Altruda F. 2002. Hemopexin: structure, function, and regulation. *DNA Cell Biol.* 21(4):297–306
58. Karnaukhova E, Owczarek C, Schmidt P, Schaer DJ, Buehler PW. 2021. Human plasma and recombinant hemopexins: heme binding revisited. *Int. J. Mol. Sci.* 22(3):1199
59. Delanghe JR, Langlois MR. 2001. Hemopexin: a review of biological aspects and the role in laboratory medicine. *Clin. Chim. Acta* 312(1–2):13–23
60. Smith A, Morgan WT. 1979. Haem transport to the liver by haemopexin. Receptor-mediated uptake with recycling of the protein. *Biochem. J.* 182(1):47–54
61. Smith A. 2011. Mechanisms of cytoprotection by hemopexin. In *Handbook of Porphyrin Science with Applications to Chemistry, Physics, Materials Science, Engineering, Biology and Medicine*, Vol. 15, ed. KM Kadish, KM Smith, R Guilard, pp. 217–355. Singapore: World Sci.
62. Smith A, Morgan WT. 1981. Hemopexin-mediated transport of heme into isolated rat hepatocytes. *J. Biol. Chem.* 256(21):10902–9
63. Ascenzi P, Bocedi A, Visca P, Altruda F, Tolosano E, et al. 2005. Hemoglobin and heme scavenging. *IUBMB Life* 57(11):749–59

64. Theret L, Jeanne A, Langlois B, Hachet C, David M, et al. 2017. Identification of LRP-1 as an endocytosis and recycling receptor for β1-integrin in thyroid cancer cells. *Oncotarget* 8(45):78614–32
65. Smith A, Hunt RC. 1990. Hemopexin joins transferrin as representative members of a distinct class of receptor-mediated endocytic transport systems. *Eur. J. Cell Biol.* 53(2):234–45
66. Vinchi F, Gastaldi S, Silengo L, Altruda F, Tolosano E. 2008. Hemopexin prevents endothelial damage and liver congestion in a mouse model of heme overload. *Am. J. Pathol.* 173(1):289–99
67. Wagener FADTG, Scharstuhl A, Tyrrell RM, Von den Hoff JW, Jozkowicz A, et al. 2010. The heme-heme oxygenase system in wound healing; implications for scar formation. *Curr. Drug Targets* 11:1571–85
68. Candelaria PV, Leoh LS, Penichet ML, Daniels-Wells TR. 2021. Antibodies targeting the transferrin receptor 1 (TfR1) as direct anti-cancer agents. *Front. Immunol.* 12:12:607692
69. Steere AN, Byrne SL, Chasteen ND, Mason AB. 2012. Kinetics of iron release from transferrin bound to the transferrin receptor at endosomal pH. *Biochim. Biophys. Acta Gen. Subj.* 1820(3):326–33
70. Aisen P, Leibman A, Zweier J. 1978. Stoichiometric and site characteristics of the binding of iron to human transferrin. *J. Biol. Chem.* 253(6):1930–37
71. Mayle KM, Le AM, Kamei DT. 2012. The intracellular trafficking pathway of transferrin. *Biochim. Biophys. Acta Gen. Subj.* 1820(3):264–81
72. Chen-Roetling J, Regan RF. 2016. Haptoglobin increases the vulnerability of CD163-expressing neurons to hemoglobin. *J. Neurochem.* 139(4):586
73. Chen-Roetling J, Ma S-K, Cao Y, Shah A, Regan RF. 2018. Hemopexin increases the neurotoxicity of hemoglobin when haptoglobin is absent. *J. Neurochem.* 145(6):464–73
74. Li H, Rybicki AC, Suzuka SM, von Bonsdorff L, Breuer W, et al. 2010. Transferrin therapy ameliorates disease in β-thalassemic mice. *Nat. Med.* 16(2):177–82
75. van Straaten S, Biemond BJ, Kerkhoffs J-L, Gitz-Francois J, van Wijk R, van Beers EJ. 2018. Iron overload in patients with rare hereditary hemolytic anemia: evidence-based suggestion on whom and how to screen. *Am. J. Hematol.* 93(11):E374–76
76. Baek JH, Yalamanoglu A, Gao Y, Guenster R, Spahn DR, et al. 2017. Iron accelerates hemoglobin oxidation increasing mortality in vascular diseased guinea pigs following transfusion of stored blood. *JCI Insight* 2(9):e93577
77. Ascenzi P, di Masi A, Fanali G, Fasano M. 2015. Heme-based catalytic properties of human serum albumin. *Cell Death Discov.* 1:15025
78. Taverna M, Marie A-L, Mira J-P, Guidet B. 2013. Specific antioxidant properties of human serum albumin. *Ann. Intensive Care* 3(1):4
79. Loban A, Kime R, Powers H. 1997. Iron-binding antioxidant potential of plasma albumin. *Clin. Sci.* 93(5):445–51
80. Miller YI, Felikman Y, Shaklai N. 1995. The involvement of low-density lipoprotein in hemin transport potentiates peroxidative damage. *Biochim. Biophys. Acta Mol. Basis Dis.* 1272(2):119–27
81. Neuzil J, Stocker R. 1994. Free and albumin-bound bilirubin are efficient co-antioxidants for α-tocopherol, inhibiting plasma and low density lipoprotein lipid peroxidation. *J. Biol. Chem.* 269(24):16712–19
82. Liumbruno G, Bennardello F, Lattanzio A, Piccoli P, Rossettias G. 2009. Recommendations for the use of albumin and immunoglobulins. *Blood Transfus.* 7(3):216
83. Vermeulen Windsant IC, de Wit NCJ, Sertorio JTC, van Bijnen AA, Ganushchak YM, et al. 2014. Hemolysis during cardiac surgery is associated with increased intravascular nitric oxide consumption and perioperative kidney and intestinal tissue damage. *Front. Physiol.* 5:340
84. Achkar R, Chiba AK, Zampieri-Filho JP, Pestana JOM, Bordin JO. 2011. Hemolytic anemia after kidney transplantation: a prospective analysis. *Transfusion* 51(11):2495–99
85. Petz LD. 2005. Immune hemolysis associated with transplantation. *Semin. Hematol.* 42(3):145–55
86. Norman TE, Chaffin MK, Johnson MC, Spangler EA, Weeks BR, Knight R. 2005. Intravascular hemolysis associated with severe cutaneous burn injuries in five horses. *J. Am. Vet. Med. Assoc.* 226(12):2039–43
87. Endoh Y, Kawakami M, Orringer EP, Peterson HD, Meyer AA. 1992. Causes and time course of acute hemolysis after burn injury in the rat. *J. Burn Care Rehabil.* 13(2, Part 1):203–9

88. Taketani S. 2005. Aquisition, mobilization and utilization of cellular iron and heme: endless findings and growing evidence of tight regulation. *Tohoku J. Exp. Med.* 205(4):297–318
89. Worthington MT, Cohn SM, Miller SK, Luo RQ, Berg CL. 2001. Characterization of a human plasma membrane heme transporter in intestinal and hepatocyte cell lines. *Am. J. Physiol. Liver Physiol.* 280(6):G1172–77
90. Latunde-Dada GO, Simpson RJ, McKie AT. 2006. Recent advances in mammalian haem transport. *Trends Biochem. Sci.* 31:182–88
91. Noyer CM, Immenschuh S, Liem HH, Muller-Eberhard U, Wolkoff AW. 1998. Initial heme uptake from albumin by short-term cultured rat hepatocytes is mediated by a transport mechanism differing from that of other organic anions. *Hepatology* 28(1):150–55
92. Taketani S, Immenschuh S, Go S, Sinclair PR, Stockert RJ, et al. 1998. Hemopexin from four species inhibits the association of heme with cultured hepatoma cells or primary rat hepatocytes exhibiting a small number of species specific hemopexin receptors. *Hepatology* 27(3):808–14
93. Jennifer B, Berg V, Modak M, Puck A, Seyerl-Jiresch M, et al. 2020. Transferrin receptor 1 is a cellular receptor for human heme-albumin. *Commun. Biol.* 3(1):621
94. Hellman NE, Gitlin JD. 2002. Ceruloplasmin metabolism and function. *Annu. Rev. Nutr.* 22:439–58
95. Ramos D, Mar D, Ishida M, Vargas R, Gaite M, et al. 2016. Mechanism of copper uptake from blood plasma ceruloplasmin by mammalian cells. *PLOS ONE* 11(3):e0149516
96. De Domenico I, Ward DM, di Patti MCB, Jeong SY, David S, et al. 2007. Ferroxidase activity is required for the stability of cell surface ferroportin in cells expressing GPI-ceruloplasmin. *EMBO J.* 26(12):2823–31
97. de Silva D, Aust SD. 1992. Stoichiometry of Fe(II) oxidation during ceruloplasmin-catalyzed loading of ferritin. *Arch. Biochem. Biophys.* 298(1):259–64
98. Samokyszyn VM, Miller DM, Reif DW, Aust SD. 1989. Inhibition of superoxide and ferritin-dependent lipid peroxidation by ceruloplasmin. *J. Biol. Chem.* 264(1):21–26
99. de Silva DM, Aust SD. 1993. Ferritin and ceruloplasmin in oxidative damage: review and recent findings. *Can. J. Physiol. Pharmacol.* 71(9):715–20
100. Dubick M, Barr J, Keen C, Atkins J. 2015. Ceruloplasmin and hypoferremia: studies in burn and non-burn trauma patients. *Antioxidants* 4(1):153–69
101. Dauberschmidt R, Mrochen H, Förster I, Stumpe C, Dressler C, et al. 1991. Changes in ceruloplasmin activity and lactate concentration in patients at high risk of acute organ system failure. *Clin. Chim. Acta* 199(2):167–72
102. Cunningham JJ, Lydon MK, Emerson R, Harmatz PR. 1996. Low ceruloplasmin levels during recovery from major burn injury: influence of open wound size and copper supplementation. *Nutrition* 12(2):83–88
103. Chun RF. 2012. New perspectives on the vitamin D binding protein. *Cell Biochem. Funct.* 30(6):445–56
104. Meier U, Gressner O, Lammert F, Gressner AM. 2006. Gc-globulin: roles in response to injury. *Clin. Chem.* 52(7):1247–53
105. Piktel E, Levental I, Durnaś B, Janmey P, Bucki R. 2018. Plasma gelsolin: indicator of inflammation and its potential as a diagnostic tool and therapeutic target. *Int. J. Mol. Sci.* 19(9):2516
106. Peddada N, Sagar A, Garg R. 2012. Plasma gelsolin: a general prognostic marker of health. *Med. Hypotheses* 78:203–10
107. Smith D, Janmey P, Sherwood J, Howard R, Lind S. 1988. Decreased plasma gelsolin levels in patients with *Plasmodium falciparum* malaria: a consequence of hemolysis? *Blood* 72(1):214–18
108. Pires IS, Belcher DA, Hickey R, Miller C, Badu-Tawiah AK, et al. 2020. Novel manufacturing method for producing apohemoglobin and its biophysical properties. *Biotechnol. Bioeng.* 117(1):125–45
109. Hargrove MS, Whitaker T, Olson JS, Vali RJ, Mathews AJ. 1997. Quaternary structure regulates hemin dissociation from human hemoglobin. *J. Biol. Chem.* 272(28):17385–89
110. Pires IS, Savla C, Palmer AF. 2020. Poly(ethylene glycol) surface-conjugated apohemoglobin as a synthetic heme scavenger. *Biomacromolecules* 21(6):2155–64
111. Pires IS, Hammond PT, Irvine DJ. 2021. Engineering strategies for immunomodulatory cytokine therapies: challenges and clinical progress. *Adv. Ther.* 4(8):2100035

112. Munoz CJ, Pires IS, Baek JH, Buehler PW, Palmer AF, Cabrales P. 2020. Apohemoglobin-haptoglobin complex attenuates the pathobiology of circulating acellular hemoglobin and heme. *Am. J. Physiol. Circ. Physiol.* 318(5):H1296–307
113. Belcher DA, Munoz C, Pires IS, Williams AT, Cabrales P, Palmer AF. 2020. Apohemoglobin-haptoglobin complexes attenuate the hypertensive response to low-molecular-weight polymerized hemoglobin. *Blood Adv*. 4(12):2739–50
114. Waks M, Beychok S. 1974. Induced conformational states in human apohemoglobin on binding of haptoglobin 1–1. Effect of added heme as a probe of frozen structures. *Biochemistry* 13(1):15–22
115. Munoz CJ, Pires IS, Jani V, Palmer AF, Gopal S, Cabrales P. 2022. Apohemoglobin-haptoglobin complex alleviates iron toxicity in mice with β-thalassemia via scavenging of cell-free hemoglobin and heme. *Biomed. Pharmacother.* 156:113911
116. Buzzi RM, Owczarek CM, Akeret K, Tester A, Pereira N, et al. 2021. Modular platform for the development of recombinant hemoglobin scavenger biotherapeutics. *Mol. Pharm.* 18(8):3158–70
117. Pires IS, Govender K, Munoz CJ, Williams AT, O'Boyle QT, et al. 2021. Purification and analysis of a protein cocktail capable of scavenging cell-free hemoglobin, heme, and iron. *Transfusion* 61(6):1894–907
118. Pires IS, Palmer AF. 2020. Tangential flow filtration of haptoglobin. *Biotechnol. Prog.* 36(5):e3010
119. Fasano M, Curry S, Terreno E, Galliano M, Fanali G, et al. 2005. The extraordinary ligand binding properties of human serum albumin. *IUBMB Life* 57:787–96
120. Stamler JS, Jaraki O, Osborne J, Simon DI, Keaney J, et al. 1992. Nitric oxide circulates in mammalian plasma primarily as an *S*-nitroso adduct of serum albumin. *PNAS* 89(16):7674–77
121. Rungatscher A, Hallström S, Linardi D, Milani E, Gasser H, et al. 2015. *S*-nitroso human serum albumin attenuates pulmonary hypertension, improves right ventricular-arterial coupling, and reduces oxidative stress in a chronic right ventricle volume overload model. *J. Heart Lung Transplant.* 34(3):479–88
122. Orie NN, Vallance P, Jones DP, Moore KP. 2005. *S*-Nitroso-albumin carries a thiol-labile pool of nitric oxide, which causes venodilation in the rat. *Am. J. Physiol. Circ. Physiol.* 289(2):H916–23
123. Minneci PC, Deans KJ, Shiva S, Zhi H, Banks SM, et al. 2008. Nitrite reductase activity of hemoglobin as a systemic nitric oxide generator mechanism to detoxify plasma hemoglobin produced during hemolysis. *Am. J. Physiol. Circ. Physiol.* 295(2):H743–54
124. Zorzi A, Linciano S, Angelini A. 2019. Non-covalent albumin-binding ligands for extending the circulating half-life of small biotherapeutics. *Medchemcomm* 10(7):1068–81