Blood Substitutes

Andre F. Palmer[1] and Marcos Intaglietta[2]

[1]William G. Lowrie Department of Chemical and Biomolecular Engineering, The Ohio State University, Columbus, Ohio 43210; email: palmer.351@osu.edu

[2]Department of Bioengineering, University of California, San Diego, La Jolla, California 92093; email: mintagli@ucsd.edu

Annu. Rev. Biomed. Eng. 2014. 16:77–101

First published online as a Review in Advance on April 16, 2014

The *Annual Review of Biomedical Engineering* is online at bioeng.annualreviews.org

This article's doi: 10.1146/annurev-bioeng-071813-104950

Keywords

red blood cell substitutes, oxygen therapeutics, polymerized hemoglobin, poly(ethylene glycol)-conjugated hemoglobin, vesicle-encapsulated hemoglobin, supraperfusion, plasma expanders

Abstract

The toxic side effects of early generations of red blood cell substitutes have stimulated development of more safe and efficacious high-molecular-weight polymerized hemoglobins, poly(ethylene glycol)-conjugated hemoglobins, and vesicle-encapsulated hemoglobins. Unfortunately, the high colloid osmotic pressure and blood plasma viscosity of these new-generation materials limit their application to blood concentrations that, in general, are not sufficient for full restoration of oxygen-carrying and -delivery capacity. However, these materials may serve as oxygen therapeutics for treating tissues affected by ischemia and trauma, particularly when the therapeutics are coformulated with antioxidants. These new oxygen therapeutics also possess additional beneficial effects owing to their optimal plasma expansion properties, which induce systemic supraperfusion that increases endothelial nitric oxide production and improves tissue washout of metabolic wastes, further contributing to their therapeutic role.

Contents

1. INTRODUCTION

Historically, the development of artificial red blood cell (RBC) substitutes focused on restoring blood oxygen-carrying capacity by introducing solutions of hemoglobin (Hb) or perfluorocarbons (PFCs) into the systemic circulation. In practice, this approach is preceded by remedying the volume component of blood loss via transfusion of plasma expanders such as saline, Ringer's lactate, plasma, and albumin solutions. Surprisingly, the oxygen-carrying and plasma-expansion aspects of

RBC substitute development remained compartmentalized until recently, when it became evident that an oxygen-carrying RBC substitute must also be a good plasma expander. This realization is significant because excellent plasma expansion can compensate for some of the limitations of oxygen-carrying fluids, whereas adequate oxygen-transport capacity does not compensate for poor plasma expansion and indeed can enhance the toxicity of Hb-based transfusion materials. This review analyzes how RBC substitutes respond to the physiological deficits arising from blood loss as well as how they address and correct the corresponding decrease in blood oxygen-carrying and -delivery capacity.

2. THE NEED FOR RBC SUBSTITUTES

Health-care systems are highly dependent on blood to prevent or treat anemia, and the continuous availability of blood is necessary to sustain a high standard of medical care worldwide. One of the principal regulators of the global blood supply, the World Health Organization (WHO), has codified recommendations for blood collection, processing, and storage, thus setting guidelines for standardized procedures.

In the United States, blood usage is approximately 14 million units per year. This supply is obtained from approximately 8 million volunteer donors and is transfused to about 3.5 million individuals yearly (1). At present, the supply is adequate for the current US population, and shortages are seldom reported.

The average volume of a unit of donated blood in the United States is approximately 450 mL; worldwide, it ranges from 200 to 500 mL, with the donation volume in China (200 mL) trending upward (2). Healthy, unpaid volunteers constitute the majority of blood donors in highly developed countries, where the blood supply is used primarily by older cardiovascular surgery patients, victims of trauma or sepsis, and individuals being treated for various malignancies (3). By contrast, the majority of blood donors in developing and transitional countries are friends, family, and paid blood donors, and their blood is used primarily to treat obstetric complications as well as maternal and infant anemia (4).

Globally, approximately 107 million blood donations are made per year, with half of this amount being collected in developed countries, which account for only 15% of the world's population. The average number of blood donations in the United States, Western Europe, Australia, and South Korea is approximately 4.4 per 100 persons per year (5). As a donation rate of less than 1% of the population per year is considered inadequate for the practice of modern medicine, worldwide access to the standard level of medical practice in developed countries would require at least 300 million blood donations, which indicates a present global deficit of about 200 million blood donations each year.

The possibility of meeting this demand is unlikely, as it would also require economic conditions that make the cost of a unit of blood affordable. This cost is estimated to be $\$211 \pm 38$ in the United States (2011) when purchased from a supplier and becomes as high as \$522–1,183 per unit when all associated costs with a transfusion are considered (6).

2.1. Factors and Trends in Blood Usage

Global blood usage is projected to increase with population growth and improvement in economic conditions. Whether this trend will parallel that in the developed world depends on the outcome of competing demographic and technological developments. The principal expected change is an increase in blood usage due to an aging population. Individuals older than 65 years of age use 43% of all donated blood in the United States. This group accounted for 12.8% of the population in

2009 and is expected to grow to 17.9% of the population in 2025. This demographic shift also impacts blood donation, as it shrinks the available donor population (7).

At present, RBCs can be stored refrigerated for up to 42 days after collection according to criteria set forth by the US Food and Drug Administration (FDA), which stipulate that 75% percent of transfused RBCs must survive in the circulation 24 h after transfusion. However, there is increasing evidence that short- and long-term patient survival after cardiac and cancer surgery (8, 9) decreases with increasing RBC ex vivo storage time and number of transfused units (10). These outcomes are associated with immunomodulation due to transfusion-related proinflammatory or immunosuppressive effects (11). Furthermore, RBC storage–induced damage impacts the rheological properties of blood (12) and microvascular function (13), inducing pathologies. Since RBC storage–induced damage appears after 14 days of RBC storage (14), limiting the ex vivo RBC storage duration for better short- and long-term outcomes would also decrease blood availability.

Decreasing surgical blood loss through advances such as minimally invasive, or laparoscopic, surgery may result in as much as a 3-fold decrease in the number of units of blood transfused (15). However, meta-analyses comparing laparoscopic outcomes with those of conventional approaches, although showing a decrease in the number of units of blood transfused, do not show significance (16). Whether this is intrinsic to the procedures or because laparoscopic surgery is still at an early stage remains to be determined. Furthermore, laparoscopic surgery is now complemented with or replaced by robotic surgery, and comparative outcomes have yet to be evaluated (17).

Another factor that could lower blood demand is reductions in the transfusion trigger, or Hb concentration, that prompts the need for a blood transfusion and in the transfusion target, or transfusion end point. One analysis of blood use in intraoperative transfusions showed that 76.0% of patients were transfused with 1–3 units of blood (representing 44.2% of all the blood used) and that 11.7% received approximately 30% of all the blood used (18), showing that a small fraction of the population uses a large portion of the blood supply. In this study, the overall mean Hb trigger was 8.4 ± 1.5 g/dL, compared with current recommendations of 6–7 g/dL (19). As most blood usage occurs in hospitals, lowering the transfusion trigger would significantly reduce blood usage.

At present, RBC transfusion is the fastest-growing common medical procedure that occurs in hospitals in the United States (20); however, the use and supply of blood in advanced countries appear to be constant, sufficient, and stable. The number of units of blood transfused in 2011 declined 8.2% ($p < 0.001$) relative to 2008, and the available supply of blood exceeded the demand by 725,000 units (21).

2.2. Morbidity and Mortality Due to Contamination

An initial incentive for the development of RBC substitutes stemmed from concern over contamination of the blood supply by the human immunodeficiency virus (HIV) and its transmission by blood transfusion. This, along with the threat of parallel transmission of the hepatitis C virus (HCV), led to intense scrutiny of the blood supply and initiation of strict transfusion surveillance based on detailed donor history and serological tests. This approach was successful, and presently most known transmissions of disease occur during the time window between the donor being infected and when the infectious agent can be detected in the blood. This time window is progressively being narrowed.

The present risks of transmission of viral diseases in the United States are, for example, about 1/350,000 units of transfused blood for hepatitis B and C infection, 1/1,390,000 for HCV, and 1/2,000,000 for HIV. There has been no record of transfusion-transmitted syphilis for more than 30 years (22). Transmission of other pathogens such as cytomegalovirus and parvovirus B19 is of concern, although their transmission profiles are similar to that of syphilis (23). Overall, the risk

of viral contamination of blood by known pathogens is within the 6-sigma margin. However, the emergence of new viral threats should be expected. Additionally, the risk statistics apply only to fully tested blood, but as much as 10% of the global blood supply is untested.

The most common side effect of blood transfusion is an allergic reaction to the plasma proteins present in the donated blood. Usually, the only symptoms are hives and itching. Fever occurring within 24 h of transfusion is also frequent and is a response to the presence of white blood cells in the donated blood. These reactions are easily treated and are compensated for by the undeniable positive effects of, for example, treating anemia. A more serious reaction, transfusion-related acute lung injury (TRALI), which causes difficulty in breathing lasting 2–3 days, has an incidence of about 1/5,000 transfusions and has a 5–10% mortality rate.

Other side effects include hemolytic reactions; graft-versus-host disease; and those due to infections caused by bacteria, viruses, and parasites, such as babesiosis, malaria, Lyme disease, Chagas disease, dengue, and filariasis. However, as potential donors are questioned about their travel histories and health, infections from these sources are rare.

2.3. Morbidity and Mortality Due to RBC Transfusion but Unrelated to Disease Transmission

RBC transfusion is necessary in critically bleeding and profoundly anemic patients; however, scholars are increasingly questioning both whether it is indicated in anemic patients with stable hemodynamics and what type of patient benefits from RBC transfusion (24). In a recent critical review of this paradigm, Isbister (24) stated, "It is not known for sure if a blood supply that is safer than ever necessarily means that blood transfusions are safe for patients." This contention is supported by a large body of studies including meta-analyses showing that RBC transfusions are directly associated with increased risk of morbidity and mortality (25, 26), and there is little evidence of a derived benefit (27).

Many studies demonstrate strong associations between blood transfusions and bleeding, which leads to decreased survival of transfused patients (28) who are being treated for acute coronary syndrome (29). However, these statistical findings do not establish a cause and effect link between bleeding and transfusion, as bleeding is independently associated with mortality, and establishing causation in bleeding and its treatment by transfusion is complex (25).

Nadir Hb in hospitalized patients following myocardial infarction (MI) with Hb >8 g/dL has predicted increased mortality. In one study, after risk adjustment, anemic patients receiving blood were 50% more likely to die within the follow-up period (6–48 months) than anemic patients who avoided blood transfusion (30).

Mechanistically, blood transfusion increases blood oxygen-carrying capacity. However, this does not translate into increased tissue oxygenation and, indeed, often decreases it (31). This decrease has been attributed to stored RBCs being low in 2,3-diphosphoglycerate (2,3-DPG), and this deficit, in turn, causes Hb to increase its oxygen affinity. However, this is a small and overestimated effect (32).

A partial list of factors implicated in the increase in mortality following blood transfusion includes predisposition to postoperative infection (33), duration of RBC storage (8), strengthened association between blood transfusion and vulnerability to malignancy (34), and loss of nitric oxide (NO) activity in banked blood, which impairs the vasodilatory response to hypoxia as well as promotes RBC adhesion to pulmonary endothelium (35). A meta-analysis of the relation between risk of death and transfusion of older, stored RBCs concluded that "newer blood if used exclusively might save lives" and that "current blood banking storage practices may not adequately protect patients" (36).

3. PHYSIOLOGICAL DEFICITS DUE TO BLOOD LOSS

The oxygen supply deficit due to hemorrhage can be broadly separated into several categories: anemia due to RBC deficit, simultaneous deficit of circulating blood volume and oxygen-carrying capacity, and local ischemia in which tissue pathology or injury prevents otherwise normal blood from supplying oxygen to tissues. Ideally, a transfusion fluid would be matched to the causes and extent of each of these issues.

3.1. Anemia and the Transfusion Trigger

In hospital settings, a lack of oxygen-carrying capacity manifests in a reduction in RBC count, measured by the hematocrit (Hct) or Hb concentration, and patient blood volume is controlled by fluid administration using plasma expanders. The major concern driving the decision of whether to correct lost oxygen-carrying capacity with a blood transfusion is the danger of the patient developing heart or brain ischemia. Tolerance to anemia depends in part on volemic conditions and is better tolerated in normovolemia than hypovolemia, as the related hemodilution increases cardiac output (37).

RBC transfusion corrects anemia and is used to ensure adequate oxygen delivery. However, the physiological rationale for this is not clear, particularly for transfusions of one and two units of RBCs, which account for a major fraction of overall blood use. As correction of anemia by RBC transfusion involves an increase in blood volume, the amount of infused RBCs is limited by considerations of hypervolemia, which, in turn, limit how much the oxygen-carrying capacity of blood can be increased.

The physiological effects of hypervolemic infusion of RBCs in an anemic individual are not well known, although they are generally perceived to be beneficial by practitioners and patients. Beneficial responses may not, however, be related entirely to the increase in intrinsic oxygen-carrying capacity, which in most cases is less than 10–15%. Responses are also a function of patient status, and reports indicate that anemic intensive care patients do not benefit from blood transfusions (38).

Experimental studies show that the normovolemic increase of blood viscosity in a healthy specimen can cause a paradoxical increase in cardiac output and decrease in peripheral vascular resistance (39), effects not attributable to changes in oxygen-carrying capacity because they also occur using nonoxygen-carrying RBCs (40). This suggests that the increase in blood viscosity is a factor contributing to the efficacy of RBC transfusion (41).

Considerations of the transfusion trigger for the treatment of anemia have important implications for the design of RBC substitutes, as they must be able to increase blood Hb concentration and, possibly, viscosity; that is, they must be hypooncotic, so that they are not diluted by autotransfusion upon introduction into the blood stream.

3.2. Hemorrhage and Hemorrhagic Shock

Hemorrhagic shock is due to simultaneous loss of blood oxygen-carrying capacity and blood volume, in which the circulation tolerates a comparatively large loss of intrinsic oxygen-carrying capacity but collapses functionally after small losses of circulating blood volume. The loss of circulating blood volume implies some form of trauma in which blood loss is a common denominator. Although there is a very large body of knowledge on the etiology and treatment of hemorrhagic shock, there are still many unknowns, including the actual volume and type of fluid to be used during resuscitation. Resuscitation strategies vary from hypovolemic treatments

("running the patient dry") to supranormal volume restitution through the infusion of large quantities of saline-based solutions (42).

Experimental studies of prolonged hemorrhagic shock have shown that maintenance of microvascular perfusion and particularly functional capillary density (FCD) requires achievement of a threshold blood/plasma viscosity (43) at which maintenance of FCD differentiates between survival and nonsurvival in conditions of prolonged hemorrhagic shock even though oxygen-carrying capacity and tissue oxygen levels are the same (44). To date, studies of the human microcirculation remain limited, and analysis of its functionality can be inferred only indirectly or by comparison to studies in animal models, which respond differently to injury and treatment. [This discrepancy was recognized by a 2008 FDA/NIH conference that concluded that the effects of RBC substitutes observed in preclinical animal studies did not reproduce those found in clinical trials (45).]

Important consequences of hemorrhage are acidosis, significantly decreased cardiac output due to lowered cardiac contractility, and impaired kidney function. Compensation of acidosis is therefore required in shock treatment. Acidosis is, in part, related to imbalances in intra- and extracellular osmolarity that lead to cellular swelling, which affects function in pancreatic, liver, heart, and endothelial cells. Endothelial swelling has a widespread effect, as it promotes RBC trapping in capillaries, lowering FCD and hindering RBC passage and therefore oxygen delivery (46).

The proposed rationale for introducing oxygen carriers significantly smaller in size compared with RBCs such as liposomes and molecular solutions of oxygen carriers is to circumvent microscopic obstructions of the circulation that RBCs cannot bypass. However, this approach is of doubtful efficacy because hemorrhagic shock also causes vasoconstriction and restriction of blood flow. This, in turn, reduces microvascular perfusion velocity, allowing more time for the partial pressure of blood oxygen to equilibrate with that of the anoxic tissue, thereby causing oxygen to be prevalently unloaded in the precapillary regions and not in the capillary system.

3.3. Ischemia, Inflammation, and Reperfusion Injury

Correction of oxygen supply deficits due to interruption of blood perfusion poses important problems in transfusion, as sudden reoxygenation induces reperfusion injury that affects tissue and patient recovery owing to the generation of reactive oxygen and nitrogen species.

Ischemia reperfusion injury induces cellular damage via the production of oxidative molecular species, whose activity is not neutralized by endogenous antioxidants (47). As RBC and Hb biochemistries modulate the effects of reactive molecular species, there is growing interest in endowing RBC substitutes with similar properties, thereby introducing therapeutic components that address free radical injury. This interest has shifted the development of RBC substitutes to that of oxygen therapeutics that also address reperfusion injury, inflammation, cellular ischemic damage, etc., in addition to increasing the oxygen-carrying capacity of blood.

Development of oxygen therapeutics could, in principle, help in reducing and controlling not only the issues caused by hypoperfusion and ischemia but also the toxicity of Hb solutions; however, this approach poses formidable problems because there is little agreement on the efficacy of antioxidants in preventing ischemia reperfusion injury. It should also be noted that worms that produce an excess of free radicals live longer, an effect that is abolished by the administration of antioxidants (48).

4. RBC SUBSTITUTE DEVELOPMENT TO DATE

The quest for RBC substitutes has engaged academic laboratories and industry for three decades; however, none of the results are definitive, and the majority of products proposed either have

failed in clinical trials or are still in early preliminary testing. Several publications (49–53) have recently reviewed the field and shown the following:

1. A practical oxygen carrier that is intrinsically not toxic is not available. The available selection is restricted to different types of Hbs (human, bovine, and annelid) and PFCs. It is extraordinarily difficult to control the natural toxicity of Hb from mammalian sources when it is formulated as a solution outside of the protective internal environment of the RBC. This intrinsic toxicity has been only partially eliminated from recombinant Hb and through the use of annelid Hb as well as co-transfusion of haptoglobin. PFCs, though totally inert, must be transported by emulsions based on phospholipids, which introduce comparatively large amounts of lipids in the circulation (many grams for a moderate transfusion), a process similar to adding a large amount of cholesterol to the circulation.
2. Allogeneic RBC transfusion is the standard of care; however, it has never been subjected to testing by clinical trials (54), which renders comparison to RBC substitutes and evaluation of their risks difficult. This difficulty of comparison is illustrated by consideration of the causes that led to failure of RBC substitutes in clinical trials. As an example, all products were perceived or found to cause hypertension, which was considered to be a significant risk factor. However, hypertensive interventions are the standard of care in many forms of resuscitation, and experimental studies have found such interventions to be more beneficial than nonhypertensive approaches.
3. It is difficult to test for long-term (1–5 years) morbidity and mortality, which are critical parameters of comparison between the standard of care and proposed RBC substitutes.
4. There is a lack of physiological information on the effect of blood and RBC substitute transfusion and the effects of blood storage at the microvascular level, where many important effects take place. Many RBC substitutes have undergone perfunctory tests in the microcirculation, but at least one product that failed after allegedly having been tested in 10 clinical trials (49) was never investigated for its functional effects in the microcirculation.
5. There is an imperfect understanding of the biophysical properties of blood, particularly regarding (*a*) the role of blood viscosity (55) and how this interacts with heart function (56), (*b*) the regulation of vasodilatation and perfusion by RBCs, and (*c*) the role of blood oxygen affinity and the effects of blood storage on microvascular perfusion (13).
6. There is a lack of quantitative understanding of the oxygen gradients needed to oxygenate ischemic tissues.

5. RESULTS FROM PLASMA EXPANDER AND RBC SUBSTITUTE RESEARCH

5.1. The Role of Viscosity

Nonoxygen-carrying plasma expanders precede the use oxygen-carrying RBC substitutes for the treatment of blood loss, and the transition between resuscitation regimes is determined by Hb level, based on experience and patient status. However, the transition from nonoxygen-carrying plasma expansion resuscitation to oxygen-carrying fluid resuscitation is carried out without information on the extent of the functional impairment of the microcirculation upon reaching the switchover point or transfusion trigger.

Experimental studies show that at the transfusion trigger, there is a significant reduction of microvascular flow owing to reduced blood viscosity, microvessel wall shear stress (WSS), and endothelial production of NO (57). This flow reduction results in capillary collapse, hindering oxygen delivery while toxic metabolic waste by-products accumulate in the tissue.

Comparative experimental transfusion studies with plasma expanders and blood show that restoration of oxygen transport capacity in hemorrhaged hamsters may not cause the initial therapeutic benefit, as using fresh blood, or nonoxygen-carrying blood equilibrated with carbon monoxide, and blood whose Hb is converted to methemoglobin (metHb) yields the same result (58). Therefore, it appears that blood transfusion induces resuscitation by restoring physiological deficits unrelated to loss of oxygen-carrying capacity, such as the normalization of blood viscosity.

Microvascular studies comparing the ability of plasma expanders to restore plasma and blood viscosity following 50% blood loss and 1 h of induced shock show that lowering blood viscosity only partially corrects microvascular impairment, whereas high-viscosity alginates, dextrans, poly(ethylene glycol) (PEG)-conjugated albumin (PEG-albumin), and polymerized albumin yield significantly greater recovery of FCD (59, 60). Extensive clinical studies have focused primarily on outcome, providing little information on the mechanisms underlying the results (61).

5.2. The Role of Plasma Expander–Induced Supraperfusion

Plasma expansion reduces the Hct and therefore blood viscosity, increasing blood flow velocity owing to the reduction of peripheral vascular resistance. However, the related physiological benefit is limited because significantly lowering blood viscosity lowers blood vessel WSS, and adequate levels of WSS are needed for producing NO via mechanotransduction (62) in the endothelium and for maintaining arteriolar dilatation to increase capillary pressure and sustain FCD (63).

The Hct varies throughout the circulatory system and is half the systemic value in the capillaries. The systemic blood viscosity ranges from 4–6 cP, and the capillary blood viscosity is close to the plasma blood viscosity, 1.2 cP, since the blood viscosity–Hct relationship is nonlinear (64). Therefore, hemodilution with a plasma expander whose viscosity is twice that of normal plasma can significantly lower blood viscosity in the systemic circulation while increasing blood viscosity in the capillaries. The very large endothelialized surface area of capillaries and increased blood viscosity significantly increase NO bioavailability, causing vasodilatation and increased tissue perfusion.

Tissue perfusion can be further augmented by manipulating the shear-thinning property of blood, whereby blood viscosity is lowered in the high-shear-rate regions of the circulation, such as the blood vessel walls. Resuscitation fluids such as PEG-albumin and polymerized albumin solutions exhibit this property: increased WSS in the microcirculation with lowered energy expenditure in the systemic circulation. Because these fluids also induce hemodilution, their effect is to further increase WSS and NO production, establishing a highly beneficial condition of supraperfusion (65, 66).

5.3. The Effect of Plasma Expansion Properties on Blood Transfusion Recovery

A recent experimental study of hemorrhagic shock showed that outcome in terms of microvascular function is dependent on the type of plasma expander used prior to initiating oxygen-carrying capacity restoration with blood transfusion (67). Results were optimal using 4% PEG-albumin and progressively worse for hydroxyethyl starch and homologous plasma. This indicates that microvascular outcome due to blood transfusion is dependent on the reaction of the organism to the initially used plasma expander, and this dependency is probably common to all oxygen carriers.

5.4. The Role of Plasma Expansion Properties in RBC Substitutes

Newly developed plasma expanders that induce supraperfusion increase the oxygen-delivery capacity of blood, as the arrival of oxygenated fluid (i.e., plasma, RBCs, and oxygen carriers) to

the capillaries is a function of transit losses after blood leaves the lungs. These losses oxygenate nonvital tissue regions and deprive oxygen from areas only oxygenated by capillaries. These losses are also flow-velocity dependent, being significantly reduced at high flow rates because as oxygen convection increases, diffusion remains comparatively constant, causing more oxygen to arrive to the capillaries (68). Therefore, supraperfusion may be seen as an additional increase in blood oxygen-carrying capacity, decreasing the need for an oxygen carrier.

Supraperfusion is also associated with increased FCD, which in combination with increased perfusion improves removal of tissue waste metabolites. This latter effect in combination with increased WSS and production of NO reduces inflammatory conditions and leukocyte activation, further improving tissue perfusion (57). Thus supraperfusion-inducing plasma expanders yield oxygen therapeutic–like properties without actually physically increasing blood oxygen content.

6. CURRENT RBC SUBSTITUTE DEVELOPMENT

Currently, there is no approved oxygen-carrying RBC substitute in the United States or Europe. A PEG-conjugated Hb product, MP4OX (Sangart Inc., San Diego, CA), was tested in Phase III clinical trials in Europe over the past few years, but it was not found to be effective and its development was not pursued. Four products based on chemically modified molecular Hb with roughly similar biophysical properties were tested in clinical trials in the United States and Europe within the past decade, but they did not satisfy safety requirements. There is ample literature that analyzes these products and the causes for their lack of success.

A common feature of the above-mentioned four modified molecular Hb products is that their Hb is configured as a small molecule, which results in low-viscosity solutions. Furthermore, their moderate colloid osmotic pressure (COP) allows for comparatively high plasma Hb concentrations. Molecular Hb solutions with small molecular radii are intrinsically vasoactive regardless of the chemical modification introduced, as they scavenge NO (69), thereby causing systemic hypertension. However, this is not uniformly negative, as moderately anemic animals (18% Hct) transfused with different concentrations of Oxyglobin® (Biopure Corp., Cambridge, MA) (70) showed significantly improved blood gas parameters for low concentration infusions (Hb: 4 g/dL) but not for high concentration infusions (Hb: 8 g/dL).

Nonvasoactive PEG-Hb solutions are formulated at concentrations of 4 wt% with a COP of 50 mm Hg, which effectively limits their intravascular concentration by autotransfusion as well as their potential vasoactivity. Furthermore, as COP increases nonlinearly with Hb concentration, it is not practical to infuse higher Hb concentrations because autotransfusion further limits their low oxygen-carrying capacity.

Vasoactivity is also counteracted by very-large-size molecular constructs such as vesicle-encapsulated Hbs, annelid Hbs, and high-molecular-weight polymerized Hbs as a result of increased WSS and additional NO generation by mechanotransduction (62).

7. OXYGEN THERAPEUTICS

Currently, the replacement of systemic blood oxygen-delivery capacity has not been attained. However, given the oxygen partial pressure dependence of Hb oxygen saturation, Hb molecules can be designed to target oxygen delivery to anoxic areas. Furthermore, features can be added to the carrier to treat associated local cellular dysfunctions. This approach is embodied in products

designated as oxygen therapeutics and is a promising area of development for treating local ischemia and trauma.

7.1. PEG-Conjugated Hb

MP4OX is an oxygen therapeutic product in advanced development. It was designed for ischemic rescue therapy and perfusion and oxygenation of tissues at risk of injury owing to ischemia and hypoxia in hemorrhagic shock. MP4OX (formerly MP4; Hemospan) is a hexa-PEGylated human Hb (HbA) with high oxygen affinity ($P_{50} \sim 4.5$ mm Hg), low cooperativity, and low Bohr effect that lowers vasoactivity due to arteriolar overoxygenation autoregulatory effects. This product has been subjected to extensive experimental and clinical testing and, being effective in maintaining microvascular function, has generally been found to present an excellent safety and efficacy profile (71).

Results obtained with MP4OX are paradoxical, since it scavenges NO similarly to all other Hb-based oxygen carriers (HBOCs) (69) and delivers oxygen if the tissue has reached a level of hypoxia significantly beyond that at which medical practice prescribes a blood transfusion. The explanation for these anomalies is that PEG-Hbs, like PEG-albumin, are excellent plasma expanders and therefore improve oxygen-delivery capacity (65).

7.2. The Addition of Carbon Monoxide

A common feature of the present oxygen-therapeutics approach to RBC substitute development is the low concentration of Hb in the transfusion solution. In addition, moderate carbon monoxide (CO) saturation has been introduced for mitigating ischemia-induced injuries in the PEG-Hb product SANGUINATE™ (4.5 wt% PEGylated bovine Hb in hypertonic saline; Prolong Pharmaceuticals LLC, Plainfield, NJ) and in MP4CO (4 wt% PEG-HbA in saline; Sangart Inc., San Diego, CA).

The potential beneficial effects of small dosages of CO on cardiovascular performance are well established in experimental models (58). Introducing CO dissolved in saline significantly increases tissue perfusion (72). Resuscitation using either RBCs or CO-saturated RBCs shows equally rapid recovery, which both supports the hypothesis that oxygen delivery is not the critical immediate component of shock resuscitation and highlights the importance of the nature of the initial plasma expansion and the necessity of restoring WSS. Furthermore, saturation of Hb with CO reduces oxidation-related damage, which mitigates RBC storage–induced damage.

7.3. The Addition of Oxygen Free Radical Scavengers

The effect of reactive oxygen species that develop in the tissues as a result of inflammation, ischemia, and trauma can be neutralized by the introduction of nitroxides such as the free radical scavenger TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl), which reduces blood pressure in rodent models of hypertension and mitigates endothelial dysfunction (73). This mediator reduces both toxicity due to superoxide production and NO scavenging due to the presence of acellular Hb in the circulation (74). SynZyme Technologies LLC (Irvine, CA) developed polynitroxylation of αα-fumaryl cross-linked Hb [polynitroxylated PEGylated Hb (PNPH), VitalHeme] as an oxygen therapeutic multifunctional product (75) that transports oxygen and has antioxidant properties and plasma expansion properties owing to PEGylation. Preclinical data show that PNPH also has neurovascular protective properties (76). PNPH should have a low or nonexistent

toxicity profile; however, it combines materials of substantial cost—namely, PEG, bovine Hb, and TEMPOL—which could limit its use as an RBC substitute in transfusion medicine.

8. CELLULAR HBOCs

In order to prevent Hb from interacting directly with the vasculature, it can be encapsulated inside the aqueous core of vesicles. The short circulatory half-life of liposome-encapsulated Hb (LEH) and vesicle aggregation and fusion during storage (77) were resolved by incorporating PEG on the vesicle surface (PEG-LEH), thus causing steric hindrance, increasing circulatory persistence, reducing interactions with blood plasma components, and increasing storage shelf life.

PEG-LEH half-life is a direct function of PEG molecular weight (MW) (78). As the maximum MW is restricted by the formation of PEG-lipid micelles (79, 80), the optimal formulation in rabbits is 5-kDa PEG with a circulatory half-life of about 48 h (78). However, because PEG molecules smaller than 20 kDa are less effective in suppressing complement activation (81–83), there is a balance between optimizing circulatory half-life and biocompatibility of PEG-LEHs.

Hb encapsulated inside vesicles composed of amphiphilic diblock copolymers (i.e., polymersomes) can potentially extend its circulatory half-life beyond that of PEG-LEHs. Amphiphilic diblock copolymers containing a hydrophilic block (PEG) and a hydrophobic block (84–86) form vesicles, which facilitate absolute control of the hydrophobic membrane thickness and size of the PEG corona (87, 88). Palmer's group (86) showed that polymersome-encapsulated Hb reduced the rate of oxygen offloading and NO dioxygenation compared with cell-free Hb and PEG-LEH, potentially mitigating extravasation, overoxygenation, and NO scavenging toxicity. However, the clinical utility of polymersome-encapsulated Hb as an RBC substitute is limited owing to low Hb encapsulation (<1 g/dL) (85, 89), which restricts oxygen-carrying capacity. Hence, PEG-LEHs remain the most viable option for cellular HBOC development.

Tsuchida's group has been at the forefront of in vivo validation of PEG-LEHs. For example, Sakai et al. (90) demonstrated that the HBOC molecular dimensions are inversely proportional to their vasoactivity, showing that vasoconstriction and hypertension decreased with increasing HBOC dimensions at the same Hb dose, with PEG-LEH exhibiting no side effects. This result was replicated for varying-sized polymerized Hbs (91), independently verifying the importance of HBOC dimensions in reducing Hb toxicity.

The major route of PEG-LEH clearance is through the spleen and liver. Transfusion of PEG-LEHs elicited increased plasma lipid levels that reduced to baseline levels after 7 days (92) and splenohepatomegaly that reverted to baseline levels after several weeks (93). These effects can be controlled by removing PEG-LEHs from the plasma through centrifugation and ultrafiltration before the particles are cleared by the reticuloendothelial system (94).

Overall, cellular oxygen carriers have a longer circulation half-life and lower diffusivity than acellular Hb and allow coencapsulation of allosteric effectors and antioxidants, mimicking the internal RBC environment. In addition, encapsulated Hb is stable in its tetrameric form because of its high intracellular concentration, which also presents a formidable diffusion barrier to gaseous binding/release, rendering it vasoinactive (95–100).

8.1. Problems with Acellular Hbs

All of the problems associated with previous generations of RBC substitutes can be attributed to removing Hb from the protective environment of the RBC. RBCs possess (*a*) enzymes that prevent Hb oxidation (101–103), (*b*) a cell membrane to reduce interactions with NO (104), (*c*) allosteric effectors to modulate oxygen delivery (105), and (*d*) high internal Hb concentrations

(~300 mg/mL) that minimize Hb dimerization (106). Furthermore, Hb is an unstable pro-oxidant that causes a myriad of side effects. As mentioned previously, Hb encapsulation avoids these side effects. However, this also suggests that it may be worthwhile to develop an oxygen carrier that avoids using mammalian Hbs altogether and that promotes the use of naturally acellular Hb, such as the erythrocruorin of earthworms (LtEc).

9. EARTHWORM AND MARINE WORM Hbs

Earthworms (*Lumbricus terrestris*) lack RBCs. Therefore, LtEc naturally evolved without the protection of RBCs to solve many of the problems facing modern oxygen carriers. LtEc is a macromolecular complex of 144 heme-containing globin subunits and 36 nonheme linker proteins held together by a dense network of intermolecular disulfide bonds and electrostatic interactions (107–109). It is extremely stable (28-h half-life in 1.75-M urea) (110), and its large size (~3.6-MDa MW, ~30-nm diameter) prevents extravasation. The heme groups in LtEc have less solvent exposure than mammalian Hbs, providing a physical barrier to entry of oxidative species (107, 108, 111). LtEc is also highly resistant to oxidation and has a positive redox potential (+112 mV), in contrast to the negative redox potential of HbA (−50 mV). Consequently, HbA is more prone to oxidation (Fe^{2+} to Fe^{3+}) and is much more likely to receive electrons and maintain the Fe^{2+} form, as antioxidants in human plasma (1-mM ascorbic acid) reduce up to 97% of oxidized LtEc (112–114). Autoxidation rate constants for LtEc are not available, but values from another annelid Hb [erythrocruorin of *Arenicola marina* (AmEc), 0.005 h^{-1}] are much lower than those of HbA (0.014 h^{-1}) (112). Therefore, LtEc should elicit much less oxidative damage compared with previous generations of acellular oxygen carriers.

LtEc may also have a significantly reduced rate of NO scavenging. Interestingly, it has been suggested that AmEc binds rather than scavenges NO (115), thereby reducing the oxidative stress, tissue toxicity, vasoconstriction, and systemic hypertension that are associated with acellular oxygen carriers. The low-resolution (3.5 Å) crystal structure of LtEc suggests that aromatic residues at the B10 position should block NO dioxygenation (109). The oxygen affinity of LtEc is similar to that of human blood, and it has some superoxide dismutase activity that protects it from free radicals (116). Preliminary studies show that LtEc delivers oxygen, is vasoinactive, and does not elicit systemic hypertension or immune responses in a top-load model (89).

Large Hb superstructures are also in the circulation of *A. marina* and consist of globin and nonglobin linker chains that also form complexes with a MW of 3.6 MDa (117). This large complex is used to produce the oxygen-carrying therapeutic HEMOXYCarrier® (Hemarina S.A., Morlaix, France) (115). This therapeutic is reported to have natural antioxidant properties in the presence of natural superoxide-dismutase-like enzymes (118) and a somewhat lower NO binding rate than HbA. Its administration into rodents (4 g/dL in saline) caused a transient increase in blood pressure that was not statistically different from that found after administering saline (119).

10. PFC-BASED OXYGEN CARRIERS

PFCs are extremely inert and stable synthetic fluorine–substituted hydrocarbons, which have high oxygen/CO_2 solubility (~20 times greater oxygen solubility than water). Their thermal stability is primarily due to the strong chemical bonds between carbon and fluorine atoms, one of the strongest single bonds in molecular compounds. The high gas-dissolving capacity of PFCs is a result of the weak Van der Waals interactions between PFC molecules. As the intermolecular forces between PFCs are weak, liquid PFCs behave like gas-like fluids (53) and easily dissolve gases including oxygen, CO_2, N_2, and NO.

The difference between oxygen association with HBOCs and PFCs depends on whether the oxygen molecule is covalently bound to the carrier molecule or physically dissolved within it. For HBOCs, oxygen is covalently bound to the heme group of the Hb molecule (120). PFCs are inert and extremely hydrophobic molecules not subject to oxidation reactions, and their ability to physically dissolve gases obeys Henry's law.

The extreme hydrophobicity of PFCs leads to very low solubility in water. Consequently, the only way to utilize PFCs as an oxygen carrier in aqueous solution is to emulsify them with a surfactant in order to form an emulsion. The principal difficulties in utilizing PFCs as an appropriate oxygen carrier lie in the identification of appropriate, biocompatible, and excretable PFCs and emulsifiers, as well as in engineering stable biocompatible emulsions (120).

At a given dose of PFC, a linear relationship exists between the amount of oxygen dissolved in the fluid phase of the PFC dispersion and the partial pressure of oxygen. Therefore, high concentrations of dissolved oxygen in PFC-based oxygen carriers are available for diffusion into tissues only at high partial pressures of oxygen. Hence, high inspired-oxygen concentrations are required for physiological oxygen delivery from PFCs (121).

The PFC Fluosol-DA (Green Cross Corp., Osaka, Japan) was approved by the FDA in 1989 as an adjunct for oxygen delivery during angioplasty. However, patients treated with Fluosol-DA in clinical trials had adverse physiological reactions, and it was removed from the market in 1994 (122–124). Second-generation PFC products include Oxyfluor™ (HemaGen/PFC, Waltham, MA), Oxygent™ (Alliance Pharmaceutical Corp., San Diego, CA), and Perftoran™ (SPC-Perftoran, Moscow, Russia). In Phase I clinical trials, Oxyfluor showed side effects (125), and its development was terminated (126). Clinical trials of Oxygent were terminated owing to increased incidence of stroke in coronary bypass patients. Perftoran has been approved in Russia and tested clinically in Mexico (127). Other PFC products in development include PHER-O2™ (Sanguine Corp., Pasadena, CA), and Oxycyte™ (Oxygen Biotherapeutics Inc., formerly Synthetic Blood International Inc., Morrisville, NC) (126).

11. STRATEGIES TO MITIGATE THE SIDE EFFECTS OF ACELLULAR Hb

Increasing the molecular radius of HBOCs by various chemical modification strategies can mitigate the deleterious side effects of previous generations of HBOCs. Polymerization of Hb with difunctional cross-linking reagents represents a simple strategy that can resolve previous concerns, as polymerized Hbs (PolyHbs) are inherently larger in size than tetrameric Hb. The increase in HBOC size should prevent the undesired extravasation/interaction of Hb through/with the blood vessel wall and prolong circulatory half-life (128). Other strategies to increase the molecular size of HBOCs mentioned above include conjugation of PEG to the surface of Hb (71) and encapsulation of Hb in PEG-conjugated vesicles (99). Both of these strategies are costly to implement because of the additional components (e.g., PEG and lipids) used.

In parallel with other approaches to reduce the side effects of transfused Hb, Olson's group at Rice University is taking a mechanistic biophysics approach to alleviate the hypertensive effect of HBOCs. This group has designed recombinant Hbs with low NO scavenging rates via site-directed mutagenesis of the distal heme pocket (129). This approach reduces vasoconstriction and hypertension; however, scaling up production is expensive, and transfusion of recombinant Hb still results in oxidative tissue toxicity owing to extravasation.

An alternative approach showed that continuous breathing of NO negates the hypertensive effect in animals transfused with either $\alpha_2\beta_2$ or commercially available small-sized PolyHbs (130, 131). This approach constitutes another strategy for reducing the NO-scavenging ability of Hb.

However, it increases metHb levels with increasing NO concentration in blood, which decreases RBC and HBOC oxygen-carrying capacity (130, 131), and as in the case of the recombinant engineering approach, small-sized PolyHbs are still able to extravasate into the tissue space, thus eliciting oxidative toxicity.

12. Hb POLYMERIZATION AS A STRATEGY TO MITIGATE VASCULAR SIDE EFFECTS

In order to prevent the side effects commonly associated with early commercial HBOCs, researchers have focused on preventing HBOC passage through the endothelial cell–cell junctions of blood vessels and reducing the proximity of Hb to the endothelium. This approach is expected to reduce endothelial-derived NO scavenging, reduce vasoconstriction and hypertension, and reduce oxidative tissue toxicity (132, 133). Several companies are developing PolyHb solutions.

12.1. Oxyglobin® and Hemopure®

OPK Biotech LLC (Cambridge, MA) developed Oxyglobin® and Hemopure®, which consist of glutaraldehyde-polymerized bovine Hb (PolybHb) with an average MW of 200 kDa and 250 kDA, respectively (**http://www.opkbiotech.com/company/about.php**). Glutaraldehyde forms intramolecular cross-links within the Hb tetramer and intermolecular cross-links between neighboring tetramers (134). Although approved for veterinary use in the US (135), Oxyglobin® elicits both vasoconstriction and hypertension in vivo (136). It also induces oxidative stress, damaging blood–brain barrier endothelial cells, and elicits cellular apoptosis in vivo and iron deposition in endothelial, neural, and renal tissues (137, 138).

Hemopure® is composed of 2% unpolymerized bovine Hb (bHb) compared with 31% in Oxyglobin® (139) and is reported to elicit less hypertension. However, administration before, during, and after elective aortic surgery caused hypertension in patients, and clinical trials highlighted the vulnerability of elderly orthopedic surgery patients to negative vascular side effects, indicating that even with significantly less unpolymerized bHb, Hemopure® still presents safety risks (139–143).

12.2. PolyHeme®

Northfield Laboratories Inc. (Evanston, IL) developed a glutaraldehyde-polymerized pyridoxal phosphate cross-linked HbA product known as PolyHeme®, which had an average MW of 150 kDa and reduced oxygen affinity (135, 144). PolyHeme® was administered to trauma, surgery, and hemorrhagic shock patients and did not increase blood pressure to unsafe levels. However, it showed negative side effects in animal studies, inducing vasoconstriction in lambs (145) and organ failure and death in hemorrhaged rats (146). Negative vascular responses are not surprising considering that Oxyglobin® and Hemopure®, two larger-sized PolybHbs, displayed similar negative side effects in vivo. Production was discontinued after ethical questions were raised over the consent requirements of the final clinical trial (49, 147, 148).

12.3. Hemolink™

Hemosol Inc. (Toronto, Canada) developed the *O*-raffinose cross-linked HbA product Hemolink™ (135, 149), which had a wide range of MWs (149). It caused hypertension in coronary artery surgery patients (150–152) in Phase II and III clinical trials. And although it had less

renal toxicity in vivo and less NO reactions in vitro compared with HbA, it induced hypertension in rats as well (153). Thus, despite Hemolink™ having a low affinity for NO in vitro, there was still evidence of systemic hypertension (153). Production was discontinued owing to the negative side effects found in clinical trials (49).

12.4. OxyVita™

All of the aforementioned commercial PolyHbs that reported such data had MWs ranging between 150 and 250 kDa. They all exhibited some form of vasoconstriction, hypertension, and/or oxidative stress, which makes it reasonable to assume that these PolyHbs are still able to extravasate and scavenge NO despite their larger size compared with Hb. Therefore, as Oxyglobin® induces oxidative stress, similarly sized HBOCs may possess similar potential for oxidation (137, 138) and may, therefore, support the development of nonextravasating ultrahigh-MW PolyHbs.

In light of this knowledge, OXYVITA Inc. (New Windsor, NY) developed a high-MW RBC substitute known as OxyVita™ (**http://www.oxyvita.us/**) (154–156), which is an ultrahigh-MW zero-link PolybHb with a reported MW of 42 MDa. OxyVita™ is synthesized by cross-linking the β-globin chains of bHb with bis(3,5-dibromosalicy)-adipoate and using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide to polymerize the bHb tetramers (154–156). OxyVita™ caused low levels of vasoconstriction, which did not lead to hypertension in animal studies. These benefits have been attributed to the large size of the PolybHb, which prevents its extravasation into the tissue space, thereby reducing the vascular side effects common to many commercial oxygen carriers. Despite this major advantage, OxyVita™ possesses a high oxygen affinity, which limits its potential for oxygenating tissue, like the case of MP4OX (154, 156).

13. OVERVIEW OF COMMERCIAL PolyHbs

Many of the commercial PolyHbs discussed above failed Phase III clinical trials even before their potential for negative side effects was investigated at the cellular and microvascular levels. Investigations into the gaseous ligand binding/release kinetics and the potential to induce vasoconstriction, systemic hypertension, and oxidative stress could have foreshadowed the results of Phase III clinical trials. The wide differences in MW and molecular dimension between Oxyglobin®, Hemopure®, PolyHeme®, Hemolink™, OxyVita™, and PEG-Hb-based products highlight the molecular diversity of potential products and the need for a systematic study of the effect of HBOCs' size on their efficacy and safety.

14. SYSTEMATIC STUDY OF PolyHb MW ON SAFETY PROFILE

Palmer's group pioneered the systematic study of the biophysical properties and in vivo responses of variable-MW glutaraldehyde PolybHbs (91, 157–161). In one study, they used the quaternary state of bHb to control the oxygen affinity of PolybHb solutions (159). The tense (T) and relaxed (R) quaternary states of Hb could be induced by simply fully deoxygenating and fully oxygenating the bHb solution, respectively. This facilitates synthesis of T-state and R-state PolybHbs by polymerizing T-state and R-state bHb, respectively, with glutaraldehyde (159). As expected, negative vascular effects were reduced when the PolybHb's MW was greater than 500 kDa, echoing the findings with ultrahigh-MW OxyVita™. An important distinction between OxyVita™ and the high-MW T-state PolybHbs is the high P_{50} of the T-state PolybHbs and the low P_{50} of OxyVita™ (91, 154, 156). Previous work demonstrated that high-P_{50} HBOCs deliver oxygen to tissues more readily than low-P_{50} HBOCs do (92). This was corroborated experimentally by means of direct

microvascular-tissue-oxygen measurements, which showed that T-state PolybHb delivered more oxygen to tissues in a hamster chamber window model versus R-state PolybHb (157).

In order to elucidate the effect of PolybHb size on oxidative tissue toxicity, a small library of T-state PolybHbs was synthesized and evaluated in vivo (158). Kidney iron deposition and hypertension decreased in proportion to increased PolybHb size. Similarly, circulatory half-life increased as a function of increasing PolybHb size.

15. CONCLUDING REMARKS

The need for RBC substitutes arises not only from impending shortages or viral contamination but also because allogeneic RBCs, properly screened for known transmissible viral and infectious diseases, do not have short- and long-term safety profiles commensurate with modern expectations.

Decades of HBOC research has culminated in the development of oxygen therapeutics, materials that can deliver oxygen as well as provide treatment for the effects of injury and hypoxia. However, the oxygen-carrying capacity of these materials is minimal by comparison to that attained by blood transfusion and is generally insufficient to compensate for oxygen losses that occur in the circulation prior to blood arriving to the capillaries.

Additionally, the plasma expander properties of most RBC substitutes are similar or inferior to currently available plasma expanders, which are not optimal for preserving microvascular function during resuscitation. This is a missed opportunity for optimizing the performance of RBC substitutes, which can be achieved by engineering large-sized Hb molecules.

Hb toxicity is inherent in all small-sized constructs derived from mammalian Hbs. However, PEG conjugation and polymerization of Hb into large superstructures counteract NO scavenging by increasing blood vessel WSS and the production of vasodilators. The high COP of PEG-Hb limits its concentration in plasma owing to autotransfusion, and PolyHb possessing low COP significantly increases plasma viscosity, also limiting plasma concentration and the ability to increase blood oxygen-carrying capacity. The effect of PolyHb use is comparable to increasing blood Hb by 1–2 g/dL, which is equivalent to levels attainable with supraplasma expansion using nonoxygen-carrying colloids of significantly less toxicity.

Although it has not been empirically shown that the additional anti-inflammatory properties conferred by conjugation or formulation of HBOCs with ROS scavengers are useful, such features may be beneficial in some injuries. Inflammation is a natural response to injury, which significantly increases perfusion, thereby rapidly extracting ROS from the tissue for delivery to the detoxification organs while facilitating extraction of other toxic metabolites.

Shock resuscitation also causes the rapid upregulation of genes of many defense pathways and consequent alterations of enzyme levels, a problem not addressed in the design of oxygen therapeutics that could be as important as controlling inflammation. Furthermore, in oxygen therapeutic formulations, the relationship between antioxidant, anti-inflammatory activity, and oxygen-carrying capacity is fixed a priori, rather than being of controllable dosage for each treatment strategy.

The effectiveness of RBC substitutes for use in transfusion and treatment is nullified if they do not restore microvascular function. This restoration process, although mechanistically a simple problem of fluid mechanics, is a complex biological and medical problem, which, because it is not amenable to direct observation, is seldom addressed in RBC substitute development and transfusion medicine.

Regardless of the present imperfect understanding of resuscitation from blood loss, the field has advanced to the extent that RBC substitutes are a viable option when safe blood products are not accessible. In terms of demand, there is clearly an important need for a product with a

better safety profile than stored blood. Vesicle encapsulation of Hb mitigates the shortcomings of acellular Hbs and presents a logical approach toward mimicking RBC function. Thus, it may ultimately became the technological base for future RBC substitutes.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

We acknowledge support from National Institutes of Health grants R01HL078840, R01DK070862, and P01-HL071064.

LITERATURE CITED

1. Greening DW, Glenister KM, Sparrow RL, Simpson RJ. 2010. International blood collection and storage: clinical use of blood products. *J. Proteomics* 73:386–95
2. Wang J, Guo N, Guo X, Li J, Wen G-X, et al. 2010. Who donates blood at five ethnically and geographically diverse blood centers in China in 2008. *Transfusion* 50:2686–94
3. Seifried E, Mueller MM. 2011. The present and future of transfusion medicine. *Blood Transfus.* 9:371–76
4. Morley S. 2009. Red blood cell transfusions in acute paediatrics. *Arch. Dis. Child. Educ. Pract. Ed.* 94:65–73
5. Cobain TJ, Vamvakas EC, Wells A, Titlestad K. 2007. A survey of the demographics of blood use. *Transfus. Med.* 17:1–15
6. Shander A, Hofmann A, Ozawa S, Theusinger OM, Gombotz H, Spahn DR. 2010. Activity-based costs of blood transfusions in surgical patients at four hospitals. *Transfusion* 50:753–65
7. Seifried E, Klueter H, Weidmann C, Staudenmaier T, Schrezenmeier H, et al. 2011. How much blood is needed? *Vox Sang.* 100:10–21
8. Koch CG, Li L, Sessler DI, Figueroa P, Hoeltge GA, et al. 2008. Duration of red-cell storage and complications after cardiac surgery. *N. Engl. J. Med.* 358:1229–39
9. Shiba H, Ishida Y, Wakiyama S, Iida T, Matsumoto M, et al. 2009. Negative impact of blood transfusion on recurrence and prognosis of hepatocellular carcinoma after hepatic resection. *J. Gastrointest. Surg.* 13:1636–42
10. van de Watering L, Lorinser J, Versteegh M, Westendord R, Brand A. 2006. Effects of storage time of red blood cell transfusions on the prognosis of coronary artery bypass graft patients. *Transfusion* 46:1712–18
11. Sparrow RL. 2010. Red blood cell storage and transfusion-related immunomodulation. *Blood Transfus.* 8(Suppl. 3):s26–30
12. Baskurt OK, Meiselman HJ. 2003. Blood rheology and hemodynamics. *Semin. Thromb. Hemost.* 29:435–50
13. Tsai AG, Hofmann A, Cabrales P, Intaglietta M. 2010. Perfusion versus oxygen delivery in transfusion with "fresh" and "old" red blood cells: the experimental evidence. *Transfus. Apher. Sci.* 43:69–78
14. Berezina TL, Zaets SB, Morgan C, Spillert CR, Kamiyama M, et al. 2002. Influence of storage on red blood cell rheological properties. *J. Surg. Res.* 102:6–12
15. Vicente JR, Croci AT, Camargo OP. 2008. Blood loss in the minimally invasive posterior approach to total hip arthroplasty: a comparative study. *Clinics* (*Sao Paulo*) 63:351–56
16. Smith TO, Blake V, Hing CB. 2011. Minimally invasive versus conventional exposure for total hip arthroplasty: a systematic review and meta-analysis of clinical and radiological outcomes. *Int. Orthop.* 35:173–84
17. Troisi RI, Patriti A, Montalti R, Casciola L. 2013. Robot assistance in liver surgery: a real advantage over a fully laparoscopic approach? Results of a comparative bi-institutional analysis. *Int. J. Med. Robot.* 9:160–66

18. Frank SM, Savage WJ, Rothschild JA, Rivers RJ, Ness PM, et al. 2012. Variability in blood and blood component utilization as assessed by an anesthesia information management system. *Anesthesiology* 117:99–106
19. Napolitano LM, Kurek S, Luchette FA, Anderson GL, Bard MR, et al. 2009. Clinical practice guideline: red blood cell transfusion in adult trauma and critical care. *J. Trauma* 67:1439–42
20. Wier LM, Pfuntner A, Maeda J, Stranges E, Ryan K, et al. 2011. *HCUP Facts and Figures: Statistics on Hospital-Based Care in the United States, 2009*. Rockville, MD: Agency Healthc. Res. Qual.
21. Whitaker BI. 2013. *The 2011 national blood collection and utilization survey report*. OMB 0990–0313, US Dep. Health Hum. Serv., Washington, DC
22. Stramer SL. 2007. Current risks of transfusion-transmitted agents: a review. *Arch. Pathol. Lab. Med.* 131:702–7
23. Allain JP, Stramer SL, Carneiro-Proietti AB, Martins ML, Lopes da Silva SN, et al. 2009. Transfusion-transmitted infectious diseases. *Biologicals* 37:71–77
24. Isbister JP. 2012. Comparing apples with oranges. *Vox Sang.* 103:359–60
25. Isbister JP, Shander A, Spahn DR, Erhard J, Farmer SL, Hofmann A. 2011. Adverse blood transfusion outcomes: establishing causation. *Transf. Med. Rev.* 25:89–101
26. Hofmann A, Ozawa S, Farrugia A, Farmer SL, Shander A. 2013. Economic considerations on transfusion medicine and patient blood management. *Best Pract. Res. Clin. Anaesthesiol.* 27:59–68
27. Shander A, Fink A, Javidroozi M, Erhard J, Farmer SL, et al. 2011. Appropriateness of allogeneic red blood cell transfusion: the international consensus conference on transfusion outcomes. *Transfus. Med. Rev.* 25:232–46.e53
28. Rao SV, Jollis JG, Harrington RA, Granger CB, Newby LK, et al. 2004. Relationship of blood transfusion and clinical outcomes in patients with acute coronary syndromes. *JAMA* 292:1555–62
29. Doyle BJ, Rihal CS, Gastineau DA, Holmes DR Jr. 2009. Bleeding, blood transfusion, and increased mortality after percutaneous coronary intervention: implications for contemporary practice. *J. Am. Coll. Cardiol.* 53:2019–27
30. Aronson D, Dann EJ, Bonstein L, Blich M, Kapeliovich M, et al. 2008. Impact of red blood cell transfusion on clinical outcomes in patients with acute myocardial infarction. *Am. J. Cardiol.* 102:115–19
31. Casutt M, Seifert B, Pasch T, Schmid ER, Turina MI, Spahn DR. 1999. Factors influencing the individual effects of blood transfusions on oxygen delivery and oxygen consumption. *Crit. Care Med.* 27:2194–200
32. Raat NJ, Verhoeven AJ, Mik EG, Gouwerok CW, Verhaar R, et al. 2005. The effect of storage time of human red cells on intestinal microcirculatory oxygenation in a rat isovolemic exchange model. *Crit. Care Med.* 33:39–45;238–89
33. Bernard AC, Davenport DL, Chang PK, Vaughan TB, Zwischenberger JB. 2009. Intraoperative transfusion of 1 U to 2 U packed red blood cells is associated with increased 30-day mortality, surgical-site infection, pneumonia, and sepsis in general surgery patients. *J. Am. Coll. Surg.* 208:931–39
34. Sugita S, Sasaki A, Iwaki K, Uchida H, Kai S, et al. 2008. Prognosis and postoperative lymphocyte count in patients with hepatocellular carcinoma who received intraoperative allogenic blood transfusion: a retrospective study. *Eur. J. Surg. Oncol.* 34:339–45
35. Zhu H, Zennadi R, Xu BX, Eu JP, Torok JA, et al. 2011. Impaired adenosine-5′-triphosphate release from red blood cells promotes their adhesion to endothelial cells: a mechanism of hypoxemia after transfusion. *Crit. Care Med.* 39:2478–86
36. Wang D, Sun J, Solomon SB, Klein HG, Natanson C. 2012. Transfusion of older stored blood and risk of death: a meta-analysis. *Transfusion* 52:1184–95
37. LeLubre C, Vincent JL. 2011. Red blood cell transfusion in the critically ill patient. *Ann. Intensive Care* 1:43
38. Saugel B, Klein M, Hapfelmeier A, Phillip V, Schultheiss C, et al. 2013. Effects of red blood cell transfusion on hemodynamic parameters: a prospective study in intensive care unit patients. *Scand. J. Trauma Resusc. Emerg. Med.* 21:21
39. Martini J, Carpentier B, Chávez Negrete A, Frangos JA, Intaglietta M. 2005. Paradoxical hypotension following increased hematocrit and blood viscosity. *Am. J. Physiol. Heart Circ. Physiol.* 289(5):H2136–43

40. Salazar Vázquez BY, Cabrales P, Tsai AG, Johnson PC, Intaglietta M. 2008. Lowering of blood pressure by increasing hematocrit with non–nitric oxide–scavenging red blood cells. *Am. J. Respir. Cell Mol. Biol.* 38:135–42
41. Salazar Vázquez BY, Martini J, Chávez-Negrete A, Cabrales P, Tsai AG, Intaglietta M. 2009. Microvascular benefits of increasing plasma viscosity and maintaining blood viscosity: counterintuitive experimental findings. *Biorheology* 46:167–79
42. Cotton BA, Guy JS, Morris JA Jr, Abumrad NN. 2006. The cellular, metabolic, and systemic consequences of aggressive fluid resuscitation strategies. *Shock* 26:115–21
43. Cabrales P, Intaglietta M, Tsai AG. 2005. Increase plasma viscosity sustains microcirculation after resuscitation from hemorrhagic shock and continuous bleeding. *Shock* 23:549–55
44. Kerger H, Saltzman DJ, Menger MD, Messmer K, Intaglietta M. 1996. Systemic and subcutaneous microvascular Po2 dissociation during 4-h hemorrhagic shock in conscious hamsters. *Am. J. Physiol. Heart Circ. Physiol.* 270:H827–36
45. Silverman TA, Weiskopf RB. 2009. Hemoglobin-based oxygen carriers: current status and future directions. *Transfusion* 49:2495–515
46. Mazzoni MC, Borgström P, Intaglietta M, Arfors KE. 1989. Lumenal narrowing and endothelial cell swelling in skeletal muscle capillaries during hemorrhagic shock. *Circ. Shock* 29:27–39
47. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. 2007. Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol.* 39:44–84
48. Yang W, Hekimi S. 2010. A mitochondrial superoxide signal triggers increased longevity in *Caenorhabditis elegans*. *PLoS Biol.* 8:e1000556
49. Chen JY, Scerbo M, Kramer G. 2009. A review of blood substitutes: examining the history, clinical trial results, and ethics of hemoglobin-based oxygen carriers. *Clinics* (*Sao Paulo*) 64:803–13
50. Napolitano LM. 2011. Acute traumatic hemorrhage and anemia. In *Chemistry and Biochemistry of Oxygen Therapeutics: From Transfusion to Artificial Blood*, ed. A Mozzarelli, S Bettati, pp. 79–106. Chichester, UK: Wiley
51. Grethlein SJ, Rajan A, Sandler SG, Talavera F, Conrad ME, et al. 2012. *Blood substitutes*. Medscape, WebMD, New York. **http://emedicine.medscape.com/article/207801**
52. Cabrales P, Intaglietta M. 2013. Blood substitutes: evolution from noncarrying to oxygen- and gas-carrying fluids. *ASAIO J.* 59:337–54
53. Winslow RM. 2006. *Blood Substitutes*. London/Burlington, MA: Elsevier/Academic. 576 pp.
54. Sazama K. 2007. The ethics of blood management. *Vox Sang.* 92:95–102
55. Cabrales P, Intaglietta M, Tsai AG. 2007. Transfusion restores blood viscosity and reinstates microvascular conditions from hemorrhagic shock independent of oxygen carrying capacity. *Resuscitation* 75:124–34
56. Chatpun S, Cabrales P. 2010. Effects of plasma viscosity modulation on cardiac function during moderate hemodilution. *Asian J. Transfus. Sci.* 4:102–8
57. Tsai AG, Acero C, Nance PR, Cabrales P, Frangos JA, et al. 2005. Elevated plasma viscosity in extreme hemodilution increases perivascular nitric oxide concentration and microvascular perfusion. *Am. J. Physiol. Heart Circ. Physiol.* 288:H1730–39
58. Cabrales P, Tsai AG, Intaglietta M. 2007. Hemorrhagic shock resuscitation with carbon monoxide saturated blood. *Resuscitation* 72:306–18
59. Salazar Vázquez BY, Wettstein R, Cabrales P, Tsai AG, Intaglietta M. 2008. Microvascular experimental evidence on the relative significance of restoring oxygen carrying capacity versus blood viscosity in shock resuscitation. *Biochim. Biophys. Acta* 1784:1421–27
60. Messmer C, Yalcin O, Palmer AF, Cabrales P. 2012. Small-volume resuscitation from hemorrhagic shock with polymerized human serum albumin. *Am. J. Emerg. Med.* 30:1336–46
61. Santry HP, Alam HB. 2010. Fluid resuscitation: past, present, and the future. *Shock* 33:229–41
62. Martini J, Cabrales P, Tsai AG, Intaglietta M. 2006. Mechanotransduction and the homeostatic significance of maintaining blood viscosity in hypotension, hypertension and haemorrhage. *J. Intern. Med.* 259:364–72
63. Cabrales P, Tsai AG, Intaglietta M. 2004. Microvascular pressure and functional capillary density in extreme hemodilution with low- and high-viscosity dextran and a low-viscosity Hb-based O_2 carrier. *Am. J. Physiol. Heart Circ. Physiol.* 287:H363–73

64. Salazar Vázquez BY. 2012. Blood pressure and blood viscosity are not correlated in normal healthy subjects. *Vasc. Health Risk Manag.* 8:1–6
65. Sriram K, Tsai AG, Cabrales P, Meng F, Acharya SA, et al. 2012. PEG-albumin supraplasma expansion is due to increased vessel wall shear stress induced by blood viscosity shear thinning. *Am. J. Physiol. Heart Circ. Physiol.* 302(12):H2489–97
66. Yalcin O, Wang Q, Johnson PC, Palmer AF, Cabrales P. 2011. Plasma expander viscosity effects on red cell-free layer thickness after moderate hemodilution. *Biorheology* 48:277–91
67. Hightower CM, Salazar Vázquez BY, Cabrales P, Tsai AG, Acharya SA, Intaglietta M. 2013. Plasma expander and blood storage effects on capillary perfusion in transfusion after hemorrhage. *Transfusion* 53:49–59
68. Mirhashemi S, Ertefai S, Messmer K, Intaglietta M. 1987. Model analysis of the enhancement of tissue oxygenation by hemodilution due to increased microvascular flow velocity. *Microvasc. Res.* 34:290–301
69. Rohlfs RJ, Brunner E, Chiu A, Gonzales A, Gonzales ML, et al. 1998. Arterial blood pressure responses to cell-free hemoglobin solutions and the reaction with nitric oxide. *J. Biol. Chem.* 273:12128–34
70. Cabrales P, Tsai AG, Intaglietta M. 2008. Balance between vasoconstriction and enhanced oxygen delivery. *Transfusion* 48:2087–95
71. Vandegriff KD, Winslow RM. 2009. Hemospan: design principles for a new class of oxygen therapeutic. *Artif. Organs* 33:133–38
72. Hangai-Hoger N, Tsai AG, Cabrales P, Suematsu M, Intaglietta M. 2007. Microvascular and systemic effects following top load administration of saturated carbon monoxide-saline solution. *Crit. Care Med.* 35:1123–32
73. Wilcox CS, Pearlman A. 2008. Chemistry and antihypertensive effects of tempol and other nitroxides. *Pharmacol. Rev.* 60:418–69
74. Buehler PW, Baek JH, Lisk C, Connor I, Sullivan T, et al. 2012. Free hemoglobin induction of pulmonary vascular disease: evidence for an inflammatory mechanism. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 303:L312–26
75. Kentner R, Safar P, Behringer W, Wu X, Henchir J, et al. 2007. Small volume resuscitation with tempol is detrimental during uncontrolled hemorrhagic shock in rats. *Resuscitation* 72:295–305
76. Hsia CJC, Ma L. 2012. A hemoglobin-based multifunctional therapeutic: polynitroxylated pegylated hemoglobin. *Artif. Organs* 36:215–20
77. Sakai H, Tomiyama KI, Sou K, Takeoka S, Tsuchida E. 2000. Poly(ethylene glycol)-conjugation and deoxygenation enable long-term preservation of hemoglobin-vesicles as oxygen carriers in a liquid state. *Bioconjug. Chem.* 11:425–32
78. Phillips WT, Klipper RW, Awasthi VD, Rudolph AS, Cliff R, et al. 1999. Polyethylene glycol-modified liposome-encapsulated hemoglobin: a long circulating red cell substitute. *J. Pharmacol. Exp. Ther.* 288:665–70
79. Shimada K, Matsuo S, Sadzuka Y, Miyagishima A, Nozawa Y, et al. 2000. Determination of incorporated amounts of poly(ethylene glycol)-derivatized lipids in liposomes for the physicochemical characterization of stealth liposomes. *Int. J. Pharm.* 203:255–63
80. Photos PJ, Bacakova L, Discher B, Bates FS, Discher DE. 2003. Polymer vesicles in vivo: correlations with PEG molecular weight. *J. Control Release* 90:323–34
81. Szebeni J, Spielberg H, Cliff RO, Wassef NM, Rudolph AS, Alving CR. 1997. Complement activation and thromboxane secretion by liposome-encapsulated hemoglobin in rats in vivo: inhibition by soluble complement receptor type 1. *Artif. Cells Blood Substit. Immobil. Biotechnol.* 25:347–55
82. Moghimi SM, Szebeni J. 2003. Stealth liposomes and long circulating nanoparticles: critical issues in pharmacokinetics, opsonization and protein-binding properties. *Prog. Lipid Res.* 42:463–78
83. Mosqueira VC, Legrand P, Gulik A, Bourdon O, Gref R, et al. 2001. Relationship between complement activation, cellular uptake and surface physicochemical aspects of novel PEG-modified nanocapsules. *Biomaterials* 22:2967–79
84. Arifin DR, Palmer AF. 2005. Polymersome encapsulated hemoglobin: a novel type of oxygen carrier. *Biomacromolecules* 6:2172–81
85. Rameez S, Alosta H, Palmer AF. 2008. Biocompatible and biodegradable polymersome encapsulated hemoglobin: a potential oxygen carrier. *Bioconjug. Chem.* 19:1025–32

86. Rameez S, Banerjee U, Fontes J, Roth A, Palmer AF. 2012. The reactivity of polymersome encapsulated hemoglobin with physiologically important gaseous ligands: oxygen, carbon monoxide and nitric oxide. *Macromolecules* 45:2385–89
87. Lee JC, Bermudez H, Discher BM, Sheehan MA, Won YY, et al. 2001. Preparation, stability, and in vitro performance of vesicles made with diblock copolymers. *Biotechnol. Bioeng.* 73:135–45
88. Ahmed F, Pakunlu RI, Srinivas G, Brannan A, Bates F, et al. 2006. Shrinkage of a rapidly growing tumor by drug-loaded polymersomes: pH-triggered release through copolymer degradation. *Mol. Pharm.* 3:340–50
89. Elmer J, Zorc K, Rameez S, Zhou Y, Cabrales P, Palmer AF. 2012. Hypervolemic infusion of *Lumbricus terrestris* erythrocruorin purified by tangential-flow filtration. *Transfusion* 52:1729–40
90. Sakai H, Hara H, Yuasa M, Tsai AG, Takeoka S, et al. 2000. Molecular dimensions of Hb-based O_2 carriers determine constriction of resistance arteries and hypertension. *Am. J. Physiol. Heart Circ. Physiol.* 279:H908–15
91. Cabrales P, Sun G, Zhou Y, Harris DR, Tsai AG, et al. 2009. Effects of the molecular mass of tense-state polymerized bovine hemoglobin on blood pressure and vasoconstriction. *J. Appl. Physiol.* 107:1548–58
92. Cabrales P, Sakai H, Tsai AG, Takeoka S, Tsuchida E, Intaglietta M. 2005. Oxygen transport by low and normal oxygen affinity hemoglobin vesicles in extreme hemodilution. *Am. J. Physiol. Heart Circ. Physiol.* 288:H1885–92
93. Taguchi K, Urata Y, Anraku M, Watanabe H, Kadowaki D, et al. 2009. Hemoglobin vesicles, polyethylene glycol (PEG)ylated liposomes developed as a red blood cell substitute, do not induce the accelerated blood clearance phenomenon in mice. *Drug Metab. Dispos.* 37:2197–203
94. Sakai H, Sato A, Takeoka S, Tsuchida E. 2009. Mechanism of flocculate formation of highly concentrated phospholipid vesicles suspended in a series of water-soluble biopolymers. *Biomacromolecules* 10:2344–50
95. Sakai H, Hara H, Yuasa M, Tsai AG, Takeoka S, et al. 2000. Molecular dimensions of Hb-based O_2 carriers determine constriction of resistance arteries and hypertension. *Am. J. Physiol. Heart Circ. Physiol.* 279:H908–15
96. Sakai H, Masada Y, Onuma H, Takeoka S, Tsuchida E. 2004. Reduction of methemoglobin via electron transfer from photoreduced flavin: restoration of O_2-binding of concentrated hemoglobin solution coencapsulated in phospholipid vesicles. *Bioconjug. Chem.* 15:1037–45
97. Sakai H, Sato A, Takeoka S, Tsuchida E. 2007. Rheological properties of hemoglobin vesicles (artificial oxygen carriers) suspended in a series of plasma-substitute solutions. *Langmuir* 23:8121–28
98. Sakai H, Sou K, Horinouchi H, Kobayashi K, Tsuchida E. 2010. Hemoglobin-vesicle, a cellular artificial oxygen carrier that fulfils the physiological roles of the red blood cell structure. *Adv. Exp. Med. Biol.* 662:433–38
99. Sakai H, Sou K, Tsuchida E. 2009. Hemoglobin-vesicles as an artificial oxygen carrier. *Methods Enzymol.* 465:363–84
100. Chang TM. 2000. Artificial cell biotechnology for medical applications. *Blood Purif.* 18:91–96
101. Yubisui T, Matsuki T, Tanishima K, Takeshita M, Yoneyama Y. 1977. NADPH-flavin reductase in human erythrocytes and the reduction of methemoglobin through flavin by the enzyme. *Biochem. Biophys. Res. Commun.* 76:174–82
102. Kuma F. 1981. Properties of methemoglobin reductase and kinetic study of methemoglobin reduction. *J. Biol. Chem.* 256:5518–23
103. Scott MD, Lubin BH, Zuo L, Kuypers FA. 1991. Erythrocyte defense against hydrogen peroxide: preeminent importance of catalase. *J. Lab. Clin. Med.* 118:7–16
104. Liu X, Miller MJ, Joshi MS, Sadowska-Krowicka H, Clark DA, Lancaster JR Jr. 1998. Diffusion-limited reaction of free nitric oxide with erythrocytes. *J. Biol. Chem.* 273:18709–13
105. Bunn HF, Briehl RW. 1970. The interaction of 2,3-diphosphoglycerate with various human hemoglobins. *J. Clin. Invest.* 49:1088–95
106. Chiancone E. 1968. Dissociation of hemoglobin into subunits. II. Human oxyhemoglobin: gel filtration studies. *J. Biol. Chem.* 243:1212–19
107. Royer WE Jr, Sharma H, Strand K, Knapp JE, Bhyravbhatla B. 2006. *Lumbricus* erythrocruorin at 3.5 Å resolution: architecture of a megadalton respiratory complex. *Structure* 14:1167–77

108. Fushitani K, Matsuura MS, Riggs AF. 1988. The amino acid sequences of chains *a*, *b*, and *c* that form the trimer subunit of the extracellular hemoglobin from *Lumbricus terrestris*. *J. Biol. Chem.* 263:6502–17
109. Strand K, Knapp JE, Bhyravbhatla B, Royer WE Jr. 2004. Crystal structure of the hemoglobin dodecamer from *Lumbricus* erythrocruorin: allosteric core of giant annelid respiratory complexes. *J. Mol. Biol.* 344:119–34
110. Sharma PK, Kuchumov AR, Chottard G, Martin PD, Wall JS, Vinogradov SN. 1996. The role of the dodecamer subunit in the dissociation and reassembly of the hexagonal bilayer structure of *Lumbricus terrestris* hemoglobin. *J. Biol. Chem.* 271:8754–62
111. Stellwagen E. 1978. Haem exposure as the determinate of oxidation-reduction potential of haem proteins. *Nature* 275:73–4
112. Harrington JP, Kobayashi S, Dorman SC, Zito SL, Hirsch RE. 2007. Acellular invertebrate hemoglobins as model therapeutic oxygen carriers: unique redox potentials. *Artif. Cells Blood Substit. Immobil. Biotechnol.* 35:53–67
113. Dorman SC, Harrington JP, Martin MS, Johnson TV. 2004. Determination of the formal reduction potential of *Lumbricus terrestris* hemoglobin using thin layer spectroelectrochemistry. *J. Inorg. Biochem.* 98:185–88
114. Dorman SC, Kenny CF, Miller L, Hirsch RE, Harrington JP. 2002. Role of redox potential of hemoglobin-based oxygen carriers on methemoglobin reduction by plasma components. *Artif. Cells Blood Substit. Immobil. Biotechnol.* 30:39–51
115. Rousselot M, Delpy E, La Rochelle CD, Lagente V, Pirow R, et al. 2006. *Arenicola marina* extracellular hemoglobin: a new promising blood substitute. *Biotechnol. J.* 1:333–45
116. Liochev SI, Kuchumov AR, Vinogradov SN, Fridovich I. 1996. Superoxide dismutase activity in the giant hemoglobin of the earthworm, *Lumbricus terrestris*. *Arch. Biochem. Biophys.* 330:281–84
117. Zal F, Green BN, Lallier FH, Vinogradov SN, Toulmond A. 1997. Quaternary structure of the extracellular haemoglobin of the lugworm *Arenicola marina*: a multi-angle-laser-light-scattering and electrospray-ionisation-mass-spectrometry analysis. *Eur. J. Biochem./FEBS* 243(1–2):85–92
118. Thuillier R, Dutheil D, Trieu MT, Mallet V, Allain G, et al. 2011. Supplementation with a new therapeutic oxygen carrier reduces chronic fibrosis and organ dysfunction in kidney static preservation. *Am. J. Transplant.* 11:1845–60
119. Tsai AG, Intaglietta M, Sakai H, Delpy E, La Rochelle CD, et al. 2012. Microcirculation and NO-CO studies of a natural extracellular hemoglobin developed for an oxygen therapeutic carrier. *Curr. Drug Discov. Technol.* 9:166–72
120. Riess JG. 2001. Oxygen carriers ("blood substitutes")—raison d'etre, chemistry, and some physiology. *Chem. Rev.* 101:2797–920
121. Kjellström BT. 2003. Blood substitutes: Where do we stand today? *J. Intern. Med.* 253:495–97
122. Hill SE. 2001. Oxygen therapeutics: current concepts. *Can. J. Anesth.* 48:S32–40
123. Klein HG. 2002. Blood substitutes: how close to a solution? *Oncology* 16:147–51
124. Stowell CP. 2002. Hemoglobin-based oxygen carriers. *Curr. Opin. Hematol.* 9:537–43
125. Winslow RM. 2003. Current status of blood substitute research: towards a new paradigm. *J. Intern. Med.* 253:508–17
126. Keipert PE. 2003. Oxygen therapeutics ("blood substitutes"): Where are they, and what can we expect? *Adv. Exp. Med. Biol.* 540:207–13
127. Verdin-Vasquez RC, Zepeda-Perez C, Ferra-Ferrer R, Chavez-Negrete A, Contreras F, Barroso-Aranda J. 2006. Use of perftoran emulsion to decrease allogeneic blood transfusion in cardiac surgery: clinical trial. *Artif. Cells Blood Substit. Immobil. Biotechnol.* 34:433–54
128. Riess JG. 2006. Perfluorocarbon-based oxygen delivery. *Artif. Cells Blood Substit. Immobil. Biotechnol.* 34:567–80
129. Doherty DH, Doyle MP, Curry SR, Vali RJ, Fattor TJ, et al. 1998. Rate of reaction with nitric oxide determines the hypertensive effect of cell-free hemoglobin. *Nat. Biotechnol.* 16:672–76
130. Yu B, Raher MJ, Volpato GP, Bloch KD, Ichinose F, Zapol WM. 2008. Inhaled nitric oxide enables artificial blood transfusion without hypertension. *Circulation* 117:1982–90
131. Yu B, Volpato GP, Chang K, Bloch KD, Zapol WM. 2009. Prevention of the pulmonary vasoconstrictor effects of HBOC-201 in awake lambs by continuously breathing nitric oxide. *Anesthesiology* 110:113–22

132. Alayash AI. 2004. Oxygen therapeutics: Can we tame haemoglobin? *Nat. Rev. Drug Discov.* 3:152–59
133. Palmer AF. 2006. Molecular volume and HBOC-induced vasoconstriction. *Blood* 108:3231–32
134. Chang TM. 1998. Modified hemoglobin-based blood substitutes: crosslinked, recombinant and encapsulated hemoglobin. *Vox Sang.* 74(Suppl. 2):233–41
135. Day TK. 2003. Current development and use of hemoglobin-based oxygen-carrying (HBOC) solutions. *J. Vet. Emerg. Crit. Care* 13:77–93
136. Tsai AG, Cabrales P, Manjula BN, Acharya SA, Winslow RM, Intaglietta M. 2006. Dissociation of local nitric oxide concentration and vasoconstriction in the presence of cell-free hemoglobin oxygen carriers. *Blood* 108:3603–10
137. Butt OI, Buehler PW, D'Agnillo F. 2011. Blood-brain barrier disruption and oxidative stress in guinea pig after systemic exposure to modified cell-free hemoglobin. *Am. J. Pathol.* 178:1316–28
138. Butt OI, Buehler PW, D'Agnillo F. 2010. Differential induction of renal heme oxygenase and ferritin in ascorbate and nonascorbate producing species transfused with modified cell-free hemoglobin. *Antioxid. Redox Signal.* 12:199–208
139. Rice J, Philbin N, Light R, Arnaud F, Steinbach T, et al. 2008. The effects of decreasing low-molecular weight hemoglobin components of hemoglobin-based oxygen carriers in swine with hemorrhagic shock. *J. Trauma* 64:1240–57
140. Kasper SM, Walter M, Grune F, Bischoff A, Erasmi H, Buzello W. 1996. Effects of a hemoglobin-based oxygen carrier (HBOC-201) on hemodynamics and oxygen transport in patients undergoing preoperative hemodilution for elective abdominal aortic surgery. *Anesth. Analg.* 83:921–27
141. LaMuraglia GM, O'Hara PJ, Baker WH, Naslund TC, Norris EJ, et al. 2000. The reduction of the allogenic transfusion requirement in aortic surgery with a hemoglobin-based solution. *J. Vasc. Surg.* 31:299–308
142. Jahr JS, Mackenzie C, Pearce LB, Pitman A, Greenburg AG. 2008. HBOC-201 as an alternative to blood transfusion: efficacy and safety evaluation in a multicenter phase III trial in elective orthopedic surgery. *J. Trauma* 64:1484–97
143. Freilich D, Pearce LB, Pitman A, Greenburg G, Berzins M, et al. 2009. HBOC-201 vasoactivity in a phase III clinical trial in orthopedic surgery subjects—extrapolation of potential risk for acute trauma trials. *J. Trauma* 66:365–76
144. Sehgal LR, Gould SA, Rosen AL, Sehgal HL, Moss GS. 1984. Polymerized pyridoxylated hemoglobin: a red cell substitute with normal oxygen capacity. *Surgery* 95:433–38
145. Yu B, Shahid M, Egorina EM, Sovershaev MA, Raher MJ, et al. 2010. Endothelial dysfunction enhances vasoconstriction due to scavenging of nitric oxide by a hemoglobin-based oxygen carrier. *Anesthesiology* 112:586–94
146. Handrigan MT, Bentley TB, Oliver JD, Tabaku LS, Burge JR, Atkins JL. 2005. Choice of fluid influences outcome in prolonged hypotensive resuscitation after hemorrhage in awake rats. *Shock* 23:337–43
147. Moore EE, Moore FA, Fabian TC, Bernard AC, Fulda GJ, et al. 2009. Human polymerized hemoglobin for the treatment of hemorrhagic shock when blood is unavailable: the USA multicenter trial. *J. Am. Coll. Surg.* 208:1–13
148. Kipnis K, King NM, Nelson RM. 2006. Trials and errors: barriers to oversight of research conducted under the emergency research consent waiver. *IRB: Ethics Hum. Res.* 28:16–19
149. Adamson JG, Moore C. 1998. Hemolink™, an *o*-raffinose crosslinked hemoglobin-based oxygen carrier. In *Blood Substitutes: Principles, Methods, Products, and Clinical Trials*, ed. TMS Chang, pp. 62–81. Basel, Switz.: Krager Landes Syst.
150. Cheng DC, Mazer CD, Martineau R, Ralph-Edwards A, Karski J, et al. 2004. A phase II dose-response study of hemoglobin raffimer (Hemolink) in elective coronary artery bypass surgery. *J. Thorac. Cardiovasc. Surg.* 127:79–86
151. Greenburg AG, Kim HW. 2004. Use of an oxygen therapeutic as an adjunct to intraoperative autologous donation to reduce transfusion requirements in patients undergoing coronary artery bypass graft surgery. *J. Am. Coll. Surg.* 198:373–85
152. Hill SE, Gottschalk LI, Grichnik K. 2002. Safety and preliminary efficacy of hemoglobin raffimer for patients undergoing coronary artery bypass surgery. *J. Cardiothorac. Vasc. Anesth.* 16:695–702

153. Lieberthal W, Fuhro R, Freedman JE, Toolan G, Loscalzo J, Valeri CR. 1999. *O*-raffinose cross-linking markedly reduces systemic and renal vasoconstrictor effects of unmodified human hemoglobin. *J. Pharmacol. Exp. Ther.* 288:1278–87
154. Matheson B, Kwansa HE, Bucci E, Rebel A, Koehler RC. 2002. Vascular response to infusions of a nonextravasating hemoglobin polymer. *J. Appl. Physiol.* 93:1479–86
155. Bucci E, Kwansa H, Koehler RC, Matheson B. 2007. Development of zero-link polymers of hemoglobin, which do not extravasate and do not induce pressure increases upon infusion. *Artif. Cells Blood Substit. Immobil. Biotechnol.* 35:11–18
156. Jia Y, Alayash AI. 2009. Effects of cross-linking and zero-link polymerization on oxygen transport and redox chemistry of bovine hemoglobin. *Biochim. Biophys. Acta* 1794:1234–42
157. Cabrales P, Zhou Y, Harris DR, Palmer AF. 2010. Tissue oxygenation after exchange transfusion with ultrahigh-molecular-weight tense- and relaxed-state polymerized bovine hemoglobins. *Am. J. Physiol. Heart Circ. Physiol.* 298:H1062–71
158. Baek JH, Zhou Y, Harris DR, Schaer DJ, Palmer AF, Buehler PW. 2012. Down selection of polymerized bovine hemoglobins for use as oxygen releasing therapeutics in a guinea pig model. *Toxicol. Sci. Off. J. Soc. Toxicol.* 127:567–81
159. Palmer AF, Sun G, Harris DR. 2009. The quaternary structure of tetrameric hemoglobin regulates the oxygen affinity of polymerized hemoglobin. *Biotechnol. Prog.* 25:1803–9
160. Buehler PW, Zhou Y, Cabrales P, Jia Y, Sun G, et al. 2010. Synthesis, biophysical properties and pharmacokinetics of ultrahigh molecular weight tense and relaxed state polymerized bovine hemoglobins. *Biomaterials* 31:3723–35
161. Zhou Y, Jia Y, Buehler PW, Chen G, Cabrales P, Palmer AF. 2011. Synthesis, biophysical properties, and oxygenation potential of variable molecular weight glutaraldehyde-polymerized bovine hemoglobins with low and high oxygen affinity. *Biotechnol. Prog.* 27:1172–84