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BLOOD SUBSTITUTES: EVOLUTION FROM NON-CARRYING TO OXYGEN AND GAS CARRYING FLUIDS

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Abstract

The development of oxygen (O_2) carrying blood substitutes has evolved from the goal of replicating blood O_2 transports properties to that of preserving microvascular and organ function, reducing the inherent or potential toxicity of the material used to carry O_2, and treating pathologies initiated by anemia and hypoxia. Furthermore, the emphasis has shifted from blood replacement fluid to "O_2 therapeutics" that restore tissue oxygenation to specific tissues regions. This review covers the different alternatives, potential and limitations of hemoglobin based O_2 carriers (HBOCs) and perfluorocarbon based O_2 carriers (PFCOCs), with emphasis on the physiological conditions disturbed in the situation that they will be used. It describes how concepts learned from plasma expanders without O_2 carrying capacity can be applied to maintain O_2 delivery and summarizes the microvascular responses due to HBOCs and PFCOCs. This review also presents alternative applications of HBOCs and PFCOCs namely: 1) How HBOC O_2 affinity can be engineered to target O_2 delivery to hypoxic tissues; and 2) How the high gas solubility of PFCOCs provides new opportunities for carrying, dissolving and delivering gases with biological activity. It is concluded that current blood substitutes development has amplified their applications horizon by devising therapeutic functions for oxygen carriers requiring limited O_2 delivery capacity restoration. Conversely, full, blood-like O_2 carrying capacity re-establishment awaits control of O_2 carrier toxicity.

Keywords

Plasma expander; oxygen; artificial blood; hemoglobin; perfluorocarbon; gas carriers; artificial organs

In the United States allogeneic RBC transfusion has long been considered an important treatment option for patients suffering from blood loss.[1] However, the recent emergence of infectious agents such as the H1N1 influenza virus and others have the potential of placing the blood supply at risk.[2] Currently, the American Red Cross tests donated blood for hepatitis B and C viruses, human immunodeficiency virus (HIV), human T-cell lymphotropic virus, syphilis, West Nile virus and the agent of Chagas disease.[3-6] As a result, the safety of the blood supply in terms of transfusion transmitted diseases is quite good. However, as new infectious agents emerge, the unit of blood cost increases, since additional screening tests may have to be conducted before blood can be distributed to health care providers. Of greater concern is that donated blood may contain infectious agents yet to be

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identified.[4] Moreover, short- and long-term transfusion-related adverse events are among the costliest contributors to health care expenditures.[7] In addition, there are new concerns regarding the safety of blood transfusions following extended durations of storage (i.e., the storage lesion).[8-9] To further compound the problem, the availability of human blood is even more limited in emergency situations such as wars or natural disasters.[10] Therefore, it has been a long-term goal to develop an efficacious and safe universal blood substitute (i.e., O_2 bridge) for use in transfusion medicine.

Hemoglobin (Hb) is the protein responsible for storage and transport of O_2 and other gaseous ligands in RBCs.[11] Hb is also the precursor for synthesis/formulation of Hb based O_2 carriers (HBOCs) that can be used as RBC substitutes.[12-14] It is not surprising that the first HBOC to be developed as a RBC substitute consisted of stroma-free Hb.[15-16] However, transfusion of acellular Hb leads to several major side-effects.[17-21] In the circulation extracellular tetrameric Hb ($\alpha_2\beta_2$) easily dissociates into two pairs of $\alpha\beta$ dimers[18-19], which are extremely prone to oxidation[21] and enhanced renal excretion.[18, 22-23] The process of Hb oxidation to methemoglobin (metHb) promotes unfolding of the globin chains and releases cytotoxic heme into the circulation, leading to kidney tubule damage and eventual renal failure.[18-19] MetHb can also generate harmful reactive O_2 species (ROS)[17] that can damage cell membranes and oxidize nucleic acids and proteins.[20] Therefore, the presence of extracellular Hb in the circulation can elicit tissue toxicity due to heme release and ROS generation, causing vasoconstriction and systemic hypertension by various mechanisms.[24-27] These include scavenging nitric oxide (NO) generated by endothelial cells, which acts as a vasodilator to the surrounding smooth muscle cells[24-25] and an “autoregulatory” response in which extracellular Hb facilitates O_2 transport in the lumen of the blood vessel over oxygenating the surrounding tissues, thereby eliciting vasoconstriction in order to reduce blood flow.[26-27] Regardless of the exact mechanism for the development of vasoconstriction and systemic hypertension, stroma-free Hb can be modified in order to eliminate or reduce these adverse effects.

Physiological deficits due to blood losses

Historically, blood substitutes have been formulated to address the deficit of blood’s intrinsic oxygen carrying capacity (O_2CC) resulting from the loss of red blood cells (RBCs), however the decrease of circulating RBCs and the associated blood volume are not the only factors affected by blood loss. Studies of the biomechanics of the systemic circulation and the microcirculation have shown that blood fluid mechanical properties are optimized to maintain blood flow and to uniformly perfuse the capillary network.[28] Currently, this can only be accomplished by the transfusion of fresh blood, a commodity whose increasing scarcity drives the development of blood substitutes. In order to design a blood replacement fluid, it is essential to understand the changes in physiological conditions underlying the need for blood transfusion, since this fluid will have to address these changes and restore O_2CC.

Blood loss lowers blood pressure, and most vascular beds initiate reflexes aimed at maintaining pressure via vasoconstriction. The combination of low blood pressure and vasoconstriction reduces the hydrostatic pressure at the arteriolar end of the capillaries. Low capillary pressure affects fluid movement across the capillary membrane (filtration). Therefore, blood losses reduce capillary blood pressure where fluid filters from plasma into the tissues and increases capillary pressure in the region of the capillary where fluid is reabsorbed, causing a net gain of fluid to the plasma. This fluid “autotransfusion” increases the severity of the anemia induced by blood losses. The most apparent deficits associated with blood loss are:

Intrinsic oxygen carrying capacity

The intrinsic O_2CC is the amount of O_2 contained in a volume of blood. It comprises O_2 carried by Hb and that dissolved in plasma. For saturated blood this amounts to 20 ml O_2/dl blood carried by RBCs and 0.2 ml by plasma (at Hct 45%). Blood losses change these proportions, however O_2 carried by plasma never becomes a prominent factor, even in conditions of 100% O_2 inhalation since the partial pressur of O_2 (pO_2) of hemodiluted blood reaching the tissue is much lower due to the O_2 transit losses.

Blood viscosity

The viscosity imparted to blood by blood substitutes is the most overlooked parameters in the design of blood substitutes. This arises in part from the perception embodied in medicine since antiquity[29] that lowering blood viscosity is beneficial, an axiom formerly applied throughout the practice of medicine. Deliberate or un-intended adherence to this concept cause blood substitutes viscosity to have plasma-like viscosity of the order 1.5 cP even when formulated at 14 g Hb/dl. Infusion of a fluid with low viscosity decreases blood viscosity, beyond the effect of reducing the concentration of RBCs. A moderate reduction of blood viscosity can have positive effects due to the increase in flow and perfusion which maintain WSS constant, as the increased flow compensates for the decreased viscosity. However, further lowering Hct is not compensated by a corresponding increase in blood flow because the heart is unable to sustain the increased workload, as the diluted blood delivers less O_2 to the heart muscle.[30] As a consequence WSS decreases, leading to vasoconstriction and reduced blood flow. Therefore, the positive effects of lowering viscosity are limited to Hct levels that maintain heart function, increased blood flow and maintenance of WSS sufficient to prevent the increase of vascular hindrance, a process that can be delayed by increasing plasma viscosity during hemodilution.

Vessel wall shear stress (WSS)

Shear stress is the product of blood viscosity and shear rate determined by the flow velocity profile. It has a principal cardiovascular role since it causes the endothelium to produce NO via mechanotransduction, modulating peripheral vascular resistance and blood flow. Hemorrhage depletes circulating blood volume, lowers blood pressure throughout the circulation including the capillaries, promoting fluid absorption from interstitial spaces, which continues until plasma colloidal osmotic pressure lowers to the new average capillary pressure. This process lowers blood viscosity and reduces the viscous component of vascular resistance.[29] Upon fluid resuscitation from hemorrhagic shock, blood flow increases, although it is not able to restore WSS, due to limitations in the increase in cardiac output and therefore in blood flow velocity. The decrease in WSS lowers mechanotransduction, hinders the production of NO by the endothelium, and prevents normal vascular signaling and regulation[31-33] and the production of vasorelaxants that modulate vessel diameter, which determines the geometric component of vascular resistance and blood flow.

Cell free layer (CFL)

The CFL separates the RBC blood column from the vessel wall, i.e., the endothelial cell membrane. This hydrodynamic layer is generated by axial migration due to resulting from the flexibility of RBCs that cause their displacement toward the center of the flow stream.[34-37] The presence of the CFL contributes to the reduction of flow resistance by decreasing blood viscosity near the vessel wall. Hemodilution decreases blood viscosity and increases the CFL width, further decreasing flow hindrance.[38] In this scenario using low viscosity (1.0 - 1.3 cP) plasma expanders decreases WSS because of the increased CFL width, while plasma viscosity which in normal conditions is in the range of 1.0 to 1.3 cP is mostly unchanged.[38]

NO distribution

The physiological effects due to hemodilution tend to lower the NO concentration in the arteriolar wall, causing vasoconstriction and a pro-inflammatory environment.[39] Vasoconstriction lowers blood flow and WSS, decreasing vascular mechanotransduction, a sequence of events that is aggravated if hemodilution is associated with the presence of acellular Hb or HBOCs in the circulation.[39-40] Hb undergoes several reactions reducing smooth muscle NO levels.[41-42] The extent of the physiological consequences of Hb-associated NO scavenging depends on whether Hb is localized within RBCs or circulates as acellular Hb due to hemolysis or infusion of HBOCs.[41-47] Hb scavenges NO, and effectively block NO bioactivity, resulting in vasoconstriction and transient hypertension.[48-50] In the presence of acellular Hb, NO is quenched through an NO dioxygenation reaction and by the formation of the “dead end” (highly stable and relatively non-reactive) ferrous heme-nitrosyl form.[49, 51-53] The NO dioxygenation reaction involves the efficient reaction of NO with oxygenated hemes to form met (ferric) heme and nitrate.[42, 54-55] Therefore, the NO reactions with Hb will depend on the redox and ligation states of the heme iron modulated by O_2 levels.[49, 53, 56] Scavenging of NO by acellular Hb has been viewed as a major source of HBOC induced toxicity, which has in part limited the development of these materials for clinical use.[49-50, 57] The relevance of the NO dioxygenation reaction as a contributor to HBOC-induced toxicity was compellingly demonstrated through the use of recombinant Hbs engineered to slow down NO dioxygenation reaction rates.[54-55, 58] Implications of HBOCs induced NO scavenging and vasoconstriction will be analyzed for various HBOCs later in this review.

ATP release from erythrocytes

RBCs have been shown to enable the controlled, nonlytic release of adenosine-5′-triphosphate (ATP), not involving cell membrane rupture in response to mechanical stimuli.[59] They release ATP following exposure to mechanical deformation, reduced O_2 tension, or acidosis.[60] These are conditions to which erythrocytes are exposed in the vasculature, e.g., when passing through constricted vessels or vessels in contracting striated muscle.[61] Once in the extracellular medium, extracellular ATP triggers different cellular responses by interacting with receptors on the cell surface, while at the same time its concentration is controlled by the activities of one or more ectonucleotidases.[62] Although intracellular ATP signaling is well known, there is comparatively little information on the mechanism of ATP release. Since the RBC spectrin-actin cytoskeleton appears to control ATP mechanosensitive release,[59] it is likely that blood viscosity is a factor affecting vascular and RBC ATP signaling to maintain perfusion and oxygenation. Significant decreases in Hct as well as WSS could therefore be a factor in lowering ATP bioavailability.

The combination of these deficits causes the decrease in O_2 delivery capacity of the circulation and the initiation of microvascular dysfunction, a condition characterized by the decrease of functional capillary density (FCD). This microvascular parameter measures the circulation’s capacity for maintaining uniform tissue oxygenation and the adequate extraction of metabolic by-products from the tissue. Notably microvascular dysfunction is mostly independent of tissue oxygenation, and is present when Hct is reduced by 50%, which is the nominal reduction of RBC concentration that signals the need to restore O_2 carrying capacity.

Sensing and regulation of the O_2 supply at the local level in the microcirculation has undergone a profound transformation since the finding that RBCs are not only O_2 carriers but also O_2 sensors.[63] One of the mechanisms to sense O_2 concentration by the RBCs is based on the release ATP in conditions of increased shear stress and decreased O_2 tension. Once released from RBCs ATP forms adenosine stimulating the endothelium to produce the

vasodilator NO. ATP is a signaling molecule that can stimulate the ATP-sensitive P2Y-purinergic receptor in the plasma membrane of vascular smooth muscle and endothelial cells. It contributes to blood pressure regulation since the PY2 purinergic receptor modulates the production of NO and therefore mediates vasoconstriction and dilatation.[64-65] Studies in vitro, in experimental models and in humans have shown that RBC-derived NO is a factor in regulating the match between O_2 demand and O_2 supply through the control of vessel diameter. Furthermore, this regulation is present at various Hct levels[66] and extends to other flow regulation processes such as thermoregulation.[67]

Novel HBOC formulations include ATP-adenosine and glutathione (GSH) cross-linked to Hb (ATP-ADO-GSH-Hb), which can delivery O_2 and stimulate erythropoiesis.[68-69] In children with sickle-cell anemia, a single injection of ATP-ADO-GSH-Hb stimulated erythropoiesis.[68] ATP-ADO-GSH-Hb's erythropoietic activity and O_2 carrying capacity can reduce the need for supplementary transfusions. The chemical modification with ATP, adenosine, and GSH does not interfere with Hb O_2 transport capacity, and provides the Hb molecule with properties that counteract the intrinsic toxic effects of Hb, namely vasoconstriction, oxidative stress, and inflammation.[70] In this HBOC, the ATP stabilizes the Hb tetramer and prevents its dimerization, and adenosine allows the creation of Hb oligomers,[70] in addition ATP and adenosine counteract the vasoconstrictive and proinflammatory properties of acellular Hb, and prevents platelet aggregation.[70] Lastly, GSH shields heme from reactive O_2 species (ROS), and introduces a electronegative charge onto the surface of Hb reducing renal excretion, extravasation, and phagocytociss.[70] Studies by Simoni et al. investigated the potential of ATP-ADO-GSH-Hb as an erythropoiesis stimulating agent.[68-70]

Blood rheology and plasma expansion

The viscosity of blood is primarily determined by its exponential dependence on Hct. As blood flows in a vascular network of branching arterial blood vessels, the presence of the CFL becomes an increasingly significant volume compartment. Therefore, the tube Hct in blood vessels progressively decreases from the systemic value, about 45% in men and 42% in women, to about 10% in the capillary circulation.[71] This phenomenon is known as Fahraeus effect, which is due to the motion of RBCs away from the vessel wall in microvessels (and small tubes),[72] which decreases blood effective viscosity near the vessel wall (the Fahraeus-Lindqvist effect).[73] This decreases the Hct near the vessel wall and compacts cells in the vessel core, producing a non-uniform Hct in the microvascular cross-section.[74] Since blood close to the vessel wall supplies side branches, the feeding Hct of these branches has a lower Hct, an effect known as "plasma skimming" that becomes more significant as blood vessel diameter decreases.[74] As a consequence, capillary Hct is low (0 - 20%) and capillary blood viscosity is only slightly higher than plasma viscosity while systemic blood viscosity averages about 4.5 cP. Given the exponential relationship between blood viscosity and Hct, and the dependence of blood effective viscosity on vessel diameter, hemodilution with a fluid of a plasma-like viscosity primarily lowers the effective blood viscosity in large blood vessels. Hemodilution has a lesser effect on blood viscosity in micro-vessels and has practically no effect in capillaries.

Blood viscosity is a determinant of peripheral vascular resistance and therefore cardiac output and blood pressure. The two-phase nature of blood (RBCs and plasma) and RBC deformability and aggregability cause blood to exhibit a varying non-Newtonian viscosity as it passes through the different vascular compartments. These factors also cause blood to be shear-thinning, whereby its viscosity decreases as the shear rate increases.[75] Blood shear thinning properties redistribute shear rate dependent energy expenditure from the bulk of the

flow (RBC core) to the periphery (CFL), increasing WSS. This effect is in part counteracted by the increase in CFL width.[38]

Blood rheology and hemodilution

Blood losses entail hemodilution, since the organism shifts fluids into the circulation from the tissue compartment. Correcting blood loss with a solution further lowers Hct, i.e., hemodilutes the circulation. To date, plasma expander that are used to treat hypovolemia and to reinstate blood volume, have plasma-like viscosity and are Newtonian fluids. An exception is polyethylene glycol (PEG) conjugated albumin (PEG-Alb), presently under development, which when mixed with diluted blood increases its natural shear thinning behavior, i.e., the slope of the dependence of shear stress vs. shear rate increases as the shear rate decreases, according to measurements in a conventional cone and plate viscometer. This leads to blunter velocity profiles, increased WSS and production of NO[76] and lower vascular resistance due to vasodilatation. However, the exact mechanism by which PEG-Alb affects blood viscosity is not presently known.

The PEG-Alb molecule has a large exclusion volume, which significantly increases the colloidal osmotic pressures (COP) of the albumin molecule per se, limiting its plasma concentration and therefore the blood viscosity obtained when introduced in the circulation.[76] There is presently little information on the aggregability induced by PEG-Alb or its intrinsic pseudoplasticity.

Hemodilution with PEG-polymers

PEG-Alb is a remarkable plasma expander that yields close to normal microvascular function even on conditions of extreme hemodilution.[77] The same effect has been obtained with significantly higher viscosity fluids such as dextran 500 kDa and alginates.[78] Both types of plasma expansion provide near optimal resuscitation from hemorrhagic shock induced by the experimental exsanguination of 50% blood volume.[79-80] The principal difference between plasma expanders is that dextran 6% 500 kDa (6 cP) and 1% alginate (7 cP) hemodiluted blood have comparatively high viscosity compared to 4% PEG-Alb (1.8 cP). The high viscosity causes an additional load on heart function that is absent with PEG-Alb.[30] PEG-Alb is a low viscosity plasma expander, that when mixed with blood increases blood flow driven by a constant pressure gradient or decreased the pressure drop under constant flow conditions.[76] Blood diluted with PEG-Alb restores blood's shear thinning behavior at low Hct leading to different hemodynamic effects at various locations within the circulation. In particular the inherent hemodilution associated with its introduction and it is shear thinning endowing properties reduces blood viscosity in the heart, therefore reducing its workload.[30, 81]

The effect of polymerization

Solution viscosity is a direct function of the solute dimension (molecular structure and mass) and concentration, therefore polymerization of globular proteins provides the means for increasing solution viscosity without increasing COP. Polymerizing Hb increases O_2CC of the solution.[82] Hemoglobin polymers of up to about 30 MDa have been synthesized and occur in nature. Natural acellular Hb of terrestrial and marine worms is configured in this manner.[83] Hemoglobin from the marine worm Arenicola marina is being developed as an HBOC[84] and has properties similar to the synthesized zero linked polymeric Hb OxiVita[85] developed by Prof. Bucci's group.[86]

The rheology of hemoglobin vesicles

A different means for introducing Hb in the circulation is to encapsulate it in vesicles (HbV). This technology was developed by the group formerly directed by Prof. Tsuchida[87] and consists in encapsulating Hb in 250 nm diameter phospholipid vesicles coated with PEG. This approach allows high Hb concentrations, making the O_2CC of the PEG-HbV suspension similar to blood. PEG-HbV requires suspension in a solution whose osmolarity, COP and solution viscosity can be adjusted. PEG-HbVs were tested using different plasma expanders including recombinant human serum albumin (rHSA), dextran 70 kDa, modified gelatin 45k Da, and hydroxyethyl starch 140 kDa. The HbV suspension in rHSA solution was nearly Newtonian, while PEG-HbV suspension in hydroxyethyl starch, dextran and gelatin made non-Newtonian suspensions due to PEG-HbV aggregation at a HbV concentration of 16% w/v (Hb = 10 g/dL and lipids 6 g/dL, which corresponds to a Hb volume fraction of about 60% v/v).[88]

The rheological component of hemorrhagic shock resuscitation

Blood is generally transfused to treat hemorrhage when Hct or Hb are reduced by 50%. This approach is undeniably successful in restoring O_2CC; however, it is questionable if this is the sole origin of the resulting improvements. Resuscitation from hemorrhagic shock with non-O_2 carrying fresh blood saturated with carbon monoxide or whose Hb was completely converted to methemoglobin was not different by comparison to fresh blood.[89-90] In this scenario, blood provides resuscitation by restoring blood properties independent of O_2CC, where the most conspicuous effect is the significant increase of blood viscosity. Since the decision to transfuse blood is frequently driven by medical experience, and there is evidence that the intervention provides an acute health benefit, it is possible that increasing blood viscosity of a hemodiluted individual is beneficial per se. This suggests that the transfusion trigger in many instances is a “blood viscosity trigger”. Furthermore, if the principal purpose of a blood transfusion is to restore blood viscosity, this would not be the optimal use of this resource.

Can plasma expansion increase oxygen delivery?

Plasma expanders do not carry O_2 per se, therefore increased tissue O_2 in hemodilution, if it occurs, must depend on effects equivalent to increasing O_2CC. Through the combination of vasodilatation and hemodilution, plasma expanders like PEG-Alb, dextran 500 kDa and alginates induce a condition of supra-perfusion, whereby microvascular flow is significantly increased, in some cases up to 50% above baseline.[77] Oxygen delivery is the resultant of blood flow or convection through the vasculature and diffusion from the vasculature. Increasing blood flow increases convection while diffusion remains comparatively constant, with the net effect that more O_2 arrives to the capillaries.[91] Experimental studies show that supra-perfusion is associated with a significant increase in vessel wall NO concentration.[39, 76]

Interplay between NO levels and O_2 utilization

Endothelial NO synthase can affect O_2 metabolism at the mitochondrial level.[92] Studies in conscious dogs at rest and during exercise show that NO inhibition increases O_2 consumption independently of changes of skeletal muscle blood flow.[93] Positron emission tomography measurements of human skeletal muscle blood flow and uptake shows that NO formation inhibition enhances resting muscle O_2 uptake by 20%. Conversely, cell respiration is inhibited where inducible NO synthase increases NO generation.[94] Angiotensin-converting enzyme inhibitors (enalapril) increases intrarenal O_2 tension by decreasing O_2 consumption through stimulation of NO synthesis.[95] Dietary nitrate supplements NO production independently of NO synthase.[96] Thus, increasing circulating

nitrite increases NO bioavailability.[97] Nitrite and nitrate are reduced in physiological conditions to NO. Dietary nitrates have been reported to reduce O_2 consumption during maximal arm and leg exercise.[98] Therefore, increasing NO concentration should lower O_2 metabolism and reduce O_2 demand, which can be equivalent to increasing O_2CC.

Oxygen carrying plasma expanders

Increasing O_2CC of a volume replacement fluid is in principle as simple as substituting albumin with Hb in a human serum albumin-based plasma expander, since albumin and Hb are globular proteins of virtually identical solution properties. This can be accomplished across species for Hb due to the lack of Hb antigens, which is not the case for albumin precluding the use of the bovine albumin source in humans. However, albumin and Hb are structurally very different molecules, since albumin is a folded protein, while Hb is a tetramer with 4 identical subunits that can relatively easily dis-aggregate in solution, creating problems for kidney function. These limitations were recognized early on, leading to various approaches to stabilize the tetramer by crosslinking its subunits, or polymerizing the Hb molecule.[99-101]

The pressor effect of hemoglobin solutions

Regardless of the source and method of stabilization of molecular Hb, its introduction into the circulation causes hypertension, assumed to be one of the causes for the negative outcome of clinical trials with HBOCs. However, it is questionable whether this is directly and uniquely due to the pressor effect. It should be noted that hemorrhage and other forms of shock are treated with vasopressors[102], and there are reports proposing that the Hb pressor effect could be beneficial[103-104] in the management of hemorrhage, providing immediate treatment independently of other pathologies.[105] Systemic pressure is routinely increased during resuscitation by giving fluid and using vasopressor agents when fluid alone is not sufficient. However, the effect of vasopressors does not necessarily transmit central pressure to the periphery, and can lead to necrosis and multi organ failure as seen clinically when vasopressors dosage is exceeded using noradrenaline or arginine-vasopressin.[102, 106]

The principal finding in studies of hemorrhage resuscitation with a viscogenic plasma expander (Hextend, Hospira, IL) PEG-Alb and the vasoactive polymerized bovine Hb HBOC-301 (OPK Biotech, Cambridge, MA) was that HBOC-301 transfusion in a hamster window chamber model of hemodilution prior to a major bleed yields 100% survival after one hour.[104] This result is identical to that obtained using PEG-Alb previously reported[107] to also yield the same final blood pressure and pH. The same procedure carried out with Hextend reduces survival rate. FCD was the principal microhemodynamic difference, while O_2 distribution evidenced anoxia in all groups. Thus, as in previous studies[108], outcome appears to be determined by FCD rather than tissue O_2[109-110], a direct consequence of maintaining normal capillary hydraulic pressure, requiring the transmission of central blood pressure, which is the rationale for restoring central blood pressure in resuscitation.[110-111]

Hypertension due to using cross linked Hb solutions has been associated with focal necrosis[112] as a result of vasoconstriction due to NO-scavenging.[113] Notably, there is no evidence in the literature that the use of vasopressors in the treatment of hemorrhagic shock leads to micronecrosis. Studies show that vasoconstriction is effective in transmitting central pressure to the capillary circulation, suggesting that pressure elevation by vasoconstriction “leaks through” to the capillary circulation, providing a beneficial effect.

Microhemodynamic effects of vasoactive hemoglobin solutions

The effect of HBOCs vasoactivity in hemorrhage resuscitation was also studied as a function of Hb concentration administering HBOC-301, at 4 and 13 g/dL concentration by weight,

and comparing this with an albumin solution of the same COP. It was found that even though this HBOC is vasoactive, it improved survival when used in small dosage in comparison to a non-O_2 carrying colloidal fluid such as albumin used at higher dosages.[114-115] The balance between vasoconstriction and increased O_2CC was also found to have an optimal value when HBOC-301 was used to remedy extreme hemodilution, providing improved FCD at a specific plasma Hb concentration, FCD being lower at higher and lower dosages.[116] The extent of blood exchange was also found to be a factor in determining tissue conditions. During hemodilution, studies where 80% of blood was substituted with vasoactive Hb, tissue oxygenation was significantly impaired due to an apparent increase of vessel wall O_2 metabolism.[117]

These results show that the effects of vasoactivity are strongly related to free Hb concentration in the circulation. The range of free Hb concentration where vasoactive HBOCs are beneficial is low; therefore, the gain in O_2CC is probably not physiologically relevant. These findings contribute to explain the lack of side effects noted with the use of PEG-Hb based fluid replacement fluids.[118-119] They scavenge NO being potential vasoconstrictors; however, their high COP precludes their achieving high blood concentrations. They cause modest increases of O_2 transport while fully exerting their beneficial effects as plasma expanders, a property that they share with PEG-Alb.[120]

Hemoglobin polymerization

Glutaraldehyde has been widely employed to non-specifically cross-link/polymerize Hb.[121-124] Two polymerized Hbs (PolyHb) have undergone phase III clinical trials. Hemopure® (OPK Biotech, Cambridge, MA) consists of polymerized bovine Hb (bHb) with a P_{50} (i.e. O_2 affinity) of 38 mm Hg and average MW of 250 kDa with < 2% $\alpha_2\beta_2$. [14, 125-127] In contrast, PolyHeme®(Northfield Laboratories Inc., Evanston, IL) consists of a pyridoxylated polymerized human Hb with a P_{50} of 28 - 30 mm Hg, and average MW of 150 kDa with < 1% $\alpha_2\beta_2$. [12, 128-129] These PolyHbs were commercially developed, however hypertension and safety and toxicity issues attributed to vasoactivity and oxidative events[130] were critical impediments for their further clinical use in the U.S.[131-132]

Unmodified Hb interactions with the vascular endothelium or sub-endothelium may include NO scavenging, increased facilitated diffusion of O_2 to surrounding tissues, and oxidative reactions.[133] The vasoconstriction and hypertension elicited by these small-sized PolyHbs indicates that these materials are capable of extravasating into the interstitial space scavenging NO before it can reach the smooth muscle cells, or facilitating O_2 diffusion to the blood vessel wall due to their small size.[125, 129] This has led to the development of larger sized PolyHbs that limit these effects. Polymerized bovine Hb (bHb) in the low O_2 affinity state (low P_{50} PolybHb) with the highest cross-link density (50:1, i.e., molar ratio of glutaraldehyde to bHb) yielded no vasoconstriction and was least hypertensive when compared to other PolybHb with lower molar ratios of glutaraldehyde to bHb.[101] Vasoconstriction and blood pressure were inversely proportional to the absolute MW of low P50 PolybHb (L-PolybHb) solutions. Additionally, the ability to engineer the O_2 affinity of PolybHbs allowed for targeted and regulated O_2 delivery to tissues in the microcirculation under extreme hemodilution (i.e., anemic conditions).[134] Specifically, high MW L-PolybHb improved tissue oxygenation versus high MW high O_2 affinity PolybHb (H-PolybHb).[134] In another study, high MW L-PolybHb exhibited longer circulatory half-life and reduced oxidation *in vivo* versus high MW H-PolybHb.[100]

In summary, engineering PolyHbs with tunable O_2 affinity can regulate the extent of vasoconstriction, hypertension, tissue oxygenation and circulatory half-life. Yu et al. showed that lowering the $\alpha_2\beta_2$ content in PolybHb solutions reduced the hypertensive effect

observed *in vivo.*[135] This result is supported by a recent study showing that reducing the $\alpha_2\beta_2$ content of PolybHb solutions from 31% (Oxyglobin®, OPK Biotech) to 2% (Hemopure®, OPK Biotech) significantly reduced the hypertensive effect observed *in vivo.*[136] It is important to note that Oxyglobin® is chemically identical to Hemopure®, except that the latter has undergone extensive purification to remove the majority of $\alpha_2\beta_2$ from solution. Therefore, in light of this wide body of literature, synthesis/formulation of PolybHbs with large MWs and no $\alpha_2\beta_2$ should enhance Hb compartmentalization within the vascular space, extend retention times and limit vasoconstriction/hypertension as well as tissue oxidative injury. This novel but simple design addresses two potential mechanisms for the development of vasoconstriction and hypertension upon administration of PolyHbs and may optimize circulation times for therapeutic applications of extended duration.

Current studies lead to a new strategy for developing RBC substitutes based on molecular engineering the appropriate plasma expander properties into the HBOC.[110, 137-141] An optimal O_2 carrier plasma expander should possess: high molecular volume (i.e., high MW) compared to $\alpha_2\beta_2$ (i.e., > 64 kDa) and high viscosity compared to plasma (i.e., >1.2 cP, however it should not increase blood viscosity above baseline after infusion).[142-143] As previously mentioned, the first property will prevent extravasation of the PolyHb through the blood vessel wall and will therefore limit/prevent vasoconstriction, systemic hypertension and oxidative tissue toxicity. Interestingly, the second property of PolyHb solutions elicits mechanotransduction in the endothelium, generating NO and consequently dilating blood vessels.

ATP releasing HBOCs

Linking ATP with Hb provides a means for simultaneously carrying and delivering O_2 while counteracting the vasoconstrictive effects of Hb.[70] Exploitation of this regulatory mechanism is the basis the HBOC develop by HemoBioTech, Inc. (Dallas, TX) using the method of “pharmacologic cross-linking” developed by Simoni and collaborators at Texas Tech University.[70] This material uses bovine Hb cross-linked intramolecularly with ATP and inter-molecularly with adenosine, and is conjugated with reduced glutathione (GSH). This configuration is proposed to simultaneously regulate blood vessel tone and counteract vasoconstriction and Hb pro-inflammatory effects, while GSH prevents Hb extravasation shielding heme from NO and ROS.[70] This material is being commercially developed and has entered the regulatory process in the US. This HBOC presents a combination of biochemical features that address the toxicity issues found in many Hb based materials. It remains to be established what is its maximal and most effective dose and plasma expansion properties.

Large Hb olygomers

Circulatory retention time and the hypertensive effect of acellular Hb appear to be a direct function of the molecular dimension of the modified Hb molecule.[48] Very large O_2 transporting HBOC molecules are presented in organisms such crustaceans and worms whose Hb is not contained within a cell.[144] This configuration can be obtained by oligomerization of Hb, an approach that was used by Bucci and colleagues.[86] Their intra-molecularly cross-linked bovine Hb is obtained by means of a chemistry referred to as “zero length cross linking” that yields a low O_2 affinity Hb polymer with 36 nm hydrodynamic radius and average molecular weight 17 MDa.[86]

This large “super-polymeric” Hb called “zero link Hb”, is commercialized by OXYVITA, Inc. (New Windsor, NY), with the name of OxyVita. Its large molecular dimensions are accompanied by increased solution viscosity and the attenuation of Hb’s vasoconstrictive activity and extravasation.[86] This formulation has also shown to be highly resistant to

dissociation by comparison to natural acellular polymeric Hbs found in the terrestrial and marine organisms.[85] Furthermore, OxyVita has a comparatively high heme stability.[145] Preclinical studies of the regulation of cerebral blood flow by Koheler et al. show that there are no adverse effects due to the substitution of blood with OxyVita.[146]

Naturally occurring large Hb polymers

The circulatory system of the marine invertebrate *Arenicola marina* transports and stores O_2 by means of a large Hb polymer consisting of globin and non-globin linker chain complexes with total molecular weight 3.6 MDa and large O_2 binding capacity.[147] This Hb is harvested and used to produce the O_2 carrying therapeutic HEMOXYCarrier® by Hemarina S.A., Morlaix, France.[148] Its NO binding rate is somewhat lower than human Hb while CO binding rate is somewhat greater.[84] Evaluation of HEMOXYCarrier® in rodent preparations showed that administration of 40 mg/mL Hb solution in saline caused a transient increase of blood pressure not statistically different from that due to administering saline.[84] This HBOC is reported to be stable over a wide range conditions (ionic compositions, osmolarities, and oxidative environments) and to have natural antioxidant properties in the presence of natural superoxide dismutase like enzymes.[149]

Tempol conjugated hemoglobin

Nitroxides limit the formation of toxic hydroxyl radicals and are effective in neutralizing the effects of ROS in mammalian tissues.[150] An extensively studied nitroxide is Tempol (4-Hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl) which can be administered intravenously reducing blood pressure in hypertensive rodent models and correcting for the effects of endothelial dysfunction.[150-151] Tempol is a free radical scavenger that can treat the toxicity due to excessive superoxide production and NO depletion arising from the presence of acellular Hb or the infusion of HBOCs.[152] SynZyme Technologies LLC (Irvine, CA) has introduced polynitroxylation of αα-fumaryl Hb as an approach to attenuate the vasoconstrictive activity of acellular Hb, exploiting its antioxidant properties.[153] Their multifunctional product polynitroxylated pegylated Hb (PNPH) transports O_2, has antioxidant properties and plasma expansion (hyper-colloid) properties due to pegylation.[154] Preclinical data shows that PNPH also has neurovascular protective properties.[155] PNPH should have a low or non-existent toxicity profile, however it combines three materials that at present have substantial costs, namely PEG, bovine Hb and tempol, therefore its use as blood substitute for transfusion medicine could be limited. Conversely it should be successful as an O_2 therapeutic.

Pegylation of hemoglobin

Conjugation of Hb with PEG (PEGylation) pioneered by Enzon Inc. (Piscataway Township, NJ) introduced a fundamentally new approach for dealing with some of the shortcomings of free Hb in plasma.[156] In the original formulation, bovine Hb was conjugated to 10 copies of 5 kDa PEG by means of a urethane linkage (PEG-isocyanate), yielding a molecule with a very large hydrodynamic volume. As a consequence, COP and viscosity were significantly increased relative to the unPEGylated molecule, with a significantly reduced pressor effect. Ajinomoto Inc. (Tokyo, Japan) developed a human PEG Hb which is pyridoxylated, oligomerized and surface decorated with 3 kDa PEG using an isopeptide linkage. Its P50 was 20 mmHg and it showed a transient hypertension upon transfusion.[157]

Sangart Inc. (San Diego, Ca) developed the product Hemospan™ using 2-iminothiolation based extension arm facilitated (EAF) PEGylation formulated as a 4 gm% solution using a propyl maleimide linkage.[158] This PEGylated Hb, currently called MP4OX, was extensively studied in rodents[118] and large animals[159], and is now in clinical trials in Europe.[160] Its plasma expansion characteristics are similar to those obtained with high viscosity plasma

expanders, even though its viscosity, similar to that of PEG-Alb, is considerably lower.[161] Extensive microcirculatory studies have shown that PEG-Hb maintains and at times improves microvascular function when used to restore blood volume after blood losses and to preserve volume during hemodilution, a result attributed to PEG-Hb optimal plasma expansion properties and high O_2 affinity.

The viscosity, molecular size and COP of PEG-Hb are similar to those of PEG-Alb, since Hb and albumin have virtually the same molecular weight and are both globular proteins. When PEG-Alb or PEG-Hb are mixed with blood they yield similar rheological properties. However, PEG-Hb lowers perivascular NO due to NO scavenging.[40] The high COP of PEG-Hb prevents achieving blood concentrations that are sufficiently high to sustain the O_2 metabolic demand of mammalian organisms. However, its high O_2 affinity, low viscogenicity, and supra-plasma expansion due to the low plasma concentration results in targeting O_2 delivery to ischemic tissues.[162] PEG-Hb scavenges NO, but does not cause vasoconstriction or hypertension.[40, 163] This result is probably due the low concentration that PEG-Hb achieves in the circulation, since its high COP pulls fluid from the interstitial space decreasing the concentration in plasma. In addition, PEG-Hb compensates for the NO scavenged by increasing the generation of NO due to increased WSS and its enhanced nitrite to NO reductase.[164]

NO generation from nitrite by HBOCs

Nitrite may be an important reagent in the production of bioactive NO via Hb in RBCs or from acellular Hb in the circulation. A central role for nitrite in NO production from Hb emerged when it was shown that deoxy Hb can catalyze the conversion of nitrite to NO though a nitrite reductase reaction.[47] In this reaction, penta-coordinate ferrous heme reacts with nitrite to produce NO and ferric heme.[165] Furthermore, the rate of the reaction is allosterically controlled.[166] Studies on chemically modified Hbs[167-168], sol-gel encapsulated Hbs[169], and Hb dimers bound to haptoglobin[170] all indicate that R state deoxy hemes manifest at a faster initial rate by a factor of approximately 10 over the corresponding T state deoxy hemes.[170-171] Nitrite reduction by Hb reaction is determined by a balance between the two quaternary structures of the Hb tetramer, Hb in the oxygenated and deoxygenated conformation. The redox potential of oxygenated Hb favors nitrite reductase reaction, while deoxygenated Hb provided the unligated heme sites necessary for nitrite binding, supporting the role for nitrite reductase by Hb as a sensor and effector of hypoxic vasodilation.[164] This allosterically responsive behavior links this reaction with hypoxic vasodilation. As part of the mechanism, a half-oxygenated R state Hb would have maximum NO generating efficacy since it represents a compromise between accessible reactive deoxy heme sites and the higher reactivity associated with the R state: Too much deoxygenation results in T state formation and too much oxygenation results in too few sites available for the reaction with nitrite.[166] The importance of this reaction is also apparent from HBOC studies. Hbs with the highest rates of nitrite reductase typically show the lowest levels of induced vasoconstriction due to significantly reversed NO inhibition.[166] Although NO is a potent vasodilator that produces significant effects at low concentrations, acellular Hb with high nitrite reductase may still causes vasoconstriction since it is difficult for NO to escape from Hb.

NO and NO precursors have been used to compensate for the NO consumed by HBOCs and to reduce vasoconstriction, including nitroglycerin (NTG). NTG was administered during fluid resuscitation from hemorrhagic shock using polymerized Hb, to attenuate the hypertensive, vasoconstrictive and toxic effects of acellular Hb.[172] NTG produces vasodilatation being a weak NO donor (an organic nitrates that require a three-electron reduction to be converted into NO).[173] NTG did not eliminate vasoconstriction; however, it

elevated acellular MetHb levels, reducing O_2 delivery to tissues. Similarly, when sodium nitrite was used in fluid resuscitation from hemorrhagic shock with polymerized Hb vasoconstriction was attenuated only with high doses of nitrite.[174] The nitrite dose effective in preventing hypertension, increased MetHb levels, reduced platelet function and increased pulmonary complications (edema and congestion).[174-175]

Pure NO inhalation was used to treat NO depletion.[176-177] Inhaled NO prevented pulmonary and systemic hypertension induced by polymerized Hb.[176] However, inhaled NO reacts quickly with Hb (NO dioxygenation reaction) in the lung and increases MetHb levels without restoring the NO bioavailability to peripheral tissues. The largest family of NO donors currently in use is NONOates, also known as diazeniumdiolates. NONOates decomposition rate depends on pH, and temperature.[178-179] NONOates have been used to mitigate the vascular inflammation induced by plasma Hb during malaria infection.[180] NONOates have limited application to control HBOCs induced NO depletion, due to short half-life and increased formation of MetHb.

Alternatives to prevent vasoconstriction induced by HBOCs include adjunct therapy with Sildenafil, a phosphodiesterase type 5 inhibitor; however, it partially prevented some of the hemodynamic changes induced by acellular Hb.[181] Lastly, NO releasing nanoparticles (NOnps) have been used to restore NO bioavailability after infusion of polymerized Hb.[182] The slow release of NO from circulating NOnps, allows small amounts of NO to diffuse abluminally, thus reducing the effects of NO scavenging and limiting MetHb formation.[33, 182-183]

Conclusions from the use of these various approaches to supplement NO in the presence of acellular Hb in the circulation are: 1) NO and NO precursors react with Hb reducing O_2 capacity and producing MetHb; 2) NO supplementation has to be systemic; 3) A combination of NO supplementation and enhanced sensitivity to NO with phosphodiesterase inhibitors may reduce the amount of NO and MetHb formation while counteracting acellular Hb vasoconstriction.

Transport of NO in the form of S-nitrosoHb of by HBOCs

The potential role of Hb as a source of bioactive NO started with the proposal that Hb could transport and deliver NO in an allosterically controlled manner through the formation of S-nitrosoHb (SNO-Hb).[184] SNO-Hb is a derivative of Hb in which the reactive thiols associated with the two β93 Cys undergo S-nitrosation.[185-187] Both the formation of the β93 Cys SNOs and the efficacy for the transfer or release of these SNO associated NOs were proposed to be sensitive to the quaternary structure of the Hb tetramer.[188] Although SNO-Hb has been observed *in vivo*, its role remains controversial. The ability of PEGylated Hbs without the reactive thiols to function as vasodilators indicates that SNO-Hb formation is not a requirement for this activity.[164, 189] Similarly, there are studies showing that transport of NO bioactivity by RBCs, including hypoxic vasodilation, does not require SNO-Hb participation.[190-192] Thus SNO-Hb may function to facilitate RBC-generated NO bioactivity, but it is not essential.[96, 193] Since SNO-containing molecules are likely to be the basis for the long lived transportable NO bioactivity, the more intriguing and as yet unanswered question is through what reactions can Hb create reactive species capable of S-nitrosation of reactive thiols on either Hb or other thiol-containing peptides. HBOCs can stabilize the effects of NO. The release of NO, or its metabolites, from the SNO-Hb was facilitated by addition of GSH, which aids in the decomposition of S-nitrosothiols.[194] SNO-Hb significantly reduced, but did not eliminate microvascular damage. Acellular Hb derivates based on S-nitrosylated PEG-modified Hb (SNO-PEG-Hb), deliver O_2 and NO. SNO-PEG-Hb modification increase Hb molecular mass, and when administrated to

anesthetized rats caused a hypertensive reaction, while SNO-Hb and SNO-PEG-Hb did not raise blood pressure.[195] The mayor limitation of SNO-Hb formulation is the limited stability of the compounds. The SNOHb mechanism is intriguing and thought provoking. Several reports have already generated useful discussions and will stimulate further experimentation and possibly clinical trials, benefiting transfusion medicine and leading to better care for patients.[196]

Pefluorocarbon based oxygen carriers (PFCOCs)

Perfluorocarbons (PFCs) are chemically and biologically inert, and can dissolve large amounts of O_2 and other gases. However, PFCs have low water and lipid solubility. Their gas solubility reflects the very low intermolecular interactions (fluorine's low polarizability translates into low van der Waals forces) within the PFC. The basic difference between O_2 transport by PFCs and Hb is that PFCs dissolve, whereas the Hb bind O_2. In the case of Hb, a strong bond is established between O_2 and the heme. In the case of PFCs, there is only a physical weak equilibrium between concentration and solubility. The difference between Hb and PFC O_2 transport is clearly shown by the differences in profiles of the O_2 content curves as a function of pO_2, i.e., sigmoidal for Hb vs. linear for PFCOC (**Figure 2**). With PFCOCs, there is no saturation and no possibility for chemical binding, and as O_2 is released, carbon dioxide (CO_2) is absorbed. Oxygen dissolved in PFCOC is immediately available to tissues; furthermore, dissolution and release to tissues can increase when temperature decreases.

PFCs are hydrophobic and must be emulsified for intravenous use. It is currently feasible to produce stable PFCs emulsions with particles of median diameter < 0.2 μm.[197] PFCs, as opposed to Hb, are among the most inert organic materials. PFCs were initially developed for handling extremely corrosive uranium fluorides. PFCOC are not subject to oxidation, and there is no indication that any sort of chemical modification occurs under conditions of processing, storage, and use. PFCOC can typically be heated to 300 °C and higher for several days without detectable changes[198]. Therefore, appropriately formulated PFCOCs can be terminally heat-sterilized.[197, 199]

PFCs are a synthetic material available in bulk at modest costs. Therefore, PFCOC could in principle lead to a convenient, largely available, economic, pathogen-free and storable O_2 carrier. However, emulsification of PFCs for parenteral administration requires phospholipids. PFCs droplets size is less than 0.5 micrometers and is coated with a surfactant that serves as emulsifier and stabilizes the suspension. PFCs emulsions are produced at concentrations ranging from 20% to 120% weight/volume (PFCs specific gravity is ~2). Osmolarity of the suspending media is independent of PFCOC concentration and is adjusted by the addition of tonicity agents. Principal challenges in the development of infusible PFCOC include: i) selecting the appropriate PFC (easily eliminable, highly pure and easy to emulsify); ii) preparing stable emulsions (small-sized, heat-sterilizable and well tolerated surfactant); and iii) counteracting molecular diffusion, responsible for particle size growth over time.[200-201]

HBOCs carry O_2 via a reversible chemical reaction between Hb and O_2, while PFCOs carry O_2 by dissolving the O_2, thus PFOCs O_2CC is limited by the O_2 concentration.[202] PFCOC emulsion's O_2CC is about twice that of plasma at room air, and about 10 times less than blood Hb.[203] The limited O_2CC of PFCOCs emulsion can be overcome by increasing the fraction of inspired O_2 (FiO_2).[202] Effects due to superposition of hyperoxia on the changes of circulating fluid composition are additional variables in the analysis of the effectiveness of PFCOCs.[202-203]

Few PFCs are acceptable for parenteral use because of their slow excretion and emulsion stability. It has been determined that *in vivo* PFCs excretion is mostly determined by their MW, rapid excretion corresponding to the lower MWs, while emulsion stability requires PFCs with higher MWs, conditions that cannot be satisfied simultaneously. Additionally, vapor pressure, which also depends on MW, is an important parameter, which can favor retention of air in the alveoli, resulting in increased pulmonary residual volume (also known as pulmonary gas trapping). To avoid this phenomenon, the vapor pressure of the final PFCOC phase at body temperature should not exceed about 10 mmHg.[204-206] The interaction of PFCOC with some colloids (dextrans and gelatins) clinically used as plasma expanders triggers the activation of leukocytes during hemodilution.[207] This study also reported that PFCOC has a minor interaction with hydroxyl ethyl starch (HES), but it did not impair microvascular perfusion.[207]

Oxygen transport with PFCOCs

Loading and releasing O_2 and others gases from PFCOCs to tissues is not subordinated to any change in conformation and does not require the assistance of an allosteric effector. In the case of O_2, the van der Waals interactions between O_2 and PFCOC molecules are an order of magnitude lower than Hb and O_2 reactions, resulting in higher extraction rates and ratios.[208] In normal conditions with central arterial pO_2 of 100 mmHg and venous pO_2 of 35 mmHg, PFCOC emulsions can release 65% of O_2, compared to about 30% for Hb in the RBCs. Oxygen release from PFCOCs is effective at any physiologically relevant partial pressure, rendering a cooperativity-like effect unnecessary. Likewise, O_2 release by PFCOCs is not dependent on pH and is not adversely affected by temperature.[209] Since PFCOCs undergo no oxidation or other modification over time, their O_2 uptake and release characteristics are not affected by storage or during circulation. Introducing a PFCOC emulsion into the circulation is akin to increasing the O_2 solubility of blood's plasma compartment. When Hb and a PFCOC are present in the circulation simultaneously, the PFC will always release its O_2 first, thus conserving the Hb bound O_2 until it is released to the hypoxic tissues.[210] A valuable consequence of PFCOCs, following Henry's law, is that the O_2 content of a PFCOC emulsion can be increased several fold by increasing FiO_2, which is simple to do in a rescue vehicle and critical care or surgical settings.

Oxygen diffusion from the RBCs into tissues is driven by the blood/tissue pO_2 gradient.[211] Thus, the rates of both oxygenation and deoxygenation of Hb solutions are limited by diffusion and governed by the O_2 gradient between internal and external spaces.[212] Diffusion should be facilitated when the RBC membrane is absent and in the presence of numerous small size (as compared to RBCs) highly mobile O_2 reservoirs. The CFL between the RBC column and the endothelium is particularly large during anemic or in hemodiluted states. Cell free Hb molecules, Hb-loaded liposomes and PFCOC droplet fill the CFL in large numbers, increase O_2 content and potentially facilitate O_2 diffusion by providing numerous "stepping stones" or dynamic chains of particles over which O_2 can travel. Much more numerous than RBCs, such particles also offer an area several orders of magnitude larger for gas exchange. A near-wall excess of the smaller particles is likely to develop in smaller vessels, as the shear rate gradient creates a force which counteracts dispersion forces and tends to move elastic RBCs away from the vessel wall.

The large O_2 gradients caused at high FiO_2 associated with the use of PFCOCs provide a strong driving force for O_2 diffusion from the PFCOC droplets to the tissues. The movement of emulsion microdroplets in the blood stream was proposed to create dynamic chains of particles; hence, channels which would help transfer O_2 from the RBCs to tissues.[206] PFCs deliver O_2 due to their physical characteristics and the convective delivery to tissues. By reason of their small particle size, PFC emulsions penetrate collateral capillaries of an

ischemic microcirculation, both supplying oxygenation and possibly restoring flexibility of acidotically stiffened erythrocytes by reinstituting aerobic metabolism. This theory represents a new concept in O_2 delivery and may define a new class of therapeutic agents that can improve tissue O_2 supply in pathological states involving low O_2 delivery and ischemia.[213] The rationalization that small PFCOC droplets (< 0.2 μm diameter) augmented O_2 delivery because they are able to pass through narrow microvascular channels by comparison with large RBCs (7 - 8 μm diameter) has been discarded.[214] Infusion of PFCOC emulsion during partial ischemia did not have an effect on oxygenation, and resulted in trapping of the droplets in ischemic tissue, which increased tissue damage.[214-215] The study of systemic and microvascular O_2 exchange proposed that PFCOCs and increased FiO_2 increases O_2 delivery to the tissue; however, the remnant Hb delivers most of the O_2 since PFCOCs allow RBCs to remain partially oxygenated when they arrive to the tissue.[210]

PFCOC O_2 transport capacity was investigated in the microcirculation hamster window chamber model during extreme hemodilution.[210] Pentaspan (10%, B. Brown, Medical, Irvine, CA, HES, 200 kDa MW) was used as a plasma expander to reduce Hct to 18% by two isovolemic hemodilution steps. A third step reduced the Hct to 11% and was completed with either HES or Oxycyte™ (Synthetic Blood International, Inc., Costa Mesa, CA). Comparisons of HES only hemodiluted animals vs. animals that received 4.2 g/kg of emulsion were made at normoxia ($FiO_2 = 0.21$) and hyperoxia ($FiO_2 = 1.0$). Systemic and microvascular O_2 delivery for PFCOC was 25% (normoxia) and 400% (hyperoxia) higher than for HES. The combination of PFCOCs and hyperoxic ventilation delivered O_2 to the tissue without causing vasoconstriction or impairing microvascular perfusion. Positive acid-base balance, restoration of mean arterial pressure and cardiac output suggested correspondence between microvascular and systemic events. PFCOC and increased FiO_2 increased systemic O_2 delivery and extraction when compared to a plasma expander due to the higher plasma O_2 content when PFCOC is present. In the microcirculation, O_2 delivery was also increased by PFCOC, although O_2 was mostly released from RBC Hb.[210]

Transport of other gases with PFCOC

The capacity for PFCOCs to dissolve nitrogen (and air) may find applications in the treatment of decompression sickness and for protection from neurologic damage caused by air microemboli during cardiopulmonary bypass surgery.[216-217] The solubility of xenon in PFCOCs can be exploited for magnetic resonance imaging (MRI). Both Hb and PFCOCs transport NO but by different mechanisms, which change the availability of the gas.[218-219] During cardiopulmonary bypass (CPB) volatile anesthetics can be added to the oxygenator to provide anesthesia regulated systemic vascular resistance and reduce hormonal responses to CPB. The rate of wash-in and wash-out of volatile anesthetics via oxygenators depends on their solubility in blood. Two important factors affect the solubility of volatile anesthetics: hypothermia increases solubility and crystalloid hemodilution decreases it. PFCOC unique physicochemical characteristics, chemically inert with high solubility, provide the ideal media to dissolve larger quantities of gases. Volatile anesthetics also have a higher solubility in perfluorodecalin, the main component of Fluosol™ (Alpha Therapeutic Corp., Los Angeles, CA), a first-generation emulsion.[220] Fluosol is the only PFCOC approved to date by the U.S. Food and Drug Administration (FDA) and regulatory agencies in 8 other countries.[197] Fluosol was not intended to reduce or replace allogenic blood transfusions. Fluosol which was approved for use during cardiac angioplasty increased mycoardial oxygenation, prevented ST segment elevation, and preserved the ejection fraction.[221] Fluosol was used for 3 years (1989 to 1992) in more than 40,000 patients and was discontinued due to problems with emulsion stability arising from rewarming the frozen suspension prior to infusion.

Transport of carbon dioxide

Intravascular CO_2 transport relies on several mechanisms, including physical dissolution in plasma, carbonic anhydrase induced transformation into bicarbonate and chemical binding consequent to reaction with the Hb. About 25% of total CO_2 is transported by RBCs, which affects the Bohr Effect. As CO_2 affects pH and respiratory acid-base regulation the high solubility of CO_2 PFCOCs (typically 3 to 5 times higher than for O_2), infusion of PFCOCs may affect the amounts of CO_2 transported by red cells and plasma, reduce carbonic acid levels and affect blood O_2 transport. Clinical and experimental result have shown a tendency to faster recovery of acid balance when PFCOCs are in circulation.[210, 222-224]

Volatile anesthetics transport by PFCOCs

The question therefore arises whether clinically relevant volumes of PFCOC might significantly increase the blood solubility of volatile anesthetics. Studies on the solubility of desflurane, sevoflurane, and isoflurane were performed using human blood *in vitro* mixed with clinical concentrations of perflubron based PFCOC emulsion, Oxygent™ (Alliance Pharmaceutical Corp., San Diego, CA) which has 3 times greater concentration than Fluosol (30% vs. 10% by volume; 60% vs. 20% by weight). At PFCOC concentrations equivalent to *in vivo* doses of 1.8 to 5.4 g/kg, the amount of sevoflurane carried by blood increased by a factor of 2.6, whereas desflurane did not increase (0.9 times).[225] However, their study concluded that this finding lacked apparent clinical implications.[225]

Nitric oxide transport by PFCOCs

Nitric oxide (NO) is enzymatically produced by various types of cells and is a central mediator in the cardiovascular system. As a free radical, NO is highly unstable *in vivo*. Its primary targets include heme proteins such as guanylyl cyclase and free radical species. NO is readily oxidized by O_2 in an aqueous media and mainly converted into nitrite anion. The partition coefficient of NO for PFCOC emulsion is defined as the ratio of the equilibrium concentrations between the aqueous and PFCOC phases. Partition coefficient of NO for Perftoran was found to be ~200.[226] The sequestration of NO by PFCOC emulsion, without the presence of O_2 to account for oxidation, was significant. Thus, the partition coefficient indicates that PFCOC micelle can increase micellar concentrations of NO to a higher level than an aqueous solution, and readily collect NO from regions with overproduction.

The reaction of NO and O_2 with thiols results in thiol nitrosalation through the intermediary dinitrogen trioxide (N_2O_3) formed from the combination of NO with NO_2.[226] In the presence of PFCOC (hydrophobic compartments) NO should be quickly sequestrated by PFCOC micelles. The high local concentration of NO within the hydrophobic compartment leads to the acceleration of NO oxidation and the formation of nitrosative N_2O_3 from the combination of NO with NO_2.[226] According to the theory of micellar catalysis[227], the rate of NO oxidation and S-nitrosothiols formation depends on the volume of the hydrophobic phase. Larger or smaller amounts of PFCOC could be progressively less effective as a tool for modulating plasma NO bioavailability.[226] Consistently, thiols are in excess relative to NO, and a large fraction of NO_2 should react with thiols rather than NO itself. However, it remains unclear whether intrinsic plasma NO or S-nitrosothiols play a significant role in cardiovascular processes. Notably, the administration of a large dose of PFCOC caused vasoconstriction, reduction of erythrocyte velocity in postcapillary venules and increased venular leukocyte sticking, which is probably due to rapid sequestration of NO.[226-227]

Oxygen transport with perfluorocarbon microbubbles

PFCOC microbubbles have been developed and reported to dissolve clinically relevant amounts of O_2 when administered in dosages that are about 1/500 of usual quantities in

which PFCOCs are administered to provide O_2CC.[228-229] The presence of gas bubbles in the circulation is generally considered medical anathema. However, it has been proposed that the diameter of these intravascular bubbles will remain subcapillary in size and they will transport significant amounts of O_2. Intravascular microbubbles volume-stabilized by dodecafluoropentane gas are formed when a dodecafluoropentane-emulsion is injected into the circulation at normothermic conditions. The dodecafluoropentane has a boiling point of 29 °C at atmospheric pressure and the particles in this emulsion undergo a phase shift and form bubbles when warmed in the bloodstream. Initial bubble size depends on the size of the emulsion particles but, theoretically, the bubbles undergo size variations as they exchange O_2, nitrogen and CO_2 by diffusion in the lungs and the tissues.[228-231]

Future research objectives of PFCOC emulsions aim to prolong the intravascular persistence, reduce phagocytosis by macrophages, and increase stability and surface-modified PFCOCs. Substantial efforts are currently being devoted to investigating targeted PFCOCs for the purpose of molecular imaging - i.e., detection of molecular markers, such as proteins and other cell-surface receptors, characteristic of given pathologies.[232] Such emulsions also have potential for site-directed drug delivery and monitoring of therapy. Important potential target pathologies include inflammation, atherosclerosis, tumor-related angiogenesis, and thrombi.

Engineering an oxygen carrying plasma expander

The products that went through clinical trials reflect the physiological know-how of the 1970s, when their effects could not be analyzed at the level of microscopic blood vessels, and the blood flow regulation by NO was unknown.[233] Commercial developments disregarded the microvascular autoregulation theory and assumed that the endothelium is a passive cellular lining responsible of preventing extravasation of plasma proteins.[234] High resolution methods for measuring microvascular perfusion and tissue pO_2 became available at the beginning of the 1990s,[235-236] and when these were applied to the analysis of the effects of HBOCs and PFCOCs, they elucidated several misperceptions and principles used to design the first generation of artificial O_2 carriers.[237-238] Studies showed that O_2 is delivered by the arterioles and the capillaries, and that this rate of delivery was modulated by the consumption of O_2 in the arteriolar wall. Vasoconstrictor effects were found to increase O_2 consumption of the arterioles limiting tissue oxygenation.[239] This was particularly true for Hb solutions formulated with small molecules, such as alpha-alpha-crosslinked Hb, which also presented another of the presumed required properties, namely low viscosity.[240]

The vasoconstrictor effect of HBOCs has been attributed to NO scavenging by acellular Hb, since NO is quenched through an NO dioxygenation reaction as described before in this review.[48-50] However, other mechanisms may be involved or superimposed to the NO scavenging by Hb.[27, 113]

The distribution of oxygen near the endothelium is in part determined by the size/molecular mass of the HBOC molecule and the presence of the endothelial glycocalyx which creates an unstirred layer next to the vascular wall. Acellular HBOCs increase the concentration of O_2 near the endothelium since they bring O_2 bound to Hb into the CFL[101] an effect that may trigger autoregulatory vasoconstriction aimed at preventing the oversupply of O_2 to the tissue.[241]

The endothelial glycocalyx permeability to macromolecules decreases as a function of the distance from the endothelial surface due to the decreasing density and motion of its molecular branches, being impermeable to molecules > 70 kDa [242]. This decreases scavenging of NO and O_2 delivery by large molecular Hb configurations, relative to the

smaller Hb based HBOCs. Large HBOCs also have a higher solution viscosity, increasing WSS dependent NO production and ATP release from the remaining erythrocytes, which can counteract NO consumption by the HBOC.[243]

Oxygenation of hypoxic tissues is increased by the use of high O_2 affinity HBOCs which prevent O_2 release in arterioles and maintain O_2 bound to Hb until the HBOC arrives to regions of the microcirculation with low pO_2.[162, 244] Lastly, it is critical how much HBOC or PFCOC is necessary to obtain the desired effects. According to present experimental evidence none of the current formulation of HBOC or PFCOC can be administered in sufficient amounts to preserve aerobic metabolism of all tissues without toxic effects.[245] On the other hand, HBOCs appear to be able to restore oxygenation to O_2-deficient tissues, which otherwise may be damaged by ischemia-reperfusion-reoxygenation.

Controlling the problems associated with ischemia-reperfusion injury has proven difficult with HBOCs[246-248] since the associated pathogenesis is strongly linked to oxidative stress and the formation of ROS, contributing to vascular inflammation and endothelial dysfunction.[249] Restoration of vascular levels of NO has been shown to sustain mitochondrial and cellular function, thus reducing oxidative damage.[247, 250] In the case of HBOCs, the maintenance of the ferrous form of Hb is necessary for O_2 transport and preventing oxidation to metHb (non-O_2 carrying), which leaves Hb void of efficacy and prone to degradation.[251]

Conclusions

Our knowledge of the fluids necessary for the treatment of blood loss is still under development. This is in part due to the quest for a blood substitute having begun much before the discovery of the role of NO, our understanding of how the microcirculation manages O_2 distribution, and the role of shear stress in cardiovascular hemostasis. The substitution of blood with an equivalent fluid could be defined as a process of reducing the transfusion trigger, with the goal of postponing the introduction of blood to treat blood loss. This criterion suggests exploiting additional mechanism for achieving this goal, such as reducing O_2 demand and improving the efficiency of O_2 delivery. These effects can be obtained in the pre-transfusion setting, when the volume component of blood loss is treated with plasma expanders. Discarding this possibility places the entire burden of restoring O_2CC on the O_2 carrier, increasing its concentration and the potential for the development of significant negative side effects.

An optimal O_2 carrier has not yet been devised, and banked blood is probably not adequate in this context.[252] The properties of native blood may be unattainable, particularly because of the difficulty of reproducing the physical and chemical interplay between native blood and the vascular endothelium. Nevertheless, attaining this goal with blood substitutes may be aided by recognizing the importance of maintaining the functionality of the microcirculation, which is significantly dependent on adequate levels of O_2, NO and hemodynamic interactions with the vascular endothelium.

The Center for Biologics Evaluation and Research (CBER) is the review body for the FDA in the arena of biology has implemented a comprehensive safety evaluation process for O_2 carrying plasma expanders. These points encompass characterization of the product, animal safety testing, and human studies and address the theoretic concerns of Hb solutions raised previously (including pulmonary and systemic hypertension, organ dysfunction, oxidative tissue injury, synergy with bacterial pathogens, and immunomodulation). Documenting a direct clinical endpoint for O_2 carrying plasma expanders is challenging because these endpoint were never established for RBCs.

In conclusion it is proposed that the initial step in the design of blood substitutes is the optimization of the plasma expander properties of proposed O_2 carrying fluids in terms of their fluid mechanical, solution and biochemical properties, for the eventual integration of plasma expansion and gas transport properties. Fulfillment of these conditions allows for subsequently tailoring the properties of the O_2 carrier and select between Hb molecular solutions, encapsulated Hb and fluorocarbons, including their potential as generalized gas carriers.

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List of abbreviations

CFL	Cell free layer
COP	Colloidal osmotic pressure
CPB	Cardiopulmonary bypass
FCD	Functional capillary density
FiO_2	Fraction of inspired oxygen
HSA	Human serum albumin
HBOC:	Hemoglobin based oxygen carrier
HES	Hydroxyl ethyl starch
Hb	Hemoglobin
HbV	Hemoglobin vesicle
MetHb	Methemoglobin
MW	Molecular weight
NO	Nitric oxide
O_2CC	Oxygen carrying capacity
PEG	Polyethylene glycol
PEG-HbV	Polyethylene glycol covered hemoglobin vesicle
PFC	Perfluorocarbon
PFCOC	Perfluorocarbon based oxygen carrier
pO_2	Partial pressure of oxygen
RBC	Red blood cell
rHSA	Recombinant human serum albumin
ROS	Reactive oxygen species
WSS	Wall shear stress

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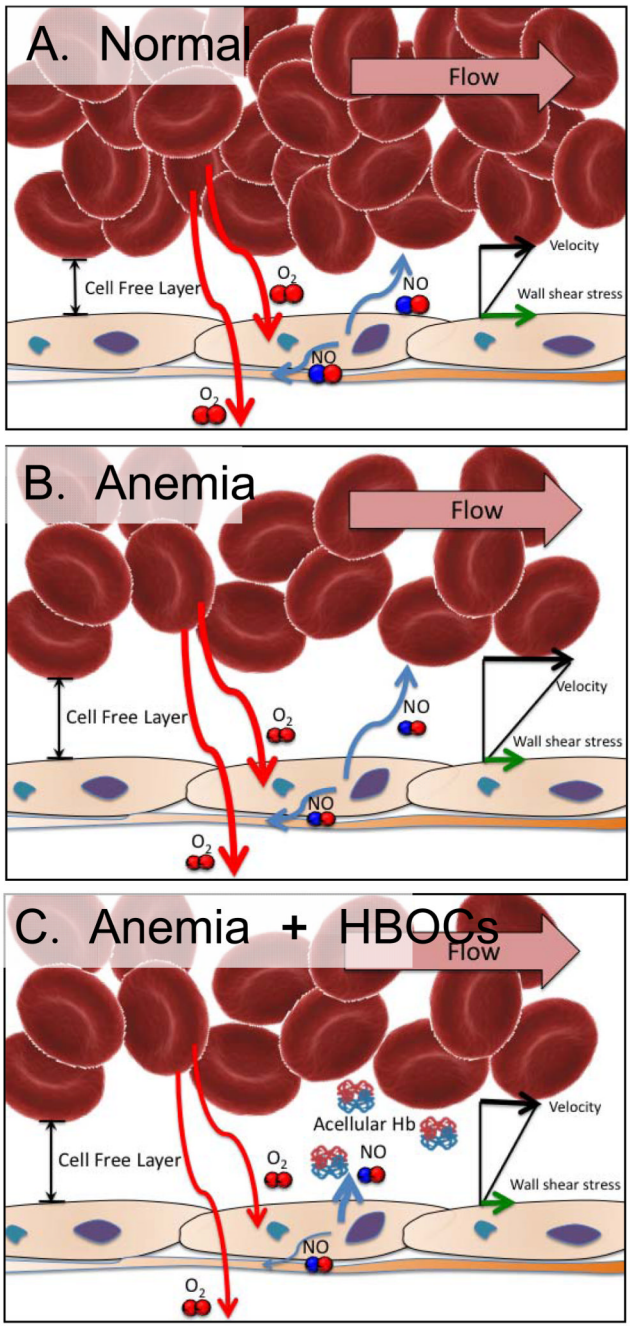

Figure 1.

In physiological conditions (A. normal), blood flow provides O_2 and mechanical signals to the vasculature, which serve to self-regulate local blood flow. After severe blood losses (B. anemic), anemia or any condition that originates a need for blood transfusion, oxygenation and vascular shear stress are compromised, thus the local vascular regulatory process becomes impaired. Consequently, when HBOCs are transfused (C. Acellular Hb in circulation), they do not achieve their intended goal since they only increase O_2CC without reversing the hypoperfusion generated pre-transfusion. Even once perfusion is recovered, vascular regulatory processes are not fully functional, such as eNOS, which becomes uncoupled and instead of generating NO produces superoxide, contributing to oxidative

stress and triggering an inflammatory cascade. The additive role of oxidative and nitrosative stress results from increased NO release from inducible NOS (iNOS) sources, including tissues and macrophages/monocytes. The NO generated by iNOS is especially metabolic to peroxynitrite, which is cytotoxic to tissues.

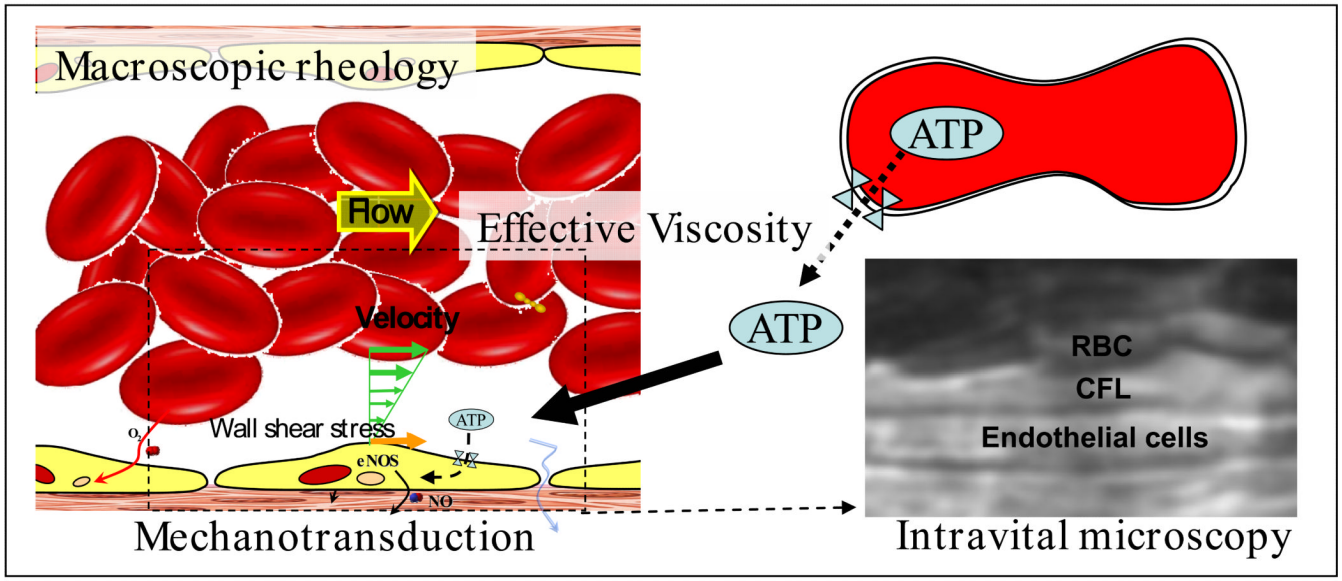

Figure 2.
Oxygen content for whole blood, Perfluorocarbon emulsion (PFCOC, Oxycyte™, Synthetic Blood International, Inc., Costa Mesa, CA) and plasma as a function of O_2 partial pressure.

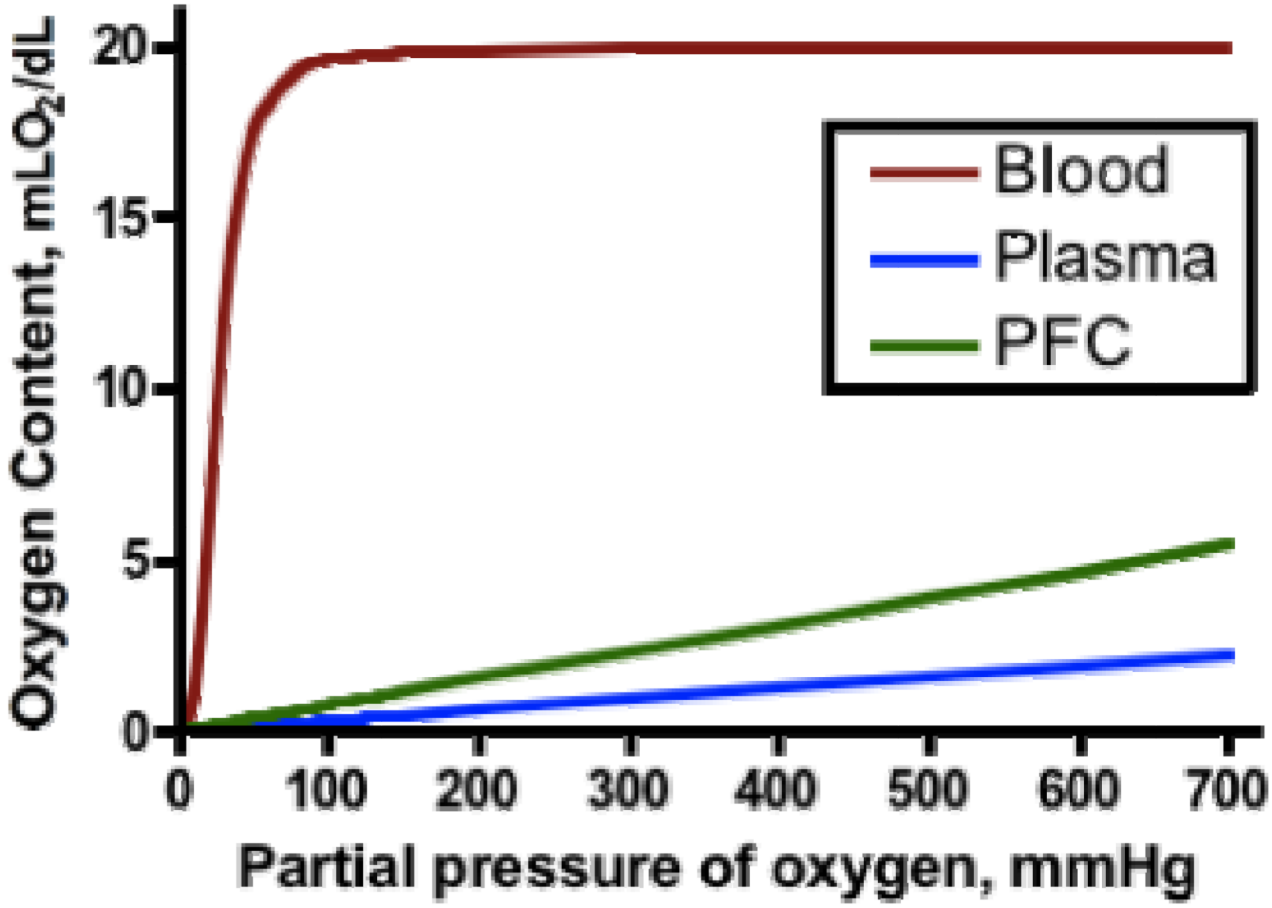

Figure 3.

Universal O_2 carrying solutions may replace the O_2 carrying capacity and O_2 transport functions of allogeneic blood. These O_2 carriers will also prevent the many complications associated with blood transfusions. The two platforms to develop O_2 carrying solutions include perfluorocarbon (PFC) based O_2 carriers (PFCOCs) and hemoglobin based O_2 carriers (HBOCs). PFCOCs are emulsions of PFC with high O_2 solubility. HBOCs are chemically modified natural or recombinant Hb or encapsulated Hb, they include large HBOC like PEG decorated Hb vesicles (PEG-HbVs), and small HBOC such as, polymerized Hb (PolyHbs) and PEG surface conjugated Hb (PEG-Hb). Various design strategies for PFCOCs and HBOCs have resulted in vasoconstriction and the development of systemic hypertension. The root cause of this effect stems from the ability of acellular Hb to extravasate, scavenge NO and hyper-oxygenation of the vessel wall. Increasing the molecular size of HBOCs prevents extravasation, increases plasma viscosity and O_2 carrying capacity without increasing colloidal oncotic pressure.