The Design of Blood Substitutes: Oxygen Carriers

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Hospitals throughout the world currently rely solely on allogeneic blood (also referred to as donor blood) for transfusions. Estimates put worldwide blood demand somewhere between 75 and 90 million units per year [1]. Canada's requirements account for 800 000 units per year and the United States for 12 million[2,3]. One unit of blood represents approximately a volume of 450 mL. Based on current requirements and donations, a shortage of 4 million units is expected by 2030 in the United States alone[1]. Furthermore, the fact that blood is donated may lead to the false assumption that it is cheap. Given that the average patient requires 4.6 units for a transfusion, and the cost is approximately $500 US per unit, a more economical alternative to allogeneic blood is desirable[1,2].

Ever since the creation of the first blood banks in the 1930s, scientists have been striving to increase the shelf-life of donated blood. The use of sodium citrate first permitted blood banking by preventing blood from clotting. Further advances, such as the use of a modified citrate-phosphate-dextrose anticoagulant solution which is supplemented with adenine, have extended the shelf-life of donated blood. The introduction of plastic storage bags has also resulted in a reduction in bacterial infections, improving the safety of allogeneic blood [1]. However, despites significant advances, the shelf-life of blood products is still very limited as red blood cells (RBC) can only be stored for a maximum period of 42 days[2,3].

Storage also affects the efficacy of red blood cells. Storage lesions result in a decrease in pH, hemolysis changes in RBC deformability, formation of microaggregates, release of vasoactive substances and denaturation of proteins. However, the most significant change observed in stored RBC is an increase in the affinity of hemoglobin for oxygen. Under normal circumstances, the affinity for oxygen is reduced by the presence of an allosteric effector, 2,3-diphosphoglycerate (2,3-DPG) facilitating O_2 release and delivery to tissues [1]. The concentration of 2,3-DPG decreases over time and almost none is left within 2 weeks. As a result affinity for oxygen increases, resulting in a release of only 5% of bound O_2 compared to about 25% in fresh blood. Transfusions of stored blood are therefore not initially very effective in delivering O_2. This undesirable effect can be partially reversed by the addition of 2,3-DPG. However, there is a period of about 24 hours before O_2 delivery increases[1].

Another concern with the use of allogeneic blood is its safety. Donated blood requires type- and cross-matching (ABO blood group system) to minimize chances of adverse transfusion reactions[3]. There have also been reports of mild reactions associated with allogeneic transfusions including fever, chills, pain, and discomfort in as many as 1 in 30 patients A transfusion-mediated immunosuppressive effect has also been observed, therefore increasing the chance of an infection. Although blood screening has significantly decreased infectious risks, there is still a 1 in 30 000 – 100 000 chance of contracting Hepatitis C from a blood transfusion. It is still unclear whether other diseases, such as Creutzfeld-Jakob disease, are transmissible through transfusions.

In order to address the issues related to allogeneic blood use, the scientific community has been intensively developing blood substitutes that could replace the O_2 carrying capability of the RBC. These alternative oxygen carriers need to be able to load O_2 rapidly while in the pulmonary capillary bed, deliver O_2 to all organs, have no physiological activity, be pathogen-free, have no blood group (ABO) antigens, have a long shelf life, be user friendly, exhibit long intravascular

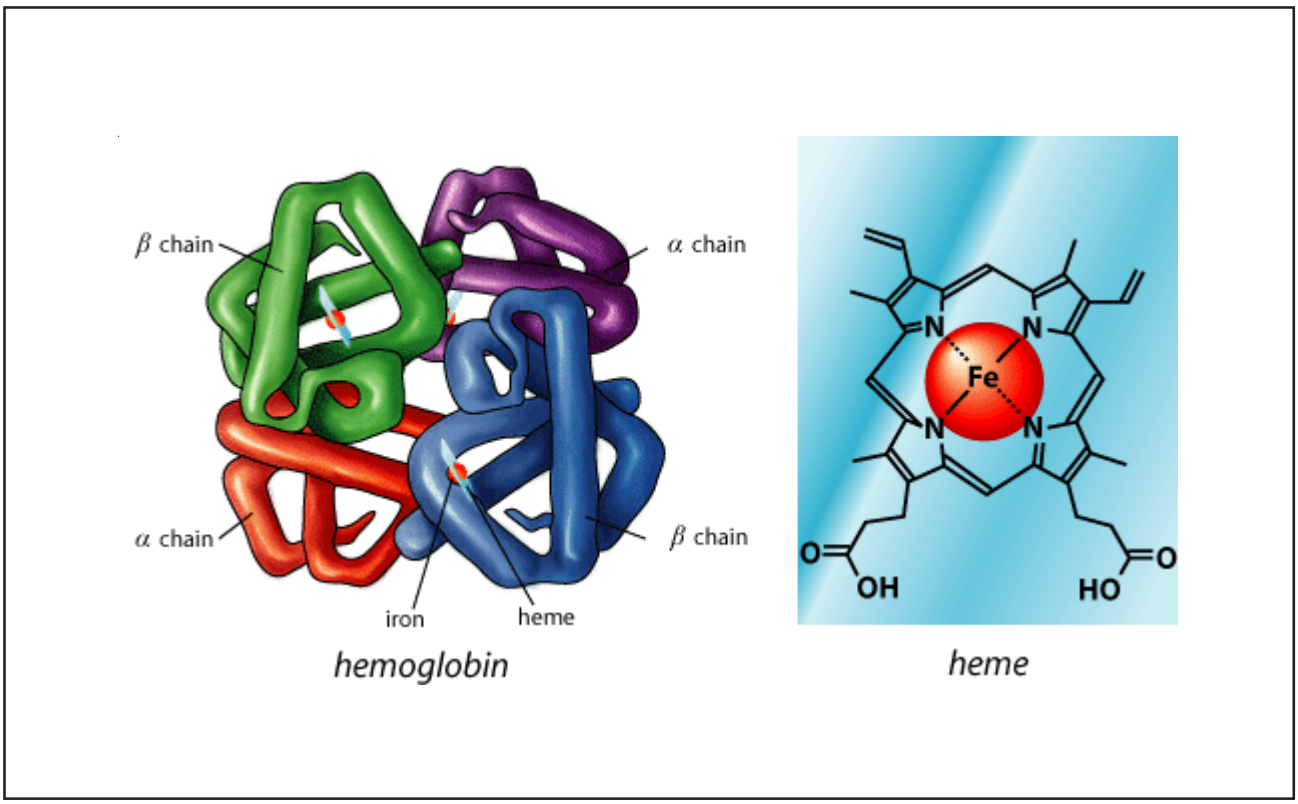

Figure 1. A hemoglobin carries four heme groups, one in each subunit.

half-lives and be produced in large quantities at a low cost[1,4]. The development of the oxygen carriers can be summarized in two distinct branches. The "biomimetic" approach involves the use of hemoglobin (Hb), the RBC intracellular protein responsible for O_2 transport. The second approach is referred to as "abiotic" as it involves the use of perfluorocarbons (PFC), a substance foreign to living organisms, in which O_2 is highly soluble.

"Biomimetic" Approach - Hemoglobin-based Oxygen Carriers (HBOC)

A. Cell-free Hemoglobin

To many scientists, the logical approach in the development of an RBC substitute was the use of Hb, which is nature's way of transporting O_2 to the tissues of warm-blooded animals[1]. Hb consists of four polypeptidic globin subunits: two alpha and two beta, which have 141 and 146 amino acid residues respectively. While the alpha/beta dimer is fairly stable, the two alpha/beta dimers are only loosely associated with each other. Hb has a molecular weight of 64.5 kDa. It has a nearly spherical shape with a diameter of 5.5 nm. Each protein subunit is centered on an iron(III) protoporphyrin IX (also referred to as a heme) prosthetic group. The prosthetic group is held in place by several hydrogen bonds and hydrophobic interactions. The Hb protein is able to bind cooperatively to 4 O_2 molecules as a result of a change in conformation from the tensed (T-form) deoxyHb to the relaxed (R-form) oxyHb, which has a higher O_2 affinity. Furthermore, it has an allosteric effector 2,3-DPG that reduces its affinity for O_2 by stabilizing the T-form.

The first problem encountered with the infusion of cell-free Hb was the presence of residual stroma (membrane of RBC) as a result of the Hb extraction procedure[6]. It was therefore thought that the use of purified Hb solutions, in which all RBC antigens would have been removed to avoid the antigenicity inherent to the cell membrane, would be safe. However, several other problematic issues arose. First, cell-free Hb is a very fragile biomolecule. Dissociation of the tetramer into alph/beta-dimers will occur, especially at low Hb

concentrations. Typical Hb solutions concentrations range from 60-80 g/L, while in RBC it is much higher at 300 g/L [1]. Once dissociated, the dimers are rapidly cleared by renal filtration and may cause renal toxicity[6]. Due to its small size, cell-free Hb was also able to escape from the vasculature. As a result, cell-free Hb had a very short intra-vascular half-life.

Furthermore, due to the loss of its allosteric inhibitor (2,3-DPG) during the extraction process, cell-free Hb solutions demonstrated a higher affinity for O_2, making it an ineffective molecule for delivering O_2 to tissues. There was also significant oxidation of Hb (Fe^{2+}) into metHb (Fe^{3+}), which binds irreversibly to O^2. In the RBC, this is not a significant problem as enzymatic systems are present to reduce metHb back to Hb. Cell-free Hb was also found to be able to bind to nitric monoxide (NO). This affects the balance of oxygen and nitrogen which can result in vascular disorders. NO is known to have a vasodilating effect. Therefore, its removal by cell-free Hb can result in vasoconstriction. Cell-free Hb could be further studied as aa potential treatment for hypotension due to this property[1].

B. Modifying Cell-Free Hemoglobin

In order to solve some of the issues encountered with cell-free Hb, the protein's structure was analyzed for possible reaction sites. The strongest nucleophiles of Hb were found to be the sulfhydryl and amino groups. Utilizing reactions that the body uses to alter Hb, scientists set out to specifically modify Hb in order to solve the issues seen with cell-free Hb such as its high affinity for oxygen.

C. Pyridoxalation

As stated previously, one of the main undesirable characteristics of cell-free Hb was that its affinity for O_2 was too high. This problem was primarily due to the loss of its allosteric effector, 2,3-DPG, during the Hb extraction from the RBC. Initial experiments involved the addition of 2,3-DPG back into the cell-free Hb solution. Unfortunately, these attempts were ineffective as the allosteric effector was rapidly cleared from circulation. A different strategy was undertaken, where a reaction was made between deoxyHb and pyridoxal phosphate (a natural coenzyme related to vitamin B6 and an analogue of the allosteric effector)[1]. The overall result of the reaction was a decrease in the affinity of O_2 binding.

D. Intramolecular Cross-Linking

Preventing the Hb tetramer's dissociation was also a major concern in order to suppress renal filtration. Since the alpha/beta dimers were relatively stable, the goal of this modification was to cross-link the two alpha (a-a) or beta (B-B) subunits and stabilize the association of the two alpha/beta dimers. In addition to preventing tetramer dissociation, cross-linking, in some cases, also reduced the affinity of Hb for O_2. The most popular cross-linkers are DBBF and nor-2-formylpyridoxal 5-phosphate (NFPLP). This reaction is also influenced by the conformation of the Hb protein. For example, when DBBF was used with oxyHB (R-form), cross-linking occurred between the two alpha subunits. However, when used with deoxyHb (T-form), cross-linking was present between the two beta subunits. The increased stability provided through intramolecular cross-linking also allowed Hb solutions to be pasteurized.

E. Polymerization

Polymerization, also referred to as intermolecular cross-linking, increases the size of molecules through the formation of Hb oligomers. Multiple Hb proteins are linked together through the use of dialdehydes such as glutaraldehyde or glycoaldehyde. The increase in size is significant as the oligomers produced have molecular weights greater than 500 kDa compared to the 64.5 kDa unpolymerized Hb tetramers. As a result of this modification, the protein's oncotic pressure is reduced. The increase in size also prevents its rapid excretion, prolonging the Hb plasma half-life as it is less likely to leak into the interstitial spaces. Unfortunately, any unreacted Hb tetramers generate excessive viscosity, oncotic pressure and O_2 affinity. It is therefore extremely importance to obtain high polymerization yields. Otherwise, the unreacted tetramers need to be separated. The intravascular half-life of polymerized Hb solutions has been reported in the range of 10 to 15 hours [1].

F. Conjugation and Pegylation

Conjugation of Hb implies the covalent binding of Hb to a biocompatible polymer, such as a polysaccharide, in order to increase its overall size and achieve similar improvements to those made using polymerization. In the case of pegylation, multiple polyethylene glycol (PEG) chains are grafted to the Hb protein as an effective way of increasing the molecule's size. Its radius therefore increases from 3 nm to about 15 nm once pegylated with 6 PEG chains. However, this does not reduce the oncotic pressure as extensive hydration of the PEG chains occurs [1].

G. Genetic Engineering of Hemoglobin

Genetic engineering offers an alternative to chemically modified Hb. In order to address the problems related to the use of cell-free hemoglobin, site-directed mutagenesis has been used. Modifications have been made to increase the tetramer's stability and decrease its affinity for O_2. Future genetic manipulations may be able to solve other issues such as the oxidation of Hb into metHb, reaction rate with nitric monoxide and the short circulation half-life [1].

H. Encapsulation

The encapsulation of Hb, whether in its natural or modified form, is based on the idea of recreating some of the properties of a RBC without the presence of blood group antigens. Encapsulated Hb is often referred to as "hemosome". The process involves the encapsulation of Hb in lipid vesicles (liposomes) using a solution of phospholipids. This approach allows for the engineering of specific membrane properties. For example, the use of negatively charged lipids has been found to limit aggregation between hemosomes. Alteration of the bilayer membrane composition may also allow for better diffusion of O_2 and CO_2. Further modifications, such as membrane polymerization, have reduced Hb leakage and increased liposome stability[1].

I. Current HBOCs in Development

There are currently several hemoglobin-based oxygen carriers in development. Almost all of the chemical modifications presented previously have been used in the development of the various HBOCs with the exception of encapsulation, which is still in the early stages of laboratory experimentation[7]. The source of Hb is usually either human or bovine with the exception of one rHb produce in *E. coli*.

An undesirable side effect has been reported with the use of most HBOCs that has been dubbed the "pressor effect". This is characterized by an increase in arterial pressure and vascular resistance. At this point, there is hope that the encapsulated Hb or genetically engineered Hb may be able to overcome these side effects [1].

"Abiotic Approach": Perfluorocarbons as Oxygen Carriers

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A. PFC Properties

The interest in PFCs as possible oxygen carriers dates back to 1966 when two scientists named Clark and Gollan demonstrated that a mouse could survive by "breathing" while immersed in an oxygen saturated solution of fluorobutyltetrahydrofurane (FX-80)[9]. PFCs have exceptional chemical and biological inertness. No enzymatic system is known to digest PFC and no bacteria are known to feed on them. Their properties are mainly due to the strength of the carbon-fluorine bond which results in strong intramolecular covalent bonding. The weak intermolecular interactions allow the formation of "holes", which can accommodate large volumes of oxygen and other non-polar gases.

B. PFC Preparation

Due to the extreme hydrophobicity of PFC, they cannot be administered directly to a patient. Therefore, PFC must be emulsified prior to administration. The emulsion needs to be highly stable and able to withstand sterilization. Typical surfactants used for the emulsion process are generally derived from egg yolk phospholipids as they have been widely used in the pharmaceutical industry. Negatively charged

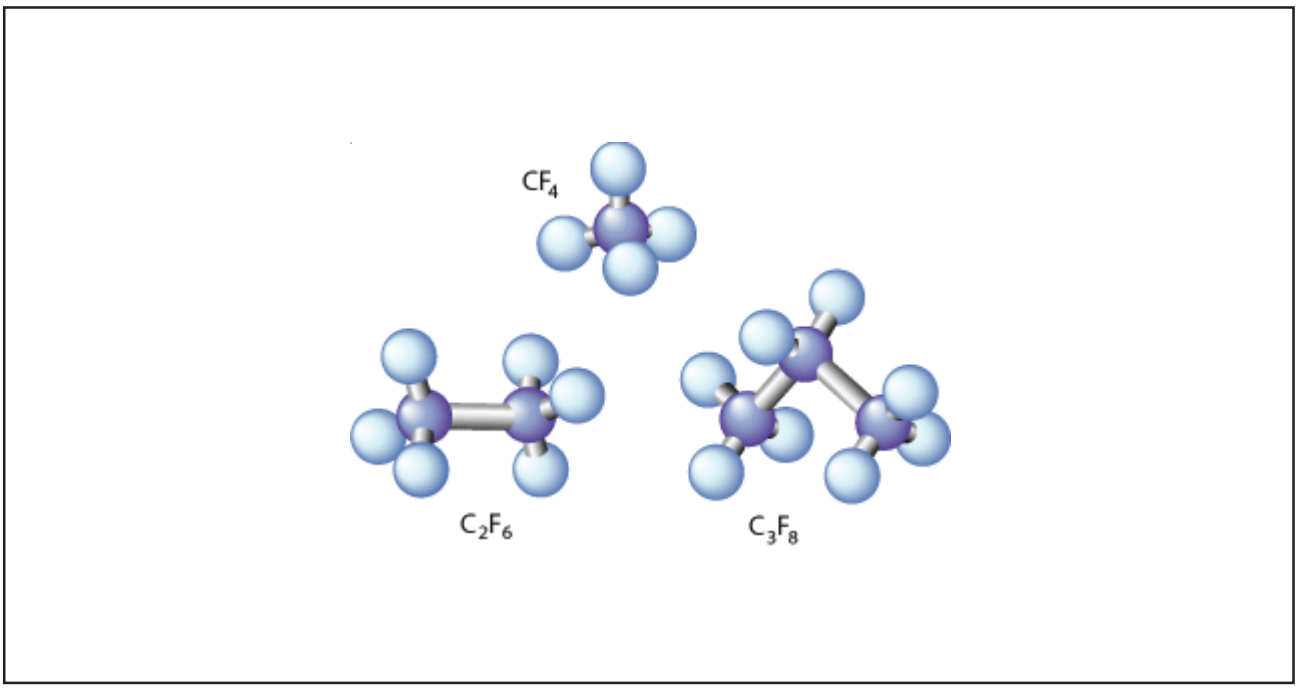

Figure 2. Structures of various perfluorocarbons.

phospholipids have been found to minimize the flocculation (formation of aggregates within solution) of PFC droplets. The droplets formed through emulsion must be very small (<0.2 um) for two reasons. First, smaller droplets improve oxygen transport as a result of reduced mass transfer resistance. Second, since larger droplets are eliminated by phagocytosis, the smaller droplets will have an increased circulatory half-life.

C. Stability vs. Body Retention Time

The use of high molecular weight (MW) PFCs also improves the stability of the emulsion. Also, the body retention time increases exponentially with MW. At MW approaching 700, the organ retention half-time reaches about 1000 days, which is clearly unacceptable. On the other hand, low MW PFCs have high vapor pressures that can cause pulmonary complications. However, the excretion rates can be increased by improving the solubility of PFCs in lipids.

D. PFC in Development

The first commercial PFCs preparation, called Fluosol-DA, was approved by the FDA in 1989 for use in percutaneous transluminal coronary angioplasty. However, due to its complex preparation (it required the mixing of 3 different solutions) and its limited application, manufacturing was halted in 1994.

In 1997, a second PFC preparation was approved in Russia called Perftoran. Currently there is a third PFC solution is clinical testing called Oxygent. Oxygent is made using Perflubron (perfluorooctyl bromide). It is now in a phase III trial for cardiac surgery application. PFC can be manufactured at a large scale and high purity (>99.9). However, as for HBOC, PFCs have a short circulation half-life of less than a day [1,10].

Conclusion

Due to the short circulation half-life of both hemoglobin-based and perfluorocarbon oxygen carriers, which is currently inferior to 24 hours, they

are far from perfect blood substitutes. However, they could be of use as an alternative to allogeneic blood in procedures such as surgeries where their oxygen-transport capacity is only required for a few hours.

The main advantages of HBOC are found in their high capacity for O_2. They also function optimally at physiological partial oxygen pressures, have a prolonged shelf-life of years compared to 42 days for RBC and no RBC antigens. However, these hemoglobin-based blood substitutes demonstrate vasoactivity, auto-oxydation of Hb into metHb and their supply is limited as they depend on outdated blood from blood banks or animals with the exception of recombinant Hb.

In the case of PFCs, the main advantages are found in the large scale production capacity and the low cost of production. They also have a prolonged shelf-life measured in years, minimal infectious risk and immunogenicity. The main drawbacks of PFCs include their emulsion form and the quick elimination of large emulsion droplets through phagocytosis. They also work optimally at greater partial oxygen pressure than can be expected under physiological conditions. They have a low capacity for O_2 under physiological conditions.

References

1. Riess, J. G. Oxygen Carriers ("Blood Substitutes") - Raison d'Etre, Chemistry, and Some Physiology. *Chem. Rev.* **101**, 2797-2919 (2001).

2. Canadian Blood Services. (2004). *What Should I Know?* Retrieved August 17, 2004 from: www.bloodservices.ca

3. Cohn, S. M. Blood substitutes in surgery. *Surgery* **127(6)**, 599-602 (2000).

4. Reid, T. J. Hb-based oxygen carriers: are we there yet? *Transfusion* **43**, 280-287 (2000).

5. Rourke, B. (1999). *Hemoglobin Structure*. Retrieved August 17, 2004 from: http://www.ags.uci.edu/~bcrourke/Blood_Fluids/sld016.htm

6. Creteur, J., Sibbald, W., Vincent, J.-L. Hemoglobin solutions - Not just red blood cell substitutes. *Crit Care Med.* **28(8)**, 3025-3034 (2000).

7. Teramura, Y., Kanazawa, H., Sakai, H., Takeoka, E., Tsuchida, E. Prolonged Oxygen-Carrying Ability of Hemoglobin Vesicles by Coencapsulation of Catalase in Vivo. *Bioconjugate Chem*. **14,** 1171-1176 (2003).

8. CBC News Online. (2003). *Hemosol stops cardiac trial to review safety data* Retrieved August 17, 2004 from: www.cbc.ca/story/business/national/2003/03/13/hemosol030313.html

9. Clark, L. C. and Gollan, F. Survival of mammals breathing organic liquids equilibrated with oxygen at atmospheric pressure. *Science* **152**, 1755-1756 (1966).

10. Stowell, C.P., Levin, J., Spiess, B., Winslow, R. M. Progress in the development of RBC substitutes. *Transfusion* **41**, 287-299 (2001).

11. Lowe, K. C. Perfluorochemical respiratory carriers: benefits to cell culture systems. *Journal of Fluorine Chemistry* **118**, 19-26 (2002).

12. Geankoplis, C. J. *Transport Processes and Unit Operation* (3rd Ed.) 586-587 (Prentice Hall, Toronto, 1993) .