# Design of Recombinant Hemoglobins for Use in Transfusion Fluids

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• Blood substitute • Hemoglobin • Myoglobin
• Nitric oxide • Oxygen carrier • Transfusion

The need for transfusion fluids to treat a variety of clinical conditions is continually increasing. The use of acellular hemoglobin (Hb) solutions as a red blood cell (RBC) replacement is being evaluated.[1–5] Evolution maximizes the best conformational and functional characteristics of proteins as related to their natural in vivo function, and the role of the Hb molecule is best expressed when contained within the erythrocytes. The transformation of such a protein into an acellular oxygen carrier necessitates the introduction of functional and conformational modifications to optimize the characteristics of the protein to the different environmental conditions. In physiologic conditions, the oxygen affinity of the erythrocytes ($P_{50}$ = 28 Torr) is lower than that of acellular Hb ($P_{50}$ = 18 Torr). Solutions of stroma-free Hb contain tetrameric (molecular weight [MW] 64 kDa) and dimeric (MW 32 kDa) molecules at equilibrium. Due to the rapid filtration of the low-molecular-weight dimers through the kidneys, the retention time of infused acellular Hb is short.[6] In addition, Hb molecules can filter through the endothelium and scavenge nitric oxide (NO) from the interstitial fluid, producing the vasoconstriction observed upon administration of acellular Hb solutions.[7,8] Moreover, acellular Hb in the plasma facilitates the delivery of bound oxygen.[9,10] Under normal conditions, the increased perivascular $PO_2$ is thought to contribute to vasoconstriction and the associated increase in arterial pressure.[11,12] This vasoconstriction may prevent excessive oxygen delivery to the tissues and free radical formation. However, persistent constriction of upstream arterioles under conditions of hemorrhagic shock or partial ischemia due to facilitated precapillary oxygen loss by an Hb-based oxygen carrier (HBOC) can exert a counterproductive effect of RBC perfusion of the downstream capillary network.[13,14]

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The following characteristics are necessary for an effective HBOC: (1) absence of renal filtration, (2) absence of NO scavenging effects, (3) stability toward auto-oxidation and heme loss, and (4) calibrated oxygen delivery. Clearly the development of an effective HBOC is a monumental task that will require contributions from basic science, animal experimentation, and clinical applications.

Chemical modifications are used to transform human and bovine Hbs into blood substitutes and large scale productions have been implemented. The first HBOCs, designed to have an oxygen affinity similar to blood and not to dissociate into dimers, were generated by introducing cross-links in the central cavity as the $\alpha$-$\alpha$ fumaryl (HemAssist; Baxter Healthcare Corp., Deerfield, Illinois)[15] or by introducing pyridoxal phosphate to decrease the oxygen affinity combined with polymerization using glutaraldehyde to increase the molecular size (Polyheme; Northfield Laboratories Inc., Evanston, Illinois; Hemopure; Biopure Corp., Cambridge, Massachusetts).[16–19] More recent products are the zero-link bovine Hb (ZL-HbBv), which has large polymers formed in the absence of residual chemicals (Oxyvita, New Windsor, New York),[20] and Hb conjugated with polyethylene glycol (PEG) (Hemospan, Sangart; Sanguinate, Prolong Pharmaceuticals).[21–23] These more recent products have a high oxygen affinity, which may act to minimize vasoconstriction of the arterioles as a regulatory mechanism for excess oxygen delivery. In addition, solutions of pegylated Hb incorporate the characteristics of a plasma volume expander due to their high viscosity and oncotic pressure. The efficacy and potential adverse effects of these products for therapeutic applications are under rigorous scrutiny.[24]

## RECOMBINANT HEMOGLOBIN

As an alternative approach to chemically modifying naturally occurring Hb, recombinant technology can be applied to the development of unique HBOCs. Hb can be expressed in microorganisms, and this approach eliminates the use of proteins of mammals, which may be in limited supply or may be infected by viruses or other pathogens. This technology offers the possibility for construction of mutant molecules that incorporate specific conformational and functional characteristics in the absence of chemical modifications.

Hemoglobin is a tetrameric protein composed of two structurally similar subunits, $\alpha$ and $\beta$, assembled through two different interfaces, $\alpha_1$ $\beta_1$ and $\alpha_1$ $\beta_2$. Each subunit contains eight $\alpha$-helices (A–H) that form a pocket containing the heme. Tetrameric Hb is present in two states at equilibrium, deoxygenated (T) and oxygenated (R). Correct assembly of this protein requires expression of native $\alpha$- and $\beta$-globins and their assembly and folding into the final quaternary structure upon combination with the heme. The first expression systems were developed by Kiyoshi Nagai and Hans Christian Thogersen[25] and adapted by Clara Fronticelli and colleagues.[26] In these systems, $\beta$-globin was expressed as a fusion protein with blood coagulation factor Xa. Cleavage of the isolated fusion protein released the authentic $\beta$-globin, which assembled into tetrameric Hb in the presence of native $\alpha$-subunits and the heme. In a similar way, recombinant $\alpha$-globin was expressed and assembled using native $\beta$-subunits.[27,28] Expression of soluble Hb was subsequently achieved by coexpressing the soluble $\alpha$ and $\beta$-chains in *Escherichia coli*, and several expression systems and purification procedures have been described.[29–31] The recombinant Hbs (rHbs) have functional characteristics similar to the natural protein and thus are apt to be molded for therapeutic applications as HBOCs.

A problem encountered in the development of rHbs as HBOCs is their low expression yield. Production of rHb in an amount sufficient for animal testing in an academic