Engineering Blood Cells and Proteins as Blood Substitutes: A Short Review

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Abstract In this brief review, basic principles and recent progresses on the development of therapeutic substitutes for major blood components are briefly discussed with primary focus on the red cell substitutes. © KSBB

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INTRODUCTION

Blood is a complex biologic fluid composed of cellular or formed elements, proteins, and other components many of which provide critical functions. For patients with acute anemia or deficient/inadequate levels of hemostatic factors, transfusion of normal donor blood or an appropriate component is a lifesaving treatment. However, donor blood has limitations: limited supply, requires typing and crossmatching, a short storage life, and a risk of disease transmission (*e.g.*, hepatitis, AIDS). A substitute for red cells or other blood component that can overcome these limitations would be highly desirable. Basic principles and recent progresses on the development of red cell substitutes (RCSs), artificial platelets, recombinant clotting factors, and artificial antibodies are discussed with a primary focus on the RCSs.

Red Blood Cell Substitutes

Because red blood cells in the blood deliver essential oxygen to tissues and organs, in acute anemia caused by traumatic hemorrhage or other causes, lost oxygen carrying capacity must be promptly restored or could be fatal. Allogeneic donor blood (red cell) transfusion is effective but has limitations mentioned above. For this reason, search for a RCS has been ongoing for decades but only recently have a few candidates reached active clinical testing [1].

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Engineered Red Blood Cells

Blood types are generally classified based on presence of AB and Rh antigens on the red cell membrane surface. In most transfusions, simple ABO/Rh blood typing is sufficient to match appropriate donor blood. Occasionally, however, patients with rare red cell antigens do present difficulty in identifying matching blood donors. More often, compatibility problems occur in patients who must receive chronic transfusions (*e.g.*, sickle cell anemia or thalassemia patients). In such patients, alloimmunization against minor red cell antigens makes it difficult to find appropriate donors. There have been efforts to create universal donor red cells. The red cell surface antigens were neutralized or masked using polyethylene glycol (PEG) or its derivatives. Covalent binding of PEGs to RBCs do appear to mask the RBC surface antigens thereby opening possibilities of transfusion of heterologous or even xenogeneic RBCs. In one recent study [2], Type A or B human RBCs modified with methoxyPEG showed decreased anti-A or anti-B antibody binding. Furthermore, methoxyPEG modified sheep RBCs were resistant to phagocytosis by human peripheral blood monocytes. In another approach, red cell A or B antigens were enzymatically removed by exoglycosidase treatment but was only partially successful [3]. These 'stealth' red cell approaches may obviate incompatibility problems but are yet to deal with issues of limited supply and potential disease transmission.

Hemoglobin-based Oxygen Carriers (HBOC)

Hemoglobin (Hb) is a natural oxygen carrier protein normally present in the red cells. Stroma-free Hb (SFH), a purified Hb solution free of red cell membrane stroma as cell-free Hb in a physiologic solution, can still reversibly bind and deliver oxygen. Because there is no antigenic red cell membrane in SFH solution, it can be used as a universal resuscitation fluid for recipients with any blood type. This SFH, however, was found to have two perceived shortfalls:

SFH was perceived to have too high an oxygen affinity (P50 of 10~15 mmHg versus 26~28 mmHg for normal red cell Hb) and too short an intravascular circulation half-time ($T_{1/2}$ < 1.5 h) to be useful. Acellular free SFH has a higher oxygen affinity than native intra-erythrocytic Hb because 2,3-diphosphoglycerate (DPG) normally present in the red cells is lost during purification. The high oxygen affinity was perceived as detrimental to optimal oxygen offloading to tissues. Acellular tetrameric Hb ($\alpha_2\beta_2$) in solution readily dissociates into αβ dimers that are easily filtered through the kidneys and excreted in the urine. The HBOCs in clinical testing today are Hbs chemically or genetically "engineered" to produce desirable oxygen offloading characteristics and an extended circulation half-time. Key approaches of HBOCs currently in development as red cell substitutes are briefly described below.

Stabilized Hbs (Tetrameric Hbs)

One way to alleviate the high oxygen affinity of SFH is through the use of functional DPG analogs that specifically attach to the DPG pocket. For example, the negatively charged pyridoxal-5′-phosphate (PLP) is a DPG analog that specifically interacts with the positively charged amino residues of the DPG binding site (*e.g.*, Lys 82β, His2β, and His143β) [4]. The reactive aldehyde group of PLP is used to crosslink PLP to the primary amino groups of the DPG pocket (valines of β1 and β2) via a Schiff reaction which is then reduced forming a covalent bond. Pyridoxylated SFH (PLP-Hb) has a near normal oxygen affinity (P_{50} = 22~24 mmHg) but is still dissociable into αβ-dimers susceptible to renal excretion because there is no inter-dimeric crosslinkage. Using a DPG analog with bifunctional aldehydes (*e.g.*, 2-nor-2-formyl pyridoxal-5′-phosphate; NFPLP), low oxygen affinity Hb with an inter-dimeric linkage between the two primary valines of β1 and β2 subunits (β stabilized Hb) was produced [5]. Hb can also be stabilized using an α-specific crosslinker bis(3,5-dibromo salicyl)-fumarate (DBBF or diaspirin) to produce 99α1-99α2 crosslinked Hb (DCLHb). DCLHb has been shown to have a P50 of 30 mmHg and substantially longer intravascular circulation time than unmodified SFH.

Polymerized Hbs

Intravascular retention times of HBOCs can be further increased by intermolecular crosslinking (polymerization) of stabilized Hbs using bi- or poly-functional crosslinkers. For example, PLP-Hb can be polymerized using glutaraldehyde, a bifunctional nonspecific crosslinker, to produce poly(PLP-Hb) with $t_{1/2}$ of over 30 h following a partial exchange transfusion in adult baboons [6]. PolyHeme® (Northfield Labs., Chicago, IL, USA), a poly(PLP-Hb) product, is being tested in Phase III clinical trial in the USA. A low oxygen affinity oligomeric HBOC with desirable circulation characteristics has been produced using ring-opened raffinose (o-raffinose), a hexafunctional crosslinker [7]. With this unique crosslinker prior intra-molecular stabilization is unnecessary. At 10 g Hb/dL, this o-raffinose HBOC delivers 4.3 mL O_2/dL under the normal arterio-venous oxygen gradient, close to the nor-

mal oxygen delivery capacity of blood. The o-raffinose modified Hb (Hemolink® by Hemosol, Inc., Toronto, Canada) was shown to reduce blood transfusion requirement in patients undergoing coronary artery bypass surgery [8]. An alternative approach to circumvent the high oxygen affinity and limited availability of human SFH is to utilize naturally low O_2 affinity bovine Hb as a starting material [9]. Bovine Hb has a naturally low oxygen affinity compared to human Hb (P_{50} = 30~32 mmHg). It is not DPG dependent and can be directly polymerized without prior modification to achieve desired the oxygen affinity and circulation time [10]. Potential immunogenicity and transmission of animal borne disease such as bovine spongiform encephalopathy (BSE) could be of concern with these products.

Conjugated Hbs

Another way to increase $t_{1/2}$ of a HBOC is to conjugate Hb with a macromolecule [11]. Human or bovine Hb conjugated with PEG appears to protect the molecule from renal excretion [12,13]. More recently, MalPEG-Hb (MP4 or Hemospan® by Sangart Corp. San Diego, CA, USA), a different type of PEG modified human Hb with an average molecular weight (MW) of 95 kilo daltons (kD), has been developed [14]. This product is reported to have improved hemodynamic and circulatory properties and is being tested in Phase II clinical trials in the USA.

To reduce oxygen radical mediated damages, a polyHb conjugated with superoxide dismutase and catalase has also been developed [15,16].

Hemoglobin/Heme Vesicles

Human or animal SFH encapsulated in phospholipid vesicles (liposomes) was developed as a potential red cell substitute but encountered difficulties due to high Hb oxidation rate and a short intravascular half-life. Recently, totally synthetic heme imbedded between two lipid bilayers (lipid-heme vesicles) has been developed [17]. A stable fat mircrosphere suspension has been achieved by emulsifying triglycerides with lipid-heme as a surfactant. These lipid-heme products are reported to have heme concentration and reversible O_2 binding close to that of normal blood. More recently, Hb and red cell enzymes (*e.g.*, met Hb reductase, SOD, CAT, and others) encapsulated in nanometer size biodegradable polymer (polylactide or polyglycolides) vesicles has been developed [18]. Unlike the lipid vesicles, these nanocapsules could be prepared permeable to glucose and other molecules needed for metHb reduction.

Hybrid Hbs

Recently, synthetic porphinatoiron (II) complexes [*e.g.*, FepivP (Im), FecycP(His)] conjugated to a recombinant human serum albumin (rHSA) have been developed [19]. In this albumin-heme hybrid approach, as many as 8 pophinatoiron (II) complexes could be absorbed to a rHSA molecule. These albumin-heme hybrids showed reversible oxygen binding under physiologic conditions. Of these, rHSA-FecycP(Im) appears to be promising as a red cell substitute since it has shown to have a P50 similar to native erythro-

cytic Hb and intravascular circulation time greater than 36 h in anesthetized rats [20]. It would be of some advantage to use a natural plasma protein like HSA as a component of an oxygen carrier solution as it is without undue adverse physiologic effects.

Recombinant/Transgenic Hbs

With recent advances in recombinant DNA technologies, native or specifically modified Hbs have been produced from microorganisms (*E. coli*, yeast, *etc.*), transgenic plants or animals [21-23]. However, the effectiveness and economics of these approaches are yet to be revealed.

Perfluorocarbon Based Oxygen Carriers

Current PFBOCs are generally stable emulsions of one or more perfluorocarbons in aqueous media using various emulsifying agents (surfactants) such as Pluronic-68®, egg yolk phospholipids, and triglycerides. In some cases, a colloidal agent (*e.g.*, hydroxyl ethyl starch or HES) is added to balance colloidal osmotic effect. After extensive exploratory experiments with various perfluorochemicals, several perfluorocabon emulsions have been developed as potential oxygen carrying red cell substitutes and are in various developmental stages.

The first PFBOC developed was Fluosol-DA® (Green Cross Corp., Japan), a 20% (w/v) co-emulsion of perfluorodecalin and perfluorotripropylamine with egg yolk phospholipid and Pluronic-68® as emulsifying agents was approved for clinical use but withdrawn soon after introduction. Because emulsions contain much less perfluorochemicals per volume compared with pure liquids, the amount of oxygen they could dissolve is also less. For examples, breathing ambient air and with the normal arterial and venous oxygen tensions (100 and 40 mmHg, respectively), Fluosol-DA® could deliver only 0.4 mL oxygen per 100 mL. To meet the metabolic oxygen demand, the fraction of O_2 in the inspired gas (FiO_2) patients were required to breathe were 100% oxygen (*i.e.*, FiO_2 = 1.0), a situation to be avoided clinically due to the adverse effects of elevated oxygen concentration on the lungs (*e.g.*, oxygen toxicity).

Recently, Oxygent® (Alliance Corp. San Diego, CA, USA), a stable 60% (w/v) emulsion of perfluorooctyl bromide (perflubron) has been developed using egg yolk phospholipid as the sole emulsifying agent. Under normal arterial and venous oxygen tensions, Oxygent® can unload as much as 1.3 mL oxygen per 100 mL, a remarkable improvement in oxygen delivery capacity (ODC). Yet, the oxygen delivery capacity of Oxygent® is less than 30% of normal blood (5 mL O_2/100 mL blood at 15 g Hb/dL) and may still require oxygen enriched air breathing to ensure adequate oxygen delivery. This product has been tested in Phase II/III clinical trials in the USA. and other countries but the trials were suspended due to higher than expected rate of stroke in treated patients [24]. Of note, a new PFBOC formulated as stable microbubbles has been developed. It is reported to dissolve a sufficient amount of O_2 so that it can be used for ultra small volume (1/500 of typical perfluorocarbon dose) resuscitation applications [25].

Substitutes for Hemostatic Factors

Hemostatsis is a physiologic regulatory mechanism that senses vascular damage and controls bleeding to prevent further blood loss and ward off invading pathogens. Hemostasis is achieved by the concerted interactions of vascular tissues, platelets and plasma coagulation factors. Blood clot (stabilized crosslinked fibrin) forms via sequential activation of intrinsic (contact) or extrinsic (tissue factor) clotting mechanisms.

Artificial Platelets

Platelets when activated form aggregates on to the damaged blood vessels and provide an active membrane surface on which fibrin and other activated clotting factors attach forming clot plug. In thrombocytopenia (less than 20% of normal), it is generally recommended that platelets be transfused. However, platelet concentrates can only stored for 24 h and may contain transmissible pathogens. To avoid disease transmission and increase availability, artificial platelets are being developed. Artificial platelet are either particles derived from human platelets or synthetic/semisynthetic particles that express platelet surface receptors, ligands or clotting factors (*e.g.*, GPIb, GPIIb, GPIX, vWF, and fibrinogen). For example, fibrinogen coated albumin capsules and polymerized albumin particles coated with recombinant GPI1/Iia were shown to correct experimental thrombocytopenia [26,27].

Recombinant Coagulation Factors

Congenital or acquired deficiencies in coagulation factors result in coagulopathy in most humans. For these patients, transfusion of fresh frozen plasma (FFP) and/or cyoprecipitate fraction is the mainstay of current factor replacement therapy. For example, in the early 1970s, commercial factor VIII and FIX concentrates from pooled human plasma became available for treatment of hemophiliacs. However, pooled plasma could transmit infectious pathogens although these risks have decreased substantially over the last decade due to improvements in donor screening, improved test assays, and viral inactivation. In addition, transfusion of blood products causes immunologic complications including serum sickness and acquired antibodies against specific coagulation factors. These problems have lead to development of recombinant clotting factors as safer treatments for bleeding patients.

With advancement of recombinant DNA and expression technology in the past 15~20 years, recombinant coagulation factors aimed at treating patients with congenital factor deficiencies (*e.g.*, hemophilia A, B). Production of recombinant coagulation factors is complicated by requirement of efficient host-cell expression system and need for post-translational modifications. For example, FVII, FIX, and prothrombin all require post-translational γ-carboxylation for biologic activity requiring use of mammalian cell cultures such as Chinese hamster ovary cells or baby hamster kidney cells [28]. FVIII is a high MW protein (~300 kDa) which also requires post-translational glycosylation. Therefore, production of these factors is complicated and less economical than proteins that

can be produced in bacteria or yeast.

Of note, some recombinant human clotting factors (*e.g.*, Factor VIIa, VIII, and IX) have already been successfully produced and licensed for treatment of hemophiliac patients.

Artificial Antibodies

Antibodies (immunoglobulins) are essential components of blood that protect and defend against pathogen invasion and infection. Antibodies raised in animals or animal sources against specific pathogen are already in wide use to prevent/treat serious infectious diseases (*e.g.*, vaccines against meningitis, small pox, and influenza). However, although animal antibodies are effective, they could cause severe allergic reactions (anaphylactic shock). To avoid such reactions, human antibodies should be developed and used. Recent advances in genetic engineering technology enabled us to produce completely human antibodies for therapeutic purposes. For example, using a combinatorial infection technique (phage technique), a very large human Ig-Vgene library approaching 10^{11} in diversity was created with human germline V-gene segment and synthetic joining segments [29]. From this library, antibodies against a plethora of antigens could be produced with high affinities. Human antibodies could also be obtained from transgenic animals that carry a restricted set of human immunoglobulin genes. With immunization and immortalization, human hybridoma with high affinities could be obtained. Therapeutic antibodies with two antigen binding specificities (bi-specific antibodies and diabodies) can also be produced. Diabody directed against surface Ig idiotype of lymphoma and the T-cell CD3 co-receptor expressed in *E. coli* was shown to be effective in recruiting T-cells to kill lymphoma cells *in vitro* [30]. With rapid advances in genomics, proteinomics, and rational design methods, it would soon be possible to create on-demand artificial antibodies with desired specificities and affinities. Such technology would help develop prevention and treatment against fast evolving highly virulent infectious pathogens (*e.g.*, influenza and SARS viruses).

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