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A COMPREHENSIVE INSIGHT INTO ARTIFICIAL BLOOD

A. V. More *, S. Y. Sayyed, P. G. Morankar, S. R. Churi, S. S. Gawli, R. A. Karande and R. Y. Gorade

St. John Institute Of Pharmacy and Research, St. John Technical Campus, Palghar - 401404, Maharashtra, India.

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Correspondence to Author:
A. V. More

Assistant Professor,
St. John Institute Of Pharmacy and Research, St. John Technical Campus, Palghar - 401404, Maharashtra, India.

E-mail: apoorvamore8@gmail.com

ABSTRACT: In hospitals, blood transfusions are a common occurrence. Because blood-related products are limited and have a range of adverse side effects, researchers have worked on artificial blood components for nearly 40 years. It is crucial to develop effective and selective alternatives for natural blood products. Artificial blood is a useful idea in transfusion medicine wherein clearly defined compounds perform the functions of oxygen transport and distribution in the body to enhance allogeneic human blood transfusion. Several molecules are created over a long period of time to achieve this goal, and ongoing modifications are performed in the pursuit of the ideal blood substitute. Currently, perfluorocarbons (PFC) or haemoglobin derived from outdated human/bovine blood (Hemoglobin Based Oxygen Carriers; HBOC) are used to manufacture artificial blood. The focus of this review paper is to provide a comprehensive view of different developed products that can be used as blood alternatives.

INTRODUCTION: Blood is a type of connective tissue made up of white blood cells, red blood cells, platelets, and plasma. It serves several purposes in the body. Plasma is an extracellular material composed of water, salts, and various proteins that, along with platelets, promotes blood clotting. Membrane proteins react with air and harden to prevent further bleeding. White blood cells are in charge of defending the body. They seek out invading organisms or materials and work to reduce their impact on the body. The red appearance of blood is caused by red cells. One billion red blood cells can be found in just two drops of blood.

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These cells are in charge of transporting oxygen and carbon dioxide throughout the body. They are also responsible for the phenomenon of "typing". Proteins that the body recognizes are found on the membranes of these cells. As a result, a person can only use blood compatible with their type. At the moment, artificial blood products are only intended to replace the function of red blood cells. It might even be better to refer to the products being developed now as oxygen carriers rather than artificial blood [1].

History: The search for a suitable substitute for human blood began in the 17th century when Sir Christopher Wren proposed using ale, wine and opium as blood substitutes. Other substances include urine, plant resins, and sheep. Previously, blood, milk (for the treatment of Asiatic cholera), and salt solutions were tried. Blood transfusion became a safer and more established medical procedure as a result of Landsteiner's ground breaking research on the various blood groups [2, 3].

As understanding of oxygen transport and delivery of RBCs improved and the need for type-specific allogeneic transfusion became clear, the groundwork for developing artificial blood began in the early 1900s. Amberson *et al*. reported the first infusion of cell-free hemoglobin for resuscitation in a patient with postpartum hemorrhage in 1949.

Animal experiments that collected free hemoglobin by lysing red blood cells and transfusing the unmodified products resulted in renal failure, coagulopathy, complement activation, antigenicity, histamine release, iron deposition, and vasoconstriction. The presence of red cell stroma in the product was later found to be the cause of the toxicity [4-6].

Present Status: As artificial red blood cells, materials such as perfluorocarbon emulsion and modified hemoglobin have been evaluated and clinically used, but none have proven satisfactory in function and safety. Hemoglobin vesicles (HbV) are high-concentration hemoglobin encapsulated in a phospholipid bilayer hence analogous to erythrocytes, which are currently under investigation in Japan and are the safest and most promising for practical use. While effective utilization of hemoglobin from expired donated blood is being proposed, recombinant hemoglobin will most likely be used in the future.

Blood group substances, proteins other than hemoglobin, and viruses (if present) are completely removed from erythrocytes by heating and filtration during the hemoglobin purification process. Re-encapsulation utilizing a stable lipid membrane ensures that the product can be stored in liquid form for two years at room temperature (in contrast to current erythrocyte preparations, which can be stored for three weeks with refrigeration after blood drawing). When stored as a dry powder, the product can be kept for a longer period. These are widely regarded as significant advantages of the artificial blood product [7].

Need for Artificial Blood: Due to a lack of blood supply, a country's minimum bloodstock is 1% percent of the country's total population in need. In India, a total of 4 million units of blood have been collected. In the case of 10% million requirements, it can only meet 40% of the units required. Recent reports show that blood donation has increased from 4.4 million units to 9.3 million. There is a risk of chronic and infectious diseases like HIV, Hep B, Hep C, and others being transmitted during blood transfusions from one person to another, as well as other blood-borne diseases that can be prevented / stopped by the use of artificial blood. Mistransfusion can also occur due to error. For a standard blood transfusion process, the cost of collecting the blood, screening it, storing it with all requirements, and administering it is quite high [8].

Ideal Characters:

- Safe to use.
- In the human body, they are compatible.
- Capable of transporting and releasing oxygen where needed.
- Is devoid of pathogens and toxins that would produce an immune system.
- Blood substitutes, also known as synthetic blood, are available.
- Currently referred to as "oxygen carriers."
- This is due to their inability to mimic many of the other functions of blood. They are devoid of cells.
- The primary function is to replace lost blood.

Composition and Types:
Composition of Artificial Blood:

Perfluoro-octyl bromide - 28%

1. Fo-9982 - 12%
2. Yolk lecithin - 2.4%
3. DSPE-50H - 0.12%
4. Distilled water - 57.48%

Types of Blood Substitutes:

1. Perfluorocarbon (PFC) emulsions.
2. Hemoglobin based oxygen carriers (HBOC’s).

The following year, Sloviter and Kamimoto created the first physiologically adjusted PFC emulsion, allowing for an intravascular infusion of PFCs; Geyer conducted the first blood substitution experiment in which a PFC assumed the oxygen transport function in live rodents, replacing the red blood cells of rats with a poloxamer stabilized perfluorotributy lamine (FTBA) emulsion and successfully keeping the animals alive for approximately 8 hours while breathing Because it was eliminated within a week of administration, perfluorodecalin (FDC) drew more attention from the scientific community [9, 10].

Perfluorocarbon Emulsions: Scientists began their studies of artificial blood using perfluorocarbons, so these substances are regarded as the first artificial blood generation. Perfluorocarbons are colorless, clear liquids that are nontoxic, have a low boiling temperature, and are chemically inert. Its chemical structure is primarily made up of fluorine and carbon atoms. Clark first explained the perfluorocarbon oxygen-carrying ability in 1966; PFCs have cyclic or straight hydrocarbon chains. The straight shape is better at carrying oxygen than the cyclic shape. PFC has the general chemical formula C_nF_{2n+2} [11 – 14].

PFCs are synthetic and biologically inert compounds composed of fluorinated hydrocarbon aqueous emulsions capable of storing large amounts of dissolved oxygen. Because perfluorocarbons are not hydrosoluble, they are administered as perfluorocarbon emulsions. The PFC particles are about 0.2 microns in diameter (1/40th of RBC size), with a perfluorocarbon core and a thin lecithin phospholipids as a coating [15 – 17].

Perfluorocarbons are oxygen carriers that are synthesized. They are chains of eight to ten hydrocarbon molecules with fluorine replacing the hydrogen. Perfluorocarbons are chemically and biologically inert. They possess high gas-dissolving properties. Perfluorocarbons are not miscible with water and must be brought into an emulsion before use. Perfluorocarbons have a short half-life of 12-18 hours, but are only cleared from the body weeks later, preventing multiple doses in a short time span. The reticuloendothelial system initially takes up perfluorocarbons. They form droplets of emulsion, broken down and taken up by blood, which are again bound to lipids. They are then transported to the lungs and expelled through exhalation. This entire procedure takes several weeks. It is also required that oxygen be inspired to a supraphysiological level with 100 percent oxygen during product infusion because perfluorocarbons carry oxygen proportionally to the inspired oxygen. Instead of the sigmoidal curve of human hemoglobin, perflurocarbons have a linear relationship with the partial pressure of oxygen and the oxygen content. To be effective, they require a partial pressure of oxygen greater than 300 mm Hg [18, 19].

The first blood substitute approved by the FDA for phase III trials was Fluosol-DA-20, a perfluorocarbon-based product manufactured by Green Cross Corporation in Osaka, Japan. The FDA approved it for use during PTCA in 1989, to reduce myocardial hypoxia distal to the angioplasty balloon. Fluosol-DA is the only product that has received full FDA approval. Regardless, the product was withdrawn in 1994 due to its limited success, poor sales figures, the complexity of use, and side effects (anaphylaxis, pulmonary hyperinflation and interference with PMN function) [20]. To modify a human or an animal, Hb is one of the ways to create a blood substitute, which helps to solve the problem of a lack of blood replacement; another way is to use materials that are superior to Hb. As a result, fluorocarbon chemicals were developed to replace the function of Hb [21 – 22].

Advantages of PFC: The benefit of PFCs is that they do not interact with oxygen and allow for easy transportation of oxygen to the human body. The oxygen supply in plasma has increased, as has the level in plasma. Physical parameters such as pH and temperature are reduced in blood circulation, making PFCs a better option for blood circulation.

Disadvantages of PFC: Because PFCs cannot remain in the aqueous phase for an extended period, they must be prepared as emulsions. Because PFCs absorb oxygen passively, patients must breathe at a linear rate to ensure tissue oxygenation. A high fraction of inspired oxygen is required.

The platelets count in the blood is reduced, resulting in flu-like symptoms. Adverse effects of PFCs may also occur if continuous doses are consumed. It will also impair neutrophil function and trigger allergic reactions [23].

Generations of PFC:

First Generation: The first acceptable product-based PFC substance was developed in Japan and tested in the United States in November 1979. This product is referred to as "Fluosol-DA". This product contains emulsifying agents made from egg yolk phospholipid and pluronic-68. Fluosol-DA is an emulsion of Perfluoro-decaline (PFD) and perfluorotri- propylamine (FTPA) [24]. The per-fluorotri propylamine (FTPA) has a high degree of stability, and PFC is its main component and oxygen carrier. Their life spans differ from one another. PFC has a half-life of 3 to 6 hours, whereas FTPA stays in the body for months [25]. People must breathe pure oxygen through a mask or in a hyperbaric chamber for it to carry a sufficient amount of oxygen [26]. Red blood cells can carry more oxygen at 37°C than Fluosol-DA, which can only carry about 7.2 percent. This product had several side effects that caused it to be rejected, and its production was halted in 1994. These side effects include: Full activation of Fluosol-DA by emulsion factor causes a pulmonary reaction; this effect can be avoided with steroid injection; however, emulsion storage is difficult [27, 28].

Second Generation: Fluosol paved the way for further research into PFCs. Reiss and Le Blanc et al. describe the following eligible features of the second generation of PFCs:

- ✓ High ability to resolve oxygen.
- ✓ Short survival in tissue and faster secretion.
- ✓ Few side effects.
- ✓ Raising purity.
- ✓ Large existence and massive manufacturing [29].

West has focused on Perfluoro-octyl bromide ($C_8F_{17}Br$), Perfluoro-decyl bromide ($C_{10}F_{21}Br$), and Perfluoro-dichloro-octane ($C_8F_{16}C_{l2}$), which are linear perfluorocarbons, in the development of second-generation emulsions of perfluorocarbons [30].

Although oxyfluor and oxygen have been subjected to safety testing and have served as the foundation for clinical testing by authorities, most of these studies have not been declared by scientific proprietary. These products have been linked to several negative side effects, including Flu marks are attributed to the working of phagocytic cells of the retiendothelial system, so a tiny particle (sized between 0.1- 0.2 m) is not discovered by the retiendothelial system as large particles larger than 0.2 m are. They also cause the moderate temperature to rise [31, 32].

Furthermore, difficulties in introducing the functional dose of Oxyfluor management and oxegent management increase the risk of stroke. Nonetheless, it has been demonstrated that no reaction between PFCs product and blood contents occurred, except a minor change in clotting factors. PFCs cause moderate thrombocytopenia (a ten percent to fifteen percent decrease in platelets) [33 - 35]. Perfluorocarbons of the Third Generation Synthetic Blood International in Costa Mesa, California, pastures Oxycyte as a third-generation curative oxygen carrier Perfluorocarbon.

It is used to provide oxygen to the body's tissues and transport carbon dioxide to the lungs, expelling it from the body. When compared to hemoglobin, Oxycyte can carry five times as much oxygen as haemoglobin. It has been accepted for use in phase II, possibly for wound care, skill cell crisis, and heart attack. However, more research is needed to ensure that this product can be used as artificial blood. Perftoran is another Perfluorocarbons product that has been approved for human use, while others, such as PHER-O2, are still in the works [36].

TABLE 1: SUMMARY OF PFC PRODUCTS [37]

PFC product	Generation	Approval	Clinical Trials
Fluosol-DA-20	1st generation	Approved by FDA	Stop in 1994
Oxygent	2nd generation	Doesn't accepted due to its side effects	Phase III clinical trial because of its stability
Oxycyte	3rd generation	No acceptance in countries	Phase III clinical trials
Perftoran	3rd generation	Approved in Russia in 1996	For human use in Russia
PHER-O	3rd generation	NO acceptance	Pre-clinical trials

Hemoglobin-Based Oxygen Carriers (HBOC's): In the 1930s, the first effort at using a Hb-based O_2 carrier was made. HBOCs are semisynthetic systems that use natural haemoglobin (Hb) as the oxygen carrier, either in chemically modified cell-free solutions, conjugated and cross-linked with polymers and protective enzymes, or encapsulated within microparticulate or nanoparticulate vehicles [38]. The source of hemoglobin is either human, obtained from outdated stored blood or bovine blood or genetically engineered [39]. Oxygen binds covalently to these compounds as they do to naturally occurring hemoglobin. They were designed to fulfill the following purposes:

1. Inherent decreased oxygen affinity to increase tissue unloading.
2. Prolonged intravascular retention.
3. Decreased colloidal osmotic activity.
4. Absence of renal toxicity [40].

Currently, transfusions of whole blood, as well as various isolated components, are clinically approved for applications in civilian and battlefield trauma (*e.g.,* in Damage Control Resuscitation), surgical settings (*e.g.,* transplants), chronic and acute anemia's, and disease-associated, drug-induced or congenital bleeding disorders [41].

Hb-based oxygen carriers (HBOCs) provide the same excellent oxygen-carrying properties as red blood cells while overcoming some drawbacks of donor blood. RBCs are made up primarily of haemoglobin (Hb), the molecule responsible for oxygen delivery. Intravascular administration of free Hb, on the other hand, has serious side effects, which have been linked to its dissociation into dimers, which causes too-short circulation periods and renal toxicity. Due to their small size, Hb molecules can also extravasate across capillary walls, where they operate as nitric oxide (NO) scavengers. Because NO is a vasodilator, its scavenging results in vasoconstriction, systemic hypertension, and higher mortality rates [9, 42]. As a result, much effort has been concentrated on producing Hb-based oxygen carriers (HBOCs) to avoid Hb's toxicity while using its superior oxygen-carrying and delivery characteristics. Because they have potentially unlimited availability, are compatible with all blood types, present no risk of disease transmission, can be prepared in sterile conditions, and have a long storage shelf life, these semi-synthetic systems can overcome most limitations of donor blood. To date, HBOCs have mostly been made by chemically altering Hb (such as cross-linking, polymerization, or conjugation to polymers) or encapsulating it within a carrier vehicle. The later method attempts to replicate the biological condition in which Hb is contained within the RBC membrane [43].

While chemical modification of Hb can stabilize the tetramer, covalent modification can impair cooperativity, decreasing the tetramer's capacity to bind and release oxygen. On the other hand, encapsulation platforms protect Hb by avoiding contact with external stimuli and other blood components while allowing other compounds to be included in the carrier system. Hb has been effectively encapsulated within liposomes, polymersomes, hydrogels, and metal-organic framework-based particles, among other encapsulation platforms currently being studied [44].

Manufacturing processes of HBOC involve the extraction of hemoglobin and thereafter stabilisation with cross-linking as tetramers or polymerization (using glutaraldehyde or o-raffinose), or conjugation with polyethylene glycol or encapsulation in phospholipid vesicles before mixing into an electrolyte solution. The Hb is isolated from outdated human or bovine RBCs using cell lysis, purified using sterile filtration and chromatographic methods, and sterilized (*e.g.,* by low heat). HBOC can be stabilized by cross-linking or polymerization with bigger molecules like polyethylene glycol, dextran, or polyoxyethylene, which increases the intravascular "dwell" period significantly (24 - 48 hours). Newer molecules are being developed with antioxidants like superoxide dismutase or catalase cross-linked to the haemoglobin structure. Incorporating antioxidants help reduce the severity of ischaemic reperfusion injury in conditions like stroke, myocardial infarction or organ transplantation.

The following groups of hemoglobin solutions are available based on strategies for enhancing stability:

Surface Modified Haemoglobin (PEG Hb, PHP, Haemospan): They're made by attaching large molecules like polyethylene glycol to lysine groups on the surface. The solution's viscosity and oncotic pressure are increased due to this modification. These solutions have a moderate tendency to elicit vasoconstriction. Because of their small size, haemoglobin molecules can pass through small vessels and oxygenate areas that RBCs can't reach. As a result, they can be used to treat stroke patients, increase tumour cell susceptibility to radiation or chemotherapy, and the vasopressor effects can be used to treat hypotension following septic shock. Antibody generation is also less common among them.

Intramolecular Cross-Linked Haemoglobin (Hemassist, r-Hb1-1, r-Hb 2-0): The tetrameric stabilization and prevention of renal filtration is accomplished by intermolecular cross-linking between the two α and the two β subunits using a site-specific crosslinker which cross-links the two α or the two β chains. The affinity of haemoglobin for oxygen is also reduced by cross-linking. 3,5-dibromosalicyl fumarate (DBBF) and nor-2-formylpyridoxal 5-phosphate (NFPLP) are two commonly utilised cross-linkers.

Polymerized Haemoglobin (Polyheme, Hemopure, Hemolink): Reagents like glutaraldehyde are used to link the surface amino acid groups. This compound has been reported to have negligible negative effects. This substance has been transfused multiple times on humanitarian use basis. In patients having infrarenal aortic reconstruction, polymerized haemoglobin administration resulted in a 27 percent reduction in allogenic blood transfusion.

Liposomes Encapsulated Haemoglobin: Purified haemoglobin is re-encapsulated in a lipid membrane that is stable. The liposomes contain a stroma-free haemoglobin solution and 2,3 diphosphoglycerate or inositol hexaphosphate as a gelatinous fluid and are composed of phospholipid bilayers with cholesterol molecules added for increased rigidity and mechanical stability. UV light or redox inhibitors are employed to produce polymerization of unsaturated phospholipids for enhanced stability. Liposomes can also be stabilized by coating them with polymers.

The physiochemical characteristics and circulation lifespan of encapsulated haemoglobin (also known as haemosomes) can be controlled. Modifications of the liposomes can be used to change the P50 of the modified haemoglobin, allowing for easier oxygen unloading. Co-encapsulating, an allosteric effector like pyridoxal 5'-phosphate, can change oxygen affinity. Negatively charged lipids are often seen in their capsules, which prevents them from aggregating.

Encapsulation also prevents hemoglobin denaturation and improves biodistribution. Polyethylene glycol modification extends their half-life, makes them water soluble, reduces antigenicity and improves site-specific targeting. These vesicles are immune-compatible since they are made up of pure haemoglobin or lipids. Hemoglobin nanocapsules composed of biodegradable polymers such as polylactide have been developed. Polylactide is broken down in the body into water and carbon dioxide, thus, it doesn't build up in the reticuloendothelial system.

Limitations of HBOC's: Before widespread support for HBOC's, a number of considerations must be considered. RBCs have no colloidal osmotic pressure, although haemoglobin (and other plasma proteins) have it. As a result, cellular haemoglobin can change intravascular volume and operate as a plasma expander. The circulation half-life of HBOC is less than that of regular RBCs. Most HBOC stays in the bloodstream for 20 - 30 hours, whereas whole blood transfusions last 34 days.

They produce free radicals in the body by releasing free haemoglobin and breakdown products such as haem and iron. HBOC's oxidative characteristics cause methemoglobin concentrations to increase. The most common source of haemoglobin is outdated human blood, which is in limited supply. As a result, bovine blood must be used to obtain haemoglobin. Bovine haemoglobin has the possibility to harbour the prion pathogen that causes bovine spongiform encephalopathy (BSE) (Creutzfeldt-Jakob disease). To tackle this difficulty and secure a consistent supply of haemoglobin in the future, scientists attempted to genetically engineer bacteria to produce a recombinant source of human haemoglobin.

Recombinant Haemoglobin (Optro): In species like *E. coli* and yeast, recombinant DNA technology can be used to produce modified haemoglobin. To prevent disassociation into dimers and maintain oxygen affinity, some portions of the amino acid sequence of human haemoglobin are altered. The haemoglobin gene is then introduced into *E. coli* cells using a plasmid vector.

The expression of these genes triggers the production of haemoglobin proteins. This method reduces the risk of disease transmission *via* haemoglobin derived from human or bovine sources. However, the high expense of these procedures is a serious obstacle.

Advantages of HBOC's: They are available in larger quantities and can be sterilized using the pasteurization method. It can be stored for a long time. It can be administered rapidly, even without typing or cross-matching. They're also useful in the military.

Benefits of HBOC's: It is more efficient and effective at distributing oxygen. It has a long shelf life. There is no requirement for refrigeration. All receptors are universally compatible with it. It's one of the ready-to-use products. It immediately offloads oxygen after injection.

Disadvantages of Hb-based Oxygen Carriers: In humans, they have not been found to be safe. It has a shorter half-life in circulation. It is chiefly responsible for the release of free radicals into the body. Certain physiological structures, particularly the gastrointestinal tract, are disrupted.

Adverse Effects of Hb-based Oxygen Carriers: Renal toxicity, neurotoxicity, platelet aggregation, antigenicity, and pancreatic and liver enzyme elevation are all side effects. GI side effects, hyperactivity/hypertension, nephrotoxicity, coagulopathy, and immunological suppression are also common [45].

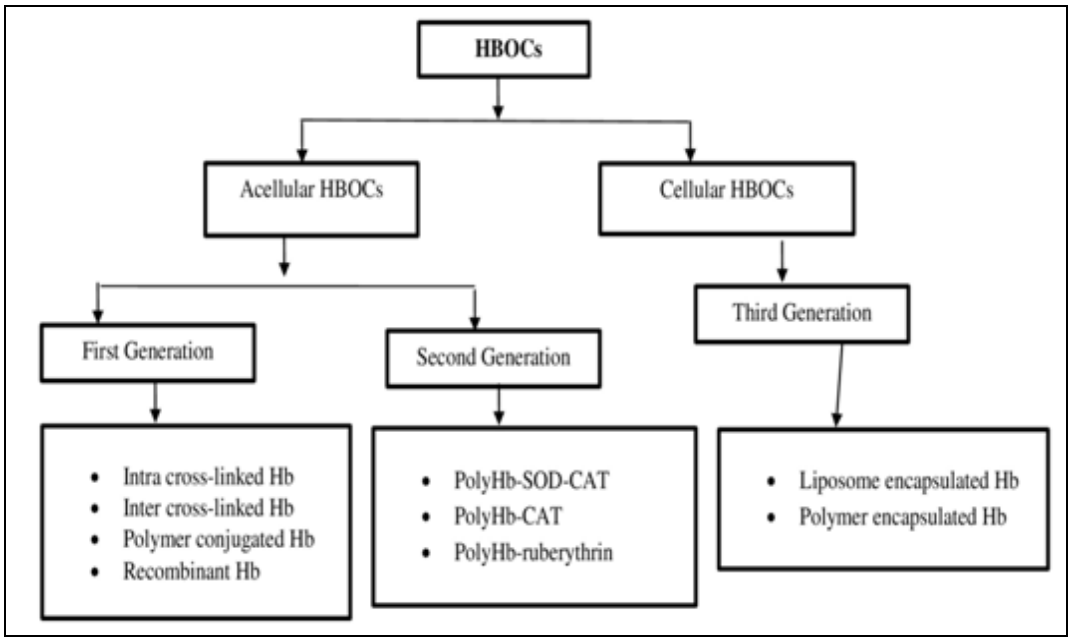

FIG. 1: CLASSIFICATION OF HBOC's

Developed Products: The first generation of blood substitutes produced were the stroma-free hemoglobin (SFH) products. SFHs were prepared by lysis of packed RBCs forming soluble hemoglobin. This mixture was then centrifuged to remove the bulk of the red cell stroma, producing SFH. SFH's could be prepared by either ultrafiltration or crystallization. SFH prepared by ultrafiltration was characterized by a substantially lower content of residual membrane phospholipids and a more restricted protein composition. This preparation was also free of vasoconstrictor and contractility-depressant actions on the *ex-vivo* perfused heart. In contrast, crystallization-produced SFH resulted in a less well-purified product of both phospholipid and protein constituents. Thus, it was likely to generate denatured protein aggregates during storage and exhibited vasoconstrictor and

contractility-depressant activity, which could vary significantly from batch to batch. These findings indicated that preparative methodology based on ultrafiltration and size exclusion yielded SFHs that were superior to those produced by the crystallization method. The second-generation HBOC's were pyridoxilated hemoglobin-polyoxyethylene conjugates (PHPCs) prepared through the chemical modification of SFH. These products have been designed to prevent the major disadvantages of SFHs reported in multiple studies: increased O_2 affinity, short circulatory half-life, and nephrotoxicity. PHPC is prepared after acquiring SFH; the free hemoglobin is pyridoxilated (*i.e.*, the addition of vitamin B6) to adjust the O_2 affinity and later conjugated with α-carboxymethyl-ω-carboxymethoxy-polyethylene to increase the molecular weight and ensure a longer circulatory half-life.

The O_2 affinity of such cross-linked hemoglobin was found to be higher (*i.e.,* lower P50, the PO_2 at which hemoglobin is half saturated with O_2) compared to native human RBCs, and it remains in a deoxy (T) state with the utilization of 2,3-DPG analogs. The second-generation HBOCs that have been developed are PolyHeme® (Northfield Laboratories, Evanston, IL), Hemopure® (also referred to as HBOC-201; Hemoglobin Oxygen Therapeutics LLC, Souderton, PA) and HemoLink™ (Hemosol Inc, Mississauga, Canada). However, many of these products have been discontinued owing to the observed adverse events in clinical trials.

Hemopure, however, is approved by the South African drug council for the treatment of anemia dating back to 2001, and the product is available in the United States for qualified patients (such as Jehovah's Witnesses who do not accept blood) with life-threatening anemia under the FDA's expanded (compassionate use) access program. The third-generation blood substitutes include hemoglobin cross-linked between the α chains with bis(dibromosalicyl) fumarate (DBBF) or αα-hemoglobin. This product was developed at the Letterman Army Institute of Research (LAIR) in San Francisco, CA. Similarly, Baxter International Corporation (Deerfield, IL) developed HemAssist®, adiaspirin-crosslinked hemoglobin (DCLHB). These developed products were more homogenous to support the major toxicological and physiological experiments and have O_2 affinity similar to that of blood. They were made using outdated human or bovine blood, washing the RBCs with sterile saline to remove all the traces of plasma and then subjecting them to hypertonic lysis. The remaining membrane material was filtered out, allowing the purified hemoglobin to be put toward the cross-linking reaction. To maintain specific cross-linking between lysine 99α residues, an allosteric effector (2,3 DPG pocket) was added to keep the hemoglobin in the deoxygenated (T) state. In the final stage, the reagent DBBF was added; this mixture was then heated to remove unreacted hemoglobin and pathogens. The final product was formulated in either Ringer's lactate or Ringer's acetate and could be stored frozen at -20^{o}C for up to one year. These products, however, cannot regulate the oxidative state caused by iron in their heme group, and their phase III clinical trials have shown increased mortality with their use.

Limitations: As mentioned above, several serious adverse events have resulted in the premature termination of HBOC clinical trials. The following is a succinct description of these key limitations. Vasoconstriction leading to tissue hypo-oxygenation and subsequent systemic hypertension and pulmonary hypertension is the most feared limitation of HBOCs. Studies have shown that NO-scavenging by free hemoglobin has been the major factor mediating vasoconstriction. NO regulates endothelial mechanisms of smooth muscle relaxation by limiting the production of a vasoconstrictor known as endothelin. HBOCs are carried in the plasma and, therefore, free to cross through the endothelium; thereby this free hemoglobin has the potential to consume larger amounts of NO to regulate the vascular smooth muscle tone. Other proposed mechanisms of HBOC-induced vasoconstriction include the release of norepinephrine from adrenergic regulation of the peripheral nerves and neurally-mediated distortion of chronotropic and inotropic myocardial responses and the disturbed neural response in maintaining smooth muscle tone. Vasoconstriction to a certain extent has been noted to be less severe in polymerized hemoglobin preparations.

The phenomenon of vasoactivity and dysregulated smooth muscle tone may manifest clinically in the form of gastrointestinal distress, flu-like symptoms, and nephrotoxicity. Compared to RBCs, HBOCs lack the remarkable free-radical scavenging system, which includes enzymes superoxide dismutase and hemoglobin reductase. Moreover, free hemoglobin can participate in a number of reactions that could potentially produce toxic free radicals. The iron in free hemoglobin can oxidize to form methemoglobin (HFe^{3+}) which reacts with NO to disturb the regulation of smooth muscle tone. Also, HBOCs can induce dysregulation of other physiologic functions at the vascular endothelium by extracellular hemoglobin. Free hemoglobin gets oxidized and produces unneutralized H_2O_2, which oxidizes ferrous and ferric hemoglobins to Fe (IV)-ferryl hemoglobin and oxyferryl hemoglobin, respectively.

Ferryl hemoglobin can react with H_2O_2, yielding free iron and other heme degradation products. Fe (III) hemoglobin produced during hemoglobin autoxidation also readily releases heme, an additional source for oxidative stress and oxidative reactions in the plasma. The pro-inflammatory effects of heme and oxyferryl have also been shown to increase the risk of further oxidative stress. Methemoglobin is also the degradation product of hemoglobin, the production of which is physiologically held in control by the enzyme methemoglobin reductase in the erythrocyte.

Therefore, HBOCs have the propensity to produce highly-oxidative free radicals, capable of inducing cell damage and halting other biochemically essential processes. Substances do not normally cross the intact blood-brain barrier unless there is injury or a diseased state in which the sanctity of the blood-brain barrier is compromised. For example, in the case of head injury, cerebral hemorrhage, or ischemic injury to the brain, free hemoglobin in the HBOCs may cross the barrier and act as a potential neuro-excitatory toxin. In addition, HBOCs may cause reperfusion injury due to their high O_2 content [46]. One of the successful examples of HBOC is called Erythromer, which was developed in 2016. While previous HBOCs had failed to deliver oxygen with minimal toxicity efficiently, Erythromer was able to emulate natural red blood cells by encapsulating purified haemoglobin within nanoparticles. Using a novel 2,3-Bisphosphoglyceric acid (2,3-DPG) shuttle, it could control oxygen capture and release effectively. It is responsive to pH change, and its affinity for oxygen shifts at different locations in the body, ensuring optimal oxygen transfer. Previous HBOC had been shown to trap the vasodilator NO and cause vasoconstriction, but Erythromer used a novel polymer shell that does not interfere with vascular tone.

Moreover, it offers two more advantages over blood transfusion. First, it can be freeze-dried and converted into a solid powder. Thus, it is capable of long-term storage, and it has a prolonged shelf-life. After 3 months of dry storage, less than 10% change was observed in its properties such as size, zeta potential, and polydispersity.

It is a significant advantage over normal blood, which has a limited shelf life. For example, red blood cells can be stored for at most 35 days. Secondly, it does not require blood typing, which means that one formulation is universal for all humans regardless of blood type. This offers benefits over usual blood transfusion in situations such as emergencies and a lack of necessary equipment, where the patient's blood type is unknown. Initial *ex-vivo* and *in-vivo* studies have shown that Erythromer has overcome challenges in mimicking red blood cells and can perform normal red blood cell activities. Erythomer has shown positive results in rat studies but to ensure its safety, larger-scale *in-vivo* studies such as rabbits or human clinical trials are required [47]. Phospholipid vesicles or liposome encapsulating concentrated human Hb (Hb-vesicle, HbV) have been developed as O_2 carriers. The HbV cellular structure (particle diameter, ca. 250 nm) has characteristics similar to those of natural RBCs because both have lipid bilayer membranes that prevent direct contact of Hb with blood components and the endothelial lining. Reasons for the Hb encapsulation in red blood cells (RBCs) include:

A decreased high viscosity of Hb and a high colloidal osmotic pressure. Prevention of Hb removal from the blood circulation and preservation of the chemical environment in RBCs, such as the concentration of phosphates (2,3-DPG,

ATP, *etc*.) and other electrolytes [48]. Hemoglobin transports oxygen from the lungs to the rest of the body's tissues. Hemoglobin-based artificial blood takes advantage of this natural function. Unlike PFC compounds, which rely on dissolving, oxygen forms a covalent bond with hemoglobin. Because these hemoglobin products are not contained in membrane-like whole blood, the blood type problem is eliminated. Raw hemoglobin, however, cannot be utilised since it will break down into smaller, toxic compounds in the body. There are also issues with haemoglobin stability in a solution. The challenge in developing artificial blood based on haemoglobin is to alter the haemoglobin molecule to eliminate these issues. Hemoglobin is stabilised *via* a variety of methods. Chemically cross-linking molecules or employing recombinant DNA technologies to generate modified proteins are options. In solutions, these modified hemoglobins are stable and soluble. These alterations should theoretically result in products that can carry more oxygen than our own red blood cells. The first of these products is expected to be available within one to two years. Several companies are now working on the development of a safe and effective artificial blood substitute. All of the blood substitutes have their own set of restrictions. Most hemoglobin-based products, for example, only last 20 to 30 hours in the body. This compares to entire blood transfusions, which last 34 days. Furthermore, these blood substitutes do not have the same ability as blood to fight diseases and clot. As a result, present artificial blood technologies will only be useful for short-term blood replacement. New materials to carry oxygen in the body are expected to be discovered. Longer-lasting products, as well as products that perform blood's other functions, should be developed [49]. Most HBOCs can no more last than 20-30 hrs. Thus, these are called as short-term blood transfusions. As a result, longer-lasting products that can run over a body for extended periods of time will be developed in the future, and this advancement will have a larger range of applications [50]. Currently, none of the products that have been developed to date have been successful in getting FDA approval for use in clinical settings due to discouraging adverse events during phase II and III studies, though hemoglobin-based oxygen carrying (HBOC) agents have been approved and used clinically in two countries outside of the United States (Russia and South Africa).

TABLE 2: SUMMARY OF HB BASED PRODUCTS [51, 52]

Type of HBOC	Product Name	Biogenesis
Cellular Hb-based oxygen carriers	Neo Red Cell	Haemoglobin
Cellular Hb-based oxygen carriers	Hemoglobin vesicle (Hb V)	Carbonyl Human Haemoglobin
Cellular Hb-based oxygen carriers	liposome-encapsulated hemoglobin (LEH)	Outdated human RBC
Cellular Hb-based oxygen carriers	polymer-encapsulated hemoglobin (PEH)	Human and Bovine haemoglobin
Acellular Hb-based oxygen carriers	Hemopure	Glutaraldehyde bovine Haemoglobin
Acellular Hb-based oxygen carriers	Diaspirin cross-linked Hb (DCLHb) or HemAssist	Human hemoglobin
Acellular Hb-based oxygen carriers	Oxyglobin	Bovine hemoglobin
Acellular Hb-based oxygen carriers	PolyHeme	Glutaraldehyde, pyridoxal human Hb

CONCLUSION: In the event of an emergency patient during surgery when blood loss is greater, artificial blood will prove to be the most reliable substitute for blood transfusion. Although it does not substitute blood, it does help to take up space and carry some important gases. It is also known as blood surrogate or blood substitute. As it transports oxygen to tissues, it can sustain life until patients' own red blood cells can be generated again or until they can get a transfusion of banked blood. It will prevent issues with misleading negative findings from natural blood. HBOCs have the capacity to perform a dual role in addition to the basic physiological function of blood, such as assisting a therapy or attacking various pathogens. In the future, lab-grown synthetic blood can revolutionize medical care by providing a far-reaching solution to keeping people in need supplied with blood regardless of type or donor. To assess the scalability to ensure manufacturing reproducibility, consistent composition and, safety, and therapeutic benefits, extensive *in-vitro* and *in-vivo* testing of various approaches is necessary. A coordinated effort involving biomedical and materials engineers, haematologists, immunologists, and the necessary regulatory agencies is required to achieve the desired result. Although human trials for synthetic blood are about to commence, there is

still a long way to go before it can be used on a large scale. Admittedly, humanity's future could be changed by these advancements.

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