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Review

A new era in oxygen therapeutics? From perfluorocarbon systems to haemoglobin-based oxygen carriers

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ABSTRACT

Blood transfusion is the key to life in case of traumatic emergencies, surgeries and in several pathological conditions. An important goal of whole blood or red blood cell transfusion is the fast delivery of oxygen to vital organs and restoration of circulation volume. Whole blood or red blood cell transfusion has several limitations. Free haemoglobin not only loses its tetrameric configuration and extracts via the kidney leading to nephrotoxicity but also scavenges nitric oxide (NO), leading to vasoconstriction and hypertension. PFC based formulations transport oxygen *in vivo*, the contribution in terms of clinical outcome is challenging. The oxygen-carrying capacity is not the only criterion for the successful development of haemoglobin-based oxygen carriers (HBOCs). This review is a bird's eye view on the present state of the PFCs and HBOCs in which we analyzed the current

Abbreviations: ATP, adenosine triphosphate; B-PEG-Hb, bovine pegylated-haemoglobin; bis-Mal-PEG2000, bis(maleidophenyl)-PEG2000; CO, carbon monoxide; CO_2, carbon dioxide; DBBF, bis-(3,5-dibromosalicyl)-fumarate; deoxyHb, deoxyhaemoglobin; DPG, diphosphoglycerate; DPPC, 2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine; EAF, extension arm facilitated; E. coli, Escherichia coli; FDA, Food and Drug Administration; GU-HP-Hb, glutaraldehyde-polymerized human placenta haemoglobin; Hb, haemoglobin or hemoglobin; HBOC, haemoglobin-based oxygen carrier; HBOCs, haemoglobin-based oxygen carriers; HIF-α, hypoxia-inducible factor 1-alpha; HIF-β, hypoxia-inducible factor 1-beta; HO-1, heme oxygenase-1; IgM, immunoglobin M; LHb, liposome-encapsulated haemoglobin; MAP, mean arterial pressure; MNBs, micro-nanobubbles; $MnCO_3$, manganese carbonate; MP4CO, pegylated human haemoglobin-based carbon monoxide; mPEG-PLA-mPEG, methoxy poly(ethylene glycol)-b-poly(*L*-lactide); N_2, nitrogen gas; NFPLP, 2-*nor*-2-formylpyridoxal phosphate; NO, nitric oxide; O_2, oxygen gas; O-R-Hb, O-raffinose cross-linked haemoglobin; oxyHb, oxyhaemoglobin; p50, oxygen half-saturation; PC, phosphatidylcholine; PEG, poly(ethylene glycol); PEG-COHb, carboxy form of poly(ethylene glycol)-haemoglobin; PEG-DSPE, 1, 2-Distearoyl-sn-glycero-3-phosphoethanolamine-poly(ethylene glycol); PEG-Hb, poly(ethylene glycol)-haemoglobin; PFC, perfluorocarbon; PFCs, perfluorocarbons; PLA, polylactic acid; PLGA, polylactic-co-glycolic acid; PolyHeme®, human polymerized haemoglobin; PPHb, polynitroxylated PEGylated haemoglobin; pPolyHb, glutaraldehyde-polymerized porcine haemoglobin; PS, phosphatidylserine; RBC, red blood cells; rHb, recombinant haemoglobin; rHb 1.1, first generation of recombinant haemoglobin; rHb 2.0, second generation of recombinant haemoglobin; TLR4, toll-like receptor 4; Val, valine; ZO-1, zonula occludens-1 or tight junction protein-1..

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modifications made or which are underway in development, their promises, and hurdles in clinical implementation.

1. Introduction

Considering the use of donated blood products is relatively safe nowadays, there are some inherent problems in allogeneic blood transfusions. Transmission of infectious diseases, compatibility issues, cost of blood processing, wastage during storage over extended period of time, sterilization, post-operatory complications, immunosuppression due to transfusions, requirement of an extremely skilled person for transfusion, use of glycerol to store the blood cells, and eventually low availability of units when disaster strikes are just some of the reasons that have encouraged decades of the search for alternatives to whole blood transfusion [1–6]. Furthermore, it is crucial to restore the blood volume immediately in case of loss of 30 to 40% of the blood volume. This ensure that the oxygen support to the tissue does not compromise. The whole blood transfusion is naturally the first choice to replace the lost blood, because it matches all the natural components which are normally present in the blood. However, because of the obvious concerns of the immediate availability and safety issues, efforts are now being invested to produce artificial oxygen carrier that can substitute and restore the normal blood functions.

Artificial oxygen carriers have emerged as an alternative to allogeneic blood transfusions. It decreases the risk of disease transmission and avoids not only incompatibility problems, but also transports and delivers oxygen to organs and tissues and acts as an anti-ischemic agent in a variety of pathogenic conditions that compromise tissue oxygenation [7,8]. Ideally the blood substitute should not trigger immune response and should not transmit infections. Furthermore, it should be easy to make, readily available, not depend on the availability of the whole blood, have the long half-life, and be stable at room temperature and above all, should be universally acceptable to be use in emergencies.

Artificial oxygen carriers require haemoglobin (Hb), which is generally sourced from bovine or humans. Other types of oxygen carriers without haemoglobin, like perfluorocarbon (PFC) emulsions, have been examined for their oxygen delivery capacity. However, some formulations are found to be potentially toxic to the renal system and have been associated with immune system inhibition and increased pulmonary and systemic blood pressure, gastrointestinal irritability, and inefficient blood supply to the tissues [9]. Additionally, oxygen carriers are usually called blood substitutes, although these compounds do not replace all its components and do not cover all blood functions, such as nutrient transport, coagulation, or immune response [10–13]. They are synthetic solutions with the ability to bind, transport, and deliver oxygen to any tissue or organ that needs it. However, these characteristics are not enough for medicinal and clinical uses. Ideally, these systems should not interfere with capillary circulation or interact with the immune system; instead, they should have the ability to access all areas of the human body, be metabolized and eliminated quickly, maintaining adequate blood pressure [14]. Based on its intrinsic characteristics, as discussed earlier, oxygen carriers are categorized into haemoglobin-based oxygen carriers (HBOCs) and perfluorocarbons (PFCs). In warm-blooded animals, the modified Hb method (i.e., HBOCs) follows the natural way of oxygen delivery to the tissues. This approach is based on the reversible binding of the diatomic oxygen molecule to the metal-centered coordination complexes. Whereas in the perfluorocarbons approach, oxygen is dissolved in the inert perfluorocarbons in the presence of the emulsifying agent.

Nevertheless, substantial efforts and resources are required to develop a product to address the global shortage of the blood and safety issues of PFCs and HBOCs. Efforts are also required to refine the existing products and develop next generation products based on the existing ones.

Because the research in the development of the oxygen transporters mostly address the clinical issues involving emergencies, surgeries, and very sick patients, it is therefore not very surprising that insufficient efforts are directed towards resolving the performance issues of these products. Therefore, in the present article we critically reviewed the available artificial oxygen transporters, their pre-clinical development, clinical performance, potential adverse effects, and future directions.

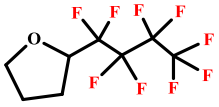
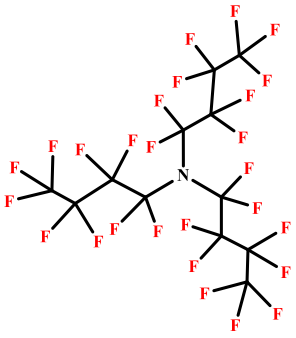
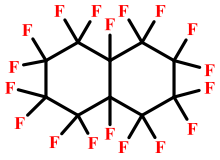
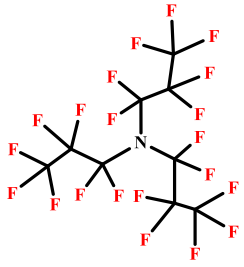
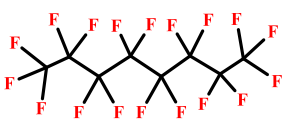
2. Perfluorocarbon's (PFCs) derivatives

Due to their chemical and physical properties, perfluorinated compounds have a long history of industrial and biomedical applications [15]. Their industrial and commercial applications includes, refrigerant agents, aerosol propellants, foam-blowing agents, solvents, polymers, and even in fire extinguishers. In medicine, fluorine-containing compounds are used in orthopedic implants, replacement for vascular structures, inhalation anesthetics (one of the most important contributions considering that prior to 1940 commonly used anesthetics were inflammable compounds e.g. cyclopropane and diethyl ether), anti-inflammatory agents, synthetic drugs and steroids [16–21]. Chemically, PFC liquids contain chains of 8 to 10 carbon, where hydrogen atoms are completely replaced with fluorine atoms to get C-F polar bonds. These liquids have special characteristics like water immiscibility, chemical inertness, higher density than water, and comparatively higher solubility for respiratory gases, making them a unique vehicle to deliver respiratory gases. Because of these characteristics, PFC emulsions in normal saline solution have been extensively studied over the past six decades as an artificial oxygen-carrier vehicle. PFC emulsions have the potential to replace Hb to supply oxygen to vital organs in case of an emergency. The discovery that PFC fluids can largely solubilize gases such as oxygen (O_2) and carbon dioxide (CO_2) led to their evaluation as vehicles for the transport of respiratory gas [22–25]. Moreover, PFCs were the first synthetic compounds tested as oxygen carriers [26].

PFCs compounds are halogenated molecules obtained from linear, cyclic, or polycyclic anthropogenic hydrocarbons. Common PFCs are chemically inert, extraordinarily hydrophobic, and stable at elevated temperatures. These materials also present high chemical resistance and low coefficients of friction. These characteristics are observed because the fluorine nucleus, being the most electronegative of all elements, has a high ionization potential energy, considerably larger electron affinity, low polarizability, and van der Waals interactions, which dramatically change the stability, lipophilicity, and bioavailability of the resulting compound, when compared to hydrogen [27–31].

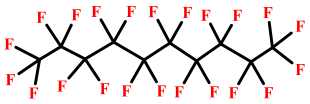
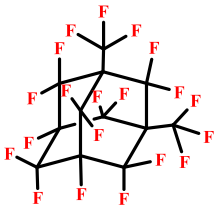
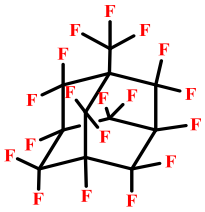
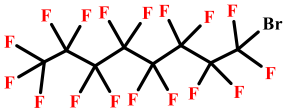
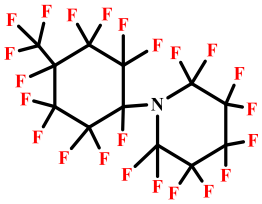
Single carbon-fluorine C–F bond is the strongest single bond in organic chemistry (147 kJ/mol and 170 kJ/mol stronger than C–C and C–Cl respectively) being able to strengthen adjacent aliphatic bonds, e.g., aliphatic C–C bond in hexafluoroethane is 42 kJ/mol stronger than the same bond in ethane molecule [28]. As a result of all the fluorine characteristics discussed above, PFCs backbone adopts a helical chain orientation, with C-F dipoles distributed axially around the chain helix, rather than the usual planar zigzag configuration observed in hydrocarbons [29–31]. The capacity of PFCs to dissolve large amounts of gases is explained due to the absence of accessible low energy molecular orbitals capable of binding gases as O_2, CO_2, N_2, or NO. As denser fluorine atoms generate a repellent sheath that covers and protects the perfluorinated backbone against reagents, gases occupy intermolecular spaces within the PFC. Moreover, the solubility of O_2 in PFCs is inversely related to temperature and increases linearly with a partial pressure, in contrast with the sigmoid curve of O_2 in Hb, so the amount of gas dissolved depends upon the PFC concentration and its solubility coefficient for the gas [32]. Due to their extreme hydrophobicity, PFCs are not

Table 1
Available PFCs: characteristics and their uses.

Names	Chemical structure and formula - Molecular weight (MW) - CAS	Formulation	Uses
Perfluorobutyl tetrahydrofuran, FX-80, FC-80, F-2-butyl Tetrahydro Furan	 $C_8H_7F_9O$ MW: 290.13 CAS: 26446-59-3	Initially pure, used as an emulsion afterwards (20% PFC) with Krebs-Ringer bicarbonate buffer solution, using bovine serum as surfactant.	Used pure in the first experiment (Clark) and by Sloviter as an emulsion afterwards, solving the problem with salts and metabolites transportation [41].
Perfluoro tributylamine, FC-47, F-tri-*n*-butylamine, FTBA, F-43, Fluosol-43, Oxypherol ®	 $C_{12}NF_{27}$ o $N(C_4F_9)_3$ MW: 671.09 CAS: 311-89-7	Emulsion (12% PFC) with Pluronic F-68 as surfactant.	Highly stable. Used in experiments with rats, completely replacing blood with the emulsion; retained in the liver. Limited animal survival [41].
Perfluoro decalin, Fluosol-DC, F-decalin, PFD	 $C_{10}F_{18}$ MW: 462.08 CAS: 306-94-5	Emulsion (10% PFC) with Pluronic F-68 as surfactant.	Used in experiments with monkeys; has a better and faster excretion [41].
Perfluoro tripropylamine, FTPA, F-tripropyl amine	 C_9NF_{21} o $N(C_3F_7)_3$ MW: 521.07 CAS: 338-83-0	Emulsion based on Fluosol-DC, with 30% total PFC.	Improved emulsion stability, longer half-life in organs. Had to be frozen for shipping and reconstituted prior to use [42].
Perfluoro-*n*-octane, PFO, octadeca fluorooctane, FC-77	 C_8F_{18} MW: 438.06 CAS: 307-34-6	Emulsion with Pluronic F-68 or Vitrum egg phospholipid.	Used to test perfusion media in dogs, retained in liver and kidney. Later, used to maintain viability for up to 37 days of fish sperm, promoting oxygen and/or nutrient uptake [43,44].

(*continued on next page*)

Table 1 (*continued*)

Names	Chemical structure and formula - Molecular weight (MW) - CAS	Formulation	Uses
Perfluoro decane, PFD	 $C_{10}F_{22}$ MW: 538.07 CAS: 307-45-9	Emulsion with Pluronic F-68	Used to test perfusion media in dogs and rats [43].
F-dimethyl adamantane, FMD, PP-9, F-DMA, F-1,3-DMA	 $C_{12}F_{20}$ MW: 524.11 CAS: 36481-20-6	Lecithin emulsified	Long tissue residence, initially tested to prevent central nervous tissue ischemia, increasing oxygen delivery [45].
F-methyl adamantane, PFDMA, Perfluoro (1-methyl adamantane), F-MA	 $C_{11}F_{18}$ MW: 474.09 CAS: 60096-00-6	Emulsion with Vitrum egg phospholipid	Initially used in the preclinical test as an oxygen carrier. Shorter tissue residence than F-DMA. Highly stable under 4 °C [46].
Perfluoro octylbromide, PFOBT, Perflubron	 $C_8F_{17}Br$ MW: 498.96 CAS: 425-25-2	Emulsion with lecithin	Can be stored in cold for 2 years. Increases blood oxygenation levels on tissue. Associated with improved myocardial recovery post-bypass [47].
Perfluoro-*N*-(4-methylcyclohexyl)-piperidine, PFMCP, FMCP, Perftoran, Ftorosan	 $C_{12}F_{23}N$ MW: 595.09 CAS: 86630-50-4 (PFC), 99752-82-6 (Emulsion)	Emulsion with 11%-14% of PFC (PFMCP + perfluorodecaline) with poloxamer as emulsifier and F-68 as surfactant.	Initially was an emulsion with 15.2% of perfluorodecaline (PFD) and 7.6% of PFMCP. Extensively used clinically in Russia, Mexico, South Africa, Kazakhstan, Ukraine, and Kirghiz Republic. Later was changed to 14% of PFD and 6% of PFMCP; stable for 1 month at low temperature; half-life of 90 days in human organs [48].

miscible in water or plasma, low molecular weight PFCs are gaseous and can be aspirated, but higher molecular weight PFCs are generally liquids that must be emulsified for *in vivo* applications [33]. Nowadays, because of the well-established synthetic routes that allow the production of PFCs and availability of tested emulsifying agents, it is possible to generate a stable PFC nanoemulsion.

As PFCs deliver oxygen due to its gases solubilizing ability, their delivery capacity is relative to the arterial oxygen pressure, and to be effective, it needs high arterial oxygen pressure (> 300 mmHg) [34]. Higher arterial blood tension-based oxygen delivery was confirmed in a

Fig. 1. Chronological developments of PFCs based oxygen carriers: from 1949 to present. [53–124]

clinical trial of the marketed formulation of PFC (e.g. Fluosol-DA® and Fluosol-43®). The study was conducted in severely anemic patients before surgery. It was observed that when the arterial pressure was around 101 mmHg, oxygen delivery was low, but as the patient was administered with pure oxygen (arterial oxygen tension of 361 mmHg), oxygen consumption was found to increase by around 24% [34]. Moreover, Fluosol- DA® was approved by the FDA for coronary transluminal angioplasty and it was withdrawn from the market due to low oxygen transport capability and deficient stability.

Very recently, a study was conducted to analyze the effect of PFCs formulation on hypoxia, sepsis-induced renal tubular epithelial cells injury, and renal CD133^{+} progenitor differentiation. The plasma of the septic patients was found to potentiate the renal cell apoptosis along with downregulation of overall oxidative metabolism, reduction of

Fig. 1. (*continued*).

albumin uptake, and downregulation of ZO-1, a cell junction protein. This hallmark was even found to be substantially reduced by the PFC emulsion. PFCs emulsions were also found to enhance the viability of tubular epithelial cells along with the induction in the expression of insulin and hepatocyte growth factors [35a]. For intravenous therapeutic use, PFC preparations need to be formulated into the form, which should be acceptable *in vivo* by blood [35b]. Emulsification of PFCs is one of the options, but the side effects associated with emulsifier agents already have limited its use [36]. Another feasible option is the formation of the PFC core in an albumin shell. Tsuchida *et al.* have first demonstrated the role of albumin in the manufacture of Hb based artificial oxygen transporter [37]. This formulation has shown the desired properties, including appropriate oxygen binding, and releasing features, absence of pathogens, no blood antigen, highly stable on long term storage and biocompatibility [37]. Wrobeln *et al.* have developed and evaluated nanoparticles with an albumin shell and perfluorodecalin core [38]. Administration of these nanoparticles to the healthy rats was found to be very well tolerated except few doses dependent side effects. Later, they proved the functionality of these nanocapsules in Langendorff-heart [39].

Furthermore, tumor hypoxia has been associated with the formation of new vessels and cell survival. It is also linked with the resistance of cancer cells towards chemo, photo, and radiotherapy. Maintenance of normoxia conditions could reverse the situation and could sensitize the cancer cells towards cancer therapy. Oxygen delivery to the tumor cells is considered as a viable option. Various PFCs formulations are widely investigated for their role as oxygen carriers in sensitizing the cancer cell towards cancer therapy. Zhou *et al.* have reported the development of PFC and etoposide loaded hollow magnetic nanoparticles [40]. These nanoparticles were designed to deliver the anticancer drug to the cancer cell and, at the same time, improve the oxygen status inside the cell. Besides, these nanoparticles were found to significantly reduce the hypoxic condition and increased its susceptibility towards the anticancer activity of etoposide [40].

Some oxygen carriers approved by the FDA are based on PFC and are currently being investigated for oxygen delivery to tumors. Table 1

summarizes the different basic PFCs commercially available to support the development of PFC based oxygen carriers.

Additionally, PFCs are quickly eliminated from the vascular space by the reticuloendothelial system and stay in organs like the spleen and liver for weeks [34]. Prolonged stays in the liver limits the possibility to repeat the dosing of PFCs in a short time [49] and pharmaceutical formulation stability is another limitation of PFCs. In clinical trials, emulsion instability was reported, which makes it compulsory to store them frozen [34,50]. Dosing and formulation stability issues are addressed using advanced formulation techniques, but oxygen delivery at high arterial blood tension is an inherent characteristic and remains an important challenge in PFCs based oxygen delivery. Furthermore, PFC based formulations transport oxygen *in vivo*, the contribution in terms of clinical outcome is presently under investigation. It is noteworthy that Perftoran (Vidaphor), the first generation of PFCs product, is used in Russia, Mexico (under the name of Perftec), South Africa, Kazakhstan, Ukraine, and Kirghiz Republic. Moreover, Perftoran has been used to improve plastic surgery, to avoid rejection of transplant, to treat various occlusion vessels pathologies, among others. Additionally, Oxygent and Oxycyte products are still available commercially (Oxygen Biotherapeutics, Inc., NC, USA and Alliance Pharmaceutical Corp., CA, USA, respectively). Oxycyte has been studied to overcome spinal cord injury in swine models [51]. However, from the available clinical trial data, PFCs as an artificial oxygen transporter can expand the options available for red blood cells, especially for eliminating the risk of allogeneic blood transfusion (Fig. 1.). With the current advances in science, the utility of additional PFCs products in real clinical settings is around the corner [51,52].

3. Haemoglobin-based oxygen carriers (HBOCs)

HBOCs try to mimic the oxygen and nutrient transport functions of red blood cells. Their aim is to act as an alternative to the blood or red blood cell transfusion to eliminate the risk of pathogen transmission, blood group matching, blood shortage, and stability issues. To date, there is no perfect alternative to blood transfusion, something which could replace all its functions. Cell-free Hb does not behave like Hb enclosed in the cell membrane. They have several issues like high oxygen affinity, high elimination rate, nephrotoxicity, vasoconstriction, etc. To overcome these issues, several options like recombinant Hb, cross-linked Hb, PEGylated Hb, and liposomal Hb have been proposed. Such products could be critical to improving clinical trial outcomes of cardiovascular disorders, trauma victims, and patients undergoing surgical procedures by replacing oxygen and nutrient transport functions of red blood cells artificially. It is generally understood that an artificial approach cannot carry out the numerous complex functions of blood. The potential advantages of the artificial oxygen transporters not only include the universal transfusion without matching the antigen groups, but also ready availability, long term stability, and lack of infection are other key advantages [125].

Transport of oxygen in the blood is performed by the major protein of red blood cells, the haemoglobin [126]. Each Hb subunit has an iron II (Fe^{2+}) atom in a porphyrin ring, which is the site where the oxygen binds, and its affinity is principally controlled by the 2,3-diphosphoglycerate (2,3-DPG) molecule that changes the Hb conformation by increasing its oxygen tension, known as T state. When oxygen binds to the iron atom, 2,3-DPG is released, and the oxygen affinity increases, changing to R state [127,128]. Early development of oxygen carriers involved the use of stroma-free Hb solutions. Unfortunately, stroma-free Hb from red blood cells cannot be used as an oxygen carrier itself since the extracted Hb tetramers tend to dissociate into α-β dimers that are rapidly excreted by the kidneys and trigger a nephrotoxic secondary action [129].

In the last five decades, different methods have been developed to prevent these problems by chemically modifying and stabilizing the Hb molecule, with the aim to have a better oxygen release, e.g., intramolecular cross-links were used to stabilized the tetramer, while the high oxygen affinity has been reduced using 2,3-DPG analogs or by combining intramolecular cross-linked/oxygen affinity modifier molecules as 2-*nor*-2-formylpyridoxal phosphate (NFPLP) and bis-(3,5-dibromosalicyl)-fumarate (DBBF) [130].

HBOCs are generally based on the modifications of Hb purified from human or bovine blood [13]. Based on the functionalization process, HBOCs could be classified into:

- polymerized Hb,
- cross-linked Hb,
- polyethylene glycol conjugated Hb,
- liposome-encapsulated Hb, and
- recombinant Hb

Functionalization is generally aimed to inhibit renal filtration by preventing tetramer dissociation, increase the oxygen affinity of Hb, and to increase the molecular weight and size to avoid renal filtration [131]. Modifications to reduce the renal clearance include intramolecular crosslinking, intermolecular cross-linking with bifunctional agents [132–135], and large-molecular-weight polymers to increase the circulation time [132,134].

3.1. Chemical modifications of Hb for effective oxygen transport

Various limitation of the use of whole blood or red blood cell for transfusion leads to the search for HBOCs. Eliminating side effects of free Hb, enhancing the self-life, and circulation time is the rational thinking behind resource investment in the development of HBOCs. Several chemical modifications dealing with the reduction of the toxicity and improvement in the efficiency of HBOCs has been studied and reported in the literature. The following section of the review deals with the discussion of various chemical modifications carried out to improve the acceptability of HBOCs.

3.1.1. Pyridoxalation of Hb-oxygen affinity modulation

The Hills Coefficient (n_H) is a measure of cooperativity in a binding process, providing a way to quantify interaction between ligands, and denotes the shift between the different Hb conformations. A n_H of 2 reflects cooperative oxygen binding, and 1 demonstrates the negative cooperativity between protein subunits [136]. The typical normal value of 2,3-DGP concentration in red blood cells (RBC) is around 5 mmol/L. Moreover, oxygen half-saturation (p50) of normal human blood is around 27 mmHg, and this is the optimal value for HOBCs development. 2,3-DGP bound to the deoxyHb and stabilized it in the peripheral site where oxygen levels are low and required the oxygen release. Cell-free and some chemically modified Hb lose 2,3-DGP activity, and hence such Hbs have higher oxygen affinity and thereby, low release rate. Most of HBOCs are based on acellular Hb, except liposome encapsulated Hb, so mimicking such ability is a difficult task. Some cross-linking methodologies are available in the literature to stabilize the Hb in T state (deoxy state), and the use of bovine or recombinant haemoglobin (rHb) for HBOCs preparations is also possible due to the fact that bovine Hb works similarly to human Hb, at very low levels of 2,3-DGP. This means that stromal free bovine Hb has low oxygen affinity as compared to stromal free human Hb [137].

The undesirable character of cell-free Hb is the high oxygen affinity due to the loss of 2,3-DGP. Unsuccessful attempts were made to restore the original oxygen binding of cell-free Hb by merely adding the 2,3-DGP [138]. Pyridoxalation of Hb improved the Hb function by binding to the same site where 2,3-DGP links and it occurs at the *N* terminal group of the β-chain when the reaction is carried out on deoxyHb and at the *N* terminal of α chain reaction carried out on oxyHb. Residue binding, bridge formation, gelation, and conformation arrangement are also similar to the 2,3-DPG binding [139,140].

3.2. Intramolecular and intermolecular cross-linking of Hb / rational thinking behind the cross-linking Hb molecules

Reducing the nephron toxicity due to the dissociation of Hb tetramer is the principal aim of the cross-linked Hb. Advanced medical techniques, along with organ transplants further enhanced the need for blood and its components. An artificial oxygen transporter capable not only of transporting the oxygen but also reconstituting the volume is the most sought-after medical discovery. To avoid the complexities associated with it, the Hb solution was considered as the more feasible approach. Use of Hb has several advantages as compared with the whole blood due to these solutions are more useful in emergencies. Moreover, repeated Hb transfusion to maintain the steady state of transfused Hb could pose a severe hazard to the patient with the history of renal disorder [141,142]. Therefore, the idea of encapsulated Hb of the stromal-free Hb in lipid membrane, without antigen, would not only increase the circulation time of Hb by reducing the renal excretion but could also eliminate the need for blood group matching.

Chang proposed the first concept of Hb-based oxygen transporter in 1964, the renal toxicity, and a few other adverse events were reported in phase I clinical trial by Savitsky *et al.* in 1978 [143,144]. All the shortcomings of the stromal free oxygen were attempted to overcome by modified Hb, including 1) Molecular-based modified products like polymeric, crosslinked, recombinant, and conjugated Hb and 2) Nanotechnology-based modified Hb products which include encapsulated or liposomal Hb.

The early idea of modified Hb was available in the 1970s; however, research interest developed considerably only after the toxicity of stromal-free Hb, and the possibility of HIV and hepatitis transmission was reported. The primary focus of the early work was on the function and stability of synthetic membrane, permeability, membrane fusion, physicochemical properties of the lipid bilayer, prevent renal excretion, etc. Several interesting approaches to modified Hb are investigated, which are discussed below.

3.2.1. Molecular-based modified Hb products

Toxicity is the critical hurdle in HBOCs development. Free Hb, unlike cellular Hb, undergoes irreversible damage, which not only disturbs its oxygen-carrying capabilities but also makes them more toxic. Stabilization of acellular Hb molecules using chemical modification approaches like irreversible cross-linking of the monomers of Hb and conjugating the cross-linked Hb molecules with inert high molecular weight compounds are few of the first generation molecular-based modification of the Hb molecules.

3.2.1.1. Cross-linked modified products. In addition to the nephrotoxicity and vasoconstriction, another major issue with the acellular Hb is the oxidation of the iron atom inside Hb. In the absence of a cell membrane, Hb undergoes autoxidation from iron II (Fe^{2+}) to iron III (Fe^{3+}) (methaemoglobin). Methaemoglobin does not bind with oxygen, thereby limiting the oxygen transport capability of Hb, which can lead to the ischemic condition in the tissues [145]. Cross-linking of Hb is aimed to solve some of the problems associated with unmodified stroma-free Hb. Cross-linking of Hb involves chemically linking α and β chains of Hb to impart stability in the cell-free tetramer. Such modifications were also found to increase the half-life of Hb. In spite of advancements in cross-linking and improvement in stability, side effects like vasoconstriction are still a significant challenge.

Highly purified Hb found to be more prone to the oxidative degradation when exposed to the plasma containing hydrogen peroxide, which is the major oxidizing agent present in the blood. For example, Kulger *et al.* prepared *N,N*'-5,5'-bis[bis(3,5-dibromosalicyl)isophthalyl] terephthalamide and this is a multifunctional agent that is useful to cross-link inter and intra monomers of tetramer. Moreover, Bis'Hb forms when deoxyHb reacts with *N,N*'-5,5'-bis[bis(3,5-dibromosalicyl) isophthalyl]terephthalamide. This cross-linked product was found to have low oxygen affinity, but the simultaneous reduction in cooperative based oxygen binding was also observed [146]. Gourianov *et al.* and Kluger *et al.* modified this agent and reported the synthesis of tetrakis acylphosphate esters and its derivatives [147,148]. Hb cross-linked with this agent has shown cooperative based oxygen binding but lower Hill coefficients as compared to the native Hb.

Alagic *et al.* developed a dual functional protein [149] to combine the oxygen transport capability of Hb and superoxide radical catalyzing ability of superoxide dismutase. The product was found to have less cooperative based oxygen binding, but the radical catalyzing ability of superoxide dismutase remains the same [149]. Cross-linking Hb in dendritic assembly was reported by Hu *et al.* [150]. This cross-linking produces dendritic products with similar cooperative based oxygen-binding as of human Hb [150].

Diaspirin, bis(o-carboxyphenyl) succinate was also shown to have cross-linked the Hb subunits [151,152]. Walder *et al.* reported two esters of dibromosalicyl acid via bis(3,5-dibromosalicyl) succinate and DBBF as a potential acetylating agent [153]. Diaspirin cross-linked Hb was later checked for their immunogenicity in patients enrolled in phase II and III clinical trials by Patel *et al.* All the patient specimens (preinfusion and postinfusion) of the clinical trial confirmed the lack of preexisting antibodies to diaspirin cross-linked Hb and the absence of antibodies after exposure to this new biologic entity [154].

Site-specific cross-linking of Hb and its relationship with activity was studied by various research groups [155–157]. Jones *et al.* managed to make double-crossed linked Hb [156] and Walder *et al.*, developed an efficient Hb-based oxygen carrier [157]. The oxygen affinity of double cross-linked Hb was found to retain significant cooperativity with a Hill coefficient of 2.3 compared with 3.0 for unmodified Hb [156]. Chatterje *et al.*, used bis(3,5-dibromosalicyl)fumarate to form the fumaryl bridge between Lys-99α1 and Lys99 α2, spanning the central cavity of the tetramer of deoxyHb. Similar to Jones *et al.*, Chatterjee's cross-linked Hb retained highly cooperative oxygen binding. These examples suggest that the DBBF could be used to cross-link Hb both in the oxy and deoxy states at β and α chains [158].

Hbs tetramers are held together with the help of noncovalent bonds. Covalent or noncovalent modifications of Hb are the preferred method of shifting the Hb S (abnormal Hb) conformational equilibrium toward the oxygenated state. Hence, covalent modification of the terminal amino residue of the beta chain is an attractive target because this site overlaps with the binding site of 2,3-DPG. A few of the covalent modification approaches include Schiff base formation between an aldehyde and the terminal α-NH_2 group. Other classes of covalent approaches include cyanate and the aspirin reaction products. May *et al.* confirmed the relationship of Hbs carbamylation (with cyanate) with its increased oxygen affinity [159]. In an attempt to make the clinically useful antisickling agents, aspirin was used to acetylate the Hb of the sickle cell by Klotz *et al.* [160] Acetylated Hb was found to have a higher oxygen affinity as compared to the unacetylated one. A few of the significant advantages of aspirin is that it is an old, very well-tolerated drug with the additional benefit of prostaglandin inhibition. Prostaglandin has been positively associated with cell sickling [161]. Unfortunately, aspirin was found not to be an effective antisickling agent [162]. However, this investigation has brought the focus on the new chemical compound, which has the potential of further exploitation for the development of clinically relevant antisickling agents.

Hb dissociate into dimers when it is placed outside the erythrocyte and in solution. Two dimers come together to form the central cavity. Several attempts have been made to cross-link the tetramer utilizing the residues within this central cavity. Few of the investigations include an extension of the aspirin base antisickling agent's approach. For example, Walder *et al.* tested bifunctional acylating agent bis(3,5-dibromosalicyl) fumarate and bis(3,5-dibromosalicyl) succinate to halt the sickling process [163]. DPG binding to the Hb regulates the oxygen affinity of the erythrocyte, which makes DPG binding site a critical target for the

development of the antisickling agent and dimer stabilization. Halogen in both the diesters makes them more lipophilic, which ease their transfer across the erythrocyte cell membrane and makes them active *in vivo*. Overall, the study directed the research focus on the 2,3-DPG binding site for the development of clinically useful antisickling and artificial oxygen transport agents [163].

3.2.1.2. Non-specific Hb cross-linking. Cross-linking Hb using agents like glutaraldehyde and oxidized sugars (raffinose and dextran) generally yield heterogeneous Hb cross-linked tetramers. Such a mix of products has different physical, chemical, and biological properties, which sometimes are the leading cause of toxicity [164]. Human or bovine Hb with a range of purity is used as a starting material for cross-linking. As acellular Hb loses 2,3-DPG, oxygen release from the modified Hb is the function of the site and nature of chemical linking.

As an example, glutaraldehyde is a most common nonspecific crosslinking agent used to prevent the Hb tetramer [165,166]. It can cross-link with a variety of amino acids of Hb molecule obtained from a human or bovine source [167].

PolyHeme® is a glutaraldehyde cross-linked pyridoxalated human Hb product which is manufactured by Northfield Laboratories. Phase III clinical trials of PolyHeme® were conducted on 714 patients, resulting in 40% of patients administered with PolyHeme®, and 35% of control group patients experiencing severe side effects [168,169]. Hemopure® is glutaraldehyde cross-linked bovine Hb manufactured by Biopure Corporation and has p50 of 36 mmHg, circulation half-life of 19 hours, and shelf life of three years. In phase III clinical trial of Hemopure® at least one adverse event, including an elevation in blood pressure, was observed. Based on this clinical observation, trials of Hemopure® were halted [170]. Another glutaraldehyde cross-linked bovine Hb is Oxyglobin®, which is produced by Biopure for veterinary use and is approved to treat canine anemia in the United States and Europe [171]. These three glutaraldehydes cross-linked Hb products possess relatively low O_2 affinities. The oxygen affinity of these HBOCs was designed to match the p50 of human Hb to transport oxygen to tissues and organs properly.

3.2.1.3. O-raffinose linked Hb. Raffinose is a trisaccharide composed of fructose, glucose, and galactose. O-raffinose cross-linked Hb (O-R-Hb) solutions are now in clinical trials as an HBOCs. Hemolink™ is a formulation tested in humans, produced by a Canadian company, Hemosol Inc. (Toronto, ON, Canada) [172]. Boykines *et al.* were among the first groups who created the O-R-Hb by cross-linking ultra-pure deoxy-Hb with O-raffinose [173].

As stromal free Hb could potentially affect tissues, organs, and cellular components of blood, a study was conducted by Leytin *et al.* to examine the effect of O-R-Hb on blood platelets in vitro [174]. No adverse effects on blood platelets could be observed when studying clusters of differentiation proteins in flow cytometry experiments, furthermore repeated dose studies in rats did not reveal immunogenic effects, underlining the overall safety of O-R-Hb [175].

Cross-linking of Hb generally locks the conformation, e.g., if the cross-linking takes place in R state, then its transformation into T state conformation is inhibited. Hence stabilization of Hb in T state using crosslinking agents is the most sought approach to make ideal HBOCs. When deoxyHb is cross-linked using O-raffinose, it not only stabilized the Hb in T state but also oligomerized the Hb. Jia *et al.* revealed that Hb cross-linked with O-raffinose maintains the T state conformation [176].

To study the safety and efficacy of O-R-Hb, phase I placebo-controlled, randomized, double-blind clinical trial was conducted on 42 normal humans [177]. O-R-Hb in a dose of 0.025 - 0.6 g/kg or Ringer's solution was injected, and volunteers were monitored for three days, and a forty two-day follow-up period was taken. Dose dependent rise in mean arterial pressure, severe to moderate abdominal pain, lower heart rate, increased serum bilirubin level and higher creatine kinase levels were the most common associated effects, whereas a minor increase in aspartate aminotransferase and alanine aminotransferase was noted in a few patients [177]. In phase II, single-blind, randomized, open-label clinical trial conducted at multiple sites in Canada and UK, analysis of dose-response of Hb raffimer in a coronary bypass surgery was reported. Hb raffimer is an o-raffinose cross-linked Hb developed by Hemosol Inc, Canada. In this trial, atrial fibrillation elevated blood pressure, and jaundice was the most cited side effect in the Hb raffimer group. Overall, this trial confirmed that the Hb raffimer is safe to use in the patients undergoing coronary artery bypass graft surgery [178]. As observed in both animals and humans, HBOCs are associated with the rise in blood pressure.

In animal studies conducted on rats, unmodified Hb was found to induce mean arterial pressure (MAP) by 14 % when compared with O-R-Hb [179,180]. Cardiac output was unaffected by O-R-Hb, but unmodified Hb was found to reduce it substantially. O-R-Hb does not affect the renal function system, but unmodified Hb is found to have adverse effects on the renal vitals [179,180]. In another study conducted on anesthetized rabbits, O-R-Hb has shown a very low effect on heart rate, MAP, cardiac output, vasoconstricting properties of Hb, abdominal aortic, and vascular resistance when compared with other modified Hbs [181]. In a separate study conducted by Wong *et al.* on anesthetized rats, similar observations were made about mean arterial pressure and heart rate [182].

Based on these clinical and preclinical animals studied of O-R-Hb it appears that its use is free of severe toxicity. O-R-Hb has no immunogenic interference in animals and humans. However, antibodies against O-R-Hb in animals are reported and it is also found to be useful when used as an alternative to blood transfusion in a murine model of malaria [183]. In conclusion, O-R-Hb based HBOCs could be the potential alternative to whole blood or blood cell transfusion. Current and planned clinical trials will further analyze the safety profile and dose regimes.

3.2.1.4. Polymerized Hb. Polymerized Hb has been considered as an essential alternative to the oxygen-carrying fluid in case of emergencies when blood is not available. The lifesaving ability of polymerized Hb has led to the development of the various polymerization methods useful for retaining the tetramer structure of Hb. Like stromal Hb, unstromal polymerized Hb should reversibly bind the oxygen to deliver it to the required tissues. In its natural form, Hb is a conjugated non-crossed link protein, an essential characteristic for the normal red blood cell (RBC) shape and function. Kent *et al.* reported a critical method for the intramolecular cross-linking of stromal free human Hb. The separation of Hb from the cell membrane can be an important step to avoid vasoconstriction. Commonly it is intramolecularly cross-linked, forming water-soluble macro-molecular stromal-free Hb [184].

Several laboratories have confirmed the vasoconstriction related side effect of HBOCs, including that of glutaraldehyde-polymerized human and bovine Hb. For example, Irwin *et al.* reported the decrease in oxygen delivery during normoxia and acute hypoxia in the rat when administered with polymerized bovine Hb [185]. Optimal dosing regimen and time interval is critical. Shen *et al.* reported the bioanalytical method to determine the polymerized porcine Hb levels over a period of time in different animal models [186]. Polymerized porcine Hb (pPolyHb) is a kind of glutaraldehyde-polymerized Hb-based oxygen carrier. Zhu *et al.* have developed pPolyHb and studied its pharmacokinetics in a rat model of exchange transfusion [187] using the versatile glutaraldehyde polymerization method for porcine Hb, and few products have already been tested in clinical trials [188–191]. Additionally, the half-life of pPolyHb was higher and found to be in non-pathological conditions, but in adverse clinical events such as trauma and anemia, the half-life of pPolyHb was found low [187]. pPolyHb has also been tested in reperfusion injury, which is considered more serious then cerebral ischemic injury [192]. pPolyHb, when administered in a rat model, was not only

found to inhibit the expression of the TNF-α and IL-1β but a substantial reduction in the cerebral infarct size and lipid peroxidase and myeloperoxidase (markers of oxidative damage) activity is also observed [192]. Overall, there was a significant reduction in the infarcted volume and improved neurological function.

The availability of universal oxygen carriers in case of emergency is an unmet challenge. The shortage of RBCs, immunologic reactions, transport, and infection transmission is the major issue. PolyHeme®, which is a Human polymerized Hb developed by Northfield Laboratories, is currently under clinical trial [193] and developed for its negligible NO scavenging activity. In clinical trials, this product was proven to be equally effective to that of RBCs which makes it a crucial candidate for the through clinical investigation [194,195]. Moor *et al.* conducted clinical trials with the aim to analyze the survival benefits of the PolyHeme® in the case of haemorrhagic shock and compared it with the classical blood resuscitated [196]. When tested on 700 patients it was observed that the resuscitation with PolyHeme® early 12 hours after injury had a similar outcome to that of classical resuscitation. However, adverse event frequency with PolyHeme® was more than compared with that of classical resuscitation, but the risk to benefit ratio was in favor of this product when whole blood is not accessible easily [196].

Similarly, Gould *et al.* also conducted the first prospective, randomized trial to analyze the beneficial advantage of PolyHeme® when compared with a whole blood transfusion [194]. It was observed that oxygen consumption from PolyHeme® was high when compared with whole blood transfusion, and it was safe to repeatedly administer the six units of PolyHeme® with observed minor adverse events. Also, PolyHeme® was also found to be safe in another clinical trial conducted by Gould *et al.* on 171 patients [194]. In a separate study conducted on 39 healthy volunteers, Gould *et al.* again confirmed the safety of polymerized Hb [197].

Another polymerized Hb product is the OxyVita®Hb. This polymerized Hb is termed as the potential substitute of the blood based on different results documented from preclinical and clinical studies [198,199]. Wollocko *et al.* led a study to analyze the resistance to heme exposure of bHb, myoglobin, and OxyVita®Hb when exposed to the denaturant like urea [200]. This observation is crucial because the heme released is associated with adverse events like oxidative stress when substituted with blood [201]. Hemopure®, which is another polymerized Hb, manufactured by OPK Biotech, was approved for clinical use in South Africa and Russia for the treatment of anemia. Furthermore, Hemopure® has been utilized in the United States to treat patients with life-threatening anemia for whom blood transfusion is recommended and who have tried all the treatment alternatives without success. The therapeutic efficiency of Hemopure® was compared with blood by analyzing their effect on microcirculation at a concentration between 4 to 12 gHb/dL [202,203]. Furthermore, another clinical study, showed that a high dose administration into injured patients does not show a vasoconstriction effect [204].

Immune response towards the acellular polymerized Hb was analyzed by Marks *et al.* in a dog model [205]. Hemorrhagic animals were administered with polymerized Hb. A significant level of antibody was detected in the test animals after the 10^{th} week when compared with the control. In contrast, Bleeker et al investigated the potential immunogenicity of human Hb polymerized using glutaraldehyde. The antibody response was analyzed in rabbit by weekly intravenous infusion of the clinically relevant dose of the rabbit Hb what was prepared in the same way as that of the human glutaraldehyde Hb. The study confirmed the weak immune response in the experimental condition [206]. Yan *et al.* studied the immune response against polymerized porcine Hb. Three inflammation indicators (C3a, IL-6 and TNF-α) were analyzed in rat model and cultured cells. The level of these three indicators were not changed, indicating no immunotoxicity for the polymerized Hb [207].

In another preclinical safety study of polymerized Hb, the cardioprotective role was analysed [208]. In this study, glutaraldehyde-polymerized human placenta Hb (GU-HP-Hb) benefit was accessed in cardiopulmonary bypass surgery in a dog model. The low dose of GU-HP-Hb was proven to be protective again cardiac ischemia when compared with the high dose, which was verified by the overall impaired cardiac function [208].

Similarly, in another study conducted by Heneka *et al.,* polymerized Hb was found to reinstate cardio and glomerular function in an endotoxin-induced animal model [209,210]. Polymerized bovine Hb (HBOC-201) was compared with Hetastarch concerning resuscitation performance in pig models [211]. For pigs treated with polymerized Hb, survival rate was 100%, animals exposed to Hetastarch survived in 88% of the cases and of the non-resuscitated control group only 63% animals survived. Tissue oxygen levels and, at the same time, mean arterial pressure was also high in polymerized Hb administered group. In conclusion, polymerized Hb groups were found to restore the cardiopulmonary function to the normal in comparison with Hetastarch group and ultimately proved better in a hemorrhagic animal model.

Belcher *et al.* analyzed the chemotherapy when polymerized Hb was transfused simultaneously. Regular polymerized Hb transfusion to the mice displaying breast cancer cells established the reduced angiogenesis, hypoxic condition, and tumor growth. Simultaneously, clearance of polymerized Hb was observed through the liver signifying lower nephrotoxicity [212]. Cytoprotective role of polymerized Hb was also observed when lipopolysaccharide induces inflammation was attenuated by it [213].

Alternatively, Ohta *et al.* developed microspheres made up of human albumin and Hb obtained from human RBCs [214]. The oxygen loading capacity and oxygen dissociation characteristics were found to be similar to the ones of red blood cells. When HeLa cells were treated with these microspheres, significant oxygen supply from the microsphere was observed [214].

Overall, polymerized Hb is found to be safe in preclinical and clinical studies. Polymerized Hb was also useful in maintaining the normoxia condition of the tumor and hence could also sensitize chemotherapy. Along with nonimmunogenic character, and less renal and vasoconstriction activity polymerized Hb is undoubtedly a potential candidate for HBOCs.

3.2.1.5. PEGylated Hb. Another vital approach includes the use of polyethylene glycol (PEG). PEG has been utilized in the development of non-immunogenic, sustained therapeutics with longer circulation time. Few PEGylated Hb have entered Phase II clinical trials, including MP4OX, MP4CO, and Hemospan of Sangart Pharmaceuticals [215–218]. The unmet challenge before the inclusion of PEGylated Hb clinical trials includes 1) Direct PEGylation of uncross linked Hb weakens the tetramers to dissociate into dimers, which ultimately reduces oxygen-binding and 2) Its increased oxygen affinity, which leads to a decreased delivery of oxygen to the tissue.

PEGylation of Hb is now considered as the newest approach to attenuate the vasoconstriction activity of acellular Hb, which is a significant hurdle in its clinical application. The earliest PEGylation was carried out of the bovine Hb [219–221]. HexaPEGylated Hb generated using 2-iminothiolane approach was served as the model for the preparation of MP4 (Hemospan), which entered in Phase III clinical trials [222].

The PEG-linked on Hb molecule was found to increase the viscosity on the molecular surface of Hb. The viscous PEG should slow down the entry and release of oxygen to and from the central cavity of the heme. The direct influence of PEG density on the tissue oxygenation is an area of research that has yet to be explored in depth. Studies related to this topic are especially important, since PEG density has shown interesting effects concerning oxygen uptake and release in seminal studies. This was proved by the variable oxygen-binding capabilities of various PEGylated Hb (e.g., PEG5K2 Hb, PEG10K2 Hb, PEG5K4 canine Hb, and PEG5K6 Hb) containing a different number of cross-linked PEG

[223–228].

Preparation of Human PEG-Hb required human adult Hb as a starting material. However, obtaining Hb from human blood is difficult due to the limited availability of outdated human blood. Bovine Hb offers a better alternative due to the availability of ample resources for mass production. Bovine PEG-Hb (B-PEG-Hb) was reported by Wang *et al.* [229]. When B-PEG-Hb was compared with human PEG-Hg, B-PEG-Hg showed higher hydrodynamic volume and was devoid of vascular activity. B-PEG-Hg was also found to recover mean arterial blood pressure in the hemorrhagic shock animal model. This observation makes B-PEG-Hba a versatile HBOC because of its high oxygen delivery capability and plasma expanding ability.

Another approach proposed by Webster *et al.* known as "Inside out method", is the reverse of previously employed PEGylation methods [230]. Inside-out PEG-Hb has also been shown to enhance structural and protein stability without significantly affecting the p50 value when compared with the native protein [230].

Despite the several benefits PEG-Hb offers, limitations associated with them can't be overlooked; this includes higher oxidative stress and its effects on the various organs. Alomari *et al.* observed a positive correlation between higher tissue damage and high oxygen-affinity of HBOC when compared to the high and low-affinity PEG-Hb products in an animal model [231]. These observations call for the development of low-affinity PEG-Hb with low NO dioxygenase reactivity.

3.2.1.6. Polynitroxylated PEGylated Hb. HBOCs further refinement derived in the polynitroxylated PEGylated Hb (PPHb). PPHb was first studied in neuroprotection, where it was found to reduce infarcts by around 53% [232]. Present generation HBOCs have vasoconstriction activities because of their NO scavenging activity. The severity of which could be explained with the decrease in posttraumatic blood flow to the vital organs, like the brain [233].

Traumatic and infarcted brain injuries have been associated with reduced levels of NO and nitric oxide synthase [234]. This explains the additional harmful effects of HBOCs in such situations. Additionally, cell-free Hb is also found to hurt neurons in cell culture. As discussed earlier, various modified Hb had been proposed to tone down the harmful side effects associated with cell-free Hb, including the addition of nitroxyl groups due to their antioxidant activity and superoxide dismutase mimetic activity.

On the other hand, PEGylation of Hb adds various beneficial effects, including limiting direct interaction of Hb with the endothelium, thereby avoiding oxygen-mediated vasoconstriction with enhanced NO synthesis [235]. PEGylation was also found to prolong the circulation time of Hb. SenZyme technologies were the first to introduce PPHb, a novel bovine HBOC based on PEG-Hb. Moreover, Shellington *et al.* reported PPHb for neuroprotection during acute brain injury and hemorrhagic hypotension in mice [233]. The proposed PPHb found to have unique neuroprotective activity, both *in vitro* and *in vivo* models, hence considered as a suitable candidate for clinical development [233]. Furthermore, transfusion of PPHb was not only found to be protective in the rat filament model of middle cerebral artery occlusion but also found to increase the perfusion in the ischemic border region and reduce the infarct volume [236]. Resuscitation with PPHb as compared to lactated Ringer's solution improved the mean arterial pressure, heart rate, and reduced intracranial pressure along with maintenance of proper potassium levels at a well-tolerated wide range dose, further supporting its clinical development [237].

Multiple therapeutic benefits of PPHb prepared from bovine PEG-Hb are observed in three indications 1) Traumatic brain injury with hemorrhagic shock, 2) Stroke, and 3) Sickle cell disease [232]. These available preclinical evidences suggest that the PPHb is the future of the Hb-based oxygen carriers, which reduces the oxidative stress, corrects inadequate blood flow, and hence could meet the FDA mandate for an HBOC with the substantial advance in therapeutic index.

3.2.2. Encapsulated HBOCs

Molecular-based crosslinked Hb are the first HBOCs which are ready for the clinical trials. Encapsulation of the purified Hb or crossed linked Hb, along with the necessary co-factors, can make HBOCs more like the red blood cells. The following section deals with the encapsulation of Hb in the lipid bilayer and the challenges it could face until it gets approval for clinical use.

3.2.2.1. Haemoglobin encapsulation. Free Hb tetramer scavenge NO, causing vasoconstriction. It is therefore advisable to re-encapsulate the Hb in a lipid bilayer, which could eliminate side effects and rule out the blood grouping step required during a conventional blood transfusion. Initial efforts were focused on relatively large semipermeable microcapsules, primarily composed of synthetic materials like nylon [143]. Arakawa *et al.* were the first to report the hemolysate microcapsules made up of poly(*N*-α, *N*-ε-*L*-lysinediylterephthaloyl) [238]. Hb encapsulation in the liposome (also called hemosomes) is mostly focused on reducing the toxicity of free Hb and on enhancing the circulation time. Although, the Food and Drug Administration (FDA) approved some liposome-encapsulated antiviral and anticancer drugs, but liposome-encapsulated Hb is still facing some clinical and pharmaceutical challenges [239–241]. These challenges include oxidation of Hb, very high encapsulation efficiency, pilot to large scale transfer, large scale dose administration, and stability. Normal red blood cells have 300 g/dL of Hb, and matching this entrapment is a difficult task. Such physicochemical interaction is a great cause of concern to the development of pharmaceutically formulations, as it limits the use of lipids, which are more prone to oxidation [242]. Liposome removal from the blood varies with the charge on the lipids. The removal rate observed is positively charged liposomes > negatively charges liposomes > neutral liposomes. However, negatively charged lipids like phosphatidylinositol was found to inhibit liposome aggregation and fusion during long term storage. The following section of the review is focused on the recent advances in the Hb encapsulation.

3.2.2.2. Liposomal Haemoglobin (LHb). To address the side effects of the free Hb, liposome-encapsulated Hb was developed as an artificial oxygen carrier. They were developed to address the urgent need for post-hemorrhage oxygen demand and volume deficit. From the physiological and anatomical prospects, liposomes are the artificial models of the cell membrane made up of various lipids. One of the applications is the encapsulation of Hb to deliver oxygen during emergency conditions and to protect its tetrameric conformation. Lipids, like the 2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), cholesterol, 1,5-*O*-dihexadecyl-D-glutamate, and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-PEG_{5000}, have been used for the encapsulation of purified, virus-free, Hb solution [243]. Liposomal encapsulation of Hb inside the lipid shell has tremendously reduced the toxic effect of acellular Hb; however, the biocompatibility of lipids used is still a critical issue [244]. Moreover, liposome was found to be stable over a period of 2 years and found to be intact in the blood circulation [245]. In the case of encapsulated Hb, it is possible to directly use it for resuscitation by following the protocol generally used for RBC transfusion. Before transfusion, encapsulated Hb needs to be mixed with a plasma expander to adjust the osmotic pressure equal to the physiological pressure [243].

One of the major advantages of encapsulated Hb over RBCs is the zero risk of blood transfusion-related viruses like HIV and hepatitis, no matching of blood group antigens, less oxidative damage, and increased shelf-life over long-term storage. Acellular Hb oxygen carrier is under development as a substituent for the red blood cells. However, the deleterious effect on kidney, vasoconstriction, and hypertension due to NO scavenging activity has hampered its clinical approval. Hence various studies were undertaken to test the hypothesis that the encapsulation of Hb tetramer inside the hydrophilic core of liposome could not only regulate the NO levels and oxygen release but also, in turn, could

regulate the vasoconstriction and hypertension. To test this hypothesis, Rameez *et al.* encapsulated bovine and human Hb in PEG conjugated liposomes [246]. In this study, oxygen dissociation, CO association, and NO dioxygenation were studied for free Hb and LHb. The most important observation was that the encapsulated Hb prevented the NO scavenging and ultimately reduced hypertension, and no changes were observed in the CO association between free Hb and liposome-encapsulated Hb [246].

Szebeni *et al.* encapsulated Hb in liposomes composed of different lipids [247]. Phosphatidylinositol (PI) containing liposomes were found to transport *in vivo* oxygen with a less adverse effect on immunity [248], and cholesterol in the lipid bilayer was found to stabilize Hb in the liposomes [247,248].

Other than the oxygen supply to the tissues and organs, LHb was also tested for its role in transporting oxygen to cultured cells. In one unique study conducted by Sakai *et al.*, Hep G2 and rat liver cells were used to study toxicity and oxygen-carrying capacity of Hb encapsulated in PEGylated liposome [249]. Cytotoxicity to Hep G2 cells was observed during the first six days of the culture, which was subsequently diminished with the addition of bovine serum albumin to the medium. On the other hand, normal rat hepatocytes did not show any adverse effect of liposomal encapsulated Hb when cultured as a monolayer. Secondly, it was observed that the improved oxygen levels by supplementing the culture with liposome-encapsulated Hb recovered the deteriorating cells [249]. Similarly, the feasibility of LHb as an oxygen transporter was studied using adult rat and primary fetal rat liver cells respectively [250]. These cells were found to be unaffected by liposome-encapsulated Hb, remarkably growth was even improved when cultivated in a perfused flat plate bioreactor under these conditions.

Clinical development of the Hb encapsulated in liposomes was supported by the various safety studies carried out. For example, lyophilized LHb infusion at 1 to 6 mL/kg of body weight not only has no detectable effects on cardiac output, total peripheral resistance, blood pressure, and heart rate for the period of 5 hours but also has no effect on various hematological parameters (RBC, platelets, and coagulation factors) including TNF-α levels. The survival rate after 7 days was also found to be a hundred percent [251,252]. Although the effect of encapsulated Hb on RBC; platelets and coagulation factors were studied for the first time, its impact on the immune system was studied by Azuma *et al.* and the antibody production was also found to be unaffected [253].

Besides, Terumo Corporation's TRM-645, a liposome-encapsulated Hb formulation, also underwent the basic safety and efficacy studies during the preclinical evaluation and entering clinical trials [254].

An immune response, like an accelerated blood clearance phenomenon, which leads to the reduction of the circulation half-life, was studied on a rat model of haemorrhagic shock, because it can be caused by the repeated administration of liposomes to the same animals [255,256]. After the initial dose of the encapsulated Hb (1400 mg/kg), rapid clearance was observed. Immunoglobin M (IgM) against liposome-encapsulated Hb was formed at day four after the first dose of liposomes, but levels reduced on day seven. Increased phagocyte activity was also observed. These results indicate that consideration of accelerated blood clearance could be very beneficial for repeated dose regimes of liposome encapsulated Hb [256].

Similarly, hexadecylcarbamoylmethylhexadecanoate-PEG-modified liposomes were evaluated for their immune response [257]. Repeated injection of modified liposome-encapsulated Hb was found to have reduced levels of anaphylatoxins C3a and C5a and thromboxane B2 in rats, nor does it have an effect on accelerated blood clearance, and no antibodies against encapsulated Hb and liposomes were detected [257]. On the other hand, to evaluate the effect of a liposomal formulation of Hb on macrophages, Azuma *et al.* analyzed the effect of empty and Hb loaded liposomes on T cell proliferation, where splenic T cell suppression was observed when rats were administered with empty and Hb loaded liposomes. The observed effect was transient, and the macrophages were found to be responsible for the T cell suppression with no change in the antibody production [253].

LHb also demonstrated its usefulness in hypohemoglobinemic condition [258]. LHb improved cardiac dysfunction during severe hemodilation. Hypoxia-inducible factor 1 α levels, which are generally high during hypoxic conditions, were also found to be low, and sympathetic nerve activity, along with its neurotransmitter was at the optimum levels. These observations suggest that cardiac dysfunction and sympathetic stimulation (epinephrine and norepinephrine) during blood loss in hemorrhagic shock was mitigated by the liposome-encapsulated Hb [258]. This study was carried out in a rat model of acute hemodilution.

The success of LHb depends on its stability in the circulation supply and tissues. However, the stability is the limiting factor in the development of a pharmaceutically acceptable formulation. Therefore, to obtain a stable LHb Liu *et al.* first made silica conjugated Hb and then nanoparticles [259].

Similarly, tissue distribution is an essential factor in the success of LHb. ^{99m}Tc-labeled-LEHSN was used to study its distribution in an anesthetized rabbit [260]. Biodistribution data indicated a distribution of 42.6% in the blood, 15.4% in the liver, 18.1% in spleen, 3.2% in the lungs, 2.4% in muscle, 1.6% in urine, and less than one percent in the kidney, brain, and heart after 20 hours of infusion [260]. 42.6 % in the blood indicate an increased circulation time of the Hb as compared to the stromal free Hb.

Interaction with the platelets is also one of the concerns in the development of LHb. To demonstrate this effect, PEG-DSPE was incorporated in the lipid membrane of the anionic and neutral liposome. PEGylation of anionic liposome found to inhibit the thrombocytopenia by 45.3%, whereas the PEGylated neutral liposomal encapsulated Hb showed the least thrombocytopenia (23.8%) [261].

Overall, LHb has no serious side-effects and is a promising approach to safely administer HBOCs. However, more work is required to optimize the lipid content of the liposomes, PEGylation effect on the circulation, release time, and effect of Hb on the stability of liposomes. Future work will be focused on these questions and towards a detailed investigation of the molecular events involved in the interaction of Hb with charged phospholipid bilayers.

3.2.2.3. Surface-modified liposomes. Surface modification of LHb can be used to enhance the circulation time of the liposome and to deliver it at the desired site. PEG derivatives, phosphatidylinositol, and polysaccharide derivatives were studied for surface modification. Phosphatidylinositol was reported to increase the circulation time from 15 to 20 hours [262]. PEG was found to increase the half-life of liposome-encapsulated Hb up to 65 hours [263,264]. Similarly, PEG conjugated liposomes significantly inhibit particle aggregation and reduce the viscosity [264].

These PEG conjugated liposomes were found to have no effect of clearance rate, no observed antibody response, and it was found to reduce the liposome aggregation considerably [257,265]. When polyethylene glycol (PEG5000)-conjugated phosphatidylethanolamine was introduced on the liposome surface and suspended in the albumin, the viscosity observed was 3.5 cP at 358 s^{-1} (Shear Rate), which is comparable to that of human blood. This is an important observation because when unmodified liposome is suspended in albumin, the viscosity observed was 37 cp at 0.58 s^{-1} [266]. To increase the circulation time, distearoyl phosphoethanolamine PEG 5000 (10 mol%) was added to the formulation of liposome-encapsulated Hb to reduce the reticuloendothelial system uptake [261]. Surface modification with PEG is also found to be associated with the reducing thrombocytopenic reaction.

It is clear that surface modification can modulate the pharmaceutical characteristics of the liposome. However, conjugating the target-specific ligand on the liposome surface can substantially enhance the cellular uptake [267–270]. In the future, to inhibit liposome clearance, reduce viscosity and nanoparticle aggregation, PEGylated liposome is the most

relevant approach. Surface modification of the LHb is the most prominent step towards a sustained and extended delivery of oxygen.

3.3. Genetic engineering of Haemoglobin

Genetic engineering is one of the most important tools to study the function and modifications of proteins. The production of mutant Hb is the best approach to study the oxygen affinity towards the globin. Several Hb mutants have been reported and clinically studied, but these mutants have restricted utility in the investigation towards the Hb functions. Genetic engineering, along with various biophysical techniques allows us to analyze the role of a particular sequence in the physiological function. Hb is one of the first few proteins which was structurally analyzed to study the concept of cell-to-cell interaction, cell-to-protein interaction, cooperative ligand binding, protein folding, and the functions of various conformers. The structure-activity relationship is still far from being completely explored. During the last two decades, there has been an explosion in Hb research. Modern tools like the CRISPR-CAS system further enhanced our ability to easily create more complicated mutants. The following section deals with the recent advances made by the recombinant Hb in HBOCs.

3.3.1. A step closer to HBOCs- Recombinant Hb- Genetically engineered Hb

NO scavenging activity, the risk of blood-transmitted viral and bacterial infections, the issue of tetramer stability, and a limited supply of human Hb prompted the search for a better source of human Hb. Use of recombinant technology to express the genetically modified Hb from *Escherichia coli* (*E. coli)* to get a fully functional Hb without the risk of blood-transmitted diseases and less NO scavenging activity is the current research focus. The most sought genetic modification includes the 1) modification which could alter the metabolism and the oxygen affinity of native Hb, 2) modifications which could prevent the dissociation of the functional Hb tetramer into dimers and 3) genetic modifications which have the least NO scavenging activity.

Scientific advancement has led to the development of highly efficient vectors and methods to produce rHb. The primary goals for the development of the suitable vector for expression are the cell-free Hb without the risk of pathogen transfer. Nagai *et al.*, Olson *et al.*, and Hoffman *et al.* developed a few of the first bacterial expression system for rHb in *E. coli* [271–274]. Hoffman *et al.* reported the use of polycistronic transcript with Tac promoter [274]. This method involves the addition of an exogenous Heme. Fronticelli *et al.* describes a plasmid similar to that of Nagai *et al.* type, which, by chemical induction, produces a β-globin fusion protein. They also proposed the feasible method to produce Hb tetramer from β-globin chains [275]. Expression of the soluble Hb is the target, and Vasseur-Godbillonn *et al.* expressed the soluble globin with high yield in *E. coli* [276]. They also co-expressed the erythroid-specific chaperone protein, which prevents the protein precipitation, specifically by binding to free α-globin. Natarajan *et al.* also described the system and conditions for the expression of the soluble rHb of the deer mouse [277]. One of the major advantages of this system is that it does not need the co-expression of the molecular chaperones, and no additional Heme incorporation step is involved.

For cooperativity-based oxygen binding and also ease of autoxidation of the heme group, Jeong *et al.* proposed three rHb with amino residue substitution [278]. These mutations were found to exhibit high cooperativity-based oxygen binding and resistance to autoxidation.

As discussed earlier, NO scavenging by stromal free Hb leads to vasoconstriction. Pancreatic hypoxia is also one of the consequences of hemorrhagic shock, which leads to the inhibition of its microcirculatory system [279]. Therefore, von Dobschuetz *et al.* tested and compared the activity of rHb 2.0, having 20-30-fold, lower the NO scavenging activity with rHb on the microhemodynamics and leucocyte activity on pancreas venules after hemorrhagic shock [280]. In conclusion, it was observed that this rHb is an effective resuscitation fluid that effectively restores the pancreatic microcirculation aftershock [280].

Baxter therapeutics rHb 2.0 was the first rHb to enter clinical trials. 20-to-30 times lower NO scavenging activity is the highlight of the rHb 2.0, and reduction in the cooperativity-based oxygen binding was one of the major disadvantages. It was observed that the total oxygen-binding capacity was unchanged. Raat *et al.* compared rHb 2.0 (second generation rHb) with rHb 1.1 (first generation rHb, a product of Somatogen Inc.), rHb having NO scavenging activity similar to that of adult Hb in a fixed pressure rat model [281]. It was observed that rHb 2.0 reduced the mean atrial pressure in pressures around 27% from the baseline, confirming the 20-to-30 times NO scavenging activity reduction of rHb 2.0. Similarly, Hermann *et al.* compared rHb 2.0 with rHb 1.1 in a rodent model of hemorrhagic shock, with a particular focus in the microcirculatory situation [282]. Resuscitation with rHb 2.0 was found to recover the mean arterial pressure with statically significant improvement in functional capillary density. rHb 1.1 was also able to restore the mean arterial pressure, but at the cost of a loss in functional capillary density [282]. Similarly, when rHb 2.0 was tested in an animal model of hemorrhagic shock, pancreatic microcirculation was found to be effectively restored [280]. This effect was attributed to its low NO scavenging activity. Rattan *et al* confirmed the effect of rHb 1.1 on gastrointestinal and internal anal sphincter smooth muscle [283].

Furthermore, the decrease in the NO scavenging and associated vasoconstriction properties with recombinant technology is feasible. Nevertheless, mutations that lead to such phenotypes could compromise the Hb stability and could enhance heme loss and related toxicities [284]. As an example, fetal Hb was vastly studied for its higher stability as compared with the adult Hb due to its reducing oxidative reactivity [285]. Moreover, *Simons et al.* compared the oxidative and functional properties of fetal rHb and adult rHb [286]. The results showed that both rHbs were expressed in *E. coli.* and there were not differences in terms of their reactivity towards NO scavenging activity, hydrogen peroxide, and autoxidation rate. Therefore, both rHb were recommended as a starting material for HBOC production [286]. Additionally, Silkstone *et al.* proposed that tyrosine mutation in Hb could reduce the heme-mediated oxidative reactivity and NO scavenging activities with the consequent enhancing stabilization [284]. This mutation in the adult human Hb was found not only to have the stabilizing effects but also to have reduced vasoactivity and hence considered as the precursor for HBOC production [284].

Additionally, rHb is not only a robust system to study any number of mutation and variation in Hb. In the near future, it has the potential to replace the whole blood or red blood cell transfusion. A few of the major challenges that rHb production need to overcome are the misfolding and denaturation of the globin molecules. Post-purification formulations like crosslinking, PEGylation, and encapsulation in liposomes also contribute to the time and cost of the final product.

Expression systems successfully exploited to produce rHb are *E. coli* and yeast cell along with a few mammals and insect cells [287]. Overall, the Hb function and structure is highly conserved through evolution. The non-protein part of Hb i.e., the Heme is common across the heme-containing Hb proteins across the diverse species of animals [288]. This peculiar character of Hb resolves the complications involved in the production of rHb. Once produced, rHb proteins can be combined with the externally supplied heme to get the functional Hb molecule [289]. Conservation of Hb function throughout the evolution also facilitates the expression of rHb in any mammals because rHb can substitute for the function of the Hb functions in other mammals, including humans [290]. Therefore, the important requirements of the recombinant production of Hb are the synthesis of a soluble form of globin proteins, their proper recovery, and recombination with heme moiety.

3.4. Lipid-coated oxygen microbubble, hollow microparticles, and polymer-based hollow microparticles

Lipid-coated microbubbles are a new class of nanoparticles that have the potential to become an important therapeutic aid in the future.

Microbubbles are composed of a gas core which is stabilized by the lipid coat. These particles have diagnostic and oxygen/drug delivery applications. Oxygen carriers like PFCs or the oxygen gas can be trapped inside the core of such particles making the microbubbles stable enough to withstand the circulation whirlpool. Such microbubble can be made targeted by linking it with targeting proteins or peptides or could trigger them to release the content at a specific pH or with ultrasound. On the other hand, polymer-based hollow microparticles are polymeric spheres with pores on its surface. They are considered to be more stable than the lipid-coated hollow particles, and hence, the recent research focus is mostly on their ability to deliver therapeutic gases and drugs.

Oxygen microbubbles have been tested for their role in sensitizing chemotherapy by increasing the oxygen levels [291]. Localized oxygen microbubble delivery to the hypoxic tumor was studied by Eisenbrey *et al.* for its effect on radiotherapy [292]. The oxygen delivery capacity of the oxygen microbubbles was also studied for its potential application in cardiac arrest, hypoxemia, and resuscitation, which is the most collective cause of mortality in critically ill patients [293,294]. Oxygen microbubble transfusion has been associated with a rapid rise in arterial blood saturation and improved survival rate in animal models of hemorrhage shock.

Moreover, lipid-based oxygen microbubbles have the potential to become an effective theragnostic agent in cancer and cardiotherapy, and most recently, for its role as an effective oxygen carrier. The first oxygen microbubbles was prepared from lipids, which are not suitable for long storage conditions and hence are unsuitable for clinical application. Similarly, the coalesce of microbubbles to form a large bubble could lead to obstruction and could be lethal to patients. The stability issues of microbubbles and potential blockage by large bubbles need to be addressed to exploit its potential application. To overcome the issue of oxygen microbubbles, Polizzotti *et al.* and Seekell *et al.* have proposed the concept of polymer-based hollow microparticles [295,296]. To develop these microparticles, they dissolved the polymer (poly(*D*,*L*-lactic-coglycolic acid) and perfluorooctyl bromide in oil emulsion and emulsified it with Pluronic F-68 [296]. These particles were stable during rapid infusion and when stored in dispersion and freeze-dried form [295]. As the oxygen delivery via oxygen microbubble and perfluorocarbon emulsions undergoes premature oxygen release and are unsuitable for long term storage, Song *et al.* addressed this limitation by developing oxygen bilayer nanobubbles [297]. These nanobubbles possessed excellent stability reducing the risk of premature oxygen release and were stored as freeze-dried powders to avoid shelf storage issues. Moreover, these nanobubbles were the first to use as an adjunct agent in cancer photodynamic therapy

Microbubbles, nanobubbles, and hollow particles have a core containing gas, which imparts them with the echogenic character [298] (Fig. 2). Furthermore, oxygen microbubbles and nanobubbles have been linked with the restoration of the normoxia condition in tumors and could be used as an adjuvant along with various types of cancer therapy [298].

These studies have confirmed that oxygen microbubbles, hollow microparticles, and polymer-based hollow microparticles are promising artificial oxygen delivery systems suitable for cell proliferation, rejuvenation of ischemic organs and tissues, and sensitization of cancer chemo, radio, and phototherapy.

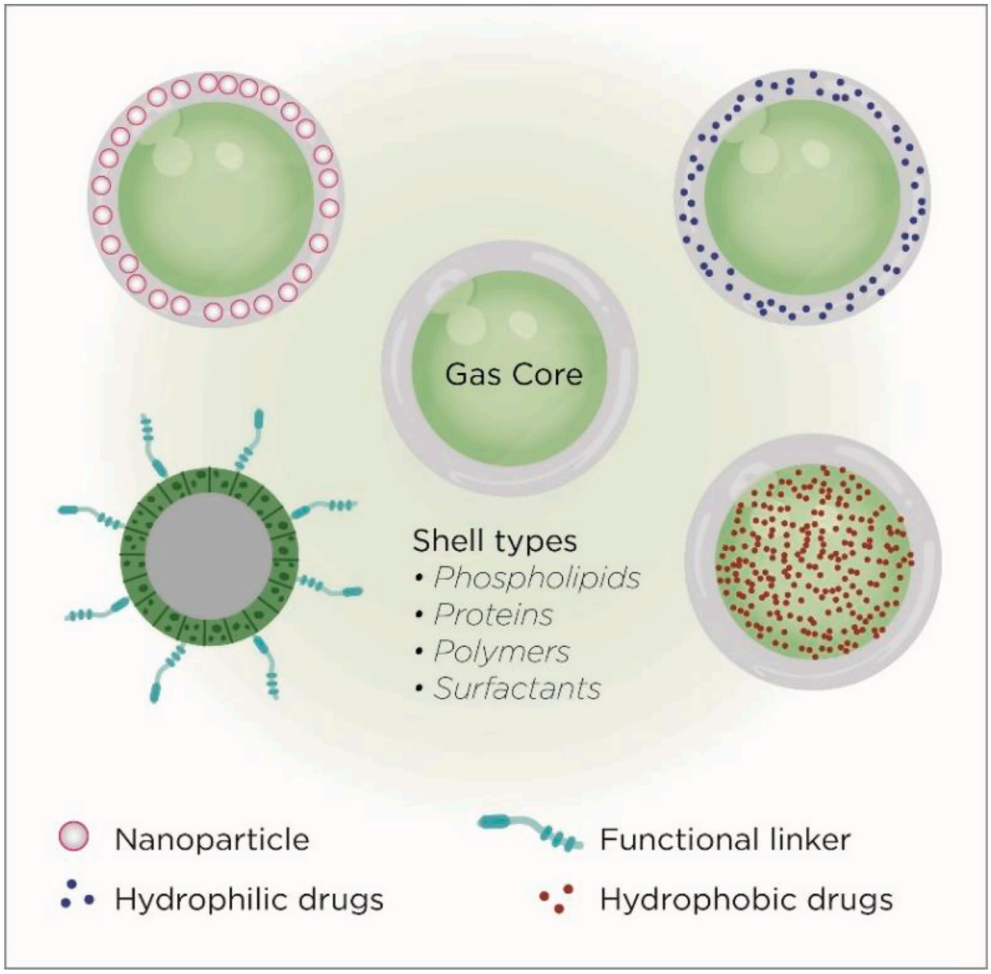


Fig. 2. Schematic of micro-nanobubbles (MNBs) and their functionalization. Adapted from Khan *et al.* [298]

4. Recent clinical development of HBOCs

The regular treatment for sickle cell anemia is based on supportive therapy. When Hb levels decrease to perform the basal metabolic functions, whole blood or blood cell transfusion is recommended. Occasionally, patients may deny a blood transfusion based on religious or cultural beliefs, and sometimes the compatible blood products are not available. To manage these circumstances, several products are under clinical development, one of which is the HBOC-201, a polymerized bovine Hb created by Biopure Corporation. This product is currently available in the USA, and it has been previously utilized to treat severe sickle cell anemia and in multi-organic failure events [299]. Due to the NO scavenging by Hb, transient hypertension due to the administration of HBOC-201 continues to be a clinical challenge. Despite these minor drawbacks, HBOC-201 is under clinical trial due to the several advantages it presents.

Another product called "HemoAct" has been clinically tested for its potential to replace the RBC. HemoAct is a Hb molecule covalently linked with the albumin protein. When tested in rats, it was found that HemoAct does not affect blood pressure. HemoAct was tested for its influence on the intrinsic and extrinsic pathways by measuring prothrombin time and activated partial thromboplastic time. When HemoAct was mixed with blood, no change in the prothrombin and activated partial thromboplastic time was observed and when it was examined *in vitro,* it showed good blood compatibility [300].

HemoCD is an artificial oxygen transporter made to replace the haemoglobin molecules [301]. It shows favorable reversible oxygen binding in aqueous solution unlike another similar kind of preparations that shows oxygen binding in anhydrous organic solvents [302]. Due to its reversible oxygen binding in aqueous solvent HemoCD is considered as one of the few artificial oxygen transporters, which could be categorized as a complete synthetic oxygen transporter. Other than the favorable oxygen-binding, it has shown widespread stability in circulation, non-toxic to cells, with no vasoconstriction effect. Despite its synthetic nature, it is not free of undesirable effects; the most noted are 1) low synthetic yield, 2) high intravenous CO binding, and 3) short circulation time due to the rapid clearance from the renal system [301,302].

HEMOXCell is another artificial oxygen carrier developed recently to supplement oxygen to the mesenchymal stem cell culture [303]. The rational thinking behind the development of HEMOXCell is the inherent problems associated with the traditional supplements, contamination and immunogenic reactions are the major cause of concern with fetal bovine serum uses. One of the limiting factors in cell culture is the proliferation of the cells and the reduction of available oxygen. HEMOXCell, which is developed by the Hemarina SA (Morlaix, France) is focused on the oxygen supply during mesenchymal stem cell culture [303,304].

Currently, Erythromer is an artificial red cell under development to substitute the red blood cells [305]. Inadequate physiological interaction of available artificial oxygen carrier with oxygen and NO scavenging are the major limiting factors in clinical development. Erythromer is designed to overcome this limitation by controlling adequate oxygen release, adding novel 2,3-DPG in the capsule along with the Hb molecules and mitigating the NO scavenging activity. In hemorrhagic shock model, Erythromer was found to have very little NO scavenging activity and to be stable over a 3-month storage period [305]. Erythromer has the greatest potential to substitute red blood cells due to its negligible NO scavenging activity and high stability in the lyophilized form.

5. Challenges in HBOCs development

Several clinical trials suggest that acellular unmodified Hb is unsafe to use, even when is highly purified. Hb sourced from bovine or humans requires downstream processing to eliminate toxicity and impart red blood cell functions. Ideal HBOCs should have stable tetramer, low oxygen affinity, cooperation-based oxygen binding, less oxidation, no vasoconstriction, and nephrotoxicity, nonimmunogenic, and no interference with normal physiological functions. Some important issues to consider in HBOCs developments are pure raw material supply, hurdles in site-specific crosslinking or modifications, high cost of large-scale manufacturing, and a stable product.

As discussed earlier, stromal-free Hb tetramer, when exposed to an internal environment, dissociates into dimers, which are readily eliminated through glomerular filtration. Rapid excretion results in short half-life and renal toxicity. It is observed that the heme molecule readily dissociates from the dimers when compared with the tetramer. Various approaches studied to impart stability to the tetrameric Hb include chemical or genetic crosslinking of the Hb protein, polymerization, crosslinking to the polymer, and encapsulation in the liposomes. These approaches reduce the Hb interactions with the endothelial layer, stabilized the tetramer, and reduce elimination via the kidney.

Altering oxygen affinity is another important goal. Acellular Hb has no 2,3-DPG, which could regulate its oxygen affinity. In the absence of 2,3-DPG, oxygen affinity towards Hb increases, leading to the reduced release inside the tissues. The higher plasma pH, as compared to the inside of RBCs, increase the affinity of Hb towards oxygen. The importance of conserving an original oxygen affinity and cooperative oxygen binding is a crucial challenge for the commercial success of the HBOCs. Emphasis is shifted to preserve the morphology of the binding site. Various chemical and recombinant modifications are found to be effective to keep the original oxygen affinity. Bovine Hb offer an effective approach to tackle this issue. Oxygen delivery by the bovine Hb is chlorine ion concentration sensitive and is independent of 2,3-DGP [306]. Point mutation for appropriate oxygen affinity and recombinant Hb expression along with 2,3-DGP are now advanced means to decrease oxygen affinity [289].

One of the principal methodologies to inhibit vasoconstriction comprise of PEGylation and encapsulation of Hb in liposomes. Oxygen and NO have almost a similar binding site in heme hence, making mutant Hb having preferential oxygen binging over the NO could be the potential approach [307].

The immunogenic response of the body towards modified Hb is a primary cause of concern. Responses like accelerated clearance on repeated dosing, macrophages accumulation in the reticuloendothelial system, suppression of T cell multiplication, decreased lymphocyte ratio, increased granulocyte ratio, less neutrophil infiltration, and more macrophage infiltration are reported [252–254]. Genetically or chemically modified Hb may induce the immune response, and proper measures should be addressed in the future development of HBOCs.

Ensuring sterility and endotoxin elimination from the final product is one of the most prominent goals to be achieved immediately. Hb cannot survive heat sterilization [308]. Denatured Hb can enhance the coagulation activity, and hence terminal sterilization of Hb is managed by filtration. Some modified Hb́s like crossed linked and PEGylated are stable and subject to the pasteurization process. However, complete elimination of endotoxins from rHb expressed in bacteria like *E. coli* is a continuous scientific challenge [309–311].

The basis of HBOCs is the modification at the specific site of Hb using selective chemistry. However, Hb is a complex molecule with multiple functional groups and several potential sites for modifications, making them a difficult task. Most of the reagents used in the modifications are functional group-specific, but due to the heterogeneous nature of Hb, site-specificity is not an easy task. Also, the specificity of the reaction depends upon the temperature, pH, and the presence or absence of cofactors. Hb functions can vary with the type and site of chemical modifications, and proper purification is the essential prerequisite to get the homogenous product.

All the above-mentioned factors are the primary reasons for the slow development of the HBOCs. Incomplete understanding of the complexities of oxygen physiology, interactions with normal physiological

functions, insufficient elucidation of the mechanism of side effects, incomplete standardized validation protocol, strict regulatory compliances, time, and investments required to develop the viable products, to name a few, are the significant other hurdles in HBOCs development. Practically, there are several obstacles; however, each barrier creates a new line of research. Over the last five decades, these challenges have significantly enhanced our knowledge about oxygen physiology, *in vitro* behavior of PFCs, and HBOCs resulting in considerable advances in the product safety, efficiency, efficacy, and potency.

6. Future considerations in the oxygen carrier's field

Although a lot of efforts were put into overcoming the problems of oxygen delivery by Hb based oxygen carriers, their toxicity has hampered their clinical application. The development of new PFCs formulations is a must to get similar features to an ideal oxygen carrier. Improving the emulsifying agent and other formulation conditions must be addressed in the search for clinical approval. To do this, interdisciplinary studies are needed to improve the safety for PFCs as oxygen carriers.

HBOCs cause clinically significant vasoconstriction, which may be advantageous in case of hypovolemic shock. However, such vascular constriction could impair local blood flow to the organs. Chemical modifications and genetic alterations play a crucial role in the effectiveness of the HBOCs, including its efficiency and side effects. Parallel investigation of the HBOCs derived from bovine or human sources on the various metabolic functions of the cells is required [312]. Mapping of the cellular events adversely affected by HBOCs would prove beneficial to design the next generation HBOCs without side effects. Polymerized and crosslinked HBOCs has significantly reduced the adverse effects of cell free Hb. Development of different polymerization techniques could further help to develop different polymeric Hb with better oxygen carrying capabilities. Polymerization was also found to reduce the ability of Hb to adjust with the pH change, which is a crucial regulator of Hb binding with oxygen. Investigation of polymerized Hb revealed that it has a reduced CO_2 binding, which confirms the modifications of Val residue, an important site for CO_2 binding [313]. Noteworthy, the site of polymerization is also crucial for the oxygen carrying ability of the polymerized Hb. Hence, one of the significant challenges could be the identification of the optimized site of the polymerized which does not affect the oxygen binding abilities of the Hb complex.

Higher heme iron oxidation and heme loss were both reported in the HBOCs and more significantly in PEGylated Hb [312,314–316]. The present Hb based products have not undertaken the effects of HBOCs exposure to the physiological condition, which may expose them to the oxidative pathways and heme loss. As oxidative damage and heme loss lead to the immunological and inflammatory reactions [317,318], it is crucial to test HBOCs against such physiological response.

All the issues mentioned above, and challenges associated with HBOCs are mostly linked with the complex chemistries involved in product formulations. Better knowledge and innovative improvements will provide a strong foundation to design and deliver safe and more efficient HBOCs [319]. For example, PPHb haemoglobin is developed to avoid the side effects associated with Hb oxidation. Erythromer is not only morphologically similar to RBCs but also has similar oxygen binding and release properties, resistance towards oxidation, and lower NO sequestration. HBOCs display different physiochemical properties based on the degree of polymerization and crosslinking methods resulting in variable oxygen binding and release properties. This also affects oxidative related side effects, heme clearance, and NO scavenging.

An alternative approach is the development of the recombinant Hb for HBOCs. Recombinant Hb based HBOCs offers the advantage of 1) natural origin for the alternative transfusion approach, 2) lower disease transmission risk, 3) better shelf-life, 4) a standardized and uniform final product, and 5) universal acceptability. Despite these advantages, sufficient efforts are not seen in the development of recombinant based HBOCs which could result in a product fit for clinical use. To date, Hb has been successfully expressed from transgenic hosts like yeast, bacteria, mice, among others [142]. The physiological stability of these products could be enhanced by the point mutation strategy. Mutagenesis in the Hb gene could 1) enhance oxygen affinity by several-fold, 2) inhibit heme oxidation and NO scavenging, and 3) resist dissociation of Hb subunits. Recombinant Hb, other than the lower side effect, could also be the source of unlimited Hb supply. Moreover, it could have universal acceptability and could be the product of choice for the patient who does not have an alternative blood product or when allogeneic blood transfusion is not the best choice or is not available. The various mutations for higher Hb stability, lower rate of iron oxidation, and heme loss studies are identified; however, the precise assembly of the mutations is not yet studied to develop the ideal Hb with the characteristics described above. In the future, proper selection of mutations to develop recombinant Hb with lower oxidation, heme loss, and NO scavenging without affecting the core properties of Hb is the immediate challenge. Consequently, this source of Hb will be the most economical way of producing a feasible oxygen carrier with no side effects.

Polymerized and crosslinked Hb or encapsulation of such Hb products requires matching with the RBC molecules in terms of physicochemical and morphological properties. Adaptation with RBC properties is crucial because most of the RBC functions are governed by such characteristics. RBC flexibility allows it to squeeze through the micro blood capillaries. This elasticity and mechanical strength are provided by the specialized erythrocyte cytoskeletal proteins called spectrin. Oxygenation of Hb leads to the greater morphological changes in the RBCs than the deoxygenated Hb. Morphological changes are dynamically absorbed by virtue of its elastic nature and allows them to pass through the microvasculature [320]. All these considerations recently lead to a focus on the development of the biomaterials for HBOCs, which could mimic RBC's size, shape, flexibility, and mechanical strength. For example, Doshi *et al.* developed Hb microparticles that mimic the flexibility and morphological characters of RBCs [321]. Haghgooie *et al.* also reported the RBC similar Hb microparticles, prepared using the stop-flow-lithography technique. They used polyethylene glycol hydrogel particles with morphology similar to RBCs [322]. Merkel *at al.* prepared RBC shaped mimetic microparticles from acrylate hydrogels. They also confirmed enhanced circulation time and biodistribution of the RBC mimetic nanoparticles by increasing the deformability of the microparticles [323]. For example, actin haemoglobin was encapsulated in the liposomes to emulate the RBC morphology [324] where the negative charges on the RBC avoid their aggregation. Moreover, Xu *et al.* developed Hb loaded polymeric nanoparticles using mPEG-PLA-mPEG, regulating the surface charge by using cationized cetyltrimethylammonium bromide and anionized sodium dodecyl sulphate [325]. Anionic nanoparticles were rapidly removed, although the cationic nanoparticles were observed to have a half-life of eleven hours.

On the other hand, obtaining Hb for HBOCs will be challenging and crucial to produce an innovative and useful product. Nowadays the utilization of recombinant Hb could provide an unlimited source of Hb. Nevertheless, it should be highlighted that human Hb function is controlled by compounds like 2,3-DPG which binds the deoxygenated Hb with more affinity than the oxygenated Hb. However, the cell-free Hb, which loses out 2,3-DPG, has much higher affinity towards the oxygen, which shifts the oxygen equilibrium curve (OEC) to the left and leads to the lower tissue oxygenation [326]. Conversely, bovine Hb is not dependent on the 2,3-DPG for its oxygen affinity; rather, it depends on the chloride ions, which are abundantly available in human blood. Bovine Hb also has much higher stability at higher temperatures during isolation and processing [327]. Therefore, from the viewpoint of availability, stability, and oxygen transport capacity, bovine Hb offers several advantages over human Hb. A product approved for veterinary use called Hemopure® is developed from bovine Hb. HBOCs developed from

such sources need to be further studied for their immunogenic properties. Besides Hb based HBOCs, compounds like PFCs are also underdoing preclinical and clinical testing, but none of them are yet classified as safe for clinical use. Cell-free donor independence Hbs are also under development from stem cells [328,329]. Innovative research work is also directed towards the development of donor independent RBCs. The translation of this research to the patient who needs it is the immediate challenge. It requires overcoming the issue of ethical approvals for preclinical and clinical studies, consistency in the final product, scaling up the pilot to large-scale production, etc.

7. Conclusions

In a nutshell, the PFCs have been the object of multiple clinical trials, their use in clinical treatment is suggested in several studies and approved by the FDA in some cases. Most of them are not used today due to problems associated mainly with the formulation regardless of the known PFC capability to capture and transport oxygen and other gases.

Moreover, the use of PFCs is a different approximation to oxygen carriers and brings a new perspective to conditions associated with the lack of oxygen on different systems.

Additionally, the development of an Hb based oxygen transporter is the most sought-after discovery in haematology. Hb can be readily available from the outdated blood from blood banks and can be chemically modified for use in clinical emergencies. Bovine Hb is also cross-linked, PEGylated, and encapsulated in liposomes to study its clinical applications [8]. The use of rHb has eliminated the risk of infection and provides the tools to modify the globin protein to study the structure base oxygen-binding research.

In fact, the development of HBOCs has faced several challenges in the past; most important of them are the severe side effects of acellular Hb. Nephrotoxicity of the dissociated tetramer, hypertension mediated by the NO scavenging, and inflammation are the major ones. In this review, several approaches like crosslinking, polymerization, conjugation, PEGylation, encapsulation of Hb in liposomes are described. As discussed, recombinant technology has already provided the means to produce stable Hb, which has minimum NO scavenging activity and an extended half-life. In early clinical trials, these rHb 1.1 and rHb 2.0 are found to be safe with no nephrotoxicity and less vasoactivity. Infarct side effects like fever, GIT problems, and mild hypertension were reported in the patients receiving low to high doses of experimental Hb but some of these drawbacks are because of the endotoxin, which could be best rectified by proper purification of the product. The most important approach to avoid these side effects is the production of recombinant Hb with specific mutations [281]. Similarly, several studies are underway to predict the required mutation to convert the Hb to a chloride regulated oxygen carrier instead of 2,3-DPG [330]. If successful, this approach has the potential to offer the new modified Hbs to produce HBOCs.

One of the primary causes of the short life of Hb is its oxidation. Attempts are underway to identify the mutation in Hb, which could reduce the oxidation of Heme in solution [331–333]. Attempts are also underway to alter the Hb oxidation by site-directed mutation to change the heme pocket morphology and confirmation [331]. NO, CO_2, and O_2 bind to the Hb via heme pocket, and future mutations and chemical modifications in the globin proteins will be focused on the differentiation between these gases.

Practice points

- Features of an ideal oxygen carrier: no impact on circulation and blood pressure, immunological inertness, easy uptake, distribution, metabolism, and elimination.
- Linear increase of oxygen solubility within the intermolecular spaces of the PFCs depends on temperature and pressure
- Sigmoidally dependent oxygen solubility in haemoglobin its Fe atom is controlled by the 2,3-diphosphoglycerate metabolite.
- Severe anemic patients administrated with Fluosol have resulted in a 24% increase in oxygen uptake.
- Perftoran it is used widely in Russia, Mexico, South Africa, Kazakhstan, Ukraine, and Kirghiz Republic. It was also used in México from 2005 to 2010. Over 35.000 patients have been treated over the world. Later was commercialized in the USA for acute anemia in animals
- PFCs have seen limited clinical use due to the side effects associated with the emulsifying agents.
- The administration of Oxygent and acute norvomolemic hemodilution have reduced the need for red blood cell transfusion in non-cardiac surgery patients.
- Clinical trials have confirmed the lack of diasparin cross-linked Hb antibodies before and after the infusion.
- Hemopure® can cause elevated blood pressure and it was approved for clinical use in South Africa and Russia.
- Hemopure® has been utilized to treat patients in the USA under the FDA's Expanded Access Program (EAP)
- Currently, Oxyglobin was approved for the treatment of canine anemia.
- Oxycyte has been used for lung injury in veterinary treatments.

Research agenda

- More complete clinical studies considering Oxycyte (PFCs) and the safety and dose regimes of O-raffinose cross-linked Hb are necessary.
- The impact of PEGylation on the tetramer formation of non-cross linked Hb should be studied, focusing on the improvement of their oxygen binding properties.
- Further research regarding the morbidity and mortality associated with different encapsulated HOBCs is needed.
- Preclinical evidence suggests a great potential of PPHb, but studies regarding its impact on oxidative stress and blood flow are essential.
- Optimization of the lipid content of the liposomes in LHb is required.
- Complementary interdisciplinary studies considering the safety of clinical use of artificial oxygen carriers are desirable.

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Declaration of Competing Interest

No conflicts to disclosure.

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