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Stepping stones to the future of haemoglobin-based blood products: clinical, preclinical and innovative examples

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There is an enormous demand for blood transfusions in daily clinical practices since blood products, and especially red blood cells (RBCs), can significantly improve survival. However, donor-derived RBCs have also important limitations as a result of their insufficient availability, the need for typing and cross-matching, short self-life or risk of pathogenic contamination. Thus, as a result of the unique oxygen-transport ability of hemoglobin (Hb), Hb-based oxygen carriers (HBOCs) have attracted a lot of attention for the development of RBCs surrogates able to provide tissue oxygenation. Here, we highlight the progress in the development of HBOCs, placing the focus on the different examples that have undergone exhaustive pre-clinical and clinical evaluation. Additionally, we also provide a comprehensive review of very recent and innovative examples depicting the pipeline of the next generation of blood substitutes.

1. Introduction

The transfusion of whole blood but also of various isolated components (e.g., packed red blood cells (RBCs), platelet concentrates or plasma) is an indispensable and common clinical procedure. Specifically, blood transfusions are widely employed in conditions of serious blood disorders such as anaemia, haemophilia or for patients receiving tumour chemotherapy.¹ Additionally, blood transfusions are also extensively used in the context of traumatic injuries and surgical operations (e.g., transplants).²

Despite its many advantages, the use of donor blood has several limitations and risks. For example, there is a limited availability since only 10% of eligible donors are actually donating blood.³ Unfortunately, this blood short supply is expected to get worse due to increased population growth and aging. What is more, only 7% of the donors have blood that can be immediately used in emergencies (i.e., “universal” O-negative blood).³ Thus, before blood can be transfused, there is a need for cross-matching and blood group typing which can cause fatal delays in emergency situations. Blood-based products have a short storage lifetime of a maximum of 42 days in refrigerated conditions and 1 day at room temperature. This short self-life makes it impossible to create large stockpiles to be used in acute disasters (e.g., earthquakes, plane crashes or terrorist attacks). The need for refrigerated conditions creates major logistical challenges when donor blood is needed in austere and remote battlefield or civilian locations.^{4–6}

⁶ Also, both RBCs and platelets develop storage lesions over time affecting their stability, physiology as well as their in vivo circulation time.^{7,8} For example, while RBCs can circulate in the bloodstream for about 120 days, this time is reduced to only 28 days for donor RBCs. Blood products are also a transmission vector for infectious diseases such as human immunodeficiency (HIV) virus, Zika virus and hepatitis B or C. This results in extensive and costly screening prior to transfusion.^{3,9} Lastly, of the 75 million units of RBCs that are collected worldwide, 13 million are currently not being tested for HIV.¹⁰

While research efforts are focusing on resolving these issues to enhance the availability and applicability of donor-derived blood products,^{11–14} in parallel, a robust volume of research is being devoted to create semi- or fully-synthetic artificial oxygen (O₂) carriers. It is envisioned that such an approach will revolutionize the practices in blood banking and transfusion medicine.¹⁵ Artificial O₂ carriers are being specifically designed to substitute and even amplify the functions of endogenous blood components. Additionally, such a (semi)-synthetic platform will have several advantages over donor blood including: sufficient availability, since it can be produced at a large scale; prolonged storage across a wide range of environmental conditions, thus having easy storage and portability; absence of disease transmission, since they could be sterilized without compromising their functionality; universal application since they are absent of blood group antigens thus avoiding the need for typing and cross-matching.^{16,17}

In this review, we first provide an overview of the biology of RBCs and haemoglobin (Hb). Next, we feature the most successful and recent examples of different Hb-based oxygen carriers (HBOCs). The different examples will be classified depending on their development status (i.e., marketed products, clinical or preclinical developments). The most novel and innovative designs will also be discussed at the end of the section.

2. RBCs and Hb

RBCs, also known as erythrocytes, haemocytes or red corpuscles, are the most prevalent cell type since they constitute approximately 84% of all the cells of our body.¹⁸ In an adult healthy person, every second, the bone marrow produces and releases into the circulation between 2 and 3 million RBCs.¹⁹

RBCs are flexible oval disks of about 7.5 - 8.7 μm in diameter and around 2 μm in thickness (**Scheme 1A**). They display a biconcave shape in order to increase their surface area for optimal O_2 diffusion. RBCs are also highly flexible with a Young's modulus of only 10-20 kPa. This deformability allows them to change their shape and morphology when passing through the smallest blood vessels (i.e., capillaries of 5-10 μm in diameter).^{20,21} Thanks to a two-dimensional spectrin network located in the cytosolic side of their membrane, RBCs are highly viscoelastic. This allows them to preserve their mechanical integrity following cyclical deformation in the microvasculature. Importantly, when oxygenated, RBCs are significantly more deformable than when they are in a deoxygenated state, thus making it possible to squeeze through and deliver O_2 to the microvasculature.

Furthermore, the size, shape and flexibility of RBCs determine their hemodynamic distribution in the blood flow. In mid to large blood vessels, RBCs are located in the center of the parabolic flow field. In the small capillaries, RBCs are distributed throughout the vessels for efficient O_2 exchange.²² Each RBC remains in circulation for about 120 days and, the clearance of old or damaged RBCs, is carried out by macrophages residing in the liver and the spleen.²³

Since O_2 is approximately 20 times less soluble in the aqueous phase than carbon dioxide (CO_2), an O_2 transport system is required to achieve the high O_2 demands of our body.³ This function is conducted by Hb, which is the main component of RBCs. Hb is responsible for the transport of 98% of all the O_2 while the remaining 2% is dissolved in blood plasma. In an adult human, around 250 million Hb molecules with a weight of 27-31 pg can be found in each RBC. This accounts for one third of the total volume of a RBC. In fact, to allow for a high Hb loading, mature human RBCs do not have a nucleus and are missing most of the organelles present in other eukaryotic cells (e.g., mitochondria or Golgi apparatus). Hb is a tetrameric protein with a molecular weight of 64.5 kDa. As suggested by its name, Hb is constituted by "heme groups" (non-protein part) and "globins" (protein part). Specifically, Hb is composed of two alpha and two beta subunits ($\alpha_1\beta_1\alpha_2\beta_2$) and each subunit contains a heme group and a polypeptide globulin chain (**Scheme 1B**). The alpha globulin chains consist of 141 amino acid residues and the beta chains are slightly bigger with 146

Scheme 1. A) i) Schematic illustration of red blood cells (RBCs) travelling from the lungs (oxygen (O_2)-loading site) to the tissues where O_2 is released. The RBCs also carry carbon dioxide (CO_2) from the tissues and to the lungs for excretion. Schematic representation of RBCs circulating within a blood vessel (ii) and showing their diameter and thickness (iii). B) Multi-scale representation of RBCs and haemoglobin (Hb). Hb is composed by two alpha (red) and two beta (blue) subunits constituting the "globins" (protein part). Each globin contains a heme group in the centre (non-protein part) which changes conformation from an oxygenated (oxy-Hb) to a deoxygenated (deoxy-Hb) state.

amino acids.²⁴ The heme group is comprised by a protoporphyrin ring with an iron ion bound to the centre, which is responsible for O₂ transport. Since each iron ion can carry one O₂ molecule, a total of four O₂ can be transported by each Hb. Importantly, for O₂ transport, the iron ion must be in the reduced state (ferrous form: Fe²⁺).

Throughout the bloodstream, Hb is exposed to different partial O₂ pressures (pO₂) and the O₂ binding kinetics to Hb are positively cooperative. This means that, a small variation in pO₂, results in a large change in the amount of O₂ captured or released by Hb. Thus, in the lungs, where there is a high pO₂, Hb predominates in the relaxed state (R-state) displaying a high affinity for O₂ (**Scheme 2A**). In this R-state, Hb readily binds to O₂ (oxy-Hb). As a result, within the lung capillaries, Hb is nearly saturated with around 98% of its O₂-binding sites occupied. However, as blood transits from the lungs to the tissues with a lower pO₂, Hb changes its conformation to the tense state (T-state). In the T-state, also known as the unbound state (deoxy-Hb), Hb has a low affinity towards O₂, thus promoting its release into the tissues.²⁵

An O₂ dissociation curve (ODC) is obtained by plotting the percentage of saturated Hb (y) versus the pO₂ (x) (Scheme 2B). Such a curve displays a sigmoidal shape highlighting that O₂ binding takes place in a positively cooperative fashion. The ODC shows a plateau for high pO₂ indicating complete saturation of Hb. Several physiological factors can influence the O₂ binding kinetics to Hb and this will result on shift for the ODC. Specifically, an increase in the CO₂ partial pressure (pCO₂), the concentration of the allosteric effector 2,3-diphosphoglycerate (2,3-DPG), the temperature or a decrease in pH (acidity), promote the T-state and the release of O₂ into the tissues. In contrast, lower pCO₂, 2,3-DPG concentration, temperature or higher pH (alkalinity), will result on a leftward shift, promoting the R-state and consequently enhancing the O₂ binding.²⁶ The p50, which is defined as the pO₂ at which Hb is 50% saturated with O₂, is a good indication of Hb's affinity for O₂. Thus, for high p50 values, Hb's affinity for O₂ decreases while low p50 values result on a left shift of the ODC and the corresponding high O₂ affinity. The p50 of Hb encapsulated within RBCs corresponds to 26.6 mmHg.^{27,28}

Scheme 2. A) Schematic representation of the different conformations of haemoglobin (Hb) depending on the partial oxygen (O₂) pressure (pO₂). In the tense state (T-state) or unbound state (deoxy-Hb), Hb has a low affinity towards O₂ promoting its release into the tissues. However, when the first molecule of O₂ binds to Hb, a conformational change in the Hb's structure increases the O₂ affinity favouring the binding of more O₂ molecules and facilitating the transition to the relaxed state (R-state) where Hb binds to O₂ (oxy-Hb). Several physiological factors such as temperature (T), pH, concentration of 2,3-diphosphoglycerate (2,3-DPG) or partial carbon dioxide pressure (pCO₂) facilitate either the T- or the R-state. B) Representation of Hb O₂ dissociation curve with the characteristic sigmoidal shape highlighting Hb's positive cooperativity. The plateau reached at high pO₂ indicates complete saturation of Hb. The p50 indicates the pO₂ at which Hb is 50% saturated with O₂.

REVIEW

The amount of CO_2 , pH (as concentration of proton ions (H^+)) and the resulting O_2 affinity, are highly interrelated parameters (**Scheme 3**). CO_2 can be transported in the bloodstream in three different ways. A small part of CO_2 can be dissolved in blood plasma ($\sim 10\%$) (Scheme 3A) while, the majority of the CO_2 ($\sim 85\%$), is transported as bicarbonate (HCO_3^-), which is water soluble. The enzyme carbonic anhydrase (CA) within RBCs is responsible for catalysing the hydration of CO_2 into HCO_3^- while releasing a H^+ (Scheme 3B).²⁹ A small CO_2 amount ($\sim 5\%$) can be transported bound to Hb by forming carbaminohaemoglobin ($\text{CO}_2\text{-Hb}$) (Scheme 3C). Upon binding of CO_2 to Hb, an additional H^+ is generated. The association of the two H^+ with the imidazole groups of the histidine amino acids within Hb, is responsible for Hb's conformational change and the O_2 unloading into the tissues (Scheme 3D).³⁰ Such a phenomenon is known as the "Bohr effect". Importantly, this binding of excess H^+ onto Hb's histidine moieties, also mitigates the corresponding acidosis and its detrimental effects. While the as-produced HCO_3^- diffuses out of the RBCs following a concentration gradient; chloride anions diffuse in, in order to maintain the electrical neutrality (Scheme 3E).³¹ This phenomenon where O_2 affects Hb's affinity for both CO_2 and H^+ is known as the "Haldane effect" and plays a central role in regulating the O_2 and CO_2 transport as well as the pH in the bloodstream.

Scheme 3. Schematic illustration of the oxygen (O_2) and carbon dioxide (CO_2) between tissues, red blood cells (RBCs) and alveoli showing the Bohr (brown lines) and Haldane (blue lines) effect. CO_2 is transported in the bloodstream dissolved in plasma (A), as bicarbonate (HCO_3^-) (B) or bound to haemoglobin (Hb) forming carbaminohaemoglobin ($\text{CO}_2\text{-Hb}$) (C). The Bohr effect explains Hb's ability to decrease O_2 affinity while increasing the affinity for both CO_2 and proton ions (H^+). In the peripheral circulation, where there is a high CO_2 partial pressure, the diffusion of CO_2 into the RBCs is facilitated. Inside the RBCs, CO_2 reacts with water (H_2O) to generate HCO_3^- and H^+ in a reaction catalysed by the enzyme carbonic anhydrase (CA) (B). The resulting H^+ readily bind to oxy-Hb (HbO_2) promoting a change in its conformation from the R- to the T-state and releasing O_2 to the tissues (D). HCO_3^- is transported outside the RBCs following a concentration gradient and chloride anions (Cl^-) enter in the RBC in order to maintain the electrical neutrality (E). The Haldane effect explains the ability of Hb to bind O_2 displacing CO_2 and H^+ . Specifically, in the pulmonary circulation, the high O_2 partial pressure facilitates the O_2 diffusion into the RBCs where readily binds to Hb (D). This binding results on a conformational change in Hb's from the T- to the R-state facilitating H^+ release. The generated H^+ reacts with HCO_3^- to form CO_2 and H_2O (B) in a reaction catalysed by CA. CO_2 diffuses out of the RBC to be exhaled in the lungs. $\text{HCO}_3^-/\text{Cl}^-$ anion exchanger enables the entrance of HCO_3^- to the RBC in order to compensate the decrease in HCO_3^- concentration (E). Cl^- is transported out of the RBC to maintain electrical neutrality.

Within RBCs, Hb is exposed to different endogenous and exogenous agents oxidizing it to methemoglobin (metHb). Within metHb, the heme iron is in its oxidized ferric state (Fe^{3+}) and is not able to bind O_2 . Every 24 hours, 3% of all Hb is oxidized into metHb producing reactive oxygen species (ROS) as by-products. However, this process is accelerated under oxidative stress conditions or in the settings of increased O_2 delivery. In particular, hydrogen peroxide (H_2O_2) can react with both Fe^{2+} and Fe^{3+} Hb, resulting in heme degradation and the subsequent release of the free iron ions. These free iron ions can take part, together with H_2O_2 , in the Haber-Weiss or Fenton reaction generating hydroxyl radicals ($\text{OH}\cdot$).³⁰ These resulting ROS have a very short life since they rapidly participate in a wide variety of reactions increasing toxicity. As an example, ROS can react with nitric oxide (NO) producing metHb and nitrate (NO_3^-).³² NO being an important signalling molecule and a natural vasodilator, its depletion from the bloodstream results in vasoconstriction and the subsequent cardiovascular complications.³³ Fortunately, natural RBCs are well equipped with mechanisms to prevent oxidation. In particular, cytochrome-b5 reductase using NADH as a co-factor, maintains metHb levels below 1%.³⁴ The enzyme methHb reductase, which also uses the NADH co-factor, has the ability to revert Hb's auto-oxidation. Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) prevent ROS generation and thus contribute to RBCs function and integrity. RBCs also present high concentrations of antioxidant vitamins (ascorbic acid, α -

tocopherol) and other antioxidant compounds such as reduced glutathione (GSH) or ferritin, which can scavenge free iron ions preventing the formation of $\text{OH}^\cdot$. Importantly, failure to reverse metHb formation not only diminishes the O_2 -carrying capacity of RBCs but also impairs Hb's positive cooperativity resulting in an increased affinity for O_2 for the remaining ferrous heme groups. This also leads to dysregulated vascular tone and inflammatory reactions.²

3. Hb-based O_2 carriers (HBOCs)

HBOCs are semi-synthetic systems that make use of the excellent O_2 carrying and delivering properties of natural Hbs. The idea is to eliminate the toxic effects of free Hb to create a RBC surrogate to be used when donor RBCs are not available. The toxicity of acellular Hb mainly results from the tetramer dissociation into dimers and monomers, which can then permeate in between the endothelial cells lining our blood vessels into the smooth muscle tissue.^{35,36} There, they rapidly scavenge NO promoting the subsequent cardiovascular problems resulting from vasoconstriction.³⁷ Additionally, free Hb affects the osmolarity of blood thus leading to alteration of blood volumes and associated side effects. Therefore, as a result of all these shortcomings, a substantial amount of research has been devoted to stabilizing the Hb molecule while also optimizing its functionality. The two main approaches developed so far involve the chemical modification of Hb or its encapsulation within a protective carrier shell. **Scheme 4** shows a time line of the main HBOCs developed so far which will be discussed in the next sections. Both strategies aim at eliminating, or at least minimizing, Hb's dissociation and extravasation as well as its rapid clearance by the kidneys. At the same time, extended blood circulation lifetimes should also be achieved. Importantly, Hb's modification should not compromise its O_2 loading-releasing abilities.

The chemical-modification approaches developed so far involve: Hb cross-linking, polymerization and conjugation to polymers. The different Hb subunits can be cross-linked intramolecularly while polymerized Hb can be fabricated by employing bifunctional cross-linking reagents. By using bifunctional crosslinkers, the Hb molecules are linked to each other increasing the overall molecular size. Chemically-modified HBOCs can also be obtained by conjugation to biocompatible polymers such as poly(ethylene glycol) (PEG) or poly(oxy ethylene).

Encapsulation strategies aim to mimic the in vivo situation, where Hb is encased in a protective RBC membrane. By entrapping Hb within a carrier system, Hb is prevented from dissociation into dimers and monomers but also from extravasation into the sub-endothelial smooth muscle tissue. Encapsulation also reduces Hb's high viscosity and high colloidal osmotic pressure. Additionally, Hb remains protected from direct contact with other blood components and the surrounding tissues.^{38,39} Furthermore, an encapsulation platform allows for the incorporation of additional active compounds within the same carrier. For example, an antioxidant multi-enzyme system can be incorporated together with Hb in order to avoid or revert Hb's oxidation into non-functional metHb. However, employing an encapsulation platform has the problem of rendering a low Hb content as compared to the chemically-modified HBOCs which are mainly composed by Hb.³² Additionally, Hb encapsulating platforms can also impede the diffusion of O_2 in and out of the carrier. Encapsulation platforms offer a broad range of different structures and materials and, therefore, they require an exhaustive optimization and characterization. **Table 1** depicts the main advantages and drawbacks for the two strategies.

Scheme 4. Time line of the different HBOCs discussed in this review: marketed products showing the date of marketed approval; clinical developments with the date when the clinical trials were started; innovative designs showing the date of the first publication; discontinued HBOCs showing the date of the first publication and when the clinical trials were terminated; and the preclinical developments showing the date when they were first evaluated in in vivo models

3.1. Marketed products

After decades of research and, despite the fact that several products have entered clinical trials, only two HBOCs are currently on the market. This relatively low success is a result of cardiovascular complications and renal dysfunction observed for the treatment groups during clinical trials.⁴⁰ The two approved products are based on chemically-modified HBOCs. While one of them is approved for human use (Hemopure, Biopure Corp., now OPK Biotech, Boston, MA, USA), the other one (Oxyglobin, also from Biopure) has only received veterinary regulatory clearance.

Hemopure. Hemopure (HbO₂ Therapeutics, USA) is a type of polymerized Hb (polyHb) created using glutaraldehyde as the bifunctional crosslinking agent. Each polyHb is constituted by four or five Hb molecules yielding a total molecular weight of 250 kDa and a p50 value of 38 mm Hg (**Figure 1A**).⁴¹ By increasing the size, extravasation is reduced and the circulatory life increased to over 24 h (as compared to 6 h for cell free Hb). For this HBOC, bovine Hb instead of human is used. Making use of bovine Hb has several advantages since it is widely available and it can be used by certain religious groups refusing donor blood transfusions such as the Jehovah's Witness.⁴² Hemopure was clinically approved in South Africa (in 2001) and in Russia (in 2012) for the treatment of acutely anemic patients.^{43,44} Hemopure clinical trials showed a decreased need for blood transfusions during cardiac surgery however, increased occurrence of cardiac events (i.e., arrhythmia or cardiac infarction), were also observed. As a result, clinical trials with Hemopure were terminated by the Food and Drug Administration (FDA) in 2008.⁴⁰ Nonetheless, since 2013, Hemopure is being administrated also in the USA to patients with life-threatening anemia for whom allogeneic blood transfusion is not an option.²⁸ Importantly, within these "compassionate use" programs, survival of the patients is clearly and significantly higher when treated with Hemopure.⁴⁵

Oxyglobin. Oxyglobin is an analogous product to Hemopure also produced by the same company (HbO₂ Therapeutics, USA).² While both products are chemically identical, Hemopure has undergone thorough purification steps to remove the majority of low molecular weight products from the solution.^{46,47} Oxyglobin is currently approved for veterinary use in both the US and the EU.²

Table	Chemically modified HBOCs		Encapsulated HBOCs	
	Advantages	Disadvantages	Advantages	Disadvantages
Fabrication process	<ul style="list-style-type: none"> • Straight Forward • Low cost 	<ul style="list-style-type: none"> • Likelihood of Hb dissociation • Altered O₂ affinity 	<ul style="list-style-type: none"> • Prevention of Hb dissociation • Prevention of direct contact between Hb and blood components 	<ul style="list-style-type: none"> • Complex • High cost • Hindered O₂ diffusion
Physical Properties	<ul style="list-style-type: none"> • Possibility to surface functionalize 	<ul style="list-style-type: none"> • Small size 	<ul style="list-style-type: none"> • Possibility to surface functionalize • Possibility of incorporation of additional active compounds • Closer mimicry of biological RBCs 	<ul style="list-style-type: none"> • Broad size distribution
Hb loading capacity	<ul style="list-style-type: none"> • High Hb loading 			<ul style="list-style-type: none"> • Challenges with high Hb loading • Batch to batch variation with loading capacities
Anatomical and physiological characteristics	<ul style="list-style-type: none"> • Increased circulation in comparison to free Hb 	<ul style="list-style-type: none"> • Possible extravasation associated with the small size of the HBOC • Possible hypertensive and/or adverse GI responses 	<ul style="list-style-type: none"> • Increased circulation times in comparison to free Hb. • Prevent extravasation • Reduced colloidal osmotic pressure • Reduced Hb viscosity • Reduced hypertensive responses • Reduced uptake by macrophages 	<ul style="list-style-type: none"> • Broader range of materials requiring more comprehensively designed in vitro and in vivo models to detect adverse events
Industrial Scalability	<ul style="list-style-type: none"> • Easy to scale-up 			<ul style="list-style-type: none"> • Difficult to scale-up

Table 1. The table lists the various advantages and disadvantages of using chemical modification and encapsulation as the strategy for HBOC fabrication

3.2. Clinical developments

MP4 (aka Hemospan). Bioconjugation of Hb has also been considered as a strategy to increase its stability as well as the in vivo circulation time by reducing immune recognition.^{48,49} PEG is one of the most attractive polymers for bioconjugation due to its biocompatibility and lack of immunogenicity.⁵⁰ Thus, human Hb has been conjugated to PEG by a maleimide linker.^{28,51} The so called MP4 (previously known as Hemospan, Sangart Inc., San Diego, CA) has a molecular weight of 96 kDa and a p50 value of 6 mm Hg.⁵² However, due to a relatively low Hb content (of 4.2 g dL⁻¹), MP4 is being developed as an oxygen carrier to improve the supply of oxygen rather than a blood surrogate for the full replacement of blood.⁵³ Importantly, MP4 did not promote vasoconstriction in animal models and, the clinical trials that have been conducted so far, have shown that MP4 is an efficient oxygen delivery system well tolerated in humans.^{54,55}

Interestingly, MP4 is currently also being investigated as a carbon monoxide (CO) carrier (MP4-CO) since CO can also bind to the heme group (Hb-CO).⁵⁶ While CO is known as a toxic gas, in very low doses, it acts as a signaling molecule.⁵⁷ Important properties such as anti-inflammatory, antiapoptotic or antiproliferative have been attributed to CO. What is more, CO has the ability to prevent and reverse the polymerization of Hb S, thus inhibiting the distortion of sickled RBCs.⁵⁸ The potential of MP4-CO in the treatment of sickle cell disease has been demonstrated in a transgenic mouse model, where administration of MP4-CO reduced microvascular stasis after hypoxia or reperfusion.⁵⁹

Sanguinate. PEGylated Hb has also been employed to develop Sanguinate (Figure 1B). This HBOC has a molecular weight of 120 kDa and a p50 value of 7–16 mm Hg. Sanguinate also employs Hb-CO. This CO bound to the Hb heme group is released within minutes of administration and its thought to avoid vasoconstriction as a result of its anti-inflammatory properties.⁶⁰ Sanguinate has undergone pre-clinical evaluation in a transfusion rat model where significant reduction of the infarct volume could be observed for the treatment group.⁶¹ Sanguinate is currently in Phase I and II clinical trials for sickle cell disease and in Phase I for the treatment of acute anemia. Promising results in terms of safety profile and oxygenation parameters are being demonstrated.^{2,3} Sanguinate is also currently available under an emergency investigational new drug protocol where it has enabled the survival of several patients refusing blood transfusion due to religious reasons.^{60,62,63}

Hemo₂Life and organ transplantation. Hemo₂Life is constituted by unmodified cell-free Hb. However, this HBOC employs Hb extracted from lugworms, which is not packed within RBCs or any other type of membrane (Figure 1C). This lugworm Hb has a molecular weight of 3600 kDa and a p50 value of 7 mm Hg. As a result of its large size, each Hb can transport 38× more O₂ molecules than mammal Hb. Additionally, Hemo₂Life displays intrinsic SOD-like activity thus having the ability to deplete ROS.⁶⁴ Interestingly, Hemo₂Life has been evaluated both in pre-clinical animal models and clinical trials but in the context of organ transplantation. As a result of the mismatch between the demand and the supply of viable transplantable organs, research efforts have been focusing on developing solutions for organ preservation and extracorporeal resuscitation. Prolonged periods of ischemia are inherent to harvesting and transplanting donor organs thus resulting in ischemia-reperfusion injuries.⁶⁵ This, in turn, enhances the risk of early-graft dysfunction and reduced long-term survival. Current standard preservation techniques include hypothermic storage in a preservation fluid containing electrolytes, amino acids and other compounds designed to preserve cell integrity. However, the lack of oxygen results in the depletion of adenosine triphosphate (ATP) and the building-up of toxic substances within the cells (i.e., adenosine, inosine and hypoxanthine).^{10,65} As a result of ATP depletion, a large number of cellular processes are disrupted.⁶⁶ Additionally, the inactivation of the sodium and potassium ATPase pumps results in the accumulation of calcium, sodium and water promoting cellular swelling.⁶⁷ Such an anaerobic metabolism promotes the decrease of intracellular pH which, in turn, causes lysosomal instability with the subsequent activation of lytic enzymes causing cellular damage. All in all, this cellular malfunction resulting from prolonged organ preservation, can lead to the failure of the graft.^{10,65} Thus, the development of artificial oxygen carriers hold a lot of potential to create novel preservation solutions in the context of organ transplantation. In this context, Hemo₂Life is one of the most advanced artificial oxygen carriers for organ transplantation purposes. Pre-clinical evaluation in animal models have demonstrated the ability of Hemo₂Life to improve the storage of donor rat hearts and pig lungs.^{68,69} transplantation. Additionally, Phase I clinical trials of kidney transplantation, have confirmed the safety of Hemo₂Life for both patients and grafts (Figure 1C).²⁸ Importantly, significantly less delayed graft function and a shorter renal function recovery was observed for the treatment groups. Currently, Phase II and III clinical trials are being conducted.

Fig. 1. A) Hemopure development. Haemoglobin is extracted from bovine red blood cells (RBCs). This extracellular tetramer dissociates into its dimers which are crosslinked via glutaraldehyde. Four to five tetramers are crosslinked together rendering Hemopure with a molecular weight of 250 kDa. B) Schematic illustration of Sanguinate, which is a poly(ethylene glycol) (PEG)ylated haemoglobin-based oxygen carrier. C) i) Haemoglobin M101 extracted from lugworm, *Arenicola marina* which is used for the production of HEMO₂Life®. This oxygen carrier is used to oxygenate tissues prior to organ transplantation. ii) Kaplan-Meier estimate curves of cumulative probability to achieve creatinine levels below 250 $\mu\text{mol L}^{-1}$ of two groups of 25 paired kidneys preserved in cold storage in the presence or absence (contralateral) of HEMO₂Life®. There was a significant difference in time to achieve the event between the two groups. Adapted with permission from ¹⁴⁶. Copyright 2020 The American Society of Transplantation and the American Society of Transplant Surgeons.

The 2008 meta-analysis and the need of incorporating antioxidant functions. Research in blood surrogates spans several decades. In fact, major interest in developing synthetic O₂ carriers took place in the 1980s, due to the fear to contaminated blood products owing to the HIV crisis. However, despite the efforts, no product is currently clinically approved for human use neither in the USA nor in Europe. Only Hemopure has been approved in South Africa and Russia for human use and, under special circumstances, it may be administered for compassionate use in the USA and Europe.² This limited success is partly a result of a meta-analysis of 16 clinical trials for five different HBOCs that were seeking FDA approval. Such meta-analysis, which was conducted in 2008, indicated increased risk of myocardial infarction and death in the treatment groups.⁴⁰ Although this study was statistically well executed, it has received much criticism in terms of the analysis design.⁷⁰ While the pre-clinical animal studies of the different HBOCs had been conducted in healthy, normal young animals; many of the clinical trials involved populations with elderly, diabetic, atherosclerotic and hypertensive patients.⁷¹ Thus, much of the criticism was directed to the fact that the meta-study did not take into account the

patients pre-existing conditions since, actually, many of the discontinued products did well in healthy patients that underwent elective surgery.¹⁵ Additionally, a prominent feature of the treatment groups with pre-existing conditions, is the presence of endothelial dysfunction, including impaired NO response and exaggerated endothelin response.⁷⁰ Thus, the administration of HBOCs with undesirable NO scavenging ability could have exacerbated rather than corrected these pre-existing conditions. As such, this meta-analysis emphasized the need for a better understanding of the benefits and drawbacks of the different HBOCs both at fundamental physiological and mechanistic levels.² As a result of the research conducted in recent years, it is now widely accepted that, the cardiovascular complications of the clinical trials, were a result of the NO scavenging abilities of Hb, heme-mediated oxidative side reactions as well as to the O₂ over supply.⁷² This fact would explain the differences observed between the pre-clinical studies conducted in healthy animals and the clinical studies.

Below we comment on the different HBOCs that have been discontinued following clinical evaluation.

Discontinued HBOCs Following Clinical Evaluation. *HemAssist.* HemAssist (Baxter, USA), also known as Diaspirin or DBBF-Hb, consists of intramolecular crosslinked human Hb. Specifically, the lysine groups of the two alpha-subunits of the Hb tetramer in the deoxy conformation (Lys99 from $\alpha 1$ and Lys99 from $\alpha 2$) are crosslinked by acylation using bis-(3,5 dibromosalicyl)-fumarate.^{73–76} Such a product displayed a p50 of 30 mm Hg which is very close to the p50 of Hb entrapped within natural RBCs (i.e., P50 of 26 mm Hg). Compared to cell free Hb, HemAssist showed a substantial increase in circulation residence time (i.e., of 12 h as compared to less than 6 h for free Hb). While HemAssist had been extensively evaluated in preclinical models and also phase I and II clinical studies, phase III clinical trials were discontinued due to a 72% increase in mortality rate for the treatment groups as compared to the saline control.^{77,78}

Optro. Optro (Somatogen, USA) also consists of intramolecular cross-linked Hb. In this product, recombinant Hb was used. Specifically, the deoxy form of the low oxygen affinity mutant Hb Presbyterian (β N109K) was expressed in *Escherichia Coli*.⁷⁰ Interestingly, the mutations had been incorporated to render a highly-stable Hb that does not dissociate into $\alpha_1\beta_1$ dimers under normal physiological conditions.⁷⁹ Furthermore, this recombinant Hb also displayed reduced NO scavenging ability.⁷⁹ The two alpha-subunits of the tetramer were next crosslinked by glycine.^{3,80} Similarly to HemAssist, Optro displayed a p50 value also close to that of Hb entrapped within biological RBCs. Phase I and II clinical trials in elective surgeries were conducted and finally discontinued as a result of hypertensive effects.⁷⁰

PolyHeme. PolyHeme (Northfield Laboratories, Evanston, IL) is composed by polyHb. As opposed to Hemopure, which is also a polyHb-based product, PolyHeme employs pyridoxylated human Hb instead of bovine Hb. PolyHeme was evaluated in several cardiac surgery and trauma/emergency clinical trials.^{77,81} However adverse effects were observed and attributed to vasoconstriction and oxidative stress.⁴⁶ As a result, PolyHeme was rejected by the FDA in 2009 and Northfield Laboratories were closed down.¹⁰

HemoLink. HemoLink (Hemosol Inc, Mississauga, Ontario, Canada) is another type of polyHb. For this product, the raffinose trisaccharide was employed both as an intra- and intermolecular crosslinker. HemoLink displayed a p50 of 38–40 mmHg and a non-cooperative behavior as shown by a Hill coefficient of one.⁷⁰ This is opposed to biological RBCs, where Hb displays oxygen-binding cooperativity as shown by the Hill coefficient values in the range of 2.5–2.9.⁷⁰ Phase II clinical trials were conducted in 2003, where the administered HemoLink demonstrated the ability to reduce the need for a subsequent autologous blood transfusion as compared to the pentastarch control.⁸² However, also with this product, hypertension, myocardial infarction and also gastrointestinal side effects, were observed during the clinical trials resulting in HemoLink being discontinued.¹⁰

PHP. PHP is a type of pyridoxylated crosslinked and bioconjugated Hb developed by Apex Bioscience (USA). However, in this case, Hb is bioconjugated to poly(oxy ethylene) instead of PEG. PHP was evaluated in clinical trials as an oxygen carrier but also as a NO scavenger to correct hypotensive effects in response to septic shock.^{83,84} However, as a result of several safety concerns, PHP did not received FDA approval and was discontinued.

3.3. Preclinical developments

As a result of the failure in clinical trials of the abovementioned HBOCs, research efforts have focused in refining their design to address the toxicity issues. Thus, the following section will focus on novel and advanced HBOCs that have already been evaluated in pre-clinical animal models. The different HBOCs will be classified in two different categories: HBOCs that have been prepared employing either a chemical modification approach or an encapsulation strategy.

Chemically-modified HBOCs. *OxyVita.* OxyVita consists of inter- and intra-molecularly crosslinked bovine Hb but, in this case, carbodiimide chemistry is used instead of crosslinkers.²⁸ Thus, the Hb tetramers are linked to each other by amide bonds rendering a Hb macropolymer with a very low oncotic pressure. Also as a result of its large molecular weight (~17.000 KDa), extravasation into the smooth muscle tissue following intravenous administration is prevented.⁸⁵ Studies in mice have shown how OxyVita can

deliver O₂ to ischemic tissues more efficiently than biological RBCs. Specifically, diminished damage was observed when OxyVita was used to salvage ischemic brain tissue in stroke mice models as compared to biological RBCs.⁸⁶ OxyVita has also been evaluated in a rat hemorrhagic shock set-up mimicking the initial medical treatment of severely injured patients prior arriving to the hospital.⁵³ In this context, OxyVita was able to prolong the rats survival for at least three hours employing relatively small volumes of resuscitation fluid.⁸⁷ It is also worth noticing that OxyVita has also demonstrated to be stable at all ambient temperatures, while also being able to be stored in its lyophilized form at any temperature for at least 5 years. Additionally, OxyVita also has a shelf life of up to twelve months at room temperature in liquid form.⁵³

HemoTech. HemoTech is a novel type of bovine polyHb. The novelty of this product lies in co-polymerizing Hb together with pharmacologically active compounds. In particular, the Hb molecules are linked to ATP, adenosine and GSH. While GSH was incorporated to protect the heme group from NO and ROS, open ring ATP was introduced to regulate the vascular tone through purinergic receptors. Open ring adenosine was incorporated to counteract the potential vasoconstriction promoted by Hb, since adenosine stimulates the adenosine receptors which result in vasodilation, moderate inflammatory reactions and prevention of platelet aggregation. HemoTech has a molecular weight below 500 kDa, a p50 of 20 mm Hg and Hill coefficient of 1.8. Pre-clinical studies in several animal models have shown the ability of HemoTech to reduce vasoconstriction following a hemorrhage. Additionally, after administration, no adverse nephrotoxic, neurotoxic, oxidative or inflammatory reactions could be detected.^{88,89} Early-phase clinical studies have been conducted in the Democratic Republic of Congo, where it has been shown that HemoTech is an efficient oxygen carrier for the treatment of sickle cell diseases, acute and chronic anemia, coronary artery disease, traumatic brain injury and stroke.³

VitalHeme. VitalHeme (Synzyme Technologica LLC, Irvine, California, USA) consists of carboxylated PEG-Hb containing nitroxide moieties (**Figure 2Ai**). Such a polynitroxylated PEG-Hb (PNPH) displays both SOD- and CAT-like activity. Thus, VitalHeme has the potential to reduce oxidative stress and to decrease the ROS-mediated cytotoxicity of free Hb. Additionally, the PEG side chains provide a “hydrating shell” with a marked oncotic effect that is useful in resuscitation.⁹⁰ The pre-clinical studies have shown the ability of VitalHeme to provide neurovascular protection in a mice model reproducing pre-hospital resuscitation of traumatic brain injury together with hemorrhagic shock (**Figure 2Aii**).⁹¹ Neurotoxicity studies were also conducted in vitro, demonstrating preservation of cell viability only for the PNPH group (**Figure 2Aiii**).

HemoAct. HemoAct is an innovative approach where human serum albumin (HSA) is conjugated to human Hb. Such a conjugation is conducted by covalently reacting Hb's surface lysines with HSA's cysteine-34 using α -succinimidyl- ϵ -maleimide as a cross-linker.⁹² By doing so, a cluster constituted by a Hb tetramer wrapped around by two or three HSA molecules (Hb-HSA₂ and Hb-HSA₃) is obtained (**Figure 2Bi**). Pre-clinical evaluation of HemoAct showed a circulation life-time of 18.5 hours in rats (**Figure 2Bii** and **iii**). Histopathologic evaluation indicated absence of organ damage 7 days post-administration.⁹³ However, their ability as an O₂ carrier in animal models still remains to be demonstrated.

PolyHb and antioxidant enzymes. Worth mentioning in this context is the work by Chang et al.^{94,95} As early as in 1998, the authors cross-linked the CAT and SOD enzymes to polyHb. The resulting polyHb-SOD-CAT could be successfully freeze-dried, sterilized and stored at ambient temperature for 320 days. Furthermore, it also displayed antioxidant properties. Pre-clinical evaluation was also conducted on a rat model of intestinal ischemia-reperfusion injury, which demonstrated that polyHb-SOD-CAT decreased the formation of oxygen radicals as compared to polyHb only. In follow up work, the same group incorporated the CA enzyme into the formulation to create polyHb-SOD-CAT-CA.⁹⁶ Such a HBOC was not only able to carry O₂ but also CO₂, since it had the ability to lower the tissue pCO₂ in a haemorrhagic shock rat model.⁹⁷

The Chang group also pioneered the fabrication of an O₂ carrier also displaying platelet-like activity.⁹⁸ By crosslinking fibrinogen (Fg) to Hb, the polyHb-Fg complex was obtained. The in vitro characterization showed how polyHb-Fg yielded clotting times similar to those of whole blood. In contrast, polyHb alone, showed significantly higher clotting times. The in vivo evaluation also gave very encouraging results since, in an exchange transfusion rat model, polyHb-Fg also showed a clotting time of 1-2 min, which is very similar to that of whole blood. However, the clotting time for polyHb was very high (of about 10 min).

Fig. 2. A) i) Schematic of polynitroxylated poly(ethylene glycol) (PEG)-ylated haemoglobin (PNPH). A haemoglobin molecule is functionalised with nitroxides (12–14 per molecule) and PEG chains of about 8–10 kDa forming an outer shell. The resulting PNPH has a molecular radius of approximately 6.8 nm and a molecular weight of 256 kDa. Adapted with permission from ⁹⁰. Copyright 2012 John Wiley and Sons. ii) Mean arterial pressure readings over time depending on the administered resuscitation fluid: PNPH, Hextend (HEX) and Lactated Ringer's (LR). HEX (solution of colloids) and LR (isotonic fluid) correspond to the resuscitation fluids used in the civilian and military standards of care, respectively. The time points of 0 and 30 min correspond to experiment times of 90 and 120 min, respectively. Data indicate mean $\pm$ SEM at each time point, $n = 5$ per group ($* p < 0.05$, analysis of variance). Adapted with permission from ⁹¹. Copyright 2011 by the Society of Critical Care Medicine and Lippincott Williams & Wilkins. B) i) Schematic illustration of the synthetic route to create clusters of Hb and human serum albumin (HbX-HSA_m) using heterobifunctional cross-linkers (X). Adapted with permission from ⁹². Copyright 2013 American Chemical Society. ii) Relative plasma concentration of radiolabelled Hb-HSA₃ cluster (¹²⁵I-HemoAct), ¹²⁵I-HSA and ¹²⁵I-Hb at 3, 10, 30 min and 1, 3, 6, 12 and 24 h after the infusion in Wistar rats. Each data point represents the mean $\pm$ SD ($n = 6$). $**p < 0.01$ vs. ¹²⁵I-HSA. iii) Different pharmacokinetic parameters following intravenous administration of ¹²⁵I-HemoAct, ¹²⁵I-HSA and ¹²⁵I-Hb in Wistar rats. Half-life ($t_{1/2}$); mean residence time (MRT); clearance (CL_{total}); volume of distribution at steady state (V_{dss}); area under the curve (AUC). Each value represents the mean $\pm$ SD ($n = 6$). $**p < 0.01$ vs. ¹²⁵I-HSA. Adapted with permission from ⁹³. Copyright 2015 Springer Nature open access.

Encapsulation Strategies. Hb entrapment within micro- or nano-sized (up to 500 nm) encapsulation platforms has emerged as a promising strategy. Hb encapsulation offers several advantages. Firstly, it mimics more closely the physiological encapsulated state of Hb within biological RBCs. Secondly, by protecting Hb from being in direct contact with plasma, increased circulation times, reduced hypertensive response and uptake by the mononuclear phagocyte system (MPS) can be achieved. Lastly, encapsulation platforms offer the possibility to incorporate other functional compounds such as antioxidant enzymes or allosteric effector molecules within the same carrier. In fact, the concept of Hb encapsulation was presented in the 1950s and 1960s in a pioneering work by Chang and collaborators.² The authors were able to encapsulate, not only Hb, but also reductive enzymes within a polymeric micron-sized carrier shell. While, on a first study, cellulose nitrate was used to fabricate the carrier shell, in follow up work, PEG-poly(Lactide) was used as a

biocompatible and biodegradable material.^{99,100} With such a carrier, the authors were also able to co-encapsulate not only SOD, CAT and CA enzymes but also the 2,3-DPG allosteric effector.^{101,102} However, these Hb-loaded microcarriers, which were termed “hemoglobin corpuscles”, were rapidly removed from the circulation by the MPS. Regrettably, reducing the size of the microcarriers to about 1 μm in diameter resulted in only a slight improvement on the vascular residence time.²

Since then, much work has been conducted towards improving the circulation lifetime by making use of different polymers, phospholipids or surface coatings and below we comment on the few systems that have undergone pre-clinical evaluation.

ErythroMer. Human Hb has also been encapsulated together with the reductant leuko-methyleneblue and a pH sensitive allosteric effector. The so-called ErythroMer (KaloCyte, St. Louis MO, USA) consists of toroidal-shaped nanoparticles of about 200 nm in diameter. Such nanoparticles, which are also known as nanobialys, are fabricated by the self-assembly of an amphiphilic polymeric system of polyethylene imine (PEI) grafted to palmitic acid. Leuko-methylene blue encapsulation provides a reductive environment able to retard the rate of methHb conversion. The pH-sensitive allosteric modifier 2-[4-[[[(3,5-dimethylanilino) carbonyl]methyl]phenoxy]-2-methylpropionic acid (also known as RSR-13) was incorporated too. RSR-13 promotes the O_2 release of Hb at lower pH, thus increasing O_2 availability to hypoxic tissues.¹⁰³

ErythroMer has been evaluated in murine models of hemorrhagic shock and in a hemodilution model of 70% blood exchange. The results have shown promising oxygen transport in vivo.^{28,104} Additionally, ErythroMer can be efficiently lyophilized and reconstituted in saline prior administration. Despite the progress, biocompatibility, vascular residence time and immunotoxicity still need to be thoroughly evaluated.

Hb Vesicles. Liposome encapsulated Hb, also known as Hb vesicles (HbVs), are structures with a core of concentrated Hb wrapped in a phospholipid membrane (**Figure 3A**).³⁸ The optimized formulation employs dioctadecadienoyl-*sn*-glycero-3-phosphatidylcholine (DODPC) as the main phospholipid for their preparation. Importantly, by γ -irradiation-induced radiolysis of water molecules within the vesicles, $\text{OH}^\cdot$ ions are generated. Such radicals promote the intermolecular polymerization of the dienoyl groups of the DODPC, which results in highly stable HbVs. Thus, HbVs can be successfully freeze-thawed, freeze-dried and reconstituted in saline.^{2,38} The optimal HbV formulation has been functionalized by 5 kDa PEG in order to reduce opsonization and the subsequent elimination by the MPS. PEG modification has rendered HbVs with extended circulation times of about 60 h in some animal models.^{105–107}

HbVs have a size of 250–280 nm in diameter and a p50 ranging from 9 to 30 mm Hg, which can be adjusted by the co-encapsulation of pyridoxalphosphate.¹⁰⁸ Each HbV contains about 30,000 Hb molecules with a 30% encapsulation efficiency. HbVs have similar ODCs and release kinetics than biological RBCs. Additionally, Hb encapsulation within the vesicles results in reduced NO scavenging due to the extra surface lipid bilayer. The large size of the vesicles as compared to cell-free Hb, avoids Hb's glomerular clearance thus reducing the subsequent nephrotoxicity. HbVs have been extensively studied in pre-clinical animal models. Specifically, their potential as oxygen therapeutics has been evaluated in animal models of haemorrhagic shock or for the oxygenation of ischemic tissues (**Figure 3B**).^{107,109} Additionally, HbVs have also been evaluated in the context of organ perfusion, extracorporeal membrane oxygenation priming or to provide oxygen in in vitro models of two-dimensional cell cultures.^{108,110} Despite the promising results as therapeutic O_2 carriers, issues with pharmacokinetics and complement-mediated immune responses still need to be addressed.³⁸ Other important drawbacks of this technology is their broad size distribution and the batch-to-batch variation in Hb-encapsulation efficiencies.

Other Encapsulation Platforms. Other types of vesicles constituted by different phospholipids than DODPC have also been evaluated.¹¹¹ For example, Hb has been encapsulated within phosphatidylcholine and cholesterol-based vesicles also functionalized by a PEG coating. The resulting Hb-loaded vesicles have been evaluated in hemodiluted beagle dogs where whole blood had been replaced by iso-oncotic starch. Importantly, the animals in the vesicles group survived significantly longer after completion of the treatment as compared to the control group that received saline only.¹¹² Other liposome-encapsulated Hbs known as “neo red cells” and “neohemocytes” have been developed and evaluated in vivo. In particular, the neo red cells showed enhanced O_2 delivery capacity as compared to natural RBCs in total cardiopulmonary bypass in an animal study.¹¹³ In another study, Hb was encapsulated together with enzymes extracted from outdated human RBCs within neo red cells.¹¹⁴ The allosteric effector inositol hexaphosphate, the NAD co-enzyme and the required substrates for the enzymes (i.e., adenine and inosine) were also co-encapsulated. In vitro evaluation showed that methHb formation was reduced from 1% h^{-1} to 0.4% h^{-1} upon incorporation of the enzymatic reduction system. These enzyme-loaded neo red cells were also assessed as a perfusate for cardiopulmonary bypass during hypothermia. This was done employing an in vitro artificial lung system where the O_2 transporting efficiency could be demonstrated.

There are other Hb encapsulation platforms that have been fabricated to serve as O_2 carriers however, due to early developments, a pre-clinical evaluation still remains to be conducted. For instance, Hb has also been encapsulated with polymersomes. Polymersomes are the polymeric analogues of lipidic vesicles, fabricated by the self-assembly of amphiphilic block-copolymers. Advantages of polymersomes over liposomes include higher stability both in storage and in vivo.¹¹⁵ Their size and thickness can be easily tailored by the right choice of the constituting amphiphilic polymers. Polymersomes can also be produced in large quantities

Fig. 3. A) Schematic illustration of the preparation of haemoglobin (Hb)-loaded vesicles (HbV). Hb is extracted from outdated nucleic acid amplification testing-inspected red blood cells (RBCs) provided by the Japanese Red Cross. The concentrated carbonyl haemoglobin (HbCO) is purified by pasteurization and nanofiltration for utmost safety from infection. Adapted with permission from ¹⁰⁸. Copyright 2017 MDPI. B) In vivo evaluation of HbV. Survival rate (i), change in body weight (ii), daily food intake (iii) and running wheel revolutions (iv) for mice undergoing a 40% blood exchange with: HbV and recombinant human serum albumin (rHSA) (HbV/rHSA); mouse RBCs (mRBCs) and rHSA (mRBC/rHSA) or Lactated Ringer's (LR). The results show differences in survival rate, $n = 10$, (i) percent change in body weight ($n = 10$ initially in each group, $n = 5$ after the 1st postoperative day (POD) in the rHSA group) (ii) daily food intake ($n = 10$ initially each group, $n = 5$ after the 1st POD in the rHSA group) (iii) and daily number of running wheel revolutions ($n = 5$ between the baseline period and the 7th POD in the indicated study groups) (iv). Values are shown as the mean $\pm$ SD. * $P < 0.05$ versus baseline (Student's t -test), # $P < 0.05$ versus the rHSA group (Dunnett's test). Adapted with permission from ¹¹⁰. Copyright 2017 PLoS One open access.

and, following Hb encapsulation, they show similar O_2 affinity to human RBCs. Hb-loaded polymersomes are also stable in saline for several months and in blood for at least five days.^{116,117} However, due to a thicker shell as compared to the phospholipidic counterparts, research efforts should focus in evaluating the O_2 diffusion in and out of the system.^{2,3} Another encapsulation strategy consists of entrapping Hb within polymeric micelles. Using this approach, Hb has been incorporated into the micelles by covalently reacting carbonyl moieties of the polymer with Hbs amino groups.⁵⁰ On a different approach, Hb was incorporated onto the micelle's surface (Figure 4A).¹¹⁸ The resulting Hb-loaded micelles had the ability to reversibly bind and release O_2 in an in vitro set up (Figure 4B). Additionally, no detrimental effects on blood components could be observed in vitro (Figure 4C). Polymeric particles in the micron range have also been evaluated. For example, by co-precipitating Hb and calcium carbonate ($CaCO_3$), followed by glutaraldehyde cross-linking and $CaCO_3$ dissolution, Hb-loaded microparticles have been prepared.¹¹⁹ With a similar approach, Hb was co-precipitated with $MnCO_3$ followed by the immediate addition of HSA for encapsulation and stabilization of the particles.¹²⁰ Such particles demonstrated reduced vasoconstriction, low immunogenicity and did not interfere with platelet activation. Additionally, they successfully delivered O_2 in an in vitro (mouse) afferent arteriole model. While these systems have shown considerable promise toward their application as RBC surrogates employing in vitro models, follow up in vivo evaluation is essential in order to assess their full potential.

Fig. 4. A) Schematic illustration of polymeric micelles with haemoglobin (Hb) bound onto the surface. The insert shows the chemical structure of the triblock co-polymer used to assemble the micelles. The polymer mPEG-b-PMAC-b-PCL is constituted by a methoxypoly(ethylene glycol) (mPEG) block, a 5-methyl-5-acryloyloxymethyl-1, 3-dioxan-2-one (PMAC) block and poly caprolactone (PCL). B) Dynamic light scattering results and C) transmission electron microscopy image of the micelles. D) UV-vis spectra of the micelles and free haemoglobin (Hb) in different atmospheres. E) Morphology of RBC mixed with different solutions observed by optical microscopy (bar = 20 μm). Adapted with permission from ¹¹⁸. Copyright 2016 John Wiley and Sons.

3.4. Novel and innovative designs

Clinical studies of HBOCs have been mainly conducted in the context of trauma or major surgery. Within this framework, HBOCs did not show a general benefit in terms of avoidance or reduction of RBC transfusions or 28-day mortality.¹²¹ On the contrary, enhanced risk of myocardial infarction and death was observed in the treatment groups.³ These adverse effects were attributed to the vasoconstriction resulting from NO scavenging. Due to this toxicity issues, in 2008, all HBOCs were put on hold by the FDA.² However, several lessons have been learned from the failure of this first generation of HBOCs and the new generations of HBOCs are currently minimizing NO binding and heme-mediated side reactions. Additionally, due to the relatively short circulation life-times of the reported HBOCs as compared to human RBCs (i.e., of about 120 days) the focus has shifted from “RBCs substitutes” to “oxygen therapeutics”. The aim is to highlight the important role of these products in situations where donor blood is not available (e.g., remote locations, emergency in an ambulance, far-forward military settings). Thus, in the following section, we comment on recent and innovative approaches developed to address the aforementioned limitations of the first reported HBOCs. The chosen examples have been thoroughly characterized *in vitro* in terms of morphology, stability, cytotoxicity and biofunctionality. However, due to early development, pre-clinical evaluation in relevant animal models still remains to be conducted.

Chemically-modified HBOCs. A novel HBOC with the ability to counteract oxidative stress is composed by polyHb crosslinked to a Tempol compound with an amine group (4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl, NH_2 -Tempol).¹²² Tempol is a nitroxide compound with the ability to shuttle between the nitroxide radical, the reduced hydroxylamine and the oxidized oxoammonium cation form. Such polyHb-Tempol was able to eliminate $\text{O}_2^{\cdot -}$ -induced oxidative stress in an endothelial cell in vitro model indicating SOD-like activity. Other antioxidative compounds incorporated to polyHb include ferulic acid (FA) which is able to quench oxygen free radicals.¹²³ While FA was successfully incorporated to generate an FA-modified Hb, the antioxidant properties of such a HBOCs still remain to be demonstrated.

On an innovative approach, the HemoAct product consisting of Hb-HSA core-shell nanoclusters was also equipped with antioxidant platinum nanoparticles (PtNPs) (Figure 5A).⁹³ The PtNPs were incorporated within the clefts of the HSA units of the clusters where they were acting as reductive catalytic entities and were able to deplete $\text{O}_2^{\cdot -}$ radicals and H_2O_2 which, in turn, prevented the formation of metHb (Figure 5B and C).

Another innovative approach, to reduce NO scavenging, the Hb cysteine residues of Hb β -subunits have been S-nitrosilated. The aim was to endow Hb with NO producing abilities.¹²⁴

Fig. 5. A) i) Schematic illustration of the Hb-HSA₃ cluster which is constituted by a haemoglobin (Hb) molecule at the centre and three covalently linked human serum albumins (HSA) located at the periphery. The cluster also incorporates platinum nanoparticles (PtNPs) located into the external albumin units yielding an Hb-HSA₃(PtNP) cluster. ii) The thiol residue (Cys-34) of HSA and the surface Lys group of Hb are covalently connected through a crosslinker. iii) The PtNPs are able to conduct superoxide dismutase (SOD)-like and catalase-like activities. B) Residual hydrogen peroxide (H_2O_2) over time following incubation with: HSA, a solution of the HSA-PtNP complex, PtNPs, Mn(III)-terakis(N-methylpyridinium) porphyrin (Mn-TMPyP) and the natural catalase enzyme. C) MetHb conversion over time of several Hb-containing solutions in the presence of H_2O_2 . Adapted with permission from ¹⁴⁷. Copyright 2014 PLoS One open access.

Encapsulation strategies. Encapsulation strategies have also addressed the challenge of avoiding heme-mediated side reactions by offering antioxidant protection. Specifically, several recent studies have focused in encapsulating Hb within a polydopamine (PDA) shell. PDA is formed by the self-polymerization of dopamine (DA) at slightly basic conditions and in the presence of O_2 . PDA can be deposited onto practically any substrate independent of size and shape.¹²⁵ Importantly, PDA has barely any reaction with underlying functional groups, thus preserving Hb's structure and functionality.¹²⁶ Additionally, PDA also holds a redox character which has been used to protect Hb from oxidation into metHb. In particular, Wang et al. coated single Hb molecules rendering Hb-PDA (**Figure 6A and B**).¹²⁶ Such a PDA coating was able to inhibit metHb formation as well as heme oxidation to ferryl (Fe^{4+}) Hb. Additionally, the Hb-PDA particles were able to scavenge free radicals and ROS. They were also able to suppress intracellular ROS generated by incubating cardiomyocytes with H_2O_2 (**Figure 6C**). Micron-sized Hb particles fabricated by co-precipitation have been also successfully coated by PDA.¹²⁷ In this study, the PDA-coated particles showed twice as much functional Hb content than their uncoated counterparts. This was attributed to PDA's antioxidant properties, which minimized metHb conversion. PDA-coated Hb particles fabricated by co-precipitation were also reported by Hu et al.¹²⁸ In this study, the PDA coating minimized Hb leakage from the particles also in flow conditions. Additionally, as a result of the antioxidant PDA coating, the particles were able to reduced oxidative injury in an in vitro model using HUVEC cells. We also recently reported the fabrication of PDA-coated NPs fully made of Hb employing the electrospray technique.¹²⁹ The ability of the PDA-coating in minimizing metHb conversion was also demonstrated. In this study, the Hb-NPs were further functionalized by a PEG coating and the low-fouling properties of the PEGylated Hb-NPs was evaluated in terms of decreased protein adsorption.

Fig. 6. A) Photographic images of Tris-HCl buffer, a solution of haemoglobin (Hb) and a suspension of polydopamine (PDA)-coated Hb (Hb-PDA). B) Transmission electron microscopy images of Hb-PDA. C) Ability of Hb-PDA to scavenge intracellular reactive oxygen species (ROS). Specifically, H9c2 cells were incubated with Hb-PDA for 12 h at 37 °C followed by exposure to hydrogen peroxide (H_2O_2) for 120 min. Then, the cells were observed by confocal microscopy and analysed by flow cytometry making use of the fluorescent probe DCFH-DA. Adapted with permission from ¹²⁶. Copyright 2017 American Chemical Society.

Metal-organic frameworks (MOFs) are also an interesting material for the fabrication of HBOCs. MOF are self-assembled structures constituted by metal ions or clusters connected by organic linkers and they display well-defined, crystalline pore structures.^{130–132} Their well-defined cages, which can be adjusted by the right choice of metal ions or clusters and organic linkers, have been used to encapsulate a wide range of biomolecules such as proteins,¹³³ enzymes^{134,135} and DNA.¹³⁶ As a result of their high void space and pre-defined cavities, MOF-based NPs have been used for Hb encapsulation. A first report employed the so-called zeolitic imidazole framework-8 (ZIF-8).¹³⁷ Due to this ZIF-8 coating, the encapsulated Hb exhibited enhanced stability upon exposure to several harsh conditions such as alkaline treatment, oxidation, high temperature or even an enzymatic environment. What is more,

following evaluation in a murine model, the Hb-loaded ZIF-8 NPs displayed extended circulation times and reduced nonspecific distribution in normal organs as compared to free Hb. The ZIF-8 NPs were also evaluated in a mice model of haemorrhagic shock where enhanced survival time was observed for the treatment group. We also employed MOF-NPs for Hb encapsulation. Specifically, we used the so-called porous coordination network (PCN)-333(Al) which is composed of trivalent metal species (i.e., Al^{3+}) coordinated to the organic linker triazine-2,4,6-triyl-tribenzoic acid.¹³⁸ PCN-333 was chosen since such a MOF-NP displays one of the highest void volumes and largest cages. As a result, PCN-333 acts as a single molecule traps for Hb thus minimizing Hb's aggregation and leaching.¹³⁹ We demonstrated preservation of the excellent O_2 binding and releasing properties of Hb following encapsulation within MOF-NPs. Additionally, to achieve extended circulation lifetimes in the blood stream, the Hb-loaded MOF-NPs were camouflaged within erythrocyte derived cell membranes (Figure 7A). Such an approach is based on the "self-marker" biomolecules embedded within the lipid bilayer of natural RBCs.^{140,141} Successful coating by the cell membranes was demonstrated by the decreased protein adsorption and cell uptake by mouse macrophages for the coated MOF-NPs (Figure 7B and C).

Mesoporous silica NPs (MSNs) have been successfully employed for Hb encapsulation.¹⁴² In such a study, Hb was encapsulated inside pores with an average size of 10 nm. The Hb-loaded MSNs were next coated by a lipid layer-containing PEG. However, the O_2 -binding and releasing properties of the encapsulated Hb still remain to be evaluated.

Particles with comparable size and shape to biological RBCs have also been fabricated. She et al. fabricated microparticles with similar shape and size than RBCs by making use of calcium hydroxide biconcave particles which were used as templates.¹⁴³ The biconcave templates were subsequently coated by alternating layers of polyallylamine and GA and, following dissolution of the core template, capsules with a size and shape resembling RBCs were obtained.

The DeSimone group employed the particle replication in non-wetting templates (PRINT) technique to fabricate PEG-based particles of 6.3 μm in diameter and 1.8 μm in height.¹⁴⁴ Importantly, the high elasticity of biological RBCs was also taken into account. Although the RBCs size is around 7.5 μm in diameter, they routinely traverse blood capillaries smaller than 5 μm . This is a result of their high flexibility with a Young moduli of only ~26 kPa.³² Actually, inspired by the elasticity of RBCs, soft polymeric particles have been engineered. Examples include PEG-based nanoparticles displaying a 130-fold lower modulus than RBCs.¹⁴⁵ DeSimone employed a low crosslinking strategy which resulted in extremely deformable particles with the ability to pass through pores half as wide as the diameter of the particles employing a microfluidics device. Hb was incorporated by post-encapsulation and the authors were able to demonstrate preservation of the O_2 -binding and releasing properties as shown by UV-vis experiments.

Fig. 7. A) Schematic illustration of the preparation of haemoglobin (Hb)-loaded metal organic framework (MOF)-based nanoparticles (MOF^{Hb}-NPs) with two different stealth layers, namely: a poly(ethylene glycol) (PEG) coating or a coating with membranes extracted from fresh red blood cells (RBC-M). B) Normalized mean fluorescence intensity (nMFI) of MOF^{Hb}-NPs with either a PEG (MOF^{Hb}/PEG-NPs) or a RBC-M (MOF^{Hb}/M-NPs) coating incubated in the presence of fluorescently labelled bovine serum albumin (BSA-FITC) or immunoglobulin G (IgG-FITC). C) Cell uptake efficiency (CUE) of RAW 264.7 (i) and HUVEC (ii) cells incubated with MOF^{Hb}-NPs with the different coatings. * $p \leq 0.05$; ** $p \leq 0.001$, *** $p \leq 0.0005$, **** $p \leq 0.0001$. Adapted with permission from ¹³⁸. Copyright 2020 Royal Society of Chemistry.

4. Future perspectives and outlook

Blood, an intricate liquid composed of millions of chemicals and cells, is crucial for our survival. As such, for several decades, scientists have focused in replicating some of its properties in an attempt to create a blood surrogate. In particular, research efforts intensified in the 1980s, as a result of the HIV crisis, which emphasized how donor blood products could be the perfect source for spreading disease. As such, the creation of a blood surrogate is of utmost importance for countries with high HIV incidences. Additionally, novel infectious agents including Ebola, H1N1 (swine flu) or COVID-19 have emerged since the 1980s. A substitute for donor blood is also highly needed in emergency situations or in the context of natural (e.g., earthquakes) or man-made (e.g., plane crash, terrorist attacks) disasters. In this context, an important class of blood substitutes are the Hb-based oxygen carriers, which aim to take advantage of the oxygen transport properties of RBCs while overcoming some of its drawbacks since HBOCs will display: enhanced availability (by large-scale manufacture), universal applicability (by the absence of RBCs blood group antigens), absence of contamination risks (due to sterilization) and extended shelf life (stored in stable freeze-dried powder form).

Although research efforts have focused in developing a wide variety of HBOCs making use of diverse approaches and designs with some of them advancing to clinical trials, currently, no oxygen-carrying blood surrogate has been approved for human use in Europe or the US. Serious side effects were the main reason for not meeting the criteria for being approved by the FDA or EMEA. In particular, a meta-analysis of data from later stage clinical trials conducted in 2008, revealed that treatment with HBOCs resulted in enhanced risk of myocardial infarction and death. Such adverse events have been attributed to their renal clearance and the associated nephrotoxicity, NO scavenging and the resulting vasoconstriction and heme-mediated oxidative reactions.

As a result of the shortcomings of this first generation of blood substitutes, we know now that this is a complex field that needs much basic research to validate the proposed issues and theories. Only by obtaining such an important information, it will be possible to develop effective and safe Hb-based blood surrogates. Approaches to address these side toxicities are on the way. In particular, novel HBOCs involve Hb's chemical modification (e.g., HemoTech) or its encapsulation within a protective carrier shell (e.g., Hb vesicles, PDA-coatings, Erythomer). Studies to evaluate their pharmacokinetics and pharmacodynamics, ability for tissue oxygenation and in vivo safety are currently being conducted and time will tell whether the toxicity issues of the first generation of HBOCs have been successfully mitigated.

Despite the progress, another important concern is their short vasculature residence time. While donor RBCs circulate in the bloodstream for about 28 days, circulation life times of HBOCs that are currently being developed are only in the range of 12 to 48 h. This highlights the fact that HBOCs might find an application in situation-specific scenarios when donor blood is not available. Thus, the more recent studies on HBOCs have shifted the focus from blood replacement to oxygen therapeutics. Such artificial oxygen carriers may find applications in pre-hospital settings, en route scenarios or to provide emergency care in remote civilian and battlefield locations. Within this framework, a clinical study design should systematically compare the administration of HBOCs to the relevant "standard of care" in pre-hospital trauma (i.e., administration of saline or plasma expanders instead of donor blood).

While the production of a liquid able to replace for donor blood is still a dream, the current development of HBOCs may pave the way towards the creation of a first generation of safe oxygen carriers to be used as a bridging solution making possible the management of bleeding in situations where donor blood is not available (e.g., in emergency prehospital and en route scenarios or remote locations).

Conflicts of interest

There are no conflicts to declare.

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