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Recent and Prominent Examples of Nano- and Microarchitectures as Hemoglobin-Based Oxygen Carriers

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ABSTRACT

Blood transfusions, which usually consist in the administration of isolated red blood cells (RBCs), are crucial in traumatic injuries, pre-surgical conditions and anemias. Although RBCs transfusion from donors is a safe procedure, donor RBCs can only be stored for a maximum of 42 days under refrigerated conditions and, therefore, stockpiles of RBCs for use in acute disasters do not exist. With a worldwide shortage of donor blood that is expected to increase over time, the creation of oxygen-carriers with long storage life and compatibility without typing and cross-matching, persists as one of the foremost important challenges in biomedicine.

However, research has so far failed to produce FDA approved RBCs substitutes (RBCSs) for human usage. As such, due to unacceptable toxicities, the first generation of oxygen-carriers has been withdrawn from the market. Being hemoglobin (Hb) the main component of RBCs, a lot of effort is being devoted in assembling semi-synthetic RBCS utilizing Hb as the oxygen-carrier component, the so-called Hb-based oxygen carriers (HBOCs). However, a native RBC also contains a multi-enzyme system to prevent the conversion of Hb into non-functional methemoglobin (metHb). Thus, the challenge for the fabrication of next-generation HBOCs relies in creating a system that takes advantage of the excellent oxygen-carrying capabilities of Hb, while preserving the redox environment of native RBCs that prevents or reverts the conversion of Hb into metHb. In this review, we feature the most recent advances in the assembly of the new generation of HBOCs with emphasis in two main approaches: the chemical modification of Hb either by cross-linking strategies or by conjugation to other polymers, and the Hb encapsulation strategies, usually in the form of lipidic or polymeric capsules. The applications of the aforementioned HBOCs as blood substitutes or for oxygen-delivery in tissue

engineering are highlighted, followed by a discussion of successes, challenges and future trends in this field.

KEYWORDS

Encapsulation platforms; hemoglobin; hemoglobin-based oxygen carriers; red blood cells substitutes; oxygen delivery

1. INTRODUCTION

Blood transfusions, which in most cases involve the administration of isolated red blood cells (RBCs), are crucial in several settings such as traumatic injuries (e.g., in accidents or natural disasters), in a pre-surgical context (e.g., transplants or trauma surgery) or for treatment of chronic and acute anemias.[1,2] Blood transfusion has been a routine and safe procedure over the last century since, under normal circumstances, donor blood is the best replacement for blood. However, blood transfusions from donors have some limitations and risks such as: a limited supply with a worldwide acute shortage that is expected to get worse due to an ageing population;[3] the need of typing and matching before RBCs can be transfused to patients due to the RBCs membranes blood group antigens, which can cause fatal delays in emergency situations; the short lifetime of RBCs by standard storage methods (one day stability when stored at room temperature and 42 days at 4 °C); or the fact that RBCs cannot be sterilized to remove infective agents such as hepatitis viruses, HIV and other potential emerging infective agents which could result in contaminated donor blood.[4]

The primary biological function of natural RBCs is to transport oxygen (O_2) from the O_2 -rich lungs to O_2 -poor tissues (Scheme 1Ai). Therefore, there is a significant clinical interest in creating synthetic analogues of RBCs, the so-called RBC substitutes (RBCSs), which can mimic the biological functions of natural RBCs while, simultaneously, overcoming some of its drawbacks. Unlike natural RBCs, RBCSs can be sterilized by pasteurization, ultrafiltration and other chemical means to remove microorganisms responsible for AIDS or hepatitis, amongst others, thus reducing biological risks. Furthermore, RBCSs do not possess RBC blood group antigens, eliminating the need for cross-matching or typing which saves time and facilities, a

crucial factor in emergency situations. What is more, RBCs can be readily lyophilized and stored for a long time in a dried powder form, which can be reconstituted with salt solution the moment is needed.[5,6] To this end, a large volume of research has been carried out in the area of synthetic RBCs.[1,7]

There are three main types of RBCs that have undergone extensive preclinical and some clinical research, including perfluorocarbon (PFC)-based emulsions, iron (Fe^{2+})-containing porphyrins and hemoglobin (Hb)-based oxygen carriers (HBOCs). HBOCs make use of either bovine Hb or cell-free human Hb (hHb) from outdated blood. They take advantage of the excellent O_2 -carrying and releasing properties of Hb to create RBCs, and are the focus of this review. For a detailed overview of PFC-based emulsions and Fe^{2+} -containing porphyrins, the reader is referred to excellent reviews on these fields.[1,8–10]

Hb is the main component of RBCs, but a RBC also contains a multi-enzyme system to prevent the conversion of Hb into non-functioning methemoglobin (metHb), as well as the allosteric effector 2,3-diphosphoglycerate (2,3-DPG), which promotes O_2 release to the tissues.[4] Natural RBCs also carry out the important function of carbon dioxide (CO_2) transport from the tissues to the lungs for excretion. While around 20% of CO_2 is carried by Hb, the remaining 80% of CO_2 is transported in the plasma as bicarbonate (HCO_3^-). The conversion of CO_2 into HCO_3^- is catalyzed in RBCs by the enzyme carbonic anhydrase (CA).[11]

Hb is a tetramer with two globular α and two globular β polypeptide subunits ($\alpha_1\beta_1\alpha_2\beta_2$), and each subunit contains a heme group consisting of a porphyrin ring structure with Fe^{2+} bound to the center (Scheme 1Aii). Each heme group is a single O_2 -binding site and, thus, each Hb molecule has the capacity to carry four O_2 molecules. Hb is in the “oxy” state (oxy-Hb) when it carries O_2 , which is also known as the relaxed form of Hb (R-state), where it has a high O_2 -

affinity structure (Scheme 1Aiii). When Hb releases O_2 , it undergoes a conformational change to the “deoxy” state (deoxy-Hb), also known as the tense form of Hb (T-state), where the quaternary structure of Hb has low affinity towards O_2 (Scheme 1Aiii). Diverse factors such as low pH, high CO_2 and high 2,3-DPG concentrations at the tissue level favor the T-state, which releases O_2 into the tissues. Similarly, high pH, low CO_2 and low 2,3-DPG concentrations facilitate the R-state, where Hb prefers O_2 binding. The amount of O_2 bound to Hb at any time is related to the partial pressure of oxygen (pO_2) to which Hb is exposed. In the lungs, pO_2 is typically high and O_2 binds readily to Hb (R-state). As RBCs move to other body tissues with a lower pO_2 , Hb releases O_2 into the tissue (T-state). The shape of the O_2 dissociation curve (ODC) of Hb is sigmoidal (Scheme 1B), which results from the interaction of bound O_2 with incoming O_2 molecules. Hb's affinity for O_2 increases as successive molecules of O_2 bind, due to an induced conformational change in its structure.[6] The p_{50} value refers to the pO_2 at which Hb is 50% saturated with O_2 ($Hb_{(p50)} = 26.5 \text{ mmHg}$) (Scheme 1B).[12]

The transport of O_2 requires O_2 reversibly bound to ferrous Hb ($Hb(Fe^{2+})$). Although oxy- $Hb(Fe^{2+})$ is a very stable molecule, it slowly auto-oxidizes to oxy- $Hb(Fe^{3+})$, the so-called metHb,[13] which is unable to bind O_2 and, furthermore, leads to dysregulated vascular tone and inflammatory reactions.[8] Thus, natural RBCs possess mechanisms to prevent or reverse the oxidative denaturation of Hb into metHb by making use of a multi-enzyme system that includes cytochrome- b_5 reductase, superoxide dismutase (SOD), glutathione peroxidase and catalase (CAT).[13–15]

Apart from the lack of an antioxidant multi-enzyme system and the allosteric effector 2,3-DGP, Hb alone cannot be used for infusion in the body because, when outside the RBCs, the $\alpha_1\beta_1\alpha_2\beta_2$ tetramer breaks down into dimers ($\alpha_1\beta_1$ and $\alpha_2\beta_2$) and monomers (α_1 , β_1 , α_2 , β_2) causing

toxicity by means of several pathways (Scheme 1C).[4] One mechanism for the removal of extracellular oxy-Hb involves the plasma protein haptoglobin (Scheme 1C, 1).[16] Oxy-Hb dimers form complexes with haptoglobin which are subsequently recognized by the mononuclear phagocyte system (MPS), followed by macrophage endocytosis by the CD163 receptor and removal in the liver and spleen.[17] When large amounts of extracellular oxy-Hb are present, upon saturation of haptoglobin, the remaining oxy-Hb dimers and monomers get oxidized into metHb products (Scheme 1C, 2). This oxidation of oxy-Hb into metHb, a part of provoking the loss of the O₂-carrying ability of Hb, generates a series of free radicals and reactive oxygen species (ROS) such as superoxide radicals (O₂^{•-}) and hydrogen peroxide (H₂O₂),[11,18–20] which subsequently react with nitric oxide (NO) converting it into nitrate (NO₃⁻) (Scheme 1C, 3). Being NO a natural vasodilator, its depletion from the bloodstream generates vasoconstriction and the associated cardiovascular complications (e.g., myocardial infarction) and increased mortality.[21] Furthermore, H₂O₂ can oxidize both oxy-Hb and metHb into ferryl Hb (Hb(Fe⁴⁺)) leading to the release of both the heme group and Fe⁴⁺ ions (Scheme 1C, 4).[20] While Fe⁴⁺ is sequestered by transferrin, the heme group binds to the plasma glycoprotein hemopexin (Scheme 1C, 5).[16,22] Next, the heme groups are released from hemopexin to be degraded in the liver while the hemopexin glycoprotein is recycled back.[22] When the plasma protein scavenging pathways are saturated, the oxy-Hb dimers and monomers will also start scavenging NO from the bloodstream, which will result in the formation of NO₃⁻ and the oxidized Hb products metHb and Hb(Fe⁴⁺) (Scheme 1C, 6). MetHb and Hb(Fe⁴⁺) will subsequently release the heme groups which will permeate into the endothelial cells lining the blood vessels generating an inflammatory response (Scheme 1C, 7).[23,24] Further accumulation of oxy-Hb in the bloodstream will be filtered out by the kidney glomeruli and reabsorbed by the tubules (Scheme

1C, 8).[25] Overloading this system will result in oxidative damage and cell death within the kidneys. Additionally, upon dissociation of the oxy-Hb tetramer, the oxy-Hb dimers and monomers will be able to penetrate in between the endothelial cells lining the blood vessels due to their small size (Scheme 1C, 9).[21,26] Following extravasation into the sub-endothelial layer, the oxy-Hb dimers and monomers can react again with NO present in the underlying smooth muscle cells which will result in conversion to metHb products together with NO_3^- . This depletion of the NO present in the smooth muscle cells will cause endothelial dysfunction, irregular muscle functioning (e.g., vasoconstriction, contraction, and systemic hypertension) and intravascular platelet activation.[16,27] Platelet activation together with the vasoconstriction can, furthermore, cause blood clot formation.

Due to all the aforementioned toxic effects generated by the dissociation of Hb into dimers and monomers, the challenge when developing HBOCs lies in creating a system able to take advantage of the Hb O_2 -carrying capabilities while avoiding dissociation of the Hb tetramer and preserving the redox environment of RBCs that minimizes the conversion of Hb into non-functional metHb and all the associated toxic effects. The research efforts to achieve this goal have so far gone through three different generations of HBOCs.[4,28] While the first generation focused on the polymerization or cross-linking of Hb to prevent tetramer dissociation and renal filtration, the second generation of HBOCs enhanced their functionality by incorporating antioxidant enzymes to minimize the conversion of Hb into non-functional metHb. While the first and second generations entail the Hb's chemical modification, the last or next generation of HBOCs involve the encapsulation of Hb within a synthetic membrane. In the next sections, we will review HBOCs with emphasis in recent examples from the last 2-3 years. The different approaches will be classified in the two main strategies that have been developed so far: the

chemical modification of Hb (i.e., the first and second generation of HBOCs) or the encapsulation of Hb within a protective shell (i.e., the last generation of HBOCs) (Scheme 2). The different strategies will be further classified in nano or microarchitectures, depending on the size of the as-prepared HBOCs. It is important to note that, unless stated otherwise, the Hb employed to assemble the different HBOCs is from bovine descent.

2. CHEMICAL MODIFICATIONS

Hb cannot be administered alone due to its unacceptable short circulation times (> 6 h) as well as to the associated toxicities due to the dissociation of the Hb tetramer. To overcome these drawbacks, several chemical modification approaches have been employed as a strategy.[1,4,21,29]

2.1. Nanoarchitectures

Mainly, three types of chemically modified Hb generating molecular structures in the nano-range have been developed to date: cross-linked Hb, polymerized Hb and Hb conjugated to polymers (Table 1).

Intramolecular cross-linked Hb was the first approach to avoid dissociation of the tetramer by creating chemical cross-links between the α and β chains of the tetramer, which gave rise to commercial products such as HemAssist (Baxter Healthcare Corporation, U.S.A.).[30] HemAssist, which consisted in diaspirin cross-linked hHb, showed an increase in circulation time to up to 12 h (in contrast to < 6 h for native Hb).[30] However, due to increased mortality rates in human patients as compared to the control group, all cross-linked Hb-based products have been discontinued.[10,31]

Several bi-functional cross-linking reagents have also been used to polymerize Hb. As such, glutaraldehyde (GA) and *o*-raffinose have been used to create different polyHb products. HemoPure (Biopure Corporation, U.S.A.), which consists of Hb polymerized by GA, is sold in South Africa to treat acute anemia and is the only FDA approved HBOC in the U.S.A. and Europe for veterinary use under the name of Oxyglobin (Biopure Corporation, U.S.A.).[32] Other polyHb products such as PolyHeme (hHb, Northfield Laboratories, Inc., U.S.A.)[33] or HemoLink (hHb, Hemosol BioPharma, Canada),[34] have been discontinued due to unacceptable toxicities.[10]

Finally, conjugation of Hb to other molecules has been considered as an approach to create HBOCs. As an example, Hb has been conjugated to polyethylene glycol (PEG) forming an Hb-polymer conjugate with reduced recognition by the MPS and with increased viscosity and osmotic pressure.[35] Some of the PEGylated products reached the market, such as Hemospan (hHb, Sangart, Inc., U.S.A.), Pyridoxalated Hemoglobin Polyoxyethylene (hHb, Apex Bioscience, U.S.A.), or PEG-Hb (Enzon, U.S.A.). However, these products have also demonstrated a statistical increase in the risk of death and myocardial infarction in the treatment groups,[36] and they have, therefore, been withdrawn from the market.[10]

Despite the disappointing outcomes of all these chemically modified Hb products, there are useful lessons to be learned from this first generation which can help to develop a safer HBOCs.[37] As an example, the vasoconstriction caused by the chemically modified Hb-based products has been partly attributed to the infiltration of modified Hb into the endothelium of blood vessels, probably due to incomplete cross-linking. This results in oxidative damage as well as in the scavenging of NO. Since NO is responsible for relaxation of the underlying smooth muscle,[25] NO removal causes potent microvascular vasoconstriction, which subsequently

results in cardiovascular compromise (i.e., myocardial infarction) and increased mortality (Scheme 1C).[10,36,38–41]

It is also important to note that, despite having been withdrawn from the market, several polymerized Hb products (e.g., PolyHeme, Hemopure and Oxyglobin) induced limited NO sequestration as compared to unmodified Hb. This fact is attributed to the increased particle size of polyHb, which limits its interaction with the vascular endothelium.[25] Similarly, polymerization of Hb with PEG (e.g., Hemospan) also produces larger particles as compared to cross-linked or free native Hb which, in turn, results in minimal vasoconstriction as well as longer circulatory half-lives.[10,42,43]

One of the current research directions towards the development of chemically modified HBOCs in the nano-range involves the conjugation of Hb with dextran (Dex). Since Dex is a natural, biocompatible, hydrophilic polymer which has the capacity to prevent thrombosis and shock, it is very attractive for the creation of HBOCs and has recently been employed for the fabrication of Hb-Dex conjugates.[44–46] Wang *et al.*[47] have recently developed an Hb-Dex conjugate of around 30 nm in diameter with lower oxidative toxicity as compared to other Hb-polymer conjugates. Since one of the leading obstacles for the development of Hb-polymer conjugates is the fact that Hb can be easily oxidized to metHb, which results in oxidative stress and vasoconstriction, the authors prepared the Hb-Dex conjugate while protecting the critical moieties of the heme group. The rationale behind their approach lies in the fact that many of the reported Hb-polymer conjugates, including Hb-Dex but also hHb-PEG[48] and polyHb,[49] are prepared by modifying the thiol moiety of the amino acid residue Cys-93(β) which can unfavorably influence the heme pocket of the β chain. This modification of the heme pocket increases the autoxidation rate, which subsequently increases the affinity towards O₂ and

significantly decreases the p50. Hb oxidation onto metHb causes a leftward shift in the ODC due to impaired metHb cooperativity.[50,51] Therefore, Hb was conjugated to Dex while protecting the Cys-93(β) residue. As expected, the Hb-Dex conjugates showed significantly higher hydrodynamic volume and higher viscosity than native Hb. Furthermore, the authors could successfully demonstrate that protection of Cys-93(β) played a critical role in decreasing the autoxidation rate of Hb-Dex by comparing it to the autoxidation rate of the non-protected counterpart.

Also, making use of Dex to create Hb-polymer conjugates, Wei *et al.*[52] reported the synthesis of Dex-based nanogels. Nanogels are particularly well suited as Hb carriers their high water content which translates into high deformability, allows them to remain in circulation for extended amounts of time.[53] For the assembly of Hb-containing nanogels, the authors synthesized an amphiphilic graft co-polymer based on Dex, succinic anhydride (SA) and dopamine (DA) conjugated to Hb (Figure 1A). This Dex-SA-DA graft co-polymer could self-assemble into nanogels just by adjusting the pH to 2. Next, the nanogels were cross-linked *in situ* employing sodium periodate to cross-link the DA moieties by oxidation. Finally, the Dex's moieties of the nanogels were functionalized with aldehyde groups to allow for Hb's conjugation *via* Schiff base reaction between the aldehydes of the functionalized Dex and the Hb's amino groups. Transmission electron microscopy (Figure 1B) and scanning electron microscopy (SEM) (Figure 1C) images showed that the Hb-nanogels (HbNGs) had a diameter of ~260 nm. It was possible to demonstrate the gas binding capacity of the HbNGs by UV-vis analysis (Figure 1D). The maximum absorption peak of HbNGs at different wavelengths when exposed to different gases, could shift reversibly. This fact demonstrates that the HbNGs are able to bind and release different gas ligands freely while retaining Hb's bioactivity. The O₂ affinity of the HbNGs was

assessed by an ODC and it revealed a p50 of about 14 mmHg, which indicates that the HbNGs exhibit a higher O₂ affinity as compared to native Hb encapsulated within RBCs (p50 = 26.5 mmHg). High O₂ affinity (low p50) is an important feature for HBOCs, since high O₂ affinity avoids vasoconstriction caused by O₂ oversupply in the precapillary arterioles.[54] Since Hb will slowly autoxidize into non-functional metHb, the authors also assessed the HbNGs half-life ($t_{1/2}$) (Figure 1E), showing that the HbNGs exhibited a similar $t_{1/2}$ as native Hb at 37 °C. In the same study, the authors also showed that the Dex-based HbNGs exhibited good biocompatibility as demonstrated by cell viability assays. It was also confirmed that the HbNGs do not interact with blood components and will, therefore, not cause a detrimental effect after being introduced in the bloodstream.

Dex has also been chosen as a component to create stimuli-responsive Hb-polymer conjugates by co-polymerizing it with poly(*N*-isopropylacrylamide) (PNIPAAm). PNIPAAm is one of the most prominent stimuli-responsive polymers reported to date since it displays a reversible phase transition with temperature in aqueous solution.[55,56] To create thermo-responsive Hb-polymer conjugates, Wang *et al.*[57] synthesized a Dex-graft-PNIPAAm (Dex-g-PNIPAAm) co-polymer that displayed a reversible change of solubility in response to temperature. Since above the lower critical solution temperature (LCST), which is ~32 °C, the PNIPAAm block experiences a transition from a hydrophilic to a hydrophobic conformation, the Dex-g-PNIPAAm co-polymer could self-assemble into stable micelles. The micelles, which were ~100 nm in diameter, were composed of PNIPAAm as the hydrophobic core and Dex as the hydrophilic shell.[58] Incorporation of Hb into the co-polymer, which was conjugated to the Dex moieties of the co-polymer, decreased the hydrodynamic diameter to ~50 nm. This fact could be attributed to the additional hydrogen-bonding interactions within the core. Interestingly, the authors demonstrated

that, although at room temperature ($T < \text{LCST}$) the autoxidation rate of native Hb was much slower than the autoxidation rate of the Hb-Dex-g-PNIPAAm conjugates, when increasing the temperature to $T > \text{LCST}$, the results were different. At 37 °C ($T > \text{LCST}$), the autoxidation rate of native Hb was faster than the Hb-Dex-g-PNIPAAm conjugates. This result was explained by the authors by the fact that, when the polymer conjugates self-assembled into micelles, they provided a better protection to the oxy-Hb molecules, thus improving their stability in air.

On a very original study, Bu *et al.*[59] designed an Hb-polymer conjugate functionalized with specific recognition moieties for cancer cells, which was designed for the treatment of hypoxia in tumors. The authors synthesized the amphiphilic block co-polymer poly[2-(methacrylamido) glucopyranose]-*block*-poly(methacrylic acid)-*block*-poly(butylmethacrylate) (PMAG-*b*-PMAA-*b*-PBMA) where the first block consists of glucose units that are able to recognize cancer cells *via* the glucose transporter isoform 1 (GLUT1). Upon self-assembly of the amphiphilic polymer into micelles, Hb was conjugated to the PMAG-*b*-PMAA-*b*-PBMA polymer through a condensation reaction between the amino groups of Hb and the carboxyl groups of the PMAA block. The Hb-based micelles displayed a uniform size of ~147 nm. Importantly, the Hb-based micelles demonstrated excellent capacity for O₂ binding, as shown by their UV-vis spectrum after being oxidized by O₂ which displayed the characteristic oxy-Hb peak at 412 nm, thus confirming the transformation of deoxy-Hb into oxy-Hb. Additionally, *in vitro* cell experiments showed high cell viability for both kidney cells (the control cell line) and cancer cells, therefore demonstrating the biocompatibility of the Hb-based micelles. In addition, the Hb-based micelles actively recognized cancer cells *via* GLUT1, as shown by the high cellular uptake of doxorubicin-loaded Hb-based micelles by cancer cells as compared to kidney cells. These results demonstrate the potential of the Hb-based micelles in tumor therapy.

Despite this progress in the development of Hb-conjugates, all described products still lack the ability to regulate the autoxidation of oxy-Hb. The oxidative state of the iron of the heme groups will unavoidably result in the irreversible conversion of oxy-Hb into metHb which, apart from not being able to deliver O₂ at physiological pO₂ tissue levels, also generates toxic free radicals.[10,20,60–62]

To prevent or reverse the conversion of oxy-Hb into metHb, natural RBCs contain antioxidant enzymes such as CAT and SOD. Thus, in an attempt to minimize metHb conversion, these enzymes have been cross-linked to polyHb to form polyHb-SOD-CAT conjugates.[63,64] The as-prepared conjugate was able to reduce the oxidative stress produced by ROS, thus demonstrating its potential in reducing oxidative damage in injured and ischemic tissues.[8,65,66]

Based on the previous approach and with the aim to create a RBCS with three augmented RBCs functions, namely O₂ transport, ROS removal, and elimination of CO₂, Bian *et al.*[67] employed polyHb where the Hb molecule was cross-linked to the antioxidant enzymes SOD and CAT, and also to the CA enzyme, which is responsible for the conversion of ~ 80% of the CO₂ into H₂O and HCO₃⁻. The polyHb-SOD-CAT-CA conjugate was synthesized by cross-linking SOD, CAT and CA to polyHb employing GA (Figure 2A). The authors chose to work with a derivative of polyHb since it has been previously shown that polyHb can delay the need for blood transfusion for up to 12 hours.[39] Furthermore, although polyHb has only been authorized for routine use in countries such as South Africa, where the side effects caused by polyHb are not considered as serious as compared to having HIV-contaminated donor blood or to not having donor blood at all, so far, polyHb is the only approved HBOC.[1,68] The authors conducted an *in vivo* study with a severe hemorrhagic shock rat model to which two thirds of the

blood volume had been removed generating a mean arterial blood pressure (MAP) of only 30 mmHg. This was followed by the reinfusion of polymerized stroma-free hemolysate (polySFHb), polySFHb-SOD-CAT-CA, and several control solutions (sham control, blood, lactated Ringer's solution, polyHb, and polyHb-SOD-CAT) for 60 minutes (Figure 2B). The results demonstrated that the MAP decreased after reinfusion with the lactated Ringer's solution (ii, 43.3 ± 2.8 mmHg), while was maintained after reinfusion with blood (iii, 91.3 ± 3.6 mmHg), polyHb (iv, 82.6 ± 3.5 mmHg), polySFHb (v, 85.0 ± 2.5 mmHg), polyHb-SOD-CAT (vi, 86.0 ± 4.6 mmHg), and polySFHb-SOD-CAT-CA (vii, 87.5 ± 5 mmHg). Although there were no significant differences in the MAP when injecting polySFHb-SOD-CAT-CA, polyHb-SOD-CAT, polySFHb or polyHb, the authors also measured the partial pressure of CO₂ (pCO₂) upon applying the different resuscitation fluids (Figure 2C). The relevance of pCO₂ has been demonstrated, since increasing levels of intracellular pCO₂ are associated to a more severe myocardial ischemia[69] as well as to higher mortality rates.[70] Interestingly, the results showed that polySFHb-SOD-CAT-CA was able to reduce tissue pCO₂ from 98.0 ± 4.5 mmHg to 68.6 ± 3.0 mmHg (Figure 2Cvii), demonstrating that polySFHb-SOD-CAT-CA was significantly more effective in reducing the pCO₂ levels than all the other resuscitation fluids (lactated Ringer's solution (ii, 98.0 ± 4.5 mmHg), blood (iii, 79.1 ± 4.7 mmHg), polyHb (iv, 90.1 ± 4.0 mmHg), polySFHb (v, 77.0 ± 5.0 mmHg) and polyHb-SOD-CAT (vi, 90.9 ± 1.4 mmHg)). The authors also demonstrated that polySFHb-SOD-CAT-CA could be freeze-dried, pasteurized and stored for 320 days at room temperature, all of them crucial parameters to create successful RBCs.

Another strategy employing enzymes to minimize oxidative stress and, therefore, the metHb conversion, was employed by Hathazi *et al.*[71] The authors conjugated Hb to the non-heme

peroxidase rubrerythrin (Rbr), which is an enzyme with the ability to reduce H_2O_2 to H_2O without involving free radicals or ferryl iron, which is usually formed in peroxidase and catalase reactions. Thus, with the aim of using a safer enzyme than heme-based peroxidases or catalases, the authors polymerized Hb with Rbr using GA to form polyHb-Rbr. UV-vis characterization showed that polyHb-Rbr accumulated significantly less Fe^{4+} than polyHb, thus demonstrating the ability of Rbr to protect against oxidative stress, which may result in reduced toxicity *in vivo*. Furthermore, cell viability studies using endothelial cells revealed slightly better performance of polyHb-Rbr as compared to polyHb, showing the potential for a better protective effect towards Hb autoxidation *in vivo*.

A different approach to avoid the toxic effects of polyHb was developed by Simoni *et al.*[72,73] The authors reported a product named HemoTech, where Hb had been cross-linked intramolecular by adenosine-5'-triphosphate (ATP), intermolecular by adenosine and further combined with reduced glutathione (GSH). With this composition, ATP prevented Hb dimerization while regulating the vascular tone through purinergic receptors; adenosine promoted the polymerization of Hb while simultaneously counteracting the vasoconstrictive and pro-inflammatory properties of Hb by stimulating the adenosine receptors; and GSH shielded the heme group from NO and ROS while blocking Hb's extravasation and glomerular filtration by lowering its isoelectric point. Preclinical studies and an older clinical proof-of-concept study have already been performed, and the results suggest that HemoTech can work as an effective HBOC able to treat several conditions such as blood loss, anemia or ischemic vascular conditions.[72]

A very creative approach to minimize the conversion of oxy-Hb into metHb was developed by Tomita *et al.*[74] The authors employed a previously reported core-shell structure where Hb was

located at the core and conjugated to human serum albumin (HSA) which was located in the periphery (Figure 3A1-2). The assembly was conducted by cross-linking the amino groups of the lysines on the Hb's surface to the free cysteines of HSA. The heterobifunctional cross-linker *N*-succinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate was employed to conduct the reaction and three HSA molecules were conjugated to each Hb.[74] These Hb-(HSA)₃ nanoclusters are envisaged to have low risks of rapid clearance and extravasation, since HSA shows low permeability in the vasculature walls due to the electrostatic repulsion between the negatively charged HSA's surface and the negatively charged membrane of the endothelial cells.[75] An important consideration for this system is that flavin derivatives can bind strongly to the exterior of the HSA units of the core-shell structure (Figure 3Ai). The relevance of flavin derivatives lies in that they are electron mediators in biological systems and, upon being reduced by the co-factor nicotinamide adenine dinucleotide (NADH), they can act as reducing agents and convert metHb back to oxy-Hb (Figure 3B). The authors showed the reduction of metHb after addition of NADH using the flavin derivatives lumiflavin (LF) and flavin mononucleotide (FMN) (Figure 3C). LF, which is smaller and more hydrophobic than FMN, binds more tightly into the HSA pocket and showed increased metHb reduction rates as compared to FMN. Another modification of these Hb-(HSA)₃ core-shell nanoclusters where platinum nanoparticles (PtNPs) were embedded into the HSA pockets was also reported (Figure 3Aii).[76] PtNPs can perform SOD and CAT activities, since they can convert O₂^{•-} into H₂O₂ plus O₂, and H₂O₂ into O₂ plus H₂O and, as such, they have the ability to minimize the autoxidation of Hb to metHb. The results demonstrated higher CAT activities for the HSA-PtNP complexes as compared to the free PtNPs, marking the importance of the PtNP-protein complexes. Furthermore, metHb levels were measured for different Hb-(HSA)₃ cluster combinations in aqueous H₂O₂ solutions (Figure 3D).

Incorporation of PtNPs into the Hb-(HSA)₃ nanoclusters showed good protection towards the oxidation of Hb as compared to the different controls. In a different study, the authors evaluated the potential of Hb-(HSA)₃ nanoclusters as an HBOC *in vivo*.^[77] The hemodynamic and pharmacokinetic properties were analyzed and compared to that of cross-linked Hb (XLHb). The authors first demonstrated that there was no unfavorable interaction between the Hb-(HSA)₃ nanoclusters and the different blood components. Next, the potential of the Hb-(HSA)₃ nanoclusters as HBOCs was investigated by analyzing the MAP after their injection in rats (Figure 3E). The results show that the injection of Hb-(HSA)₃ nanoclusters displayed the same response as an HSA injection, which had a non-vasopressor effect, showing only slight MAP alternation after injection. In contrast, upon injection of XLHb an immediate increase in MAP was observed followed by urinary excretion only 10 minutes after injection. The circulation $t_{1/2}$ was also investigated using iodine-125 labeling (Figure 3F). While XLHb had a $t_{1/2}$ of only 0.53 hours, HSA had a $t_{1/2}$ of 11.0 hours, while Hb-(HSA)₃ had a $t_{1/2}$ of 18.5 hours. This increase in circulation time was ascribed to the increased size and negative surface charge of the Hb-(HSA)₃ nanoclusters as compared to bare HSA. The *in vivo* studies also demonstrated that there were no negative side effects or toxic reactions in the major organs after injection. Other articles published using the same Hb-albumin core structure, but employing serum albumins from feline and canine origin, showed the potential of this HBOCs product for veterinary use.^[78,79]

Despite the toxicities displayed by many of the chemically modified nano-sized HBOCs, which has resulted in the withdrawal from clinical trials and/or the market of most of the products, chemically modified HBOCs have been thoroughly studied and, as a result, we have learned several lessons from their shortcomings, which are now being addressed in the most recent studies. Promising results arise from the strategies that aim at limiting the autoxidation of

Hb into metHb, thus decreasing the toxicity caused by ROS. However, although most of this new generation of chemically modified HBOCs with Hb protection towards oxidation have been extensively evaluated for their O₂-binding capacity, redox properties and *in vitro* stability, rigorous *in vivo* studies are still needed in order to establish their *in vivo* applicability.

2.2 Microarchitectures

Parameters such as the shape, stiffness and surface chemistry strongly influence the particles circulation time.[80] For example, elongated or disc-shaped particles have shown to have longer circulation times as compared to their spherical counterparts,[81,82] and carriers with high deformability which allows them to flow through small vesicles have also shown extended circulation times.[83,84] However, the chemical modification of Hb renders spherical particles. When considering the size for spherical carriers it is noted that, while particles larger than 5 μm can block the circulation at high concentrations, particles in the range of 1-3 μm can be strongly phagocytosed.[85,86] Therefore, for spherical HBOCs that are expected to be injected intravenously sizes in the submicron range are the preferred ones. Nonetheless, micron-sized particles can have applications in other fields that do not require intravenous administration such as tissue engineering (Table 1).[87,88]

Despite the fact that Hb-based biomaterials are expected to have great potential in both *in vitro* tissue engineering and wound healing applications, the field is still in its infancy with few reports having been published to date. For *in vitro* tissue engineering, O₂ delivery is a crucial parameter specially for the fabrication of large tissue analogues due to the “size-barrier effect”, which hinders the transport of nutrients.[89] For *in vivo* wound closures, lowering the O₂ tension during the regeneration process is a critical phenomenon affecting both the final architecture, as well as the functionality of the restored part. Therefore, the presence of a scaffold that releases O₂

locally during a wound healing process would help to maintain suitable levels of tissue oxygenation.[90]

One of the most recent examples of chemically modified micron-sized HBOCs for tissue engineering applications was reported by Paciello *et al.*[91] The authors conjugated hHb to the surface of biocompatible and biodegradable gelatin microparticles (GM) with a size in the range of 75–150 μm , with the aim of producing an Hb-based O_2 depot (GM-Hb). For the GM-Hb preparation, two different conjugation strategies were employed to link the Hb molecules to the surface of the GMs (Figure 4A). The authors demonstrated that the conjugation strategy employed affected the adsorption and release of O_2 . When employing the first strategy (strategy 1, GM-Hb-1), Hb was conjugated to GM by means of the formation of a polyurethane linker while, when making use of the second strategy (strategy 2, GM-Hb-2), the conjugation took place *via* amide bond formation. To form the polyurethane linker in strategy GM-Hb-1, urethane functions through the use of *N,N'*-disuccinimidyl carbonate (DSC) activation were employed,[92,93] since DSC is highly reactive toward primary amines and hydroxyls.[94] By using this method, Hb was conjugated to the surface of GMs without compromising the Hb's secondary structure. In contrast, to conduct strategy GM-Hb-2, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) chemistry was employed. EDC chemistry is known to induce the auto cross-linking of Hbs through amide bonds which, in turn, induces the formation of large Hb clusters.[95–97] Those large clusters affect the equilibrium between the R- and T-state of the quaternary structure of Hb.[98,99] As a consequence the O_2 -binding and release properties, which are mediated by the structural changes of Hb, could be compromised.

The functionality of GM-Hb-1 and GM-Hb-2 was evaluated by conducting O_2 -uptake/release experiments. For the O_2 uptake experiments, the cell medium was oxygenated (initial $\text{pO}_2 =$

100%, 0.21 atm) and nitrogen (N₂)-treated samples were loaded into the medium. The decay of the solution's pO₂ was measured over time to analyze the O₂ uptake by both GM-Hb-1 and GM-Hb-2 (Figure 4B). Even though GM-Hb-2 displayed a higher Hb content, the results demonstrated lower O₂-absorption capacity for this strategy, a fact that could be explained by the steric hindrance for the Hb inner units due to the Hb-cluster formation and the cross-links among the Hb subunits. Next, the solutions were treated with N₂ (initial pO₂ = 0%) and the O₂ release from GM-Hb-1 and GM-Hb-2 was measured over time (Figure 4C). The results showed different release kinetics for the two different strategies, with 67% and 100% O₂ release with respect to the loaded O₂ for GM-Hb-1 and GM-Hb-2, respectively (Figure 4B, 24 hours). Interestingly, the authors also demonstrated that GM-Hb-1 were able to work under repeated charge and discharge cycles. To assess the functionality of the GM-Hb-1 in a cellular context, the GM-Hb-1 were embedded within a disk-shaped collagen 3D cell culture. The setup allowed nutrients and O₂ transport along the disk thickness (2 mm). After 48 hours of incubation, the 3D cell cultures were transversally cut and the cell viability was assessed by live/dead fluorescence staining (Figure 4D). Fluorescence microscopy images showed the living cells (green) and dead cells (red) throughout the cut section, demonstrating increased cell viability and proliferation rate in the cell culture loaded with GM-Hb-1 as compared to the control (spheres also showed in green). The cell viability fraction (Figure 4E) confirmed these findings, since GM-Hb-1 displayed good cell viability throughout the 3D cell culture after 48 hours. These results demonstrate the potential of the system to work as an O₂ reservoir in 3D cell cultures to overcome the critical issue of the high O₂ demand in the early stages of the fabrication of tissue analogues *in vitro*. [100]

Knirsch *et al.* also assembled Hb-loaded GM.[101,102] Although an application for such microparticles still remains to be identified, the authors managed to incorporate polyHb, which

had been previously cross-linked making use of PEG bis(*N*-succinimidyl succinate), into 1.37 μm gelatin microcapsules.

3. ENCAPSULATION STRATEGIES

In the body, native Hb is protected by the membrane of RBCs. This encapsulation strategy allows to entrap a multi-enzyme system able to avoid or reverse the conversion of Hb into metHb without chemical modification of the enzymes secondary structure, which can alter the Hb's O_2 -binding and releasing properties. Additionally, by enclosing Hb within a protective shell, Hb is prevented from both its rapid removal from circulation and from extravasation through the vessel walls. Encapsulation also reduces Hb's high viscosity and high colloidal osmotic pressure while protecting it from direct contact with surrounding tissues and blood components.[2,103] Therefore, due to the aforementioned advantages, several approaches have been investigated to encapsulate Hb within synthetic carriers.[10]

3.1 Nanoarchitectures

The advent of nanoparticle technologies has revolutionized the field of drug delivery by entrapping all kinds of active compounds within delivery vehicles, thus protecting the compounds from plasma-induced effects, increasing their circulation time and, furthermore, allowing for their sustained delivery to cells, tissues and organs. Naturally, the concept of encapsulation within nano-sized architectures has also been adapted to create HBOCs entrapping Hb within biomaterials-based carrier vehicles (Table 2).[8]

The concept of Hb encapsulation was first introduced by the Chang group[38,104] a few decades ago by making use of a double-emulsion-solvent evaporation technique, where Hb was entrapped within poly(lactic acid) (PLA), poly(lactic acid-*co*-glycolide) (PLGA), PLA-PEG, and

poly(isobutyl cyanoacrylate) as matrix polymers. Essential enzymes that avoid the conversion of Hb into MetHb, and CA, which allows for CO₂ transport, could also be entrapped within the polymeric shell.[38] Furthermore, the as-prepared polymeric particles had diameters that made them suitable for intravenous delivery (70-200 nm). However, they suffered from low encapsulation efficiency, a parameter that was later on improved by Meng *et al.*[105] by making use of a w/o/w double-emulsion-solvent-diffusion process, which yielded a 90% Hb encapsulation efficiency.

In the following years, the concept of Hb encapsulation employing polymeric nanoparticles has been expanded to a wide range of polymer systems which usually involve the use of amphiphilic co-polymers such as poly(ϵ -caprolactone)-PLA (PCL-PLA), PCL-PEG, PEG-PLA-PEG or PLGA-PEG co-polymers that self-assemble to form the so-called polymersomes.[106–109] Polymersomes are considered as the synthetic analogues of liposomes, but with improved chemical and mechanical stability, since their membranes are significantly thicker than those of liposomes. Importantly, polymersomes offer more flexibility in the choice of several parameters such as the vesicle size, membrane thickness or drug-encapsulation efficiency. These parameters can be tuned by the right choice of the constituting polymers and a proper selection and adjustment of the polymers molecular weight and concentration. Furthermore, polymersomes can be designed to be both biocompatible and biodegradable.[110] The polymersomes surfaces can be functionalized with targeting moieties and they can also be composed of PEG polymers to further improve their *in vitro* stability, increase their *in vivo* bioavailability and prolong their circulation in blood.[110,111] The field of Hb encapsulation within polymeric vehicles has been continuously growing and, herein, we will illustrate the most up to date and appealing examples.

Lu *et al.*[112] were the first to conduct *in vivo* O₂ delivery of Hb encapsulated within a polymer shell. They fabricated nanoparticles made of PLGA-PEG. PEG was chosen as a co-polymer due to its low fouling properties, which may create particles that evade the MPS system thus exhibiting longer circulation times, while PLGA was chosen due to its elastic modulus (which plays an important role in RBCs mimicking),[84] biodegradability, and biocompatibility.[113] The Hb-loaded PLGA-PEG nanoparticles were prepared by the double emulsion method. The authors conducted a thorough investigation of the composition of the organic solvent, the emulsifier and the ratio between co-polymer and Hb to optimize the system. The authors were then able to report Hb-loaded PLGA-PEG nanoparticles displaying sufficient and effective capacity for O₂ supply, negligible leakage, blood compatibility, and a viscosity comparable to that of whole blood. What is more, the Hb-loaded PLGA-PEG nanoparticles were also evaluated *in vivo* and, by means of a controlled hemorrhage mice model, the authors demonstrated their efficiency in *in vivo* oxygenation. These results highlight the potential of the Hb-loaded PLGA-PEG nanoparticles in the biomedical field.

Hb-loaded polymer nanoparticles have also been employed to explore their potential in the treatment of ischemic tissue reperfusion in hemorrhagic shock. Li *et al.*[114] fabricated nanoparticles consisting of a hetero triblock co-polymer containing a hydrophilic PEG chain and hydrophobic biodegradable PCL and PLA chains. The authors synthesized a series of PEG-PCL-PLA-PEG co-polymers by varying their molecular weights, followed by optimization of the polymer/Hb ratio. The as-prepared nanoparticles were ~800 nm in size and had an Hb content comprising above 60% of the total dehydrated nanoparticles weight. Although the Hb dry weight content in natural RBCs is substantially higher (~90%), the Hb-loaded nanoparticles demonstrated undamaged O₂-binding capacity and O₂ affinity (p50 = 21.6 mmHg). Furthermore,

the authors explored the potential of this system *in vivo* to evaluate its efficacy on ischemia reperfusion injury by injecting the Hb-loaded polymer nanoparticles into an acute anemia rat model. The results demonstrated that Hb-loaded polymer nanoparticles were efficient in mitigating the lethal shock symptom and were also able to reduce short-term mortality.

Recent studies involving Hb encapsulation within polymeric nanoparticles include the work by Li *et al.*[115] With the aim to correlate the polymer composition with important parameters of HBOCs such as morphology, encapsulation efficiency, non-specific protein adsorption, Hb leakage or hemocompatibility, the authors conducted a study where Hb was loaded within polymer shells of three different compositions. In particular, PCL, PEG-*block*-poly(allyl glycidyl ether) (functionalized with mercaptopropionic acid)-*block*-PCL (PEG-PAGE(MPA)-PCL) and the blend of the two polymers, was studied. The results demonstrated that the amphiphilic PEG-PAGE(MPA)-PCL co-polymers showed higher stability in blood and no interference with blood components as compared to the hydrophobic PCL. Both properties are a central prerequisite to avoid premature recognition by the MPS system. The authors also conducted a thorough study to identify the emulsification parameters that allow to maintain the bioactivity of Hb, since it is well known that Hb's functionality can be damaged by mechanical forces, or by the existence of an oil/water interface.[116] The authors demonstrated that both a reduced emulsification speed together with a suitable organic solvent such as ethyl acetate, could maintain the quaternary structure and subsequent functionality of the encapsulated Hb.

On a very creative work, Wang *et al.*[117] encapsulated Hb within a polydopamine (PDA) shell. DA polymerizes at basic conditions ($\text{pH} > 7.5$) and in the presence of O_2 to form PDA, which can deposit onto virtually any substrate independent of size and shape.[118] The structure of PDA consists of a mixture of DA and indole units at various oxidation states.[119] However,

the bulk of the PDA structure consist of indole units, since ring closure of the aminoethyl chain takes place upon oxidation. The DA and indole units polymerize at their benzo moiety into PDA oligomers, which are subsequently stacked using hydrogen bonds, π - π stacking and charge transfer.[119,120] Unlike the previously reported chemically-modified strategies, PDA has barely any chemical reaction with functional groups of Hb, thus preserving Hb's structure and activity.[117,118] PDA holds great promise in biomedical applications due to its excellent hemo- and biocompatibility since, PDA coatings, have demonstrated to preserve the viability or proliferation of many kinds of mammalian cells, and have also shown to not promote tissue damage, inflammation or fibrosis.[121–123] Furthermore, PDA also holds a redox character by means of its quinone groups due to the release of electrons during the spontaneous oxidation process of the different PDA constituting oligomers.[118] By coating Hb with a PDA shell, the authors aimed to endorse Hb with oxidative protection towards the conversion of Hb into non-functional metHb (Figure 5A). Using a Fe^{3+} reducing antioxidant power assay, it was shown that PDA-Hb has the ability to reduce Fe^{3+} into Fe^{2+} (absorbance at 598 nm), while Hb does not have this ability (Figure 5B). The authors also showed that PDA-Hb nanoparticles were efficient in scavenging 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS^+) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals, as shown by their respective radical-scavenging assays. The amount of ABTS^+ radicals (absorbance at 734 nm) decreased in presence of Hb-PDA (Figure 5C). Similarly, a decrease in absorbance at 520 nm, which is the characteristic wavelength of the DPPH radical, was shown in the presence of Hb-PDA (Figure 5D). No decrease in absorbance at 520 nm is observed for DPPH radical incubated with Hb only, thus demonstrating the capabilities of PDA to scavenge radicals. The authors also demonstrated exceptional ROS scavenging properties *in vitro* by Hb-PDA, a very important feature since, ROS such as H_2O_2 ,

would be generated by Hb during its autoxidation to metHb.[20] Cardiomyoblasts were prematurely damaged with H_2O_2 to generate intracellular ROS, after which the cells were incubated with PDA-Hb particles. The fluorescent probe dichlorofluorescein-diacetate, which is able to permeate through the cells and is subsequently converted into the fluorescent dichlorofluorescein product (DCF) by ROS-mediated oxidation, was monitored by fluorescence microscopy and flow cytometry (Figure 5E). Cells treated with PDA-Hb showed weaker fluorescent intensity signal, demonstrating their ability to suppress the intracellular H_2O_2 -induced ROS. In a follow-up study,[124] the authors employed the PDA coating to diminish the effect of H_2O_2 -induced apoptosis. By monitoring fluorescence intensity readings the authors demonstrated the suppressive effect of Hb-PDA on H_2O_2 -induced apoptosis, which had been previously induced through changes in the membrane potential of mitochondria. Furthermore, the authors were also able to show that the PDA layer thickness did not affect the O_2 -release capacity. Lastly, Wang *et al.* demonstrated that the PDA-Hb particles had a high O_2 affinity ($p50 = 13.86 \text{ mmHg}$), low interaction with blood components and low cytotoxicity, thus making them good candidates as HBOCs.

While in the previous examples the Hb-loaded polymer nanoparticles have been prepared by the self-assembly of amphiphilic co-polymers (except for the PDA-coated Hb), the Layer-by-Layer (LbL) assembly technique, which consists on the deposition of alternating interacting polymers onto a (sacrificial) template, allows for the fabrication of hollow capsules or core-shell particles with specific shapes, sizes, material compositions and functionality.[125–128] As such, this technique has also been employed to prepare Hb-based hollow capsules and particles. One of the most recent examples was reported by Chen *et al.*[129] who created Hb- and CAT-loaded nanotubes to combat oxidative stress. The authors claimed advantages of using nanotubes as

compared to nanospheres. For instance, by making use of nanotubes several functionalities can be introduced independently by functionalizing the inner and the outer surface,[130] while the open-end terminals allow for quick loading. Furthermore, nanotubes have a longer circulating time than spherical particles in the bloodstream.[81,131] The Hb-based nanotubes were prepared by the alternating deposition of Hb and dialdehyde heparin (DHP), which interact by covalent binding, onto a polycarbonate (PC) membrane which was removed at the end of the assembly to render hollow nanotubes. After removal of the PC membrane, the CAT enzyme was introduced by using DHP as the cross-linker to covalently attach the enzyme (Figure 6A).[129] CAT was attached to the inner part of the nanotubes in order to be protected from the external environment, and (Hb/DHP)₅-CAT nanotubes were obtained. Interestingly, Hb was not used to transport O₂, but to scavenge radicals instead. It has also been demonstrated that Hb overexpression suppresses oxidative stress in pathological cells due to the Hb's ability to attenuate H₂O₂-induced oxidative stress by acting as an antioxidative peroxidase.[132] Additionally, the (Hb/DHP)₅-CAT nanotubes displayed autofluorescence capacity after excitation at 405 and 488 nm, where the nanotubes showed fluorescence at 460-490 nm (Figure 6B), at 510-540 nm (Figure 6C), and at 570-600 nm (Figure 6D), thus allowing them to be detected and tracked without having to conjugate them to any external fluorophore. This autofluorescence was attributed to the n- π^* transition of C=N bonds between the amino groups of Hb and the aldehyde groups of DHP. The authors evaluated the ability of (Hb/DHP)₅-CAT nanotubes to remove H₂O₂ *in vitro* by assessing the cell viability under oxidative stress. Liver cells were cultured in the presence of different concentrations of H₂O₂, with or without (Hb/DHP)₅-CAT nanotubes, and the results demonstrated a higher cell viability for the cells cultured in the presence of the nanotubes (Figure 6E).

Lastly, efforts have also been made to encapsulate hHb within lipidic vesicles (HbV), commonly known as liposomes, which already started being investigated in the 1980s.[133–135] hHb-loaded liposomes have shown longer circulation times (~ 24 hours) than free Hb (> 6 hours).[136,137] This circulation time can be further dramatically increased by PEGylating their surface (up to 65.2 hours and up to 72 hours in rabbits and monkeys, respectively).[138–140] Surface functionalization with PEG further augments the liposomes hemodynamic properties as well as their biocompatibility.[139,141–143] Liposomes have shown to be able to encapsulate Hb with almost the same O_2 affinity as native RBCs ($p50 = 28$ mmHg), a fact that can be attributed to the liposomes biomimetic phospholipid bilayer membrane which resembles natural RBCs membranes.[144] Also, Hb entrapment within liposomes reduces the NO scavenging, thus diminishing the vasoconstriction effects and it also prevents glomerular clearance of Hb, subsequently reducing renal toxicity.[2,136] Additionally, liposomes can have shelf-storage lives of two to three years in liquid state (under N_2) and even longer when freeze-dried.[1,144] Since the early days in the 1980s, Hb-loaded liposomes have undergone considerable research in preclinical animal models for potential use as RBCs in several fields, including transfusion and resuscitative approaches in perioperative settings, hemorrhagic shock and hemodilution incidents, oxygenation of ischemic tissues and in transplantation of tissues and organs.[2,10,136,139,141,145] The current optimized HbV product, aside from having an O_2 affinity similar to that of Hb in RBCs, has an extremely high Hb concentration ($hHb = 10$ g dL $^{-1}$, lipids = 6 g dL $^{-1}$) which is similar to the Hb concentration in native RBCs (14 g dL $^{-1}$).[2,136] HbVs contain about 30 000 Hb molecules encapsulated within one PEGylated liposomal vesicle of ~ 250 nm in diameter (RBCs contain 250 million Hb molecules).[2,136] Research is currently being conducted towards improving Hb-loaded liposomes for potential clinical

translation.[2,146–148] Recent advances in the development of Hb-loaded liposomes include the usage of a non-phospholipidic anionic lipid to increase the Hb encapsulation efficiency and to reduce the host immune response to liposomes containing anionic phospholipids.[149] Utilizing the novel non-phospholipid amphiphilic hexadecylcarbamoyl-methylhexadecanoate-conjugated PEG of 2000 Da to further improve the liposomes tolerance against host immune response, has also been explored.[150–153] A very recent example of HbVs development was reported by Yadav *et al.*, who prepared HbVs constituted by dipalmitoylphosphatidylcholine (DPPC), anionic N_1 -(2-aminoethyl)- N_4 -hexadecyl-2-tetradecyl succinamide (HDAS), cholesterol, vitamin E, and HDAS-PEG (2000 Da).[152–154] The vesicles were ~215 nm in size (Figure 7A), contained 7.2 g dL⁻¹ of hHb and displayed a p50 of 31.9 mmHg. The authors induced a hemorrhagic shock into rats by removing 45% of their blood volume and measured different parameters before shock (baseline), immediately after inducing the hemorrhagic shock, after 6 hours of hemorrhagic shock (HS) and after resuscitation with saline or the as-prepared HbVs. While the heart rate, MAP, and corticosterone (a stress indicator) levels showed no significant difference between saline and HbVs resuscitation, a difference in tissue inflammation and toxicity could be observed for the different resuscitation fluids. Cardiac dysfunction was measured using cardiac troponin, a specific marker for cardiac injury; skeletal muscle metabolism was measured by an isoform of troponin, which is only generated by skeletal muscle injury; renal insufficiency was measured by creatinine, which is normally removed by glomerular filtration; and lastly, liver injury was investigated using the plasma levels of alanine transaminase (ALT), which is a marker for hepatic parenchyma injury, and the scavenger receptor CD163, indicating the activation of the Kupffer cells in the MPS. For the cardiac and muscle dysfunction, both saline and HbV resuscitation fluids could reduce the troponin

concentrations to similar concentrations as before inducing hemorrhagic shock (control) (Figure 7B-C). In contrast, for the renal insufficiency, HbV resuscitation could reduce the creatinine levels, while the saline solution still had a significant difference with the HS (Figure 7D). Additionally, a significant decrease in both ALT and CD163 was observed when administering HbVs as compared to the saline control (Figure 7E-F). Thus, these results demonstrate both lower renal insufficiency and liver injury when HbVs were employed as resuscitation fluids as compared to the saline control, demonstrating the potential of HbVs acting as an O₂ carrier able to prevent tissue inflammation and toxicity.

Although these studies have shown significant potential for HbVs working as RBCs, liposomes still present several shortcomings such as a broad size distribution, variations in Hb-encapsulation efficiencies, variable pharmacokinetics, and variable clearance profiles by the MPS.[1,2,147,148,155] Other important drawbacks of liposomes as RBCs include the fact that phospholipids are expensive and, furthermore, they may induce peroxidation in ischemia reperfusion injuries. Additionally, the lipid bilayer membrane is impermeable to some reducing agents present in the circulating blood that are crucial to avoid or minimize the conversion of Hb into methHb.[38,103,156]

3.2 Microarchitectures

The concept of encapsulating Hb within a microparticle was presented as early as the 1960s and 1970s by the Chang group.[157–159] The authors encapsulated not only Hb, but also relevant enzymes present in native RBCs such as CAT or CA, within a polymeric microparticle which was first made of collodion (cellulose nitrate) and later changed to PEG-PLA.[157] These Hb-loaded microcapsules showed a similar ODC as Hb enclosed within native RBCs and also retained the enzymatic activity of the entrapped enzymes.[157,158] However, one of the main

shortcomings of these microarchitectures was their rapid removal from blood circulation by the MPS.[8]

Recent examples to construct micron-sized architectures encapsulating Hb involve the fabrication of hollow microcapsules employing the LbL technique which include the alternate deposition of Hb and GA, which interact through covalent interactions, onto PLGA particles or polystyrene microparticles.[84,103] In another study, Hb-based microcapsules were prepared by the deposition onto sacrificial manganese carbonate templates of alternating layers of Hb and DHP which interact covalently.[160] In this work, DHP was used both as one of the wall components but also as a cross-linker to avoid the use of the toxic GA cross-linker. Similarly to the (Hb/DHP)₅-CAT nanotubes described in section 3.1, these Hb/DHP capsules were also fluorescent, a feature attributed to the $n-\pi^*$ transition of C=N bonds due to the Schiff base reaction between the Hb/DHP multilayers.[161] This property could be very favorable to track and monitor the Hb-capsules without the need to attach foreign fluorochromes.

So far, all the previously reported Hb encapsulating systems display a spherical morphology, however, it is noted that native RBCs possess a biconcave discoid morphology, with a diameter of $\sim 7\ \mu\text{m}$ and a thickness of $\sim 2\ \mu\text{m}$, and are also highly flexible (with a Young's modulus of $\sim 26\ \text{kPa}$).[162,163] These properties allow RBCs to change both their shape and morphology when passing through the microvascular circulation.[164,165] Oxy-Hb renders RBCs significantly more deformable than deoxy-Hb, a fact that enables RBCs to pass through the microvasculature transporting O₂. Also, the size, shape and deformability of RBCs are known to influence their hemodynamic distribution in the blood flow. While in mid to large vessels the RBCs flow in the center of the parabolic flow field, in small vessels and capillaries RBCs are distributed throughout the vessels for efficient O₂ exchange.[8,166,167] Thus, in recent years a substantial

amount of effort has been devoted in mimicking not only the functionality but also the structure of native RBCs, since both parameters are highly interconnected.

First attempts in mimicking the biconcave RBC-shaped structure of native RBCs were conducted by Doshi *et al.*, [84] who made use of templates with a biconcave RBC-shaped structure. In particular, the authors employed PLGA particles of $\sim 1\ \mu\text{m}$ diameter and 400 nm of shell thickness, which were subsequently collapsed into biconcave spheres with a diameter of $\sim 7\ \mu\text{m}$. The authors coated the PLGA templates by the alternating deposition of hHb and bovine serum albumin (BSA), which interact *via* electrostatics. After PLGA core removal, RBC-shaped, Hb-based capsules were obtained.

Alternatively, Yu *et al.* [168] prepared RBCs-shaped microparticles by co-precipitation of Dex together with Hb and calcium hydroxide ($\text{Ca}(\text{OH})_2$), followed by GA cross-linking (Figure 8A). The as-prepared microparticles exhibited a biconcave discoidal morphology and a size of $\sim 6.5\ \mu\text{m}$. SEM images show pure $\text{Ca}(\text{OH})_2$ particles are hexagonal prismatic crystals in shape (Figure 8B), while upon addition of Dex and Hb during the reaction process, particles with a biconcave discoidal morphology are obtained (Figure 8C). The Hb content in the microparticles corresponded to the 68% of the Hb content in native RBCs. The carrying–releasing O_2 properties of the microparticles were studied employing UV-vis absorption and electrochemical techniques. The UV-vis absorbance spectrum (Figure 8D) of the as-prepared $\text{Ca}(\text{OH})_2$ -Hb microparticles treated with O_2 displayed the three characteristic peaks of oxy-Hb, namely, at 406 nm (the Soret peak) and at 540 nm and 578 nm. After N_2 treatment, as expected, the Soret peak shifted to 429 nm and a single peak appeared at 558 nm, which corresponds to the characteristic deoxy-Hb peaks. The O_2 uptake and release by $\text{Ca}(\text{OH})_2$ -Hb was further investigated using differential pulse voltammetry (DPV) and cyclic voltammetry. The DPV results (Figure 8E) show

microparticles carrying and releasing O₂ for five consecutive cycles. The values were normalized (normalized ratio = DPV peak current of N₂ or O₂ treatment / DPV peak current of initial N₂ saturation treatment) and showed that the microparticles displayed a better O₂-binding reversibility as compared to free Hb which, the authors speculate, could be due to the high Hb encapsulation efficiency within the microparticles. Furthermore, even though a slight decrease in O₂ uptake was observed throughout the cycles, the Ca(OH)₂-Hb microparticles showed 7 times higher values of O₂ uptake as compared to free Hb after the five cycles. Additionally, the authors demonstrated that the O₂-binding and releasing properties were maintained at different temperatures, ranging from 5 °C to 50 °C.

Worth mentioning in this context is the work by Duan *et al.*[169] who fabricated Hb-based microparticles for a different purpose than the synthesis of RBCSs. Their study was aimed at removing uranyl ions (UO₂²⁺), an important radionuclide, from radiation-contaminated blood. The contamination of blood by UO₂²⁺ is attributed to the high binding coordination as well as to the electrostatic interactions of Hb with UO₂²⁺. With the aim to eliminate UO₂²⁺ from blood, the authors fabricated magnetic Hb microparticles employing iron oxide (Fe₃O₄)-loaded porous calcium carbonate (CaCO₃) particles. The particles were subsequently coated by the LbL technique with alternating layers of Hb and dialdehyde PEG which interact covalently to create CaCO₃-(Hb/PEG)₅ microparticles (Figure 9A).[169] The magnetic properties of Fe₃O₄ facilitated the trapping of the Hb-based microparticles from blood with the help of a magnet (Figure 9B). The Hb/PEG layers increased the capacity of UO₂²⁺ loading as compared to bare Fe₃O₄-loaded particles from 5% to up to 90% (Figure 9C), which showed that the capacity to adsorb UO₂²⁺ is mainly due to the Hb coating, demonstrating the high affinity of Hb towards UO₂²⁺.

Another recently adopted approach to prepare polymeric HBOCs is the Shirasu porous glass (SPG) membrane emulsification technique.[170,171] Lai *et al.*[172] were the first to employ the SPG technique to prepare emulsion droplets containing bovine Hb and BSA, which were cross-linked by GA. The authors employed the SPG technique since, by means of the narrow pore-size distribution of the SPG membrane, it enables the preparation of monodispersed and size-controlled emulsions that can be obtained in 1 μm increments.[170,173] The microparticles had a diameter of 4-5 μm and showed to be biocompatible, as demonstrated by a cell viability assay with a liver cancer cell line. Furthermore, the authors conducted an ODC of the Hb-based microparticles which was left shifted as compared to the ODC of native Hb, thus indicating an increase in O_2 affinity for the SPG microparticles.

Since one of the main challenges in tissue engineering is to achieve sufficient O_2 supply for regeneration of a compelling amount of tissue,[174] the authors also evaluated the potential of the as-prepared Hb-BSA microparticles for tissue engineering applications.[175] Employing HBOCs for tissue engineering purposes is of special relevance for liver cells, since they have a high demand for O_2 , [176–179] and it has been previously demonstrated that supplementing the circulating cell culture with HBOCs enhances the hepatocytes metabolism.[180] Thus, taking advantage of the precise control of the microparticles size enabled by the SPG technique, the optimum size of the Hb-BSA microparticles to perform as HBOCs in tissue engineering applications was evaluated. The study involved the preparation of Hb-BSA microparticles with varying diameters ranging from 1.2 to 18.3 μm , which were evaluated with macrophage, liver cancer, and endothelial cell lines to assess the optimum size of the O_2 depot. The authors demonstrated that cell uptake of the microparticles was only observed for macrophages exposed to Hb-BSA microparticles of diameters below 5 μm while uptake of the microparticles by liver

cancer and endothelial cells was not observed for any of the studied diameters. The authors put special emphasis in evaluating the biocompatibility of Hb-BSA microparticles of varying diameters since, good biocompatibility is a crucial factor for the applicability of any HBOCs. The results demonstrated that the biocompatibility of the Hb-BSA microparticles increased with decreasing microparticle diameter for the three studied cell lines.

4. CONCLUSIONS

In this review, we have featured the most recent advances in the design and assembly of HBOCs, which can be classified in two different groups. The first group involves chemically modified HBOCs, where Hb has been modified either by cross-linking its four sub-units, by polymerizing it with other Hb molecules, or by conjugating Hb to a different polymer. These systems comprise the first and second generation of HBOCs and, as such, they have been studied most extensively and have provided important lessons to implement for the upcoming generations of HBOCs. Furthermore, chemically modified HBOCs are mainly constituted by Hb, and possible copolymerized enzymes, thus rendering high Hb encapsulation per particle. However, covalent modification of Hb affecting its secondary structure, could also result in the loss of functionality. The second group comprises the so called third generation of HBOCs and entails the encapsulation of Hb within a protective shell that can be either of lipidic (i.e., liposomes encapsulating Hb) or polymeric nature. The latter mainly involving the encapsulation of Hb within carrier shells composed of amphiphilic polymers (i.e., polymersomes encapsulating Hb), or polymer shells assembled by the LbL technique, where Hb is either entrapped in the capsules core or is a component of the polymeric wall. The main advantage of using an encapsulating platform as opposed to chemically modifying Hb relies in the preservation of Hb's secondary structure and, in turn, its functionality. Such an approach, furthermore, offers a broad

variety of possible particle structures – ranging from spherical assemblies to nanotubes – each one with its own tailored properties. However, this custom-made encapsulating platforms entail that each system has to be optimized individually. Another drawback is that encapsulation platforms often afford low encapsulation efficiencies as compared to the chemically-modifying strategies. Furthermore, the encapsulating protective shell can hinder the O₂ diffusion from the Hb to the tissue. As such, prior to clinical trials, encapsulation strategies require a more exhaustive characterization as compared to the approaches involving Hb's chemical modification.

The academic developments highlighted throughout the review are highly successful and include examples where the HBOCs display O₂-binding and releasing properties very close to the ones of Hb encapsulated within natural RBCs, high Hb loading efficiency, incorporation of a reducing system that minimizes the conversion of Hb into metHb, minimization of NO-scavenging by Hb, thus diminishing the associated vascular and toxic side effects, or the extremely long storage shelf-lives achieved by some HBOCs products. However, although each of this single achievements is of utmost importance in its own rights, none of the HBOCs reported to date has been able to address all the aforementioned challenges. Additionally, despite the multiple and diverse strategies that have been developed, and the increasing efforts that have been undertaken in the field, which is shown by the increasing number of scientific publications in recent years, several challenges still remain to be solved towards a translation into the clinic.

While is noted that most of the reported HBOCs systems have been thoroughly characterized either in test tub or *in vitro* examples employing small animal models remain scarce. Thus, the first challenge relies in obtaining HBOCs exhibiting a precise and durable functionality *in vivo*, not only in the context of the specific functions of RBCs, but also regarding their potential side

effects and immune response. Thus, it is important to take into account that, when making use of foreign materials such as polymers, they will need to be removed from the body once they have conducted their required function for the desired time-period. The clinical usefulness of the HBOCs will be limited if their constituting materials remain in the body for a long time after administration, potentially causing long-term toxicity and immunogenicity, which may limit their potential clinical applications. It is also of utmost importance to evaluate the immunological response (e.g., MPS clearance) that HBOCs will encounter upon intravenous administration, which may involve a more advanced design of the HBOCs to achieve the desired physicochemical properties, especially since reasonably long circulation times are required. Recent studies have demonstrated the impact of physicochemical parameters such as shape, size, surface charge or mechanical modulus towards influencing the biodistribution and function of RBCs.[181,182] Only robust research of the HBOCs in standardized preclinical animal models, followed by a rigorous clinical evaluation of these systems, can lead to fully RBCs substitutes for blood transfusion applications in the future. The clinical translation of these HBOCs will only occur with the collaborative efforts of chemists, materials scientists, and blood biology experts together with trauma clinicians.

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Table 1. Several research approaches for the creation of chemically modified hemoglobin-based oxygen carriers.

	Formulation	Size	Hb content	p50 (mmHg)	Characterization and properties	Ref
	Hb in RBC	2 × 7 μm	92% ^a	24-30	• Stable for 42 days (4 °C)	[12,183]
	Free hHb	~65 kDa	100% ^a	15	• Rapid intravenous dissociation and clearance • Toxicity	[184]
Chemically modification of Hb - Nanoarchitectures						
Cross-linked Hb	Diaspirin cross-linked Hb	69 kDa	100% ^a	33	• Marketed products: HemAssist (Baxter Healthcare Corp.) • Circulation time: 12 hours • Increased mortality rates • Discontinued products	[10,31,184]
Polymerized Hb (polyHb)	GA polymerized Hb	100-500 kDa	100% ^a	38	• Marketed products: Hemopure (Biopure) • Shelf-life: > 3 years (room temperature) • Approved in South Africa • Approved for veterinary use (U.S.A. and Europe) • Circulation time: 19 hours	[32,185,186]
	polyHb-SOD-CAT-CA	> 450 kDa	-	19.92	• Shelf-life: 320 days (room temperature) • Preclinical studies (in rats) • Maintained MAP after reinfusion in hemorrhagic shock model • Reduction of pCO ₂ levels after reinfusion in hemorrhagic shock model	[67,187,188]
	polyHb-Rbr	≤ 600 kDa	-	8.9	• <i>In vitro</i> studies only • Better biocompatibility than polyHb	[71]
	polyHb-ATP-AD-GSH	< 500 kDa	-	20	• Product: Hemotech (TTUHSC) • Clinical proof-of-concept study (pediatric sickle cell anemia patients) • Preclinical studies (in multiple animal models) • Circulation half-life: 24 hours (in monkeys)	[73,189]

					<ul style="list-style-type: none"> • No nephron-, neuron-, and oxidative toxicity • Effective erythropoietic response in sickle cell anemia patients 	
Hb-polymer conjugates	Hb-PEG	~120 kDa	-	10-20	<ul style="list-style-type: none"> • Marketed products: Hemospan (Sangart), Pyroidoxalated Hb polyoxyethylene (Apex Bioscience), PEG-Hb (Enzon) • Circulation time: 43-66 hours (in humans) • Increased mortality rates • Discontinued products 	[36,184,190,191]
	hHb-Dex		-		<ul style="list-style-type: none"> • Preclinical studies (in dogs) • Circulation time: 1.9 days 	[192]
	Hb-SA-DA - Dex	320 nm ^d 260 nm ^e	25-55% ^b 7-33% ^c	14	<ul style="list-style-type: none"> • <i>In vitro</i> studies only • Biocompatibility (0.25 mg mL⁻¹) • Hemocompatibility 	[52]
	Hb-Dex-g-PNIPAAm	~50 nm ^d ~30 nm ^e	-	-	<ul style="list-style-type: none"> • Test tube characterization only • Thermoresponsive particle size • Decreased Hb autoxidation rate (at 37 °C) 	[57]
	PMAG- <i>b</i> -PMAA-Hb- <i>b</i> -PBMA	147 nm ^d 73 nm ^e	10.7% ^b	-	<ul style="list-style-type: none"> • <i>In vitro</i> studies only • Biocompatibility (0.25 mg mL⁻¹) • Selective uptake of doxorubicin-loaded PMAG-<i>b</i>-PMAA-Hb-<i>b</i>-PBMA by cancer cells 	[59]
	Hb-(HSA) ₃ clusters	265 kDa	25% ^a	9	<ul style="list-style-type: none"> • Preclinical studies (in rats) • Circulation half-life: 18.5 hours • Hemocompatibility • No side effects or toxicity in vital organs 	[74,76,77]
Chemical modification of Hb - Microarchitectures						

	Gelatin-encapsulated poly(Hb-PEG)	1.37 μm^d ~750 nm ^e	25.0% ^c	-	<ul style="list-style-type: none"> • Test tube characterization only • Irreversible O₂ reduction of polyHb 	[101,102]
	Gelatin-hHb microparticles	75-150 μm^e	3.9-4.5% ^a 60-70% ^c	-	<ul style="list-style-type: none"> • <i>In vitro</i> studies only • Microparticles as O₂-reservoirs in 3D cell cultures (tissue engineering applications) 	[91]

Hb: hemoglobin, hHb: human Hb, RBC: red blood cell, p50: partial pressure with 50% oxygenated Hb, GA: glutaraldehyde, SOD: superoxide anhydrase, CAT: catalase, CA: carbonic anhydrase, MAP: mean arterial blood pressure, pCO₂: partial pressure of carbon dioxide, Rbr: ruberitritin, ATP: adenosine-5'-triphosphate, AD: adenosine, GSH: reduced glutathione, TTUHSC: Texas Tech Uni. Health Sc. Center, PEG: poly(ethylene glycol), Dex: dextran, SA: succinic anhydride, DA: dopamine, PNIPAAm: poly(*N*-isopropylacrylamide), PMAG-*b*-PMAA-*b*-PBMA: poly[2-(methacrylamido) glucopyranose]-*block*-poly(methacrylicacid)-*block*-poly(butylmethacrylate), HSA: human serum albumin, O₂: oxygen. a) Weight content, b) Conjugation ratio: $C_{\text{Hb}} / (C_{\text{total}} - C_{\text{Hb}}) * 100\%$, c) Encapsulation efficiency: $(\text{Hb}_{\text{total}} - \text{Hb}_{\text{free}}) / \text{Hb}_{\text{total}} * 100\%$, d) Hydrodynamic size, and e) size by electron microscopy.

Table 2. Several encapsulation approaches for the creation of hemoglobin-based oxygen carriers.

[illegible]

Biconcave	(hHb/BSA) ₄ /hHb capsules		-	-	<ul style="list-style-type: none"> • Test tube characterization only • High deformability (elastic modulus = 92.8 ± 42 kPa) • O₂ carrying capacity similar to RBCs 	[84]
	Ca(OH) ₂ -Hb particles	6.5 μm^e	27% ^b 55.3% ^c	-	<ul style="list-style-type: none"> • Test tube characterization only • Improved O₂ binding and release properties 	[168]
Spherical	(Hb/DHP) ₅ capsules	3.4 μm^d	-	-	<ul style="list-style-type: none"> • <i>In vitro</i> studies only • Biocompatibility (0.11 mg mL⁻¹) • Low uptake by macrophages • Hemocompatibility 	[160]
	CaCO ₃ /(Hb/PEG) ₅ microparticles	> 5 μm^e	-	-	<ul style="list-style-type: none"> • Test tube characterization only • Potential of Hb-containing capsules for UO₂²⁺ removal in radiation-contaminated blood 	[169]
	Hb-albumin microparticles	4-5 μm^e	30-70% ^b	5-12	<ul style="list-style-type: none"> • <i>In vitro</i> studies only • Cytotoxic oxy-Hb containing microparticles (20 mg mL⁻¹) • Increased size decreases uptake by macrophage 	[172,175]

Hb: hemoglobin, hHb, human Hb, p50: partial pressure with 50% oxygenated Hb, PLA: poly(lactic acid), PEG: poly(ethylene glycol), PLGA: poly(lactide-co-glycolide), PAGE(MPA): poly(allyl glycidyl ether) (functionalized with mercaptopropionic acid), MAP: mean arterial blood pressure, PCL: poly(ϵ -caprolactone), DPPC: dipalmitoylphosphatidylcholine, Chol: cholesterol, DHSG: 1,5-O-dihexadecyl-N-succinyl-L-glutamate, DSPE: 1, 2-distearoyl-sn-glycero-3-phosphatidyl-ethanolamine HDAS: hexadecylcarbamoylmethylhexadecanoate, VitE: vitamin E, BSA: bovine serum albumin, O₂: oxygen, Ca(OH)₂: calcium hydroxide, DHP: dialdehyde heparin, CaCO₃: calcium carbonate. a) Weight content, b) Conjugation ratio determined by means of Hb/ (total weight) *100 and c) Encapsulation efficiency: (Hbtotal - Hbfree) / Hbtotal *100%, d) Hydrodynamic size, and e) size by electron microscopy.

Scheme 1. Schematic representation of the red blood cells (RBCs) structure, oxygen (O₂)-binding capacity and intravascular hemoglobin (Hb). A) RBCs intravenous O₂ uptake and release (i), structure of the Hb tetramer (ii) and oxy-Hb or R-state (left) and deoxy-Hb or T-state (right) of the heme group (iii). B) The O₂ dissociation curve of Hb with the p50 value (dotted line) at 26.5 mmHg O₂ partial pressure (pO₂). C) Intravascular dissociation of Hb. 1) Oxy-Hb is dissociated into its dimers and monomers which bind to haptoglobin, followed by endocytosis via the CD163 receptor and degradation in liver and spleen. 2) Oxy-Hb dimers and monomers can also auto-oxidize into metHb products and the complementary reactive oxygen species (ROS) which results in nitric oxide (NO) depletion by converting it into nitrate (NO₃⁻) (3). Next, the oxy-Hb oxidation products (i.e., metHb and ferryl Hb) promote the unfolding of the globin

chains releasing the heme groups (4). The free heme groups bind to hemopexin to be released again and degraded by the macrophages while the hemopexin is recycled back (5). 6) When the plasma protein mechanisms are saturated, oxy-Hb will also scavenge NO resulting in metHb, Hb(Fe⁴⁺) and NO₃⁻ production. Unbound metHb products can penetrate through the endothelial cells membrane causing an inflammatory response (7). Additional intravenous oxy-Hb dimers and monomers are removed by kidney filtration (5). 9) Finally, oxy-Hb dimers and monomers can also extravasate in between the endothelial cells lining the blood vessels to scavenge the NO of the underlying smooth muscle cells which results in inflammation, smooth muscle impairment, and platelet activation.

Scheme 2. Schematic representation of different hemoglobin (Hb)-based oxygen carriers (HBOCs) discussed in the review. HBOCs are classified depending if Hb has been chemically modified to be attached to a carrier (top) or if it has been encapsulated (bottom). RBC: red blood cell, SOD: superoxide dismutase, CAT: catalase, CA: carbonic anhydrase, PEG: polyethylene glycol, PDA: polydopamine.

Figure 1. Hemoglobin-based nanogels (HbNGs) synthesis and characterization. A) The HbNGs assembly starts with the synthesis of a co-polymer consisting of dextran (Dex), succinic anhydride (SA) and dopamine (DA). The Dex-SA-DA co-polymer self-assembles into NGs at acidic pH (1). Next, the NGs are cross-linked by oxidizing the DA moieties with sodium periodate (NaIO₄) and functionalizing the Dex component with aldehyde groups (2). Hb is then incorporated through covalent bonding between the amino groups of Hb and the Dex's aldehyde groups (3). B) Transmission and scanning (C) electron microscopy images of the HbNGs. Scale bars of 500 nm. D) UV-vis spectra of HbNGs exposed to carbon monoxide (CO-HbNGs), nitrogen (Deoxy-HbNGs) and oxygen (Oxy-HbNGs). E) Stability of Hb and HbNGs at 37 °C with the corresponding half-lives (dotted lines). Adapted from [52], with permission from Elsevier.

Figure 2. Hemorrhagic shock model for cross-linked hemoglobin (Hb) or stroma-free hemolysate (SFHb) with superoxide dismutase (SOD), catalase (CAT), and carbonic anhydrase (CA). A) Schematic representation of the polyHb-SOD-CAT-CA. B) The mean arterial blood pressure (MAP) and tissue CO₂ partial pressure (pCO₂) (C) were measured after reinfusion of different fluids (ii-vii) injected 90 min after inducing a hemorrhagic shock that had generated a MAP of 30 mmHg. i) Sham control without hemorrhagic shock. Adapted from [67], with permission from Informa Healthcare USA, Inc.

Figure 3. Hemoglobin (Hb)-albumin clusters assembly and metHb reduction. A) Hb was modified with the *N*-succinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate heterobifunctional cross-linker (1) after which human serum albumin (HSA) was attached to obtain Hb-(HSA)₃ nanoclusters (2). Flavin derivatives could be bound to a subdomain of the HSA (i), or a platinum nanoparticle (PtNP) could be bound within the cleft of one HSA unit (ii). B) Diagram of metHb reduction by nicotinamide adenine dinucleotide (NADH) using flavin derivatives. C) Dependence of initial rate (v_0) of metHb reduction on NADH concentration using the flavin derivatives lumiflavin (LF) or flavin mononucleotide (FMN) bound to the Hb-(HSA)₃ clusters. Reprinted with permission from [74]. Copyright 2013 ACS Publication. D) MetHb

levels of Hb-(HSA)₃, Hb-(HSA)₃(PtNP) clusters, and a mixture of the different components over time in a 20 μ M hydrogen peroxide solution. Hb sham without hydrogen peroxide. Adapted with permission from [76]. PLoS ONE open access. D) Difference in mean arterial pressure (Δ MAP) after intravenous injection of HSA, Hb-(HSA)₃, and cross-linked Hb (XLHb) into rats. ** $p < 0.01$ vs. XLHb, stopped after 10 minutes due to urinary excretion. E) Relative blood plasma concentration of iodine-125 (I^{125})-labeled HSA, Hb-(HSA)₃, and XLHb. ** $p < 0.01$ vs. I^{125} -HSA. Reprinted with permission from [77]. Nature Scientific reports open access.

Figure 4. Synthesis and characterization of hemoglobin (Hb) coated gelatin microparticles (GM), GM-Hb. A) Reaction scheme of GM where (1) the surface was activated with *N,N'*-disuccinimidyl carbonate (i) followed by Hb coupling (ii) using polyurethane linkage (GM-Hb-1), and (2) where 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide was employed to couple Hb to the GM using amide bond formation (GM-Hb-2). B) Oxygen uptake and release (C) over time by measuring the partial oxygen pressure (pO_2) of cell medium without GM (control) with bare

GM, GM-Hb-1 or GM-Hb-2. D) Fluorescence microscopy image of a 3D cell culture after 48 h of incubating cells only (top) and cells with embedded GM-Hb-1 (bottom). The green and red spots depict living and dead cells, respectively. GM-Hb-1 appear as big green circles. Scale bars of 100 μm . E) Cell viability fraction in the 3D cell culture after 48 h of incubating cells only, cells with bare GMs, and cells with GM-Hb-1. * $p < 0.01$. Adapted with permission from [91].

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Figure 5. Oxidative protection of hemoglobin (Hb) and reactive oxygen species (ROS) scavenging by a polydopamine (PDA) coating. A) Schematic illustration of Hb coated with PDA (1) which, upon being in contact with free radicals and ROS, (2) get reduced by the PDA coating thus preserving Hb's oxygen-carrying capacity (3). B) UV-vis absorption spectra of 2,4,6-tripyridyl-s-triazine (TPTZ) incubated with Hb-PDA and Hb only. The peak at 598 nm corresponds to ferrous-TPTZ. C) A 2,2-azido-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS⁺) solution, with a characteristic peak at 734 nm, incubated with Hb-PDA and Hb only. D) A 2,2-

diphenyl-1-picrylhydrazyl (DPPH) solution, with a characteristic peak at 520 nm, incubated with Hb-PDA and with Hb only. E) Intracellular ROS scavenging activity of Hb-PDA in H₂O₂-induced damaged cells employing dichlorofluorescein-diacetate (DCF) as fluorescent probe for ROS activity. Overlay of bright field and fluorescence microscopy images (right panel) and the corresponding flow cytometry histograms (left panel) of cells only (control, top image), cells incubated with H₂O₂ (middle image) and cells incubated with both H₂O₂ and Hb-PDA (bottom image). Adapted with permission from [117]. Copyright 2017 American Chemical Society.

Figure 6. Hemoglobin (Hb)-based nanotubes for oxidative stress treatment. A) The nanotube fabrication starts with a sacrificial template containing 400 nm-sized pores through which Hb and dialdehyde heparin (DHP) are deposited in alternating layers (1). The assembly is then followed by plasma cleaning and dissolution of the template (2) to obtain nanotubes which are then coated with catalase (CAT) on the inner wall (3). Confocal laser scanning fluorescence images of the as-prepared Hb-based nanotubes excited at 405 and 488 nm and read at 460-490 nm (B), 510-540 nm (C) and 570-600 nm (D). Scale bars of 10 μ m. E) Cell viability readings of HepG-2 cells incubated with decreasing amounts of oxygen peroxide (H₂O₂) in the presence or

absence of the CAT-loaded Hb-based nanotubes. Adapted with permission from [129].

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Figure 7. Hemoglobin (Hb)-loaded vesicles (HbVs) in a hemorrhagic shock model. A) Electron microscopy image of HbVs. Scale bar of 500 nm. The effect of HbVs resuscitation on tissue toxicity measured by plasma levels of cardiac troponin (B), skeletal muscle troponin (C), creatinine (D), alanine transaminase (ALT) (E), and CD163 receptor (F). Plasma levels were recorded at baseline (control), after 6 hours of induction of the hemorrhagic shock (HS) and after saline or HbVs resuscitation. $p < 0.05$ vs. control*, HS and saline. Adapted with permission from [153]. Copyright 2016 Elsevier B.V.

Figure 8. Biconcave hemoglobin (Hb)-based microparticles. A) Schematic illustration showing Hb co-precipitation with dextran and calcium chloride (1) to form calcium hydroxide (Ca(OH)_2)-Hb microparticles, which are then cross-linked with glutaraldehyde (2). Scanning electron microscopy images of pure Ca(OH)_2 (B) and Ca(OH)_2 -Hb microparticles (C). Scale bars of 5 μm . D) UV-vis absorbance spectra of oxygenated, deoxygenated, and reoxygenated Ca(OH)_2 -Hb microparticles. E) Normalized ratio of differential pulse voltammetry peak currents of Ca(OH)_2 -Hb microparticles and free Hb to several cycles of oxygen and nitrogen treatment. Adapted with permission from [168]. Copyright 2017 Elsevier B.V.

Figure 9. Magnetic hemoglobin (Hb)-based microparticles for the removal of radioactive uranyl ion (UO_2^{2+}). A) Schematic illustration of the microparticles assembly. The Hb-based microparticles are fabricated by encapsulating iron oxide (Fe_3O_4) nanoparticles into porous calcium carbonate particles (1) followed by the alternating deposition of Hb (2) and dialdehyde poly(ethylene glycol) (DA-PEG) layers (3). Upon addition of the desired number of bilayers (4) the microparticles are used to deplete UO_2^{2+} (5). B) Images showing the Fe_3O_4 -loaded (Hb/PEG)₅ microparticles in UO_2^{2+} -contaminated blood (left), and the magnetically assisted separation process (right). C) Percentage of UO_2^{2+} absorption by the Fe_3O_4 -loaded (Hb/PEG) microparticles depending on the number of deposited Hb/PEG bilayers. Reprinted with permission from [169]. Copyright 2014 John Wiley and Sons.

Highlights

- Overview of the recent achievements for the creation of artificial red blood cells
- Artificial red blood cells are crucial due to the short lifetime of donor blood
- Artificial red blood cells assembled by encapsulating or conjugating hemoglobin