



UNIVERSITÀ DI PARMA

ARCHIVIO DELLA RICERCA

University of Parma Research Repository

From hemoglobin allostery to hemoglobin-based oxygen carriers

This is the peer reviewed version of the following article:

Original

From hemoglobin allostery to hemoglobin-based oxygen carriers / Faggiano, S.; Ronda, L.; Bruno, S.; Abbruzzetti, S.; Viappiani, C.; Bettati, S.; Mozzarelli, A.. - In: MOLECULAR ASPECTS OF MEDICINE. - ISSN 0098-2997. - 84:(2022), p. 101050.101050. [10.1016/j.mam.2021.101050]

Availability:

This version is available at: 11381/2906360 since: 2024-11-25T12:48:42Z

Publisher:

Elsevier Ltd

Published

DOI:10.1016/j.mam.2021.101050

Terms of use:

Anyone can freely access the full text of works made available as "Open Access". Works made available

Publisher copyright

note finali coverpage

(Article begins on next page)

31 May 2025

Molecular Aspects of Medicine

From Hemoglobin Allostery to Hemoglobin-Based Oxygen Carriers

--Manuscript Draft--

Manuscript Number:	MAM-D-21-00074R1
Article Type:	SI: Haemoglobin and Myoglobin
Keywords:	hemoglobin; hemoglobin-based oxygen carriers; blood substitutes; allostery; Cooperativity; Oxygen transport
Corresponding Author:	Andrea Mozzarelli University of Parma Parma, PARMA ITALY
First Author:	Serena Faggiano
Order of Authors:	Serena Faggiano Luca Ronda Stefano Bruno Stefania Abbruzzetti Cristiano Viappiani Stefano Bettati Andrea Mozzarelli
Manuscript Region of Origin:	Europe
Abstract:	<p>Hemoglobin (Hb) plays its vital role through structural and functional properties evolutionarily optimized to work within red blood cells. i.e., the tetrameric assembly, well-defined oxygen affinity, positive cooperativity, and heterotropic allosteric regulation by protons, chloride and 2,3-diphosphoglycerate. Outside red blood cells, the Hb tetramer dissociates into dimers, which exhibit high oxygen affinity and neither cooperativity nor allosteric regulation. They are prone to extravasate, thus scavenging endothelial NO and causing hypertension, and cause nephrotoxicity. In addition, they are more prone to autoxidation, generating radicals. The need to overcome the adverse effects associated with cell-free Hb has always been a major hurdle in the development of substitutes of allogeneic blood transfusions for all clinical situations where blood is unavailable or cannot be used due to, for example, religious objections. This class of therapeutics, indicated as hemoglobin-based oxygen carriers (HBOCs), is formed by genetically and/or chemically modified Hbs. Many efforts were devoted to the exploitation of the wealth of biochemical and biophysical information available on Hb structure, function, and dynamics to design safe HBOCs, overcoming the negative effects of free plasma Hb. Unfortunately, so far, no HBOC has been approved by FDA and EMA, except for compassionate use. However, the unmet clinical needs that triggered intensive investigations more than fifty years ago are still awaiting an answer. Recently, HBOCs "repositioning" has led to their successful application in organ perfusion fluids.</p>
Suggested Reviewers:	<p>William A Eaton eaton@niddk.nih.gov World leader in Hb allostery</p> <p>Maurizio Brunori maurizio.brunori@uniroma1.it World leader in hemoglobin</p> <p>Paul Buehler PBuehler@som.umaryland.edu World expert in blood substitutes</p> <p>Christopher Cooper ccooper@essex.ac.uk World expert in hemoglobin and hemoglobin modifications for blood substitutes</p>

	Abdu Alayash alayash@cber.fda.gov Leading expert in hemoglobin toxicity and blood substitutes
Opposed Reviewers:	
Response to Reviewers:	

Dear Editor

On behalf of my colleagues, I am submitting the revised manuscript “From Hemoglobin Allostery to Hemoglobin-Based Oxygen Carriers” for the special issue of the Antonini and Brunori book anniversary. We have extensively revised the manuscript following the pertinent and valuable comments of reviewers. We really thank the reviewers for their comments. A point-by-point reply to reviewers’ comments is reported in the “reply to reviewer” file.

I hope that the revised version of our manuscript is suitable for publication in Molecular Aspects of Medicine.

With best regards

Andrea Mozzarelli

From Hemoglobin Allostery to Hemoglobin-Based Oxygen Carriers

Serena Faggiano^{1,2}, Luca Ronda^{2,3}, Stefano Bruno¹, Stefania Abbruzzetti⁴,
Cristiano Viappiani⁴, Stefano Bettati^{2,3,5} and Andrea Mozzarelli^{1, 2*}

¹Department of Food and Drug, University of Parma, Parma, Italy

²Institute of Biophysics, National Research Council, Pisa, Italy

³Department of Medicine and Surgery, University of Parma, Parma, Italy

⁴Department of Mathematical, Physical and Computer Sciences, University of Parma, Parma, Italy

⁵National Institute of Biostructures and Biosystems, Rome, Italy

*Corresponding author

E-mail: andrea.mozzarelli@unipr.it

Full address: Institute of Biophysics, National Research Council, Via Moruzzi 1, 56124 Pisa, Italy

Keywords: hemoglobin, hemoglobin-based oxygen carriers, blood substitutes, allostery, cooperativity, oxygen transport

Abstract. Hemoglobin (Hb) plays its vital role through structural and functional properties evolutionarily optimized to work within red blood cells. i.e., the tetrameric assembly, well-defined oxygen affinity, positive cooperativity, and heterotropic allosteric regulation by protons, chloride and 2,3-diphosphoglycerate. Outside red blood cells, the Hb tetramer dissociates into dimers, which exhibit high oxygen affinity and neither cooperativity nor allosteric regulation. They are prone to extravasate, thus scavenging endothelial NO and causing hypertension, and cause nephrotoxicity. In addition, they are more prone to autoxidation, generating radicals. The need to overcome the adverse effects associated with cell-free Hb has always been a major hurdle in the development of substitutes of allogeneic blood transfusions for all clinical situations where blood is unavailable or cannot be used due to, for example, religious objections. This class of therapeutics, indicated as hemoglobin-based oxygen carriers (HBOCs), is formed by genetically and/or chemically modified Hbs. Many efforts were devoted to the exploitation of the wealth of biochemical and biophysical information available on Hb structure, function, and dynamics to design safe HBOCs, overcoming the negative effects of free plasma Hb. Unfortunately, so far, no HBOC has been approved by FDA and EMA, **except for compassionate use**. However, the unmet clinical needs that triggered intensive investigations more than fifty years ago are still awaiting an answer. Recently, HBOCs “repositioning” has led to their successful application in organ perfusion fluids.

1. Introduction. The structure, dynamics, function, and regulation of proteins are evolutionarily optimized to work in specific cell compartments, and their improper localization can lead to toxicity. In this respect, hemoglobin (Hb) is paradigmatic, as oxygen affinity, tetramer stability, oxidation state and cooperativity are perfectly tailored to work as an oxygen carrier within red blood cells (RBCs), but wholly unsuitable for this function when free in the plasma, where these properties are profoundly altered. In fact, cell-free Hb undergoes dissociation to dimers that show increased propensity to autoxidation, absence of cooperativity and high oxygen affinity. In addition, both free Hb tetramers and dimers scavenge nitric oxide (NO) generated by endothelial cells, causing hypertension. *In vivo*, Hb dimers released from aged RBCs are trapped by haptoglobin and directed to degradation by the reticuloendothelial system (di Masi et al., 2020).

The compartment-specific activity of Hb has been investigated in the attempt to design Hb-based oxygen carriers (HBOCs), protein therapeutics intended to deliver oxygen to tissues as an alternative to RBC transfusions for “oxygen therapeutics”. To efficiently work in the plasma and to avoid adverse effects, HBOCs were developed using either chemically and/or genetically modified HbA to alter the functional and structural properties of unmodified Hb. The fine-tuning of these properties has relied on the enormous amount of information arisen by the decades-long effort to dissect the complex reactivity and conformational flexibility of Hb. Indeed, Hb and its monomeric homolog myoglobin (Mb) were investigated in detail in the 20th century, triggering the development of several biophysical and biochemical methods that later became generally available to understand protein structure, dynamics, and function. These include i) x-ray protein crystallography, pursued by Max Perutz and John Kendrew (Kendrew et al., 1960), ii) stopped-flow techniques, pursued by Quentin Gibson (Gibson and Roughton, 1955), laser flash photolysis, exploited by William Eaton and coworkers (Henry et al., 1997; Hofrichter et al., 1983) and Mossbauer spectroscopy, pursued by Fritz Parak (Parak, 1988). This vast body of experimental work was cleverly and clearly reviewed and presented in the Antonini and Brunori book in 1971 (Antonini and Brunori, 1971). For Hb and Mb scientists, this book has been the “Bible” for the last 50 years. In our laboratory, we have a single

copy, acquired by Prof. Gian Luigi Rossi when he carried out kinetic studies on HbA reactivity in collaboration with Bob Noble (Noble et al., 1972). The book was a unique source of information and data comparison for many of us. More recent valuable sources on Hb are Imai's book on "Allosteric Effects in Hemoglobin", published in 1982 (Imai, 1982), and the Bunn and Forget book on "Hemoglobin: Molecular, Genetic and Clinical Aspects", published in 1986 (Bunn and Forget, 1986). In addition, Hb allostery has been discussed in several reviews appeared over the years (Bellelli and Brunori, 2011; Eaton et al., 2007; Eaton et al., 1999; Miele et al., 2013). Since the inspiring Antonini and Brunori book was published, Hb has been further investigated with a kaleidoscopic pattern of different biochemical and biophysical techniques, leading to a structural and functional characterization unparalleled to that reached for any other protein. **In addition, both Hb and Mb have been genetically engineered for the understanding of the role of almost each amino acid in dictating their role in structural and functional properties and for tailoring reactivity towards oxygen, NO, oxidative agents in the development of HBOCs (Dou et al., 2002; Olson et al., 1997).**

In this review, we will describe the structural and functional properties of Hb as an oxygen carrier, with the aim to underline the molecular features that need to be modified for the design of HBOCs. We will show that protein engineering and chemical modifications have allowed to develop HBOCs with oxygen-carrying properties suitable for working outside RBCs. Depending on the fine-tuning of their properties, these products have been proposed for different applications, with recent examples of "repositioning" as solutions for organ preservation and plasma expander CO carriers. Therefore, Hb represents a clear example of how the detailed structural and functional characterization of a protein, apparently well beyond the needs of biotechnological applications, turned out to be an invaluable starting point to design life-saving therapeutics.

1.1. Hemoglobin structure, a brief outlook. Hb is a tetramer formed by two homologous subunits, referred to as α and β chains (Figure 1). The β chains contain eight helices (numbered A to H), while

the α chains have only seven. Each monomer contains a heme moiety. An Fe^{2+} ion at the center of the tetrapyrrole ring is coordinated to a His residue, called proximal His, and reversibly binds oxygen. This event triggers a series of tertiary conformational changes that destabilize the deoxyHb structure. The HbA tetramer exhibits two distinct quaternary states, called T, for "tense", and R, for "relaxed", with the former stabilized by salt bridges and thermodynamically favored in the absence of oxygen, and the latter thermodynamically favored when oxygen is bound (Figure 1). Functional properties of Hb depend on the relative population of quaternary states as well as on the relative populations of tertiary states endowed with different reactivity, t and r (see chapter 1.2 below) (Henry et al., 2002).

Studies by Eaton (Eaton, 1980) and Thornton and colleagues (Pillai et al., 2020) have dissected the Hb structure and have assigned specific functional roles to each globin portion, and, in

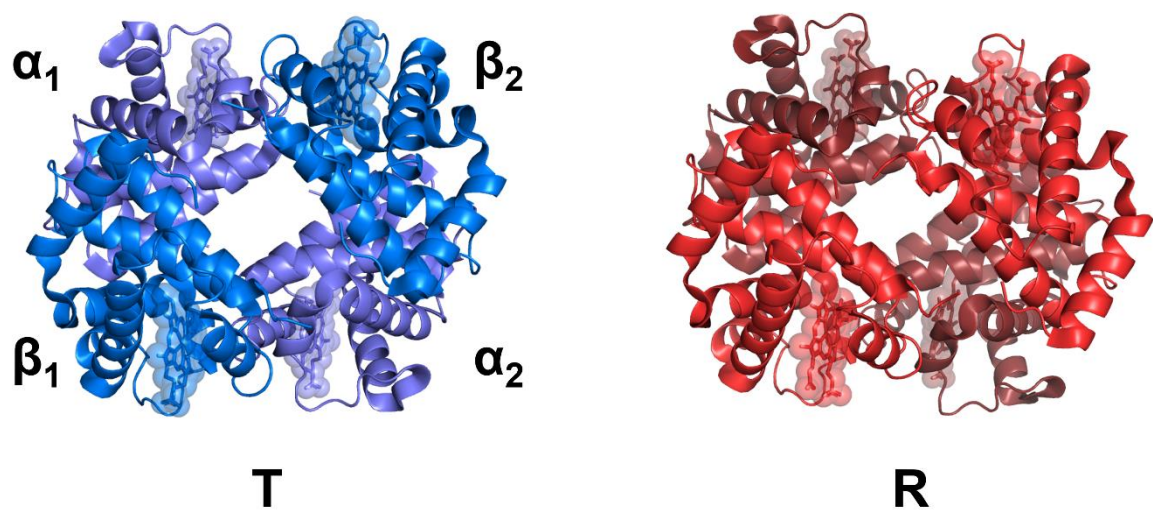


Figure 1. Structure of tetrameric deoxy T-state (left) and oxy R-state HbA (right). Subunits are shown with different colors.

parallel, to the three exons contained in the globin genes. Specifically, the amino acids associated with the three coding sequences are distributed to each of Hb functions: i) heme contacts essential for oxygenation, ii) α_1 - β_2 contacts essential for cooperative dimer, iii) α_1 - β_1 contacts essential for cooperative tetramer, iv) Bohr effect, essential for the modulation of oxygen loading and unloading from lung to tissues, and v) 2,3-diphosphoglycerate (DPG) binding, essential for oxygen affinity regulation (Eaton, 1980). Furthermore, it was shown that tetrameric Hb evolved from an ancient

monomer and a more recent noncooperative homodimer with high oxygen affinity. Two surface substitutions are sufficient to markedly reduce oxygen affinity and to confer cooperativity, suggesting a linkage between the oxygen binding site and the oligomerization interface (Pillai et al., 2020).

1.2. Hemoglobin allostery: state of the art. Hb function is under two allosteric regulations: a homotropic effect associated with the cooperative oxygen binding, and heterotropic effect, mediated by binding of effectors (e.g.: protons, CO₂, chloride ions and organic phosphates) at sites other than the heme pocket. The latter accounts for different physiological roles of Hb, beyond oxygen transport from the lungs to peripheral tissues, contributing to control pH and scavenging of CO₂. A so complex behavior intrigued scientists, fascinated by the idea of building a comprehensive model able to describe and predict how homotropic and heterotropic effectors modulate Hb ligand binding properties. Both mechanisms of regulation impact on Hb function *in vivo* and their understanding is key for the design and optimization of HBOCs with tailored properties.

Historically, homotropic effects have attracted earlier attention, starting from the sequential model originally proposed by Linus Pauling in the 1930s (Pauling, 1935) and later developed by Koshland, Nemethy and Filmer (Koshland et al., 1966). Sequential models predict continuous affinity changes upon binding of subsequent oxygen molecules to the same tetramer, implying cooperativity within the same quaternary state. On the contrary, concerted models assume a saturation-dependent equilibrium between pre-existing high and low-affinity states with perfect functional symmetry among the four subunits within a tetramer, which would exclude cooperativity in the absence of quaternary relaxations. The most famous concerted model is that proposed by Monod, Wyman, and Changeux (MWC) (Monod et al., 1965), largely motivated by the pioneer structural studies of Perutz and coworkers showing a different quaternary arrangement of fully oxygenated and deoxygenated subunits (Muirhead and Perutz, 1963; Perutz et al., 1964). Other models were proposed over the years, including the Ackers "Symmetry Code" implying cooperativity within the T state (Ackers et al.,

1992). Since all these models well describe the observed oxygen binding curves, the characterization of single Hb conformations by blocking or slowing down conformational transitions resulted a valuable approach to validate allosteric models. Controversies among models were settled in favor of MWC mainly thanks to oxygen binding studies in T-state Hb crystals carried out by Eaton, Mozzarelli and coworkers, showing lack of cooperativity in the absence of quaternary relaxations (Mozzarelli et al., 1997; Mozzarelli et al., 1991; Rivetti et al., 1993). Starting from the mid '90s, even more convincing evidence in favor of the concerted model arose from functional studies carried out by several groups on Hb encapsulated in wet, nanoporous silica gels (Bettati and Mozzarelli, 1997; Bruno et al., 2001; Jones et al., 2012; Jones et al., 2014; Juszczak and Friedman, 1999; Khan et al., 2000; Samuni et al., 2004; Samuni et al., 2006; Schiro and Cupane, 2007; Shibayama and Saigo, 1995). Encapsulation in silica gel offers two major advantages over crystallization. First of all, conformational changes are not hampered, but slowed down by orders of magnitude, with uncoupling of tertiary and quaternary relaxations that allow to interrogate the spectroscopic properties of intermediate conformations by UV-vis absorbance, Raman, near-infrared and circular dichroism spectroscopy (Das et al., 1999; Khan et al., 2000; Ronda et al., 2006; Schiro and Cupane, 2007; Viappiani et al., 2004). Moreover, in the absence of crystal lattice constraints, tertiary effects induced by heterotropic regulators are maintained (Bettati and Mozzarelli, 1997; Bruno et al., 2001; Viappiani et al., 2004). Overall, no cooperativity in oxygen binding was observed within the T and R quaternary state for Hb gels. However, the observation of functional heterogeneity within the T state, extensively investigated by Yonetani, Poyart and others (Ackers et al., 1992; Kister et al., 1987; Lalezari et al., 1990; Marden et al., 1990; Tsuneshige et al., 2002; Yonetani et al., 2002; Yonetani and Tsuneshige, 2003), raises the issue of a long-known deficiency of the MWC model, which postulates symmetry of the four subunits. This would allow heterotropic effectors to act on the equilibrium between T and R quaternary states, but with no effect on their respective equilibrium constant for oxygen binding, K_T and K_R . It should be noted that by the mid '60s, when Monod and coworkers formulated their model, heterotropic effects on Hb had been only partially investigated, and that the authors were

perfectly aware of the over-simplification of their model, which was just intended to explain in the easiest, more elegant and general way the cooperative behavior of multi-subunit proteins.

Over the last decades, the wide functional range of T and R conformations has been described in terms of continuous or discrete distributions of tertiary species accommodated within the two individual quaternary states (Bettati et al., 1998; Friedman, 1985; Lukin et al., 2003; Peterson et al., 2004; Rivetti et al., 1993; Yonetani et al., 2002; Yonetani and Tsuneshige, 2003). These findings motivated Eaton and coworkers to propose an extension of the MWC model, the Tertiary-Two State (TTS) model (Henry et al., 2002). According to the TTS, oxygen and heterotropic effectors can modulate the equilibrium between pre-existing, low- and high-reactivity tertiary conformations (called *t* and *r*, respectively), that populate both quaternary states. The tertiary conformation is the only determinant of oxygen affinity of individual subunits. Ligation and the R structure favor the highly reactive *r* state (significantly populated in liganded R, but also liganded T), while T state and negative heterotropic effectors favor *t* (significantly populated in deoxygenated T, but also deoxygenated R). Cooperativity still arises from the quaternary equilibrium, thus preserving the fundamental postulate of MWC (no cooperative effects are present within T and R conformations), but oxygen affinity can span the same range in T and R. The TTS model received experimental support by CO rebinding kinetic experiments on Hb gels (Viappiani et al., 2004; Viappiani et al., 2014; Henry et al., 2015). It was shown that only two functionally different conformations of the subunits exist both in unliganded and liganded states, regardless the quaternary structures.

As for the structural basis of functional differences between high and low affinity states, these have been proposed to be mediated by the so-called "allosteric core", the region containing the proximal His, the FG corner and part of the F helix that translates structural changes following heme ligation to the $\alpha 1$ - $\beta 2$ and $\alpha 2$ - $\beta 1$ interface (Gelin et al., 1983). Although different structural and dynamic bases have been put forward over the decades to explain Hb allostery (recently reviewed, e.g., in (Henry et al., 2015), by Shibayama (Shibayama, 2020) and Ho and coworkers (Yuan et al.,

2015)), an ultimate description still awaits the resolution of key missing structures: those of oxygenated and deoxygenated Hb in the high-affinity T state (deoxy(Tr) and oxy(Tr) in the framework of the TTS model) and low-affinity R state (deoxy(Rt) and oxy(Rt)). Progress in cryo-electron microscopy, with the great advantage over crystallographic techniques to make feasible solving structures of heterogeneous conformations, might allow to tackle this issue in the future.

The relevant conclusion from these studies for the development of an HBOC is that Hb oxygen affinity can be modulated by altering the equilibrium distribution of *r* and *t* tertiary states **within each quaternary state. However, so far, this key observation has not yet been exploited for the development of tailored HBOCs.**

2. Towards Hemoglobin-based Oxygen Carriers: chemical and genetic strategies to modulate oxygen affinity, allostery, oxidation propensity, and NO reactivity. In the last decades, artificial oxygen carriers have been developed to tackle clinical needs not met by allogeneic blood transfusions, and, particularly, to treat: i) hemorrhagic events outside a hospital setting, ii) patients that refuse human blood for religious reasons, iii) patients that are immunoreactive to all blood types, iv) pre-term babies that need transfusions with oxygen properties different from adult blood, and v) patients that need transfusions in Third World countries where healthy blood donors and national blood banks are rare. **As reported by the World Health Organization's Global Program for Blood Safety, out of the 118.5 million blood donations collected globally in 2018, 40% are from countries with high Human Development Index (HDI), where 16% of the world population live. In addition, 48 out of 171 countries do not possess a national blood policy (<https://www.who.int/news-room/fact-sheets/detail/blood-safety-and-availability>). The median blood donation rate in high-HDI countries is 31.5 donations per 1000 people. This compares with 15.9 donations per 1000 people in upper-middle HDI countries, 6.8 donations per 1000 people in lower-middle HDI countries, and five donations per 1000 people in low-HDI countries. For 62 countries fewer than 10 donations per 1000 people are reported and, among**

these, 34 countries are in the WHO African Region (<https://www.who.int/news-room/fact-sheets/detail/blood-safety-and-availability>).

The development of a blood substitute was initially thought to be easily achievable. However, more than fifty years of investigations, many preclinical, phase I, phase II and even phase III clinical trials have led to no FDA- and EMA-approved blood substitute (Dolgin, 2017). Nevertheless, two products, Hemopure® and Sanguinate® have received the FDA approval for compassionate use in patients that cannot receive or refuse transfusions (see below). In addition, a perfluorocarbon derivative, Fluosol-DA was approved by FDA in 1989 and used in Jehovah's Witness patients (Krafft and Riess, 2009). Its production ended in 1994 due to lack of use and a second-generation of perfluorocarbon, Oxygent, proceeded through phase III trials but was never FDA licensed (Krafft and Riess, 2009).

Failures of hemoglobin-based oxygen carriers are due to two main issues: i) HbA works properly only when confined within RBCs, and ii) the allosteric properties are associated with the integrity of the tetrameric state and the presence of allosteric effectors. Both these conditions are lost when HbA is free in the plasma, where no allosteric effectors are present, and diluted Hb dissociates into dimers that exhibit high affinity and no cooperativity. In addition, dimeric HbA free in the plasma undergoes accelerated oxidation due also to heme loss, generating oxygen radicals, which in turn cause oxidative stress. HbA oxidation in the plasma is not counterbalanced by the enzymatic systems present within RBCs. Moreover, cell-free HbA extravasates easily, scavenging endothelial NO, and thus causing hypertension. Finally, HbA as a dimer, in contrast to tetramers, is filtered in the renal glomerulus, causing nephrotoxicity (Figure 2A).

To overcome limitations, different approaches of Hb modification have been exploited, including protein decoration with polyethylene glycol (PEG) to increase the hydrodynamic radius and avoid extravasation, intermolecular crosslinking to prevent tetramer dissociation, encapsulation in synthetic envelopes or different nano- or microarchitectures (Figure 2B) (Bobofchak et al., 2003; Devineau et al., 2018; Inayat et al., 2006; Jahr et al., 2012; Jansman and Hosta-Rigau, 2018; Kocian and Spahn,

2008; Meng et al., 2018; Mozzarelli et al., 2010; Winslow, 2006a). The development of the first HBOCs aimed at mimicking the properties of RBCs in terms of oxygen binding, autoxidation rate, and reactivity with NO. More recently, HBOCs have been considered as "oxygen therapeutics" for emergency treatment, and it is now debated whether functional properties close to those of RBCs are clinically relevant (Belcher et al., 2018; Dolgin, 2017; Mozzarelli et al., 2010). Nevertheless, the modulation of the oxygen-binding properties, autoxidation rates, and NO reactivity is still an active field of investigation to obtain HBOCs endowed with different functional properties, possibly to be used for specific clinical applications and therapeutic treatments. This variety of functional properties might be more easily achieved by engineering Hb via genetic approaches rather than chemical modifications (Figure 2B) (Benitez Cardenas et al., 2019; Cooper et al., 2020; Varnado et al., 2013).

One caveat of genetic methods for HBOC production is the relative low yield of purified material from expression systems, leading to a high cost per unit of blood (Graves et al., 2008).

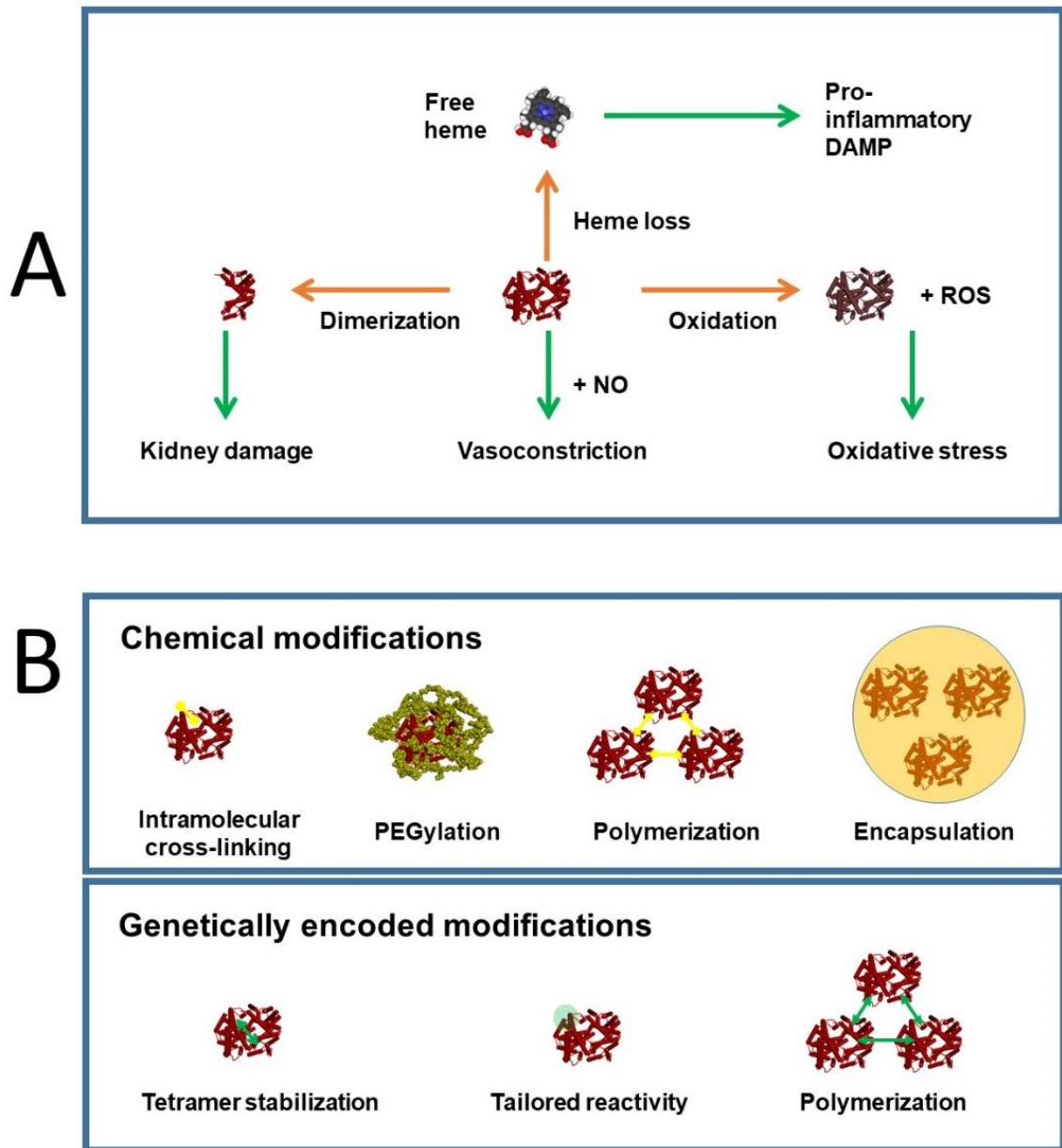


Figure 2: A. Potential toxic effects caused by cell-free Hb. B. Strategies for the development of HBOCs: chemical and genetically encoded modifications to reduce dimer formation, increase of the overall size of the protein or modification of Hb reactivity towards NO.

Key questions in the development of effective HBOCs are: i) is there a unique ideal oxygen affinity for an HBOC or should it be dependent on the specific HBOC clinical use? and ii) are tailored HBOC

functional properties more “easily” achieved by chemical or genetic methods or by a combination of both methods? These questions are still awaiting a definite answer. As discussed in the next sections, developed HBOCs are endowed with oxygen affinity that can be higher, similar, or even lower than Hb oxygen affinity within RBCs (Table 1). This is somewhat surprising as all products are designed to deliver oxygen to tissues in hemorrhagic events, orthopedic surgery, ischemic episodes or sickle cell crises. The rationale for a high affinity HBOC is that oxygen is released only to peripheral tissues where oxygen tension is low and no triggering of signals controlling vascular tension occurs due to oxygen release in the pre-peripheral circulation (Winslow, 2008). The rationale for HBOCs with an oxygen affinity close or lower than that of Hb in RBCs is that this oxygen affinity is physiologically relevant for oxygen delivery to peripheral tissues (Jahr et al., 2008; Moore et al., 2009). Noteworthy to mention that for all products, allosteric properties are almost completely abolished, impairing the well-known critical function of Hb for oxygen release from lungs to tissues. Whereas genetic manipulation of Hb has demonstrated to be able to generate almost a continuum of individual functional properties of Hb (Olson et al., 1997), difficulties have raised due to the concomitant alteration of other properties, given the tight and complex interaction network of the protein matrix.

2.1. Modulation of the oxygen-binding properties. Several modification chemistries of Hb have tackled the need to control the oxygen-binding properties of HBOCs by modulating the tertiary and quaternary conformational equilibria of the tetramer.

2.1.1 Control of oxygen binding properties in PEGylated HBOCs. HBOCs consisting of PEGylated Hbs take advantage of the extensive use of polyethylene glycol (PEG) to conjugate protein therapeutics and increase their half-life and reduce extravasation (Roberts et al., 2002). PEGylation of Hb yields products endowed with significantly different oxygen binding properties, depending on the chemistry of conjugation, the ligation state, and the residues involved in the modification (Table 1). Several researchers have worked towards the characterization of all these variables, including

Acharya and coworkers (Li et al., 2006; Manjula et al., 2000; Manjula et al., 2005), Winslow and coworkers (Winslow, 2004; Young et al., 2005), Perrella and coworkers (Caccia et al., 2009; Iafelice et al., 2007; Portoro et al., 2008), Kluger and coworkers (Lui et al., 2008; Lui and Kluger, 2009), and our group (Alomari et al., 2018; Portoro et al., 2008; Ronda et al., 2011).

The PEGylation chemistry employed in the preparation of HBOCs has proven crucial in modulating oxygen affinity, mainly because it dictates which residues are modified. As direct PEGylation of HbA with urethane linkages produces extensive PEGylation with little control over the oxygen-binding properties (Bradley et al., 1994), an approach consisting in the thiolation of amino groups followed by their PEGylation was later introduced (Li et al., 2009; Li et al., 2006). The resulting HBOCs exhibited oxygen binding properties that depend on whether PEGylation is carried out under aerobic or anaerobic conditions. A drawback of thiol-directed chemistries is that the PEGylation of residue Cys93(β) was suggested to perturb the heme-binding pocket. For this reason, its protection was pursued through several chemical approaches, leading to different conclusions regarding its relevance in the control of HBOCs properties (Le Coeur et al., 2015; Manjula et al., 2003; Wang et al., 2014). Residue Cys93(β) is also involved in NO binding under aerobic conditions. The physiological relevance of the SNO-Hb complex in hypoxia vasodilation via the reversible release of a SNO derivative during deoxygenation was proposed by Stamler (see for a review (Premont et al., 2020)). An alternate view, suggested by Gladwin (Gladwin, 2017) proposes the formation of NO from nitrite by deoxyHb, leading to hypoxic vasodilation. Furthermore, since Cys93(β) is only reactive in the R state, blocking it by bulky groups hinders the R to T transition, stabilizing the R state and increasing oxygen affinity.

As PEGylation at the N-terminal Val1(α) and Val1(β) was shown to induce the destabilization of Hb tetramers, their carboxymethylation and propylation before PEGylation was pursued (Hu et al., 2009; Hu et al., 2011; Hu et al., 2012).

The ligation state of Hb at the time of PEGylation also proved critical in dictating the oxygen-binding properties of the final product. By PEGylating oxy-HbA, Winslow and colleagues obtained

a HBOC endowed with no cooperativity and oxygen affinity close to isolated dimers and R state Hb (Caccia et al., 2009; Iafelice et al., 2007; Portoro et al., 2008; Winslow, 2005). The PEGylation of deoxy-HbA, on the other hand, resulted in a low-affinity, high-cooperativity product (Euro-PEG-Hb) (Caccia et al., 2009; Portoro et al., 2008). We compared high- and low-affinity PEGylated HbA in a Guinea pig transfusion model, demonstrating a correlation between oxygen affinity and oxidative stress (Alomari et al., 2018).

PEG was also used in the preparation of phospholipids bilayer vesicles containing purified HbA for the development of red cell mimics (Sakai et al., 2001). Hb vesicles exhibiting p_{50} of 8 and 29 torr were obtained by modulating the amount of pyridoxal 5-phosphate as allosteric effector. In an hamster model, it was found that vesicles containing higher affinity Hb led to a higher oxygen release (Sakai et al., 2005).

2.1.2 Control of oxygen binding properties in polymerized HBOCs. HBOCs have been obtained by introducing crosslinks between dimers to prevent dissociation. Since the first observations on diaspirin-crosslinked HbA (Chatterjee et al., 1986; Walder et al., 1979), it was concluded that crosslinking chemistry is crucial in modulating the oxygen-binding properties. For example, Hb raffimer (Hemolink™) is based on intramolecular/intermolecular HbA crosslinked with oxidized raffinose (Scatena and Giardina, 2001) and its affinity for oxygen is lower than that of Hb inside RBCs (Table 1) (Caron et al., 1999; Scatena and Giardina, 2001). HbA polymerized with glutaraldehyde and then pyridoxylated (PolyHeme®, Northfield) exhibits a low affinity (Table 1) (Gould et al., 1998). As for PEGylated HBOCs, the conditions of the reaction, as well as the ligation state of HbA, were investigated (Zhang et al., 2011).

2.1.3 Control of oxygen binding properties by using non-human Hbs. Bovine Hb (bHb) naturally exhibits a low affinity for oxygen and was therefore proposed as a starting point for preparing low-affinity HBOCs. Hemopure® (Biopure, now HbO2 Therapeutics) (and its most recent version called HBOC-201), a glutaraldehyde-polymerized bovine Hb, exhibits an oxygen affinity lower than human

RBCs (Table 1) (Jahr et al., 2008). It has been authorized for compassionate use in patients that cannot be transfused for any clinical reason or refuse transfusions such as Jehovah's Witnesses. Furthermore, Hemopure® is approved in South Africa to treat anemia in adult surgical patients, and in Russia for acute anemia, irrespective of etiology. A partially purified Hemopure®, called Oxyglobin® was approved by FDA and EMA for veterinary use (Harris and Palmer, 2008). Hemopure® is also used in organ perfusion solutions (see 4.2).

As for PEGylated HBOCs and glutaraldehyde-polymerized HbA, the ligation state of Hb during the reaction strongly affected the oxygen-binding properties (Buehler et al., 2010; Zhou et al., 2011). bHb has been intramolecularly crosslinked with ATP and intermolecularly linked with adenosine (Simoni et al., 2014; Simoni et al., 2012) and conjugated to dextran upon Cys93(β) protection (Wang et al., 2017). A more recent approach consisted in the decoration of CO-bHb with PEG, leading to the product Sanguinate® (Abuchowski, 2016, 2017). Clinical trials were carried out for the use of Sanguinate® to treat sickle cell crises (Misra et al., 2017) and animal studies for the use as resuscitation fluid (Guerci et al., 2020). This product was granted the designation of orphan drug for sickle cell patients as well as compassionate use in severe anemia when transfusions were not possible or refused (McConachie et al., 2020). However, concern about the benefits of its use in such a complex clinical frame as sickle cell disease was raised (Alayash, 2017).

Recent studies (Gu et al., 2021) report on the development of a series of HBOCs obtained from cross-linked bHb PEGylated in the either the T or R state upon tangential flow filtration to separate high and low molecular weight forms. For both forms, the R state PEG bHb products exhibit p50s close to 1 torr, whereas the T state PEG bHb exhibits p50 of 20 torr. For all products, cooperativity is close to 1 (Belcher et al., 2017; Gu et al., 2021).

Another HBOC derived from purified bHb is the zero-linked polymeric hemoglobin, OxyVita®Hb obtained from the formation of pseudopeptide bonds between carboxyl groups and amino groups on

the surface of bovine cross-linked tetrameric Hb molecules (Table 1) (Harrington et al., 2011). Studies are ongoing to investigate oxidation stability (Wollocko et al., 2017a) and formulation (Wollocko et al., 2017b).

A cluster was produced by cross-linking bHb with human albumin molecules, named HemoAct (Table 1) (Tomita et al., 2013). HemoAct safety, evaluated in rats, was found acceptable (Haruki et al., 2015). Using as crosslinking agent *N*-succinimidyl 3-maleimidopropionate (Funaki et al., 2019), a product with a p50 of 9 torr and a Hill coefficient of 1.4 was obtained, lower than the control HbA exhibiting a p50 of 23 torr and a Hill coefficient of 2.6. This suggests a stabilization of the R state due to constraints to the transition to the T state, as lysine residues were modified.

Besides bHb, crocodile Hb, *Trematomus bernacchii* Hb and erythrocrutorin of the earthworm *Lumbricus terrestris* have also been considered as starting points for low-affinity HBOCs due to their specific oxygen binding properties (Coppola et al., 2011; Elmer et al., 2012; Jani et al., 2017; Komiya et al., 1995; Rajesh et al., 2018; Roamcharern et al., 2019; Spivack et al., 2018). More recently, cell-free Hb from *Arenicola marina* has been proposed as a blood substitute. This lugworm Hb has a high molecular weight (3600 kDa), with a p50 of 7 mmHg (Table 1) (Mallet et al., 2014; Varney et al., 2021), and is prepared by the company Hemarina with the name Hemo2Life® M101. This product has been tested as a preservation fluid for storing healthy and marginal kidney before transplantation in a preclinical porcine model (Kaminski et al., 2019; Thuillier et al., 2011).

2.1.4 Control of oxygen binding properties by non-natural Hb variants. It was suggested to produce recombinant Hb variants by introducing mutations capable of modulating oxygen affinity (Benitez Cardenas et al., 2019; Cooper et al., 2020; Dou et al., 2002; Olson et al., 1997; Ronda et al., 2008; Varnado et al., 2013), in particular through the stabilization of high or low-affinity conformations, the alteration of the interactions between oxygen and amino acid side chains in the heme pocket and the modification of heme accessibility (Birukou et al., 2010).

Hb Polytaur is a chimeric autopolymerizing recombinant Hb engineered to both increase the p50 of HbA and its size through autopolymerization via formation of disulfide bonds (Bobofchak et al., 2003; Faggiano et al., 2011). It is formed by human α chains and bovine β chains, intended to lower the affinity of Hb. Mutations Cys104Ser(α), Cys93Ala(β), and Ser9Cys(β) were introduced to limit polymerization to Ser9Cys(β). The autopolymerized product exhibited a p50 of 16-18 mmHg under physiological conditions, values close to Hb inside RBCs.

Recently, a double mutant (Cys93Ala(β)/Ala19Cys(α)) was generated and decorated with PEG20000 Da. This HBOC showed oxygen affinity and cooperativity close to HbA (Cooper et al., 2021; Cooper et al., 2020).

Towards the development of stable Hb tetramers, a combination of two α_2 and β_2 homodimers was obtained from a fusion gene containing two α chains or two β chains, coupled via a specific linker (Panetta et al., 2008). The modification increased the oxygen affinity, due to a destabilization of the T quaternary state, and did not increase the autoxidation rate (Panetta et al., 2008).

2.2. Modulation of the autoxidation rates and NO dioxygenase reactivity. HBOCs are prone to oxidation to metHb or ferryl species. These species do not bind oxygen and promote oxidative reactions of proteins, lipids, DNA, which correlate to the oxidative stress observed upon administration (Mollan and Alayash, 2013; Strader and Alayash, 2017). Indeed, the chemical modifications used in the preparation of HBOCs increase Hb propensity to autoxidation and production of $O_2^{\cdot -}$ and H_2O_2 (Mollan and Alayash, 2013). Antioxidants like ascorbate mediate Hb reduction (Cooper et al., 2008), but their plasma concentration in humans is limited by dietary intake (Rumsey and Levin, 1998). **The extensive mutagenesis studies carried out by Olson and coworkers on Mb and Hb have allowed to pinpoint residues affecting oxygen affinity as well as oxidation stability (Benitez Cardenas et al., 2019; Dou et al., 2002). Fetal Hb (HbF) is more stable and less prone to autoxidation in comparison to HbA (Graves et al., 2008), suggesting its use as a precursor of HBOCs. Recombinant human fetal**

Hb and HbA were prepared and evaluated in terms of oxidative stress (Simons et al., 2018). Differences in oxidative reactivity were observed but neither form was superior to the other for the preparation of effective HBOC (Simons et al., 2018).

Another oxidatively stable HBOC was prepared based on the observation that the naturally occurring K82D variant (Hb Providence) is more resistant towards oxygen peroxide degradation with respect to wild-type HbA (Strader et al., 2017). The effects of genetic cross-linking of Hb Providence on autoxidation, heme loss, and reactions with H_2O_2 were compared with HbA and found that cross-linking and mutation confer higher stability to oxidative reactions, thus suggesting that Hb Providence is a valuable source for the development of HBOCs (Strader et al., 2017). It was also found that in Hb Providence Cys93(β) is less prone to oxidation with respect to HbA (Jana et al., 2020).

HbA as well as Mb, under oxidative conditions, form ferryl heme iron and protein-based free radicals. Ferryl reduction can occur either via direct electron transfer from a reducing agent to heme edges or via a protein-based pathway that involves tyrosine residues (Reeder et al., 2008). Based on the observation that in HbA tyrosine Tyr42(α) is involved in the electron transfer between endogenous antioxidants and the ferryl heme, and that a tyrosine residue is absent in the equivalent position of the β subunits, the recombinant Hb variant Phe41Y(β) was designed to improve the reduction rates of β subunits (Silkstone et al., 2016). It was found that mutation decreases heme-mediated oxidative reactivity and enhances NO bioavailability (Silkstone et al., 2016). Several other variants introducing tyrosine residues in both subunits were generated to minimize Hb pro-oxidant activity, maintaining Hb oxygen affinity in a range compatible with oxygen delivery to tissues (Cooper et al., 2019). Among them, Thr84Tyr(β) variant was found to increase the rate of heme reduction by ascorbate, restoring Hb function. HEK cells showed reduced membrane damage upon treatment with the Thr84Tyr(β) variant in comparison to HbA (Cooper et al., 2019). When this variant was PEGylated

and administered to mice, it was found to be retained in the circulation longer than PEGylated HbA (Cooper et al., 2019).

The reactivity of Hb with NO is also a concern in the design of HBOCs as cell-free Hb is prone to NO scavenging, which causes hypertension and also results in Hb oxidation (Alayash, 2019; Doherty et al., 1998; Eich et al., 1996; Olson et al., 2004). NO-induced oxidation was found to be similar in unmodified, polymerized, crosslinked, or PEGylated Hbs, suggesting that the NO scavenging effect observed for HBOCs is mostly associated with their extracellular localization rather than different reactivity towards NO (Meng et al., 2018). However, the thorough investigation of recombinant variants (Doherty et al., 1998; Fronticelli and Koehler, 2009; Frost et al., 2018; Graves et al., 2008) has indicated that some residues are particularly involved in the NO dioxygenase activity, opening the way to produce HBOCs based on recombinant Hbs endowed with lower reactivity towards NO (Doherty et al., 1998; Cooper et al., 2019).

Instead of altering Hb reactivity, the reduction of adverse effects of reactive nitrogen and oxygen species has also been tackled by incorporating antioxidant functions in HBOCs. In the late 1990s, Chang and coworkers crosslinked the enzymes catalase and superoxide dismutase (SOD) to a polyHb (D'Agnillo and Chang, 1998; Powanda and Chang, 2002). Lugworm Hb (Hemo2Life®) shows also SOD-like activity (Mallet et al., 2014).

3. Clinical trials on HBOCs. Several HBOCs were tested in preclinical and clinical trials, and some of them, such as PolyHeme®, Hemopure® and Hemospan® (Sangart), HemAssist® (Baxter), Hemolink™ (Hemosol), reached phase III. However, no products have so far obtained approval by either FDA or EMA, whereas HBOC-201, the most recent version of Hemopure®, is approved in Russia and in South Africa for anemic patients undergoing surgery and is used in the USA for patients in critical conditions that refuse transfusions, under investigational or expanded access/compassionate use (Khan et al., 2020). Here we briefly summarize the latest phase III clinical trials. More information is found in several books on HBOCs published over the years (Chang, 1997;

Chang, 1992; Kim and Greenburg, 2013; Mozzarelli and Bettati, 2011; Winslow et al., 1996; Winslow, 2006b).

PolyHeme® was tested on more than 700 patients in USA as a first treatment for hemorrhagic events vs blood expanders at ambulances and vs transfusions in emergency rooms. Results indicated that PolyHeme® was not superior with respect to controls when survival was used as therapeutic index. Therefore, FDA stated that the risk:benefit assessment of the product in trauma was unfavorable. Moreover, ethic issues were raised since consensus was not collected due to the unconscious patient conditions, and safe transfusions were available in emergency rooms (Dube et al., 2019).

HBOC-201 was tested vs transfusions using severe adverse effects as therapeutic index. Results showed an increase in severe adverse effects possibly associated with greater anemia (Dube et al., 2019). For both HBOC-201 and PolyHeme® clinical evidence indicated significant levels of cardiovascular dysfunction (Chen et al., 2009).

A Hemospan® (MP4OX) phase III clinical trial was carried out to determine if the product was able to prevent hypotension in hip arthroplasty more than a colloidal plasma expander, and to reduce the incidence of operative and postoperative complications including organ dysfunction and failure until follow-up at one month following surgery (<https://clinicaltrials.gov/ct2/show/NCT00421200>). This study followed a randomized, single-blind, increasing dose safety trial selecting orthopedic surgery patients with spinal anesthesia (Olofsson et al., 2008). It should be pointed out that the aim of these trials was not to demonstrate Hemospan® efficacy as an HBOC but as an oxygen-carrying plasma expander. Results indicated that Hemospan® significantly reduced the incidence of hypotensive episodes in patients undergoing hip arthroplasty, but the adverse event profile did not support use in routine low-risk surgical patients (Olofsson et al., 2011).

Another HBOC, HemAssist®, was tested in a phase III clinical trial in USA but the trial was suspended when a significant increase in mortality rates as compared to patients treated with blood products was observed (Chen et al., 2009; Jahr et al., 2007).

Phase III clinical trials were suspended in 2003 also for Hemolink™, since patients treated with this product suffered from adverse cardiac side effects (Sen Gupta, 2019).

In 2008 Natanson et al. (Natanson et al., 2008) carried out a meta-analysis of 16 clinical trials associated with five products. Results showed a significantly increased risk of death and myocardial infarction in patients treated with HBOCs. This study put an end to a period where three major USA companies, Northfield, Biopure and Sangart, intensively and parallelly tried to develop a safe HBOC. However, in spite of the so far unsuccessful investigations for the development of a safe HBOC, transfusional medicine still urgently needs a drug that i) is able to deliver oxygen, ii) can be stored at room temperature for prolonged periods, iii) can be administered without any blood group matching and iv) can effectively replace RBC transfusions when blood is not available (Pusateri et al., 2019).

4. HBOC "repositioning". The extensive studies carried out for the development of HBOCs led to a deeper understanding of Hb function and physiology, and oxygen supply requirements as well as NO roles in controlling blood vessel tension. Nevertheless, all HBOCs are products that load and unload oxygen depending on oxygen pressures. For this key function, they have been exploited for supplying oxygen or other ligands, such as CO, to isolated organs or tissues.

4.1 CO delivery based on HBOCs. A CO derivative of Hemospan®, MP4CO (Belcher et al., 2013), can deliver CO that at low concentrations shows positive effects including anti-apoptosis, anti-inflammatory, anti-oxidative and anti-proliferative actions (Taguchi et al., 2020). Transgenic mice expressing HbS, the Hb responsible for the sickle cell disease in humans, were treated with MP4CO as a strategy to deliver CO and, thus, to induce the expression of heme oxygenase-1 that inhibits microvascular stasis in sickle mice. In addition, it was also demonstrated that administration of MP4CO induces the expression of the cytoprotective Nrf2 transcriptional regulator of heme oxygenase-1 (Belcher et al., 2013). **In the case of Sanguinate®, manufactured as CO derivative and**

as such used for the treatment of sickle cell patients (see above, 2.1.3), the CO-Hb derivative was the only product developed and investigated (Abuchowski, 2016).

4.2 Organ perfusion fluids based on HBOCs. Every day, in the US and EU, about 50 people die because of the lack of healthy organs to be transplanted. Moreover, the rate of opt-outs from organ donation is increasing, reaching for example in Italy, in 2020, the record rate of 34%, with the consequential increase in mortality for conditions that otherwise would be treatable. Furthermore, not all the organs used lead to a successful transplantation, worsening the scenario. By the end of 2019, over 58.000 patients were on a waiting list for organ transplantation in Europe and, on yearly average, about 3-4% of the patients die before being transplanted (Vanholder et al., 2021). For example, in 2019 in Italy, patients in the kidney transplant list were 8615 and only 3813 were transplanted (data from the Italian National Center Transplant). Moreover, only a small proportion of donated kidneys were eligible for transplant (1379 out of 2766), indicating that organs are very susceptible to ischemia and damage after death. Since the need for organs is significantly larger than the availability of ideal donors, criteria for donor acceptance have been expanded and led to the use of extended criteria donation (ECD) after circulatory death (DCD), but this is still not enough. For these reasons, the development of new methods for improving organ preservation is a very active field. Whereas the most used method for organ preservation is the normothermic perfusion with crystalloid solutions and RBCs, recently HBOCs have been explored as an alternative for the improvement of oxygen supply and conservation of optimal metabolic activity. A key advantage of HBOCs over RBCs is their ability to diffuse in every organ capillary given the very reduced size (nanometers vs microns). For instance, HBOCs were employed in machine perfusion of liver and kidney grafts after prolonged cold ischemia (Mahboub et al., 2020) and were efficacious at improving organ condition of the liver graft in animal experiments (Bhattacharjee et al., 2020). It was shown that more oxygen was extracted when HBOC-201 was used with respect to RBCs in a human model of normothermic machine

perfusion for liver (Laing et al., 2017) and kidney grafts (Aburawi et al., 2019). Ten discarded human livers were exposed to a continuous cycle of hypo- and normothermic perfusions using HBOC-201 (Boteon et al., 2019). Results indicated that the procedure leads to a mitigation of the oxidative-mediated tissue injury and an enhancement of hepatic energy stores, similarly to an interrupted combined protocol, but with a simpler and more clinically applicable procedure (Boteon et al., 2019). Similar positive results were obtained in a study where hypo- and normothermic perfusions were sequentially applied to seven livers in order to preserve mitochondria activity as well as hepatic functions, respectively (de Vries et al., 2019). During the normothermic phase, HBOC-201 was used for supplying oxygen. Given the optimal metabolic conditions, five livers were transplanted and 100% were still in place after three months (de Vries et al., 2019). In a collaborative study we are carrying out with the University of Bologna, a combination of bovine crosslinked HBOC and mesenchymal stromal cell extracellular vesicles was used for human kidney normothermic perfusions, obtaining very positive results for the preservation and improvement of marginal organs (unpublished data). Because of this new game-changing application, HBOCs specifically designed for *ex vivo* perfusion machines are currently being investigated, although most of the products used so far are derivatives or HBOCs envisaged for *in vivo* administration as blood substitutes.

Bibliography

Abuchowski, A., 2016. PEGylated Bovine Carboxyhemoglobin (SANGUINATE): Results of Clinical Safety Testing and Use in Patients. *Advances in experimental medicine and biology* 876, 461-467.

Abuchowski, A., 2017. SANGUINATE (PEGylated Carboxyhemoglobin Bovine): Mechanism of Action and Clinical Update. *Artificial organs* 41 (4), 346-350.

Aburawi, M.M., Fontan, F.M., Karimian, N., Eymard, C., Cronin, S., Pendexter, C., Nagpal, S., Banik, P., Ozer, S., Mahboub, P., Delmonico, F.L., Yeh, H., Uygun, K., Markmann, J.F., 2019.

Synthetic hemoglobin-based oxygen carriers are an acceptable alternative for packed red blood cells in normothermic kidney perfusion. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 19 (10), 2814-2824.

Ackers, G.K., Doyle, M.L., Myers, D., Daugherty, M.A., 1992. Molecular code for cooperativity in hemoglobin. *Science* 255 (5040), 54-63.

Alayash, A.I., 2017. Hemoglobin-Based Blood Substitutes and the Treatment of Sickle Cell Disease: More Harm than Help? *Biomolecules* 7 (1).

Alayash, A.I., 2019. Mechanisms of Toxicity and Modulation of Hemoglobin-based Oxygen Carriers. *Shock* 52 (1S Suppl 1), 41-49.

Alomari, E., Ronda, L., Bruno, S., Paredi, G., Marchetti, M., Bettati, S., Olivari, D., Fumagalli, F., Novelli, D., Ristagno, G., Latini, R., Cooper, C.E., Reeder, B.J., Mozzarelli, A., 2018. High- and low-affinity PEGylated hemoglobin-based oxygen carriers: Differential oxidative stress in a Guinea pig transfusion model. *Free radical biology & medicine* 124, 299-310.

Belcher, D.A., Banerjee, U., Baehr, C.M., Richardson, K.E., Cabrales, P., Berthiaume, F., Palmer, A.F., 2017. Mixtures of tense and relaxed state polymerized human hemoglobin regulate oxygen affinity and tissue construct oxygenation. *PLoS One* 12 (10), e0185988.

Belcher, D.A., Ju, J.A., Baek, J.H., Yalamanoglu, A., Buehler, P.W., Gilkes, D.M., Palmer, A.F., 2018. The quaternary state of polymerized human hemoglobin regulates oxygenation of breast cancer solid tumors: A theoretical and experimental study. *PLoS One* 13 (2), e0191275-e0191275.

Belcher, J.D., Young, M., Chen, C., Nguyen, J., Burhop, K., Tran, P., Vercellotti, G.M., 2013. MP4CO, a pegylated hemoglobin saturated with carbon monoxide, is a modulator of HO-1, inflammation, and vaso-occlusion in transgenic sickle mice. *Blood* 122 (15), 2757-2764.

Bellelli, A., Brunori, M., 2011. Hemoglobin allostery: variations on the theme. *Biochimica et biophysica acta* 1807 (10), 1262-1272.

Benitez Cardenas, A.S., Samuel, P.P., Olson, J.S., 2019. Current Challenges in the Development of Acellular Hemoglobin Oxygen Carriers by Protein Engineering. *Shock* 52 (1S Suppl 1), 28-40.

Bettati, S., Mozzarelli, A., 1997. T state hemoglobin binds oxygen noncooperatively with allosteric effects of protons, inositol hexaphosphate, and chloride. *The Journal of biological chemistry* 272 (51), 32050-32055.

Bettati, S., Mozzarelli, A., Perutz, M.F., 1998. Allosteric mechanism of haemoglobin: rupture of salt-bridges raises the oxygen affinity of the T-structure. *Journal of molecular biology* 281 (4), 581-585.

Bhattacharjee, R.N., Patel, S.V.B., Sun, Q., Jiang, L., Richard-Mohamed, M., Ruthirakanthan, A., Aquil, S., Al-Ogaili, R., Juriasingani, S., Sener, A., Luke, P.P.W., 2020. Renal Protection Against Ischemia Reperfusion Injury: Hemoglobin-based Oxygen Carrier-201 Versus Blood as an Oxygen Carrier in Ex Vivo Subnormothermic Machine Perfusion. *Transplantation* 104 (3), 482-489.

Birukou, I., Schweers, R.L., Olson, J.S., 2010. Distal histidine stabilizes bound O₂ and acts as a gate for ligand entry in both subunits of adult human hemoglobin. *The Journal of biological chemistry* 285 (12), 8840-8854.

Bobofchak, K.M., Mito, T., Texel, S.J., Bellelli, A., Nemoto, M., Traystman, R.J., Koehler, R.C., Brinigar, W.S., Fronticelli, C., 2003. A recombinant polymeric hemoglobin with conformational, functional, and physiological characteristics of an in vivo O₂ transporter. *American journal of physiology. Heart and circulatory physiology* 285 (2), H549-561.

Boteon, Y.L., Laing, R.W., Schlegel, A., Wallace, L., Smith, A., Attard, J., Bhogal, R.H., Reynolds, G., Perera, M., Muiesan, P., Mirza, D.F., Mergental, H., Afford, S.C., 2019. The impact on the bioenergetic status and oxidative-mediated tissue injury of a combined protocol of hypothermic and normothermic machine perfusion using an acellular haemoglobin-based oxygen carrier: The cold-to-warm machine perfusion of the liver. *PLoS One* 14 (10), e0224066.

Bradley, R., Slosberg, S., Nho, K., Czuba, B., Szesko, D., Shorr, R., 1994. Production of PEG-modified bovine hemoglobin: economics and feasibility. *Artificial cells, blood substitutes, and immobilization biotechnology* 22 (3), 657-667.

Bruno, S., Bonaccio, M., Bettati, S., Rivetti, C., Viappiani, C., Abbruzzetti, S., Mozzarelli, A., 2001. High and low oxygen affinity conformations of T state hemoglobin. *Protein science : a publication of the Protein Society* 10 (11), 2401-2407.

Buehler, P.W., Zhou, Y., Cabrales, P., Jia, Y., Sun, G., Harris, D.R., Tsai, A.G., Intaglietta, M., Palmer, A.F., 2010. Synthesis, biophysical properties and pharmacokinetics of ultrahigh molecular weight tense and relaxed state polymerized bovine hemoglobins. *Biomaterials* 31 (13), 3723-3735.

Caccia, D., Ronda, L., Frassi, R., Perrella, M., Del Favero, E., Bruno, S., Pioselli, B., Abbruzzetti, S., Viappiani, C., Mozzarelli, A., 2009. PEGylation promotes hemoglobin tetramer dissociation. *Bioconjugate chemistry* 20 (7), 1356-1366.

Caron, A., Menu, P., Faivre-Fiorina, B., Labrude, P., Alayash, A.I., Vigneron, C., 1999. Cardiovascular and hemorheological effects of three modified human hemoglobin solutions in hemodiluted rabbits. *Journal of applied physiology* 86 (2), 541-548.

Chang, T.M.S., 1997. *Blood Substitutes. Principles, Methods, Products and Clinical Trials*, Vol. 1 and 2. Karger, Basel.

Chang, T.M.S.E., 1992. *Blood substitutes and oxygen carriers*. Marcel Dekker, Inc., New York.

Chatterjee, R., Welty, E.V., Walder, R.Y., Pruitt, S.L., Rogers, P.H., Arnone, A., Walder, J.A., 1986. Isolation and characterization of a new hemoglobin derivative cross-linked between the alpha chains (lysine 99 alpha 1----lysine 99 alpha 2). *The Journal of biological chemistry* 261 (21), 9929-9937.

Chen, J.Y., Scerbo, M., Kramer, G., 2009. A review of blood substitutes: examining the history, clinical trial results, and ethics of hemoglobin-based oxygen carriers. *Clinics* 64 (8), 803-813.

Cooper, C.E., Bird, M., Sheng, X., Choi, J.W., Silkstone, G.G., Simons, M., Syrett, N., Piano, R., Ronda, L., Bettati, S., Paredi, G., Mozzarelli, A., Reeder, B.J., 2021. Stability of maleimide-PEG and mono-sulfone-PEG conjugation to a novel engineered cysteine in the human hemoglobin alpha subunit. *Front Chem* 9, 707797.

Cooper, C.E., Silaghi-Dumitrescu, R., Rukengwa, M., Alayash, A.I., Buehler, P.W., 2008. Peroxidase activity of hemoglobin towards ascorbate and urate: a synergistic protective strategy against toxicity

of Hemoglobin-Based Oxygen Carriers (HBOC). *Biochimica et biophysica acta* 1784 (10), 1415-1420.

Cooper, C.E., Silkstone, G.G.A., Simons, M., Gretton, S., Rajagopal, B.S., Allen-Baume, V., Syrett, N., Shaik, T., Popa, G., Sheng, X., Bird, M., Choi, J.W., Piano, R., Ronda, L., Bettati, S., Paredi, G., Mozzarelli, A., Reeder, B.J., 2020. Engineering hemoglobin to enable homogenous PEGylation without modifying protein functionality. *Biomaterials science* 8 (14), 3896-3906.

Cooper, C.E., Silkstone, G.G.A., Simons, M., Rajagopal, B., Syrett, N., Shaik, T., Gretton, S., Welbourn, E., Bulow, L., Eriksson, N.L., Ronda, L., Mozzarelli, A., Eke, A., Mathe, D., Reeder, B.J., 2019. Engineering tyrosine residues into hemoglobin enhances heme reduction, decreases oxidative stress and increases vascular retention of a hemoglobin based blood substitute. *Free radical biology & medicine* 134, 106-118.

Coppola, D., Bruno, S., Ronda, L., Viappiani, C., Abbruzzetti, S., di Prisco, G., Verde, C., Mozzarelli, A., 2011. Low affinity PEGylated hemoglobin from *Trematomus bernacchii*, a model for hemoglobin-based blood substitutes. *BMC biochemistry* 12, 66.

D'Agnillo, F., Chang, T.M., 1998. Polyhemoglobin-superoxide dismutase-catalase as a blood substitute with antioxidant properties. *Nature biotechnology* 16 (7), 667-671.

Das, T.K., Khan, I., Rousseau, D.L., Friedman, J.M., 1999. Temperature dependent quaternary state relaxation in sol-gel encapsulated hemoglobin. *Biospectroscopy* 5 (5 Suppl), S64-70.

de Vries, Y., Matton, A.P.M., Nijsten, M.W.N., Werner, M.J.M., van den Berg, A.P., de Boer, M.T., Buis, C.I., Fujiyoshi, M., de Kleine, R.H.J., van Leeuwen, O.B., Meyer, P., van den Heuvel, M.C., de Meijer, V.E., Porte, R.J., 2019. Pretransplant sequential hypo- and normothermic machine perfusion of suboptimal livers donated after circulatory death using a hemoglobin-based oxygen carrier perfusion solution. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 19 (4), 1202-1211.

Devineau, S., Kiger, L., Galacteros, F., Baudin-Creuzat, V., Marden, M., Renault, J.P., Pin, S., 2018. Manipulating hemoglobin oxygenation using silica nanoparticles: a novel prospect for artificial oxygen carriers. *Blood advances* 2 (2), 90-94.

di Masi, A., De Simone, G., Ciaccio, C., D'Orso, S., Coletta, M., Ascenzi, P., 2020. Haptoglobin: From hemoglobin scavenging to human health. *Molecular aspects of medicine* 73, 100851.

Doherty, D.H., Doyle, M.P., Curry, S.R., Vali, R.J., Fattor, T.J., Olson, J.S., Lemon, D.D., 1998. Rate of reaction with nitric oxide determines the hypertensive effect of cell-free hemoglobin. *Nature biotechnology* 16 (7), 672-676.

Dolgin, E., 2017. Bioengineering: Doing without donors. *Nature* 549 (7673), S12-S15.

Dou, Y., Maillett, D.H., Eich, R.F., Olson, J.S., 2002. Myoglobin as a model system for designing heme protein based blood substitutes. *Biophysical chemistry* 98 (1-2), 127-148.

Dube, G.P., Pitman, A.N., Mackenzie, C.F., 2019. Relative Efficacies of HBOC-201 and Polyheme to Increase Oxygen Transport Compared to Blood and Crystalloids. *Shock* 52 (1S Suppl 1), 100-107.

Eaton, W.A., 1980. The relationship between coding sequences and function in haemoglobin. *Nature* 284 (5752), 183-185.

Eaton, W.A., Henry, E.R., Hofrichter, J., Bettati, S., Viappiani, C., Mozzarelli, A., 2007. Evolution of allosteric models for hemoglobin. *IUBMB life* 59 (8-9), 586-599.

Eaton, W.A., Henry, E.R., Hofrichter, J., Mozzarelli, A., 1999. Is cooperative oxygen binding by hemoglobin really understood? *Nature structural biology* 6 (4), 351-358.

Eich, R.F., Li, T., Lemon, D.D., Doherty, D.H., Curry, S.R., Aitken, J.F., Mathews, A.J., Johnson, K.A., Smith, R.D., Phillips, G.N., Jr., Olson, J.S., 1996. Mechanism of NO-induced oxidation of myoglobin and hemoglobin. *Biochemistry* 35 (22), 6976-6983.

Elmer, J., Zorc, K., Rameez, S., Zhou, Y., Cabrales, P., Palmer, A.F., 2012. Hypervolemic infusion of *Lumbricus terrestris* erythrocyte purified by tangential-flow filtration. *Transfusion* 52 (8), 1729-1740.

- Faggiano, S., Bruno, S., Ronda, L., Pizzonia, P., Pioselli, B., Mozzarelli, A., 2011. Modulation of expression and polymerization of hemoglobin Polytaur, a potential blood substitute. *Archives of biochemistry and biophysics* 505 (1), 42-47.
- Friedman, J.M., 1985. Structure, dynamics, and reactivity in hemoglobin. *Science* 228 (4705), 1273-1280.
- Fronticelli, C., Koehler, R.C., 2009. Design of recombinant hemoglobins for use in transfusion fluids. *Critical care clinics* 25 (2), 357-371, Table of Contents.
- Frost, A.T., Jacobsen, I.H., Worberg, A., Martinez, J.L., 2018. How Synthetic Biology and Metabolic Engineering Can Boost the Generation of Artificial Blood Using Microbial Production Hosts. *Frontiers in bioengineering and biotechnology* 6, 186.
- Funaki, R., Kashima, T., Okamoto, W., Sakata, S., Morita, Y., M., S., T., K., 2019. Hemoglobin–Albumin Clusters Prepared Using N- Succinimidyl 3- Maleimidopropionate as an Appropriate Cross-Linker. *ACS Omega* 4.
- Gelin, B.R., Lee, A.W., Karplus, M., 1983. Hemoglobin tertiary structural change on ligand binding. Its role in the co-operative mechanism. *Journal of molecular biology* 171 (4), 489-559.
- Gibson, Q.H., Roughton, F.J., 1955. The kinetics of dissociation of the first oxygen molecule from fully saturated oxyhaemoglobin in sheep blood solutions. *Proceedings of the Royal Society of London. Series B, Biological sciences* 143 (912), 310-334.
- Gladwin, M.T., 2017. How Red Blood Cells Process Nitric Oxide: Evidence for the Nitrite Hypothesis. *Circulation* 135 (2), 177-179.
- Gould, S.A., Moore, E.E., Hoyt, D.B., Burch, J.M., Haenel, J.B., Garcia, J., DeWoskin, R., Moss, G.S., 1998. The first randomized trial of human polymerized hemoglobin as a blood substitute in acute trauma and emergent surgery. *Journal of the American College of Surgeons* 187 (2), 113-120.
- Graves, P.E., Henderson, D.P., Horstman, M.J., Solomon, B.J., Olson, J.S., 2008. Enhancing stability and expression of recombinant human hemoglobin in *E. coli*: Progress in the development of a recombinant HBOC source. *Biochimica et biophysica acta* 1784 (10), 1471-1479.

Greenburg, A.G., Kim, H.W., 2004. Hemoglobin-based oxygen carriers. *Critical care* 8 Suppl 2, S61-64.

Gu, X., Savla, C., Palmer, A.F., 2021. Tangential flow filtration facilitated fractionation and PEGylation of low and high-molecular weight polymerized hemoglobins and their biophysical properties. *Biotechnology and bioengineering*.

Guerci, P., Ergin, B., Kandil, A., Ince, Y., Heeman, P., Hilty, M.P., Bakker, J., Ince, C., 2020. Resuscitation with PEGylated carboxyhemoglobin preserves renal cortical oxygenation and improves skeletal muscle microcirculatory flow during endotoxemia. *American journal of physiology. Renal physiology* 318 (5), F1271-F1283.

Harrington, J.P., Wollocko, J., Kostecki, E., Wollocko, H., 2011. Physicochemical characteristics of OxyVita hemoglobin, a zero-linked polymer: liquid and powder preparations. *Artificial cells, blood substitutes, and immobilization biotechnology* 39 (1), 12-18.

Harris, D.R., Palmer, A.F., 2008. Modern cross-linking strategies for synthesizing acellular hemoglobin-based oxygen carriers. *Biotechnology progress* 24 (6), 1215-1225.

Haruki, R., Kimura, T., Iwasaki, H., Yamada, K., Kamiyama, I., Kohno, M., Taguchi, K., Nagao, S., Maruyama, T., Otagiri, M., Komatsu, T., 2015. Safety Evaluation of Hemoglobin-Albumin Cluster "HemoAct" as a Red Blood Cell Substitute. *Scientific reports* 5, 12778.

Henry, E.R., Bettati, S., Hofrichter, J., Eaton, W.A., 2002. A tertiary two-state allosteric model for hemoglobin. *Biophysical chemistry* 98 (1-2), 149-164.

Henry, E.R., Jones, C.M., Hofrichter, J., Eaton, W.A., 1997. Can a two-state MWC allosteric model explain hemoglobin kinetics? *Biochemistry* 36 (21), 6511-6528.

Henry, E.R., Mozzarelli, A., Viappiani, C., Abbruzzetti, S., Bettati, S., Ronda, L., Bruno, S., Eaton, W.A., 2015. Experiments on Hemoglobin in Single Crystals and Silica Gels Distinguish among Allosteric Models. *Biophysical journal* 109 (6), 1264-1272.

- Hofrichter, J., Sommer, J.H., Henry, E.R., Eaton, W.A., 1983. Nanosecond absorption spectroscopy of hemoglobin: elementary processes in kinetic cooperativity. *Proceedings of the National Academy of Sciences of the United States of America* 80 (8), 2235-2239.
- Hu, T., Li, D., Manjula, B.N., Brenowitz, M., Prabhakaran, M., Acharya, S.A., 2009. PEGylation of Val-1(alpha) destabilizes the tetrameric structure of hemoglobin. *Biochemistry* 48 (3), 608-616.
- Hu, T., Li, D., Meng, F., Prabhakaran, M., Acharya, S.A., 2011. Increased inter dimeric interaction of oxy hemoglobin is necessary for attenuation of reductive pegylation promoted dissociation of tetramer. *Artificial cells, blood substitutes, and immobilization biotechnology* 39 (2), 69-78.
- Hu, T., Li, D., Wang, J., Wang, Q., Liang, Y., Su, Y., Ma, G., Su, Z., Wang, S., 2012. Propylbenz methylation at Val-1(alpha) markedly increases the tetramer stability of the PEGylated hemoglobin: a comparison with propylation at Val-1(alpha). *Biochimica et biophysica acta* 1820 (12), 2044-2051.
- Iafelice, R., Cristoni, S., Caccia, D., Russo, R., Rossi-Bernardi, L., Lowe, K.C., Perrella, M., 2007. Identification of the sites of deoxyhaemoglobin PEGylation. *The Biochemical journal* 403 (1), 189-196.
- Imai, K., 1982. *Allosteric effects in Hemoglobin*. Cambridge University Press; Cambridge, New York.
- Inayat, M.S., Bernard, A.C., Gallicchio, V.S., Garvy, B.A., Elford, H.L., Oakley, O.R., 2006. Oxygen carriers: a selected review. *Transfusion and apheresis science : official journal of the World Apheresis Association : official journal of the European Society for Haemapheresis* 34 (1), 25-32.
- Jahr, J.S., Akha, A.S., Holtby, R.J., 2012. Crosslinked, polymerized, and PEG-conjugated hemoglobin-based oxygen carriers: clinical safety and efficacy of recent and current products. *Current drug discovery technologies* 9 (3), 158-165.
- Jahr, J.S., Walker, V., Manoochchri, K., 2007. Blood substitutes as pharmacotherapies in clinical practice. *Current opinion in anaesthesiology* 20 (4), 325-330.

Jahr, J.S., Moallempour, M., Lim, J.C., 2008. HBOC-201, hemoglobin glutamer-250 (bovine), Hemopure (Biopure Corporation). *Expert opinion on biological therapy* 8 (9), 1425-1433.

Jana, S., Strader, M.B., Alayash, A.I., 2020. The Providence Mutation (betaK82D) in Human Hemoglobin Substantially Reduces betaCysteine 93 Oxidation and Oxidative Stress in Endothelial Cells. *International journal of molecular sciences* 21 (24).

Jani, V.P., Jelvani, A., Moges, S., Nacharaju, P., Roche, C., Dantsker, D., Palmer, A., Friedman, J.M., Cabrales, P., 2017. Polyethylene Glycol Camouflaged Earthworm Hemoglobin. *PLoS One* 12 (1), e0170041.

Jansman, M.M.T., Hosta-Rigau, L., 2018. Recent and prominent examples of nano- and microarchitectures as hemoglobin-based oxygen carriers. *Advances in colloid and interface science* 260, 65-84.

Jones, E.M., Balakrishnan, G., Spiro, T.G., 2012. Heme reactivity is uncoupled from quaternary structure in gel-encapsulated hemoglobin: a resonance Raman spectroscopic study. *Journal of the American Chemical Society* 134 (7), 3461-3471.

Jones, E.M., Monza, E., Balakrishnan, G., Blouin, G.C., Mak, P.J., Zhu, Q., Kincaid, J.R., Guallar, V., Spiro, T.G., 2014. Differential control of heme reactivity in alpha and beta subunits of hemoglobin: a combined Raman spectroscopic and computational study. *Journal of the American Chemical Society* 136 (29), 10325-10339.

Juszczak, L.J., Friedman, J.M., 1999. UV resonance raman spectra of ligand binding intermediates of sol-gel encapsulated hemoglobin. *The Journal of biological chemistry* 274 (43), 30357-30360.

Kaminski, J., Hannaert, P., Kasil, A., Thuillier, R., Leize, E., Delpy, E., Steichen, C., Goujon, J.M., Zal, F., Hauet, T., 2019. Efficacy of the natural oxygen transporter HEMO2 life((R)) in cold preservation in a preclinical porcine model of donation after cardiac death. *Transplant international : official journal of the European Society for Organ Transplantation* 32 (9), 985-996.

- Kendrew, J.C., Dickerson, R.E., Strandberg, B.E., Hart, R.G., Davies, D.R., Phillips, D.C., Shore, V.C., 1960. Structure of myoglobin: A three-dimensional Fourier synthesis at 2 Å. resolution. *Nature* 185 (4711), 422-427.
- Khan, F., Singh, K., Friedman, M.T., 2020. Artificial Blood: The History and Current Perspectives of Blood Substitutes. *Discoveries* 8 (1), e104.
- Khan, I., Shannon, C.F., Dantsker, D., Friedman, A.J., Perez-Gonzalez-de-Apodaca, J., Friedman, J.M., 2000. Sol-gel trapping of functional intermediates of hemoglobin: geminate and bimolecular recombination studies. *Biochemistry* 39 (51), 16099-16109.
- Kim, H.W., Greenburg, A.G.E., 2013. Hemoglobin-Based Oxygen Carriers as Red Cell Substitutes and Oxygen Therapeutics. Springer
- Kister, J., Poyart, C., Edelstein, S.J., 1987. An expanded two-state allosteric model for interactions of human hemoglobin A with nonsaturating concentrations of 2,3-diphosphoglycerate. *The Journal of biological chemistry* 262 (25), 12085-12091.
- Kocian, R., Spahn, D.R., 2008. Haemoglobin, oxygen carriers and perioperative organ perfusion. *Best practice & research. Clinical anaesthesiology* 22 (1), 63-80.
- Komiyama, N.H., Miyazaki, G., Tame, J., Nagai, K., 1995. Transplanting a unique allosteric effect from crocodile into human haemoglobin. *Nature* 373 (6511), 244-246.
- Koshland, D.E., Jr., Nemethy, G., Filmer, D., 1966. Comparison of experimental binding data and theoretical models in proteins containing subunits. *Biochemistry* 5 (1), 365-385.
- Krafft, M.P., Riess, J.G., 2009. Chemistry, physical chemistry, and uses of molecular fluorocarbon--hydrocarbon diblocks, triblocks, and related compounds--unique "apolar" components for self-assembled colloid and interface engineering. *Chemical reviews* 109 (5), 1714-1792.
- Laing, R.W., Bhogal, R.H., Wallace, L., Boteon, Y., Neil, D.A.H., Smith, A., Stephenson, B.T.F., Schlegel, A., Hubscher, S.G., Mirza, D.F., Afford, S.C., Mergental, H., 2017. The Use of an Acellular Oxygen Carrier in a Human Liver Model of Normothermic Machine Perfusion. *Transplantation* 101 (11), 2746-2756.

- Lalezari, I., Lalezari, P., Poyart, C., Marden, M., Kister, J., Bohn, B., Fermi, G., Perutz, M.F., 1990. New effectors of human hemoglobin: structure and function. *Biochemistry* 29 (6), 1515-1523.
- Lamy, M.L., Daily, E.K., Brichant, J.F., Larbuisson, R.P., Demeyere, R.H., Vandermeersch, E.A., Lehot, J.J., Parsloe, M.R., Berridge, J.C., Sinclair, C.J., Baron, J.F., Przybelski, R.J., 2000. Randomized Trial of Diaspirin Cross-linked Hemoglobin Solution as an Alternative to Blood Transfusion after Cardiac Surgery. *Anesthesiology* 92, 646-656.
- Le Coeur, C., Combet, S., Carrot, G., Busch, P., Teixeira, J., Longeville, S., 2015. Conformation of the Poly(ethylene Glycol) Chains in DiPEGylated Hemoglobin Specifically Probed by SANS: Correlation with PEG Length and in Vivo Efficiency. *Langmuir : the ACS journal of surfaces and colloids* 31 (30), 8402-8410.
- Li, D., Hu, T., Manjula, B.N., Acharya, S.A., 2009. Extension arm facilitated pegylation of alphaalpha-hemoglobin with modifications targeted exclusively to amino groups: functional and structural advantages of free Cys-93(beta) in the PEG-Hb adduct. *Bioconjugate chemistry* 20 (11), 2062-2070.
- Li, D., Manjula, B.N., Acharya, A.S., 2006. Extension arm facilitated PEGylation of hemoglobin: correlation of the properties with the extent of PEGylation. *The protein journal* 25 (4), 263-274.
- Lui, F.E., Dong, P., Kluger, R., 2008. Polyethylene glycol conjugation enhances the nitrite reductase activity of native and cross-linked hemoglobin. *Biochemistry* 47 (40), 10773-10780.
- Lui, F.E., Kluger, R., 2009. Enhancing nitrite reductase activity of modified hemoglobin: bis-tetramers and their PEGylated derivatives. *Biochemistry* 48 (50), 11912-11919.
- Lukin, J.A., Kontaxis, G., Simplaceanu, V., Yuan, Y., Bax, A., Ho, C., 2003. Quaternary structure of hemoglobin in solution. *Proceedings of the National Academy of Sciences of the United States of America* 100 (2), 517-520.
- Mahboub, P., Aburawi, M., Karimian, N., Lin, F., Karabacak, M., Fontan, F., Tessier, S.N., Markmann, J., Yeh, H., Uygun, K., 2020. The efficacy of HBOC-201 in ex situ gradual rewarming kidney perfusion in a rat model. *Artificial organs* 44 (1), 81-90.

- Mallet, V., Dutheil, D., Polard, V., Rousselot, M., Leize, E., Hauet, T., Goujon, J.M., Zal, F., 2014. Dose-ranging study of the performance of the natural oxygen transporter HEMO2 Life in organ preservation. *Artificial organs* 38 (8), 691-701.
- Manjula, B.N., Malavalli, A., Smith, P.K., Chan, N.L., Arnone, A., Friedman, J.M., Acharya, A.S., 2000. Cys-93-beta-beta-succinimidophenyl polyethylene glycol 2000 hemoglobin A. Intramolecular cross-bridging of hemoglobin outside the central cavity. *The Journal of biological chemistry* 275 (8), 5527-5534.
- Manjula, B.N., Tsai, A., Upadhy, R., Perumalsamy, K., Smith, P.K., Malavalli, A., Vandegriff, K., Winslow, R.M., Intaglietta, M., Prabhakaran, M., Friedman, J.M., Acharya, A.S., 2003. Site-specific PEGylation of hemoglobin at Cys-93(beta): correlation between the colligative properties of the PEGylated protein and the length of the conjugated PEG chain. *Bioconjugate chemistry* 14 (2), 464-472.
- Manjula, B.N., Tsai, A.G., Intaglietta, M., Tsai, C.H., Ho, C., Smith, P.K., Perumalsamy, K., Kanika, N.D., Friedman, J.M., Acharya, S.A., 2005. Conjugation of multiple copies of polyethylene glycol to hemoglobin facilitated through thiolation: influence on hemoglobin structure and function. *The protein journal* 24 (3), 133-146.
- Marden, M.C., Bohn, B., Kister, J., Poyart, C., 1990. Effectors of hemoglobin. Separation of allosteric and affinity factors. *Biophysical journal* 57 (3), 397-403.
- McConachie, S., Wahby, K., Almadrahi, Z., Wilhelm, S., 2020. Early Experiences With PEGylated Carboxyhemoglobin Bovine in Anemic Jehovah's Witnesses: A Case Series and Review of the Literature. *Journal of pharmacy practice* 33 (3), 372-377.
- Meng, F., Kassa, T., Jana, S., Wood, F., Zhang, X., Jia, Y., D'Agnillo, F., Alayash, A.I., 2018. Comprehensive Biochemical and Biophysical Characterization of Hemoglobin-Based Oxygen Carrier Therapeutics: All HBOCs Are Not Created Equally. *Bioconjugate chemistry* 29 (5), 1560-1575.

- Miele, A.E., Bellelli, A., Brunori, M., 2013. Hemoglobin allostery: new views on old players. *Journal of molecular biology* 425 (9), 1515-1526.
- Misra, H., Bainbridge, J., Berryman, J., Abuchowski, A., Galvez, K.M., Uribe, L.F., Hernandez, A.L., Sosa, N.R., 2017. A Phase Ib open label, randomized, safety study of SANGUINATE in patients with sickle cell anemia. *Revista brasileira de hematologia e hemoterapia* 39 (1), 20-27.
- Mollan, T.L., Alayash, A.I., 2013. Redox reactions of hemoglobin: mechanisms of toxicity and control. *Antioxidants & redox signaling* 18 (17), 2251-2253.
- Monod, J., Wyman, J., Changeux, J.P., 1965. On the Nature of Allosteric Transitions: A Plausible Model. *Journal of molecular biology* 12, 88-118.
- Moore, E.E., Moore, F.A., Fabian, T.C., Bernard, A.C., Fulda, G.J., Hoyt, D.B., Duane, T.M., Weireter, L.J., Jr., Gomez, G.A., Cipolle, M.D., Rodman, G.H., Jr., Malangoni, M.A., Hides, G.A., Omert, L.A., Gould, S.A., PolyHeme Study, G., 2009. Human polymerized hemoglobin for the treatment of hemorrhagic shock when blood is unavailable: the USA multicenter trial. *Journal of the American College of Surgeons* 208 (1), 1-13.
- Mozzarelli, A., Bettati, S.E., 2011. *Chemistry and Biochemistry of Oxygen Therapeutics: From Transfusion to Artificial Blood*. John Wiley and Sons Ltd, Chichester, UK
- Mozzarelli, A., Rivetti, C., Rossi, G.L., Eaton, W.A., Henry, E.R., 1997. Allosteric effectors do not alter the oxygen affinity of hemoglobin crystals. *Protein science : a publication of the Protein Society* 6 (2), 484-489.
- Mozzarelli, A., Rivetti, C., Rossi, G.L., Henry, E.R., Eaton, W.A., 1991. Crystals of haemoglobin with the T quaternary structure bind oxygen noncooperatively with no Bohr effect. *Nature* 351 (6325), 416-419.
- Mozzarelli, A., Ronda, L., Faggiano, S., Bettati, S., Bruno, S., 2010. Haemoglobin-based oxygen carriers: research and reality towards an alternative to blood transfusions. *Blood transfusion = Trasfusione del sangue* 8 Suppl 3, s59-68.

Muirhead, H., Perutz, M.F., 1963. Structure of Haemoglobin. A Three-Dimensional Fourier Synthesis of Reduced Human Haemoglobin at 5-Å Resolution. *Nature* 199, 633-638.

Natanson, C., Kern, S.J., Lurie, P., Banks, S.M., Wolfe, S.M., 2008. Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death: a meta-analysis. *Jama* 299 (19), 2304-2312.

Noble, R.W., Rossi, G.L., Berni, R., 1972. A structural comparison of ligand-saturated hemoglobin with protoporphyrin globin. I. Reaction with bromothymol blue and sulfhydryl reagents. *Journal of molecular biology* 70 (3), 689-696.

Olofsson, C., Nygård, E.B., Ponzer, S., Fagrell, B., Przybelski, R., Keipert, P.E., Winslow, N., Winslow, R.M., 2008. A randomized, single-blind, increasing dose safety trial of an oxygen-carrying plasma expander (Hemospan) administered to orthopaedic surgery patients with spinal anaesthesia. *Transfusion medicine* 18 (1), 28-39.

Olofsson, C.I., Gorecki, A.Z., Dirksen, R., Kofranek, I., Majewski, J.A., Mazurkiewicz, T., Jahoda, D., Fagrell, B., Keipert, P.E., Hardiman, Y.J., Levy, H., Study Clinical, I., 2011. Evaluation of MP4OX for prevention of perioperative hypotension in patients undergoing primary hip arthroplasty with spinal anesthesia: a randomized, double-blind, multicenter study. *Anesthesiology* 114 (5), 1048-1063.

Olson, J.S., Eich, R.F., Smith, L.P., Warren, J.J., Knowles, B.C., 1997. Protein engineering strategies for designing more stable hemoglobin-based blood substitutes. *Artificial cells, blood substitutes, and immobilization biotechnology* 25 (1-2), 227-241.

Olson, J.S., Foley, E.W., Rogge, C., Tsai, A.L., Doyle, M.P., Lemon, D.D., 2004. No scavenging and the hypertensive effect of hemoglobin-based blood substitutes. *Free radical biology & medicine* 36 (6), 685-697.

Parak F, H.J., Nienhaus G U, 1988. Protein structural dynamics as determined by Mössbauer spectroscopy. *Hyperfine Interactions* 40, 147-157.

Pauling, L., 1935. The Oxygen Equilibrium of Hemoglobin and Its Structural Interpretation. *Proceedings of the National Academy of Sciences of the United States of America* 21 (4), 186-191.

Perutz, M.F., Bolton, W., Diamond, R., Muirhead, H., Watson, H.C., 1964. Structure of Haemoglobin. An X-Ray Examination of Reduced Horse Haemoglobin. *Nature* 203, 687-690.

Peterson, E.S., Shinder, R., Khan, I., Jucyszak, L., Wang, J., Manjula, B., Acharya, S.A., Bonaventura, C., Friedman, J.M., 2004. Domain-specific effector interactions within the central cavity of human adult hemoglobin in solution and in porous sol-gel matrices: evidence for long-range communication pathways. *Biochemistry* 43 (16), 4832-4843.

Pillai, A.S., Chandler, S.A., Liu, Y., Signore, A.V., Cortez-Romero, C.R., Benesch, J.L.P., Laganowsky, A., Storz, J.F., Hochberg, G.K.A., Thornton, J.W., 2020. Origin of complexity in haemoglobin evolution. *Nature* 581 (7809), 480-485.

Portoro, I., Kocsis, L., Herman, P., Caccia, D., Perrella, M., Ronda, L., Bruno, S., Bettati, S., Micalella, C., Mozzarelli, A., Varga, A., Vas, M., Lowe, K.C., Eke, A., 2008. Towards a novel haemoglobin-based oxygen carrier: Euro-PEG-Hb, physico-chemical properties, vasoactivity and renal filtration. *Biochimica et biophysica acta* 1784 (10), 1402-1409.

Powanda, D.D., Chang, T.M., 2002. Cross-linked polyhemoglobin-superoxide dismutase-catalase supplies oxygen without causing blood-brain barrier disruption or brain edema in a rat model of transient global brain ischemia-reperfusion. *Artificial cells, blood substitutes, and immobilization biotechnology* 30 (1), 23-37.

Premont, R.T., Reynolds, J.D., Zhang, R., Stamler, J.S., 2020. Role of Nitric Oxide Carried by Hemoglobin in Cardiovascular Physiology: Developments on a Three-Gas Respiratory Cycle. *Circulation research* 126 (1), 129-158.

Pusateri, A.E., Glassberg, E., Weiskopf, R.B., 2019. Reassessment of the Need for an Oxygen Carrier for the Treatment of Traumatic Hemorrhage When Blood is not an Option. *Shock* 52 (1S Suppl 1), 55-59.

- Rajesh, A., Zimmerman, D., Spivack, K., Abdulmalik, O., Elmer, J., 2018. Glutaraldehyde cross-linking increases the stability of *Lumbricus terrestris* erythrocytes. *Biotechnology progress* 34 (2), 521-528.
- Ratanasopa, K., Chakane, S., Ilyas, M., Nantasenamat, C., Bulow, L., 2013. Trapping of human hemoglobin by haptoglobin: molecular mechanisms and clinical applications. *Antioxidants & redox signaling* 18 (17), 2364-2374.
- Reeder, B.J., Grey, M., Silaghi-Dumitrescu, R.L., Svistunenko, D.A., Bulow, L., Cooper, C.E., Wilson, M.T., 2008. Tyrosine residues as redox cofactors in human hemoglobin: implications for engineering nontoxic blood substitutes. *The Journal of biological chemistry* 283 (45), 30780-30787.
- Rivetti, C., Mozzarelli, A., Rossi, G.L., Henry, E.R., Eaton, W.A., 1993. Oxygen binding by single crystals of hemoglobin. *Biochemistry* 32 (11), 2888-2906.
- Roamcharern, N., Payoungkiattikun, W., Anwised, P., Mahong, B., Jangpromma, N., Daduang, S., Klaynongsruang, S., 2019. Physicochemical properties and oxygen affinity of glutaraldehyde polymerized crocodile hemoglobin: the new alternative hemoglobin source for hemoglobin-based oxygen carriers. *Artificial cells, nanomedicine, and biotechnology* 47 (1), 852-861.
- Roberts, M.J., Bentley, M.D., Harris, J.M., 2002. Chemistry for peptide and protein PEGylation. *Advanced drug delivery reviews* 54 (4), 459-476.
- Ronda, L., Bruno, S., Abbruzzetti, S., Viappiani, C., Bettati, S., 2008. Ligand reactivity and allosteric regulation of hemoglobin-based oxygen carriers. *Biochimica et biophysica acta* 1784 (10), 1365-1377.
- Ronda, L., Bruno, S., Viappiani, C., Abbruzzetti, S., Mozzarelli, A., Lowe, K.C., Bettati, S., 2006. Circular dichroism spectroscopy of tertiary and quaternary conformations of human hemoglobin entrapped in wet silica gels. *Protein science : a publication of the Protein Society* 15 (8), 1961-1967.
- Ronda, L., Pioselli, B., Bruno, S., Faggiano, S., Mozzarelli, A., 2011. Electrophoretic analysis of PEGylated hemoglobin-based blood substitutes. *Analytical biochemistry* 408 (1), 118-123.

- Rumsey, S.C., Levin, A., 1998. Absorption, transport, and disposition of ascorbic acid in humans. *The Journal of Nutritional Biochemistry* 9 (3), 116-130.
- Sakai, H., Cabrales, P., Tsai, A.G., Tsuchida, E., Intaglietta, M., 2005. Oxygen release from low and normal P50 Hb vesicles in transiently occluded arterioles of the hamster window model. *American journal of physiology. Heart and circulatory physiology* 288 (6), H2897-2903.
- Sakai, H., Horinouchi, H., Tomiyama, K., Ikeda, E., Takeoka, S., Kobayashi, K., Tsuchida, E., 2001. Hemoglobin-vesicles as oxygen carriers: influence on phagocytic activity and histopathological changes in reticuloendothelial system. *The American journal of pathology* 159 (3), 1079-1088.
- Samuni, U., Dantsker, D., Juszczak, L.J., Bettati, S., Ronda, L., Mozzarelli, A., Friedman, J.M., 2004. Spectroscopic and functional characterization of T state hemoglobin conformations encapsulated in silica gels. *Biochemistry* 43 (43), 13674-13682.
- Samuni, U., Roche, C.J., Dantsker, D., Juszczak, L.J., Friedman, J.M., 2006. Modulation of reactivity and conformation within the T-quaternary state of human hemoglobin: the combined use of mutagenesis and sol-gel encapsulation. *Biochemistry* 45 (9), 2820-2835.
- Scatena, R., Giardina, B., 2001. O-raffinose-polymerised haemoglobin. A biochemical and pharmacological profile of an oxygen carrier. *Expert opinion on biological therapy* 1 (1), 121-127.
- Schiro, G., Cupane, A., 2007. Quaternary relaxations in sol-gel encapsulated hemoglobin studied via NIR and UV spectroscopy. *Biochemistry* 46 (41), 11568-11576.
- Sen Gupta, A., 2019. Hemoglobin-based Oxygen Carriers: Current State-of-the-art and Novel Molecules. *Shock* 52 (1S Suppl 1), 70-83.
- Shibayama, N., 2020. Allosteric transitions in hemoglobin revisited. *Biochimica et biophysica acta. General subjects* 1864 (2), 129335.
- Shibayama, N., Saigo, S., 1995. Fixation of the quaternary structures of human adult haemoglobin by encapsulation in transparent porous silica gels. *Journal of molecular biology* 251 (2), 203-209.
- Silkstone, G.G., Silkstone, R.S., Wilson, M.T., Simons, M., Bulow, L., Kallberg, K., Ratanasopa, K., Ronda, L., Mozzarelli, A., Reeder, B.J., Cooper, C.E., 2016. Engineering tyrosine electron transfer

pathways decreases oxidative toxicity in hemoglobin: implications for blood substitute design. *The Biochemical journal* 473 (19), 3371-3383.

Simoni, J., Simoni, G., Moeller, J.F., Feola, M., Wesson, D.E., 2014. Artificial oxygen carrier with pharmacologic actions of adenosine-5'-triphosphate, adenosine, and reduced glutathione formulated to treat an array of medical conditions. *Artificial organs* 38 (8), 684-690.

Simoni, J., Simoni, G., Wesson, D.E., Feola, M., 2012. ATP-adenosine-glutathione cross-linked hemoglobin as clinically useful oxygen carrier. *Current drug discovery technologies* 9 (3), 173-187.

Simons, M., Gretton, S., Silkstone, G.G.A., Rajagopal, B.S., Allen-Baume, V., Syrett, N., Shaik, T., Leiva-Eriksson, N., Ronda, L., Mozzarelli, A., Strader, M.B., Alayash, A.I., Reeder, B.J., Cooper, C.E., 2018. Comparison of the oxidative reactivity of recombinant fetal and adult human hemoglobin: implications for the design of hemoglobin-based oxygen carriers. *Bioscience reports* 38 (4).

Spivack, K., Tucker, M., Zimmerman, D., Nicholas, M., Abdulmalik, O., Comolli, N., Elmer, J., 2018. Increasing the stability of *Lumbricus terrestris* erythrocrucorin via poly(acrylic acid) conjugation. *Artificial cells, nanomedicine, and biotechnology* 46 (sup2), 1137-1144.

Strader, M.B., Alayash, A.I., 2017. Exploring Oxidative Reactions in Hemoglobin Variants Using Mass Spectrometry: Lessons for Engineering Oxidatively Stable Oxygen Therapeutics. *Antioxidants & redox signaling* 26 (14), 777-793.

Strader, M.B., Bangle, R., Parker Siburt, C.J., Varnado, C.L., Soman, J., Benitez Cardenas, A.S., Samuel, P.P., Singleton, E.W., Crumbliss, A.L., Olson, J.S., Alayash, A.I., 2017. Engineering oxidative stability in human hemoglobin based on the Hb providence (betaK82D) mutation and genetic cross-linking. *The Biochemical journal* 474 (24), 4171-4192.

Taguchi, K., Maruyama, T., Otagiri, M., 2020. Use of Hemoglobin for Delivering Exogenous Carbon Monoxide in Medicinal Applications. *Current medicinal chemistry* 27 (18), 2949-2963.

Thuillier, R., Dutheil, D., Trieu, M.T., Mallet, V., Allain, G., Rousselot, M., Denizot, M., Goujon, J.M., Zal, F., Hauet, T., 2011. Supplementation with a new therapeutic oxygen carrier reduces chronic fibrosis and organ dysfunction in kidney static preservation. *American journal of transplantation* :

official journal of the American Society of Transplantation and the American Society of Transplant Surgeons 11 (9), 1845-1860.

Tomita, D., Kimura, T., Hosaka, H., Daijima, Y., Haruki, R., Ludwig, K., Bottcher, C., Komatsu, T., 2013. Covalent core-shell architecture of hemoglobin and human serum albumin as an artificial O₂ carrier. *Biomacromolecules* 14 (6), 1816-1825.

Tsuneshige, A., Park, S., Yonetani, T., 2002. Heterotropic effectors control the hemoglobin function by interacting with its T and R states--a new view on the principle of allostery. *Biophysical chemistry* 98 (1-2), 49-63.

Vandegriff, K.D., Malavalli, A., Wooldridge, J., Lohman, J., Winslow, R.M., 2003. MP4, a new nonvasoactive PEG-Hb conjugate. *Transfusion* 43 (4), 509-516.

Vanholder, R., Dominguez-Gil, B., Busic, M., Cortez-Pinto, H., Craig, J.C., Jager, K.J., Mahillo, B., Stel, V.S., Valentin, M.O., Zoccali, C., Oniscu, G.C., 2021. Organ donation and transplantation: a multi-stakeholder call to action. *Nature reviews. Nephrology* 17 (8), 554-568.

Varnado, C.L., Mollan, T.L., Birukou, I., Smith, B.J., Henderson, D.P., Olson, J.S., 2013. Development of recombinant hemoglobin-based oxygen carriers. *Antioxidants & redox signaling* 18 (17), 2314-2328.

Varney, J., Rivera, A., Dong, V., Tieu, P., Zia, S., Huy, N.T., 2021. Mini-review on the properties and possible applications of therapeutic oxygen carrier Hemarina-M101. *Transfusion and apheresis science : official journal of the World Apheresis Association : official journal of the European Society for Haemapheresis* 60 (1), 103016.

Viappiani, C., Abbruzzetti, S., Ronda, L., Bettati, S., Henry, E.R., Mozzarelli, A., Eaton, W.A., 2014. Experimental basis for a new allosteric model for multisubunit proteins. *Proceedings of the National Academy of Sciences of the United States of America* 111 (35), 12758-12763.

Viappiani, C., Bettati, S., Bruno, S., Ronda, L., Abbruzzetti, S., Mozzarelli, A., Eaton, W.A., 2004. New insights into allosteric mechanisms from trapping unstable protein conformations in silica gels.

Proceedings of the National Academy of Sciences of the United States of America 101 (40), 14414-14419.

Walder, J.A., Zaugg, R.H., Walder, R.Y., Steele, J.M., Klotz, I.M., 1979. Diaspirins that cross-link beta chains of hemoglobin: bis(3,5-dibromosalicyl) succinate and bis(3,5-dibromosalicyl) fumarate. *Biochemistry* 18 (20), 4265-4270.

Wang, Q., Sun, L., Ji, S., Zhao, D., Liu, J., Su, Z., Hu, T., 2014. Reversible protection of Cys-93(beta) by PEG alters the structural and functional properties of the PEGylated hemoglobin. *Biochimica et biophysica acta* 1844 (7), 1201-1207.

Wang, Y., Zhang, S., Zhang, J., Yu, W.-L., Gao, D.-W., Wang, Q., You, G.-X., Hu, T., Zhao, L., Zhou, H., 2017. Structural, functional and physiochemical properties of dextran-bovine hemoglobin conjugate as a hemoglobin-based oxygen carrier. *Process Biochemistry* 60, 67-73.

Winslow, R.M., 2004. MP4, a new nonvasoactive polyethylene glycol-hemoglobin conjugate. *Artificial organs* 28 (9), 800-806.

Winslow, R.M., 2005. Targeted O₂ delivery by low-p50 hemoglobin: a new basis for hemoglobin-based oxygen carriers. *Artificial cells, blood substitutes, and immobilization biotechnology* 33 (1), 1-12.

Winslow, R.M., 2006a. Current status of oxygen carriers ('blood substitutes'): 2006. *Vox sanguinis* 91 (2), 102-110.

Winslow, R.M., Vandegriff, K., Intaglietta, M.E., 1996. *Blood substitutes: new challenges*. Springer.

Winslow, R.M.E., 2006b. *Blood substitutes*. Elsevier.

Winslow, R.M., 2008. Cell-free oxygen carriers: scientific foundations, clinical development, and new directions. *Biochimica et biophysica acta* 1784 (10), 1382-1386.

Wollocko, H., Anvery, S., Wollocko, J., Harrington, J.M., Harrington, J.P., 2017a. Zero-link polymerized hemoglobin (OxyVita((R))Hb) stabilizes the heme environment: potential for lowering vascular oxidative stress. *Artificial cells, nanomedicine, and biotechnology* 45 (4), 701-709.

Wollocko, H., Wollocko, B.M., Wollocko, J., Grzegorzewski, W., Smyk, L., 2017b. OxyVita((R))C, a next-generation haemoglobin-based oxygen carrier, with coagulation capacity (OVCCC). Modified lyophilization/spray-drying process: proteins protection. *Artificial cells, nanomedicine, and biotechnology* 45 (7), 1350-1355.

Yonetani, T., Park, S.I., Tsuneshige, A., Imai, K., Kanaori, K., 2002. Global allosteric model of hemoglobin. Modulation of O₂ affinity, cooperativity, and Bohr effect by heterotropic allosteric effectors. *The Journal of biological chemistry* 277 (37), 34508-34520.

Yonetani, T., Tsuneshige, A., 2003. The global allosteric model of hemoglobin: an allosteric mechanism involving homotropic and heterotropic interactions. *Comptes rendus biologies* 326 (6), 523-532.

Young, M.A., Riddez, L., Kjellstrom, B.T., Bursell, J., Winslow, F., Lohman, J., Winslow, R.M., 2005. MalPEG-hemoglobin (MP4) improves hemodynamics, acid-base status, and survival after uncontrolled hemorrhage in anesthetized swine. *Critical care medicine* 33 (8), 1794-1804.

Yuan, Y., Tam, M.F., Simplaceanu, V., Ho, C., 2015. New look at hemoglobin allostery. *Chemical reviews* 115 (4), 1702-1724.

Zhang, N., Jia, Y., Chen, G., Cabrales, P., Palmer, A.F., 2011. Biophysical properties and oxygenation potential of high-molecular-weight glutaraldehyde-polymerized human hemoglobins maintained in the tense and relaxed quaternary states. *Tissue engineering. Part A* 17 (7-8), 927-940.

Zhou, Y., Jia, Y., Buehler, P.W., Chen, G., Cabrales, P., Palmer, A.F., 2011. Synthesis, biophysical properties, and oxygenation potential of variable molecular weight glutaraldehyde-polymerized bovine hemoglobins with low and high oxygen affinity. *Biotechnology progress* 27 (4), 1172-1184.

Table 1. Oxygen properties of HBOCs.

HBOC name	p50 (torr)	Hill n	Refs.
Sanguinate	7-16	-	(Misra et al., 2014)
Oxyvita	6	1.1-1.2	(Harrington et al., 2011)
HemoAct	8-11	1.1	(Tomita et al., 2013)
Hemo2Life	7-12	-	(Mallet et al., 2014) (Varney et al., 2021)
Hemopure	40	1.4	(Jahr et al., 2008)
Oxyglobin	38	1.3	(Harris et al., 2008)
PolyHeme	26-32	1.5	(Gould et al., 1998)
Hemospan (MP4OX)	5-6	1.2	(Vandegriff et al., 2003)

Euro-PEG-Hb	14	1.5	(Portoro et al., 2008)
HemAssist	32	-	(Lamy et al., 2000)
Hemolink	30-40	1	(Greenburg and Kim, 2004)
Hb Polytaur	16-18	-	(Bobofchak et al., 2003)
Cross-linked Pegylated bHb	1 (R state PEG bHb) 20 (T state PEG bHb)	1 1	(Belcher et al, 2017) (Gu et al., 2021)

Reply to reviewers.

In normal character is the reviewer's comment; in bold, our reply and in red the text inserted in the revised manuscript

Reviewer #1: General Comments

This is an interesting review although perhaps a bit more comprehensive about the details of Hemoglobin Allostery rather than its relationship to the development of a hemoglobin based oxygen carrier. So, I think the area that will need major revision to make this paper more useful is to develop further the link between the oxygen affinity and new HBOC products. I think the questions that readers would like to know are the following

- 1 Why would you choose a particular oxygen affinity for a particular use of a product?
- 2 How would you go about designing such a product?

I think the authors have the expertise to answer these questions or at least say what the current state of play is in this area. The absence of such "joined up thinking" is currently the major flaw in this review. However, I think it is within the capabilities of the others to address this in an interesting way in a revised review.

First of all, we thank the reviewer for his/her valuable comments. We have removed the extensive part related to the Tertiary Two State (TTS) model, as, to our knowledge, no experimental work has been proposed to develop HBOCs on the basis of the different functional properties of alternative tertiary states, independent of quaternary conformation. Indeed, future activities should be directed along this line. Regarding the link between allosteric properties of Hb and HBOCs we have revised the text, highlighting studies that have addressed this issue, although the primary goal of many investigations was not to conserve allostery but to stabilize the tetramer, to reduce oxidation, and to obtain a defined oxygen affinity. As a matter of fact, no experimental answer has been provided to the first question: Why would you choose a particular oxygen affinity for a particular use of a product?.

We have inserted the following text:

“Key questions in the development of effective HBOCs are: i) is there a unique ideal oxygen affinity for an HBOC or should it be dependent on the specific HBOC clinical use? and ii) are tailored HBOC functional properties more “easily” achieved by chemical or genetic methods or by a combination of both methods? These questions are still awaiting a definite answer. As discussed in the next sections, developed HBOCs are endowed with oxygen affinity that can be higher, similar, or even lower than Hb oxygen affinity within RBCs (Table 1). This is somewhat surprising as all products are designed to deliver oxygen to tissues in hemorrhagic events, orthopedic surgery, ischemic episodes, or sickle cell crises. The rationale for a high affinity HBOC is that oxygen is released only to peripheral tissues where oxygen tension is low and no triggering of signals controlling vascular tension occurs due to oxygen release in the pre-peripheral circulation (Winslow, 2008). The rationale for HBOCs with an oxygen affinity close or lower than that of Hb in RBCs is that this oxygen affinity is physiologically

relevant for oxygen delivery to peripheral tissues (Jahr et al., 2008; Moore et al., 2009). Noteworthy to mention that for all products, allosteric properties are almost completely abolished, impairing the well-known critical function of Hb for oxygen release from lungs to tissues. Whereas genetic manipulation of Hb has demonstrated to be able to generate almost a continuum of individual functional properties of Hb (Olson et al., 1997), difficulties have raised due to the concomitant alteration of other properties, given the tight and complex interaction network of the protein matrix.“

I also have concerns that the literature review is a bit biased towards areas the authors are more comfortable with and/or more knowledgeable of. For example, there is a major gap in not addressing in more detail John Olson's extensive work on recombinant Hb and Mb, as this has proved so insightful in suggesting how you could alter the oxygen affinity or NO scavenging properties of haemoglobin. Also, I was surprised to see no mention of the product developed by Abraham Abuchowski - SANGUINATE. This PEGylated Hb is still (I think) in phase II clinical trials for sickle cell disease (as far as I know it is the only HBOC in current clinical trials though this seems to have stalled with no updates since 2018). Interestingly it is the CO form of bovine Hb that was being trialled. The use of HbCO and bovine Hb in an HBOC is a development the authors discuss in this review without mentioning the one current "live" product that uses this formulation. Sanguinate was also (like Hemopure) approved for compassionate use as a blood substitute in Jehovah's Witness patients (McConachie et al, 2020, Journal of Pharmacy Practice) and has been granted orphan drug status for treatment of sickle cell disease by the FDA. Other current "live" or semi-dormant GMP HBOC products in preclinical testing could at least be mentioned briefly in this review. These might include OxyVita, HbVesicles and HemoAct.

We fully agree with the reviewer that we missed citations of the extensive and valuable work carried out by Olson's group in pinpointing the role of individual amino acids in modulating Hb, as well as Mb, structural and functional properties. We have revised the manuscript quoting the most relevant papers by Olson and coworkers. We have also quoted HBOCs presently under investigation, all of them (with the exception of Sanguinate), still at the laboratory and preclinical levels. We have inserted the following texts:

“This variety of functional properties might be more easily achieved by engineering Hb via genetic approaches rather than chemical modifications (Figure 2B) (Benitez Cardenas et al., 2019; Cooper et al., 2020; Varnado et al., 2013). One caveat of genetic methods for HBOC production is the relative low yield of purified material from expression systems, leading to a high cost per unit of blood (Graves et al., 2008).”

“Hemopure® (Biopure, now HbO2 Therapeutics) (and its most recent version called HBOC-201), a glutaraldehyde-polymerized bovine Hb, exhibits an oxygen affinity lower than human RBCs (Jahr et

al., 2008). It has been authorized for compassionate use in patients that cannot be transfused for any clinical reason or refuse transfusions such as Jehovah's Witnesses. Furthermore, Hemopure® is approved in South Africa to treat anemia in adult surgical patients, and in Russia for acute anemia, irrespective of etiology. A partially purified Hemopure®, called Oxyglobin® was approved by FDA and EMA for veterinary use (Harris and Palmer, 2008). Hemopure® is also used in organ perfusion solutions (see 4.2).”

“A more recent approach consisted in the decoration of CO-bHb with PEG, leading to the product Sanguinate® (Abuchowski, 2016, 2017). Clinical trials were carried out for the use of Sanguinate® to treat sickle cell crises (Misra et al., 2017) and animal studies for the use as resuscitation fluid (Guerci et al., 2020). This product was granted the designation of orphan drug for sickle cell patients as well as compassionate use in severe anemia when transfusions were not possible or refused (McConachie et al., 2020). However, concern about the benefits of its use in such a complex clinical frame as sickle cell disease was raised (Alayash, 2017).

Recent studies (Gu et al., 2021) report on the development of a series of HBOCs obtained from cross-linked bHb PEGylated in the either the T or R state upon tangential flow filtration to separate high and low molecular weight forms. For both forms, the R state PEG bHb products exhibit p50s close to 1 torr, whereas the T state PEG bHb exhibits p50 of 20 torr. For all products, cooperativity is close to 1 (Belcher et al., 2017; Gu et al., 2021).

Another HBOC derived from purified bHb is the zero-linked polymeric hemoglobin, OxyVita®Hb obtained from the formation of pseudopeptide bonds between carboxyl groups and amino groups on the surface of bovine cross-linked tetrameric Hb molecules (Harrington et al., 2011). Studies are ongoing to investigate oxidation stability (Wollocko et al., 2017a) and formulation (Wollocko et al., 2017b).

A cluster was produced by cross-linking bHb with human albumin molecules, named HemoAct (Table 1) (Tomita et al., 2013). HemoAct safety, evaluated in rats, was found acceptable (Haruki et al., 2015). Using as crosslinking agent *N*-succinimidyl 3-maleimidopropionate (Funaki et al., 2019), a product with a p50 of 9 torr and a Hill coefficient of 1.4 was obtained, lower than the control HbA exhibiting a p50 of 23 torr and a Hill coefficient of 2.6. This suggests a stabilization of the R state due to constraints to the transition to the T state, as lysine residues were modified.”

“This lugworm Hb has a high molecular weight (3600 kDa), with a p50 of 7 mmHg (Mallet et al., 2014; Varney et al., 2021), and is prepared by the company Hemarina with the name Hemo2Life® M101. This product has been tested as a preservation fluid for storing healthy and marginal kidney before transplantation in a preclinical porcine model (Kaminski et al., 2019; Thuillier et al., 2011). “

Comments about specific sections of the text

Page 3

"undergoes dissociation to dimers that show increased propensity to autoxidation, absence of cooperativity, high oxygen affinity and reactivity with nitric oxide (NO)"

This sentence implies - although does not actually state - that Hb dimers react fast with NO. It is fast, but no faster than tetramers. Maybe this statement could be altered to make this clearer?

We have revised the text:

“In fact, cell-free Hb undergoes dissociation to dimers that show increased propensity to autoxidation, absence of cooperativity and high oxygen affinity. In addition, both Hb tetramers and dimers scavenge nitric oxide (NO) generated by endothelial cells causing hypertension.”

Page 11

"hemorrhagic events associated with childbirths one of the leading causes of death for African women".

This statement needs a reference. Also, it seems slightly, old fashioned, all-encompassing and (dare I say) colonial to refer to a whole continent which has widely diverse economics and healthcare. I think you need to be far more specific, both in terms of geography and the different healthcare economics in different countries.

We have revised the text:

“As reported by the World Health Organization’s Global Program for Blood Safety, out of the 118.5 million blood donations collected globally in 2018, 40% are from countries with high Human Development Index (HDI), where 16% of the world’s population live. In addition, 48 out of 171 countries do not possess a national blood policy (<https://www.who.int/news-room/fact-sheets/detail/blood-safety-and-availability>). The median blood donation rate in high-HDI countries is 31.5 donations per 1000 people. This compares with 15.9 donations per 1000 people in upper-middle HDI countries, 6.8 donations per 1000 people in lower-middle HDI countries, and five donations per 1000 people in low-HDI countries. For 62 countries fewer than 10 donations per 1000 people are reported and, among these, 34 countries are in the WHO African Region (<https://www.who.int/news-room/fact-sheets/detail/blood-safety-and-availability>).

"However, after more than fifty years of investigations, many preclinical and even phase III clinical trials have led to no FDA- and EMA-approved blood substitute"

Not strictly true as the PFC Fluosol was FDA approved as a blood substitute in 1989. In terms of HBOC, the EMA approved Oxyglobin as a Veterinary product in 2002

We have revised the text:

“The development of a blood substitute was initially thought to be easily achievable. However, more than fifty years of investigations, many preclinical, phase I, phase II and even phase III clinical trials have led to no FDA- and EMA-approved blood substitute (Dolgin, 2017). Nevertheless, two products, Hemopure® and Sanguinate® have received the FDA approval for compassionate use in patients that cannot receive or refuse transfusions (see below). In addition, a perfluorocarbon derivative, Fluosol-DA was approved by FDA in 1989 and used in Jehovah’s Witness patients (Krafft and Riess, 2009). Its production ended in 1994 due to lack of use and a second-generation of perfluorocarbon, Oxygent, proceeded through phase III trials but was never FDA licensed (Krafft and Riess, 2009).”

.”

And in a following section we have inserted the text:

“PEG was also used in the preparation of phospholipids bilayer vesicles containing purified HbA for the development of red cell mimics (Sakai et al., 2001). Hb vesicles exhibiting p50 of 8 and 29 torr were obtained by modulating the amount of pyridoxal 5-phosphate as allosteric effector. In an hamster model, it was found that vesicles containing higher affinity Hb led to a higher oxygen release (Sakai et al., 2005).“

Page 12

Figure 3 should be modified to include the view that heme loss may also be partly responsible for damage as it acts as a pro-inflammatory DAMP (damage associated molecular pattern)

Figure 3 (now Figure 2) has been modified according to reviewer's comment.

Page 13

If discussing altering betaCys93, I think you need to at least refer to the Stamler and Gladwin controversy re its SNO vs nitrite reductase activity.

We have revised the text:

“Residue Cys93(β) is also involved in NO binding under aerobic conditions. The physiological relevance of the SNO-Hb complex in hypoxia vasodilation via the reversible release of a SNO derivative during deoxygenation was proposed by Stamler (see for a review (Premont et al., 2020)). An alternate view, suggested by Gladwin (Gladwin, 2017) proposes the formation of NO from nitrite by deoxyHb, leading to hypoxic vasodilation. Furthermore, Cys93(β) is only reactive in the R state, thus blocking it by bulky groups hinders the R to T transition, stabilizing the R state and increasing oxygen affinity.”

.

Page 14

Section 2.1.2 I you need to be careful here when talking about high p50s at the same time as talking about high affinity. I would stick to one term so as not to confuse the reader or be crystal clear somewhere that a high p50 is low affinity and a low p50 is high affinity.

We have revised the text discussing changes in oxygen affinity and avoiding to confuse readers with changes of p50s. We have also inserted a table, Table 1, that reports values of oxygen affinity and Hill coefficient for the main developed HBOCs.

Page 15

"preclinical porcine model (Kaminski et al., 2019) as well as in clinical studies on kidneys."

Please provide a reference for the clinical study if it is not Kaminski et al. Otherwise put the reference at the end of the sentence if it refers to both the model and the clinical data

We have revised the text:

"This product has been tested as a preservation fluid for storing healthy and marginal kidney before transplantation in a preclinical porcine model ; Kaminski et al., 2019)."

Page 16

Based on the observation that specific residues might be responsible for the higher (Ratanasopa et al., 2016; Simons et al., 2018).

I am not sure these are the right references for these mutations. No beta subunit mutations were in either paper. I think the correct reference is likely to be one by John Olson (who also used the Providence mutation described in this paragraph much earlier than the Jana reference). Finally, the Simons paper does not conclude that recombinant foetal has higher autoxidation nor that it contributes to greater oxidative stress than adult Hb. It is in opposition to the Ratanasopa study in that respect (although Ratanasopa used native not recombinant proteins).

The Reviewer is absolutely right. These were wrong citations. We have revised the text:

"The extensive mutagenesis studies carried out by Olson and coworkers on Mb and Hb have allowed to pinpoint residues affecting oxygen affinity as well as oxidation stability (Benitez Cardenas et al., 2019; Dou et al., 2002). Fetal Hb (HbF) is more stable and less prone to autoxidation in comparison to HbA (Graves et al., 2008), suggesting its use as a precursor of HBOCs. Recombinant human fetal Hb and HbA were prepared and evaluated in terms of oxidative stress (Simons et al., 2018). Differences in oxidative reactivity were observed but neither form was not superior to the other for the preparation of effective HBOC (Simons et al., 2018)."

In the final paragraph you discuss Y42 and Y84. The former enhances ferryl reduction and the latter ferric reduction. So, the use of "particularly" is not appropriate. I think the section would benefit from a brief comment at the beginning that tyrosine residues in haemoglobin can function as electron transfer centers in their own right.

We have revised the text:

“Another oxidatively stable HBOC was prepared based on the observation that the naturally occurring K82D variant (Hb Providence) is more resistant towards oxygen peroxide degradation with respect to wild-type HbA (Strader et al., 2017). The effects of genetic cross-linking of Hb Providence on autoxidation, heme loss, and reactions with H₂O₂ were compared with HbA and found that cross-linking and mutation confer higher stability to oxidative reactions, thus suggesting that Hb Providence is a valuable source for the development of HBOCs (Strader et al., 2017). It was also found that in Hb Providence Cys93(β) is less prone to oxidation with respect to HbA (Jana et al., 2020).

HbA as well as Mb, under oxidative conditions, form ferryl heme iron and protein-based free radicals. Ferryl reduction can occur either via direct electron transfer from a reducing agent to heme edges or via a protein-based pathway that involves tyrosine residues (Reeder et al., 2008). Based on the observation that in HbA tyrosine Y42(α) is involved in the electron transfer between endogenous antioxidants and the ferryl heme, and that a tyrosine residue is absent in the equivalent position of the β subunits, the recombinant Hb variant F41Y(β) was designed to improve the reduction rates of β subunits (Silkstone et al., 2016). It was found that mutation decreases heme-mediated oxidative reactivity and enhances NO bioavailability (Silkstone et al., 2016). Several other variants introducing tyrosine residues in both subunits were generated to minimize Hb pro-oxidant activity, maintaining Hb oxygen affinity in a range compatible with oxygen delivery to tissues (Cooper et al., 2019). Among them, T84Y(β) variant was found to increase the rate of heme reduction by ascorbate, restoring Hb function. HEK cells showed reduced membrane damage upon treatment with the T84Y(β) variant in comparison to HbA (Cooper et al., 2019). When this variant was PEGylated and administered to mice, it was found to be retained in the circulation longer than PEGylated HbA (Cooper et al., 2019).”

Again paragraph 2 would benefit from citing the key Olson papers that showed that blood pressure changes correlate with rate of NO reactivity in different mutants. This strongly suggests that both the location AND the intrinsic reactivity of Hb are important.

We have revised the text:

“The reactivity of Hb with NO is also a concern in the design of HBOCs as cell-free Hb is prone to NO scavenging, which causes hypertension and also results in Hb oxidation (Alayash, 2019; Doherty et al., 1998; Eich et al., 1996; Olson et al., 2004). NO-induced oxidation was found to be similar in unmodified, polymerized, crosslinked, or PEGylated Hbs, suggesting that the NO scavenging effect observed for HBOCs is mostly associated with their extracellular localization rather than different reactivity towards NO (Meng et al., 2018). However, the thorough investigation of recombinant variants (Doherty et al., 1998; Fronticelli and Koehler, 2009; Frost et al., 2018; Graves et al., 2008) has indicated that some residues are particularly involved in the NO dioxygenase activity, opening the way to produce HBOCs based on recombinant Hbs endowed with lower reactivity towards NO (Doherty et al., 1998; Cooper et al., 2019).”

Page 18

"safe transfusions were available in emergency rooms (Dube et al., 2019)."

Though at the time I think Northfield argued the patient would benefit if offered Polyheme in the hospital, so this was the more ethical thing to do in the trial. Of course - in retrospect - this was a disastrous decision commercially and for the HBOC field as the pre hospital data showed a positive effect.

We agree that was a disastrous decision.

Page 19

"A key strategic weakness that eventually led to multiple failures was that each company had only one HBOC product in the pipeline, without any options to change or to optimize it on the basis of the results from clinical and lab studies."

I am not sure this is relevant for two reasons. First - and this is the major criticism of the study - Natanson lumped all HBOC together and so it is unlikely a second product would have helped with the regulatory process at the time. And secondly the US Navy did move on to a "secondary" product (to decrease NO scavenging as suggested by Natanson) by adding nitrite to the Biopure product (though this actually made things worse in preclinical studies).

We have deleted the sentence.

"As above reported, no HBOCs did gain approval for their primary role as a blood substitute".

None of the Natanson-criticised clinical trials - with the possible exception of Northfield - were explicitly seeking approval as a "blood substitute". Indeed, Sangart's trauma trials explicitly stated that RBC should be given in addition to MP4 according to standard best practice.

We have deleted the sentence.

page 20

See comments above about the HbCO product, Sanguinate.

We have revised the text reporting on Sanguinate product: “ In the case of Sanguinate®, manufactured as CO derivative and as such used for the treatment of sickle cell patients (see above, 2.1.3), the CO-Hb derivative was the only product developed and investigated (Abuchowski, 2016).

Reviewer #2: The manuscript by Faggiano et al. reports on the allosteric behaviour of hemoglobin and the possible chemical modifications to adapt this behaviour to the necessities of an oxygen carrying resuscitation fluid. The logical approach of the authors is sound, but the relationships between the first and second part of the manuscript may be improved, e.g. the effects of the chemical modifications described are discussed qualitatively, rather than in the very quantitative terms used to describe the details of hemoglobin oxygenation. Indeed some of the experiments and hypotheses described in the first section of the manuscript, like the "tertiary two state model" do not find any application in the second section. I understand that many of the preparations tested as blood replacement do not lend themselves to the refined experiments necessary to evaluate which allosteric model provides a strict agreement with the experimental data; but, if so, it is unclear why these models should be part of the manuscript.

We have removed the extensive part related to the Tertiary Two State (TTS) model, as, to our knowledge, no experimental work has been proposed to develop HBOCs on the basis of the different functional properties of alternative tertiary states, independent of quaternary conformation. Indeed, future activities should be directed along this line. Regarding the link between allosteric properties of Hb and HBOCs we have revised the text highlighting studies that have addressed this issue, although the primary goal of many investigations was not to conserve allostery but to stabilize the tetramer, to reduce oxidation, and to obtain a defined oxygen affinity.

Regarding the effects of the chemical modifications that are “discussed qualitatively, rather than in the very quantitative terms used to describe the details of hemoglobin oxygenation”, we feel that a clear, quantitative relationship between chemical modifications and oxygen affinity is still missing and a trial-and-error approach has been used. One attempt was carried out by us when comparing PEGylated Hb obtained under oxy or deoxy conditions (Caccia et al., 2009). It was found that PEGylation of Hb under oxy conditions generates a dimeric high oxygen affinity, non-cooperative product, whereas PEGylation under deoxy conditions generates a tetrameric product with oxygen affinity and cooperativity closer to HbA within RBCs. This experiment is reported in the manuscript. In order to provide readers quantitative information on oxygen affinities and cooperativity properties of HBOCs we have inserted a Table, Table 1, that reports values of the main developed HBOCs.

From Hemoglobin Allostery to Hemoglobin-Based Oxygen Carriers

Serena Faggiano^{1,2}, Luca Ronda^{2,3}, Stefano Bruno¹, Stefania Abbruzzetti⁴,
Cristiano Viappiani⁴, Stefano Bettati^{2,3,5} and Andrea Mozzarelli^{1, 2*}

¹Department of Food and Drug, University of Parma, Parma, Italy

²Institute of Biophysics, National Research Council, Pisa, Italy

³Department of Medicine and Surgery, University of Parma, Parma, Italy

⁴Department of Mathematical, Physical and Computer Sciences, University of Parma, Parma, Italy

⁵National Institute of Biostructures and Biosystems, Rome, Italy

*Corresponding author

E-mail: andrea.mozzarelli@unipr.it

Full address: Institute of Biophysics, National Research Council, Via Moruzzi 1, 56124 Pisa, Italy

Keywords: hemoglobin, hemoglobin-based oxygen carriers, blood substitutes, allostery, cooperativity, oxygen transport

Abstract. Hemoglobin (Hb) plays its vital role through structural and functional properties evolutionarily optimized to work within red blood cells. i.e., the tetrameric assembly, well-defined oxygen affinity, positive cooperativity, and heterotropic allosteric regulation by protons, chloride and 2,3-diphosphoglycerate. Outside red blood cells, the Hb tetramer dissociates into dimers, which exhibit high oxygen affinity and neither cooperativity nor allosteric regulation. They are prone to extravasate, thus scavenging endothelial NO and causing hypertension, and cause nephrotoxicity. In addition, they are more prone to autoxidation, generating radicals. The need to overcome the adverse effects associated with cell-free Hb has always been a major hurdle in the development of substitutes of allogeneic blood transfusions for all clinical situations where blood is unavailable or cannot be used due to, for example, religious objections. This class of therapeutics, indicated as hemoglobin-based oxygen carriers (HBOCs), is formed by genetically and/or chemically modified Hbs. Many efforts were devoted to the exploitation of the wealth of biochemical and biophysical information available on Hb structure, function, and dynamics to design safe HBOCs, overcoming the negative effects of free plasma Hb. Unfortunately, so far, no HBOC has been approved by FDA and EMA, except for compassionate use. However, the unmet clinical needs that triggered intensive investigations more than fifty years ago are still awaiting an answer. Recently, HBOCs “repositioning” has led to their successful application in organ perfusion fluids.

1. Introduction. The structure, dynamics, function, and regulation of proteins are evolutionarily optimized to work in specific cell compartments, and their improper localization can lead to toxicity. In this respect, hemoglobin (Hb) is paradigmatic, as oxygen affinity, tetramer stability, oxidation state and cooperativity are perfectly tailored to work as an oxygen carrier within red blood cells (RBCs), but wholly unsuitable for this function when free in the plasma, where these properties are profoundly altered. In fact, cell-free Hb undergoes dissociation to dimers that show increased propensity to autoxidation, absence of cooperativity and high oxygen affinity. In addition, both free Hb tetramers and dimers scavenge nitric oxide (NO) generated by endothelial cells, causing hypertension. *In vivo*, Hb dimers released from aged RBCs are trapped by haptoglobin and directed to degradation by the reticuloendothelial system (di Masi et al., 2020).

The compartment-specific activity of Hb has been investigated in the attempt to design Hb-based oxygen carriers (HBOCs), protein therapeutics intended to deliver oxygen to tissues as an alternative to RBC transfusions for “oxygen therapeutics”. To efficiently work in the plasma and to avoid adverse effects, HBOCs were developed using either chemically and/or genetically modified HbA to alter the functional and structural properties of unmodified Hb. The fine-tuning of these properties has relied on the enormous amount of information arisen by the decades-long effort to dissect the complex reactivity and conformational flexibility of Hb. Indeed, Hb and its monomeric homolog myoglobin (Mb) were investigated in detail in the 20th century, triggering the development of several biophysical and biochemical methods that later became generally available to understand protein structure, dynamics, and function. These include i) x-ray protein crystallography, pursued by Max Perutz and John Kendrew (Kendrew et al., 1960), ii) stopped-flow techniques, pursued by Quentin Gibson (Gibson and Roughton, 1955), laser flash photolysis, exploited by William Eaton and coworkers (Henry et al., 1997; Hofrichter et al., 1983) and Mossbauer spectroscopy, pursued by Fritz Parak (Parak, 1988). This vast body of experimental work was cleverly and clearly reviewed and presented in the Antonini and Brunori book in 1971 (Antonini and Brunori, 1971). For Hb and Mb scientists, this book has been the “Bible” for the last 50 years. In our laboratory, we have a single

copy, acquired by Prof. Gian Luigi Rossi when he carried out kinetic studies on HbA reactivity in collaboration with Bob Noble (Noble et al., 1972). The book was a unique source of information and data comparison for many of us. More recent valuable sources on Hb are Imai's book on "Allosteric Effects in Hemoglobin", published in 1982 (Imai, 1982), and the Bunn and Forget book on "Hemoglobin: Molecular, Genetic and Clinical Aspects", published in 1986 (Bunn and Forget, 1986). In addition, Hb allostery has been discussed in several reviews appeared over the years (Bellelli and Brunori, 2011; Eaton et al., 2007; Eaton et al., 1999; Miele et al., 2013). Since the inspiring Antonini and Brunori book was published, Hb has been further investigated with a kaleidoscopic pattern of different biochemical and biophysical techniques, leading to a structural and functional characterization unparalleled to that reached for any other protein. In addition, both Hb and Mb have been genetically engineered for the understanding of the role of almost each amino acid in dictating their role in structural and functional properties and for tailoring reactivity towards oxygen, NO, oxidative agents in the development of HBOCs (Dou et al., 2002; Olson et al., 1997).

In this review, we will describe the structural and functional properties of Hb as an oxygen carrier, with the aim to underline the molecular features that need to be modified for the design of HBOCs. We will show that protein engineering and chemical modifications have allowed to develop HBOCs with oxygen-carrying properties suitable for working outside RBCs. Depending on the fine-tuning of their properties, these products have been proposed for different applications, with recent examples of "repositioning" as solutions for organ preservation and plasma expander CO carriers. Therefore, Hb represents a clear example of how the detailed structural and functional characterization of a protein, apparently well beyond the needs of biotechnological applications, turned out to be an invaluable starting point to design life-saving therapeutics.

1.1. Hemoglobin structure, a brief outlook. Hb is a tetramer formed by two homologous subunits, referred to as α and β chains (Figure 1). The β chains contain eight helices (numbered A to H), while

the α chains have only seven. Each monomer contains a heme moiety. An Fe^{2+} ion at the center of the tetrapyrrole ring is coordinated to a His residue, called proximal His, and reversibly binds oxygen. This event triggers a series of tertiary conformational changes that destabilize the deoxyHb structure. The HbA tetramer exhibits two distinct quaternary states, called T, for "tense", and R, for "relaxed", with the former stabilized by salt bridges and thermodynamically favored in the absence of oxygen, and the latter thermodynamically favored when oxygen is bound (Figure 1). Functional properties of Hb depend on the relative population of quaternary states as well as on the relative populations of tertiary states endowed with different reactivity, t and r (see chapter 1.2 below) (Henry et al., 2002).

Studies by Eaton (Eaton, 1980) and Thornton and colleagues (Pillai et al., 2020) have dissected the Hb structure and have assigned specific functional roles to each globin portion, and, in

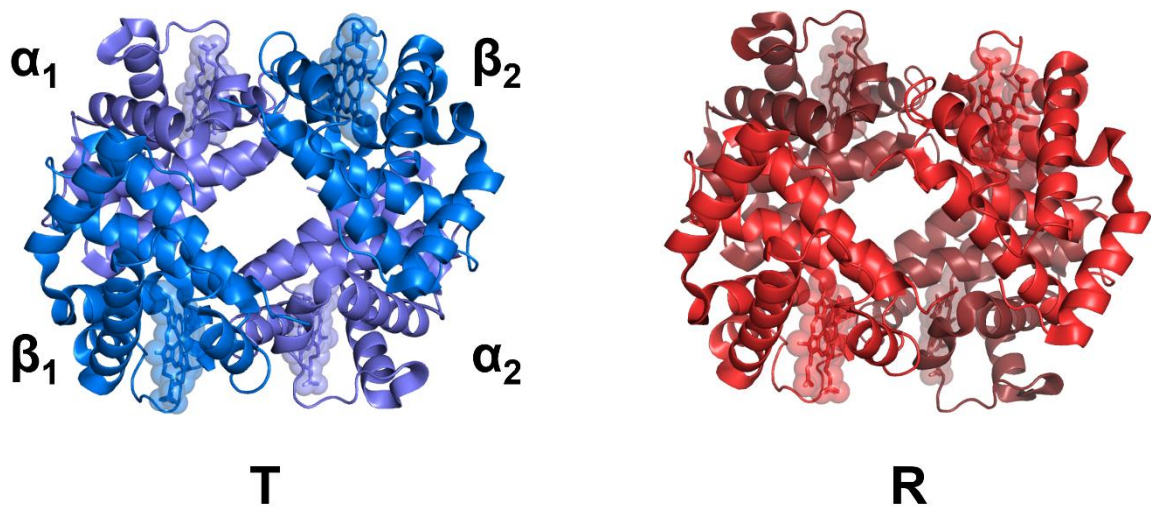


Figure 1. Structure of tetrameric deoxy T-state (left) and oxy R-state HbA (right). Subunits are shown with different colors.

parallel, to the three exons contained in the globin genes. Specifically, the amino acids associated with the three coding sequences are distributed to each of Hb functions: i) heme contacts essential for oxygenation, ii) α_1 - β_2 contacts essential for cooperative dimer, iii) α_1 - β_1 contacts essential for cooperative tetramer, iv) Bohr effect, essential for the modulation of oxygen loading and unloading from lung to tissues, and v) 2,3-diphosphoglycerate (DPG) binding, essential for oxygen affinity regulation (Eaton, 1980). Furthermore, it was shown that tetrameric Hb evolved from an ancient

monomer and a more recent noncooperative homodimer with high oxygen affinity. Two surface substitutions are sufficient to markedly reduce oxygen affinity and to confer cooperativity, suggesting a linkage between the oxygen binding site and the oligomerization interface (Pillai et al., 2020).

1.2. Hemoglobin allostery: state of the art. Hb function is under two allosteric regulations: a homotropic effect associated with the cooperative oxygen binding, and heterotropic effect, mediated by binding of effectors (e.g.: protons, CO₂, chloride ions and organic phosphates) at sites other than the heme pocket. The latter accounts for different physiological roles of Hb, beyond oxygen transport from the lungs to peripheral tissues, contributing to control pH and scavenging of CO₂. A so complex behavior intrigued scientists, fascinated by the idea of building a comprehensive model able to describe and predict how homotropic and heterotropic effectors modulate Hb ligand binding properties. Both mechanisms of regulation impact on Hb function *in vivo* and their understanding is key for the design and optimization of HBOCs with tailored properties.

Historically, homotropic effects have attracted earlier attention, starting from the sequential model originally proposed by Linus Pauling in the 1930s (Pauling, 1935) and later developed by Koshland, Nemethy and Filmer (Koshland et al., 1966). Sequential models predict continuous affinity changes upon binding of subsequent oxygen molecules to the same tetramer, implying cooperativity within the same quaternary state. On the contrary, concerted models assume a saturation-dependent equilibrium between pre-existing high and low-affinity states with perfect functional symmetry among the four subunits within a tetramer, which would exclude cooperativity in the absence of quaternary relaxations. The most famous concerted model is that proposed by Monod, Wyman, and Changeux (MWC) (Monod et al., 1965), largely motivated by the pioneer structural studies of Perutz and coworkers showing a different quaternary arrangement of fully oxygenated and deoxygenated subunits (Muirhead and Perutz, 1963; Perutz et al., 1964). Other models were proposed over the years, including the Ackers "Symmetry Code" implying cooperativity within the T state (Ackers et al.,

1992). Since all these models well describe the observed oxygen binding curves, the characterization of single Hb conformations by blocking or slowing down conformational transitions resulted a valuable approach to validate allosteric models. Controversies among models were settled in favor of MWC mainly thanks to oxygen binding studies in T-state Hb crystals carried out by Eaton, Mozzarelli and coworkers, showing lack of cooperativity in the absence of quaternary relaxations (Mozzarelli et al., 1997; Mozzarelli et al., 1991; Rivetti et al., 1993). Starting from the mid '90s, even more convincing evidence in favor of the concerted model arose from functional studies carried out by several groups on Hb encapsulated in wet, nanoporous silica gels (Bettati and Mozzarelli, 1997; Bruno et al., 2001; Jones et al., 2012; Jones et al., 2014; Juszczak and Friedman, 1999; Khan et al., 2000; Samuni et al., 2004; Samuni et al., 2006; Schiro and Cupane, 2007; Shibayama and Saigo, 1995). Encapsulation in silica gel offers two major advantages over crystallization. First of all, conformational changes are not hampered, but slowed down by orders of magnitude, with uncoupling of tertiary and quaternary relaxations that allow to interrogate the spectroscopic properties of intermediate conformations by UV-vis absorbance, Raman, near-infrared and circular dichroism spectroscopy (Das et al., 1999; Khan et al., 2000; Ronda et al., 2006; Schiro and Cupane, 2007; Viappiani et al., 2004). Moreover, in the absence of crystal lattice constraints, tertiary effects induced by heterotropic regulators are maintained (Bettati and Mozzarelli, 1997; Bruno et al., 2001; Viappiani et al., 2004). Overall, no cooperativity in oxygen binding was observed within the T and R quaternary state for Hb gels. However, the observation of functional heterogeneity within the T state, extensively investigated by Yonetani, Poyart and others (Ackers et al., 1992; Kister et al., 1987; Lalezari et al., 1990; Marden et al., 1990; Tsuneshige et al., 2002; Yonetani et al., 2002; Yonetani and Tsuneshige, 2003), raises the issue of a long-known deficiency of the MWC model, which postulates symmetry of the four subunits. This would allow heterotropic effectors to act on the equilibrium between T and R quaternary states, but with no effect on their respective equilibrium constant for oxygen binding, K_T and K_R . It should be noted that by the mid '60s, when Monod and coworkers formulated their model, heterotropic effects on Hb had been only partially investigated, and that the authors were

perfectly aware of the over-simplification of their model, which was just intended to explain in the easiest, more elegant and general way the cooperative behavior of multi-subunit proteins.

Over the last decades, the wide functional range of T and R conformations has been described in terms of continuous or discrete distributions of tertiary species accommodated within the two individual quaternary states (Bettati et al., 1998; Friedman, 1985; Lukin et al., 2003; Peterson et al., 2004; Rivetti et al., 1993; Yonetani et al., 2002; Yonetani and Tsuneshige, 2003). These findings motivated Eaton and coworkers to propose an extension of the MWC model, the Tertiary-Two State (TTS) model (Henry et al., 2002). According to the TTS, oxygen and heterotropic effectors can modulate the equilibrium between pre-existing, low- and high-reactivity tertiary conformations (called *t* and *r*, respectively), that populate both quaternary states. The tertiary conformation is the only determinant of oxygen affinity of individual subunits. Ligation and the R structure favor the highly reactive *r* state (significantly populated in liganded R, but also liganded T), while T state and negative heterotropic effectors favor *t* (significantly populated in deoxygenated T, but also deoxygenated R). Cooperativity still arises from the quaternary equilibrium, thus preserving the fundamental postulate of MWC (no cooperative effects are present within T and R conformations), but oxygen affinity can span the same range in T and R. The TTS model received experimental support by CO rebinding kinetic experiments on Hb gels (Viappiani et al., 2004; Viappiani et al., 2014; Henry et al., 2015). It was shown that only two functionally different conformations of the subunits exist both in unliganded and liganded states, regardless the quaternary structures.

As for the structural basis of functional differences between high and low affinity states, these have been proposed to be mediated by the so-called "allosteric core", the region containing the proximal His, the FG corner and part of the F helix that translates structural changes following heme ligation to the $\alpha 1$ - $\beta 2$ and $\alpha 2$ - $\beta 1$ interface (Gelin et al., 1983). Although different structural and dynamic bases have been put forward over the decades to explain Hb allostery (recently reviewed, e.g., in (Henry et al., 2015), by Shibayama (Shibayama, 2020) and Ho and coworkers (Yuan et al.,

2015)), an ultimate description still awaits the resolution of key missing structures: those of oxygenated and deoxygenated Hb in the high-affinity T state (deoxy(Tr) and oxy(Tr) in the framework of the TTS model) and low-affinity R state (deoxy(Rt) and oxy(Rt)). Progress in cryo-electron microscopy, with the great advantage over crystallographic techniques to make feasible solving structures of heterogeneous conformations, might allow to tackle this issue in the future.

The relevant conclusion from these studies for the development of an HBOC is that Hb oxygen affinity can be modulated by altering the equilibrium distribution of *r* and *t* tertiary states within each quaternary state. However, so far, this key observation has not yet been exploited for the development of tailored HBOCs.

2. Towards Hemoglobin-based Oxygen Carriers: chemical and genetic strategies to modulate oxygen affinity, allostery, oxidation propensity, and NO reactivity. In the last decades, artificial oxygen carriers have been developed to tackle clinical needs not met by allogeneic blood transfusions, and, particularly, to treat: i) hemorrhagic events outside a hospital setting, ii) patients that refuse human blood for religious reasons, iii) patients that are immunoreactive to all blood types, iv) pre-term babies that need transfusions with oxygen properties different from adult blood, and v) patients that need transfusions in Third World countries where healthy blood donors and national blood banks are rare. As reported by the World Health Organization's Global Program for Blood Safety, out of the 118.5 million blood donations collected globally in 2018, 40% are from countries with high Human Development Index (HDI), where 16% of the world population live. In addition, 48 out of 171 countries do not possess a national blood policy (<https://www.who.int/news-room/fact-sheets/detail/blood-safety-and-availability>). The median blood donation rate in high-HDI countries is 31.5 donations per 1000 people. This compares with 15.9 donations per 1000 people in upper-middle HDI countries, 6.8 donations per 1000 people in lower-middle HDI countries, and five donations per 1000 people in low-HDI countries. For 62 countries fewer than 10 donations per 1000 people are reported and, among

these, 34 countries are in the WHO African Region (<https://www.who.int/news-room/fact-sheets/detail/blood-safety-and-availability>).

The development of a blood substitute was initially thought to be easily achievable. However, more than fifty years of investigations, many preclinical, phase I, phase II and even phase III clinical trials have led to no FDA- and EMA-approved blood substitute (Dolgin, 2017). Nevertheless, two products, Hemopure® and Sanguinate® have received the FDA approval for compassionate use in patients that cannot receive or refuse transfusions (see below). In addition, a perfluorocarbon derivative, Fluosol-DA was approved by FDA in 1989 and used in Jehovah's Witness patients (Krafft and Riess, 2009). Its production ended in 1994 due to lack of use and a second-generation of perfluorocarbon, Oxygent, proceeded through phase III trials but was never FDA licensed (Krafft and Riess, 2009).

Failures of hemoglobin-based oxygen carriers are due to two main issues: i) HbA works properly only when confined within RBCs, and ii) the allosteric properties are associated with the integrity of the tetrameric state and the presence of allosteric effectors. Both these conditions are lost when HbA is free in the plasma, where no allosteric effectors are present, and diluted Hb dissociates into dimers that exhibit high affinity and no cooperativity. In addition, dimeric HbA free in the plasma undergoes accelerated oxidation due also to heme loss, generating oxygen radicals, which in turn cause oxidative stress. HbA oxidation in the plasma is not counterbalanced by the enzymatic systems present within RBCs. Moreover, cell-free HbA extravasates easily, scavenging endothelial NO, and thus causing hypertension. Finally, HbA as a dimer, in contrast to tetramers, is filtered in the renal glomerulus, causing nephrotoxicity (Figure 2A).

To overcome limitations, different approaches of Hb modification have been exploited, including protein decoration with polyethylene glycol (PEG) to increase the hydrodynamic radius and avoid extravasation, intermolecular crosslinking to prevent tetramer dissociation, encapsulation in synthetic envelopes or different nano- or microarchitectures (Figure 2B) (Bobofchak et al., 2003; Devineau et al., 2018; Inayat et al., 2006; Jahr et al., 2012; Jansman and Hosta-Rigau, 2018; Kocian and Spahn,

2008; Meng et al., 2018; Mozzarelli et al., 2010; Winslow, 2006a). The development of the first HBOCs aimed at mimicking the properties of RBCs in terms of oxygen binding, autoxidation rate, and reactivity with NO. More recently, HBOCs have been considered as "oxygen therapeutics" for emergency treatment, and it is now debated whether functional properties close to those of RBCs are clinically relevant (Belcher et al., 2018; Dolgin, 2017; Mozzarelli et al., 2010). Nevertheless, the modulation of the oxygen-binding properties, autoxidation rates, and NO reactivity is still an active field of investigation to obtain HBOCs endowed with different functional properties, possibly to be used for specific clinical applications and therapeutic treatments. This variety of functional properties might be more easily achieved by engineering Hb via genetic approaches rather than chemical modifications (Figure 2B) (Benitez Cardenas et al., 2019; Cooper et al., 2020; Varnado et al., 2013).

One caveat of genetic methods for HBOC production is the relative low yield of purified material from expression systems, leading to a high cost per unit of blood (Graves et al., 2008).

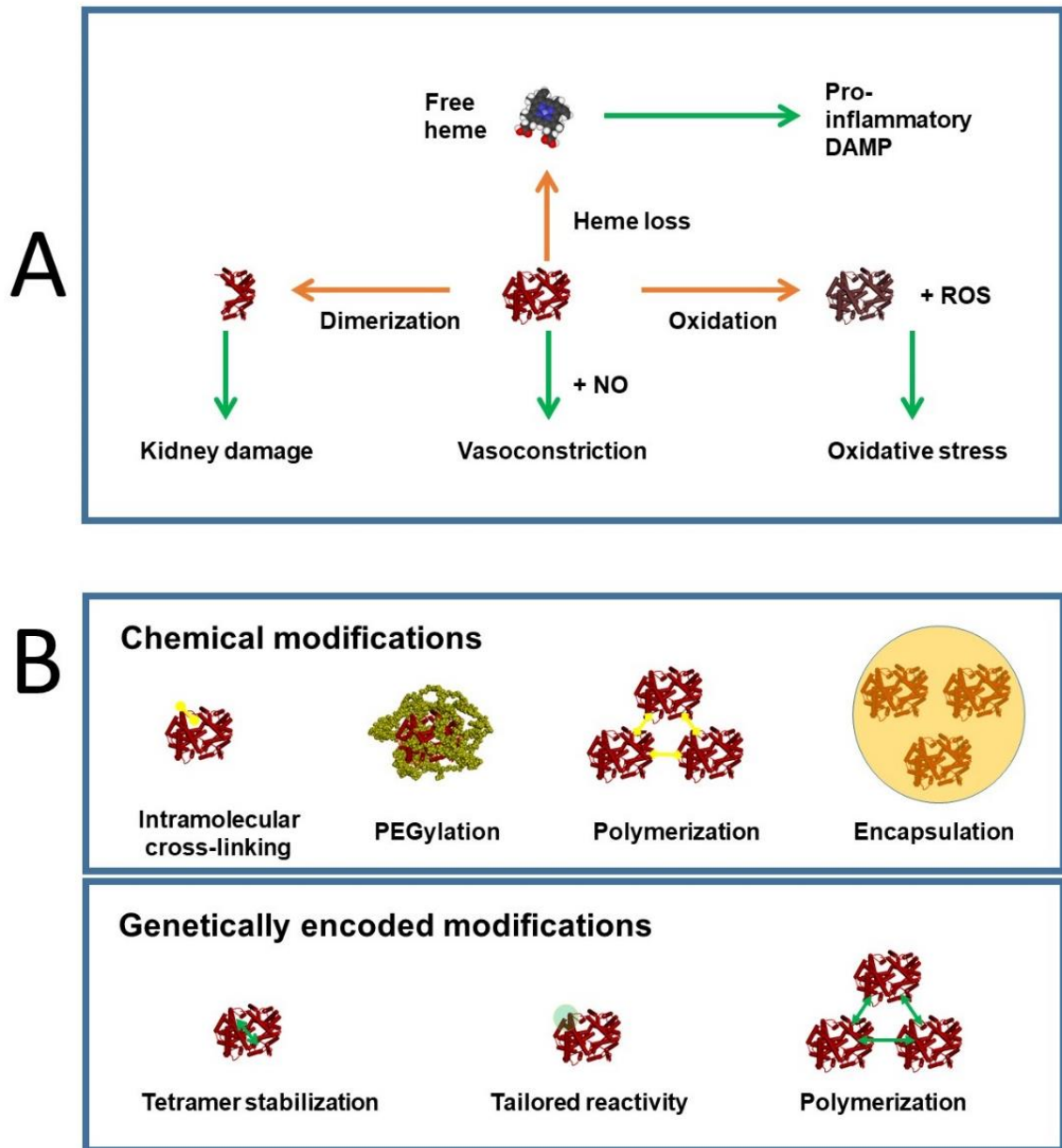


Figure 2: A. Potential toxic effects caused by cell-free Hb. B. Strategies for the development of HBOCs: chemical and genetically encoded modifications to reduce dimer formation, increase of the overall size of the protein or modification of Hb reactivity towards NO.

Key questions in the development of effective HBOCs are: i) is there a unique ideal oxygen affinity for an HBOC or should it be dependent on the specific HBOC clinical use? and ii) are tailored HBOC

functional properties more “easily” achieved by chemical or genetic methods or by a combination of both methods? These questions are still awaiting a definite answer. As discussed in the next sections, developed HBOCs are endowed with oxygen affinity that can be higher, similar, or even lower than Hb oxygen affinity within RBCs (Table 1). This is somewhat surprising as all products are designed to deliver oxygen to tissues in hemorrhagic events, orthopedic surgery, ischemic episodes or sickle cell crises. The rationale for a high affinity HBOC is that oxygen is released only to peripheral tissues where oxygen tension is low and no triggering of signals controlling vascular tension occurs due to oxygen release in the pre-peripheral circulation (Winslow, 2008). The rationale for HBOCs with an oxygen affinity close or lower than that of Hb in RBCs is that this oxygen affinity is physiologically relevant for oxygen delivery to peripheral tissues (Jahr et al., 2008; Moore et al., 2009). Noteworthy to mention that for all products, allosteric properties are almost completely abolished, impairing the well-known critical function of Hb for oxygen release from lungs to tissues. Whereas genetic manipulation of Hb has demonstrated to be able to generate almost a continuum of individual functional properties of Hb (Olson et al., 1997), difficulties have raised due to the concomitant alteration of other properties, given the tight and complex interaction network of the protein matrix.

2.1. Modulation of the oxygen-binding properties. Several modification chemistries of Hb have tackled the need to control the oxygen-binding properties of HBOCs by modulating the tertiary and quaternary conformational equilibria of the tetramer.

2.1.1 Control of oxygen binding properties in PEGylated HBOCs. HBOCs consisting of PEGylated Hbs take advantage of the extensive use of polyethylene glycol (PEG) to conjugate protein therapeutics and increase their half-life and reduce extravasation (Roberts et al., 2002). PEGylation of Hb yields products endowed with significantly different oxygen binding properties, depending on the chemistry of conjugation, the ligation state, and the residues involved in the modification (Table 1). Several researchers have worked towards the characterization of all these variables, including

Acharya and coworkers (Li et al., 2006; Manjula et al., 2000; Manjula et al., 2005), Winslow and coworkers (Winslow, 2004; Young et al., 2005), Perrella and coworkers (Caccia et al., 2009; Iafelice et al., 2007; Portoro et al., 2008), Kluger and coworkers (Lui et al., 2008; Lui and Kluger, 2009), and our group (Alomari et al., 2018; Portoro et al., 2008; Ronda et al., 2011).

The PEGylation chemistry employed in the preparation of HBOCs has proven crucial in modulating oxygen affinity, mainly because it dictates which residues are modified. As direct PEGylation of HbA with urethane linkages produces extensive PEGylation with little control over the oxygen-binding properties (Bradley et al., 1994), an approach consisting in the thiolation of amino groups followed by their PEGylation was later introduced (Li et al., 2009; Li et al., 2006). The resulting HBOCs exhibited oxygen binding properties that depend on whether PEGylation is carried out under aerobic or anaerobic conditions. A drawback of thiol-directed chemistries is that the PEGylation of residue Cys93(β) was suggested to perturb the heme-binding pocket. For this reason, its protection was pursued through several chemical approaches, leading to different conclusions regarding its relevance in the control of HBOCs properties (Le Coeur et al., 2015; Manjula et al., 2003; Wang et al., 2014). Residue Cys93(β) is also involved in NO binding under aerobic conditions. The physiological relevance of the SNO-Hb complex in hypoxia vasodilation via the reversible release of a SNO derivative during deoxygenation was proposed by Stamler (see for a review (Premont et al., 2020)). An alternate view, suggested by Gladwin (Gladwin, 2017) proposes the formation of NO from nitrite by deoxyHb, leading to hypoxic vasodilation. Furthermore, since Cys93(β) is only reactive in the R state, blocking it by bulky groups hinders the R to T transition, stabilizing the R state and increasing oxygen affinity.

As PEGylation at the N-terminal Val1(α) and Val1(β) was shown to induce the destabilization of Hb tetramers, their carboxymethylation and propylation before PEGylation was pursued (Hu et al., 2009; Hu et al., 2011; Hu et al., 2012).

The ligation state of Hb at the time of PEGylation also proved critical in dictating the oxygen-binding properties of the final product. By PEGylating oxy-HbA, Winslow and colleagues obtained

a HBOC endowed with no cooperativity and oxygen affinity close to isolated dimers and R state Hb (Caccia et al., 2009; Iafelice et al., 2007; Portoro et al., 2008; Winslow, 2005). The PEGylation of deoxy-HbA, on the other hand, resulted in a low-affinity, high-cooperativity product (Euro-PEG-Hb) (Caccia et al., 2009; Portoro et al., 2008). We compared high- and low-affinity PEGylated HbA in a Guinea pig transfusion model, demonstrating a correlation between oxygen affinity and oxidative stress (Alomari et al., 2018).

PEG was also used in the preparation of phospholipids bilayer vesicles containing purified HbA for the development of red cell mimics (Sakai et al., 2001). Hb vesicles exhibiting p50 of 8 and 29 torr were obtained by modulating the amount of pyridoxal 5-phosphate as allosteric effector. In an hamster model, it was found that vesicles containing higher affinity Hb led to a higher oxygen release (Sakai et al., 2005).

2.1.2 Control of oxygen binding properties in polymerized HBOCs. HBOCs have been obtained by introducing crosslinks between dimers to prevent dissociation. Since the first observations on diaspirin-crosslinked HbA (Chatterjee et al., 1986; Walder et al., 1979), it was concluded that crosslinking chemistry is crucial in modulating the oxygen-binding properties. For example, Hb raffimer (Hemolink™) is based on intramolecular/intermolecular HbA crosslinked with oxidized raffinose (Scatena and Giardina, 2001) and its affinity for oxygen is lower than that of Hb inside RBCs (Table 1) (Caron et al., 1999; Scatena and Giardina, 2001). HbA polymerized with glutaraldehyde and then pyridoxylated (PolyHeme®, Northfield) exhibits a low affinity (Table 1) (Gould et al., 1998). As for PEGylated HBOCs, the conditions of the reaction, as well as the ligation state of HbA, were investigated (Zhang et al., 2011).

2.1.3 Control of oxygen binding properties by using non-human Hbs. Bovine Hb (bHb) naturally exhibits a low affinity for oxygen and was therefore proposed as a starting point for preparing low-affinity HBOCs. Hemopure® (Biopure, now HbO2 Therapeutics) (and its most recent version called HBOC-201), a glutaraldehyde-polymerized bovine Hb, exhibits an oxygen affinity lower than human

RBCs (Table 1) (Jahr et al., 2008). It has been authorized for compassionate use in patients that cannot be transfused for any clinical reason or refuse transfusions such as Jehovah's Witnesses. Furthermore, Hemopure® is approved in South Africa to treat anemia in adult surgical patients, and in Russia for acute anemia, irrespective of etiology. A partially purified Hemopure®, called Oxyglobin® was approved by FDA and EMA for veterinary use (Harris and Palmer, 2008). Hemopure® is also used in organ perfusion solutions (see 4.2).

As for PEGylated HBOCs and glutaraldehyde-polymerized HbA, the ligation state of Hb during the reaction strongly affected the oxygen-binding properties (Buehler et al., 2010; Zhou et al., 2011). bHb has been intramolecularly crosslinked with ATP and intermolecularly linked with adenosine (Simoni et al., 2014; Simoni et al., 2012) and conjugated to dextran upon Cys93(β) protection (Wang et al., 2017). A more recent approach consisted in the decoration of CO-bHb with PEG, leading to the product Sanguinate® (Abuchowski, 2016, 2017). Clinical trials were carried out for the use of Sanguinate® to treat sickle cell crises (Misra et al., 2017) and animal studies for the use as resuscitation fluid (Guerci et al., 2020). This product was granted the designation of orphan drug for sickle cell patients as well as compassionate use in severe anemia when transfusions were not possible or refused (McConachie et al., 2020). However, concern about the benefits of its use in such a complex clinical frame as sickle cell disease was raised (Alayash, 2017).

Recent studies (Gu et al., 2021) report on the development of a series of HBOCs obtained from cross-linked bHb PEGylated in the either the T or R state upon tangential flow filtration to separate high and low molecular weight forms. For both forms, the R state PEG bHb products exhibit p50s close to 1 torr, whereas the T state PEG bHb exhibits p50 of 20 torr. For all products, cooperativity is close to 1 (Belcher et al., 2017; Gu et al., 2021).

Another HBOC derived from purified bHb is the zero-linked polymeric hemoglobin, OxyVita®Hb obtained from the formation of pseudopeptide bonds between carboxyl groups and amino groups on

the surface of bovine cross-linked tetrameric Hb molecules (Table 1) (Harrington et al., 2011). Studies are ongoing to investigate oxidation stability (Wollocko et al., 2017a) and formulation (Wollocko et al., 2017b).

A cluster was produced by cross-linking bHb with human albumin molecules, named HemoAct (Table 1) (Tomita et al., 2013). HemoAct safety, evaluated in rats, was found acceptable (Haruki et al., 2015). Using as crosslinking agent *N*-succinimidyl 3-maleimidopropionate (Funaki et al., 2019), a product with a p50 of 9 torr and a Hill coefficient of 1.4 was obtained, lower than the control HbA exhibiting a p50 of 23 torr and a Hill coefficient of 2.6. This suggests a stabilization of the R state due to constraints to the transition to the T state, as lysine residues were modified.

Besides bHb, crocodile Hb, *Trematomus bernacchii* Hb and erythrocrutorin of the earthworm *Lumbricus terrestris* have also been considered as starting points for low-affinity HBOCs due to their specific oxygen binding properties (Coppola et al., 2011; Elmer et al., 2012; Jani et al., 2017; Komiya et al., 1995; Rajesh et al., 2018; Roamcharern et al., 2019; Spivack et al., 2018). More recently, cell-free Hb from *Arenicola marina* has been proposed as a blood substitute. This lugworm Hb has a high molecular weight (3600 kDa), with a p50 of 7 mmHg (Table 1) (Mallet et al., 2014; Varney et al., 2021), and is prepared by the company Hemarina with the name Hemo2Life® M101. This product has been tested as a preservation fluid for storing healthy and marginal kidney before transplantation in a preclinical porcine model (Kaminski et al., 2019; Thuillier et al., 2011).

2.1.4 Control of oxygen binding properties by non-natural Hb variants. It was suggested to produce recombinant Hb variants by introducing mutations capable of modulating oxygen affinity (Benitez Cardenas et al., 2019; Cooper et al., 2020; Dou et al., 2002; Olson et al., 1997; Ronda et al., 2008; Varnado et al., 2013), in particular through the stabilization of high or low-affinity conformations, the alteration of the interactions between oxygen and amino acid side chains in the heme pocket and the modification of heme accessibility (Birukou et al., 2010).

Hb Polytaur is a chimeric autopolymerizing recombinant Hb engineered to both increase the p50 of HbA and its size through autopolymerization via formation of disulfide bonds (Bobofchak et al., 2003; Faggiano et al., 2011). It is formed by human α chains and bovine β chains, intended to lower the affinity of Hb. Mutations Cys104Ser(α), Cys93Ala(β), and Ser9Cys(β) were introduced to limit polymerization to Ser9Cys(β). The autopolymerized product exhibited a p50 of 16-18 mmHg under physiological conditions, values close to Hb inside RBCs.

Recently, a double mutant (Cys93Ala(β)/Ala19Cys(α)) was generated and decorated with PEG20000 Da. This HBOC showed oxygen affinity and cooperativity close to HbA (Cooper et al., 2021; Cooper et al., 2020).

Towards the development of stable Hb tetramers, a combination of two α_2 and β_2 homodimers was obtained from a fusion gene containing two α chains or two β chains, coupled via a specific linker (Panetta et al., 2008). The modification increased the oxygen affinity, due to a destabilization of the T quaternary state, and did not increase the autoxidation rate (Panetta et al., 2008).

2.2. Modulation of the autoxidation rates and NO dioxygenase reactivity. HBOCs are prone to oxidation to metHb or ferryl species. These species do not bind oxygen and promote oxidative reactions of proteins, lipids, DNA, which correlate to the oxidative stress observed upon administration (Mollan and Alayash, 2013; Strader and Alayash, 2017). Indeed, the chemical modifications used in the preparation of HBOCs increase Hb propensity to autoxidation and production of $O_2^{\cdot -}$ and H_2O_2 (Mollan and Alayash, 2013). Antioxidants like ascorbate mediate Hb reduction (Cooper et al., 2008), but their plasma concentration in humans is limited by dietary intake (Rumsey and Levin, 1998). The extensive mutagenesis studies carried out by Olson and coworkers on Mb and Hb have allowed to pinpoint residues affecting oxygen affinity as well as oxidation stability (Benitez Cardenas et al., 2019; Dou et al., 2002). Fetal Hb (HbF) is more stable and less prone to autoxidation in comparison to HbA (Graves et al., 2008), suggesting its use as a precursor of HBOCs. Recombinant human fetal

Hb and HbA were prepared and evaluated in terms of oxidative stress (Simons et al., 2018). Differences in oxidative reactivity were observed but neither form was superior to the other for the preparation of effective HBOC (Simons et al., 2018).

Another oxidatively stable HBOC was prepared based on the observation that the naturally occurring K82D variant (Hb Providence) is more resistant towards oxygen peroxide degradation with respect to wild-type HbA (Strader et al., 2017). The effects of genetic cross-linking of Hb Providence on autoxidation, heme loss, and reactions with H_2O_2 were compared with HbA and found that cross-linking and mutation confer higher stability to oxidative reactions, thus suggesting that Hb Providence is a valuable source for the development of HBOCs (Strader et al., 2017). It was also found that in Hb Providence Cys93(β) is less prone to oxidation with respect to HbA (Jana et al., 2020).

HbA as well as Mb, under oxidative conditions, form ferryl heme iron and protein-based free radicals. Ferryl reduction can occur either via direct electron transfer from a reducing agent to heme edges or via a protein-based pathway that involves tyrosine residues (Reeder et al., 2008). Based on the observation that in HbA tyrosine Tyr42(α) is involved in the electron transfer between endogenous antioxidants and the ferryl heme, and that a tyrosine residue is absent in the equivalent position of the β subunits, the recombinant Hb variant Phe41Y(β) was designed to improve the reduction rates of β subunits (Silkstone et al., 2016). It was found that mutation decreases heme-mediated oxidative reactivity and enhances NO bioavailability (Silkstone et al., 2016). Several other variants introducing tyrosine residues in both subunits were generated to minimize Hb pro-oxidant activity, maintaining Hb oxygen affinity in a range compatible with oxygen delivery to tissues (Cooper et al., 2019). Among them, Thr84Tyr(β) variant was found to increase the rate of heme reduction by ascorbate, restoring Hb function. HEK cells showed reduced membrane damage upon treatment with the Thr84Tyr(β) variant in comparison to HbA (Cooper et al., 2019). When this variant was PEGylated

and administered to mice, it was found to be retained in the circulation longer than PEGylated HbA (Cooper et al., 2019).

The reactivity of Hb with NO is also a concern in the design of HBOCs as cell-free Hb is prone to NO scavenging, which causes hypertension and also results in Hb oxidation (Alayash, 2019; Doherty et al., 1998; Eich et al., 1996; Olson et al., 2004). NO-induced oxidation was found to be similar in unmodified, polymerized, crosslinked, or PEGylated Hbs, suggesting that the NO scavenging effect observed for HBOCs is mostly associated with their extracellular localization rather than different reactivity towards NO (Meng et al., 2018). However, the thorough investigation of recombinant variants (Doherty et al., 1998; Fronticelli and Koehler, 2009; Frost et al., 2018; Graves et al., 2008) has indicated that some residues are particularly involved in the NO dioxygenase activity, opening the way to produce HBOCs based on recombinant Hbs endowed with lower reactivity towards NO (Doherty et al., 1998; Cooper et al., 2019).

Instead of altering Hb reactivity, the reduction of adverse effects of reactive nitrogen and oxygen species has also been tackled by incorporating antioxidant functions in HBOCs. In the late 1990s, Chang and coworkers crosslinked the enzymes catalase and superoxide dismutase (SOD) to a polyHb (D'Agnillo and Chang, 1998; Powanda and Chang, 2002). Lugworm Hb (Hemo2Life®) shows also SOD-like activity (Mallet et al., 2014).

3. Clinical trials on HBOCs. Several HBOCs were tested in preclinical and clinical trials, and some of them, such as PolyHeme®, Hemopure® and Hemospan® (Sangart), HemAssist® (Baxter), Hemolink™ (Hemosol), reached phase III. However, no products have so far obtained approval by either FDA or EMA, whereas HBOC-201, the most recent version of Hemopure®, is approved in Russia and in South Africa for anemic patients undergoing surgery and is used in the USA for patients in critical conditions that refuse transfusions, under investigational or expanded access/compassionate use (Khan et al., 2020). Here we briefly summarize the latest phase III clinical trials. More information is found in several books on HBOCs published over the years (Chang, 1997;

Chang, 1992; Kim and Greenburg, 2013; Mozzarelli and Bettati, 2011; Winslow et al., 1996; Winslow, 2006b).

PolyHeme® was tested on more than 700 patients in USA as a first treatment for hemorrhagic events vs blood expanders at ambulances and vs transfusions in emergency rooms. Results indicated that PolyHeme® was not superior with respect to controls when survival was used as therapeutic index. Therefore, FDA stated that the risk:benefit assessment of the product in trauma was unfavorable. Moreover, ethic issues were raised since consensus was not collected due to the unconscious patient conditions, and safe transfusions were available in emergency rooms (Dube et al., 2019).

HBOC-201 was tested vs transfusions using severe adverse effects as therapeutic index. Results showed an increase in severe adverse effects possibly associated with greater anemia (Dube et al., 2019). For both HBOC-201 and PolyHeme® clinical evidence indicated significant levels of cardiovascular dysfunction (Chen et al., 2009).

A Hemospan® (MP4OX) phase III clinical trial was carried out to determine if the product was able to prevent hypotension in hip arthroplasty more than a colloidal plasma expander, and to reduce the incidence of operative and postoperative complications including organ dysfunction and failure until follow-up at one month following surgery (<https://clinicaltrials.gov/ct2/show/NCT00421200>). This study followed a randomized, single-blind, increasing dose safety trial selecting orthopedic surgery patients with spinal anesthesia (Olofsson et al., 2008). It should be pointed out that the aim of these trials was not to demonstrate Hemospan® efficacy as an HBOC but as an oxygen-carrying plasma expander. Results indicated that Hemospan® significantly reduced the incidence of hypotensive episodes in patients undergoing hip arthroplasty, but the adverse event profile did not support use in routine low-risk surgical patients (Olofsson et al., 2011).

Another HBOC, HemAssist®, was tested in a phase III clinical trial in USA but the trial was suspended when a significant increase in mortality rates as compared to patients treated with blood products was observed (Chen et al., 2009; Jahr et al., 2007).

Phase III clinical trials were suspended in 2003 also for Hemolink™, since patients treated with this product suffered from adverse cardiac side effects (Sen Gupta, 2019).

In 2008 Natanson et al. (Natanson et al., 2008) carried out a meta-analysis of 16 clinical trials associated with five products. Results showed a significantly increased risk of death and myocardial infarction in patients treated with HBOCs. This study put an end to a period where three major USA companies, Northfield, Biopure and Sangart, intensively and parallelly tried to develop a safe HBOC.

However, in spite of the so far unsuccessful investigations for the development of a safe HBOC, transfusional medicine still urgently needs a drug that i) is able to deliver oxygen, ii) can be stored at room temperature for prolonged periods, iii) can be administered without any blood group matching and iv) can effectively replace RBC transfusions when blood is not available (Pusateri et al., 2019).

4. HBOC "repositioning". The extensive studies carried out for the development of HBOCs led to a deeper understanding of Hb function and physiology, and oxygen supply requirements as well as NO roles in controlling blood vessel tension. Nevertheless, all HBOCs are products that load and unload oxygen depending on oxygen pressures. For this key function, they have been exploited for supplying oxygen or other ligands, such as CO, to isolated organs or tissues.

4.1 CO delivery based on HBOCs. A CO derivative of Hemospan®, MP4CO (Belcher et al., 2013), can deliver CO that at low concentrations shows positive effects including anti-apoptosis, anti-inflammatory, anti-oxidative and anti-proliferative actions (Taguchi et al., 2020). Transgenic mice expressing HbS, the Hb responsible for the sickle cell disease in humans, were treated with MP4CO as a strategy to deliver CO and, thus, to induce the expression of heme oxygenase-1 that inhibits microvascular stasis in sickle mice. In addition, it was also demonstrated that administration of MP4CO induces the expression of the cytoprotective Nrf2 transcriptional regulator of heme oxygenase-1 (Belcher et al., 2013). In the case of Sanguinate®, manufactured as CO derivative and

as such used for the treatment of sickle cell patients (see above, 2.1.3), the CO-Hb derivative was the only product developed and investigated (Abuchowski, 2016).

4.2 Organ perfusion fluids based on HBOCs. Every day, in the US and EU, about 50 people die because of the lack of healthy organs to be transplanted. Moreover, the rate of opt-outs from organ donation is increasing, reaching for example in Italy, in 2020, the record rate of 34%, with the consequential increase in mortality for conditions that otherwise would be treatable. Furthermore, not all the organs used lead to a successful transplantation, worsening the scenario. By the end of 2019, over 58.000 patients were on a waiting list for organ transplantation in Europe and, on yearly average, about 3-4% of the patients die before being transplanted (Vanholder et al., 2021). For example, in 2019 in Italy, patients in the kidney transplant list were 8615 and only 3813 were transplanted (data from the Italian National Center Transplant). Moreover, only a small proportion of donated kidneys were eligible for transplant (1379 out of 2766), indicating that organs are very susceptible to ischemia and damage after death. Since the need for organs is significantly larger than the availability of ideal donors, criteria for donor acceptance have been expanded and led to the use of extended criteria donation (ECD) after circulatory death (DCD), but this is still not enough. For these reasons, the development of new methods for improving organ preservation is a very active field. Whereas the most used method for organ preservation is the normothermic perfusion with crystalloid solutions and RBCs, recently HBOCs have been explored as an alternative for the improvement of oxygen supply and conservation of optimal metabolic activity. A key advantage of HBOCs over RBCs is their ability to diffuse in every organ capillary given the very reduced size (nanometers vs microns). For instance, HBOCs were employed in machine perfusion of liver and kidney grafts after prolonged cold ischemia (Mahboub et al., 2020) and were efficacious at improving organ condition of the liver graft in animal experiments (Bhattacharjee et al., 2020). It was shown that more oxygen was extracted when HBOC-201 was used with respect to RBCs in a human model of normothermic machine

perfusion for liver (Laing et al., 2017) and kidney grafts (Aburawi et al., 2019). Ten discarded human livers were exposed to a continuous cycle of hypo- and normothermic perfusions using HBOC-201 (Boteon et al., 2019). Results indicated that the procedure leads to a mitigation of the oxidative-mediated tissue injury and an enhancement of hepatic energy stores, similarly to an interrupted combined protocol, but with a simpler and more clinically applicable procedure (Boteon et al., 2019). Similar positive results were obtained in a study where hypo- and normothermic perfusions were sequentially applied to seven livers in order to preserve mitochondria activity as well as hepatic functions, respectively (de Vries et al., 2019). During the normothermic phase, HBOC-201 was used for supplying oxygen. Given the optimal metabolic conditions, five livers were transplanted and 100% were still in place after three months (de Vries et al., 2019). In a collaborative study we are carrying out with the University of Bologna, a combination of bovine crosslinked HBOC and mesenchymal stromal cell extracellular vesicles was used for human kidney normothermic perfusions, obtaining very positive results for the preservation and improvement of marginal organs (unpublished data). Because of this new game-changing application, HBOCs specifically designed for *ex vivo* perfusion machines are currently being investigated, although most of the products used so far are derivatives or HBOCs envisaged for *in vivo* administration as blood substitutes.

Bibliography

- Abuchowski, A., 2016. PEGylated Bovine Carboxyhemoglobin (SANGUINATE): Results of Clinical Safety Testing and Use in Patients. *Advances in experimental medicine and biology* 876, 461-467.
- Abuchowski, A., 2017. SANGUINATE (PEGylated Carboxyhemoglobin Bovine): Mechanism of Action and Clinical Update. *Artificial organs* 41 (4), 346-350.
- Aburawi, M.M., Fontan, F.M., Karimian, N., Eymard, C., Cronin, S., Pendexter, C., Nagpal, S., Banik, P., Ozer, S., Mahboub, P., Delmonico, F.L., Yeh, H., Uygun, K., Markmann, J.F., 2019.

Synthetic hemoglobin-based oxygen carriers are an acceptable alternative for packed red blood cells in normothermic kidney perfusion. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 19 (10), 2814-2824.

Ackers, G.K., Doyle, M.L., Myers, D., Daugherty, M.A., 1992. Molecular code for cooperativity in hemoglobin. *Science* 255 (5040), 54-63.

Alayash, A.I., 2017. Hemoglobin-Based Blood Substitutes and the Treatment of Sickle Cell Disease: More Harm than Help? *Biomolecules* 7 (1).

Alayash, A.I., 2019. Mechanisms of Toxicity and Modulation of Hemoglobin-based Oxygen Carriers. *Shock* 52 (1S Suppl 1), 41-49.

Alomari, E., Ronda, L., Bruno, S., Paredi, G., Marchetti, M., Bettati, S., Olivari, D., Fumagalli, F., Novelli, D., Ristagno, G., Latini, R., Cooper, C.E., Reeder, B.J., Mozzarelli, A., 2018. High- and low-affinity PEGylated hemoglobin-based oxygen carriers: Differential oxidative stress in a Guinea pig transfusion model. *Free radical biology & medicine* 124, 299-310.

Belcher, D.A., Banerjee, U., Baehr, C.M., Richardson, K.E., Cabrales, P., Berthiaume, F., Palmer, A.F., 2017. Mixtures of tense and relaxed state polymerized human hemoglobin regulate oxygen affinity and tissue construct oxygenation. *PLoS One* 12 (10), e0185988.

Belcher, D.A., Ju, J.A., Baek, J.H., Yalamanoglu, A., Buehler, P.W., Gilkes, D.M., Palmer, A.F., 2018. The quaternary state of polymerized human hemoglobin regulates oxygenation of breast cancer solid tumors: A theoretical and experimental study. *PLoS One* 13 (2), e0191275-e0191275.

Belcher, J.D., Young, M., Chen, C., Nguyen, J., Burhop, K., Tran, P., Vercellotti, G.M., 2013. MP4CO, a pegylated hemoglobin saturated with carbon monoxide, is a modulator of HO-1, inflammation, and vaso-occlusion in transgenic sickle mice. *Blood* 122 (15), 2757-2764.

Bellelli, A., Brunori, M., 2011. Hemoglobin allostery: variations on the theme. *Biochimica et biophysica acta* 1807 (10), 1262-1272.

Benitez Cardenas, A.S., Samuel, P.P., Olson, J.S., 2019. Current Challenges in the Development of Acellular Hemoglobin Oxygen Carriers by Protein Engineering. *Shock* 52 (1S Suppl 1), 28-40.

Bettati, S., Mozzarelli, A., 1997. T state hemoglobin binds oxygen noncooperatively with allosteric effects of protons, inositol hexaphosphate, and chloride. *The Journal of biological chemistry* 272 (51), 32050-32055.

Bettati, S., Mozzarelli, A., Perutz, M.F., 1998. Allosteric mechanism of haemoglobin: rupture of salt-bridges raises the oxygen affinity of the T-structure. *Journal of molecular biology* 281 (4), 581-585.

Bhattacharjee, R.N., Patel, S.V.B., Sun, Q., Jiang, L., Richard-Mohamed, M., Ruthirakanthan, A., Aquil, S., Al-Ogaili, R., Juriasingani, S., Sener, A., Luke, P.P.W., 2020. Renal Protection Against Ischemia Reperfusion Injury: Hemoglobin-based Oxygen Carrier-201 Versus Blood as an Oxygen Carrier in Ex Vivo Subnormothermic Machine Perfusion. *Transplantation* 104 (3), 482-489.

Birukou, I., Schweers, R.L., Olson, J.S., 2010. Distal histidine stabilizes bound O₂ and acts as a gate for ligand entry in both subunits of adult human hemoglobin. *The Journal of biological chemistry* 285 (12), 8840-8854.

Bobofchak, K.M., Mito, T., Texel, S.J., Bellelli, A., Nemoto, M., Traystman, R.J., Koehler, R.C., Brinigar, W.S., Fronticelli, C., 2003. A recombinant polymeric hemoglobin with conformational, functional, and physiological characteristics of an in vivo O₂ transporter. *American journal of physiology. Heart and circulatory physiology* 285 (2), H549-561.

Boteon, Y.L., Laing, R.W., Schlegel, A., Wallace, L., Smith, A., Attard, J., Bhogal, R.H., Reynolds, G., Perera, M., Muiesan, P., Mirza, D.F., Mergental, H., Afford, S.C., 2019. The impact on the bioenergetic status and oxidative-mediated tissue injury of a combined protocol of hypothermic and normothermic machine perfusion using an acellular haemoglobin-based oxygen carrier: The cold-to-warm machine perfusion of the liver. *PLoS One* 14 (10), e0224066.

Bradley, R., Slosberg, S., Nho, K., Czuba, B., Szesko, D., Shorr, R., 1994. Production of PEG-modified bovine hemoglobin: economics and feasibility. *Artificial cells, blood substitutes, and immobilization biotechnology* 22 (3), 657-667.

Bruno, S., Bonaccio, M., Bettati, S., Rivetti, C., Viappiani, C., Abbruzzetti, S., Mozzarelli, A., 2001. High and low oxygen affinity conformations of T state hemoglobin. *Protein science : a publication of the Protein Society* 10 (11), 2401-2407.

Buehler, P.W., Zhou, Y., Cabrales, P., Jia, Y., Sun, G., Harris, D.R., Tsai, A.G., Intaglietta, M., Palmer, A.F., 2010. Synthesis, biophysical properties and pharmacokinetics of ultrahigh molecular weight tense and relaxed state polymerized bovine hemoglobins. *Biomaterials* 31 (13), 3723-3735.

Caccia, D., Ronda, L., Frassi, R., Perrella, M., Del Favero, E., Bruno, S., Pioselli, B., Abbruzzetti, S., Viappiani, C., Mozzarelli, A., 2009. PEGylation promotes hemoglobin tetramer dissociation. *Bioconjugate chemistry* 20 (7), 1356-1366.

Caron, A., Menu, P., Faivre-Fiorina, B., Labrude, P., Alayash, A.I., Vigneron, C., 1999. Cardiovascular and hemorheological effects of three modified human hemoglobin solutions in hemodiluted rabbits. *Journal of applied physiology* 86 (2), 541-548.

Chang, T.M.S., 1997. *Blood Substitutes. Principles, Methods, Products and Clinical Trials*, Vol. 1 and 2. Karger, Basel.

Chang, T.M.S.E., 1992. *Blood substitutes and oxygen carriers*. Marcel Dekker, Inc., New York.

Chatterjee, R., Welty, E.V., Walder, R.Y., Pruitt, S.L., Rogers, P.H., Arnone, A., Walder, J.A., 1986. Isolation and characterization of a new hemoglobin derivative cross-linked between the alpha chains (lysine 99 alpha 1----lysine 99 alpha 2). *The Journal of biological chemistry* 261 (21), 9929-9937.

Chen, J.Y., Scerbo, M., Kramer, G., 2009. A review of blood substitutes: examining the history, clinical trial results, and ethics of hemoglobin-based oxygen carriers. *Clinics* 64 (8), 803-813.

Cooper, C.E., Bird, M., Sheng, X., Choi, J.W., Silkstone, G.G., Simons, M., Syrett, N., Piano, R., Ronda, L., Bettati, S., Paredi, G., Mozzarelli, A., Reeder, B.J., 2021. Stability of maleimide-PEG and mono-sulfone-PEG conjugation to a novel engineered cysteine in the human hemoglobin alpha subunit. *Front Chem* 9, 707797.

Cooper, C.E., Silaghi-Dumitrescu, R., Rukengwa, M., Alayash, A.I., Buehler, P.W., 2008. Peroxidase activity of hemoglobin towards ascorbate and urate: a synergistic protective strategy against toxicity

of Hemoglobin-Based Oxygen Carriers (HBOC). *Biochimica et biophysica acta* 1784 (10), 1415-1420.

Cooper, C.E., Silkstone, G.G.A., Simons, M., Gretton, S., Rajagopal, B.S., Allen-Baume, V., Syrett, N., Shaik, T., Popa, G., Sheng, X., Bird, M., Choi, J.W., Piano, R., Ronda, L., Bettati, S., Paredi, G., Mozzarelli, A., Reeder, B.J., 2020. Engineering hemoglobin to enable homogenous PEGylation without modifying protein functionality. *Biomaterials science* 8 (14), 3896-3906.

Cooper, C.E., Silkstone, G.G.A., Simons, M., Rajagopal, B., Syrett, N., Shaik, T., Gretton, S., Welbourn, E., Bulow, L., Eriksson, N.L., Ronda, L., Mozzarelli, A., Eke, A., Mathe, D., Reeder, B.J., 2019. Engineering tyrosine residues into hemoglobin enhances heme reduction, decreases oxidative stress and increases vascular retention of a hemoglobin based blood substitute. *Free radical biology & medicine* 134, 106-118.

Coppola, D., Bruno, S., Ronda, L., Viappiani, C., Abbruzzetti, S., di Prisco, G., Verde, C., Mozzarelli, A., 2011. Low affinity PEGylated hemoglobin from *Trematomus bernacchii*, a model for hemoglobin-based blood substitutes. *BMC biochemistry* 12, 66.

D'Agnillo, F., Chang, T.M., 1998. Polyhemoglobin-superoxide dismutase-catalase as a blood substitute with antioxidant properties. *Nature biotechnology* 16 (7), 667-671.

Das, T.K., Khan, I., Rousseau, D.L., Friedman, J.M., 1999. Temperature dependent quaternary state relaxation in sol-gel encapsulated hemoglobin. *Biospectroscopy* 5 (5 Suppl), S64-70.

de Vries, Y., Matton, A.P.M., Nijsten, M.W.N., Werner, M.J.M., van den Berg, A.P., de Boer, M.T., Buis, C.I., Fujiyoshi, M., de Kleine, R.H.J., van Leeuwen, O.B., Meyer, P., van den Heuvel, M.C., de Meijer, V.E., Porte, R.J., 2019. Pretransplant sequential hypo- and normothermic machine perfusion of suboptimal livers donated after circulatory death using a hemoglobin-based oxygen carrier perfusion solution. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons* 19 (4), 1202-1211.

Devineau, S., Kiger, L., Galacteros, F., Baudin-Creuzat, V., Marden, M., Renault, J.P., Pin, S., 2018. Manipulating hemoglobin oxygenation using silica nanoparticles: a novel prospect for artificial oxygen carriers. *Blood advances* 2 (2), 90-94.

di Masi, A., De Simone, G., Ciaccio, C., D'Orso, S., Coletta, M., Ascenzi, P., 2020. Haptoglobin: From hemoglobin scavenging to human health. *Molecular aspects of medicine* 73, 100851.

Doherty, D.H., Doyle, M.P., Curry, S.R., Vali, R.J., Fattor, T.J., Olson, J.S., Lemon, D.D., 1998. Rate of reaction with nitric oxide determines the hypertensive effect of cell-free hemoglobin. *Nature biotechnology* 16 (7), 672-676.

Dolgin, E., 2017. Bioengineering: Doing without donors. *Nature* 549 (7673), S12-S15.

Dou, Y., Mailliet, D.H., Eich, R.F., Olson, J.S., 2002. Myoglobin as a model system for designing heme protein based blood substitutes. *Biophysical chemistry* 98 (1-2), 127-148.

Dube, G.P., Pitman, A.N., Mackenzie, C.F., 2019. Relative Efficacies of HBOC-201 and Polyheme to Increase Oxygen Transport Compared to Blood and Crystalloids. *Shock* 52 (1S Suppl 1), 100-107.

Eaton, W.A., 1980. The relationship between coding sequences and function in haemoglobin. *Nature* 284 (5752), 183-185.

Eaton, W.A., Henry, E.R., Hofrichter, J., Bettati, S., Viappiani, C., Mozzarelli, A., 2007. Evolution of allosteric models for hemoglobin. *IUBMB life* 59 (8-9), 586-599.

Eaton, W.A., Henry, E.R., Hofrichter, J., Mozzarelli, A., 1999. Is cooperative oxygen binding by hemoglobin really understood? *Nature structural biology* 6 (4), 351-358.

Eich, R.F., Li, T., Lemon, D.D., Doherty, D.H., Curry, S.R., Aitken, J.F., Mathews, A.J., Johnson, K.A., Smith, R.D., Phillips, G.N., Jr., Olson, J.S., 1996. Mechanism of NO-induced oxidation of myoglobin and hemoglobin. *Biochemistry* 35 (22), 6976-6983.

Elmer, J., Zorc, K., Rameez, S., Zhou, Y., Cabrales, P., Palmer, A.F., 2012. Hypervolemic infusion of *Lumbricus terrestris* erythrocyte purified by tangential-flow filtration. *Transfusion* 52 (8), 1729-1740.

Faggiano, S., Bruno, S., Ronda, L., Pizzonia, P., Pioselli, B., Mozzarelli, A., 2011. Modulation of expression and polymerization of hemoglobin Polytaur, a potential blood substitute. *Archives of biochemistry and biophysics* 505 (1), 42-47.

Friedman, J.M., 1985. Structure, dynamics, and reactivity in hemoglobin. *Science* 228 (4705), 1273-1280.

Fronticelli, C., Koehler, R.C., 2009. Design of recombinant hemoglobins for use in transfusion fluids. *Critical care clinics* 25 (2), 357-371, Table of Contents.

Frost, A.T., Jacobsen, I.H., Worberg, A., Martinez, J.L., 2018. How Synthetic Biology and Metabolic Engineering Can Boost the Generation of Artificial Blood Using Microbial Production Hosts. *Frontiers in bioengineering and biotechnology* 6, 186.

Funaki, R., Kashima, T., Okamoto, W., Sakata, S., Morita, Y., M., S., T., K., 2019. Hemoglobin–Albumin Clusters Prepared Using N- Succinimidyl 3- Maleimidopropionate as an Appropriate Cross-Linker. *ACS Omega* 4.

Gelin, B.R., Lee, A.W., Karplus, M., 1983. Hemoglobin tertiary structural change on ligand binding. Its role in the co-operative mechanism. *Journal of molecular biology* 171 (4), 489-559.

Gibson, Q.H., Roughton, F.J., 1955. The kinetics of dissociation of the first oxygen molecule from fully saturated oxyhaemoglobin in sheep blood solutions. *Proceedings of the Royal Society of London. Series B, Biological sciences* 143 (912), 310-334.

Gladwin, M.T., 2017. How Red Blood Cells Process Nitric Oxide: Evidence for the Nitrite Hypothesis. *Circulation* 135 (2), 177-179.

Gould, S.A., Moore, E.E., Hoyt, D.B., Burch, J.M., Haenel, J.B., Garcia, J., DeWoskin, R., Moss, G.S., 1998. The first randomized trial of human polymerized hemoglobin as a blood substitute in acute trauma and emergent surgery. *Journal of the American College of Surgeons* 187 (2), 113-120.

Graves, P.E., Henderson, D.P., Horstman, M.J., Solomon, B.J., Olson, J.S., 2008. Enhancing stability and expression of recombinant human hemoglobin in *E. coli*: Progress in the development of a recombinant HBOC source. *Biochimica et biophysica acta* 1784 (10), 1471-1479.

- Greenburg, A.G., Kim, H.W., 2004. Hemoglobin-based oxygen carriers. *Critical care* 8 Suppl 2, S61-64.
- Gu, X., Savla, C., Palmer, A.F., 2021. Tangential flow filtration facilitated fractionation and PEGylation of low and high-molecular weight polymerized hemoglobins and their biophysical properties. *Biotechnology and bioengineering*.
- Guerci, P., Ergin, B., Kandil, A., Ince, Y., Heeman, P., Hilty, M.P., Bakker, J., Ince, C., 2020. Resuscitation with PEGylated carboxyhemoglobin preserves renal cortical oxygenation and improves skeletal muscle microcirculatory flow during endotoxemia. *American journal of physiology. Renal physiology* 318 (5), F1271-F1283.
- Harrington, J.P., Wollocko, J., Kostecki, E., Wollocko, H., 2011. Physicochemical characteristics of OxyVita hemoglobin, a zero-linked polymer: liquid and powder preparations. *Artificial cells, blood substitutes, and immobilization biotechnology* 39 (1), 12-18.
- Harris, D.R., Palmer, A.F., 2008. Modern cross-linking strategies for synthesizing acellular hemoglobin-based oxygen carriers. *Biotechnology progress* 24 (6), 1215-1225.
- Haruki, R., Kimura, T., Iwasaki, H., Yamada, K., Kamiyama, I., Kohno, M., Taguchi, K., Nagao, S., Maruyama, T., Otagiri, M., Komatsu, T., 2015. Safety Evaluation of Hemoglobin-Albumin Cluster "HemoAct" as a Red Blood Cell Substitute. *Scientific reports* 5, 12778.
- Henry, E.R., Bettati, S., Hofrichter, J., Eaton, W.A., 2002. A tertiary two-state allosteric model for hemoglobin. *Biophysical chemistry* 98 (1-2), 149-164.
- Henry, E.R., Jones, C.M., Hofrichter, J., Eaton, W.A., 1997. Can a two-state MWC allosteric model explain hemoglobin kinetics? *Biochemistry* 36 (21), 6511-6528.
- Henry, E.R., Mozzarelli, A., Viappiani, C., Abbruzzetti, S., Bettati, S., Ronda, L., Bruno, S., Eaton, W.A., 2015. Experiments on Hemoglobin in Single Crystals and Silica Gels Distinguish among Allosteric Models. *Biophysical journal* 109 (6), 1264-1272.

- Hofrichter, J., Sommer, J.H., Henry, E.R., Eaton, W.A., 1983. Nanosecond absorption spectroscopy of hemoglobin: elementary processes in kinetic cooperativity. *Proceedings of the National Academy of Sciences of the United States of America* 80 (8), 2235-2239.
- Hu, T., Li, D., Manjula, B.N., Brenowitz, M., Prabhakaran, M., Acharya, S.A., 2009. PEGylation of Val-1(alpha) destabilizes the tetrameric structure of hemoglobin. *Biochemistry* 48 (3), 608-616.
- Hu, T., Li, D., Meng, F., Prabhakaran, M., Acharya, S.A., 2011. Increased inter dimeric interaction of oxy hemoglobin is necessary for attenuation of reductive pegylation promoted dissociation of tetramer. *Artificial cells, blood substitutes, and immobilization biotechnology* 39 (2), 69-78.
- Hu, T., Li, D., Wang, J., Wang, Q., Liang, Y., Su, Y., Ma, G., Su, Z., Wang, S., 2012. Propylbenz methylation at Val-1(alpha) markedly increases the tetramer stability of the PEGylated hemoglobin: a comparison with propylation at Val-1(alpha). *Biochimica et biophysica acta* 1820 (12), 2044-2051.
- Iafelice, R., Cristoni, S., Caccia, D., Russo, R., Rossi-Bernardi, L., Lowe, K.C., Perrella, M., 2007. Identification of the sites of deoxyhaemoglobin PEGylation. *The Biochemical journal* 403 (1), 189-196.
- Imai, K., 1982. *Allosteric effects in Hemoglobin*. Cambridge University Press; Cambridge, New York.
- Inayat, M.S., Bernard, A.C., Gallicchio, V.S., Garvy, B.A., Elford, H.L., Oakley, O.R., 2006. Oxygen carriers: a selected review. *Transfusion and apheresis science : official journal of the World Apheresis Association : official journal of the European Society for Haemapheresis* 34 (1), 25-32.
- Jahr, J.S., Akha, A.S., Holtby, R.J., 2012. Crosslinked, polymerized, and PEG-conjugated hemoglobin-based oxygen carriers: clinical safety and efficacy of recent and current products. *Current drug discovery technologies* 9 (3), 158-165.
- Jahr, J.S., Walker, V., Manoochchri, K., 2007. Blood substitutes as pharmacotherapies in clinical practice. *Current opinion in anaesthesiology* 20 (4), 325-330.

Jahr, J.S., Moallempour, M., Lim, J.C., 2008. HBOC-201, hemoglobin glutamer-250 (bovine), Hemopure (Biopure Corporation). Expert opinion on biological therapy 8 (9), 1425-1433.

Jana, S., Strader, M.B., Alayash, A.I., 2020. The Providence Mutation (betaK82D) in Human Hemoglobin Substantially Reduces betaCysteine 93 Oxidation and Oxidative Stress in Endothelial Cells. International journal of molecular sciences 21 (24).

Jani, V.P., Jelvani, A., Moges, S., Nacharaju, P., Roche, C., Dantsker, D., Palmer, A., Friedman, J.M., Cabrales, P., 2017. Polyethylene Glycol Camouflaged Earthworm Hemoglobin. PLoS One 12 (1), e0170041.

Jansman, M.M.T., Hosta-Rigau, L., 2018. Recent and prominent examples of nano- and microarchitectures as hemoglobin-based oxygen carriers. Advances in colloid and interface science 260, 65-84.

Jones, E.M., Balakrishnan, G., Spiro, T.G., 2012. Heme reactivity is uncoupled from quaternary structure in gel-encapsulated hemoglobin: a resonance Raman spectroscopic study. Journal of the American Chemical Society 134 (7), 3461-3471.

Jones, E.M., Monza, E., Balakrishnan, G., Blouin, G.C., Mak, P.J., Zhu, Q., Kincaid, J.R., Guallar, V., Spiro, T.G., 2014. Differential control of heme reactivity in alpha and beta subunits of hemoglobin: a combined Raman spectroscopic and computational study. Journal of the American Chemical Society 136 (29), 10325-10339.

Juszczak, L.J., Friedman, J.M., 1999. UV resonance raman spectra of ligand binding intermediates of sol-gel encapsulated hemoglobin. The Journal of biological chemistry 274 (43), 30357-30360.

Kaminski, J., Hannaert, P., Kasil, A., Thuillier, R., Leize, E., Delpy, E., Steichen, C., Goujon, J.M., Zal, F., Hauet, T., 2019. Efficacy of the natural oxygen transporter HEMO2 life((R)) in cold preservation in a preclinical porcine model of donation after cardiac death. Transplant international : official journal of the European Society for Organ Transplantation 32 (9), 985-996.

- Kendrew, J.C., Dickerson, R.E., Strandberg, B.E., Hart, R.G., Davies, D.R., Phillips, D.C., Shore, V.C., 1960. Structure of myoglobin: A three-dimensional Fourier synthesis at 2 Å. resolution. *Nature* 185 (4711), 422-427.
- Khan, F., Singh, K., Friedman, M.T., 2020. Artificial Blood: The History and Current Perspectives of Blood Substitutes. *Discoveries* 8 (1), e104.
- Khan, I., Shannon, C.F., Dantsker, D., Friedman, A.J., Perez-Gonzalez-de-Apodaca, J., Friedman, J.M., 2000. Sol-gel trapping of functional intermediates of hemoglobin: geminate and bimolecular recombination studies. *Biochemistry* 39 (51), 16099-16109.
- Kim, H.W., Greenburg, A.G.E., 2013. Hemoglobin-Based Oxygen Carriers as Red Cell Substitutes and Oxygen Therapeutics. Springer
- Kister, J., Poyart, C., Edelstein, S.J., 1987. An expanded two-state allosteric model for interactions of human hemoglobin A with nonsaturating concentrations of 2,3-diphosphoglycerate. *The Journal of biological chemistry* 262 (25), 12085-12091.
- Kocian, R., Spahn, D.R., 2008. Haemoglobin, oxygen carriers and perioperative organ perfusion. *Best practice & research. Clinical anaesthesiology* 22 (1), 63-80.
- Komiyama, N.H., Miyazaki, G., Tame, J., Nagai, K., 1995. Transplanting a unique allosteric effect from crocodile into human haemoglobin. *Nature* 373 (6511), 244-246.
- Koshland, D.E., Jr., Nemethy, G., Filmer, D., 1966. Comparison of experimental binding data and theoretical models in proteins containing subunits. *Biochemistry* 5 (1), 365-385.
- Krafft, M.P., Riess, J.G., 2009. Chemistry, physical chemistry, and uses of molecular fluorocarbon--hydrocarbon diblocks, triblocks, and related compounds--unique "apolar" components for self-assembled colloid and interface engineering. *Chemical reviews* 109 (5), 1714-1792.
- Laing, R.W., Bhogal, R.H., Wallace, L., Boteon, Y., Neil, D.A.H., Smith, A., Stephenson, B.T.F., Schlegel, A., Hubscher, S.G., Mirza, D.F., Afford, S.C., Mergental, H., 2017. The Use of an Acellular Oxygen Carrier in a Human Liver Model of Normothermic Machine Perfusion. *Transplantation* 101 (11), 2746-2756.

Lalezari, I., Lalezari, P., Poyart, C., Marden, M., Kister, J., Bohn, B., Fermi, G., Perutz, M.F., 1990. New effectors of human hemoglobin: structure and function. *Biochemistry* 29 (6), 1515-1523.

Lamy, M.L., Daily, E.K., Brichant, J.F., Larbuisson, R.P., Demeyere, R.H., Vandermeersch, E.A., Lehot, J.J., Parsloe, M.R., Berridge, J.C., Sinclair, C.J., Baron, J.F., Przybelski, R.J., 2000. Randomized Trial of Diaspirin Cross-linked Hemoglobin Solution as an Alternative to Blood Transfusion after Cardiac Surgery. *Anesthesiology* 92, 646-656.

Le Coeur, C., Combet, S., Carrot, G., Busch, P., Teixeira, J., Longeville, S., 2015. Conformation of the Poly(ethylene Glycol) Chains in DiPEGylated Hemoglobin Specifically Probed by SANS: Correlation with PEG Length and in Vivo Efficiency. *Langmuir : the ACS journal of surfaces and colloids* 31 (30), 8402-8410.

Li, D., Hu, T., Manjula, B.N., Acharya, S.A., 2009. Extension arm facilitated pegylation of alphaalpha-hemoglobin with modifications targeted exclusively to amino groups: functional and structural advantages of free Cys-93(beta) in the PEG-Hb adduct. *Bioconjugate chemistry* 20 (11), 2062-2070.

Li, D., Manjula, B.N., Acharya, A.S., 2006. Extension arm facilitated PEGylation of hemoglobin: correlation of the properties with the extent of PEGylation. *The protein journal* 25 (4), 263-274.

Lui, F.E., Dong, P., Kluger, R., 2008. Polyethylene glycol conjugation enhances the nitrite reductase activity of native and cross-linked hemoglobin. *Biochemistry* 47 (40), 10773-10780.

Lui, F.E., Kluger, R., 2009. Enhancing nitrite reductase activity of modified hemoglobin: bis-tetramers and their PEGylated derivatives. *Biochemistry* 48 (50), 11912-11919.

Lukin, J.A., Kontaxis, G., Simplaceanu, V., Yuan, Y., Bax, A., Ho, C., 2003. Quaternary structure of hemoglobin in solution. *Proceedings of the National Academy of Sciences of the United States of America* 100 (2), 517-520.

Mahboub, P., Aburawi, M., Karimian, N., Lin, F., Karabacak, M., Fontan, F., Tessier, S.N., Markmann, J., Yeh, H., Uygun, K., 2020. The efficacy of HBOC-201 in ex situ gradual rewarming kidney perfusion in a rat model. *Artificial organs* 44 (1), 81-90.

Mallet, V., Dutheil, D., Polard, V., Rousselot, M., Leize, E., Hauet, T., Goujon, J.M., Zal, F., 2014. Dose-ranging study of the performance of the natural oxygen transporter HEMO2 Life in organ preservation. *Artificial organs* 38 (8), 691-701.

Manjula, B.N., Malavalli, A., Smith, P.K., Chan, N.L., Arnone, A., Friedman, J.M., Acharya, A.S., 2000. Cys-93-beta-beta-succinimidophenyl polyethylene glycol 2000 hemoglobin A. Intramolecular cross-bridging of hemoglobin outside the central cavity. *The Journal of biological chemistry* 275 (8), 5527-5534.

Manjula, B.N., Tsai, A., Upadhy, R., Perumalsamy, K., Smith, P.K., Malavalli, A., Vandegriff, K., Winslow, R.M., Intaglietta, M., Prabhakaran, M., Friedman, J.M., Acharya, A.S., 2003. Site-specific PEGylation of hemoglobin at Cys-93(beta): correlation between the colligative properties of the PEGylated protein and the length of the conjugated PEG chain. *Bioconjugate chemistry* 14 (2), 464-472.

Manjula, B.N., Tsai, A.G., Intaglietta, M., Tsai, C.H., Ho, C., Smith, P.K., Perumalsamy, K., Kanika, N.D., Friedman, J.M., Acharya, S.A., 2005. Conjugation of multiple copies of polyethylene glycol to hemoglobin facilitated through thiolation: influence on hemoglobin structure and function. *The protein journal* 24 (3), 133-146.

Marden, M.C., Bohn, B., Kister, J., Poyart, C., 1990. Effectors of hemoglobin. Separation of allosteric and affinity factors. *Biophysical journal* 57 (3), 397-403.

McConachie, S., Wahby, K., Almadrahi, Z., Wilhelm, S., 2020. Early Experiences With PEGylated Carboxyhemoglobin Bovine in Anemic Jehovah's Witnesses: A Case Series and Review of the Literature. *Journal of pharmacy practice* 33 (3), 372-377.

Meng, F., Kassa, T., Jana, S., Wood, F., Zhang, X., Jia, Y., D'Agnillo, F., Alayash, A.I., 2018. Comprehensive Biochemical and Biophysical Characterization of Hemoglobin-Based Oxygen Carrier Therapeutics: All HBOCs Are Not Created Equally. *Bioconjugate chemistry* 29 (5), 1560-1575.

Miele, A.E., Bellelli, A., Brunori, M., 2013. Hemoglobin allostery: new views on old players. *Journal of molecular biology* 425 (9), 1515-1526.

Misra, H., Bainbridge, J., Berryman, J., Abuchowski, A., Galvez, K.M., Uribe, L.F., Hernandez, A.L., Sosa, N.R., 2017. A Phase Ib open label, randomized, safety study of SANGUINATE in patients with sickle cell anemia. *Revista brasileira de hematologia e hemoterapia* 39 (1), 20-27.

Mollan, T.L., Alayash, A.I., 2013. Redox reactions of hemoglobin: mechanisms of toxicity and control. *Antioxidants & redox signaling* 18 (17), 2251-2253.

Monod, J., Wyman, J., Changeux, J.P., 1965. On the Nature of Allosteric Transitions: A Plausible Model. *Journal of molecular biology* 12, 88-118.

Moore, E.E., Moore, F.A., Fabian, T.C., Bernard, A.C., Fulda, G.J., Hoyt, D.B., Duane, T.M., Weireter, L.J., Jr., Gomez, G.A., Cipolle, M.D., Rodman, G.H., Jr., Malangoni, M.A., Hides, G.A., Omert, L.A., Gould, S.A., PolyHeme Study, G., 2009. Human polymerized hemoglobin for the treatment of hemorrhagic shock when blood is unavailable: the USA multicenter trial. *Journal of the American College of Surgeons* 208 (1), 1-13.

Mozzarelli, A., Bettati, S.E., 2011. *Chemistry and Biochemistry of Oxygen Therapeutics: From Transfusion to Artificial Blood*. John Wiley and Sons Ltd, Chichester, UK

Mozzarelli, A., Rivetti, C., Rossi, G.L., Eaton, W.A., Henry, E.R., 1997. Allosteric effectors do not alter the oxygen affinity of hemoglobin crystals. *Protein science : a publication of the Protein Society* 6 (2), 484-489.

Mozzarelli, A., Rivetti, C., Rossi, G.L., Henry, E.R., Eaton, W.A., 1991. Crystals of haemoglobin with the T quaternary structure bind oxygen noncooperatively with no Bohr effect. *Nature* 351 (6325), 416-419.

Mozzarelli, A., Ronda, L., Faggiano, S., Bettati, S., Bruno, S., 2010. Haemoglobin-based oxygen carriers: research and reality towards an alternative to blood transfusions. *Blood transfusion = Trasfusione del sangue* 8 Suppl 3, s59-68.

- Muirhead, H., Perutz, M.F., 1963. Structure of Haemoglobin. A Three-Dimensional Fourier Synthesis of Reduced Human Haemoglobin at 5-5 Å Resolution. *Nature* 199, 633-638.
- Natanson, C., Kern, S.J., Lurie, P., Banks, S.M., Wolfe, S.M., 2008. Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death: a meta-analysis. *Jama* 299 (19), 2304-2312.
- Noble, R.W., Rossi, G.L., Berni, R., 1972. A structural comparison of ligand-saturated hemoglobin with protoporphyrin globin. I. Reaction with bromothymol blue and sulfhydryl reagents. *Journal of molecular biology* 70 (3), 689-696.
- Olofsson, C., Nygårds, E.B., Ponzer, S., Fagrell, B., Przybelski, R., Keipert, P.E., Winslow, N., Winslow, R.M., 2008. A randomized, single-blind, increasing dose safety trial of an oxygen-carrying plasma expander (Hemospan) administered to orthopaedic surgery patients with spinal anaesthesia. *Transfusion medicine* 18 (1), 28-39.
- Olofsson, C.I., Gorecki, A.Z., Dirksen, R., Kofranek, I., Majewski, J.A., Mazurkiewicz, T., Jahoda, D., Fagrell, B., Keipert, P.E., Hardiman, Y.J., Levy, H., Study Clinical, I., 2011. Evaluation of MP4OX for prevention of perioperative hypotension in patients undergoing primary hip arthroplasty with spinal anesthesia: a randomized, double-blind, multicenter study. *Anesthesiology* 114 (5), 1048-1063.
- Olson, J.S., Eich, R.F., Smith, L.P., Warren, J.J., Knowles, B.C., 1997. Protein engineering strategies for designing more stable hemoglobin-based blood substitutes. *Artificial cells, blood substitutes, and immobilization biotechnology* 25 (1-2), 227-241.
- Olson, J.S., Foley, E.W., Rogge, C., Tsai, A.L., Doyle, M.P., Lemon, D.D., 2004. No scavenging and the hypertensive effect of hemoglobin-based blood substitutes. *Free radical biology & medicine* 36 (6), 685-697.
- Parak F, H.J., Nienhaus G U, 1988. Protein structural dynamics as determined by Mössbauer spectroscopy. *Hyperfine Interactions* 40, 147-157.

Pauling, L., 1935. The Oxygen Equilibrium of Hemoglobin and Its Structural Interpretation. *Proceedings of the National Academy of Sciences of the United States of America* 21 (4), 186-191.

Perutz, M.F., Bolton, W., Diamond, R., Muirhead, H., Watson, H.C., 1964. Structure of Haemoglobin. An X-Ray Examination of Reduced Horse Haemoglobin. *Nature* 203, 687-690.

Peterson, E.S., Shinder, R., Khan, I., Jucyszak, L., Wang, J., Manjula, B., Acharya, S.A., Bonaventura, C., Friedman, J.M., 2004. Domain-specific effector interactions within the central cavity of human adult hemoglobin in solution and in porous sol-gel matrices: evidence for long-range communication pathways. *Biochemistry* 43 (16), 4832-4843.

Pillai, A.S., Chandler, S.A., Liu, Y., Signore, A.V., Cortez-Romero, C.R., Benesch, J.L.P., Laganowsky, A., Storz, J.F., Hochberg, G.K.A., Thornton, J.W., 2020. Origin of complexity in haemoglobin evolution. *Nature* 581 (7809), 480-485.

Portoro, I., Kocsis, L., Herman, P., Caccia, D., Perrella, M., Ronda, L., Bruno, S., Bettati, S., Micalella, C., Mozzarelli, A., Varga, A., Vas, M., Lowe, K.C., Eke, A., 2008. Towards a novel haemoglobin-based oxygen carrier: Euro-PEG-Hb, physico-chemical properties, vasoactivity and renal filtration. *Biochimica et biophysica acta* 1784 (10), 1402-1409.

Powanda, D.D., Chang, T.M., 2002. Cross-linked polyhemoglobin-superoxide dismutase-catalase supplies oxygen without causing blood-brain barrier disruption or brain edema in a rat model of transient global brain ischemia-reperfusion. *Artificial cells, blood substitutes, and immobilization biotechnology* 30 (1), 23-37.

Premont, R.T., Reynolds, J.D., Zhang, R., Stamler, J.S., 2020. Role of Nitric Oxide Carried by Hemoglobin in Cardiovascular Physiology: Developments on a Three-Gas Respiratory Cycle. *Circulation research* 126 (1), 129-158.

Pusateri, A.E., Glassberg, E., Weiskopf, R.B., 2019. Reassessment of the Need for an Oxygen Carrier for the Treatment of Traumatic Hemorrhage When Blood is not an Option. *Shock* 52 (1S Suppl 1), 55-59.

- Rajesh, A., Zimmerman, D., Spivack, K., Abdulmalik, O., Elmer, J., 2018. Glutaraldehyde cross-linking increases the stability of *Lumbricus terrestris* erythrocytes. *Biotechnology progress* 34 (2), 521-528.
- Ratanasopa, K., Chakane, S., Ilyas, M., Nantasenamat, C., Bulow, L., 2013. Trapping of human hemoglobin by haptoglobin: molecular mechanisms and clinical applications. *Antioxidants & redox signaling* 18 (17), 2364-2374.
- Reeder, B.J., Grey, M., Silaghi-Dumitrescu, R.L., Svistunenko, D.A., Bulow, L., Cooper, C.E., Wilson, M.T., 2008. Tyrosine residues as redox cofactors in human hemoglobin: implications for engineering nontoxic blood substitutes. *The Journal of biological chemistry* 283 (45), 30780-30787.
- Rivetti, C., Mozzarelli, A., Rossi, G.L., Henry, E.R., Eaton, W.A., 1993. Oxygen binding by single crystals of hemoglobin. *Biochemistry* 32 (11), 2888-2906.
- Roamcharern, N., Payoungkiattikun, W., Anwised, P., Mahong, B., Jangpromma, N., Daduang, S., Klaynongsruang, S., 2019. Physicochemical properties and oxygen affinity of glutaraldehyde polymerized crocodile hemoglobin: the new alternative hemoglobin source for hemoglobin-based oxygen carriers. *Artificial cells, nanomedicine, and biotechnology* 47 (1), 852-861.
- Roberts, M.J., Bentley, M.D., Harris, J.M., 2002. Chemistry for peptide and protein PEGylation. *Advanced drug delivery reviews* 54 (4), 459-476.
- Ronda, L., Bruno, S., Abbruzzetti, S., Viappiani, C., Bettati, S., 2008. Ligand reactivity and allosteric regulation of hemoglobin-based oxygen carriers. *Biochimica et biophysica acta* 1784 (10), 1365-1377.
- Ronda, L., Bruno, S., Viappiani, C., Abbruzzetti, S., Mozzarelli, A., Lowe, K.C., Bettati, S., 2006. Circular dichroism spectroscopy of tertiary and quaternary conformations of human hemoglobin entrapped in wet silica gels. *Protein science : a publication of the Protein Society* 15 (8), 1961-1967.
- Ronda, L., Pioselli, B., Bruno, S., Faggiano, S., Mozzarelli, A., 2011. Electrophoretic analysis of PEGylated hemoglobin-based blood substitutes. *Analytical biochemistry* 408 (1), 118-123.

Rumsey, S.C., Levin, A., 1998. Absorption, transport, and disposition of ascorbic acid in humans. *The Journal of Nutritional Biochemistry* 9 (3), 116-130.

Sakai, H., Cabrales, P., Tsai, A.G., Tsuchida, E., Intaglietta, M., 2005. Oxygen release from low and normal P50 Hb vesicles in transiently occluded arterioles of the hamster window model. *American journal of physiology. Heart and circulatory physiology* 288 (6), H2897-2903.

Sakai, H., Horinouchi, H., Tomiyama, K., Ikeda, E., Takeoka, S., Kobayashi, K., Tsuchida, E., 2001. Hemoglobin-vesicles as oxygen carriers: influence on phagocytic activity and histopathological changes in reticuloendothelial system. *The American journal of pathology* 159 (3), 1079-1088.

Samuni, U., Dantsker, D., Juszczak, L.J., Bettati, S., Ronda, L., Mozzarelli, A., Friedman, J.M., 2004. Spectroscopic and functional characterization of T state hemoglobin conformations encapsulated in silica gels. *Biochemistry* 43 (43), 13674-13682.

Samuni, U., Roche, C.J., Dantsker, D., Juszczak, L.J., Friedman, J.M., 2006. Modulation of reactivity and conformation within the T-quaternary state of human hemoglobin: the combined use of mutagenesis and sol-gel encapsulation. *Biochemistry* 45 (9), 2820-2835.

Scatena, R., Giardina, B., 2001. O-raffinose-polymerised haemoglobin. A biochemical and pharmacological profile of an oxygen carrier. *Expert opinion on biological therapy* 1 (1), 121-127.

Schiro, G., Cupane, A., 2007. Quaternary relaxations in sol-gel encapsulated hemoglobin studied via NIR and UV spectroscopy. *Biochemistry* 46 (41), 11568-11576.

Sen Gupta, A., 2019. Hemoglobin-based Oxygen Carriers: Current State-of-the-art and Novel Molecules. *Shock* 52 (1S Suppl 1), 70-83.

Shibayama, N., 2020. Allosteric transitions in hemoglobin revisited. *Biochimica et biophysica acta. General subjects* 1864 (2), 129335.

Shibayama, N., Saigo, S., 1995. Fixation of the quaternary structures of human adult haemoglobin by encapsulation in transparent porous silica gels. *Journal of molecular biology* 251 (2), 203-209.

Silkstone, G.G., Silkstone, R.S., Wilson, M.T., Simons, M., Bulow, L., Kallberg, K., Ratanasopa, K., Ronda, L., Mozzarelli, A., Reeder, B.J., Cooper, C.E., 2016. Engineering tyrosine electron transfer

pathways decreases oxidative toxicity in hemoglobin: implications for blood substitute design. *The Biochemical journal* 473 (19), 3371-3383.

Simoni, J., Simoni, G., Moeller, J.F., Feola, M., Wesson, D.E., 2014. Artificial oxygen carrier with pharmacologic actions of adenosine-5'-triphosphate, adenosine, and reduced glutathione formulated to treat an array of medical conditions. *Artificial organs* 38 (8), 684-690.

Simoni, J., Simoni, G., Wesson, D.E., Feola, M., 2012. ATP-adenosine-glutathione cross-linked hemoglobin as clinically useful oxygen carrier. *Current drug discovery technologies* 9 (3), 173-187.

Simons, M., Gretton, S., Silkstone, G.G.A., Rajagopal, B.S., Allen-Baume, V., Syrett, N., Shaik, T., Leiva-Eriksson, N., Ronda, L., Mozzarelli, A., Strader, M.B., Alayash, A.I., Reeder, B.J., Cooper, C.E., 2018. Comparison of the oxidative reactivity of recombinant fetal and adult human hemoglobin: implications for the design of hemoglobin-based oxygen carriers. *Bioscience reports* 38 (4).

Spivack, K., Tucker, M., Zimmerman, D., Nicholas, M., Abdulmalik, O., Comolli, N., Elmer, J., 2018. Increasing the stability of *Lumbricus terrestris* erythrocrucorin via poly(acrylic acid) conjugation. *Artificial cells, nanomedicine, and biotechnology* 46 (sup2), 1137-1144.

Strader, M.B., Alayash, A.I., 2017. Exploring Oxidative Reactions in Hemoglobin Variants Using Mass Spectrometry: Lessons for Engineering Oxidatively Stable Oxygen Therapeutics. *Antioxidants & redox signaling* 26 (14), 777-793.

Strader, M.B., Bangle, R., Parker Siburt, C.J., Varnado, C.L., Soman, J., Benitez Cardenas, A.S., Samuel, P.P., Singleton, E.W., Crumbliss, A.L., Olson, J.S., Alayash, A.I., 2017. Engineering oxidative stability in human hemoglobin based on the Hb providence (betaK82D) mutation and genetic cross-linking. *The Biochemical journal* 474 (24), 4171-4192.

Taguchi, K., Maruyama, T., Otagiri, M., 2020. Use of Hemoglobin for Delivering Exogenous Carbon Monoxide in Medicinal Applications. *Current medicinal chemistry* 27 (18), 2949-2963.

Thuillier, R., Dutheil, D., Trieu, M.T., Mallet, V., Allain, G., Rousselot, M., Denizot, M., Goujon, J.M., Zal, F., Hauet, T., 2011. Supplementation with a new therapeutic oxygen carrier reduces chronic fibrosis and organ dysfunction in kidney static preservation. *American journal of transplantation* :

official journal of the American Society of Transplantation and the American Society of Transplant Surgeons 11 (9), 1845-1860.

Tomita, D., Kimura, T., Hosaka, H., Daijima, Y., Haruki, R., Ludwig, K., Bottcher, C., Komatsu, T., 2013. Covalent core-shell architecture of hemoglobin and human serum albumin as an artificial O₂ carrier. *Biomacromolecules* 14 (6), 1816-1825.

Tsuneshige, A., Park, S., Yonetani, T., 2002. Heterotropic effectors control the hemoglobin function by interacting with its T and R states--a new view on the principle of allostery. *Biophysical chemistry* 98 (1-2), 49-63.

Vandegriff, K.D., Malavalli, A., Wooldridge, J., Lohman, J., Winslow, R.M., 2003. MP4, a new nonvasoactive PEG-Hb conjugate. *Transfusion* 43 (4), 509-516.

Vanholder, R., Dominguez-Gil, B., Busic, M., Cortez-Pinto, H., Craig, J.C., Jager, K.J., Mahillo, B., Stel, V.S., Valentin, M.O., Zoccali, C., Oniscu, G.C., 2021. Organ donation and transplantation: a multi-stakeholder call to action. *Nature reviews. Nephrology* 17 (8), 554-568.

Varnado, C.L., Mollan, T.L., Birukou, I., Smith, B.J., Henderson, D.P., Olson, J.S., 2013. Development of recombinant hemoglobin-based oxygen carriers. *Antioxidants & redox signaling* 18 (17), 2314-2328.

Varney, J., Rivera, A., Dong, V., Tieu, P., Zia, S., Huy, N.T., 2021. Mini-review on the properties and possible applications of therapeutic oxygen carrier Hemarina-M101. *Transfusion and apheresis science : official journal of the World Apheresis Association : official journal of the European Society for Haemapheresis* 60 (1), 103016.

Viappiani, C., Abbruzzetti, S., Ronda, L., Bettati, S., Henry, E.R., Mozzarelli, A., Eaton, W.A., 2014. Experimental basis for a new allosteric model for multisubunit proteins. *Proceedings of the National Academy of Sciences of the United States of America* 111 (35), 12758-12763.

Viappiani, C., Bettati, S., Bruno, S., Ronda, L., Abbruzzetti, S., Mozzarelli, A., Eaton, W.A., 2004. New insights into allosteric mechanisms from trapping unstable protein conformations in silica gels.

- Proceedings of the National Academy of Sciences of the United States of America 101 (40), 14414-14419.
- Walder, J.A., Zaugg, R.H., Walder, R.Y., Steele, J.M., Klotz, I.M., 1979. Diaspirins that cross-link beta chains of hemoglobin: bis(3,5-dibromosalicyl) succinate and bis(3,5-dibromosalicyl) fumarate. *Biochemistry* 18 (20), 4265-4270.
- Wang, Q., Sun, L., Ji, S., Zhao, D., Liu, J., Su, Z., Hu, T., 2014. Reversible protection of Cys-93(beta) by PEG alters the structural and functional properties of the PEGylated hemoglobin. *Biochimica et biophysica acta* 1844 (7), 1201-1207.
- Wang, Y., Zhang, S., Zhang, J., Yu, W.-L., Gao, D.-W., Wang, Q., You, G.-X., Hu, T., Zhao, L., Zhou, H., 2017. Structural, functional and physiochemical properties of dextran-bovine hemoglobin conjugate as a hemoglobin-based oxygen carrier. *Process Biochemistry* 60, 67-73.
- Winslow, R.M., 2004. MP4, a new nonvasoactive polyethylene glycol-hemoglobin conjugate. *Artificial organs* 28 (9), 800-806.
- Winslow, R.M., 2005. Targeted O₂ delivery by low-p50 hemoglobin: a new basis for hemoglobin-based oxygen carriers. *Artificial cells, blood substitutes, and immobilization biotechnology* 33 (1), 1-12.
- Winslow, R.M., 2006a. Current status of oxygen carriers ('blood substitutes'): 2006. *Vox sanguinis* 91 (2), 102-110.
- Winslow, R.M., Vandegriff, K., Intaglietta, M.E., 1996. *Blood substitutes: new challenges*. Springer.
- Winslow, R.M.E., 2006b. *Blood substitutes*. Elsevier.
- Winslow, R.M., 2008. Cell-free oxygen carriers: scientific foundations, clinical development, and new directions. *Biochimica et biophysica acta* 1784 (10), 1382-1386.
- Wollocko, H., Anvery, S., Wollocko, J., Harrington, J.M., Harrington, J.P., 2017a. Zero-link polymerized hemoglobin (OxyVita((R))Hb) stabilizes the heme environment: potential for lowering vascular oxidative stress. *Artificial cells, nanomedicine, and biotechnology* 45 (4), 701-709.

- Wollocko, H., Wollocko, B.M., Wollocko, J., Grzegorzewski, W., Smyk, L., 2017b. OxyVita((R))C, a next-generation haemoglobin-based oxygen carrier, with coagulation capacity (OVCCC). Modified lyophilization/spray-drying process: proteins protection. *Artificial cells, nanomedicine, and biotechnology* 45 (7), 1350-1355.
- Yonetani, T., Park, S.I., Tsuneshige, A., Imai, K., Kanaori, K., 2002. Global allostery model of hemoglobin. Modulation of O₂ affinity, cooperativity, and Bohr effect by heterotropic allosteric effectors. *The Journal of biological chemistry* 277 (37), 34508-34520.
- Yonetani, T., Tsuneshige, A., 2003. The global allostery model of hemoglobin: an allosteric mechanism involving homotropic and heterotropic interactions. *Comptes rendus biologiques* 326 (6), 523-532.
- Young, M.A., Riddez, L., Kjellstrom, B.T., Bursell, J., Winslow, F., Lohman, J., Winslow, R.M., 2005. MalPEG-hemoglobin (MP4) improves hemodynamics, acid-base status, and survival after uncontrolled hemorrhage in anesthetized swine. *Critical care medicine* 33 (8), 1794-1804.
- Yuan, Y., Tam, M.F., Simplaceanu, V., Ho, C., 2015. New look at hemoglobin allostery. *Chemical reviews* 115 (4), 1702-1724.
- Zhang, N., Jia, Y., Chen, G., Cabrales, P., Palmer, A.F., 2011. Biophysical properties and oxygenation potential of high-molecular-weight glutaraldehyde-polymerized human hemoglobins maintained in the tense and relaxed quaternary states. *Tissue engineering. Part A* 17 (7-8), 927-940.
- Zhou, Y., Jia, Y., Buehler, P.W., Chen, G., Cabrales, P., Palmer, A.F., 2011. Synthesis, biophysical properties, and oxygenation potential of variable molecular weight glutaraldehyde-polymerized bovine hemoglobins with low and high oxygen affinity. *Biotechnology progress* 27 (4), 1172-1184.

Table 1. Oxygen properties of HBOCs.

HBOC name	p50 (torr)	Hill n	Refs.
Sanguinate	7-16	-	(Misra et al., 2014)
Oxyvita	6	1.1-1.2	(Harrington et al., 2011)
HemoAct	8-11	1.1	(Tomita et al., 2013)
Hemo2Life	7-12	-	(Mallet et al., 2014) (Varney et al., 2021)
Hemopure	40	1.4	(Jahr et al., 2008)
Oxyglobin	38	1.3	(Harris et al., 2008)
PolyHeme	26-32	1.5	(Gould et al., 1998)
Hemospan (MP4OX)	5-6	1.2	(Vandegriff et al., 2003)

Euro-PEG-Hb	14	1.5	(Portoro et al., 2008)
HemAssist	32	-	(Lamy et al., 2000)
Hemolink	30-40	1	(Greenburg and Kim, 2004)
Hb Polytaur	16-18	-	(Bobofchak et al., 2003)
Cross-linked Pegylated bHb	1 (R state PEG bHb) 20 (T state PEG bHb)	1 1	(Belcher et al, 2017) (Gu et al., 2021)