Title page:

Artificial oxygen carriers- past, present and the future-a review of the most innovative and clinically relevant concepts

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Running title page:

Update on concepts of artificial oxygen carriers

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List of non-standard abbreviations

Artificial oxygen carriers (AOCs), Carbon monoxide (CO), Carbon Dioxide (CO_2), Oxygen (O_2), hemoglobin (Hgb) hemoglobin-based artificial oxygen carriers (HBOCs), perfluorocarbons (PFCs), perfluorocarbon-based artificial oxygen carriers (PFOCs), red blood cell concentrate (RBC), Stem cells (SCs), Sickle cell anemia (SCA), Molecular Weight (Mw)

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Abstract

Blood transfusions are daily practice in hospitals. As these products are limited in availability and have various, harmful side-effects, researchers have pursued the goal to develop artificial blood components for about 40 years. Development of oxygen therapeutics and stem cells are more recent goals. Medline, clinicaltrials.gov, clinicaltrialsregister.eu and ANZCTR were searched up to November 2017 using search terms related to artificial blood products to identify new and ongoing research of the last 5 years. For already well-known products that are, however, important to the field or relevant to gain a better understanding, the reader is punctually referred to some important articles older than 5 years. This review includes not only clinically relevant substances such as heme-oxygenating carriers (HBOCs), PFOCs, stem cells and organ conservation, but also interesting pre-clinically advanced compounds depicting the pipeline of potential new products. In- depths insights into specific benefits and limitations of each substance, including the biochemical and physiological background are included. “Fancy” ideas such as Iron-based substances, O_2-microbubbles, cyclodextranes or lugworms are also elucidated. To conclude, this systematic up-to-date review includes all actual achievements and ongoing clinical trials in the field of artificial blood products to pursue the dream of artificial oxygen carrier supply. Research is on the right track, but the task is demanding and challenging.

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Keywords

Artificial oxygen carriers, blood substitutes, stem cells, perfluorocarbon-based artificial oxygen carriers, hemoglobin-based artificial oxygen carriers

Introduction

Every day thousands of patients receive red blood cell concentrates (RBCs) in order to maintain essential functions such as oxygen (O_2) delivery(Meier J, 2016). In the recent years, state of the art was developed such as conservation of blood, anticoagulants and safety regarding infections. Nevertheless, until now blood saves life - but RBC transfusions have important side effects such as immune modulations, acute transfusion reactions, transfusion-related lung injury, volume overload or hemolytic reactions. RBCs cannot be stored without obtaining side effects called the “storage lesion”(Brunskill SJ, 2015; Tissot JD, 2017). Transfusion-associated bacterial contamination and viral infections have been reported. The incidence of cancer recessive and an increase in mortality has been reported for bladder/colon/ and gastric cancer(Sun C, 2014; Amri R, 2017; Furrer MA, 2017; Velásquez JF, 2017). And rare - but still occurring - mistransfusions may lead to severe problems.

In order to minimize the risks of RBC transfusions, Patient Blood Management programs have enabled a more careful use of blood products(Meybohm P, 2016). Demographic changes lead to more elderly people who require surgery. Therefore, artificial oxygen carriers (AOCs) may be required to enable surgery in all patients, as donated blood has also become a spare source. The actual idea in the field of AOCs has shifted during the past 40 years from “blood substitution” to “oxygen therapeutics”(Spahn, 2018). Many compounds have been developed, but *the* product has not yet been developed(Simoni, 2017). The overall aim is to provide an additional tool for physicians in clinical situations, in which blood may not be available, might not be an option (such as antibodies against blood compounds or religious reasons), or oxygen delivery is required (i.e. transplantation).

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An overview of the demands of a perfect AOC is given in Figure 1. Besides high affinity to O_2 with easy release at the tissue, high affinity to carbon monoxide (CO)/carbon dioxide (CO_2) with easy release during the lung passage are required goals. Major problems of AOCs are the induction of the inflammatory reaction of the body, hypotension or hypertension.

This review gives an overview (please see Figure 2) about the currently pre-clinically and clinically relevant AOCs, which include Hgb-based oxygen carriers (HBOCs), perfluorocarbon-based oxygen carriers (PFOCs) and stem cells (SCs). As shown in Figure 2A, PFCs are halogene-substituted compounds, while HBOCs (Figure 2B) have a central ion (most frequently iron) surrounded by tetrapyrroles. SCs can develop into RBCs or other target tissues (Figure 2C) and oxygen emulsions are useful to increase oxygen in liquids (Figure 2D). Biochemical/physiological details of PFOCs and HBOCs are lined up in the Tables “At a glance” (Table 1 and 2). A summary of the important achievements such as quality improvement prior to transplantation) with AOCs used for organ conservation is shown in Figure 3.

For a variety of other substances, please consider the Online Supplement, which includes detailed information: In Supplemental Table 1: Recent clinical trials with Hemopure; Supplemental Table 2: Clinical studies on other HBOCS (Hemolink, Polyheme, Pyridoxalated hemoglobin polyoxyethylene conjugate and Hemotech); Supplemental Table 3: Trials with hemospan/MP4OX and MP4CO; Supplemental Table 4: Clinical trials with Sanguinate; Supplemental Table 5: Sophistically engineered Hgbs (including OxyVita, Poly-Hb-tempol, Sanflow, VitalHeme, YQ23, BAEGF-Hb, PolyPHb/bPEG-Hb; Supplemental Table 6: Standard Hgb plus engineered envelope (HbVesicles, HbMP-700, ErythroMer, HbN, HbP, LEH, Hb-PDA, PDA-Hb-microcapsules, Hemoact, RBCM, Mal-PEG-ß-xl-Hb; Hemoglobin

loaded nanoliposome) and Supplemental Table 7: Other artificial blood products in preclinical stage (HemoCD, PEGLtEC, HrBOC, Cobalt-replaced myoglobin, Cobaltporphyrin-based micelles, LOM/PHMs). Please note, that per definition, free, unmodified hemoglobins (Hgb) do not belong to AOCs and have therefore not been included in this review.

Methods

Medline was searched up to July 23rd, 2018. The search terms perfluorocarbon-based oxygen carriers, perfluorocarbon based oxygen carriers, hemoglobin-based oxygen carriers, hemoglobin based oxygen carriers, artificial oxygen carriers, artificial AND blood AND substitutes, organ preservation AND perfluorocarbons, organ preservation AND hemoglobin-based artificial oxygen carriers, organ preservation AND artificial oxygen carriers, normothermic perfusion AND artificial oxygen carriers, normothermic perfusion AND perfluorocarbons, organ perfusion AND artificial oxygen carriers organ perfusion AND perfluorocarbons, natural extracellular hemoglobin, stem cells AND oxygen carriers were used.

Furthermore, the trial registers of USA, Europe and Australia (http://clinicaltrials.gov, https://www.clinicaltrialsregister.eu/ and http://anzctr.org.au were searched up to July 23rd, 2018 using the drug names DCLHb, ErythroMer, Hbmp-700, Hemopure, Hb-201, Hemoxycarrier, Hemo2life, HEMOXCell, Hemolink, Oxyvita, OxyVita Hb, Vitalheme, polynitroxylated AND pegylated AND hemoglobin, Hbvesicles, Hemoact, Hemotech, HemAssist, Polyheme, pyridoxalated hemoglobin polyoxyethylene conjugate, Hemospan, MP4OX, MP4CO, Sanguinate, Oxygent and Oxycyte. To introduce the reader into the context of artificial blood products, to point out

milestones and to explain typical problems and side-effects associated with each class of substances important and relevant key articles are cited.

Main text

Main section I: Development of artificial blood products

The development of blood transfusions started decades ago: In 1667 blood was transfused from a dog to a human(Roux FA, 2007), in 1692 from lambs to humans. On September 1st 1818, the first blood transfusion from human to human was performed by Blundell. However, only with the discovery of the ABO blood type by Landsteiner/Decastello survival improved(Greenwalt, 2005).

In parallel the development of AOCs started with the aim of (i) the elimination of whole blood-associated side effects and (ii) the unrestricted disposal of “blood” or at least parts of it (erythrocytes). Unfortunately, the pioneering work, performed in the 1930s using free Hgb extracted from human blood(Amberson et al., 1933), resulted in undesirable side effects, e.g. nephrotoxicity(Chang, 1988; Elmer et al., 2012; Cardenas et al., 2017). 27 years later the first description of AOCs in the strict sense, namely nano-bio-technologically engineered Hgb and other synthetic compounds surrounded by an artificial membrane or otherwise chemically engineered Hgb, evolved(Chang, 2012). Until now, three classes of AOCs have been defined:

- HBOCs
- PFOCs
- AOCs derived from SCs

The foci of this review are new preclinical and clinical developments within the last 5 years.

Main section II: General requirements of artificial blood products

Clinicians and researchers have physiological, biochemical and technical demands on “the perfect” artificial blood product (Figure 1). Of course the supply of the tissue with O_2 in combination with evacuation of CO_2 from the periphery are the most important ones (Figure 1). The p50 value determines the partial pressure of O_2 at which the Hgb is saturated to 50%. In a healthy adult, 26.6 mmHg (3.5 kPa) are normal. If the p50 is higher, the affinity to O_2 decreases, the standard curve shifts rightwards. A lower p50 indicates a higher affinity to O_2 with a left shift of the O_2 affinity curve. The p50 values are listed in the particular chapter and in Table 1.

Main section III: Classes of AOCs

HBOCs: Specifications, peculiarities, limitations

HBOCs are compounds consisting of natural Hgb from different organisms (Table 1).

HBOCs are attractive AOCs, as is that they are able to deliver O_2 to the tissue without an increased inspiratory O_2 concentration.

The half-life with 18-23h is much shorter than the half-life of erythrocytes with 120 days, so that repetitive doses of HBOCs would be required to maintain O_2 delivery for days (original reason for the development of HBOCs as blood substitutes). More recently, HBOCs have been designed to bridge patients safely to the clinics: to gain

time until RBC transfusion is available. In presence of flue gases they do not remain functional as CN^- or CO displace O_2 from it binding sites in the Hgb molecule and additionally components of flue gas oxidize Hgb into Met-Hgb.

HBOCs always require natural Hgb, either from outdated human RBCs, extracted from animal blood or bacteria/ yeast/ plants. Therefore, the availability of HBOCs is still dependent on people´s willingness to donate blood and the risk of infections remains (e.g. prions). In contrast, disposability of bovine blood is nearly unlimited. Instead of using *E.coli* or *S.cerevisiae*, there are now efforts to obtain recombinant Hgb(Varnado et al., 2013) from plants e.g. from *Nicotiana benthamiana*(Eriksson and Bülow, 2017) and furthermore researchers focus more and more an fetal Hgb, which is more stable than adult Hgb(Ratanasopa et al., 2016; Simons et al., 2018). Free, non-encased with any type of membrane, unprocessed mammal Hgb is associated with typical problems (Table 3 and(Cardenas et al., 2017)). Encasing in any type of membrane by crosslinking between monomers (to stable tetramers) as well as tetramers (to affect O_2 affinity or size)(Centis and Vermette, 2009), can reduce these side effects. Furthermore increasing knowledge on the influence of size and surrounding shell on the pharmacokinetic properties helped to decrease side-effects(Taguchi et al., 2017). Of note, crosslinking agents and shell material may also cause immunoreactions or increase Met-Hgb-formation(Centis and Vermette, 2009).

The novel lugworm Hgb lacks the typical side effects of immunoreaction and inflammation(Rousselot et al., 2006). Other relevant worms are oligochaetes (e.g. earthworm) containing erythrocruorin(Jani et al., 2017; Zimmerman et al., 2017) or worm-like animals such as sipunculans containing hemerythrin(Toma et al., 2018). Erythrocruorin contains 144 globin chains and heme-molecules. The mechanism of

oxygen-binding is the same as in mammal Hgb (formation of dioxygen complex). Hemerythrin that does not contain any heme group, formes hydroperoxides.

HBOC compounds recently under clinical investigation (reviewed up to 2017 in*(Njoku et al., 2015)*, *(Gupta, 2017)*)

Hemopure

- glutaraldehyde-polymerized bovine Hgb, molecular weight (Mw) of 250 kDa, p50 of 38 mmHg(Jahr et al., 2008)

The FDA terminated clinical trials because of safety concerns in 2008(Keipert, 2017). Nevertheless, many studies in phase I-III have been performed with Hemopure(Van Hemelrijck et al., 2014) (reviewed in(Chen et al., 2009; Jahr et al., 2012)), but are not listed in the clinical trials registry any more. In other countries, clinical studies have been completed or are ongoing (Supplemental Table 1). Despite the safety concerns, the substance was clinically approved in South Africa in 2001(LLC, 2014; Mer et al., 2016), in Russia in 2012(Ortiz et al., 2014) and it was provided to patients with life-threatening anemia in the United States for whom allogeneic blood transfusion was not an option (NCT01881503, NCT02684474, NCT02934282) since 2013(Lundy et al., 2014; Posluszny and Napolitano, 2014; Epperla et al., 2016; Posluszny and Napolitano, 2016; Resar et al., 2016; Gomez et al., 2017; Davis et al., 2018; Olaussen et al., 2018).

The basis of the suspension of clinical trials (the meta-analysis by Natanson and collegues(Natanson et al., 2008)) has been questioned and re-evaluated by many researchers later on revealing many methodological flaws and basically pointing out

that there is no evidence of any NO-related toxic class-effect applicable for every HBOC(Mackenzie et al., 2015; Dubé et al., 2017; Mackenzie et al., 2017). However, Hemopure-induced vasoconstriction is undisputed and meanwhile the underlying mechanisms (NO scavenging and upregulated endothelin production) have been elucidated(Cabrales and Friedman, 2013; Taverne et al., 2017).

Other HBOCs such as Hemolink or Polyheme have been developed for indications similar to Hemopure (Supplemental Table 2 and(Jahr et al., 2012)). To date, none of these therapeutics has entered the clinics. Causes are increased thirty-day mortality, hypertension and myocardial infarction(Njoku et al., 2015).

Hemospan/MP4OX

- human Hgb, conjugated with maleimide-poly(ethylene)glycol, Mw of 96 kDa, p50of 6 mmHg(Winslow, 2006)
- Hgb content of 4.2 g/dl is too low to solely supply an organism with oxygen

Hemospan, later named MP4OX, was therefore developed as O_2 therapeutic in order to improve oxygen supply rather than to fully replace blood(Jahr et al., 2012). Injection of MP4OX caused a low anti-oxidant response and tendency to extravasation into tissue in a rat model(Terraneo et al., 2017). In the last years, MP4OX was refined as a therapeutic for special occasions such as treatment of sickle cell anemia (SCA). CO prevents and reverses polymerization of hemoglobin-S and thus distortion of sickled erythrocytes(Keipert and Investigators, 2016). By MP4OX pain, severity and duration of SCA crisis can be reduced. Additionally, low-dose CO also acts as a signaling molecule to reduce inflammation and O_2 requirement as well as to prevent apoptosis in patients(Keipert and Investigators,

2016). The scientific and medical underlying mechanisms have been studied. MP4OX leads to an induction of nuclear factor erythroid 2 p45-related factor 2 and hepatic hemeoxygenase-1, as well as inhibition of nuclear factor 'kappa-light-chain-enhancer' of activated B-cells, observed in an animal model(Belcher et al., 2013) and has been reviewed in(Simoni, 2017). After unloading of CO, the compound is oxygenated in the lung and thereby transforms into MP4OX. The effects of MP4OX have been further investigated in animal models of SCA(Tsai et al., 2015). Relevant clinical trials with Hemospan/MP4OX and MP4CO are listed in Supplemental Table 3. The retrospective Phase II b study (NCT01262196) was criticized, as the authors reported “a numerically higher percentage of patients treated with MP4OX were alive and discharged from hospital at day 28 (primary efficacy endpoint) versus controls (57% vs. 50% p=0.18) (Keipert, 2017), although the study was underpowered(Keipert, 2017).

Sanguinate

- bovine Hgb crosslinked to poly(ethylene)glycol to enlarge the molecule and to hide it from the immune system, Mw of120 kDa, p50 of 7-16 mmHg(Abuchowski, 2016)
- releases CO in order to provide anti-apoptotic and anti-inflammatory properties (see MP4CO). CO additionally reduces auto-oxidation of Hgb(Abuchowski, 2016).

Clinical trials with Sanguinate are listed in Supplemental Table 4. Sanguinate is available under an emergency investigational new drug protocol. So far, it enabled

survival of only a few patients refusing transfusion due to religious reasons(Posluszny and Napolitano, 2014; Abuchowski, 2016; Resar et al., 2016).

Hemo2life

- Hgb extracted from lugworms, not packed into erythrocytes or any other membrane, Mw of 3600 kDa, p50 of 7 mmHg(Mallet et al., 2014)
- One molecule transports up to 156 molecules O_2, 38 x more than mammal Hgb, natural superoxide-dismutase-like activity that compensates for oxygen-related radicals(Mallet et al., 2014)

A clinical phase I open label trial in kidney transplantation (cold storage in Belzer University of Wisconsin versus hypothermic machine perfusion with Belzer University of Wisconsin + Hemo2life before transplantation) has been completed in February 2018 (NCT02652520). Furthermore, Hemo2life improved static storage of donor hearts prior to transplantation in a preclinical animal model(Teh et al., 2017) and early graft function after hypothermic static preservation after prolonged cold ischemia of a pig lung(Glorion et al., 2017).

<u>In the pipeline/preclinical development</u>

A lot of new research approaches evolved within the last years – all still in the preclinical status. The four most advanced compounds are OxyVita, HbVesicles, ErythroMer and HemoAct.

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OxyVita (OxyVitaHb)

- bovine Hgb inter- and intra-molecularly crosslinked leading to a homogenous globular-like molecule, MW of17 kDa.
- exists meanwhile in two subtypes, OxyVita Hb and OxyVita HbCO.(Wollocko et al., 2017)

Importantly, the Hgb tetramers are linked to each other via amide bonds without any linker-molecule(Wollocko et al., 2017). Normally, toxic linker molecules such as glutaraldehyde are necessary for these linking-reactions.

The release of free heme-iron into the circulation is low so that toxic side effects are minimized.(Wollocko et al., 2017) OxyVita was tested in different preclinical studies, among them a pre-hospital setting (mimicking initial medical treatment of severely injured patients prior to hospital) of hemorrhagic shock in rats(Jahr et al., 2012). No other HBOC developed so far provided such a success in a battle-field model of severe hemorrhage(Jahr et al., 2012).

HbVesicles

- human Hgb encapsulated by a biocompatible liposome(Azuma et al., 2017) 250-280 nm in diameter decorated with PEG_{5000}, p50 is adjusted with pyridoxalphosphate between 9-30 mmHg(Sakai, 2017)
- Depending on the Hgb core (Hb-CO or HbO_2) HbVesicles can be used as CO or as O_2 carrier
- do not contribute to the colloid osmotic pressure

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HbVesicles have been studied in preclinical animal blood exchange- and hemorrhagic shock models and were also tested in different areas such as isolated organ perfusion, ECMO priming, apnoe or 2D cell cultures(Kohno et al., 2017; Sakai, 2017).

ErythroMer

- human Hgb surrounded by a NO-attenuating polymer shell.
- Contains antioxidants (leuko-methyleneblue) + a pH-sensitive 2,3-bisphosphoglycerate shuttle resulting in a pH-sensitive O_2 affinity

So far ErythroMer was studied in a murine hemodilution model of 70% blood exchange and a rat hemorrhagic shock model(Pan et al., 2016; Kalocyte, 2017).

HemoAct

- a cluster of human Hgb covalently wrapped by a defined amount of human albumin molecules (mainly 3, Hb-HSA_3)(Haruki et al., 2015)
- intra-vascular half-life of 18.5 hours. HemoAct was well tolerated; only a transient increase in mean arterial pressure was observed in controls as well as in HemoAct-treated rats. Seven days later, no organ damage could be detected. HemoAct accumulated mainly in the liver as expected from its structure(Haruki et al., 2015)

Other interesting approaches consist of either sophistically engineered Hgb (Supplemental Table 5) or standard Hgb in a sophisticated envelope (Supplemental Table 6); these products are all in the early preclinical status.

PFOCs Specifications, peculiarities, limitations

Perfluorocarbons (PFCs) are fully halogenated, mainly fluorinated, molecules. Because of the strength of the carbon-fluorine bond, no toxic metabolites in the body are formed(Riess, 2001). Compared to water, PFCs exhibit a high solubility of respiratory gases which linearly depend on their partial pressure. In contrast to Hgb, no saturation of O_2 and CO_2 occurs. O_2 loading and unloading is two times faster than in erythrocytes and the O_2 extraction rate is 3 fold higher, as PFCs release more than 90% of the loaded O_2 to the tissue(Faithfull, 1992; Keipert et al., 1996).

In addition to respiratory gases PFCs also dissolve CO and N_2 which are relevant in the treatment of flue-gas poisonings or gas embolism/ decompression sickness(Spiess, 2009). Typical side effects of PFOCs are a decrease of the mean arterial pressure, lung damage, thrombocytopenia, flue-like symptoms in addition to poor emulsion stability and long organ retention time(Lowe, 2003; Hosgood and Nicholson, 2010). To provide compatibility with the aqueous medium blood, PFCs have to be emulsified or encapsulated for use as AOCs. Such emulsions normally display a droplet size of 100-300 nm, but are highly sensitive to flocculence and Oswald ripening when not stored frozen. Toxic emulsifiers such as Pluronic-F68 improve the stability of the formulation, but are one factor responsible for side effects such as transient hypotension, immunoreaction, activation of complement system(Kuznetsova, 2003).

In newer formulations biocompatible emulsifiers such as egg yolk phospholipids or high Mw-PFCs e.g. perfluorotributylamine or perfluoromethylcyclohexylpiperidine (however associated with long organ retention times) are used(Riess, 2005). After

uptake of the emulsion droplets into macrophages, PFCs such as perfluorodecalin or perfluorooctylbromide (possess appropriate vapor pressure) diffuse back into the blood and are, associated to lipoproteins, transported to the lung where they can be exhaled(Lowe, 2006).

Actually, there is only one PFOC, Perftoran (Perftec) which has been approved for human clinical use in Russia, Mexico, Kazakhstan, Kyrgyzstan and Ukraine(Castro and Briceno, 2010). Perftoran is now produced under standard manufacturing practice (brand name Vidaphor) with the aim to be introduced to the markets in the USA and in Europe(Fluoro2Therapeutics, 2017; Latson, 2017).

PFOCs were also used in the context of organ preservation such as brain, kidney, pancreas, liver(Okumura et al., 2017), heart either in static cold storage or for machine perfusion(Zhang and Barralet, 2017), an overview gives: (Hosgood and Nicholson, 2010). The use of PFOCs allows for RBCs-free normothermic perfusion and thus for organ regeneration prior to transplantation (Figure 3).

Compounds under clinical investigation

Currently there is only one compound, Oxygent, that has been investigated in clinical trials. Oxygent is a 60% PFC emulsion (58% perfluorooctylbromide, 2% perfluorodecylbromide, egg-yolk phospholipids). Oxygent is known since the 90s and has been studied in several clinical studies(Castro and Briceno, 2010; Spahn and Keipert, 2017). Among those, especially two phase III studies successfully showed the potential of Oxygent: The first investigated patients undergoing orthopedic surgery, who were pre-operatively normovolemic hemodiluted with colloid to a target

Hb of 9g/dl. The normovolemic hemodilution was followed by either treatment with Oxygent, autologous blood or conventional colloid when reaching a pre-defined transfusion trigger. Patients in the Oxygent group showed the longest duration of transfusion-trigger reversal, thus Oxygent was more effective than blood or colloid in stabilizing the patients and avoiding additional transfusions(Spahn et al., 1999). These results could be confirmed in a second clinical trial in patients undergoing non-cardiac surgery. Pre-operative hemodilution was followed by two doses of Oxygent. Oxygent reduced the need for blood transfusions compared to the standard care (no hemodilution, intra-operative transfusion of RBCs if indicated)(Spahn et al., 2002).

However, in 2002, Oxygent was abandoned because of safety issues in the phase III coronary artery bypass grafting trial(Keipert, 2006). In 2017, Oxygent was reproduced by Double Chrane, licensed and approved for clinical studies in China(Liu, 2017).

With the promising PFOC Oxycyte a successful phase II study was completed in 2008 in patients with traumatic brain injury (NCT00174980(Fabian, 2011)). Another phase II study on safety and efficacy of Oxycyte was started in 2009 (NCT00908063), but was terminated by the sponsor in 2014 due to lack of patient enrollment(Therapeutics, 2014). The sponsor abandoned the substance.

In the pipeline/preclinical development

Albumin-derived perfluorodecalin-filled nanocapsules showed promising results in a first *in vivo* toxicity study(Wrobeln et al., 2017a) and protected a Langendorff-heart (rat) during massive ischemia(Wrobeln et al., 2017b). Similar, a novel PFC emulsion increased myocardial O_2 delivery, improved cardiac function and generated a more physiological redox-state in a Langendorff-heart (rabbit) compared to perfusion without the PFC emulsion(Kuzmiak-Glancy et al., 2018).

Other products

Besides engineering Hgb or the surrounding shell there are other ideas such as using simply the O_2 binding porphyrin structure of Hgb (e.g. embedded in cyclodextranes (HemoCD)(Kitagishi et al., 2017)) or completely other materials such as cobalt-porphyrins (the central iron-ion in the O_2-binding structure of Hgb is replaced by cobalt) (Neya et al., 2014; Shen et al., 2016). Furthermore there have been attempts to directly introduce O_2 into particles, thus for the first time permitting for a safe and effective intravenous injection of O_2 gas which locally increases pO_2 very rapidly(Seekell et al., 2016; Black et al., 2017). All those substances (Supplemental Table 7) are still in the preclinical stage.

SCs Specifications, peculiarities, limitations

In 2006, a new cell source, that was able to differentiate into all cell types of the endo-, ecto-, or mesodermal lineages, was found by Shinya Yamanaka (Takahashi and Yamanaka, 2006). Induced pluripotent SCs can be generated from different somatic cell sources by overexpression of specific transcription factors, e.g. HOXB4 (Takahashi and Yamanaka, 2006; Schiedlmeier et al., 2007; Yu et al., 2007).

There are two approaches to use SCs in the context of AOCs (Figure 2C):

(i) differentiation of SCs into RBCs

(ii) differentiation of SCs into various target cells in an oxygenated environment.

1. SCs differentiation into RBCs

Major challenges in the use of SCs are the low retention and engraftment of transplanted cells and the adverse effects of inflammation and immunoreactions when allogeneic or xenogeneic cells are used(Van Veen T, 2015). Giarratana elucidated the quality of donated hematopoietic SCs from human donors that were developed in culture into RBCs in a proof of concept study(Giarratana et al., 2011). Challenging was not only the viability and cell deformability, but the requirement of an improved production protocol of cultured RBCs without feeder cells at reasonable costs(Giarratana et al., 2013). In order to provide the required huge amounts of RBCs, upscaling has to be improved. In addition, artificial red blood cell generation has to result in nucleus-free erythrocytes that contain only adult Hgb and no fetal Hgb.

An advantage of this technique is that cells can be produced patient-specific according to the blood phenotype of the recipient.

2. Differentiation of SCs into various target cells in an oxygenated environment

To improve SC differentiation O_2 supply to hypoxic areas should be high and the O_2 gradient formation should be reduced. The differentiation potential and cell viability should be preserved and the extracellular matrix microenvironment intact. Oxidative stress and the generation of reactive oxygen species is not desired, as hematopoetic SCs might die(Jung H, 2014). Therefore the support of AOCs in SCs exploitation displays an ideal combination.

Le Pape and colleagues(Le Pape F 2017) evaluated the ability of the HBOC, HEMOXCell, to carry O_2 for culturing human bone marrow mesenchymal SCs *in vitro* for 3D culture applications in human platelet lysate-supplemented media. HEMOXCell provoked a cell growth rate induction of 25% while the mesenchymal SCs phentoype was preserved and typical differentiation properties were maintained. In a study of 2018, the authors developed a perfusion culture method to provide similar distribution of nutrient and O_2 throughout the artificially engineered tissue, specifically for the setting of osseointegration in dental implant surgery. HEMOXCell was beneficial for the development of mesenchymal stem cells into allogenic bone substitute(Le Pape et al., 2018).

Similar effects were observed using the PFOC Fluosol-DA. The slopes of the single-dose radiation survival curves for intestinal epithelial cells and spermatogenic SCs in mice breathing air or O_2 were not significantly altered by the administration of Fluosol-DA 10 min before irradiation, and the doses to achieve an isoeffect were altered by 1.03 or less. When mice were challenged with i.v. injected Fluosol-DA tumor cells 24 h after treatment with Fluosol-DA, no increase in the number of artificial pulmonary metastases was observed(Mason KA, 1985).

Furthermore, Tang and colleagues created an oxygenated environment using a nanogel structure: They encapsulated human cardiac SCs in thermosensitive poly(N-

isopropylacrylamine-co-acrylic acid) nanogel in murine and pig models of myocardial infarction(Tang J, 2017). In contrast to conventional SCs, encapsulated human cardiac SCs did not induce an inflammatory reaction or T-cell infiltration in immunocompetent mice in contrast to xenogeneic human cardiac SCs injected in saline, which induced the immune response. The cardiac function was maintained and scar sizes reduced. The authors concluded that “thermosensitive nanogels can be used as a carrier: the porous and convoluted inner structure allows nutrient, O_2 and secretion diffusion, but can prevent SCs from being attacked by immune cells”(Tang J, 2017).

A recent publication by Cantaluppi et al. reported that the addition of PFCs to viable renal tubular epithelial cells in a renal assist device led to the differentiation of those cells towards renal progenitor cells(Cantaluppi et al., 2018).

Main section V: Discussion: Outlook –the future of AOCs

Looking at the last years, big advantages have been made in the development of AOCs (HBOCs, PFOCs, SCs). However, these years have also shown the big challenges of inflammatory reactions, conservation and O_2 affinity that have to be managed to really provide a clinically useful AOC. Economic aspects often played a role and may have caused a delay or even stopped the further development of initially successful compounds. Recently, the safety profile of the HBOC Hemopure was re-evaluated, as new, very promising HBOCs such as Sanguinate or Hemo2life emerged from the laboratories. Furthermore, biochemical and physiological properties of different HBOCs have been compared in a recent study by the US food and drug administration in order to facilitate development of novel AOCs(Meng et al.,

2018). Additionally, clinical studies with the PFOC Oxygent were resumed in 2017. And finally the efforts to make the established PFOC Perftoran (Vidaphor) available at the US and European market may bring us closer to clinically useful AOCs.

Severely anemic patients refusing transfusion because of religious grounds can benefit from AOCs. However, up to now, there are just single case reports and there is not enough evidence to generally advise their use in combination with standard care(McConachie et al., 2018).

To this end, some products have reached clinical use in selected countries. In Germany and the USA, none has reached the broad clinics.

For sure, research will continue as AOCs are urgently needed to meet the demands of the ageing population. Both, blood substitution as well as oxygen therapeutics and stem cell derivatives will be in research activities. Probably, in Europe and the USA, AOCs will be used first for *ex situ* organ preservation prior to a use as a RBCs-equivalent. Additionally, the artificial generation of personalized RBCs from adult SCs will probably be one major path forward, especially, if production of high amounts at low costs under good clinical practice-conditions can be realized. The past 40 years of research have shown us, that the development of AOCs is challenging. Many substances have been tested, but were not useful. Many projects were started, but failed. Nevertheless, the recent, novel developments indicate promising results to be expected within a sufficient period of time.

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Authorship contributions

Participated in research design: Ferenz

Performed literature research and data analysis: Ferenz and Steinbicker

Wrote or contributed to the writing of the manuscript: Ferenz and Steinbicker

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Footnotes

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Legends for Figures

Figure 1. Demands of the perfect artificial oxygen carrier

In Figure 1, the demands of a perfect AOC are depicted. A high affinity to oxygen with an easy release at the tissue is an essential adjective. High affinity to CO and CO_2 with also an easy release at the lung passage is a second required goal. Major problems of artificial components are the induction of the inflammatory reactions of the body, hypotension or hypertension.

Figure 2. Overview of different types of AOCs

To date, the following AOCs categories can be described: (A) Hgb-based oxygen carriers (HBOCs), that have a central ion (most frequently iron) atom which is oxygenated and surrounded by tetrapyroles; (B) perfluorocarbon-based oxygen carriers (PFOCs), that are halogene substituted compounds; (C) Stem cells, that can develop into different target tissues; and (D) oxygen emulsions, that are useful to increase oxygen in liquids. Structural formula in part adapted with permission from (Ferenz, 2019).

Figure 3. Organ preservation with AOCs

In organ transplantation, the major goal of AOCs is the organ retention and quality improvement prior to transplantation. The use of AOCs allows for normothermic perfusion without RBCs. Retention of AOCs or metabolites not as important as in blood substitutes, as AOCs are mainly washed out prior to transplantation.

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Tables

Table 1: At a glance: HBOCs- natural Hgb from an organism

Compounds (in bold) **abandoned** **actually investigated in clinics plus indication** *Compounds (in italy) of recent investigations, so far only in preclinical studies)*	- **Hemolink, Polyheme , DCLHb, Hemotec, rHb1.0/2.0, Hemospan/ MP4OX/ MP4CO** - **Hemopure: life-threatening anemia if blood transfusion is not an option (in the United States) since 2013** - **Saguinate : emergency protocol (so far 2 patients survived, who refused blood transfusions)** - **Hemo2life: to improve kidney quality prior to transplantation, hypothermic maschine perfusion** - *Oxyvita, HbVesicles, ErythroMer , HemoAct*
Important parameters	Examples
Misciability with blood	Unproblematic for all HBOCs
Origin of hemoglobin	1.) Human (blood donation required) or 2.) Bovine/ lugworm (risk of infection such as prions)
High range of oxygen affinity (p50)	38mmHg (Hemopure), 6mmHg (Hemospan/ MP4OX), 9-30mmHg (HbVesicles)
High carbon monoxide affinity	Some compounds are designed to deliver therapeutic, non-toxic levels of CO (example Sanguinate)
High molecular weight/ size	Surrounded by any type of membrane or crosslinked (crosslinking between monomers to stable tetramers as well as crosslinking of tetramers to molecules affecting oxygen affinity or size. Therefore huge variation (example: OxyVita 17kDa, Hemo2Life 3600kDa)
Metabolism	Crosslinking agents and shell material often cause immunoreactions or may increase methemoglobin formation
Intravascular half-life	18-23h
Recent studies performed in animals	Erythromer: rat hemorrhagic shock model, murine hemodilution model (70% blood exchange) OxyVita: preclinical studies of pre-hospital setting of hemorrhagic shock[15]
Recent studies performed in humans	Hemopure in South Africa 2001 and Russia 2012 clinically approved Hemo2Life in kidney transplantation for organ preservation, recently completed
Application areas other than substitution of blood in whole organism	HbVesicles: isolated organ perfusion, ECMO priming, 2D cell cultures[60,61] Hemo2life: isolated organ preservation
Concerns of regulatory authorities	Reviewed in 15, 43. Safety issues. Problem of HBOCs: not funcional in presence of flue gases. FDA: 20008 safety concern for Hemopure
Current to future intent	1.) Intent to get approval for Hemopure in EUROPE/GERMANY, 2.) Recombinant Hgb from plants 3.) Use the more stable fetal Hgb.
Reviews elucidating biochemistry/physiology behind HBOCs	Varnado[27,] Alayash[30,31]Cabrales and Friedman[32,] Njoku, Taguchi 33

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Table 2: At a glance: PFOCs-perfluorocarbon based oxygen carriers

Compounds (in bold) **abandoned** **actually investigated in clinics plus indication** *Compounds (in italy) of recent investigations, so far only in preclinical studies)*	**- Fluosol-DA, Oxycyte: not any more in trials since 2014** - **Perftoran (Perftec®): approved for human clinical use in Russia, Mexico, Kazakhstan, Kyrgyzstan and Ukraine[74] e.g. resuscitation from hemorrhagic shock, cardioplegia** - **Oxygent: produced, licensed and approved for clinical studies in China[77] e.g. resuscitation from hemorrhagic shock** - Albumin-derived perfluorodecalin-filled nanocapsules
Important parameters	Examples
Misciability with blood	To provide compatibility with the aqueous medium blood, PFCs have to be emulsified or encapsulated.
High oxygen affinity p50 and carbon monoxide affinity	No saturation of O_2 and CO_2 occurs, dissolubility dependent on gas partial pressure, in addition to respiratory gases PFCs also dissolve CO and N2 which are relevant in the treatment of flue-gas poisonings or gas embolism and decompression sickness[69].
High molecular weight/size	emulsified or encapsulated displaying a droplet size of 100-300 nm
Metabolism	Fully halogenated, mainly fluorinated, molecules. Strong carbon-flourine bond, no toxic metabolites are formed. Elimination: First uptake into macrophages, then diffusion into the blood, association to lipoproteins, transport to the lung, where they can be exhaled (if vapor pressure is favorable, e.g. perfluorodecalin or perfluorooctylbromide)[73,68]
Easy release of O2, CO and CO2	Oxygen loading and unloading is two times faster than in erythrocytes and the oxygen extraction rate is 3 fold higher as PFCs release more than 90% of the loaded oxygen to the tissue[66,67].
Intravascular half-life	158 min-8 Tage
Application areas other than substitution of blood in whole organism	Used for organ preservation of islets, brain, kidney, pancreas, heart either in static cold storage or for machine perfusion[76], reviewed in [70]
Recent studies performed in animals	Albumin-derived perfluorodecalin-filled nanocapsules showed promising results in a first *in vivo* toxicity study[81] and protected a Langendorff-heart (rat) during massive ischemia [82]
Studies performed in humans	Oxycyte: phase II completion in 2008 in patients with traumatic brain injury[78], phase II study on safety and efficacy in 2009 was terminated by the sponsor in 2014
Concerns of regulatory authorities	Lack of patient enrollment for clinical studies[79], long organ retention times (Riess 2002, 2005)
Current to future intent	1.) introduce Perftoran (Perftec®) as Vidaphor® to the markets in the USA and in Europe 2) Resume development of Oxygent in China
Reviews elucidating biochemistry/physiology behind PFOCs	Cabrales and Briceno, Riess

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Table 3: Typical side-effects of unprocessed mammal Hgb

problem	**complication**
dissociation into dimers(Chang, 1988; Elmer et al., 2012)	• overloading the renal tubular cells (renal failure)
NO stealing property mainly from the endothelial cell layer(Doherty et al., 1998; Olson et al., 2004; Cabrales and Friedman, 2013; Alayash, 2014)	• systemic and pulmonary vasoconstriction (myocardial damage pulmonary hypertension) • lack of mediator of thrombocyte-aggregation and -adhesion (impaired clotting) • gastrointestinal side-effects
local hyperoxia due to decreased oxygen affinity (no diffusion barrier existent)(McCarthy et al., 2001; Alayash, 2014)	• systemic hypertension
auto-oxidation(Buehler et al., 2010; Scurtu et al., 2013; Alayash, 2014)	• non-functional hemoglobin • formation of superoxide ions • altering transcriptional activity of heme oxygenase and other antioxidant enzymes

Figure 1 Demands of the perfect artificial oxygen carrier

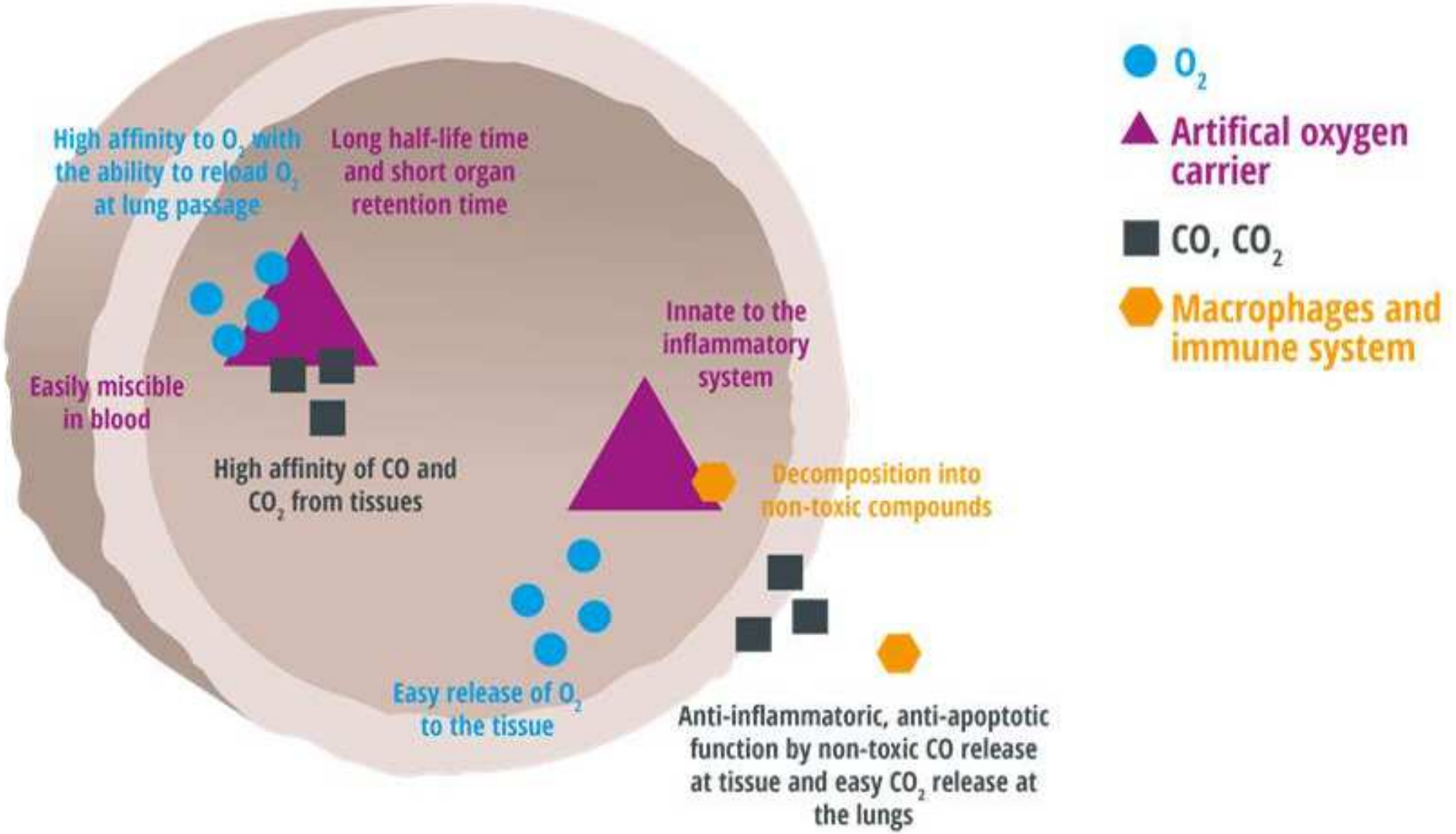


Figure 2 Overview of different types of AOCs

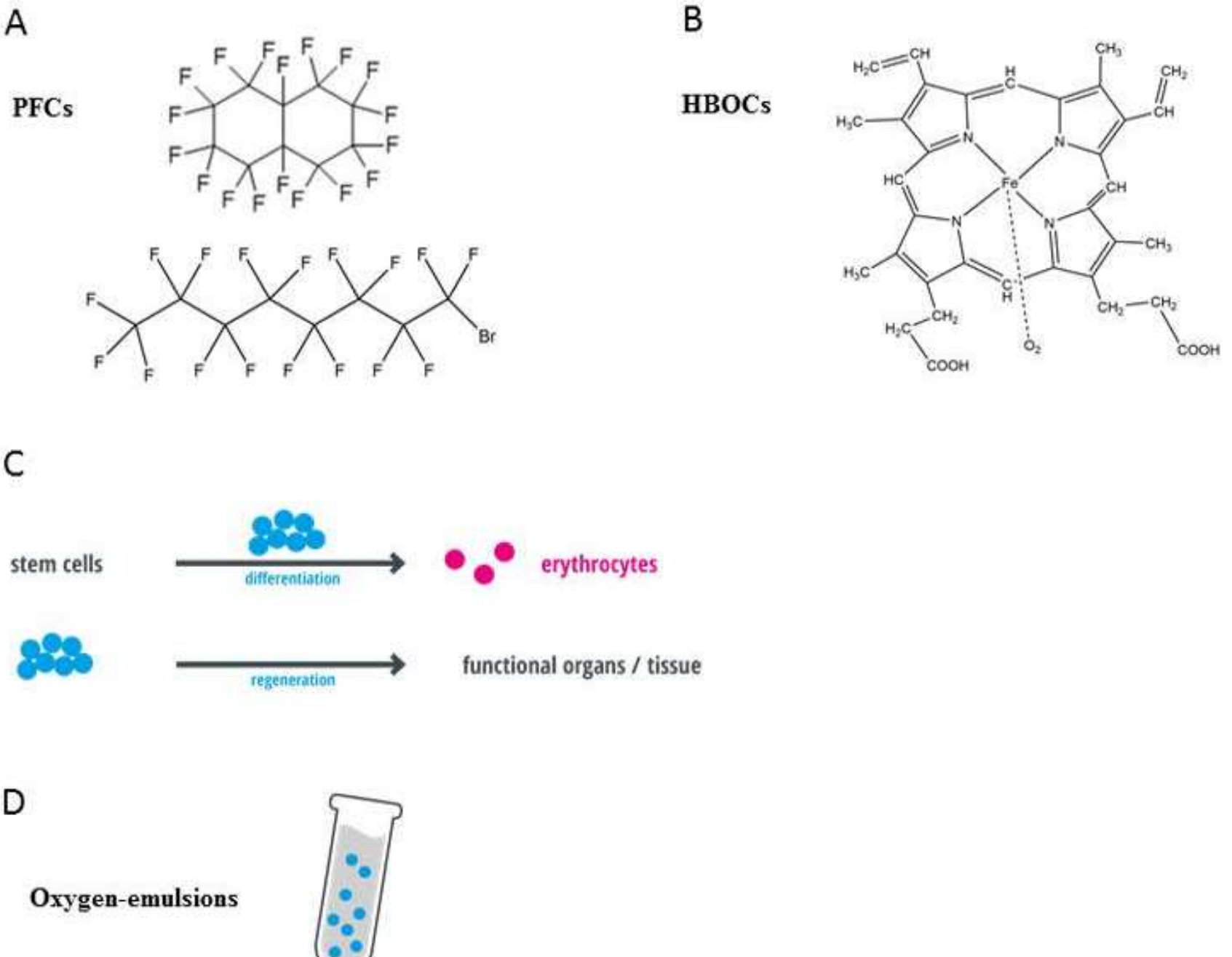
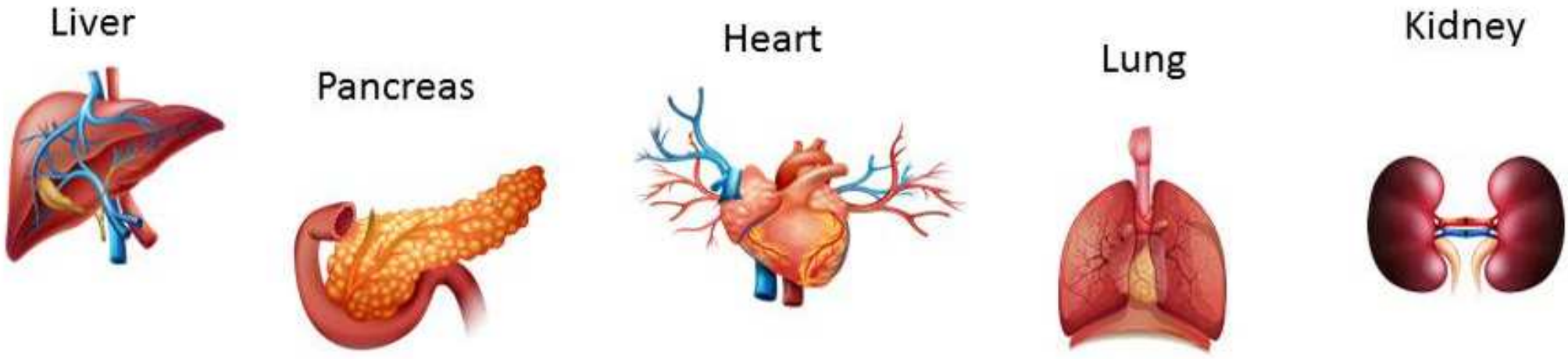


Figure 3 Organ preservation with AOCs



- Use of AOCs allows for normothermic perfusion without RBCs
- Retention of AOCs or metabolites not as important as in blood substitutes, AOCs mainly washed out prior to transplantation

⬇

Organ regeneration and quality improvement prior to transplantation

Online Supplement

Title: Artificial oxygen carriers- past, present and the future-a review of the most innovative and clinically relevant concepts

Running title: Update on concepts of artificial oxygen carriers

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Supplemental Table 1: Recent clinical trials with Hemopure

phase	trial number	locations	condition	status
II	NCT00479895	Netherlands	elective coronary revascularization	completed
II	NCT00317512	Netherlands, Belgium and Germany	elective coronary revascularization	completed with study results(Serruys et al., 2008)
II	ACTRN12615000522516	Australia	Bridging to hospital of shocked trauma patients	Ethics approval since 2013, not started yet
	2005-003639-30	UK	Tissue preservation during cardiopulmonary bypass	ongoing since 2005
II	2005-003637-41	UK	wound healing in patient with lower limp amputation	ongoing since 2005

Supplemental Table 2: Clinical studies on other HBOCs

HBOC	phase	trial number	locations	condition	status
Hemolink	II/III	NCT00038454	USA	Primary coronary artery bypass grafting in combination with intraoperative autologous blood donation	suspended since 2005
Polyheme	III	NCT00076648	USA	Bridging to hospital of shocked trauma patients	Unknown, study results(Moore et al., 2009a; Moore et al., 2009b)
Pyridoxalated hemoglobin polyoxyethylene conjugate	III	NCT00021502 2008-000504-92	USA Netherlands, Germany, Austria, UK, Spain, Belgium	Distributive shock + systemic inflammatory response syndrome	Discontinued in all countries due to low enrollment because of errors in study design. But completed as phase II(Elmer et al., 2012) study with study results(Kinasewitz et al., 2008)
Hemotech	I	Approved by the Ethics Committee of Kinshasa, Zaire (now Congo)	Congo	Sickle cell anemia in 9 children.	Completed with study results (Feola et al., 1992; Simoni et al., 2014)

Supplemental Table 3: Trials with Hemospan/ MP4OX and MP4CO

HBOC	phase	trial number	locations	condition	status
MP4OX	II	NCT00633659 2007-001538-15	Sweden	Chronic critical limb ischemia	completed prematurely ended
MP4OX	I, II	NCT00494949	Sweden	Reducing transfusion of RBCs in elective orthopedic surgery	completed with study results(Olofsson et al., 2006)
MP4OX	II	NCT00425334	USA	Reducing transfusion of RBCs in elective orthopedic surgery	completed
MP4OX	III	NCT00421200 2006-002513-12	Belgium, Czech Republic, Netherlands, Poland, Sweden, UK	Prevent hypotension in elective surgery	completed (Czech Republic, Belgium, Sweden, Netherlands with study results)(Olofsson et al., 2011) ongoing in UK (probably missing update: Sponsor terminated operations in 2013)
MP4OX	III	NCT00420277 2006-002514-35	Belgium, Czech Republic, Netherlands, Poland, Sweden, UK	Treating hypotension in elective surgery	completed in Czech Republic, Belgium, Sweden and Netherlands with study results(van der Linden et al., 2011) ongoing in UK (probably missing update: Sponsor terminated operations in 2013)
MP4OX	IIc	NCT01973504	Australia, Belgium, Brazil, France, Germany, Israel, New Zealand, Norway, South Africa, Switzerland, United Kingdom	MP4Ox in combination with standard treatment in severe trauma	2013 withdrawn prior to enrolment (probably missing update: Sponsor terminated operations in 2013)
MP4OX	IIb	NCT01262196 2010-023129-39	Australia, Austria, Brazil, Colombia, France, Germany, Israel, New Zealand, Norway, Singapore, South Africa, Spain, Switzerland, United Kingdom, Belgium, Italy	MP4Ox in combination with standard treatment in severe trauma	completed in all countries except Spain, Norway and Italy ongoing in Spain, Norway and Italy (probably missing update: Sponsor terminated operations in 2013)
MP4OX	IIa	NCT01004198 2009-013115-35	France, Germany, South Africa, UK	MP4Ox in combination with standard treatment in severe trauma	completed in all countries except France, ongoing in France (probably missing update: Sponsor terminated operations in 2013)
MP4CO	Ib	NCT01356485	France, Jamaica, Lebanon, UK	Stable sickle cell anemia	completed with study results(Keipert and Investigators, 2016)
MP4CO	II	NCT01925001 2013-001600-11	Bahrain, Belgium, Brazil, France, Lebanon, Netherlands, Qatar,	Vaso-occlusive crisis in sickle cell anemia	withdrawn prior to enrolment (Sponsor ceased operation)

			Turkey, United Kingdom		

Comment to Supplemental Table 3: Please note, that some clinical trials are still listed as "recruiting/ongoing" although the sponsor stopped funding and thereby terminated the study.

Supplemental Table 4: Clinical trials with Sanguinate

phase	trial number	Locations	condition	status
I	NCT01847222	Israel	Safety and pharmacokinetics in healthy volunteers	terminated due to completion of competing study (ACTRN126120010338 3)
I	ACTRN12612 001033831	Australia	Safety and pharmacokinetics in healthy volunteers	completed with study results(Misra et al., 2014)
I	NCT02754999	USA	Severe anemia	completed
II	NCT02323685	USA	delayed cerebral ischemia after acute aneurysmal subarachnoid hemorrhage	completed
Ib	NCT01848925	Colombia, Panama	Sickle cell disease	completed with study results(Misra et al., 2017)
I	NCT01374165	Israel	Sickle cell disease	suspended (cancelled)
II	NCT02600390	Panama, Dominican Republic	Sickle cell disease associated leg ulcer	completed
II	NCT02672540	Panama, Dominican Republic, Honduras, Colombia	Vaso-occlusive crisis in sickle cell disease	completed
II	NCT02411708	USA	Vaso-occlusive crisis in sickle cell disease	completed
II, III	NCT02490202	USA	Reduction of delayed graft function with infusion of Sanguinate prior to kidney transplantation	completed
Ib	NCT02437422	USA	Impact on humoral sensitization in end stage renal disease	completed
II	NCT02658162	Not provided	Reduction of delayed graft function in kidney transplant patients	withdrawn

Supplemental Table 5: Sophistically engineered Hgbs

product	**Idea**	**reference**
OxyVita	Linking of bovine Hgb-monomers via physiolocially present amide groups	Wollocko 2017(Wollocko et al., 2017)/ Jahr 2012(Jahr et al., 2012)
Poly-Hb-tempol	Glutaraldehyde polymerized porcine Hgb and tempol with SOD activity	Wu 2017(Wu et al., 2017)
Sanflow, VitalHeme (PNPH)	Polynitroxylated bovine Hgb	Brockman 2017(Brockman et al., 2017)'(LLC)
YQ23	Non-polymeric cross-linked tetrameric mammalian Hgb	Li, 2016(Li et al., 2017)'(Limited, 2017)
BAEGF-Hb	Antioxidative Bromoacethylethyleneglycol-ferulate-linked human Hgb	Guo, 2016(Guo et al., 2016)
PolyPHb/ bPEG-Hb	Polymerized human placenta Hgb/ pegylated bovine Hgb	Li 2015(Li et al., 2015)/ Wang, 2016(Wang et al., 2017b)

Supplemental Table 6: Standard Hgb plus engineered envelope

product	**Idea**	**reference**
HbVesicles	Human Hgb encapsulated in biocompatible liposomes	Sakai 2017(Sakai, 2017), Azuma 2017(Azuma et al., 2017), Kohno 2017(Kohno et al., 2017)
HbMP-700	Bovine Hgb in microparticles	Baeumler, 2014(Baumler et al., 2014), Kao, 2018(Kao et al., 2018)
ErythroMer	Human Hgb in tunable polymer shell, pH sensitive O_2-affinity	Pan, 2016(Pan et al., 2016; Kalocyte, 2017)
HbN	Bovine Hgb conjugated polymer micelles	Qi, 2016(Qi et al., 2016)
HbP	polymer encapsulated bovine Hgb	Lu 2016(Lu et al., 2016) and Li 2014(Li et al., 2014)
LEH	Human Hgb in liposomes	Yadav, 2016(Yadav et al., 2016), Fukui 2017(Fukui et al., 2017)
• Hb-PDA • PDA-Hb-microcapsules	• Antioxidative polydopamine-coated bovine Hgb nanocapsules • Polydopamine-coated bovine Hgb	• Wang, 2017(Wang et al., 2017a; Wang et al., 2018) • Yu, 2018(Yu et al., 2018)
Hemoact	Cluster of human Hgb + human albumin	Haruki, 2015(Haruki et al., 2015)
RBCM	RBC-like microgel particles loaded with bovine Hgb	Chen, 2012(Chen et al., 2012)
Mal-PEG-βXL-Hb	Inside-out pegylated Hgb	Webster, 2017(Webster et al., 2017)
Hemoglobin loaded nanoliposomes	Liposomes with human Hgb	Qu, 2017(Qu et al., 2017)
	Human Hgb adsorbed to silica nanoparticles	Devineau, 2018(Devineau et al., 2018)

Supplemental Table 7: Other artificial blood products in preclinical stage

product	**Idea**	**reference**
HemoCD	Iron-porphyrin/ cyclodextrin complex	Kitagishi, 2017(Kitagishi et al., 2017)
PEG-LtEC	Pegylated earthworm Hgb	Jani, 2017(Jani et al., 2017)
HrBOC	hemerythrin (from marine worms) copolymerized with glutarlaldehyde, human serum albumin or ruberythrin	Toma, 2018(Toma et al., 2018)
Cobalt-replaced myoglobin	Resulting in p50 of 37mmHg	Neya, 2014(Neya et al., 2014)
Cobaltporphyrin-based micelles	Hgb- free oxygen transporter in micelles	Shen, 2016(Shen et al., 2016)
LOMs/PHMs	Lipid-based oxygen microbubbles/Polymer hollow microparticles (stabilized thin wall)	Black, 2017(Black et al., 2017)/Seekell, 2016(Seekell et al., 2016)

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