Current status of oxygen carriers

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Heavy medical interventions in severely injured patients and complex transplantation surgery are currently performed with success, but they have increased the need of human blood. At the same time, the risk of transmission of viral diseases, the risk of errors in blood transfusion and the insufficiency of palliative treatments (blood predonation, pre- and perioperative hemodilution, perioperative blood sparing, lowering of transfusion trigger) accelerated the development of blood substitutes as alternatives to human blood.

Together with the property of carrying O_2, a blood substitute must have at least the following properties:

- free of toxicity and side effects
- adequate O_2 uptake in the lungs and adequate delivery to tissues
- sufficient half-life time in the circulation to avoid repeated administrations
- harmful and rapid excretion
- stable at room temperature, easy to store and easy to use
- easy to sterilize (to assure the absence of pathogens and viruses transmission)
- cheap to manufacture.

Intense efforts in the field of blood substitutes started up and were largely supported since 1980s by investment of the US military, which was concerned by the need of a reliable resuscitation solution that could be used immediately after injury and did not need special storage conditions. Two majors approaches were developped and are still in clinical trials: the perfluorocarbon emulsions and the solutions of free haemoglobin [1, 2, 3].

1. Perfluorocarbon emulsions

The perfluorocarbons (PFC) are synthetic cyclic or linear hydrocarbons occasionally containing oxygen or nitrogen atoms, and in which the hydrogen atoms are replaced by fluorine atoms. These solvants were produced and used for their chemical inertia in the Manhattan project during World War II. They easily solubilize and release gases without interacting with them. The quantity of gas dissolved is linearly related to its partial pressure. Perfluorocarbons are dense transparent liquids, immiscible in water.

After the famous demonstration by Clark and Golan [4] in 1966 that an animal immersed in an O_2 saturated solution of perfluorocarbon breathed normally, the perfluorocarbons were soon considered as possible red blood cells substitutes [5]. But, because the PFC are dense and not hydrosoluble, they must be emulsified for administration in the intravascular space. The properties of PFC emulsions depend on the components of the emulsion (necessary presence of surfactants), on the proportion of the various components and on the size of the emulsion particles, which influences the stability of the emulsion, the surface area available for gas exchange, the viscosity, and the intravascular half-life (linked to *in vivo* toxicity or side effects).

First generation PFC emulsions

PFC emulsions were prepared as soon as 1967 with plasma, but were not used in humans. The first generation PFC emulsion for administration to human was Fluosol-DA®, a 20% w/v solution developed by the Green Cross Corporation (Osaka, Japan). The product was made up of 2 PFC with a mixture of Pluronic F-68, egg-yolk phospholipids and glycerol as emulsifying agent [6]. It transported and delivered O_2 without major toxicity, but presented disadvantages: long tissue retention of one of its PFC components, low concentration of PFC and limited intravascular half-life both limiting the amount of transported O_2, and unsatisfactory stability. Fluosol also presented side effects, such as inhibition of white blood cells and complement activation, attributed to Pluronic [7].

Fluosol was used with success in around 300 patients who refused blood transfusion for religious reasons [8,9], and was authorized by the FDA for injection in humans (for percutaneous transluminal coronary angioplasty, PTCA). But the manufacturer stopped the preparation of Fluosol-DA for insufficient clinical use.

Second generation PFC emulsions

In the second generation emulsions, the PFC concentration is largely increased, enhancing so the O_2-carrying capacity and eliminating the dilution of patient's blood at time of administration. These emulsions are formulated "ready-for-use" in buffered saline, and present a high stability (resistance to heat sterilisation and to storage at + 4°C), due to the use of a crucial amount of egg yolk phospholipids and better emulsification techniques (high-pressure homogenization, microfluidization) [10]. The small size of their particles (mean diameter: 0.2 μm, about 1/35 of erythrocyte) allows them to easily maintain perfusion of all the capillaries of the microcirculation during states of local vasoconstriction and ischemia, when erythrocytes no longer circulate [11]. The archetypal second generation emulsion is OxygentTM (Alliance Pharmaceutical Corp., San Diego, CA), 60% w/v PFC emulsion based on the use of a linear PFC, perflubron (perfluorooctyl bromide), with particles with a mean diameter of 0.16 to 0.18 μm. This emulsion dissolves 28 ml O_2/100 g at 37°C and 750 mmHg. It can be stored at refrigerated temperature for up to two years. The presence of the terminal bromine atom lends lipophilicity to perflubron and a more rapid excretion as vapors by the lungs, limiting its persistence in tissues. Its administration is not associated with hemodynamic effects and does not activate complement, but could produce a dose-dependent and transient flu-like syndrome four to six hours after infusion, which results from the phagocytosis of the emulsion particles by macrophages.

Numerous studies were performed in humans, particularly with OxygentTM, enroling more than 500 subjects in phases I and II. An important clinical application of OxygentTM is its administration during surgery with acute normovolemic haemodilution (ANH), to allow reductions of the patient's hematocrit below currently accepted thresholds while main-

taining or improving tissue oxygenation. In a phase II study in orthopedic surgery with ANH, the use of Oxygent allows the reversal of trigger for transfusion.

Phase III studies with ANH in cardiopulmonary bypass surgery and in high-blood loss non cardiac surgery showed a greater avoidance of transfusion, but were stopped for more serious adverse events in the PFC group, results which remain debated [12]. A new phase II clinical trial in major surgery was planned in France for the end of 2006, but financial problems arrested the study.

In November 2007, phase II clinical trials in major surgery were announced in China, with the purpose to maintain haemodynamic stability and improve post-operative organ function. At the beginning of 2008, the manufacturing technology of Oxygent was transferred to Double-Crane Pharmaceuticals Co. in China, and a pilot production started. In Russia, another PFC emulsion, Perftoran, similar to Fluosol, but with improved emulsifier and low size particles (0.07 μm) is still in clinical trials with success in improving haemodynamics, and in reducing ischaemic damage and allogeneic blood transfusion [13,14]. Out of Russia, Perftoran was used in valvuloplasty surgery with ANH [15].

Advantages and inconveniences of the PFC emulsions

The advantages of PFC emulsions as blood substitute are summarized in table 1: absence of incompatibility and risk of transmission of infectious diseases, long duration of conservation, easy access, absence of metabolism and more particularly no reaction and no binding with O_2 allowing easy tissue unloading, viscosity and rheologic parameters similar to those of blood, permitting the particles to flow through swollen and/or blocked capillaries, where red blood cells might not pass. The solubility of O_2 in PFC emulsions is proportional to the partial pressure, and the O_2 transport capacity of these emulsions depends on the PFC concentration. Emulsions containing 45 to 60% PFC (weight/volume) seem ideal in terms of O_2 carriage, but the mechanisms of transport and delivery are entirely different from those of erythrocytes. The saturation curve for erythrocytes is sigmoid, and a fall in partial pressure from 150 to 50 mmHg leads to unloading of 25 % of the bound O_2. To obtain an efficiency in O_2 delivery

similar to that of oxyhemoglobin, an O_2 enriched atmosphere must be used [16], and 100 % O_2 are administered to patients receiving PFC emulsions. PFC are thus only O_2 carriers with a transport capacity greater than that of blood under hyperoxic conditions and a facilitated unloading of O_2 in the tissues. Side effects of PFC emulsions such as complement and phagocytic cells activation, principally due to the surfactant, are no longer seen with the second generation emulsions using newer surfactants. A liposome effect (coalescence of emulsion particles) was described, increasing the risk of stroke, but the question remains debated. At the opposite, beneficial anti-inflammatory effects were reported [17]. However, PFC emulsions still have a limited intravascular persistence, and there is a limitation to their use consecutive to the elimination of the emulsion particles by the reticulo-endothelial system [18]: large doses could lead to hepatic engorgement and a temporary impairment of immune defense mechanisms, a risk decreased by the small particle size of the second generation emulsions. The search for PFC in various tissues (lung, liver, spleen …) showed neither high levels of accumulation nor excessive persistence, and there are no reported toxic or side effects that could result from oxidation of the phospholipidic surfactant or from in vivo production of lysophosphatides. PFC emulsions thus remain valuable candidates as oxygen carriers.

2. Haemoglobin solutions

The first transfusion of blood occured in 1667 when the physician of the French king Louis XIV, Jean Baptiste Denis de Commercy injected 300 g of sheep blood to a young man: the assay was a success, but the physician observed emission of black urine by his young patient. Jean Denis de Commercy repeated the assay, but in 1668, a patient died after 3 “transfusions”, what led to the interdiction of blood transfusion. In 1818, James Blundell restarted human blood transfusion in several young women suffering at post-partum haemorrhage; Blundell is now considered as the “father of blood transfusion”.

• Lysed erythrocytes: the first solution used to replace blood

The first administration of a haemoglobin (Hb) solution obtained from the lysis of erythrocytes did not occur before 1898 when Von Stark [19] administered a Hb solution to patients in an attempt to treat anaemia. Rapidly, it appeared that free Hb was harmful, leading to disseminated intravascular coagulation, cardiac failure and renal toxicity. These observations and the chemical instability of Hb solutions led that line of research to be abandoned. In 1916, small quantities of free Hb were infused to human by Sellards and Minot, in order to study the clearance by the kidneys, and demonstrated signs of renal toxicity [20]. In 1937 a systemic and pulmonary vasopressor effect was described by Amberson in animals infused with lysed red blood cells, and this effect was not due to the simple expansion of circulating volume [21]. In 1949, Amberson infused 300 mL of a 6% Hb solution as a last resort to resuscitate a young woman suffering from a severe postpartum haemorrhage unresponsive to infusion of crystalloid, colloid, and homologous blood. This infusion increased the blood pressure and was associated with improved level of consciousness, what suggested that this pressor effect was beneficial [22]. Rabiner et al [23] further treated hemorrhagic shock patients with 180 to 300 mg/kg "stroma-free" hemoglobin, and Savitsky et al [24] administered 250 ml of Hb solution to volunteers with minor side effects on kidneys and cardiovascular system (first phase I clinical trial).

• Modified haemoglobin solutions: source of free Hb and chemical modifications

The major problems encountered during the early studies with free Hb were vasomotor effects, activation of the complement, kinin and coagulation systems, nephrotoxicity, interference with macrophage function, antigenic effects, histamine release, and iron deposits: they were attributed to erythrocyte membrane remnants. The renal toxicity was due essentially to the rapid breaking of the Hb molecule into its α,β dimers. Free Hb also presents an excessive affinity for oxygen (P50: 12-14 mm Hg) attributed to the absence of the allosteric effector 2,3 diphosphoglycerate (2,3-DPG). Purification of Hb from different animal species began in 1970, produced "*stroma free haemoglobin*" (SFH) and eliminated acute toxic effects of the free molecule. Chemical modifications of

the Hb molecule were designed to suppress the rapid rupture of the tetrameric molecule into its dimers and to avoid or reduce renal toxicity.

Chemical modifications of Hb molecule

The modifications which have been tried [25,26] are summarized in figure 4 [27]:

- internal stabilization of the α-α or β-β dimers by a cross-linking with polyanionic molecules (acetylation), bis-pyridoxal-5-phosphate (pyridoxylation) or dialdehydes derived from the oxidation of the cyclic structure of sugar (o-raffinose) or open ring-adenosine triphosphate (o-ATP),
- conjugation with large molecules (surface modification) with macromolecules [hydroxy-ethyl starch, polyethyleneglycol (PEG), Dextran 20] or artificial support ("nanocrystalline" beads),
- intermolecular cross-linking (polymerization) with cyanate or glutaraldehyde reagents,
- encapsulation in synthetic liposomes with co-encapsulation of other compounds such as antioxidant systems and enzymes to protect Hb from oxidation.

The modifications aim at increasing the stability and modifying the surface electric charges of Hb, in order to reduce its extravasation and increase its plasma half-life. Conjugated and polymerized Hbs have molecular weight ranging from 64,000 to 400,000 daltons and correctly deliver O_2 to tissues even when infused at low doses. The encapsulation and co-encapsulation techniques attenuate the vasoactive effects, and yield a P50 of 30 mm Hg, with a convenient kinetics of O_2 binding and delivery, but Hb liposomes have a short circulation time due to a rapid phagocytosis, with hepatic overload in relation with the infused volume.

Sources of Hb molecule

The sources of Hb are mainly human erythrocytes from outdated banked blood and bovine erythrocytes from slaughtered animal blood, both carefully purified. Free human Hb has an increased affinity for O_2 (P50: 12-14 mm Hg) compared to intracellular Hb, because it lacks the allosteric inhib-

itor 2,3-DPG [28]. Human Hb solutions have a high colloid oncotic pressure, limiting Hb concentration to 7 g/dL; they are stored in an anaerobic environment to avoid the oxidation into metHb. The bovine source is interesting for easy and cheap access, and because bovine free Hb does not require 2,3-DPG to control its affinity for O_2 and has a P50 of approximately 30 mm Hg, favouring O_2 delivery to the tissues.

Recombinant human Hb was produced in *Escherichia coli*, with the 2 α chains fused to avoid dissociation in plasma [29] or with a mutation on β chains ("Presbyterian" Hb) resulting in changes in the allosteric control mechanism and a lower affinity for O_2 [30]. This Hb variant (Optro®, Somatogen) has a P50 higher (30-33 mm Hg) than the natural Hb with an improved O_2 delivery and a plasma half-life 4 times greater than free Hb. Attempts were also made to produce recombinant Hb in yeast and transgenic plants and in transgenic animals (pigs, mice). But techniques of isolation and purification of the Hb molecule, and the scaling up of the production are still to improve.

• Clinical use of modified Hb solutions

Several companies developed free Hb solutions (table 3), which reached phase II and III clinical trials, but these solutions cannot replace the red blood cells in all their functions, since numerous components which are included in the red blood cell are lost during Hb purification. The Hb solutions have the primarily function of carrying O_2 to tissues. It is thus more accurate to design them by the terms "cell-free oxygen carriers", "Hb-based oxygen carriers (HBOCs)" or "oxygen therapeutics". They have also the function of restoring adequate volume in a large range of clinical situations with important blood loss such as cardiac surgery [31] and trauma [32]. All HBOCs have a short intravascular life-time and carry many side effects, with variable consequences (table 4). Therefore, several manufacturers stopped the clinical trials and the production of HBOCs.

Among all the HBOCs which reached the phase of clinical trials, two generations can be distinguished, the second generation of HBOCs being developed on the basis of the observations collected from the studies performed with the first one, which pointed out vasoconstriction and the gastro-intestinal symptoms.

First generation of Hb solutions

HemAssist™ (DCLHb; Baxter Healthcare Corp.) reached phase II and III clinical trials in orthopedic surgery, abdominal aortic repair surgery, major abdominal surgery and cardiac surgery. Modest results were obtained in allogeneic blood cell transfusion avoidance, and increased adverse events (hypertension, yellowing of the skin, haemoglobinuria and pancreatic suffering) and short plasma persistance of DCLHb (± 24h) did not support the routine use for transfusion avoidance [31,33]. A phase III clinical study in non-cardiac surgery was stopped early for safety concerns [34]. In trauma patients with severe haemorrhagic shock, a European "On-scene" multicentre study was prematurely arrested for lack of efficacy (mortality not significantly different in the treated group) versus the standard treatment group [35]. In a similar phase III study in patients with severe traumatic haemorrhagic shock in the USA, an increased mortality was observed [36], and subsequently, ongoing clinical trials were arrested and Baxter stopped the development of DCLHb.

For the recombinant Hb (Optro®, Somatogen Inc.), the phase II clinical study was stopped for hypertension, pyrogenicity and other adverse events [37,38]. Hemolink® (Hemosol Inc.) reached phase II and III clinical trials in high-blood-loss surgery a few years ago, but the trials were discontinued for safety problems [39], and the production of Hemolink has been terminated [40]. Encapsulated Hb in lipid vesicles were tried with success in preclinical animal studies, but there are no ongoing clinical trials with these products.

Two HBOCs are still in advanced clinical development: Hemopure® (HBOC-201) and PolyHeme®. Hemopure® was used in a phase III orthopedic surgery study, with doses ranging from 65g (± 1 RBC unit) to 325g (± 10 RBC units). A reduction of the need for allogeneic transfusion was observed, but with more adverse and serious adverse effects: gastro-intestinal events, elevated plasma levels of amylase and lipase and clearly hypertensive properties mainly in elderly patients [41,42] (table 4). Hemopure® is approved for sale in South Africa to treat acutely anemic surgical patients. A veterinary product, Oxyglobin® (HBOC-301) has been approved and is used in U.S.A. and Europe for the treatment of anemia in

dogs. The company announced that new phase II trials are being designed, but at the end of 2006, Hemopure® was still not approved by the FDA.

In phase III clinical trials (trauma and emergency surgery for aneurysm rupture), PolyHeme® up to 1,000 g in 10 L was administered with success: 50 % reduction of blood transfusion, no major concerns [43,44]. The study was halted late 2001, before completion. In the beginning of 2006, an online article of the Wall Street Journal revealed that adverse events (heart attack) were more frequent in the patients receiving PolyHeme®, but the company attributed these adverse events to an excess of total fluids given to PolyHeme® patients, and not to the product itself [45].

A multicenter phase III non-consent trial in trauma patients with severe blood loss started in January 2004 and the patient enrolment was completed in July 2006. The results published in the beginning of 2009 indicated a blood transfusion avoidance in the PolyHeme group with no difference in survival at 30 days, but higher adverse events (coagulopathy, hypertension, myocardial infarction) compared to the control group [46,47].

Second generation HBOCs: oxygen therapeutics

As it was suggested that hypertension observed with HBOCs was the consequence of NO trapping and of an arteriolar vasoconstriction in response to O_2 delivery [48], the group of Winslow [49] designed a new Hb molecule (MP4) by increasing the molecular volume of human Hb with polyethylene glycol, the affinity for O_2, the viscosity and the oncotic pressure. Animal studies showed that MP4 was safe and without haemodynamics effects.

For clinical use, MP4 (Hemospan®, Sangart Inc.) was designed as an O_2-carrying plasma expander administered at low concentration to deliver O_2 to hypoxic tissues; it is as an “oxygen therapeutics” more than a blood substitute. In phase II clinical studies [50,51], no serious adverse events could be attributed to Hemospan, and no significant hypertension was observed, but spinal anaesthesia by its hypotensive effect could have masked the hypertensive effect of Hemospan®. From these phase II studies, Hemospan® appears safe, but the doses are low (around 42g for the highest one), and despite these low doses, bradycardia and elevation of

hepatic and pancreatic enzymes are observed (this was also observed with the first generation blood substitutes). Questions remain concerning the half-time life of Hemospan® in plasma (around 20h) and the metHb production, so that studies with larger doses are expected before the inocuity (and the utility) of this new generation blood substitute can be firmly assured. Two phase III studies with Hemospan for prevention or treatment of perioperative hypotension in patients undergoing primary hip arthroplasty with spinal anaesthesia were recently completed in Europe: the publications of the detailed results are expected.

• A meta-analysis: the death blow for Hb-based blood substitutes?

A recent meta-analysis was performed on 16 randomized controlled trials involving 3711 surgical, stroke and trauma patients, with 5 haemoglobin-based blood substitutes (HemAssist, Hemopure, Hemolink, Polyheme and Hemospan) [52], used as outcome variables the data on deaths and myocardial infarctions, and demonstrated a statistically significant increase in the risk of death and the risk of myocardial infarction in the group treated with Hb-based blood substitute. From this meta-analysis and taking into account the well-demonstrated toxicity of haemoglobin out of the erythrocyte (particularly its ability to cross the endothelial barrier, to produce oxidant species, and to induce renal toxicity) [53,54,55], the use of HBOCs cannot be recommended especially in fragile (elderly and severe haemorrhagic) patients. Hope remains for Hemospan, but it is an oxygen therapeutics and not a substitute of RBC [48].

Conclusion and perspectives

Two PFC emulsions, Oxygent and Perftoran are still in clinical trials in China and Russia, with promising results in the treatment of local ischaemia and encouraging results in cardiac surgery with ANH. PFC emulsions remain thus probably valuable candidates as oxygen carriers. The HBOCs of the first generation have been used with moderate success in avoiding red blood cell transfusion, no significant results in improving survival, a short intravascular lifetime and an increase of severe side effects (hypertension, renal toxicity) compared to classical treatment. Hemopure® and PolyHeme® are the two HBOCs which remain in phase III clinical

trials, but with a questionable lack of published results. Hemospan® is still in clinical trial, but is more an oxygen therapeutics than an universal blood substitute.

A recent, perhaps promising approach is to encapsulate Hb in biodegradable nanocapsules of polylactide, which are degraded in vivo into water and carbon dioxide [56], contain around 11 g bovine Hb/dL and have O_2 carrying and delivery properties similar to that of free bovine Hb. The enzyme of the red blood cells can be co-encapsulated to protect Hb from oxidation into MetHb. However, this approach is still limited to animal studies.

Projects are on the way to obtain human Hb from micro-organisms (*Escherichia coli* and *Aspergillus niger*) or from worms, which have a polymeric Hb molecule, thus not needing chemical modification for sufficient stability in bloodstream. Successful preclinical assays seem to have been made, but technical problems of extraction remain to be solved, and there is no knowledge on the effects of worm Hb on blood pressure and on its sensibility to oxidation [3].

As a substitute for universal use blood remains necessary for urgent transfusion at the site of severe traumatic injuries, it is important to carry out more basic research for solving the problem of free Hb toxicity by oxidation and metHb formation. The most promising way remains the *in vitro* erythroid cell generation, for which important progress has been made: starting from human hematopoietic stem cells, conditions have been established for producing mature red blood cells after around 18 days of culture [57,58], but among the numerous problems which remain to be solved, the technical conditions for large scale cultures in bioreactors [59] and the control of the membrane expression of blood group systems ABO and Rh [60] will need at least a 5 to 10 year period of research.

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Tables

Table 1: Advantages and problems with perfluorocarbon emulsions used as blood substitutes

• Easy access and moderate cost, long time storage
• No acute toxicity (if small particles), no antigenicity, moderate viscosity, not metabolized
• High density, limited intravascular persistence
• Need of FiO_2 = 1: local hyperoxia?
• Phagocytosis of vesicles, RES overload, tissue persistence
• Interference with laboratory tests (opalescent plasma)
• Flu-like syndrome, platelet aggregation, digestive side-effects

Table 2: Sources of haemoglobin molecules used to prepare free haemoglobin solutions

Source of haemoglobin	Technical way of obtention
Human blood	Hb extraction from outdated donor blood and modification of Hb molecule
Cow blood	Hb extraction from slaughterhouse cow blood
Micro-organisms (genetic engineering)	Genetic modification of bacteria, fungi or plants to produce Hb
Transgenic animals	Introduction of human Hb genes in animal fœtus and production by mature individuals

Table 3: Free haemoglobin (Hb) solutions (trade name, company and main characteristic) which reached clinical trials
HBOC: haemoglobin-based oxygen carrier; PHP: pyridoxalated Hb polyoxyethylene; PEG: polyethylene glycol

Name	Company	Characteristics	Clinical trials
HemAssist	Baxter Healthcare	Cross-linked (a-a)Hb	Discontinued; safety (increased mortality)
Optro	Somatogen- Baxter	Human recombinant Hb	Discontinued; safety (hypertension)
Polyheme	Northfield Laboratories	Polymerized human Hb (glutaraldehyde, pyridoxal)	Phase III (trauma, surgery)
Hemopure (HBOC-2001)	Biopure Corp.	Polymerized bovine Hb (glutaraldehyde)	Phase III (orthopedic surgery); ® in South Africa
Hemolink	Hemosol Inc.	Polymerized cross-linked human Hb (o-raffinose)	Discontinued; safety (myocardial infarction)
PHP	Ajinomoto/Apex Bio	Conjugated hum Hb (PEG, pyridoxal)	Phase III septic shock
PEG-Hb	Enzon	PEG-Conjugated bovine Hb	Discontinued
Hemospan	Sangard	PEG-Conjugated human Hb	Phase III elective surgery

Table 4: Side-effects encountered with the administration of free haemoglobin (Hb) solutions and their possible causes

MetHb: methaemoglobin; NO: nitric oxide; ROS: reactive oxygen species; gastrointestinal tract

Side-effect of HBOC	Possible cause
Vasoconstriction (increase in systemic and pulmonary arterial pressure and vascular resistance)	NO scavenging, activation of endothelin production, direct stimulation of alpha adrenergic receptors
Cardiovascular events (myocardial infarction)	NO scavenging? Direct toxicity on organ?
Nephrotoxicity (oliguria, haematuria)	Direct toxicity leading to kidney dysfunction (tubular necrosis and obstruction)
Neurotoxicity	Direct toxicity on organ?
Increase in blood levels MetHb (ROS)	Hb autoxidation (during storage or in vivo?)
"Jaundice-like" syndrome	Hb extravasation (endothelial cells, tissues)
Bilirubinemia	Hb destruction (short intravascular lifetime, overload of plasma elimination capacity)
GI (abdominal discomfort, pain, nausea, vomiting)	Binding of NO? Direct intestinal toxicity?
Elevation of liver & pancreatic enzymes	Direct organ toxicity? NO binding?
Increased bacterial virulence	Iron supply
Interference with macrophage functions	Blocking by binding of Hb-haptoglobin complexes to receptors?
Activation of complement, kinin and coagulation cascades	NO scavenging leading to platelet aggregation?
Immunogenicity	Xenogeneic Hb, polymerized Hb
Interference with laboratory tests	"Haemolysis-like" effect