Oxygen carriers
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Three polyhemoglobins, formed by intermolecular cross-linking of hemoglobin molecules are in advanced phase III clinical trials and two conjugated hemoglobins, formed by cross-linking of hemoglobin molecules with soluble polymer, are also undergoing clinical trials. A perfluorobased emulsion is undergoing phase III clinical trials and a new recombinant human hemoglobin that does not bind to nitric oxide is also being developed. New oxygen carriers with antioxidant properties are being developed for conditions with potential for ischemia-reperfusion injuries. Third generation oxygen carriers are based on microencapsulation of hemoglobin and red blood cell enzymes either in liposomes or in biodegradable nanocapsules. This review will briefly discuss lessons learnt from the past, give an overview on the current status of selected oxygen carriers and discuss research areas in need of further development.

Keywords Blood substitutes, cross-linked hemoglobin, encapsulated hemoglobin, ischemia-reperfusion, oxygen carriers, perfluorochemicals, polyhemoglobin, radiation therapy, recombinant hemoglobin, red blood cells, transfusion

Introduction
Numerous reviews on oxygen carriers have been published in the past couple of years, presenting diverging opinions [1-3,4,5••]. Polyhemoglobin and conjugated hemoglobin (Hb) were discovered in the early 1970s [6-8]. The delay in their development and consequent use is due to a number of factors. It was first thought that Hb extracted from red blood cells (RBCs) would only need to be purified and then used as RBC substitutes. After extensive research and development by several groups, preliminary phase I clinical trials carried out in the 1980s showed that stroma-free Hb, the highly purified Hb free of RBC membrane stroma material, was toxic, and caused adverse effects on the cardiovascular system and on the kidney [9]. Each Hb molecule consists of four subunits or tetramers. When infused into the body, the molecule of Hb breaks down into half-molecules, or dimers, which can easily filter through the glomeruli of the kidney, causing renal damage. Dimers were rapidly removed from the circulation, and infusion resulted in vasoconstriction as well as other problems. Results of this clinical trial led to the erroneous conclusion that Hb, in any form, would be toxic and therefore should not be used as RBC substitutes. Therefore, there was little or no attempt to develop polyHb or conjugated Hb to solve these problems, and all the efforts concentrated on the use of an oxygen carrying chemical, perfluorocarbon. Unfortunately, clinical trials led to the conclusion that perfluorocarbon caused side effects related to complement activation; in fact, these side effects were due to the emulsifying agent.

As a result of these initial unsuccessful attempts at RBC substitutes, many at the time felt that we should depend on donor blood rather than continuing to waste time on RBC substitutes. Academic and industrial research on RBC substitutes came near to a standstill, which was a disaster when the problem of HIV in donor blood emerged in the 1980s, as nothing could replace the potentially infectious donor blood. A number of academic groups and industries urgently rushed into the research and development of modified Hb, but development of something as complicated as RBC substitutes takes time. This is the first time that industries have had to produce modified protein compounds for intravenous infusions that require as much as 1000 g or more for each infusion, and it is not surprising that after more than ten years of intensive research, oxygen carriers have only progressed to advanced phase III trials. Furthermore, as we gain experience, it is now clear that the present first generation of RBC substitutes is only an oxygen carrier; it can be very useful in substituting RBCs, but only in certain clinical conditions, such as for perioperative use. New generations of modified Hb with other properties of RBCs will be needed for other clinical conditions. The basic principles of oxygen carriers have been described in detail [10••]. This review is a discussion of the first generation oxygen carriers that have been tested clinically, as well as a discussion of areas that need further development and new generations of oxygen carriers under development.

Oxygen carriers in clinical trials
Polyhemoglobins
Sebacyl chloride was first used in the 1960s to cross-link Hb to polyHb [6,8] and later, another bifunctional agent, glutaraldehyde, was used to cross-link Hb and catalase, an RBC enzyme [7]. Glutaraldehyde cross-linked polyHb has been developed into two types of polyHb in advanced phase III trials.

Northfield Laboratories Inc developed a glutaraldehyde cross-linked pyridoxaloxadolated human polyHb (PolyHeme), now in advanced phase III trials [10••,11,12,13••]. The method involves the removal of nearly all of the cross-linked tetrameric Hb, leaving only < 1%. No gastrointestinal distress or vasoconstriction were observed, and renal and other organ functions were normal throughout the study period. Results of the studies showed that polyHb could replace the loss of corresponding volumes of RBCs. When doses were increased to 6 units (3000 ml) of polyHb, results continued to show the safety and efficacy of this preparation. Clinical trials were carried out with increasing doses of polyhemoglobin (more than 10 units or 5000 ml) [13••].

Biopure Corp investigated glutaraldehyde polymerized bovine Hb (Hemopure) [10••,13••,14-16]. Bovine Hb is easily available in large amounts and unlike human Hb, it does not require 2,3-diphosphoglycerate (2,3-DPG) or its analog pyridoxal-phosphate, and its Po2 is higher than that of human Hb. This polyHb is stable at room temperature for more than one year. Once again, in this preparation, most of
the cross-linked tetrameric Hb was removed, leaving < 5%. This product has been approved in the US for use in veterinary medicine, and for routine human clinical use in South Africa [17].

His et al worked out a new cross-linker, o-rafinoise, which also modifies the 2,3-DPG pocket, resulting in a high P50 value [18]. Hemosol Ltd has developed this into a product, o-rafinoise cross-linked human polyHb (Hemolink) [13••,18], which contains polyHb with approximately 30% of cross-linked tetrameric Hb [18]. This compound is now in advanced phase III clinical trials for coronary arterial bypass grafting surgery [13••,19].

Conjugated hemoglobin in clinical trials
Soluble conjugated Hb formed by cross-linking an individual Hb molecule to a soluble polymer, dextran, was synthesized by Wong [20]. Hb conjugated to polyoxyethylene (PHP-HT) was developed by Ajinomoto Co Inc [21], who collaborated with Apex Bioscience Inc to carry out clinical trials. No significant toxicity was reported in these trials [22]. This compound is also used for scavenging nitric oxide (NO) in the treatment of shock associated with systemic inflammatory response syndrome [22]. Enzon Inc used the soluble polymer polyethylene glycol (PEG), cross-linked to bovine Hb, and carried out clinical trials on the resulting compound (PEG-hemoglobin) [23]. Sangart Inc developed Hemospan, a novel maleamide-polyethylene glycol conjugated human Hb [13••,24]. Phase I trials were completed in Sweden and phase II trials are ongoing [13••,24].

Cross-linked tetrameric hemoglobin
Baxter Healthcare Ltd developed a diaspirin cross-linked Hb (DCLHb) that underwent clinical trials. Results of these trials led to the discontinuation of this project, with Baxter focusing on a new generation recombinant Hb [13••].

Recombinant human hemoglobin
Hoffman et al prepared recombinant tetrameric human Hb from genetically engineered Escherichia coli [25]. Somatogen Inc developed and tested the product [26]. Based on results from clinical trials, a second generation recombinant Hb is now being developed in collaboration with Baxter Healthcare, Ltd. that does not remove nitric oxide (NO) or cause vasoconstriction or other smooth muscle contraction [13••,27]. One of the advantages of recombinant human Hb is that it is a 'human' Hb that does not depend on the availability of human RBCs. Furthermore, it is already in the form of a fused Hb molecule that does not break down into dimers.

Perfluorobon-based emulsion
Oxygent (Alliance Pharmaceutical Corp/Baxter Healthcare Corp) is a 60% perfluorobon emulsion with a median particle diameter of < 0.2 µm [28,29]. The use of lecinthin as an emulsifier eliminated the adverse effects of complement activation observed in earlier studies on perfluorocarbons [10••,13••,28,29]. At a recent conference, Kiepert reported that clinical trials showed that Oxygent decreased allogeneic blood transfusion in cardiac surgery [24]. When used in conjunction with acute normovolemic hemodilution (ANH) in a multicenter European phase III study, it significantly reduced and avoided the need for red cell transfusion [24]. A phase III study cautioned the use of overly aggressive autologous blood harvesting immediately prior to cardiopulmonary bypass [24]. The company is now preparing an international phase III trial in general surgery, without autologous blood harvesting or ANH [24].

Hemoglobin-based oxygen carriers and nitric oxide
The intercellular junctions of the endothelial cell layer allow cross-linked tetrameric single Hb molecules to cross from circulating blood. Since Hb binds NO, this tetrameric Hb, on leaving the circulation, helps remove NO, which results in increased vascular tone [10]. This could account for the effects observed when using intramolecularly cross-linked tetrameric Hb and the first-generation recombinant human Hb. At high doses, effects on the nerve plexus and other smooth muscles could also be observed, resulting in gastrointestinal problems, including esophageal spasm. This could be counteracted by the use of appropriate pharmaceutical agents, including those normally used in anesthesia. Blood substitutes prepared from polyHb and conjugated Hb normally contain varying amounts of cross-linked tetrameric Hb. The smaller the amounts of remaining tetrameric component, the larger the volumes that can be infused with no vasoactivity or smooth muscle contractions. Thus, in the case of glutaraldehyde cross-linked human polyHb containing < 1% cross-linked tetrameric Hb, no vasoactivity was observed when large volumes of > 10 l were infused. As discussed earlier, the NO binding site can now be successfully blocked, using the new generation recombinant human Hb, which no longer causes vasoactivity, as observed in animal studies.

Oxygen carriers with antioxidant properties
In stroke, myocardial infarction, organ transplantation, severe sustained hemorrhagic shock and other conditions, reperfusion with oxygen carrying fluids to ischemic tissues can result in the release of superoxide and oxygen free radicals, leading to tissue injury. The enzymes superoxide dismutase (SOD) and catalase lessen these effects in RBCs, by removing superoxide and hydrogen peroxide. However, the current first-generation modified Hb, prepared from ultrapure Hb, does not contain these enzymes. These compounds have already been used safely in clinical trials, with promising results for conditions with no sustained severe ischemia. However, use in conditions with severe sustained local or general ischemia may result in a higher chance for ischemia-reperfusion injury. A number of approaches could counteract this potential problem.

We are studying the cross-linking of trace amounts of catalase and SOD to Hb to form polyHb-SOD-catalase [10••,30]. Compared to polyHb, polyHb-SOD-catalase removes significantly more oxygen free radicals and peroxides and stabilizes the cross-linked Hb, resulting in decreased oxidative iron and heme release [10••,30,31]. In the reperfusion of ischemic rat intestine, polyHb-SOD-catalase significantly reduced the increase in oxygen radicals, as measured by an increase in 3,4-dihydroxybenzoate, compared to polyHb [10••,31]. In a global cerebral ischemia-reperfusion rat model, 60 min of ischemia followed by reperfusion with polyHb resulted in disruption of blood-brain barrier and brain edema; this could be prevented by using polyHb-SOD-catalase [32].
Intramolecularly cross-linked α-α tetrameric Hb can exhibit antioxidant activity, by addition of 2,2,6,6-tetramethylpiperidinyl-1-oxyl (Tempol) to form polynitroxylated α-α-Hb (PN-α-α-Hb). This new compound acts as an antioxidant in both in vitro and in vivo assays [33].

Artificial red blood cells as a third generation oxygen carrier
Molecularly modified Hb is only an oxygen carrier, and as such, a partial substitute for RBCs. A novel approach to produce completely by artificial RBCs is now being developed as third-generation blood substitutes [6,8]. Submicron lipid membrane microencapsulated Hb [34] is being explored, especially by Rudolph's [35,36] and Tsuchida's research groups [37]. Rudolph's group has modified the surface properties, leading to a circulation half-life of about 50 h in rats [36]. In a recent conference, Tsuchida's group reported promising results of their safety and efficacy studies in animals [24]. We are developing a new system based on biodegradable polymers and nanotechnology, and polylactide membrane Hb nanocapsules of about 150 nm diameter have been developed [10••,38,39]. Polylactide is readily converted to water and carbon dioxide in the body, and therefore does not accumulate in the reticuloendothelial system. SOD, catalase, carbonic anhydrase and also multi-enzyme systems are included to prevent accumulation of methb [10••,39].

Oxygen carriers in radiation therapy for tumors
Enzon Inc carried out a phase IIb trial to evaluate the safety of PEG-conjugated Hb as an adjuvant to radiation therapy in human cancer patients [23], based on earlier experimental observations that PEG-bovine Hb oxygenates hypoxic tumor tissue and dramatically increases sensitivity to radiation therapy in laboratory models and veterinary animals. Other groups are also testing different types of oxygen carriers for the same purpose.

Melanoma is a fatal skin cancer. Meadow's research group showed that the lowering of tyrosine inhibited the growth of melanoma in culture and in animals. However, at present, there is no effective way to remove tyrosine in humans. We prepared a novel polyHb-tyrosinase solution that acts as an oxygen carrier to supply oxygen for radiation therapy and at the same time, lowers systemic tyrosine. This solution will be tested in the near future [40].

Discussion and conclusion
When will a blood substitute of any type be ready for routine clinical use? If phase III trials of the perioperative uses of oxygen carriers continue with the same positive results, this could happen soon. This would significantly decrease the strain on donor blood supplies needed for surgery. In North America alone, 8 to 9 million units of blood (4 to 4.5 million l) are used in surgery each year.

Since Hb blood substitutes do not contain blood group antigens, they can be used without cross-matching or typing. This would save much time and facilities, and would permit on-the-spot transfusions, in a similar manner to giving intravenous salt solutions. However, one should not automatically transfer the safety and efficacy results of perioperative uses to other clinical situations. Thus, in sustained severe hemorrhagic shock, reperfusion using oxygen carriers with no antioxidant activities could result in ischemia-reperfusion injury. Similarly, oxygen carriers, being solutions, would be ideal for perfusing obstructed vessels in stroke or myocardial infarction. However, one must again be very cautious when using oxygen carriers with no antioxidant properties. Unless treated almost immediately, ischemia in stroke could result in ischemia-reperfusion injuries if oxygen carriers containing no antioxidant enzymes are used [32].

After extraction from RBCs and before modification, Hb can be sterilized to an inactivate form, and microorganisms, including HIV and hepatitis viruses, can be removed. PolyHb can be stored for more than one year at 4°C, or even at room temperature; it can be lyophilized and stored for even longer periods as a stable, dried powder to be reconstituted before use. In addition to human Hb, bovine Hb and recombinant Hb can also be used. In this new millennium, we shall see rapid progress in RBC substitutes for use in transfusion medicine [5].

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