Search for the ideal blood substitute – current status

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Summary

Synthetic blood substitutes are substances produced to fulfill functions of biological blood and serve as it's replacers in diverse medical procedures. Their main function is to restore blood volume and deliver oxygen to tissues. Colloid solutions currently in use lack the ability to bind oxygen and are applied as blood serum equivalents only. Two classes of oxygen-carrying agents are being developed: hemoglobin-based oxygen carriers (HBOCs) and perfluorocarbon emulsions (PFCEs). HBOCs comprise of modified stroma-free hemoglobin and their preparation Hemopure is approved for use in the Republic of South Africa. Other HBOCs products are completing advanced clinical trials in Europe and USA. PFCs are chemical compounds with a different oxygen-binding mechanism than hemoglobin, resulting from their spatial conformation. In Russia, Mexico and China a formulation of PFC has been registered for use, however it did not get the FDA approval due to undesirable side effects. Both HBOCs and PFCs require extended clinical research before entering the market. Nevertheless, inadequate supply of fresh blood and complications associated with allogeneic blood transfusions make synthetic blood substitutes a promising alternative especially on field of emergency and military medicine.

Key words: blood substitutes, oxygen-carrying volume expanders, stroma-free hemoglobin, perfluorocarbons.

Introduction

Development of complicated surgical procedures, limited availability of donor blood for emergency medicine and multiple risks of allogeneic transfusions created an increasing need of synthetic human blood substitutes. Attempts to create a whole blood replacer with preserved ability to carry oxygen have begun in the 1960s and are still in progress. Numerous qualities of a perfect blood substitute, besides oxygen transportation, include volumetric filling of the vascular bed, exerting oncotic pressure similar to the human blood and reducing viral infections transmission. It should also appear non-toxic, non-antigenic, persist for 6 to 12h in circulation, undergo metabolism and full excretion by the kidneys. It should also remain stable while storing in room temperature and have a long half-life. Most of the available blood substitutes fill only several of those demands.

The primary group of products widely used in human and veterinary medicine are synthetic serum equivalents. They consist mostly of colloid solutions, due to their ability to increase osmotic pressure and restore blood volume more effectively than crystalloids [1] especially in hypoproteinemic states. Intensity and duration of their action depends on the molecular weight achieved in the preparation procedure. Serum substitutes are being obtained by chemical modification of natural substances (gelatin, starch) or as a result of glucose polymerization (dextran). Gelatin derivatives, used in concentration 3-5.5% show a short-lived haemodynamic effect and are quickly degraded by peptidases. More persistent products contain hydroxyethyl starch (HAS), a biopolymer resistant to alpha-amylase. It is available in 6% and 9% isotonic solutions (with addition of i.e. 0.9% sodium chloride) (HAES-STERIL, HYPER HAES, VOLUVEN, Fresenius Kabi Polska). HAS solutions have the lowest incidence of severe anaphylactoid reactions among other colloid serum substitutes [2]. Dextran is a glucose polymer obtained in biotechnological process of saccharose fermentation by lactic acid bacteria. It is characterised by a long-lasting water-binding effect, whereas it's cumulation in tissues is diminished. Dextran preparations are characterised by molecular mass dependent on the chains lenght. The most popular products are: dextran 40 kDa, 70 kDa and 1 kDa (preventing usage of large-molecule dextran to mitigate potential side effects).

Colloid volume replacers are not appropriate in case of whole blood loss, due to their inability to transport and deliver oxygen to tissues. There are two kinds of blood substitutes able to replace red blood cells and carry out their function. Hemoglobin-Based Oxygen Carriers (HBOCs) are hemoglobin derivatives whilst perfluorocarbons (PFCs) are synthetic molecules with a different mechanism of oxygen binding.

Hemoglobin-Based Oxygen Carriers

From the beginning, in search for an effective red blood replacer natural oxygen-carrying potential of stroma-free hemoglobin has been considered. Free hemoglobin maintains the ability to bind oxygen, does not require blood typing and could be sterilized in order to avoid transfer of infectious agents. However, released from erythrocytes hemoglobin has a short intravascular life. It's tetrameric structure, consisting of two kinds of polypeptide chains (alpha and beta), breaks into smaller molecules that are filtered in kidneys and eliminated from organism up to 6h after intravenous administration [3]. Absence of glycolytic metabolite of red blood cell, 2,3-diphosphoglycerate (2,3-DPG), changes affinity of oxygen to hemoglobin and impairs it's delivery to tissues [4]. Free hemoglobin increases blood pressure due to it's oncotic properties and vasoconstriction caused by nitric oxide scavenging [5]. Whereas the last effect has been advantageous in treating patients with septic shock suffering from a blood pressure decrease, the former had to be neutralized in order to use free hemoglobin as a blood substitute. It has been achieved through various chemical modification, including intramolecular cross-linking, polymerising, conjugation with larger molecules, antioxidants, enzymes and eventually placing in artificial blood cells.

Cross-linking of two alpha chains stabilized hemoglobin molecule and extended it's intravascular dwell time to 12h. The binding agent was 3,5-dibromosalicyl fumarate (DBBF). The product was called diaspirin cross-linked hemoglobin (DCLHb) or alpha alpha-hemoglobin by the U.S. Army supporting research and production of this substance. Baxter was contracted by the Army to manufacture cross-linked hemoglobin for it's internal use. They used DCLHb to release HemAssist, the first modified blood replacer that entered clinical trials.

Biotechnological methods were introduced to achieve recombinant human hemoglobin, rHb 1.1 (Optro, Somatogen). Alpha chains of rHb 1.1. were constantly bound, what prevented them from dissociation and elimination. Produced in Escherichia coli bacteria rHb 1.1 also showed a decreased oxygen affinity.

Cross-linking of free hemoglobin limited it's renal toxicity but vasoconstriction and gastrointestinal side effects, both attributed to nitric oxide scavenging activity [6,7], remained a problem. Therefore, further research on those early products had been discontinued.

In attempt to avoid considerable adverse effects, polymerized forms of hemoglobin were developed. Agents like glutaraldehyde (PolyHeme,

Northfield Laboratories Inc.; Hemopure, Biopure Corp.) or o-raffinose (HemoLink, Hemosol Corp.) link specific amino groups, resulting in molecule chains of various sizes. The post-reaction fractionisation is needed to eliminate toxic small molecules (tetramers and dimers) and unreacted free hemoglobin. Hemoglobin polymers are characterized by long intravascular life, dwelling up to 24h, oncotic pressure resembling natural blood and moderate vasoactivity.

PolyHeme is based on hemoglobin derived from outdated human blood. Besides polymerisation, it's modified with pyridoxal phosphate, which serves as 2,3-BPG and lowers the oxygen affinity of hemoglobin. PolyHeme is one of the few blood substitutes that entered phase III clinical trials [8].

Another glutaraldehyde-polymerized product, Hemopure, is manufactured from bovine hemoglobin what caused a public concern about possibility of transfering Bovine Spongiform Encephalitis (BSE) prion to human patients. It appears unjustified, for Hemopure solution undergoes ultrapurification in the production process to remove potential infectious agents. Hemopure is the only HBOC approved for use (in the Republic of South Africa). In the United States of America it's completing phase III trials [8] and has been already approved as a veterinary product called Oxypure. It has a highest hemoglobin concentration and lowest oxygen affinity among polymerized hemoglobin products. However, it showed adverse cardiovascular effects in patients undergoing preoperative haemodilution [9].

High incidence of cardiac arrest was the main cause of holding o-raffinose-bound HemoLink development [8].

Surface-modified or conjugated hemoglobin consists of stroma-free hemoglobin linked to a large-molecule polymers like dextran, polyethylene glycol (PEG) or polyoxyethylene (POE) in order to prolong it's intravascular life. Conjugates retain in circulation up to 48h and increase oncotic pressure, functioning as oxygen-carrying blood volume expanders.

Pyridoxylated hemoglobin polyoxyethylene (PHP) produced by Ajinomoto/Apex Bioscience showed a satisfying safety profile with decreased oxygen affinity (pyridoxal phosphate effect), and increased NO scavenging activity. According to the latter, it's use as an hypertensive agent is being investigated, especially in septic shock patients

PEG conjugated hemoglobin was manufactured on base of bovine (Enzon Pharm.) and human (Hemospan, Sangart Inc.) hemoglobin. Enzon product was observed to increase oxygenation of tumor tissues and therefore improve chemotherapy effects in laboratory animals [10]. Subsequently, it entered clinical trials to evaluate PEG hemoglobin action as an adjuvant to human cancer therapy. Hemospan, characterized by favourable hemodynamic stability and good toleration in human patients completed phase III trials in Europe [11].

The aim of conjugating polyhemoglobine products with antioxidant enzymes was to expand their functions and mimic normal red blood cell activity. Especially patients with ischaemic disease or undergoing organ transplantation are exposed to oxygen deficiency. After reestablishment of perfusion, oxygen-derived free radicals arise in ischaemic tissues causing substantial cell injuries, called ischaemia-reperfusion injuries. Enzymes like catalase and superoxide dysmutase, naturally associated with red blood cells, protect cells from reactive oxygen species. Superoxide dysmutase catalyzes dismutation of superoxide into oxygen and hydrogen peroxide and catalyse enhances decomposition of hydrogen peroxide to water and oxygen. Antioxidant enzymes retain their activity after glutaraldehyde polymerisation, if the specific glutaraldehyde to hemoglobin ratio is maintained [12].

In some HBOCs hemoglobin was encapsulated in lipid vesicles in attempt to create an artificial red blood cell. This method eliminated vasoconstrictive effect of free hemoglobin, it also prolonged it's persistence in circulation. However, the small size of lyposomes was associated with reticuloendothelial system activation and uptake (instead of renal elimination). Attempts to solve this problem has been made by modifying liposome surface with polyethylene glycol and therefore decreasing their recognition. It resulted in prolonging intravascular half-life of encapsulated hemoglobin to 48h [13]. In preclinical trials, form of sodium chloride isotonic solution containing suspended lyposomes has been used [14].

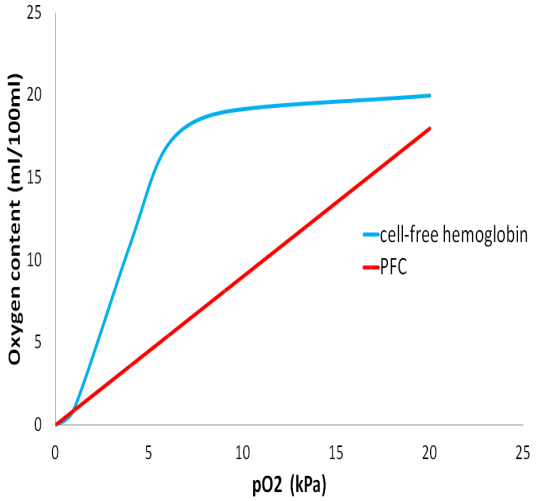

Figure 1: Oxygen dissociation curves for cell-free hemoglobin and for a perfluorocarbon.

Perfluorocarbon emulsions (PFCE)

Perfluorocarbon emulsions (PFCE) are one of the two major classes of oxygen therapeutics and potential blood substitutes, currently on the market. For religious reasons, certain groups of people are unable to accept transfusions of either blood or human and animal hemoglobin preparations. Additionally, due to their synthetic nature PFCE's have a shelf life much longer than transfused blood [15].

Perfluorocarbons (PFC) are molecules that are structurally similar to hydrocarbons, except that all or most of the hydrogen atoms are replaced with fluorine.

Extraordinary properties of these compounds come from a small diameter of fluorine atoms, their high electronegativity and high energy of C-F bond [16-18]. In contrast to hydrogen, the fluorine atoms strongly screen a carbon chain, with the result that PFCs are extremely durable, resistant to hydrolysis, photolysis, metabolic processes of the vertebrates and microbial degradation [19]. There have not yet been isolated any microorganism utilizing PFCs as a carbon source. Cases of enzymatic degradation of perfluorocarbons are not known [20]. On the other hand this results in problems with utilization and degradation of the perfluoronated compounds.

A tight packing of highly electronegative fluorine atoms causes a low surface energy. Extremely weak van der Waals interaction between molecules PFC is responsible for their very low surface tension, low viscosity and high compressibility [21]. Its consequence is a slippery and extremely resistant to wetting surface. An important property of these compounds, which gives a possibility to use them as blood substitutes, is their ability to dissolve different gases, including respiratory (CO_2 and O_2) [22].

The binding of oxygen by the PFC is entirely different than that of hemoglobin and involves the gas filling the space between the fluorine atoms, resulting from the structural arrangement of molecules. It has been shown that the solubility of gases in liquid perfluorocarbons increases in accordance with the following series: CO_2>O_2>CO>N_2 [23-24]. 100 ml of PFC can dissolve about 45 ml of oxygen, while the solubility of carbon dioxide amounts to over four times more [23].

The oxygen combines with hemoglobin in the coordination way and that phenomenon does not follow Henry's law. The organic perfluorinated compounds dissolve oxygen (and also other gases) in a way approximate to the Henry's law, and therefore depend on the pressure of the gas being in contact with the solution. (Fig. 1). This provides the ability to transport oxygen from the lungs to the tissues [24-26]. PFCs have a high solubility coefficient for oxygen, higher than the coefficient for oxygen dissolved in blood plasma. Increasing the oxygen concentration in the gas supplied to the lungs of a patient, from 21%, typical of atmospheric air, to more than 90% results in approximately 5-fold increase in the volume of oxygen dissolved in the PFC [27].

This example explains why the emulsions of PFCs have been successfully used as a replacement for blood in case of need for intensification of oxygen transport to the tissues of the patient. The general characteristic of the fluorine derivatives applied as the oxygen carriers was presented by Clark [28] and more recently by Lowe [27,29-30].

The fluorocarbon derivatives examined as blood substitutes can be divided into the following groups:

- perfluorinated alkanes of normal and branched chains e.g. 1-bromoperfluorooctane, perfluoropentane (Fig. 2a);

- perfluorinated closed chain compounds: e.g. perfluorodecaline (Fig.2b);
- perfluorinated tertiary amines, e.g. perfluorotripropylamine (Fig. 2c);
- perfluorinated heterocyclic compounds, e.g. perfluorofurane (Fig. 2d).

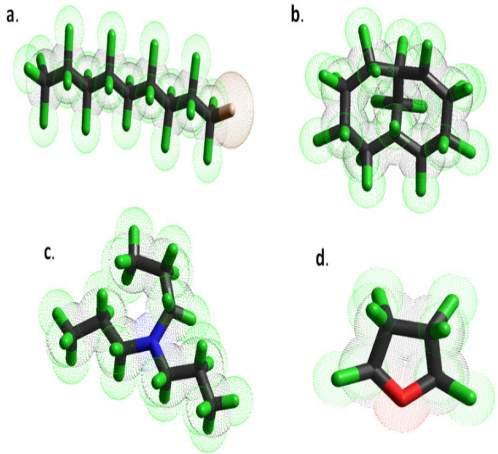

Figure 2: The spatial structures of representative perfluorocarbon compounds (Hyperchem Professional 8.0).

Oxygen is more efficiently dissolved in the aliphatic PFCs than in PCFs with the cyclic structure. It is assumed also, that the solubility of oxygen in PFCs is inversely proportional to their molecular weight, and depends on the number of fluorine atoms present in the molecule [30].

In 1966, Leland C. Clark, of the University of Cincinnati conducted a controversial experiment: a live mouse was completely submerged for several hours in a fluorobuthyltetrahydrofurane saturated with oxygen. The mouse survived the experiment, for several hours freely "breathing" oxygen dissolved in the liquid PFC. Tested in this experiment artificial ventilation with a liquid, in comparison with conventional mechanical ventilation with oxygen-enriched air, has several advantages: it eliminates the existence of the gas-liquid interface, which reduces the surface tension in the alveoli, and increases the efficiency oxygen delivery to the capillary vessels in patients with acute respiratory failure [31-32]. Moreover, liquid PFCs have a low surface tension value which enhances penetration into the bronchioles and filling their volume. In result of the research described above, perfluorocarbons have been successfully used to provide oxygen to premature infants with severe respiratory distress syndrome [32].

As oxygen carriers PFCs could be incorporated into cardioplegic solutions, used in open heart surgery. Such application improves the cardiac oxygenation and tissue metabolic status. For the same purpose PFCs may be applied to perfuse the myocardium or brain tissue in the case of myocardial infarction and strokes [33]. Besides artificial ventilation, PFCs are used in medicine as specific drug deliverers, in medical diagnostics, as a medium in transplantation organs storage and in eye surgery. PFCs could also be used in cancer therapy as agents increasing the oxygenation of tumors and consequently benefiting chemotherapy or irradiation of tumors.

More controversial is the approval of PFCEs as "blood substitutes" or, more accurately, *oxygen-carrying volume expanders*. The first studies on this topic appeared almost 70 years ago.

Perfluorocarbons have interesting physicochemical properties, however, hampering their preparations for use as blood substitutes: they are hydrophobic and besides lipophobic. This makes it necessary to administrate perluorocarbons as an emulsion (PFCE), since it does not mix with blood. It requires addition of a suitable surfactant to obtain an emulsion with appropriate particle size, with a perfluorocarbon core. In the currently investigated PFCE formulations the particles are spherical, not more than 0.2 microns in diameter, mostly coated with a egg yolk phospholipids as a surfactant [34].

The first generation of PFCE blood substitutes were fraught with serious disadvantages, which included low solubility of oxygen and consequently to poor effectiveness, instability at room temperature (storage at -20°C), induction of anaphylactic reactions [35-36]. An example of such a formulation was Fluosol-DA manufactured by Green Cross Corporation (Japan) and Alpha Therapeutics (USA). Fluosol contained a mixture of two perfluorocompounds: perfluorodecalin (Fig.2b) (14%) and perfluorotripropylamine (Fig.2c) (6%). The emulsifier was a synthetic surfactant: Pluronic F-68, egg yolk phospholipids and potassium oleate. Originally approved by the FDA (1984), but was soon withdrawn from the market (1994) because of the numer-

ous side effects [37]. Similar PFC formulation was registered in Russia in 1996 as Perftoran, in Mexico registered under the name Perftec. The preparation is similar to Fluosol, except that it uses a different emulsifier which contributes to its lower incidence of side-effects [38]. It has a PFC content of 10%/volume, can be stored for 3 years at -4 to -18°C and two weeks at 4°C. It can be thawed and re-froze up to five times [39]. A similar preparation was registered in China as Emulsion No II.

Current PFCE products are referred to as second generation PFCE's and they contain different PFC's and surfactants than the previous products. This helps to avoid disadvantages typical of the first generation of emulsion [40-43].

The basic ingredients of second generation PFCE's are:

- perflubron (1-bromoperfluorooctane, Fig. 2a), having a molar mass of 500 g/mol. The introduction of bromine into the molecule raises the lipophilic character of the compound, to facilitate penetration through the membranes;
- perfluorodecaline (Fig.2b) having a molar mass of 462 g/mol;
- small quantities of high molecular weight perfluorinated oils with high boiling point, which stabilize the emulsion;
- emulsifier – lecithin;
- perfluoro-1,3-dimorfolinopropan (to 1%) which prevents the stratification of the emulsion;

Second generation PFCE's may be stored at room temperature and sterilized. Still existing disadvantage is relatively short residence time in the vessels - 2 to 4 hours [44].

There are several PFCE formulations currently in advanced stages of clinical trials, working for FDA approval. The main problem is a requirement of PFCEs superior safety to allogeneic blood transfusions. Since the incidence of serious adverse side effects associated with blood transfusions are very low, it would be difficult to show that PFCE preparation is safer than allogeneic blood. Taking this into consideration the European Agency for the Evaluation of Medical Products (EMEA) probably will not require direct comparison of synthetic blood substitutes to allogeneic blood transfusions.

Preparations currently in advanced clinical trials are:

- Oxygent™ (Alliance Pharmaceutical Corp., with the help of Johnson and Johnson), with a median particle diameter of 0.16-0.18 microns, an optimal storage temperature of 2-8°C, and a PFC content of 60%/voume [44-47].
- Oxycyte, a second-generation PFCE similar to Oxygent™ (Synthetic Blood International SYBD) with a mean particle diameter of 0.19 microns, PFC content of 60%/volume and a typical pH of 7.1 [48].
- PHER-02 (Sanguine Corp.) that was designed to overcome the shortcomings of its predecessor: Fluosol.

Conclusions

HBOCs and PFCEs are promising equivalents of the allogeneic blood required in emergency medicine procedures. Both of them have also potential for other clinical applications, like hypoxic tumor therapy or maintenance of tissues during complicated surgery. HBOCs, with dissociation curve resembling that of the natural hemoglobin and longer intravascular life than PCFs, are more likely to enter clinical use in the future. However, there are still many concerns regarding safety profile of artificial blood substitutes, hampering their registration in Europe and USA and demanding careful clinical examination of those preparations.

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