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REVIEW ARTICLE

Mini review on Artificial Blood Substitutes: Future perspective of Perfluorocarbon based oxygen carriers

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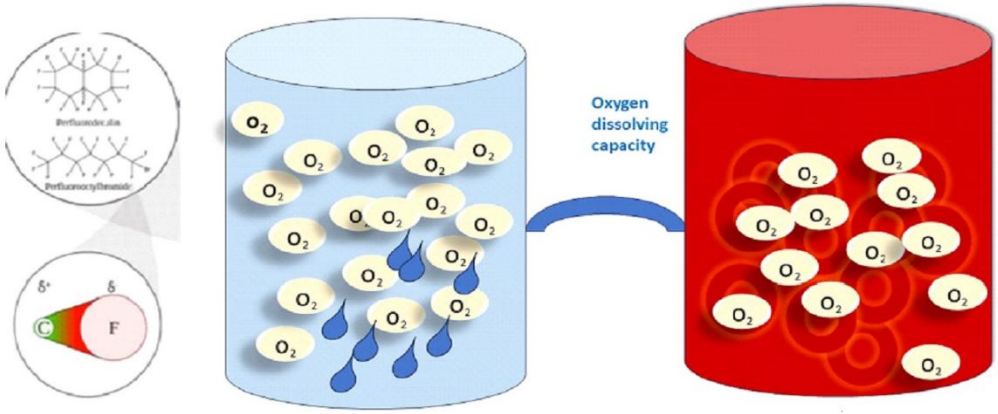
ABSTRACT

The primary cause of death in the battle field and in civilian trauma is haemorrhagic shock. Around 50% combat deaths occur due to haemorrhage, depending on the severity of bleeding medical supervision can be provided like in catastrophic haemorrhage individual death occurs before providing the medical care and it is not a prehospital combat medical management problem during warfare scenario, even to transport wounded personnel to causality centre often takes 45 minutes to one hours. The hazardous nature of the forward combat areas and hostile operational environment frequently prevents medical personnel from quickly reaching the wounded even if the transport distances are small. Moreover, injuries with penetrating battlefield trauma often have only a "platinum 5 minutes." It is very important for military medical personnel to understand their options for treating haemorrhage quickly and efficiently during combat. Early intervention and definitive treatment could save up to 30% of soldier's who die of action or of wounds.

In view of this there is a requirement for the development of synthetic blood substitutes or oxygen therapeutic agents (OTA) in order to maintain the tissue oxygenation. Blood substitutes are the substances which mimic one of the physiological functions of the blood that is transportation of oxygen and carbon dioxide throughout the body. Blood substitutes or OTAs are being clinically tested as artificial oxygen carriers to improve tissue oxygenation and also to reduce allogenic blood transfusions. Two types of blood substitutes are reported so far: 1. Haemoglobin based oxygen carriers (HBOCs) and 2. Perfluorocarbon based oxygen carriers (PFOCs). PFOCs has huge advantages like universal compatibility, no requirement of cold storage, longer shelf life and no risk of infection transmission. These products have their own limitations and they have been withdrawn from international market for clinical use because of their side effects. It is a challenging task to the scientists to develop a safe, stable and biocompatible blood substitute for combat scenarios and also for civilian applications.

Keywords: Artificial blood, Blood transfusion, Oxygen therapeutic agents (OTA), Haemoglobin based oxygen carriers (HBOCs), Perfluorocarbon based oxygen carriers (PFOCs).

Graphical Abstract



Advantages;

- Chemically and biologically inert with oxygen or other gases.
- Enhances oxygen solubility in the plasma.
- Dissolution of oxygen has no effect on temperature, pH and 2, 3 diphosphoglycerate.
- It allows easy and faster transfer of oxygen from cells to the tissues.
- It's variable oxygen carrying capacity dependent upon the FiO_2.
- Loading and unloading of oxygen is two times faster than in erythrocytes and the oxygen extraction rate is 3-fold higher since PFCs release more than 90% of the loaded O_2 to the tissue.

Introduction

Hemorrhage is the leading cause of death[1] in the battlefield and to prevent the death of soldiers from hemorrhagic shock the human donated plasma was used in place of blood. Literature says that nearly 50% of combat deaths occurs due to hemorrhage, Insufficient information was published on the combat personnel blood coagulation monitoring[2]. Along timely intervention with specific medical care we can save up to 30% of soldiers' life who die of wounds or who killed in action. In combat casualties, uncontrolled bleeding remains primary reason for mortality and fatal hemorrhage was not able to manage under combat medical. Because, when it occurs it tends to cause death before medical care can be provided[3]. In civilian conditions severely injured victims can be reached and transported by emergency medical services personnel within minutes. But, during warfare scenarios to transport injured to the hospital from battlefield takes many hours even for short distances and it is due to the hazardous nature of the forward combat areas prevents the medical rescue team from quickly reaching the wounded. Additionally, the civilian injuries are different from gunshot wounds and these civilian wounds may have a "golden hour" for treatment in comparison to combat wounds and these penetrating battlefield traumas have only "platinum 5 minutes" for treatment. Because of these challenges of treating hemorrhage during combat situation, is highly a challenging task for the medics. It is important for military medical personnel to understand their options for treating hemorrhage early as possible and most efficiently[2]. Hemorrhage control and rapid volume expansion in appropriate causalities are the main priorities in pre-hospital resuscitation of battlefield casualties[3].Until scientists develop a better understanding of the role of blood and the issues surrounding its function in the body, blood transfusion research work did not move forward. During initial days to increase plasma volume a galactoso-gluconic acid-based gum saline solution was used. In the time of world war-I, physicians tried to develop blood substitute by matching the viscosity, pH, concentration and temperature and this could have reduced the use of plasma. Whereas during 1920s some studies have reported the negative health effects of this gum solution. The use of this material was significantly diminished by the 1930s. Further, World War II has reignited an interest in the research field of blood and preparation of artificial blood substitutes. Eventually, in 1947 this led to the establishment of blood banks by the American Red Cross[4].

To replace one of the main functions of allogenic human blood transfusion i.e. transportation of oxygen and carbon dioxide throughout the body, scientists developed a unique and innovative concept called Blood substitutes. These are the key components of blood management during different pathological conditions[5]. From past few decades several formulations have been developed to achieve this objective and continuous efforts with refinements are being made in the search of the ideal synthetic blood substitutes[6-8]. A few reported haemoglobin based artificial blood was prepared using outdated prepared from haemoglobin obtained from outdated human/bovine blood (Haemoglobin Based Oxygen Carriers) or using Perfluorocarbons[9,10].

Synthetic blood substitutes are having some advantageous such as they do not require compatibility testing, are free from blood borne infections, have prolonged shelf life and do not require refrigeration.

Recently there is a high requirement of blood substitutes in the military as well as in civilian application. Hemorrhage remains the primary cause of death on the battlefield and in civilian trauma. From past few years there was an intense requirement of erythrocyte substitutes in treating military battlefield causalities and to prevent the death along with reducing the combat injury rates, wounding patterns, resuscitation doctrine and logistic requirements[4].

History

In 17th Century first time Sir Christopher Wren has started to search for a suitable compound as a blood substitute, Wren advised to use Wine, opium and ale to use as blood substitutes. Earlier they were also tried to use several other materials like milk (to treat Asiatic cholera), sheep blood, plant resins and urine[11,12]. Over the years different substances (milk, wine, sheep blood and Urine) were used as blood substitute but they are not successful[4]. Blood transfusion becomes an established and safer medical procedure, after breakthrough research of Karl Landsteiner on discovery of different blood groups in 1930 and he received noble prize for his findings. It was reported in 1947, a patient with postpartum haemorrhage received a first infusion of cell free heamoglobin for resuscitation[13]. A new type of blood substitute perfluorochemicals were used in mice experiment in the year 1966, they removed mice's blood completely and replaced with a PFC solution. They observed that mice were survived for few hours and completely recovered with their blood replacement.

Necessity of blood and blood substitutes

Blood is a very precious lifesaving commodity in the living system. Replacing it with any alternatives is impossible because blood carries so many functions in the body which was irreplaceable with any other substances[14-16]. Blood substitutes are the substances which mimic one of the physiological functions of the blood that is transportation of oxygen and carbon dioxide throughout the body. These are better called as oxygen therapeutic agents (OTA) rather than blood substitutes. More than 50% of the early trauma deaths are caused by haemorrhage shock and after severe injury nearly 30% of people die within 30 minutes[17]. There was limited effects of tourniquets and haemostatic dressings on major trauma and to stop bleeding surgical intervention is required with contusion or with laceration of parenchymal organs. However, there is lack of emergency care for trauma patients to control bleeding and medical measures at the injury sites. To minimize the bleeding and to stabilize the patient with time to carry out treatment the early fluid resuscitation is recommended[18,19]. Along with these existing treatments, blood substitutes are the lifesaving compounds which are involved in the improvement of tissue oxygenation and supports the circulatory system of wounded soldiers or patient. So, these blood substitutes are urgently required in the field of military as well as in civilian applications.

Still, blood transfusion is one of the most important treatment procedures performed to

trauma or to hospitalized patients[20]. There was an overwhelming rise for the human blood for transfusion with the remarkable increase in the number of trauma cases and surgical procedures. This leads to scarcity of blood in developing countries even though with large of number of bloods collected from donors[11]. Blood collection, processing and blood typing are being quite expensive, so it is an economic burden on the patient. Along with this, donated blood has concerns with the transmission of blood borne infections, short storage life and hypersensitive reactions. In order to alleviate the problems associated with storage of allogenic blood and increased imbalance in the demand and supply of blood, these artificial blood substitutes are the great value option for the transfusion medicine.

The 21st century is a most challenging era to the human life as increased population, occurrence of new infectious agents (Ebola and H1N1) and demographic changes demands the need for the alternative of blood as the safety issues and screening of these agents imposes further costs[21]. The risk of transmission of Human immunodeficiency virus (HIV), viral hepatitis (Hepatitis B and C viruses) and Zika virus which is recently included in the transmissible disease and a parasitic disease babesiosis may be transmitted through blood transfusion so there is always risk associated with the allogenic blood transfusion which cannot be completely ignored[22,23]. In developing countries, blood supplies are not in adequate quantity around 80% of the world's population inhabited in these countries still only 32% of blood supplies was observed with below safety standards[24]. Therefore, artificial blood or blood substitutes would be of great value for developing countries. Designing these blood substitutes involves crucial factors like size, sterility, biocompatibility and required amount of oxygen unloading at the tissue level. In this article we have further discussed the existing products, tested and reasons of failure of the developed blood substitutes and how we can overcome from these drawbacks and develop a promising blood substitute or oxygen therapeutics. In emergency hospitals, these artificial blood plays promising role in the preservation of organs for transplantation[25].

Characteristics of an ideal blood substitute:

1. It should be safe to use
2. Do not cause allergic/hypersensitive reactions
3. Should be storable at room temperature with long shelf life
4. It should be capable of transporting required amount of oxygen to the tissues
5. Cross matching, blood grouping and compatibility tests Are not necessary
6. Survival in circulation for considerable time[26, 27].

Benefits of blood substitutes over RBC's

1) Quick and greater Oxygen Distribution: The Stored blood requires 24 hours to reach complete oxygen carrying capacity due to the depletion of 2,3 diphosphoglycerate whereas, these artificial blood substitutes instantly attain full oxygen carrying capacity. The PFC molecules are able to carry 90% of oxygen due to increased extraction rates in comparison with 25-30% haemoglobin oxygen carrying capacity. Faster unloading of oxygen to tissues is due to decreased PFCs affinity for oxygen. It was assured that appropriate amount of oxygen was delivered

without causing any side effects at haemoglobin levels of 2gm/dl.

2) Longer Shelf Life: The natural blood can be stored only for approximately 35-42 days and also further refrigeration is needed whereas, these blood substitutes can be stored at room temperatures for longer periods (1-3 years) without cold storage and they are ready to use as compared to stored blood.

3) Universal Compatibility: Our immune system will not recognize these artificial blood substitutes as a foreign entity due to the removal of all the protein components. Requirement of testing compatibility based on blood groups is eliminated and we can also rule out the possibility of clerical errors which might result in mismatched transfusions.

4) Prevention of Transmission of Infectious/Anaphylactic agents: There is reduced chances of disease or viral transmission due to complete sterilization of products.

5) Inflammation, ischaemic and reperfusion injury rates are decreased,

6) Religious group/Jehovah's Witness: Blood or blood-based products not accepted this Jehovah's Witness group. These chemical-based PFC products can be accepted by this group as practical replacement to fulfil the requirement for blood transfusions[11].

Oxygen transport

Perfluorocarbon emulsions Oxygen transport/carrying characteristics are basically different in comparison to blood. The perfluorocarbon emulsions show a characteristic linear relationship with oxygen concentration and oxygen partial pressure whereas blood haemoglobin display a sigmoidal oxygen dissociation curve. Oxygen transport capacity of perfluorocarbon emulsions maximize by elevated arterial oxygen partial pressures[28]. Breathing a 100% oxygen for short duration of less than 8 hours may elevate the concerns towards oxygen toxicity. Although, there is no proof for oxygen toxicity and only after 18 hours the earliest signs of oxygen toxicity were detected[29].

Erythrocytes or Red blood corpuscles are disk-shaped and flexible cells and approximately 7–8μm in diameter they contain oxygen binding protein haemoglobin. Nearly all most of the blood vessel filled by erythrocytes and small volume of plasma occupied in the vicinity of the blood vessel along with the platelets of nearly 1μm in diameter and small particles concentrate. The distance between red blood cells increases in the capillaries, producing significant intercellular plasma gaps and it was found that capillaries are perfused by plasma only.

The perfluorocarbon nanoemulsions largely move in the larger vessels of peripheral plasma because of their small size i.e less than 0.2μm.[30,31]. Erythrocytes could not able to perfuse smallest capillaries of 4-5 diameter whereas these perfluorocarbon nanoemulsions diffuse fast in the microcirculation. Perfluorocarbon emulsions precisely exert their greatest effects in this area, because they augment local oxygen delivery much more than would be expected from the increase in oxygen content in the arterial blood (large vessel with red blood cells)[30]. Another important aspect that determines the efficacy of PFC based emulsions is the fact that all oxygen carried by the PFC is in the dissolved state, resulting in a higher oxygen partial pressure in the microcirculation and thereby

augmenting the driving pressure for the diffusion of dissolved oxygen into the tissue.

Current status

Current strategy for the preparation of blood substitutes includes industrial production, chemical isolation and recombinant biochemical technologies. Conventional synthetic blood substitutes belong to either of the two types:

I. Haemoglobin Based Oxygen Carriers (HBOCs)

II. Perfluoro Carbons Based Oxygen Carriers (PFCs).

Both the blood substitute products were clinically used but not a single product developed from red blood cells and perfluorocarbon based have proven in terms of satisfactory and safety[32,33].

I. HAEMOGLOBIN BASED OXYGEN CARRIERS (HBOCS)

HBOCs are the products of biological origin and are acquired from processed and purified haemoglobin (Hb). Haemoglobin is a ~ 64 kDa tetrameric protein molecule consisting of 2α and 2β polypeptide chains[34]. Each chain binds to single oxygen molecule due to the presence of iron associated haem group and the oxygen binding and dissociation curve is sigmoidal in shape[35]. They have the ability to bind and release oxygen and free Hb have only few hours of half-life in plasma after released from ruptured RBCs [36]. HBOCs being isolated or synthetically manufactured from haemoglobin, since Oxygen binds covalently to these compounds as they bind naturally to occurring haemoglobin [11,37]. They were designed to meet the following purposes: (1) inherent lower oxygen affinity to increase tissue unloading/oxygenation (2) prolonged intravascular retention (3) decreased colloidal osmotic activity (4) absence of renal toxicity[38,39]. During oxidative tissue injury these HBOCs are given to improve the vasoconstriction and nitric oxide (NO) scavenging[40,41]. The origin of haemoglobin is either from human blood or obtained from outdated stored blood and bovine blood or genetically engineered[37,42,43,44]. Commercially reported haemoglobin-based oxygen carriers are shown in Table 1.

Table 1: Haemoglobin based oxygen carriers

Hem Product	Manufacture	Country	Approval and Clinical trials	Reference
PolyHeme 	Northfield Laboratories (Evanston, IL) 1st	United States	Approved in South Africa; Due to safety issues, Phase II trials were discontinued	27
Hemopure 	Hemoglobin Oxygen Therapeutics LLC (Souderton, PA)	United States Europe	FDA expanded access program, life threatening anemia after exhausting all other options	

Hem Product	Manufacture	Country	Approval and Clinical trials	Reference
HemAssist	Baxter International Corporation (Deerfield, IL)	United States	Stopped at phase III trials	
Hemolink	Hemosol Inc. (Toronto, Canada)	North America	Stopped at phase II trials, Not approved	

Haemoglobin was purified from erythrocytes by following steps like heating and filtration, which remove unwanted proteins, viruses and other blood group substances are removed by heating and followed by filtration through the process of haemoglobin purification from erythrocytes. Isolated haemoglobin has been subjected to molecular modification and reconstitution in artificial blood formulation. HBOC manufacturing involves extraction of haemoglobin from the natural source and thereafter stabilisation with crosslinking agents (using glutaraldehyde or o-raffinose) or conjugation with polyethylene glycol or encapsulation in the phospholipid vesicles before mixing into an electrolyte solution [43,45,46]. Substantial increase in the intravascular "dwell" time (24-48 hours) of HBOC was found by cross linking or polymerization with larger molecules like polyethylene glycol, dextran or polyoxyethylene [47,48]. Antioxidant enzymes like superoxide dismutase or catalase are attached to the protoporphyrin structure of haemoglobin.The incorporation of antioxidants helps in reducing the severity of ischaemic reperfusion injury in conditions like stroke, myocardial infarction or organ transplantation[11,12,49,50].

Limitations of HBOC's

Haemoglobin (like other plasma proteins) exerts colloidal osmotic pressure whereas RBC's do not exert any colloidal osmotic pressure. Hence, cellular haemoglobin may change the intravascular volume and functions as a plasma expander. Half-life of HBOC's is smaller compared to normal erythrocytes. Large number of HBOCs remain in circulation for about 20-30 hours whereas whole blood transfusion lasts for 34 days. The free haemoglobin of HBOC's produces reactive oxygen species (ROS) in the body, further leads to the breakdown of haemoglobin into haem and iron and causes cell damage. Concentrations of methaemoglobin also increases due to the oxidative properties of HBOC's [51,52,53,54]. The primary option for getting haemoglobin is from outdated human blood which has a limited supply. Therefore, for the acquisition of haemoglobin bovine blood can be utilized. Although, there is a higher risk associated with Bovine haemoglobin of cultivating the prion pathogen which causes the causing bovine spongiform encephalopathy (Creutzfeldt- Jakob disease). Genetically engineered bacteria are the good choice to avoid this problem and also, we can ensure the steady availability of haemoglobin for upcoming days[11,55].

Recombinant Haemoglobin (Optro): Then, haemoglobin gene was transferred using a plasmid vector into *E.coli* cells. Haemoglobin is generated by the expression of haemoglobin genes. By this recombinant method the risk of transmission of infectious diseases by natural

haemoglobin from different human and animal origin can be terminated. High costs of this technique is a major drawback or hindrance for further exploration[27].

II. PERFLUOROCARBON (PFC) BASED PRODUCTS

PFCs are chemically and biologically inert molecules and are structurally similar to hydrocarbons compounds in which fluorine atom has replaced hydrogen and are about 100 times smaller than RBCs in size. PFCs have immense ability to dissolve gases and they are capable of carrying oxygen and carbon dioxide without binding to these gases[56,57]. Earlier PFCs are used for the treatment of respiratory distress syndrome in premature infants to provide oxygen. PFC is insoluble in water hence, emulsification of PFC by addition of lipids through high pressure homogenization is required to suspend tiny particles of PFC in the blood. The saturation of PFC takes place passively after the molecules of oxygen dissolve into molecular cavities within liquid droplets. PFC's oxygenation is connected to partial pressure of oxygen. Hence, finestoutcome are obtained if the patient is breathing 100% oxygen at the time of infusion (PaO2 > 350 mm Hg). Through the reticuloendothelial system PFC's are systematically removed from the human body. The half-life of these PFC molecules becomes reduced in dose dependent way because these are exhaled through the lungs. After administration, these PFC molecules vaporize and then are exhaled within few days. Previously one of the main disadvantages of using PFC's was the accumulation in the reticuloendothelial system. In comparison with haemoglobin, PFC's have the following advantages; (1) Chenically and biologically iInert with oxygen or other gases (2) Enhances oxygen solubility in the plasma (3) dissolution of oxygen has no effect on temperature, pH and 2, 3 diphosphoglycerate (4) it allows easy and faster transfer of oxygen from cells to the tissues (5) it's variable oxygen carrying capacity dependent upon the FiO_2. **(6)** Loading and unloading of oxygen is two times faster than in erythrocytes and the oxygen extraction rate is 3-fold higher since PFCs release more than 90% of the loaded O_2 to the tissue [58,59]. PFC based products reported in the international Market is shown in Table 2.

Table 2: PFC based products reported in the international Market

PFC Product	Generation	Country	Approval and Clinical trials	Reference
Flusol-DA-20	1st	Japan, USA	FDA, discontinued in 1994	21
Oxygent 	2nd	USA, China	Not approved, Stability and side effects	
Perfotoran	3rd	Russia	Phase III clinical trials and discontinued	

PFC Product	Generation	Country	Approval and Clinical trials	Reference
Oxycyte	3rd	Mexico	Not accepted for clinical trials	
PHER-O	3rd	USA	Pre-clinical trials	

First Generation PFC

Over 33 years ago first-generation PFC formulation i.e., Fluosol DA was developed in Japan. Prototype of this class was Fluosol DA 20%, it was made up of perfluorodecalin and perfluorotrypropylamine and Pluronic F-68 as short chain emulsifier. Perfluorodecalin involved in effective oxygen transport, Whereas, perfluorotrypropylamine acts as a balancing agent. In Japan this commercial product was used for Gastrointestinal bleeding and surgery associated blood loss[60,61]. A blood substitute formulation, Fluosol was approved by FDA for cardiac surgery. Completely oxygenated PFC is administered for the coronary angioplasty to provide oxygen delivery during surgery.

Second Generation PFC

Oxygent™ is a second-generation PFC made up of Perfluorodecalin, perfluorooctyl bromide and egg yolk phospholipids emulsifying agent. Theoxygen carrying capacity is considerably elevated than the first generation PFC's and its excretion is also faster with lower tissue retention. Clinical trials of this product show increased stroke rates in hemodiluted patients and also high cost are reasons for the discontinuation of this product[62].

Limitations of PFC's

First generation PFC was responsible for complement activation. It was observed that first generation PFC's based on lecithin showed cytotoxicity in monocytes and granulocytes (Phagocytic cells). PFC infusion causes flu by opsonisation and the patient immune system leads to phagocytosis of PFC molecules. These PFC molecules may cause oxygen toxicity in the system due to subjection of higher concentration of oxygen. There was short term decrease in the platelet count within few days of the injection of PFC molecules and gradually become normalized by seven to ten days[51]. Also, PFC products removal process from human body requires around 18-24 months. Due to excess accumulation of these PFC molcules in the reticuloendothelial system may reduce its function. Histological effects like appearances and enlargement of vacuolated histiocytes are seen in liver biopsies as it can be retained in organs[63]. Neurological complications has been reported in several human cardiac surgery cases[58].

Conclusion and future perspectives

Currently efforts are going on by many companies and research institutes for the development of a safe, stable and effective artificial blood or blood substitute product for the emergency trauma cases as well as for the battle field injuries. Although it is not a blood substitute but it helps only in tissue oxygenation and temporary support to the circulatory system until patients own RBC's generated from hematopoietic stem cells. As

allogenic blood transfusion had some negative immunological effects like delayed wound healing, increased wound infections and development of malignant diseases. The PFC solutions have high oxygen carrying capacity and individuals can survive by breathing liquid and it is termed as liquid breathing. Despite the fact that varieties of blood substitutes used for oxygen transport, delivery and volume expansion, their concurrent usage with other blood salvaging techniques can significantly reduce the requirement of allogenic blood transfusion during surgical procedures.

In the future, synthetic blood substitutes can revolutionize medical care by supplying universal compatible agents to civilian and military requirements. To assess the scalability to ensure manufacturing reproducibility, consistent composition, safety and therapeutic benefits, extensive in-vitro and in-vivo testing of various approaches is necessary. An ideal desirable blood substitutes product which should be field deployable, shelf stable and ready to use can be achieved by the coordinated effort involving biochemists, chemists, haematologists and the necessary regulatory agencies. Although in some countries human trials using synthetic blood are going on, still there is still a long way to go for a large-scale usage.

Conflict of Interest:

None.

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