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CHAPTER 1

Perspectives of Blood Substitutes

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Introduction

It has been long since a number of problems were pointed out with regard to homologous blood transfusion. They include the possibilities of viral infections such as AIDS and hepatitis, antigenic sensitization such as blood group incompatibility and GVHD (Graft Versus Host Disease), various complicated tests which are time consuming and expensive, and the limitation of blood preservation (4°C, 3 weeks). However, accidents in blood transfusion cannot be avoided entirely even with close attention. Furthermore, compared with Japan, matters are more serious in many countries where the organization of blood transfusion systems are insufficient. The development of red cell substitutes is an urgent, important, and top priority subject for the lifesaving systems equipped with artificial blood (blood substitutes) that can be supplied sufficiently, safely, and instantaneously irrespective of location and blood types in case of serious accidents.

It is no exaggeration to say that the history of blood substitutes is the history of blood transfusion. Blood transfusion other than one's own blood can be regarded as the administration of blood substitutes [1]. From the middle of the 16th century, the administration of various liquids into the blood was tested, and Blundell succeeded in conducting homologous blood transfusion (1818), and proved that death from bleeding can be avoided through blood transfusion. However, the success rate of blood transfusion in those days was too low for this method to be regarded as a means of lifesaving. The success rate of blood transfusion had dramatically risen through the discovery of ABO blood typing by Landsteiner (1901), and blood transfusion as a means of medical treatment became a focus of attention. The present blood transfusion systems were established through the establishment of the blood bank (1936) in the United States and the development of a preservation method by addition of anticoagulant, ACD solution (1943). In the middle of the 1980s, the infection through blood transfusions with HIV (human immunodeficiency virus that causes AIDS) was reported, which destroyed the reliance on blood transfusion and had tremendous impact on the blood

industry, bringing about considerable reforms; the necessity for the development of blood substitutes was strongly recognized.

Requirements for red cell substitutes

Red cell substitutes must have the capability to transport sufficient oxygen to peripheral tissues; furthermore, they must keep fulfilling their functions by staying in the blood flow for an adequate period of time. Red cell substitutes are administered when more than half of all the blood is lost making it impossible to sustain life even through the administration of plasma expanders. Thus the regulation of solution properties such as viscosity and colloidal osmotic pressure is important, which will exert influence on circulatory kinetics if not adequately regulated. For example, it has been pointed out that low-viscosity preparations will cause vasoconstriction and a decrease in functional capillary density, which will decrease oxygen transport to the peripheries (Intaglietta, Chapter 10). Other opinions have been proposed: relatively high colloidal osmotic pressure will improve microhemodynamics [2]; and excessive oxygen supply will activate the autoregulation system, which will decrease oxygen supply [3]. It is thus considered necessary to prepare appropriate solution properties in accordance with adaptation with observing peripheral microcirculatory dynamics, but no practical values have been designated.

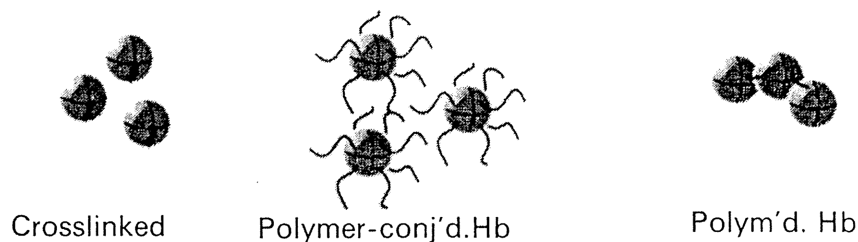
In addition, blood substitutes will not be regarded as attractive and survive in the market without effectiveness equivalent to or higher than that of homologous blood transfusion from the standpoint of safety, cost performance, and long-term preservation stability. Potentially viable preparations have been started, but will take some more time to reach completion. However, toward this purpose, several preparations have been already clinically evaluated with benefits from cutting-edge techniques, intelligence, and much support.

Properties and clinical trends of red cell substitutes

Red cell substitutes developed until now are broadly divided into two carriers (Figs. 1 and 2): hemoglobin-based oxygen carriers (1) and totally synthetic oxygen carriers (2). (1) is subdivided into acellular hemoglobin or modified hemoglobin and cellular hemoglobin or encapsulated hemoglobin; (2) is subdivided into perfluorocarbon emulsions and totally synthetic hemes. At present, modified hemoglobins and perfluorocarbon emulsions have been clinically examined in eight companies in the United States and Canada, and the others have been preclinically examined (refer to Table 1 in Chapter 2 detailing the name of the companies and types, adaptation, and the phase of clinical examination).

Acellular hemoglobin

Administration of hemoglobin, to a patient with anemia was already tried a hundred years ago by Stark [4], but this ended in failure due to serious nephrotoxicity. It was



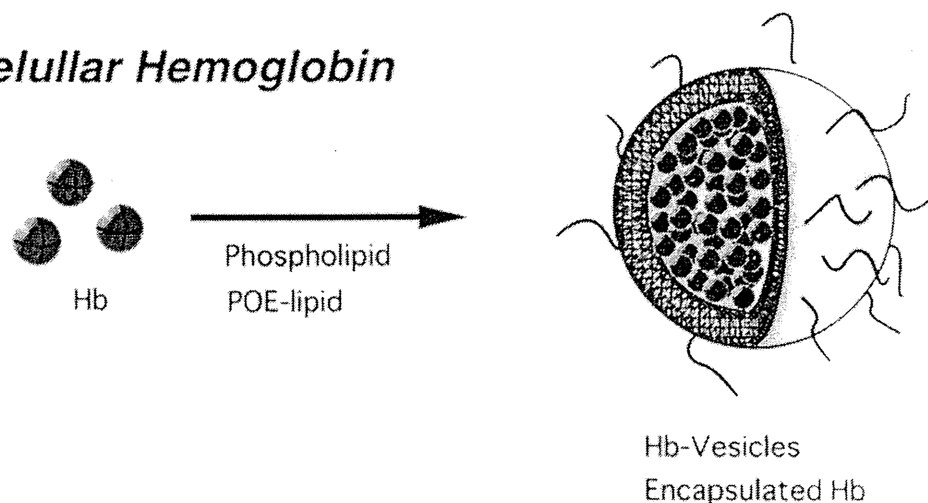


Fig. 1. Classification of Hb-based oxygen carriers.

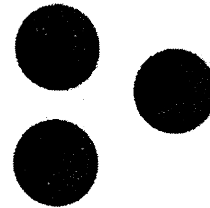
not until the series of discoveries in 1967 that the use of hemoglobin in blood substitutes was greatly advanced. Rabinar [5] significantly decreased the side effects of hemoglobin by establishing a hemoglobin purifying method that increases the elimination rate of stroma to 99%, Bunn and Jundel [6] succeeded in considerable extension of the retention time of hemoglobin by crosslinking, and Benesch et al. [7] found the decrease in the oxygen affinity for hemoglobin combined with allosteric factors such as pyridoxal 5′-phosphate. These findings established all the basics for the present fundamental strategies for hemoglobin modification.

Acellular hemoglobin is divided into four types: crosslinked hemoglobin, polymerized hemoglobin, polymer-conjugated hemoglobin, and recombinant hemoglobin. Crosslinked hemoglobin is produced by crosslinking the amino groups of the 99th lysine between the α chains of hemoglobin using fumaric acid, which has been developed as HemeAssist^{TM} by Baxter Inc. Crosslinked hemoglobin features complete homogeneity due to its simple structure, establishment of the virus-free mass production process, an adequate oxygen affinity (33 Torr), and low viscosity [8]. However, crosslinked hemoglobin has a shorter retention time in the blood compared with the other Hb preparations, and vasoconstriction was reported as a side effect. Baxter Inc. regards the HemeAssist^{TM} as a drug in Hb therapeutics [9], and has been examining its adaptation as a blood dilutant and blood pressure stabilizer in

1) Perfluorocarbon Emulsions

$CF_3(CF_2)_7Br$
Phospholipid

Emulsified



2) Totally Synthetic Hemes

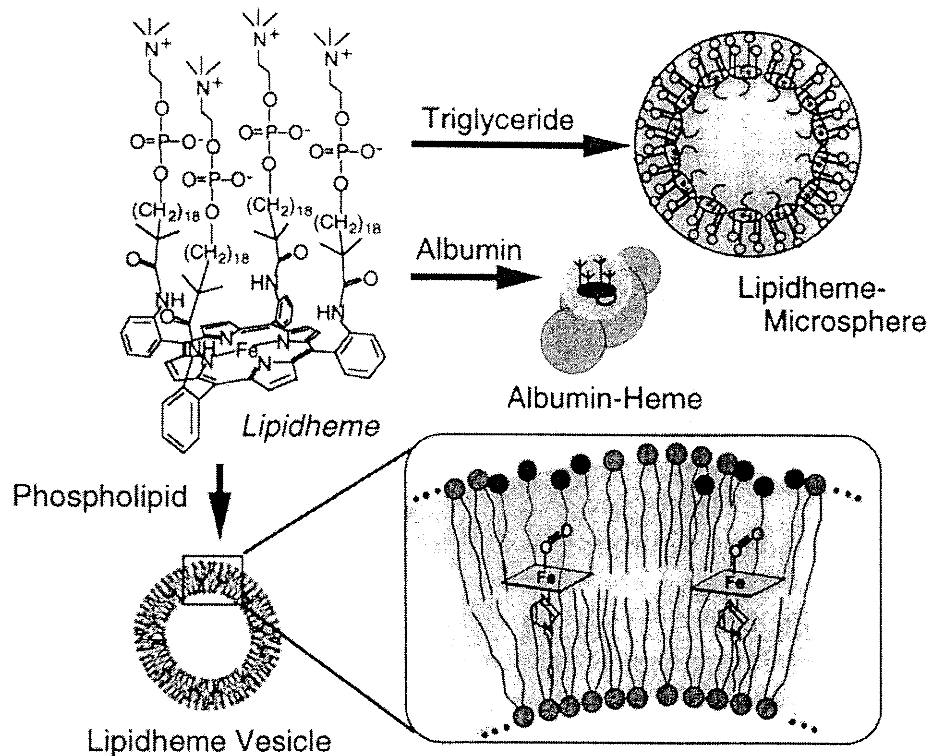


Fig. 2. Classification of totally synthetic oxygen carriers.

cerebral apoplexy, artery repair, and gastrointestinal and plastic surgery. 750 ml of HemeAssist^{TM} was administered to patients undergoing cardiac surgery, and the necessity of additional transfusion of human red cells within 24 h after surgery examined; more than half of the patients required no blood transfusion, which was considered significantly lower than the group that was administered human red cells. Also in general surgery (aortic repair, gluteal, genucubital, abdominal, and pelvic surgery), the use of HemeAssist^{TM} has been examined for the possibility of avoidance of transfusion of human red cells, or the decrease in transfused blood. This is detailed in Chapter 6. However, Baxter had stopped all clinical trials on HemAssist^{TM}, pending a review of clinical data showing the higher mortality than control, and shifted its efforts of blood substitutes research to the second generation of recombinant Hbs by purchasing Somatogen Inc. Baxter's attitude toward hemoglobin

therapeutics is interesting, but main target should not be changed from the replacement of blood transfusion.

With regard to polymerized hemoglobin, PolyHeme^{TM}, in which hemoglobin is intermolecularly crosslinked by glutaraldehyde, has been developed by Northfield Laboratories [10]. Hemolink^{TM}, in which hemoglobin is intermolecularly crosslinked by *o*-raffinose, has been developed by Hemosol Inc. [11]. Both of these are undergoing clinical examinations. Polymerized hemoglobins feature colloidal osmotic pressure similar to blood and extended retention time in blood, but they are heterogeneous and require the control of molecular weight distribution between 128 to 512 kD. As the clinical examination of PolyHeme^{TM}, it was administered up to six units (Hb 300 g) to patients who acutely bled from an injury or during operation, and the result was compared with that of blood transfusion (Chapter 4). As a result, no particular problems were recognized with regard to safety, and no additional blood transfusion was necessary within 24 h for more than half of the patients whose blood, the mean Hb concentration of which was 7.5 g/dl, was substituted with up to 4.8 g/dl of Hb derived from PolyHeme^{TM}. In accordance with this result, PolyHeme^{TM} has been claimed to be effective as a blood substitute. No renal damage or fever, and interestingly no increases in blood pressure by vasoconstriction were recognized in the clinical examination of PolyHeme^{TM}, which is considered the features of hemoglobin preparations having high molecular weight. On the other hand, the increase in blood pressure was reported for Hemolink^{TM} (Chapter 17) but small degree in comparison with crosslinked Hb, which is presumed to be due to the difference in the residual rate of low molecular weight components.

As polymer-conjugated hemoglobins, in which polyethylene glycol having low physiological activity is bound to, the surface of hemoglobin, the type using bovine hemoglobin has been developed as PEG-Hemoglobin^{TM} by Enzon [12], and the type using human hemoglobin has been developed as PHP^{TM} by Apex [13]. Polymer-conjugated hemoglobin features a long retention time in blood, low vasoconstriction effect, and relatively high colloidal osmotic pressure and solution viscosity, etc. Polymer-conjugated hemoglobins are heterogeneous, and are undergoing clinical tests as a tumor chemotherapy agent and a therapeutics for septic shock.

Recombinant hemoglobin produced by fungus bodies or *Escherichia coli* using transgenic techniques has been produced as Optro^{TM} by Somatogen Inc., and is now being examined clinically in Phase II. Intermolecular crosslinking and an adequate oxygen affinity were achieved by transforming a part of amino acid sequence of human hemoglobin. The best advantage of recombinant hemoglobin is that it can be produced homogeneously and inexhaustibly in factories, but the complexity in the purification process should be resolved to establish a payable mass production process.

In Phase I of the clinical examination, a maximum of 25 g of Optro^{TM} was administered, and no side effects such as nephrotoxicity, immune disorders, or influences on coagulation systems were recognized, but a transient increase in blood pressure up to 50 mmHg was recognized just after administration. More

than 5 g/kg of the administration caused slight dysphagia, nausea, and emesis, and deformation was recognized in the esophagus and gastrointestine [14]. A transient increase in the concentration of amylase and lipase was recognized, but no disorder was recognized in the pancreas. The aforementioned symptoms are considered to be involved with trapping of nitric oxide by acellular hemoglobin. In the first and second phases of the clinical examination, 26 g of Optro™ was administered to anesthetized patients, and the symptoms recognized in the case of volunteers in the first phase were significantly relieved. In the Phase II trial, in which Optro™ was administered up to 100 g during intraoperative blood transfusion to examine the safety and oxygen transport effect, it was reported that though a transient increase in blood pressure was recognized, no abnormality was recognized in the form of the esophagus, and no significant differences were observed in comparison with homologous blood transfusion. The application of Optro™ has been examined for acute normovolemic hemodilution in cardiac surgery and for a hematopoietic accelerating agent by the facilitation of erythropoietin generation. The recombinant preparations are detailed in Chapters 5 and 22, and their side effects are described in Chapter 9.

Cellular hemoglobin

Cellular hemoglobin or Hb vesicles have a structure in which a high concentration (> 36 g/dl) of purified hemoglobin is covered by a phospholipid bilayer membrane, which may solve the many problems in the use of hemoglobin molecules. This idea was introduced by Chang in 1957, and the development of phospholipid vesicle was advanced by the leading studies by Djordjevich and Miller [15], Hunt [16], and Farmer et al. [17] from the 1970s through the 1980s. At present, the establishment of a highly efficient manufacturing process and improvements in the properties have greatly been advanced by our group [18], and Rudolph's group [19] accumulated fundamental findings on pharmacological and physiological responses, and we have been cooperatively making preparations to advance the clinical examination.

In cellular hemoglobin, dissociation of hemoglobin tetramer to dimers is restrained because a high concentration of hemoglobin is included in Hb vesicles, and the oxygen affinity is adequately regulated and the methemoglobin formation is restrained because allosteric effectors and reduction systems are together included. The colloidal osmotic pressure is close to zero; it is thus regulatable by the addition of adequate colloids, and the viscosity can be restrained equivalent to or less than that of blood (Chapter 14). The physiological activity of hemoglobin and liberated heme can further be restrained by encapsulation into the cells. If these many advantages achieve a decrease in the cost and increase in the efficiency of capsulation process of high-purity lipid, great clinical development can be expected for cellular hemoglobin.

For example, in the examination using strips of aorta, it was proved that cellular hemoglobin showed low vasoconstriction, one hundredth of that of molecular hemoglobin. The cause of vasoconstriction by molecular hemoglobin is considered

to be trapping of nitric oxide as an endothelial derived relaxation factor (EDRF) by hemoglobin dispersed between vascular endothelium and smooth muscles. On the other hand, phospholipid vesicles cannot penetrate through blood vessel walls because they have a large particle size, 40 times of that of hemoglobin, and the binding of nitric oxide with hemoglobin is restrained due to the long mean distance from vascular endothelium because of the separating lipid membrane (Chapter 20). In addition, perfusion of acellular hemoglobin in an isolated liver causes a rapid increase in the perfusion pressure and vasoconstriction, while these symptoms are hardly recognized for cellular hemoglobin. Suematsu has pointed out that the system of trapping of carbon monoxide, which is the relaxation factor of liver vessels, is described; the sinusoid vessels of liver have holes of about 150 nm, and hemoglobin readily passes through the holes and traps carbon monoxide, which shows the significant differences between cellular and acellular structure (Chapter 19).

The results of resuscitation from hemorrhagic shock and exchange blood transfusion in the animal test using Hb vesicles are described in Chapter 14. Achievement of more than 80% of the exchange level is impossible by the administration of albumin solution alone that has no oxygen transport capacity, but all rats administered with Hb vesicles survived even with more than 90% of the exchange level. In addition, aggregation in bloodstream was effectively restrained in the system of which the surface was modified with polyethylene glycol chain, and the decrease in vascular resistance and improvement of microcirculatory dynamics were clearly recognized. A quantitative report was made on the effect of PEG chain modification on the surface of vesicles, on the basis of the measurements of the subcutaneous microcirculatory system measured by attaching a window on the dorsal skin of hamsters (Chapter 15).

Philips showed the necessity of 10 mol% PEG surface modification for the effective increase in the retention time of liposome encapsulated hemoglobin (LEH) (Chapter 12). He also observed the distribution of LEH by ^{99m}Tc labeling on the hydrophobic part of LEH lipids, and showed that 49% of administered LEH was still circulating 48 h after the administration. Szebeni (Walter Reed Army Laboratory) has been examining the influences of LEH on human complement activation to evaluate the safety of LEH [20]. The increase in C4d and Bd by the administration of LEH shows classical and the alternative pathway activation, which is due to the interaction between LEH and IgG or IgM. In addition, it was shown that IgM interacts with choline type phospholipid, and all complement activities are effectively restrained by soluble complement receptors-I (SCRI) [21]. Rudolph (Naval Research Laboratory) reported the results of the analysis focusing on the interaction between various endothelial cells and heme ingestion of acellular Hb (crosslinked Hb) and cellular Hb (LEH) in the incubation system [22]. With regard to acellular Hb, heme ingestion was particularly recognized for methemoglobin and heme oxygenase was activated with the heme ingestion, while heme was hardly ingested for cellular Hb. This is considered an advantage of cellular Hb.

Perfluorocarbon emulsions

Perfluorocarbon (PFC) solution has high oxygen solubility, but it is not miscible with water, thus PFC is emulsified with a surfactant such as phospholipid for use. PFC emulsions have several advantages such as production at a low cost, instantaneous availability in case of emergency due to long shelf stability, and no risk of infection. However, there still remain problems which must be solved, such as confirmation of the safety and metabolism, and the limit of capability including oxygen transport capacity.

The Green Cross Corp. developed the first generation of PFC emulsion, Fluosol-DA, the clinical examination of which was started in 1978; the preparation was first approved by the FDA and was introduced in the market in 1990, though the application was restricted to perfusion of colonary arteries after percutaneous transluminal angioplasty (PTCA). However, sufficient effectiveness was not obtained because of the low oxygen transporting capacity; thus its production is stopped at present.

The second generation PFCs are Oxygent™ and Oxyfluor™ developed by Alliance Pharmaceutical Corp. and HemaGen/PFC Inc., respectively, and both preparations are now under clinical examination. In the 7-ISBS session on perfluorocarbon emulsions, Riess (UCSD) and Lowe (Nottingham University), both of whom stand foremost in this field, introduced new viewpoints, in which they described the progress such as the increase in the stability of new generation PFC and in oxygen transporting capacity, the trends in clinical examinations, and various application using the properties of PFC (Chapters 7 and 25). The other PFC product; PERF-TRAN, developed by Russian Academy of Science, is clinically used for patients in trauma, ischemia, transplantation, disorder in microcirculation, etc. The small particle size of 70 nm is one characteristic of this material, however, oxygen solubility of 7 vol.% under 760 mmHg pO_2 should be improved for the replacement of blood transfusion.

Totally synthetic heme oxygen carriers

In 1983, a new totally synthetic oxygen carrier that was the first in the world to use synthetic heme was developed by our group (Fig. 2). Heme is the oxygen binding site of hemoglobin, but is bound with oxygen stably as being inserted into heme pockets. In other words, each heme molecule is inserted into a specific site in the hydrophobic heme pocket structured by globin chains, by which the sixth position of heme becomes free for oxygen coordination; dimerizing oxidization is sterically prevented, and proton oxidization is prevented. This was proven by studies actively conducted in the 1970s. Besides our group, Collman, Baldwin, Traylor, Monamenteau, and others synthesized a series of derivatives to obtain such compounds that fill the aforementioned requirements [23]; only our group succeeded in the development of the system under the idea of using amphiphatic structure and molecular assembly, in which oxygen reversibly binds and dissociates in water. For about 20 years since then, we have been studying totally synthetic oxygen carriers using heme derivatives.

Besides in the system using phospholipid bilayer membrane of vesicles, oxygen transporting capacity equivalent to that of blood was found in the system using lipid heme as the surfactant of lipid microsphere, which is clinically used in a nutritional injection, in the system in which the bilayer membrane is structured by lipid heme only, in a synthesized molecule in which alkylimidazole bound with the fifth position is directly bound to the heme part, and just recently albumin-heme in which the above molecule is bound with recombinant albumin. The use of albumin that is plasma protein is expected to be safer than the use of hemoglobin.

The high efficiency of albumin-heme as an oxygen transfusion was recognized in the examination in which rats were previously treated with 70% blood drawing exchange, then were put into a shock condition by drawing a further 40% of their blood; they were subsequently administered with albumin-heme to examine its recovery effect (Chapter 24). Albumin-heme can be produced in factories because it is 100% constituted from synthetics, and has no risk of infection. It also features long shelf stability in the state of freeze-dried powder, high oxygen transporting capacity, and the capability of surface modification and introduction of reduction systems due to the molecular assembly system.

All the materials of totally synthetic oxygen carriers are inexhaustible because they can be produced in factories, but the problem of synthesizing cost still remains to be solved [24]. New ideas and compounds are continuously being proposed, and they are now being produced and evaluated.

Future prospects for blood substitutes

New development of recombinant preparations

The methodology on the production of useful proteins and enzymes using recombinant techniques will be more and more active in the future. The oxygen affinity of hemoglobin may become facultatively regulatable, and not only intramolecularly crosslinked hemoglobin but also several types of intermolecularly crosslinked hemoglobin would be obtained by the recombinant system. Somatogen Inc. has already started studying measures to resolve the problems with Optro™, that is intramolecularly crosslinked recombinant hemoglobin, one of which is the method to synthesize polymers having specific structure by crosslinking between hemoglobin molecules to extend the retention time in blood, and the other is the method to restrain the binding between heme and nitric oxide by narrowing the space of heme pockets (Chapter 5). Recently, human hemoglobin can be obtained from transgenic tobacco plants. This technique provides an inexpensive and abundant source of biomass with avoiding the risk of infection [25] Ho (Carnegi Mellon University) examined the higher order structure of hemoglobin using NMR, in which α96Val and β108Asn were substituted with other amino acids, and he described the fundamental findings involved with oxygen affinity (Chapter 22). In order to materialize recombinant preparations, those of uniform standard must be supplied efficiently and at a low cost

through the advancement in production and purification techniques. In addition, when the amino acid sequence of protein is considerably transformed from that of human Hb, it may be recognized as a foreign element in the immune system and may express teratogepicity. Therefore, long-term careful confirmation of its safety would be necessary.

Recombinant human albumin has been already developed as one of plasma substitutes by two companies, Delta Co., Ltd and Yoshitomi Phamaceutical Inc., and the reports regarding the establishment of production process of high-purity body and the results of the Phase III clinical examination were reported in 7-ISBS. Recombinant human albumin can be regarded identical to totally pure human albumin, thus the lowering of the production cost would be the only problem remaining to be solved.

New development of cellular hemoglobin

Cellular hemoglobin that can stay in blood for a long time would appear by the progress in the development of surface modifier. On the other hand, long-term maintenance of the function as an oxygen carrier requires not only the extension of the retention time in blood but also considerable restraint of methemoglobin formation. Thus, the establishment of the system is expected in which a reduction catalyst system formed by incorporating a substrate (e.g. glucose) as a reducing agent into the inner aqueous phase reduces the methemoglobin.

New types of ideas are also proposed for cellular hemoglobin. Chang (McGill University) proposed a new cellular hemoglobin, "nanocapsule", using not lipid membranes but biodegradable polylactate membranes (Chapter 13). The use of regeneration and processing of red cells would be focused on if the methods for inactivation and stable preservation of viruses are established. Fisher (Southern California University) introduced an attempt to enable the administration of red cells to anyone by modifying the surface of red cells with PEG chains to mask the blood type (Chapter 23). It would take a long time for the accomplishment, but this attempt is expected for the applications such as rejections caused by chronic blood transfusion and treatments of chronic diseases such as sickle cell anemia. Yonetani (University of Pennsylvania) suggested that α-nitrosyl HbA in which two molecules of nitric oxide are bound to α chains of hemoglobin has a considerably decreased oxygen affinity, thus it effectively transports oxygen in peripheral tissues. At present, the effects are being discussed of the system that has increased oxygen transporting efficiency by binding nitric oxide to red cell hemoglobin.

Moreover, if the biosynthesis of red cells are made possible by the culture and cloning of hematopoietic stem cells, these red cells would be used as red cell substitute having completed functions and no risk of infection. In this case, the blood type will be O type or not be expressed. Although these substitutes are not suitable for the use in case of emergency, they would be very effective for the use in waiting operations and patients with chronic anemia.

Establishment of the evaluation system for efficacy and safety

The noninvasive microhemodynamics measurements for subcutaneous microcirculation is significantly important for instantaneous direct evaluation of administration effects of drugs, etc. In this method, which was established by Intaglietta (UCSD) [26], variations in vessel diameter and blood flow velocity are measured on their images, and bloodstream is calculated from vessel diameter and flow velocity; functional capillary density is calculated for vessels in the selected field and from the number of capillaries through which blood cells pass within the time specified. Oxygen partial pressure in subcutaneous capillaries and in local tissues are noninvasively measured from the phosphorescence lifetime of the probe dependent on oxygen concentration. These measurements clarify the influences of oxygen affinity, and solution viscosity of oxygen carriers on oxygen transportation to peripheral tissues. It was concluded from this method that red cell substitutes ought to have relatively high viscosity and colloidal osmotic pressure. It is also interesting that these conclusions challenge conventional remarks (Chapter 10).

In addition, physiological behaviors and physicochemical properties of oxygen carriers are intermixed in the case of in vivo measurement, thus quantitative measurement and evaluation are generally accompanied by difficulties. Hellums (Rice University) kinetically analyzed oxygen distribution by passing oxygen carriers through oxygen permeable capillary models of 25 or 10 μm in diameter, for the purpose of the in vitro simulation of oxygen transport by a modified Hb solution to tissues (Chapter 11) [27]. In comparison with red cells, rapid oxygen release was quantitatively indicated, and definite differences between cellular and acellular Hb were revealed. This result would be important on the grounds of the opinion that excessive oxygen transport would cause vasoconstriction due to the autoregulation function.

Although the hemoglobin molecule is a water soluble protein, it is not a plasma protein such as albumin but a protein included in cells. Considering the high physiological activity and oxygen transporting property of hemoglobin, with regard to mammals, hemoglobin may be a protein that should be included in cells. In actuality, enzyme systems exist in red cells, which eliminate harmful active oxygen generated from hemoglobin and reduce oxidized hemoglobin, and in case of hemolysis, hemoglobin transfused into plasma is rapidly excluded from the plasma and metabolized by the systems with haptoglobin, albumin, etc. At present no problematic side effects or toxicity have been reported for the results of clinical examination, but increases in blood pressure, effects of Hb as an oxidant, interaction with lipopolysaccharide, complementary activity, and induction of cytokine have been increasingly pointed out (Chapter 16), thus detailed examination will be successively required for the elucidation and fundamental resolution of the functional systems.

Hereafter, biogenic reactions involved with oxygen carriers including kinetics and roles of blood will be further clarified through progress with the accumulation and interpretation of findings obtained by in vitro and in vivo evaluations, as well as findings regarding in vivo macro and micro kinetics. In addition, selective

administration of appropriate oxygen carriers in accordance with the indication will be made possible. With regard to safety, many findings have been accumulated, but many questions still remain.

Trends in artificial platelet study

The following is the summary of the study on the development of artificial platelets at a developing stage. Artificial platelets are broadly divided into two types: platelets which receive treatment for long-term preservation, and platelet substitutes having partly platelet functions, and studies have been conducted individually. Particularly the latter includes infusible platelet membrane (IPM^{TM}), thrombosphere, and thrombored cells. Refer to Chapter 27 for details, in which Alving described the status and future prospects for artificial platelets. In addition, the importance of establishing evaluation methods has been pointed out for the development of artificial platelets. Ikebuchi (Hokkaido Red Cross Blood Center) reported in the 7-ISBS session on artificial platelets that cultured human megakaryocyte as the precursor cell of platelets contained lots of GPIIb/IIIa, a protein bound to platelets, and described the possibility of mass culture. Nishiya (Keio University) reported on the development of the vesicles that effectively form compounds with platelets through surface modification of phospholipid vesicles with tripeptide (RGD) that specifically interacts with platelets. Ikeda and Murata (Keio University) proposed phospholipid vesicles introduced with recombinant GPIb α (Chapter 28), and these vesicles were shown to interact with vWF that specifically binds to platelets under coexistence of ristocetin, which can be regarded as the result that will lead the practical development of artificial platelets.

Epilogue

International conferences on blood substitutes are held every other year, which offer a venue for the exchange of information and presentation of the latest study trends in each institute involved in the field. In the seventh International Symposium on Blood Substitute held in September 1997 in Tokyo, the present research of blood substitutes was fully covered, and the status and unsettled problems of therapies using substitutes were presented in intensive exchanges between researchers and clinicians. The conference also studied the future of this indispensable research field.

In the United States, research project grants were awarded from The National Heart, Lung, and Blood Institute of NIH (NHIBI) for five years from 1993 through 1997, and the results were reported every spring as the Current Issues in Blood Substitute Research and Development Course in University of California, San Diego.

In Japan, the Society of Blood Substitutes, Japan, was established in 1993, which is actively working for the development of this field through the publication of journals "Artificial Blood", and annual conferences. Moreover, as the project to advance front line medical research, from 1997 the Ministry of Health and Welfare started the

development project of artificial blood primarily focusing on the three substitutes, artificial red cells having oxygen transport functions, artificial platelets having hemostatic effects, and artificial globulin having immune functions (Fig. 3). Also in Brazil or Korea, national projects of a considerable scale have already started.

In particular, when considering blood transfusion systems from the global viewpoint, the lack and risk of blood transfusion is incomparably higher in developing countries compared with advanced countries. For this reason, demands for the development of safe, effective, and low cost blood substitutes are increasing. The materialization of blood substitutes that has no risk of infection or side effects during transfusion and is preservable for long periods would contribute not only to the advancement in blood projects and remedies in advanced countries but also to advances in medical and welfare activities in countries and areas where medical systems are yet to be organized. Moreover its repercussive effects would be immeasurable, such as innovations in remedies for cancer, strokes, and myocardial infarction.

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CHAPTER 14

Evaluation of the Oxygen Transporting Capability of Hemoglobin Vesicles

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Introduction

Enormous efforts have been made to develop red cell substitutes, especially those utilizing Hb to overcome problems associated with blood transfusions such as the necessity for blood typing, fear of infectious disease, alloimmunization, and graft-versus-host diseases, difficulties in storage, etc. [1–3]. Hemoglobin vesicles (HbV) or liposome-encapsulated hemoglobin (LEH) are red cell substitutes which have a cellular structure of phospholipid vesicles [4–7]. On the other hand, in acellular Hb solutions such as chemically modified Hb and recombinant Hb, clinical trials are underway at present. Though various modification has been made to overcome the issues of acellular Hb, inevitable difference between cellular red blood cell and acellular Hb has been discussed relating to irregular physiological responses such as vasoconstriction and autoregulation, Hb toxicity, and so on [8–11]. HbV with a cellular structure is expected to make solutions of those issues. In order to increase the oxygen carrying capability of HbV as an oxygen carrier, we have to consider the followings. (1) The regulation of oxygen affinity of HbV, (2) increase in the concentration ratio of Hb to total lipid components of the vesicles ([Hb]/[Lipid]), (3) chemical stabilization of an encapsulated Hb solution, namely the suppression of metHb formation (metHb has no ability to bind oxygen), and (4) high dispersibility in blood circulation. Those are summarized schematically in Fig. 1. In this chapter, the new preparation method which relates to those points, especially the construction of artificial metHb reduction system, and effect of polyethyleneglycol-modified HbV in blood circulation are described as well as in vivo evaluation of the efficacy of HbV.

New preparation method of HbV

We have been studying about how to encapsulate the concentrated Hb with thin lipid layers from the control of molecular assembling, because phospholipid vesicles with diameter larger than 0.1 μm are multilamellar vesicles and encapsulation of with

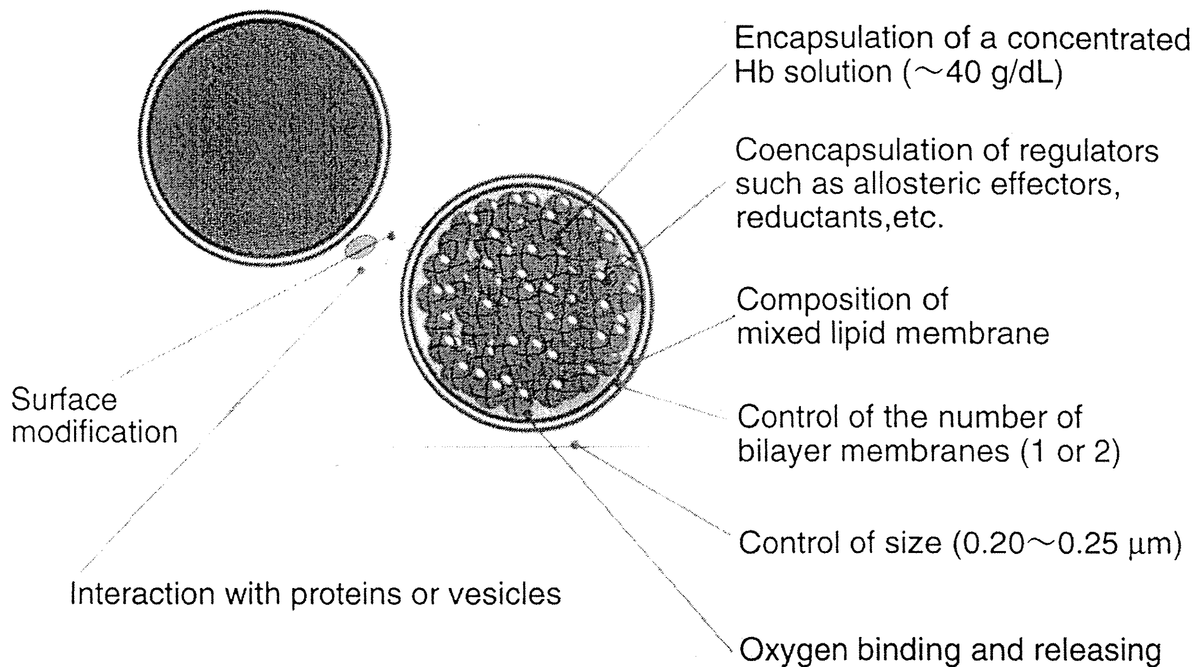

Fig. 1. Illustration of characteristics of Hb-vesicles.

concentration higher than 35 g/dl was difficult [12]. It was clarified that the pH value of Hb solutions influenced the properties of resulting HbV such as [Hb]/[Lipid] ratio, p_{50} value, and the rate of metHb formation as shown in Fig. 2 [13]. The [Hb]/[Lipid] of HbV showed the maximum value of 1.8 at pH 7.0, relating to the isoelectric point of Hb ($pI = 7.02$) at the preparation of HbV. Because the negative ζ-potential of Hb at the pH values higher than the pI causes the electrostatic repulsion between Hb molecules and the negative surface of the vesicle and results in the decrease of the [Hb]/[Lipid]. On the other hand, the lower pH reduces the negative surface potential of the vesicle and increases the number of bilayer membranes of HbV [12]. It is generally known that the metHb formation is facilitated at lower pH because of proton oxidation, however, higher pH increases the oxygen affinity to a higher value than that of RBC ($p_{50} = 27$ mmHg). Therefore, the pH value of the Hb solution should be adjusted to 7.0 in the preparation of HbV, and it is more desirable for the pH value to be adjusted to 7.4 where HbV functions as an oxygen carrier [13].

Generally, the pH control of the inner aqueous phase of HbV is difficult by simply changing the pH in the outer aqueous phase because the permeability of counter ions of H^+ and OH^- through the bilayer membrane is very low. Carbon dioxide decreases the pH of an aqueous solution in proportion to the partial pressure. From the pH measurement of the inner aqueous phase of vesicle, it was clarified that the pH change of the inner aqueous phase could be controlled by changing the partial pressure of carbon dioxide. Because a hemoglobin solution shows a buffer effect against the pH change, the relationship between pH and pCO_2 was obtained with various concentrations of Hb and various temperatures [13].

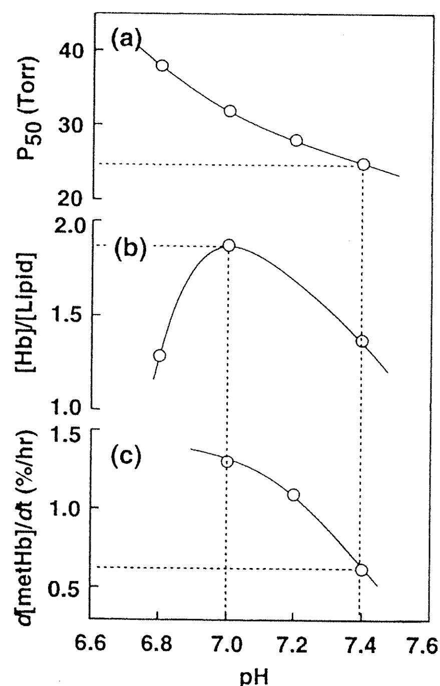

Fig. 2. Influence of the pH of Hb solutions at the preparation of HbV on (a) the oxygen affinity (p_{50}), (b) encapsulation efficiency ([Hb]/[Lipid]), and (c) the rate of metHb formation (d[metHb]/dt) of the resulting HbV.

The pH of the Hb solution used for the preparation of HbV was adjusted to 7.0 from 7.4 using N_2/CO_2 mixed gases, and HbV with a high [Hb]/[Lipid] was prepared. The pH of HbV was adjusted to 7.4 by removing the dissolved CO_2 under the reduced pressure. The resulting pH-controlled HbV with p_{50} of 27 mmHg showed a low rate of metHb formation.

Construction of artificial metHb reduction system

The common issue of Hb-based red cell substitutes is the relatively rapid metHb formation during storage or blood circulation [14,15] due to the absence of metHb reduction systems originally existing in a red blood cell. The systems include enzymatic reduction such as NADH-cytochrome b_5 and NADPH-flavin, direct reduction by glutathione (GSH) and ascorbic acid, and scavengers for active oxygen species such as superoxide disumutase (SOD) for $O_2^{\cdot -}$ and catalase for H_2O_2. HbO_2 dissociates into not only Hb and O_2, but also metHb and $O_2^{\cdot -}$. by one electron transfer [16–18]. When Hb is autoxidized to metHb, it loses its oxygen binding ability. However, the percentage of metHb in RBC is maintained at less than 0.5% of the total Hb by the systematic reduction.

Utilization of the activities of metHb reduction systems remaining in the RBC is one method to lower the metHb formation rate of encapsulated Hb [19]. However, their activity would change with the conditions of outdated red blood cell from which

hemolysate is prepared, the amounts of remaining substances, and encapsulation procedure using high shear stress. Virus inactivation using heat treatment is impossible in this case. Moreover, the mechanism of metHb reduction systems is complicated and influenced by many unknown factors. In our purification method of Hb with heat treatment, these enzymes and chemicals are denatured and then removed [20]. High purity of Hb is very important to prepare HbV containing highly concentrated Hb with high reproducibility and high efficiency. Therefore, the construction of a metHb reduction system by coencapsulation of an appropriate amount of reductants is required.

There are many reductants which can reduce the metHb to deoxyHb under anaerobic condition. However, a few of them can suppress the metHb formation of HbV under aerobic conditions; some enhance the metHb formation. This occurs because, at the beginning, there is a small percentage of metHb to be reduced in the HbV and encapsulated reductants are generally autoxidized faster than the rate of metHb formation under aerobic conditions. Furthermore, active oxygens generated from such autoxidation oxidize the Hb to metHb [21,22]. The coencapsulation effect of a series of thiols (cysteine, Cys; glutathione, GSH; homocysteine, Hcy; and acetylcysteine, Acy) was studied as reductants for HbV. Hcy and GSH showed a good suppressive effect on metHb formation, while Cys adversely accelerates the metHb formation at a rate twice that of the Hb solution without any reductants, and Acy showed no change [23,24]. At least two contributions should be considered to explain this result; one is a positive contribution of thiols to reduce the metHb; the other is a negative contribution by autoxidation of the thiols, which destroys their ability, and generated active oxygens enhance the metHb formation. The significant suppression of metHb formation of HbV by the coaddition of SOD and catalase with Cys indicates that Cys is easily oxidized by oxygen and simultaneously generates a large amount of active oxygens. On the other hand, Hcy and GSH showed the effective suppression of the metHb formation because their low rates of autoxidation exceed the low rate of metHb reduction. A suitable reductant should possess a low rate of autoxidation but a high efficiency of metHb reduction. This can be expressed from k_2app/k_1app (k_1app: the apparent rate constant of metHb reduction in anaerobic condition, k_2app: the apparent rate constant of thiol oxidation by oxygen), and its order is Acy $>$ Cys $>$ GSH $>$ Hcy. Therefore, we selected Hcy as a reductant in HbV to effectively suppress metHb formation.

It is a well-known phenomenon that there is a significant dependence of the rate of metHb formation on the oxygen partial pressure, the rate shows a maximum at a pO_2 around p_{50} of Hb, and superoxide anion generate during metHb formation [24,25]. The coencapsulation of Hcy with Hb resulted in a low rate of metHb formation in HbV (initial rate, 1%/h) in vitro at an oxygen partial pressure (pO_2) of 149 mmHg. The rate increased with decreasing pO_2, showing a maximum (2.2%/h) around $pO_2 = 23$ mmHg, and then decreased to 0%/h at 0 mmHg. At the pO_2 of 149 mmHg, the metHb formation is effectively suppressed by catalase, suggesting the generation of hydrogen peroxide from the autoxidation of Hcy, while at the pO_2