stroma-free $_S$Hb was collected in the permeate fraction. The stroma-free $_S$Hb was carbonylated and heated at 60 °C for 15 hours. During heating, denatured protein aggregated and floated in the solution. Centrifugation of the solution generated a precipitate of denatured proteins and a clear $_S$HbCO solution with the yield of 90%. Tangential flow ultrafiltration (cut-off Mw. 8 KDa) effectively concentrated the Hb solution to 42 g/dL and removed electrolytes.

After the addition of PLP to the concentrated $_S$Hb solution at different molar PLP/$_S$Hb ratio (0, 0.5, 1.0, and 2.5), it was encapsulated in phospholipid vesicles in the same manner as that described for $_H$Hb. The particle diameter was regulated well at around 250 nm, on average. The weight ratio of $_S$Hb to total lipid was 1.0–1.1.

Thermal stability of $_S$Hb

DSC thermograms of the $_S$Hb solutions showed the denaturation temperatures in the oxy, deoxy, and carbonyl states to be, 71, 81, and 83 °C, respectively (Figure 1). The deoxy $_S$Hb sample showed a smaller endothermal peak at around 70 °C, probably because of the presence of a small amount of $_S$HbO$_2$. The denaturation temperatures of $_S$Hbs resemble those of $_H$Hbs and $_B$Hbs (Table I).

Oxygen affinities of $_S$Hb and $_S$HbV

Figure 2 shows that P_{50} values for all samples increase as the molar ratio of PLP/$_S$Hb increases. It was suspected that Cl^- ions in PBS might affect oxygen affinity. Therefore, phosphate buffer (PB) without Cl^- was used as well to plot the oxygen equilibrium curves of $_H$Hb and $_S$Hb in the various PLP/$_S$Hb ratios using the Hemox analyzer. However, there was only a small difference in P_{50} values. Therefore,

Table I. Thermal stability of swine Hb ($_S$Hb) compared with those of human Hb ($_H$Hb) and bovine Hb ($_B$Hb).

	Denaturation temperature (°C)			
	HbO_2	HbCO	deoxyHb	References
$_S$Hb	71	83	81	This study
$_H$Hb	64	78	80	Sakai et al. (2002)
$_B$Hb	70	87	83	Sakai et al. (2002)

Cl^- ions do not affect the oxygen affinity of $_S$Hb solutions. Little difference was apparent in the oxygen affinity of $_S$Hb and $_H$Hb, for solutions in both PBS and PB. Without co-encapsulation of PLP, both $_S$HbV and $_H$HbV showed similar P_{50} at around 10 mmHg. With increasing amount of PLP, $_S$HbV has much higher P_{50} values (lower oxygen affinity) than the $_H$HbV solution. Hill numbers for the $_S$Hb solutions tended to decrease as the PLP/$_S$Hb ratio was increased from around 2.5 to around 2.0. The trend for Hill number values in $_S$HbV solutions differed: the Hill number increased instead as PLP/$_S$Hb ratio increases from 1.4 to 1.9.

Autoxidation rates of $_S$Hb and $_S$HbV

Figure 3 presents the time-dependent increases of metHb levels of $_S$HbO$_2$ dissolved in PBS at 37 °C in an aerobic condition in comparison to $_H$HbO$_2$. In the absence of any metHb reduction enzymatic system, the metHb level invariably increases. No significant difference was found between the two samples.

Figure 4 shows time-dependent increases of metHb levels of $_S$HbV dispersed in PBS at 37 °C in an aerobic condition in comparison to $_H$HbV. $_S$HbV showed slightly faster autoxidation rates at all PLP/$_S$Hb ratios.

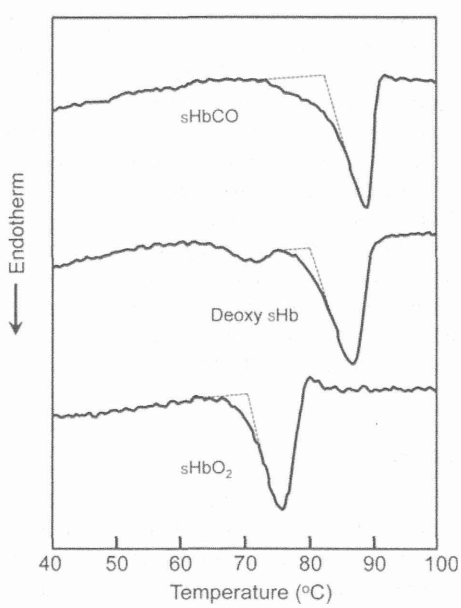

Figure 1. Calorimetric thermograms of $_S$Hbs in oxy, deoxy, and carbonyl forms. [Hb] = 10 g/dl, 60 µl, scanning rate = 1.0 °C/min.

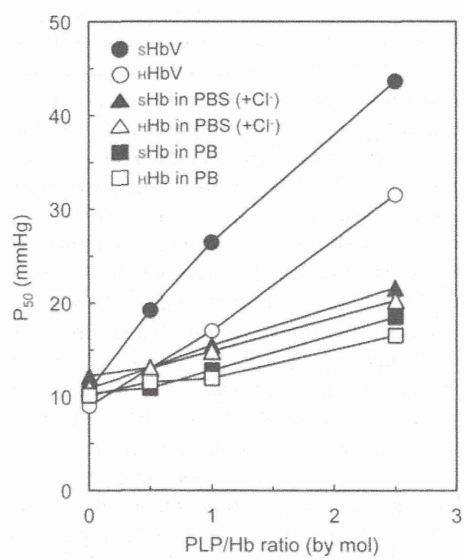

Figure 2. Oxygen affinities (P_{50} values) of $_S$Hb and $_H$Hb samples containing different amounts of pyridoxal 5′-phosphate (PLP), obtained from oxygen equilibrium curves measured using a Hemox analyzer at 37 °C. Phosphate buffered saline (PBS, pH 7.4) and phosphate buffer (PB, pH 7.4) were used for dissolving Hb to confirm the effect of Cl^- anion. HbV is dispersed in hemox buffer.

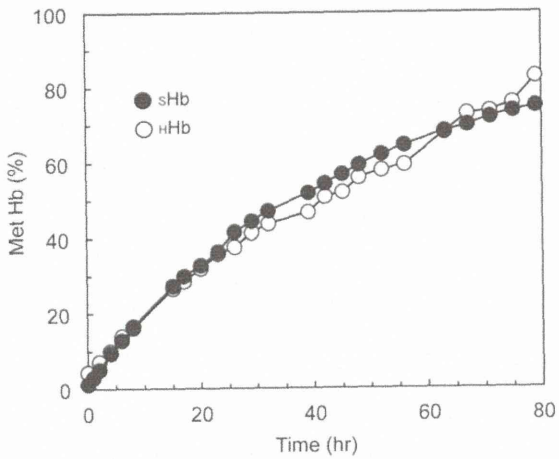

Figure 3. Autoxidation of $_{S}HbO_2$ in pH 7.4 PBS compared with that of $_{H}HbO_2$ in aerobic conditions at 37 °C.

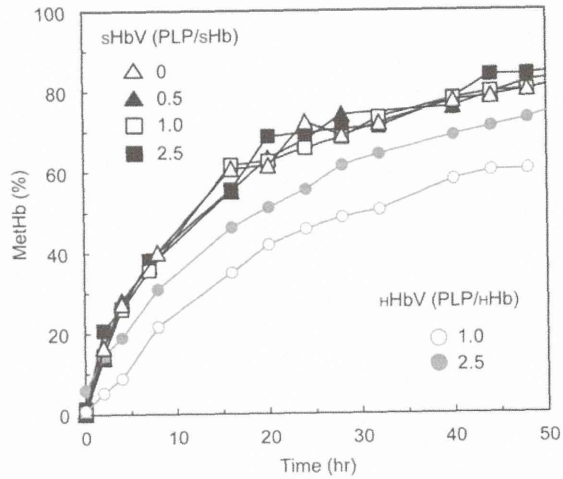

Figure 4. Autoxidation of $_{S}HbV$ in pH 7.4 PBS in comparison with $_{H}HbV$ in aerobic conditions at 37 °C.

Discussion

This study is our first preparation of Hb-vesicles by using $_{S}Hb$. The purification of $_{S}Hb$ from swine whole blood was performed effectively as we always do for human Hb ($_{H}Hb$), through stabilization by carbon monoxide (CO), pasteurization at 60 °C for 15 hours, and tangential flow ultrafiltration. Hb encapsulation was conducted in the same manner as that for $_{H}Hb$. Physicochemical characteristics of $_{S}HbV$ and $_{H}HbV$ are similar. But some differences are apparent in oxygen affinity and autoxidation rate between the two.

Both carbonyl and deoxy $_{S}Hb$ showed higher denaturation temperatures than in the oxygen bound state. This tendency is the same with $_{H}Hb$ and $_{B}Hb$. Even though oxy $_{S}Hb$ shows denaturation temperature above 60 °C, it autoxidizes to form metHb even at a lower temperature as shown in Figure 3. Actually, metHb is known to induce irreversible transformation of globin helix chains gradually. Both carbonylation and deoxygenation can prevent metHb formation effectively. This thermal stability guarantees that $_{S}HbCO$ can be heated at 60 °C for pasteurization without denaturation.

The oxygen affinities of $_{S}Hb$ in PBS (with Cl^-) and PB (without Cl^-) are similar. This tendency is similar to that of $_{H}Hb$, but not $_{B}Hb$. Reportedly $_{B}Hb$ is affected strongly by the addition of Cl^- (Fronticelli et al. 1984, Perutz et al. 1993). Mammalian Hbs are classified into two groups: those with intrinsically high O_2 affinity, and those with intrinsically low O_2 affinity (Perutz and Imai 1980). In fact, both $_{H}Hb$ and $_{S}Hb$ are examples of the former and $_{B}Hb$ of the latter group. Rodents, dogs, pigs, horses, camels, marsupials, and most primates belong to the former category. Cows, sheep, goats, deer, cats, and one primate, the lemur, belong to the latter. The high O_2 affinity of the former group is lowered in RBCs by 2,3-diphosphoglyceric acid (2,3-DPG). Therefore, $_{S}Hb$ should be mixed with pyridoxal 5'-phosphate (PLP), a substitute of 2,3-DPG.

Addition of PLP to $_{S}Hb$ before encapsulation decreases its oxygen affinity (increase P_{50} value) in the same manner as it does with $_{H}Hb$. However, after encapsulation in vesicles, $_{S}Hb$ showed much lower oxygen affinity (higher P_{50}) than $_{H}Hb$, which indicates that $_{S}Hb$ interacts with PLP more strongly than $_{H}Hb$ in a concentrated condition in the liposomal particle.

According to reports of Sinet et al. (1982) and Condo et al. (1992), $_{S}Hb$ has lower O_2 affinity (higher P_{50}) than $_{H}Hb$ has at a lower temperature, and the P_{50} of $_{S}Hb$ is less affected by temperature than $_{H}Hb$ is. These characteristics of $_{S}Hb$ are consistent with the well-known cold resistance of pigs. That presents one potential advantage of utilization of $_{S}Hb$ for a blood substitute over $_{H}Hb$ in terms of larger capacity of oxygen transport in hypothermic conditions.

Autoxidation rates of purified $_{S}HbO_2$ and $_{H}HbO_2$ are similar. However, after encapsulation in vesicles, $_{S}HbO_2$ showed higher rates than $_{H}HbO_2$. The reason remains unclear. In spite of the overall structural similarity of $_{S}Hb$ and $_{H}Hb$, small structural differences include (i) a large segmental shift of helix A of the β-subunit, (ii) slight uncoiling of the amino-terminal residues of helix B of the β-subunit and the carboxyl-terminal residue of helix E of the β-subunit, and (iii) small difference in loop regions connecting α-helices, and the eight subunit termini (Katz et al. 1994). Such a difference might affect the faster autoxidation rate, especially in a highly concentrated condition within vesicles. Autoxidation would be retarded by non-enzymatic and enzymatic methods which we reported elsewhere (Tsuchida et al. 2009).

Conclusion

Swine Hb can be purified effectively from whole blood through carbonylation, pasteurization, and tangential flow ultrafiltration. The concentrated swine Hb was encapsulated in phospholipid vesicles. Accordingly, our routine procedure to prepare HbV using human Hb is applicable to swine Hb. Despite these results, some differences exist in terms of

oxygen affinity and autoxidation rate, but sHb can be regarded as an alternative source for the production of Hb-vesicles as an artificial oxygen carrier.

Declaration of interest

Of the authors, H.S. is a holder of patents related to the production and usage of Hb-vesicles. The authors alone are responsible for the content and writing of the article.

This study was supported in part by Health and Labour Sciences Research Grants (Health Science Research Including Drug Innovation) from the Ministry of Health, Labour and Welfare, Japan, and a Supporting Project to Form Strategic Research Platform for Private Universities: Matching Fund Subsidy from Ministry of Education, Culture, Sports, Science and Technology, Japan.

References

Barnikol WKR, Burkhard O, Poetzschke H, Domack U, Dinkelmann S, Guth S, et al. 2002. New artificial oxygen carriers made of pegylated polymerized pyridoxalated porcine haemoglobin. Comp Biochem Physiol Part A. 132:185–191.

Chang TMS. 2006. Evolution of artificial cells using nanobiotechnology of hemoglobin based RBC blood substitute as an example. Artif Cells Blood Substit Immobil Biotechnol. 34:551–566.

Chang W, Chang Y, Chen Y, Sung H. 2004. Hemoglobin polymerized with a naturally occurring crosslinking agent as a blood substitute: In vivo and in vitro studies. Artif Cells Blood Substitutes Biotechnol. 32:243–262.

Condo SG, Corda M, Sanna MT, Pellegrini MG, Ruiz MP, Castagnola M, et al. 1992. Molecular basis of low-temperature sensitivity in pig hemoglobin. Eur J Biochem. 209:773–776.

Fronticelli C, Bucci E, Orth C. 1984. Solvent regulation of oxygen affinity in hemoglobin, Sensitivity of bovine hemoglobin to chloride ions. J Biol Chem. 259:10841–10844.

Hamada K, Kose T, Ohgushi T, Sakai H, Takeoka S, Nishide H, et al. 1995. Spectroscopic assay system for the determination of red cell substitutes (NRC). Artif Blood. 3:96–101 (in Japanese).

Katz DS, White SP, Huang W, Kumar R, Christanson DW. 1994. Structure determination of aquomet porcine hemoglobin at 2.8 A resolution. J Mol Biol. 244:541–553.

Lee CJ, Kan P, Chen WK. 1992. The purification and comparative analysis of hemoglobin from animal bloods. Biomater Artif Cells Immobilization Biotechnol. 20:477–488.

Matsubara T. 1987. Spectrophotometric determination of hemoglobin in blood. Tanpakushisu-kakusan-koso (Protein, Nucleic Acid and Enzyme). 32:671–674 (in Japanese).

Natanson C, Kern SJ, Lurie P, Banks SM, Wolfe SM. 2008. Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death: a meta-analysis. JAMA. 299: 2304–2312.

Olson JS, Foley EW, Rogge C, Tsai AL, Doyle MP, Lemon DD, et al. 2004. No scavenging and the hypertensive effect of hemoglobin-based blood substitutes. Free Radic Biol Med. 36:685–697.

Perutz MF, Imai K. 1980. Regulation of oxygen affinity of mammalian haemoglobins. J Mol Biol. 136:183–191.

Perutz MF, Fermi G, Poyart C, Pagnier J, Kister J. 1993. A novel allosteric mechanism in haemoglobin: structure of bovine deoxyhaemoglobin, absence of specific chloride-binding sites and origin of the chloride-linked Bohr effectin bovine and human haemoglobin. J Mol Biol. 233:536–545.

Sakai H, Hara H, Yuasa M, Tsai AG, Takeoka S, Tsuchida E, Intaglietta M. 2000. Molecular dimensions of Hb-based O2 carriers determine constriction of resistance arteries and hypertension. Am J Physiol Heart Circ Physiol. 279:H908–H915.

Sakai H, Masada Y, Takeoka S, Tsuchida E. 2002. Characteristics of bovine hemoglobin as a potential source of hemoglobin-vesicles for an artificial oxygen carrier. J Biochem. 131:611–617.

Sakai H, Sou K, Tsuchida E. 2009. Hemoglobin-vesicles as an artificial oxygen carrier. Methods Enzymol. 465:363–384.

Sakai H, Sato A, Masuda K, Takeoka S, Tsuchida E. 2008. Encapsulation of concentrated hemoglobin solution in phospholipid vesicles retards the reaction with NO, but not CO, by intracellular diffusion barrier. J Biol Chem. 283:1508–1517.

Sinet M, Bohn B, Guesnon P, Poyart C. 1982. Temperature independence of the alkaline Bohr effect in pig red cells and pig hemoglobin solutions. Biochim Biophys Acta. 708:105–111.

Tsuchida E, Sou K, Nakagawa A, Sakai H, Komatsu T, Kobayashi K. 2009. Artificial oxygen carriers, hemoglobin vesicles and albumin-hemes, based on bioconjugate chemistry. Bioconjug Chem. 20:1419–1440.

Yu B, Shahid M, Egorina EM, Sovershaev MA, Raher MJ, Lei C, et al. 2010. Endothelial dysfunction enhances vasoconstriction due to scavenging of nitric oxide by a hemoglobin-based oxygen carrier. Anesthesiology. 112:586–594.

Zhang W, Yan K, Dai P, Tian J, Zhu H, Chen C. 2011. A novel hemoglobin-based oxygen carrier, polymerized porcine hemoglobin, inhibits H_2O_2-induced cytotoxicity of endothelial cells. Artif Organs. 36:151–160.

Present Situation of the Development of Cellular-Type Hemoglobin-Based Oxygen Carrier (Hemoglobin-Vesicles)

Hiromi Sakai*[,1,2]

[1]*Waseda Bioscience Research Institute in Singapore (WABIOS), 11 Biopolis Way, #05-01/02 Helios, Singapore 138667, Republic of Singapore;* [2]*Organization for University Research Initiatives, Waseda University, Tokyo 162-0041, Japan*

Abstract: Many researchers have tested a hemoglobin (Hb) solution as a possible oxygen carrier after discovering that one Hb molecule contains four hemes that bind and release oxygen reversibly, and that blood type antigens are expressed on the surface of red blood cells (RBCs). However, various side effects emerged during the long development of Hb-based oxygen carriers (HBOCs). The physiological significance of the RBC structure is undergoing reconsideration. Fundamentally, excessive native Hb molecules are toxic, but encapsulation can shield this toxic effect. So-called liposome-encapsulated Hb or Hb-vesicles that mimic the cellular structure of RBCs have been developed for clinical applications.

Keywords: Artificial oxygen carriers, fluid resuscitation, hemoglobin, liposome, transfusion alternative.

1. CHEMICALLY MODIFIED HB AND ENCAPSULATED HB

The most abundant protein in blood is hemoglobin (Hb). The second is albumin. Hb is the oxygen binding protein that is compartmentalized in red blood cells (RBCs) with an intracellular Hb concentration of about 35 g/dL. Albumin is the major plasma protein dissolved in blood plasma. Albumin plays the important role of equilibrating the colloid osmotic pressure between blood and tissue interstitium, maintaining the circulating blood volume. Packed RBCs derived from blood donation can be stored for only 3 weeks in Japan. However, albumin is extremely stable against pasteurization at 60°C and for storage at room temperature for years. Albumin solution is routinely used in clinical settings. Quite recently, recombinant albumin was first approved for clinical use in Japan [1]. Historically, a crude Hb solution was tested as a substitute for RBCs in 1898 [2]. However, it was not successful because of various side effects. Since the 1960s, chemically modified Hb solutions have been developed. Some have progressed to clinical studies [3]. Recombinant human Hb was also tested [4]. Actually, an earthworm, as a lower organism has no RBCs, but it does have gigantic Hb molecules. Mammalians, as higher animals, have RBCs for several physiological reasons (see below, i – vi). In fact, it is not easy to create an RBC substitute with cell-free Hb solutions; although the most abundant protein in blood is Hb, it becomes toxic once released from RBCs.

A "FDA Workshop on Hemoglobin Based Oxygen Carrier" was held in Bethesda on April 29–30, 2008. The main topic was meta-analysis of clinical studies of cell-free Hb-based oxygen carriers (HBOCs) being conducted in US and Europe [5]. The examined HBOCs were from five companies, including intramolecularly crosslinked Hb, glutaraldehyde- or *o*-raffinose-polymerized human- or bovine-Hbs, and polyethylene glycol (PEG) conjugated Hbs. These molecules were designed initially to avoid the side effects of Hb molecules by increasing the molecular size, reducing extravasation, increasing the retention time in blood, and adjusting oxygen affinity. Meta-analysis of the 13 published papers and 3 other announcements of results from 3711 recipients surprisingly revealed that the treated group receiving HBOCs showed higher incidence of death and myocardial infarction than the control group receiving conventional treatment. Questions arouse whether preclinical animal experiments were insufficient, and ICH guidelines [6] are inappropriate for safety evaluation of HBOCs: companies do not disclose all clinical data and the FDA cannot collect all clinical data; possibility of methodological flaws of the analysis; clinical studies were suspended to minimize victims during clinical studies; etc. In 1990, because of severe side effects, Baxter suspended research and development of intramolecularly crosslinked Hb after spending a large budget for phase III clinical trials [7]. At that time, the researchers believed that an enlarged Hb by polymerization or polymer conjugation might solve the problems. However, polymerization or conjugation was not sufficient to eliminate all the side effects.

In fact, we believe in the physiological importance of the cellular structure of RBCs, and continue to develop Hb-vesicles (HbV) as a cellular-type HBOC [8, 9] (Fig. 1). By considering the physiological importance of RBCs, it is easy to understand the side effects of cell-free HBOCs. An RBC has a biconcave disk structure with 8 μm long-axis diameter, encapsulating about 2 million Hb molecules (Mw. 64500) at a concentration of about 35 g/dL. The physiological reasons for Hb compartmentalization in RBCs are i) shielding direct contact of toxic Hb and vasculature, ii) prevention of ex-

*Address correspondence to this author at the Waseda Bioscience Research Institute in Singapore, 11 Biopolis Way, #05-01/02 Helios, Singapore 138667, Republic of Singapore: Tel: +65-6478-9721; Fax: +65-6478-9416; E-mail: hirosakai@aoni.waseda.jp

1875-6220/12 $58.00+.00

Table 1. Similarities and Differences of RBC and HbV

Similarities:
Both RBC and HbV....
1. encapsulate concentrated Hb solution (35 g/dL).
2. co-encapsulate functional molecules such as allosteric effectors.
3. retard O_2-release and NO-binding and CO-binding of Hb.
4. have no colloid osmotic pressure.
5. are non-Newtonian viscous fluids.
6. do not induce vasoconstriction.
7. are finally captured and degraded in RES.
Differences
HbV is....
1. free from blood-type antigens and pathogens.
2. more stable than RBC (storable for two years at room temp.).
3. smaller than RBC and distributed homogeneously in plasma.
4. a versatile tool for clinical use by manipulating physical properties.

travasation of dissociated Hb dimers through renal glomeruli, and prolongation circulation time; iii) circumvention of high colloid osmotic pressure and viscosity of concentrated Hb solution; iv) co-encapsulation of electrolytes, ATP, glycolytic, and metHb reducing enzymatic systems, etc; v) reduced reaction of Hb with NO and CO as vasorelaxation factors [10, 11]; vi) Moreover, a high viscosity of blood is mainly attributable to the presence of RBCs, showing non-Newtonian fluid, which is important for blood circulation, especially in microcirculation from a physiological perspective (Table **1**).

Chang (McGill University) was the first to test encapsulation of Hb solution with a polymer membrane in 1957 [12] as one example of "artificial cells". In Japan, Kimoto *et al.* tested Hb encapsulation using polystyrene or rubber membranes in 1967 [13]. Even though their attempt was quite original, they were unsuccessful: the particle size could not be reduced to less than capillary diameter (< 4 μm). In 1964, Bangham and Horne discovered the formation of vesicles (liposomes) when phospholipid was dispersed in aqueous phase [14]. After this discovery, many researchers tested encapsulation of functional molecules in liposomes. In 1977,

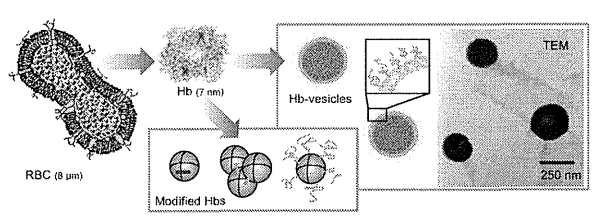

Fig. (1). Hb-based oxygen carriers (HBOCs). Hb solution is purified from outdated Hb; a concentrated Hb solution is encapsulated in phospholipid vesicles to produce Hb-vesicles. Chemically modified cell-free HBOCs such as crosslinked Hb, polymerized Hb, and PEG-conjugated Hb have been tested for clinical use in the US and Europe.

Djorjevici and Miller (University of Illinois, Chicago) reported encapsulation of Hb in liposomes, called "synthetic erythrocytes" [15]. Subsequently, many groups throughout the world attempted so-called liposome encapsulated Hb (LEH). However, those efforts were not successful because of low encapsulation efficiency, polydispersibility of particle size, and instability. The US Naval Research Laboratory aggressively developed freeze-dried powder of LEH. However, the laboratory terminated its development in the late 1990s [16].

Our group of polymer chemistry have worked to improve the encapsulation efficiency and particle size distribution from the viewpoint of molecular assembly by regulating the electrostatic and hydrophobic interactions between the components (Hb and lipids) [17]. The resulting hemoglobin-vesicles (HbV) encapsulate nearly 30,000 Hb molecules (35 g/dL Hb solution) within a 5 nm thin lipid membrane. The selection of lipids was also important for stability and biocompatibility. The starting material, Hb solution, is purified from outdated NAT-inspected red blood cells provided by the Japanese Red Cross. Bovine Hb is also available for preparation of HbV [18]. Carbonylation of Hb (HbCO) prevents metHb formation and denaturation of Hb, and enables pasteurization at 60°C for 12 hr, thereby ensuring the utmost safety for infection. HbCO encapsulated in HbV can be easily converted to HbO_2 by photodissociation using illumination of visible light under O_2 atmosphere. We formerly used polymerizable phospholipids to stabilize the resulting encapsulated Hb because it was believed that liposome had a fragile structure. However, the problem was that the polymerized liposome was so stable that it was not degraded and it remained in the liver and spleen after intravenous administration into rats. Now we use other combination of conventional phospholipid, cholesterol, negatively charged synthetic lipid [19], and PEG-conjugated phospholipid. The resulting liposome sufficiently prevents aggregation. Complete deoxygenation of the HbV suspension enables long-term storage for years at room temperature [20]. It can be said that outdated biological red cells are converted to "artificial red cells" as one "material".

One of the important physiological aspects of HbV is that it does not seem to induce vasoconstriction. Physiochemical analysis of NO reactions with HbV clarified that intracellular diffusion barrier of NO is induced by encapsulation of a concentrated Hb solution [10]. Moreover, a larger particle shows a slower lateral diffusion in an arteriole that retards the gas reaction at a vascular wall [11]. In addition, the larger particle size prevents the penetration across the perforated endothelium to approach to a space between the endothelium and the smooth muscle where NO is produced to bind to soluble guanylate cyclase. HbV does not induce hemolysis in blood circulation but it is captured by macrophages, degraded, and the components are excreted safely through urine and faces [21].

2. OXYGEN TRANSPORTING CAPACITY OF HbV AS A TRANSFUSION ALTERNATIVE

One important point when the HbV fluid is used as a transfusion alternative is that, like RBCs, the fluid shows no colloid osmotic pressure (COP) [22]. Therefore, a large dos-

age of HbV resulting in a high level of blood substitution requires the addition or co-injection of a plasma expander. For example, when HbV particles are suspended in 5%-recombinant human serum albumin (rHSA), the resulting suspension shows COP of 20 Torr and viscosity was nearly identical to that of blood (3–4 cP). These properties are important for the homeostasis of blood circulation. The small HbV particles (250–280 nm) are dispersed homogeneously in plasma phase. Co-encapsulation of an allosteric effector, such as pyridoxal 5'-phosphate (PLP) can regulate the oxygen affinity of HbV with a sigmoidal curve that closely resembles that of human blood. Ninety percent of blood exchange with HbV suspended in albumin can sustain hemodynamic and blood gas parameters and tissue oxygen tension in a rat model [23]. Moreover, a clinically relevant model of 40% blood exchange revealed that the reduced hematocrit returned to the original level in one week, and HbV captured in reticuloendothelial system (RES) was completely degraded and excreted promptly [24], indicating that HbV is useful for preoperative blood exchange or perioperative infusion in the event of hemorrhage to prevent or minimize homologous blood transfusion [25] (Fig. **2**). Although miniaturization of the cardiopulmonary bypass (CPB) circuit has reduced the priming volume, it remains insufficiently low to achieve an acceptable level of hemodilution in small patients. Homologous blood use is considered the gold standard for CPB priming in infants despite exposure of patients to potential cellular and humoral antigens. A recent experimental study of HbV suspended in rHSA as a priming solution for CPB in a rat model demonstrated that HbV protects neurocognitive function by transporting O_2 to brain tissues, even when the hematocrit is markedly reduced [26]. Results indicate that the use of HbV for CPB priming might prevent neurocognitive decline in infants caused by considerable hemodilution.

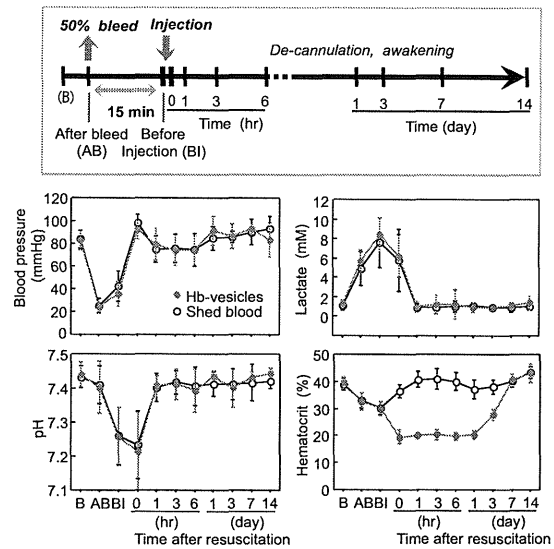

Fig. (2). Fluid resuscitation from hemorrhagic shock with hemoglobin-vesicles suspended in recombinant human serum albumin. Shock was induced by withdrawing 50% of circulating blood volume, and Hb-vesicles or shed autologous blood was injected isovolemically. Fundamentally, the resuscitative effect was almost identical, and the reduced hematocrit returned to the original level in one week.

3. OTHER POTENTIAL CLINICAL APPLICATIONS USING HbV

An important advantage of Hb-vesicles is that the physicochemical properties of HbV are adjustable, such as oxygen affinity (P_{50}, oxygen partial pressure at which Hb is half saturated with oxygen) and rheological properties. Historically, it has been widely believed that the O_2 affinity of red cell substitutes should be similar to that of RBC (25–30 Torr) using an allosteric effector or by direct chemical modification of the Hb molecules (Fig. **3**). Theoretically, this enables sufficient O_2 unloading during blood microcirculation, as can be inferred from the arterio-venous difference in the levels of O_2 saturation in accordance to an O_2 equilibrium curve. It has been expected that decreasing the O_2 affinity (increasing P_{50}) increases O_2 unloading. Regarding blood viscosity, lowered viscosity is believed to increase cardiac output and facilitate peripheral blood flow. However, this conventional assumption is being debated in the field of blood substitute research [27]. The suspension of HbV can provide unique opportunities to modify these physicochemical properties easily and to observe their physiological impacts [28].

Blood flow is much lower in ischemic tissues. Consequently, O_2 tension is very low: e.g. 5 Torr. Normal RBCs are expected to have already released O_2 before they reach the ischemic tissue. The left-shifted curve (lower P_{50}) shows that Hb does not release O_2, even in the venous side in a normal condition. However, RBC or an HBOC with a P_{50} lower than usual can carry O_2 to an ischemic tissue; so-called "Targeted Oxygen Delivery" [29]. Dr. Erni and colleagues at Inselspital Hospital of the University of Berne developed a hamster skin flap model in which the blood flow of one branch is blocked completely; the tissue becomes completely ischemic. Exchange transfusion was performed using low and high P_{50}-HbV, which revealed improved oxygenation of the ischemic part, especially with the low P_{50}-HbV [30, 31]. Collateral blood flow is expected to occur even to the ischemic part; the HbV conveys O_2 to the ischemic part via the collateral arteries. This is the first reported example demonstrating the effectiveness of HbV for an ischemic tissue, implying its applicability for other ischemic diseases [32]. In addition to the lower P_{50}, the viscosity of the HbV suspension is expected to contribute to improvement of microcirculation. The combination of HbV and dextran solution or hydroxyethyl starch solution induces flocculation of HbV, thereby rendering the suspension a non-Newtonian viscous fluid [33, 34]. The higher viscosity of the circulation fluid would increase shear stress on the vascular wall, thereby inducing vasorelaxation. A viscous fluid also pressurizes the capillaries homogeneously to improve the functional capillary density, which is beneficial for microvascular perfusion in normal cardiovascular function. But it is not known the same advantages are expected in diseased or damaged/compromised cardiovascular system.

Even though an important organ transplant law went into effect in 1997 in Japan, organ transplants are expected to

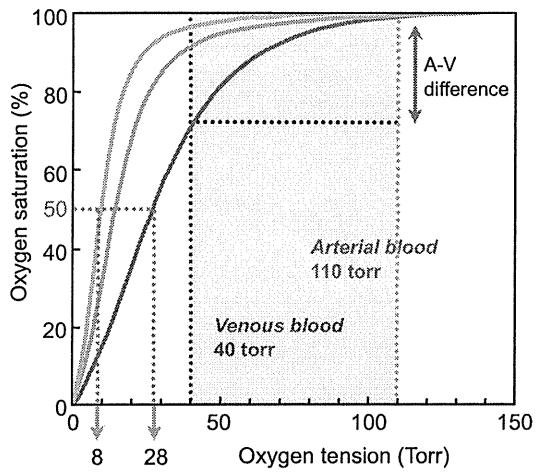

Fig. (3). Oxygen dissociation curves of Hb-vesicles. The rightmost curve shows Hb-vesicles used in the animal experiments in Figure 2. Oxygen affinity (P_{50}; the oxygen tension at which Hb is half saturated with oxygen) was 28 Torr, which is identical to that of human blood. P_{50} can be regulated easily by the addition of an allosteric effector. The leftmost curve is of Hb-vesicles without an allosteric effector, with P_{50} of about 8 Torr. In this case, Hb-vesicles would not release oxygen in a normal tissue, but can carry oxygen to an ischemic tissue and release oxygen for targeted oxygen delivery.

occur much less frequently than in the United States or Europe, probably because of the ethical and religious controversy related to the criteria for diagnosis of brain death. However, it is expected to be accepted eventually in Japan with the prevailing view of the need for transplantation and increased possession of organ donor cards. In a clinical setting of transplantation, its success is dependent in part on the prevention of ischemia-reperfusion injury after transplantation by an improved preservation condition. The representative organ preservation fluid is the University of Wisconsin (UW) solution, which comprises not only crystalloids, but also a plasma expander. The two-layer method is to dip the dissected organ at the interface of the UW solution and a perfluorocarbon (PFC) solution. Oxygen diffuses from the PFC to the organ, and the transport of nutrients and metabolites takes place through contact with the UW solution. It is speculated that there should be a limitation of the distance for diffusion of oxygen, carbon dioxide, and small molecules to maintain survival of the tissues. One idea is to use HbV as an intra-arterial perfusion fluid to carry oxygen, nutrients, and metabolites. Actually, we tested perfusion of the liver, heart, and intestine with HbV, although the purpose of those studies was to clarify the safety to these organs [35]. We confirmed the preservation of organ functions for a few hours. Our next step will be to prolong the perfusion period to the greatest extent possible. In fact, HbV can be re-oxygenated easily by perfusion through an artificial lung device. We must design the composition of HbV suspension to provide not only oxygen but also nutrients and homogeneous fluid distribution to all capillaries, which would presumably require a certain level of viscosity.

Tissue reconstruction and tissue regeneration have become popular. Cell culture requires not only a supply of oxygen and nutrition but also the removal of metabolites, which can be achieved by replacing the culturing media periodically in the case of a two-dimensional cell culturing. However, in the case of constructing a three-dimensional bulky tissue, it would require perfusion with a fluid that can serve the functions of blood in addition to angiogenesis in a regenerating tissue on a scaffold. Such functionality would necessitate the design of the composition of HbV described above. Consequently, HbV can provide unique opportunities to manipulate physicochemical properties that cannot be provided by RBCs.

4. RESEARCH AND DEVELOPMENT OF ARTIFICIAL OXYGEN CARRIERS IN JAPAN

In the 1950s, Minoshima of Hokkaido University studied "artificial blood" comprising electrolytes, colloids, and an artificial oxygen carrier of cobalt-histidine complex [36]. After the report of Clark showing a rat breathing in a perfluorocarbon liquid, a Japanese company, Green Cross, developed emulsified perfluorocarbon (Fluosol-DA). This was the first artificial oxygen carrier approved by FDA for clinical use in 1990 (although the production was terminated in 1993 because of insufficient efficacy). Ajinomoto Corp. developed PEGylated pyridoxalated Hb, which was the first HBOC approved for phase study in US in 1992 [37]. Totally synthetic heme derivatives, lipid-heme vesicles and albumin hemes, are also unique products created in Japan [9].

In fact, since around 1985, the Ministry of Health and Welfare (MHW) of Japan has supported the research of regulation of blood products (Principal investigator, Dr. Matsuhashi), where Ajinomoto's product was evaluated from the perspective of recycling outdated blood. Dr. Sekiguchi of Hokkaido Red Cross Blood Center studied pyridoxalated polymerized Hb. In 1993, a survey group of blood substitutes was organized (Principal Investigator, Dr. Sekiguchi) with support of MHW [38]. From 1997, MHW has strongly supported the research and development of blood substitutes (Health Sciences Research Grant, Artificial Blood Project) that include projects of artificial red cells, artificial platelet, artificial globulin, erythropoietic stem cells, and recombinant proteins. Since then, cellular-type Hb-vesicle (HbV) development has progressed strongly. When Japan experienced HIV-tainted-blood scandal and the disaster of the Great Hanshin-Awaji Earthquake, the government began promoting R&D of "Artificial Blood" aiming at its mass production, which is clearly stated in The Revised Pharmaceutical Affairs Act of 2002.

The development of HbV as a cellular type HBOC lags far behind that of cell-free HBOCs. Nevertheless, it is quite noteworthy that the side effects of molecular Hb and physiological importance of the cellular structure of RBCs have been recognized through R&D of artificial oxygen carriers. The Japanese Red Cross works to minimize the amount of outdated blood. However, outdated blood will never disappear. Outdated packed RBCs are a source of HbV, and strong governmental support will be necessary to establish

systems to collect such outdated RBCs efficiently throughout the country. The main difference between the conventional liposomal drug delivery system and HbV is the dosage. In fact, HbV is categorized as a new drug, and its safety must be scrutinized carefully and guaranteed by an appropriate method for its realization. The Society of Blood Substitutes, Japan (SBSJ) published guidelines for production and safety issues on artificial red cells [39]. Because of the current depression of economy and concerns on developing new drugs especially categorized as a biologic drug, Japanese pharmaceutical companies seem reluctant to develop artificial red cells. Relatively complicated production procedure of HbV in comparison with the cell-free HBOCs requires a longer time for the establishment of GMP (Good Manufacturing Practice) production method based on the experiences of laboratories. Moreover, a significant attention is paid to the safety concerns related to the large dosage of lipids of HbV. In fact, we are clarifying improved biocompatibility of HbV (less complement activation) [40], transient immunosuppressive effect [41], degradation and excretion of the components of HbV in diseased rodent models [42]. Further study is necessary using other animal species to clarify the safety of HbV before starting clinical stuies. Despite some failures for industrialization, the academic consortium in Japan is continuing research with a strong will and support from the government, aimed at eventual realization of artificial oxygen carriers that will eventually benefit human health and welfare.

ABBREVIATIONS

COP	=	Colloid osmotic pressure
CPB	=	Cardiopulmonary bypass P_{50}, oxygen partial pressure at which Hb is half saturated with oxygen
GMP	=	Good manufacturing practice
Hb	=	Hemoglobin
HBOCs	=	Hb based oxygen carriers
HbV	=	Hemoglobin-vesicles
LEH	=	Liposome encapsulated Hb
MHW	=	Ministry of Health and Welfare
PEG	=	Polyethylene glycol
PFC	=	Perfluorocarbon
PLP	=	Pyridoxal 5'-phosphate
RBC	=	Red blood cells
RES	=	Reticuloendothelial system
SBSJ	=	The Society of Blood Substitutes, Japan
UW	=	University of Wisconsin

CONFLICT OF INTEREST

Declared none.

ACKNOWLEDGEMENTS

The author greatly appreciates Emeritus Professor Eishun Tsuchida, Waseda University, for his support for the Project of Oxygen Infusion. Research of Hb-vesicles has been conducted by an academic consortium comprising many domestic and overseas research institutes. The author acknowledges the contribution of the collaborators. This research has been supported by Health and Sciences Grants from Ministry of Health Labour and Welfare, Japan; and a Grant in Aid for Scientific Research from Japan Society for the promotion of Sciences (JSPS).

DISCLOSURE

Hiromi Sakai is an inventor holding some patents related to the production and utilization of Hb-vesicles. Part of information included in this article has been previously published in IFMBE Proceedings, 2011, Volume 35, Part 19, 845-848.

REFERENCES

[1] Kobayashi K. Summary of recombinant human serum albumin development. Biologicals 2006; 34: 55-9.
[2] Von Stark G. Die resorborbarkeirt des haimatins und die bedeutungder hemoglobin-preparate. Dtsche Med Wochenschr 1898; 24: 805-8.
[3] Chang TM. Hemoglobin-based red blood cell substitutes. Artif Organs 2004; 28: 789-94.
[4] Murray JA, Ledlow A, Launspach J, Evans D, Loveday M, Conklin JL. The effects of recombinant human hemoglobin on esophageal motor functions in humans. Gastroenterology 1995; 109: 1241-8.
[5] Natanson C, Kern SJ, Lurie P, Banks SM, Wolfe SM. Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death: a meta-analysis. JAMA 2008; 299: 2304-12.
[6] FDA: Single dose acute toxicity testing for pharmaceutical; Revised guidance; Availability; Notice. Federal register, Aug. 26:43933-43935, 1996,
[7] Burhop K, Gordon D, Estep T. Review of hemoglobin-induced myocardial lesions. Artif Cells Blood Substit Immobil Biotechnol 2004; 32: 353-74.
[8] Sakai H, Sou K, Horinouchi H, Kobayashi K, Tsuchida E. Haemoglobin-vesicles as artificial oxygen carriers: present situation and future visions. J Intern Med 2008; 263: 4-15.
[9] Tsuchida E, Sou K, Nakagawa A, Sakai H, Komatsu T, Kobayashi K. Artificial Oxygen Carriers, Hemoglobin Vesicles and Albumin-Hemes, Based on Bioconjugate Chemistry. Bioconjug Chem 2009; 20: 1419-40.
[10] Sakai H, Sato A, Masuda K, Takeoka S, Tsuchida E. Encapsulation of concentrated hemoglobin solution in phospholipid vesicles retards the reaction with NO, but not CO, by intracellular diffusion barrier. J Biol Chem 2008; 283: 1508-17.
[11] Sakai H, Okuda N, Sato A, Yamaue T, Takeoka S, Tsuchida E. Hemoglobin encapsulation in vesicles retards NO and CO binding and O_2 release when perfused through narrow gas-permeable tubes. Am J Physiol Heart Circ Physiol 2010; 298: H956-65.
[12] Chang TM. "Artificial Cells" World Scientific Publishing Co. Pte. Ltd. Singapore 2007.
[13] Kimoto S, Hori M, Toyota T, Sekiguchi W. Artificial red cells. Gekachiryo 1968; 19: 324-32 (in Japanese).
[14] Bangham AD, Horne RW. Negative Staining of Phospholipids and their Structural Modification by Surface-Active Agents as Observed in the Electron Microscope. J Mol Biol 1964; 8: 660-8.
[15] Djordjevich L, Mayoral J, Miller IF, Ivankovich AD. Cardiorespiratory effects of exchange transfusions with synthetic erythrocytes in rats. Crit Care Med 1987; 15: 318-23.
[16] Flower R, Rudolph AS. Effects of free and liposome-encapsulated hemoglobin on choroidal vascular plexus blood flow, using the rabbit eye as a model system. Eur J Ophthalmol 1999; 9: 103-14.
[17] Sakai H, Sou K, Tsuchida E. Hemoglobin-vesicles as an artificial oxygen carrier. Methods Enzymol 2009; 465: 363-84.

[18] Sakai H, Masada Y, Takeoka S, Tsuchida E. Characteristics of bovine hemoglobin as a potential source of hemoglobin-vesicles for an artificial oxygen carrier. J Biochem 2002; 131: 611-7.

[19] Sou K, Tsuchida E. Electrostatic interactions and complement activation on the surface of phospholipid vesicle containing acidic lipids: effect of the structure of acidic groups. Biochim Biophys Acta 2008; 1778: 1035-41.

[20] Sakai H, Tomiyama KI, Sou K, Takeoka S, Tsuchida E. Poly(ethylene glycol)-conjugation and deoxygenation enable long-term preservation of hemoglobin-vesicles as oxygen carriers in a liquid state. Bioconjug Chem 2000; 11: 425-32.

[21] Taguchi K, Urata Y, Anraku M, *et al.* Pharmacokinetic study of enclosed hemoglobin and outer lipid component after the administration of hemoglobin vesicles as an artificial oxygen carrier. Drug Metab Dispos 2009; 37: 1456-63.

[22] Sakai H, Yuasa M, Onuma H, Takeoka S, Tsuchida E. Synthesis and physicochemical characterization of a series of hemoglobin-based oxygen carriers: objective comparison between cellular and acellular types. Bioconjug Chem 2000; 11: 56-64.

[23] Sakai H, Takeoka S, Park SI, *et al.* Surface modification of hemoglobin vesicles with poly(ethylene glycol) and effects on aggregation, viscosity, and blood flow during 90% exchange transfusion in anesthetized rats. Bioconjug Chem 1997; 8: 23-30.

[24] Sakai H, Horinouchi H, Yamamoto M, *et al.* Acute 40 percent exchange-transfusion with hemoglobin-vesicles (HbV) suspended in recombinant human serum albumin solution: degradation of HbV and erythropoiesis in a rat spleen for 2 weeks. Transfusion 2006; 46: 339-47.

[25] Sakai H, Seishi Y, Obata Y, *et al.* Fluid resuscitation with artificial oxygen carriers in hemorrhaged rats: profiles of hemoglobin-vesicle degradation and hematopoiesis for 14 days. Shock 2009; 31: 192-200.

[26] Yamazaki M, Aeba R, Yozu R, Kobayashi K. Use of hemoglobin vesicles during cardiopulmonary bypass priming prevents neurocognitive decline in rats. Circulation 2006; 114(1 Suppl): I220-5.

[27] Tsai AG, Vandegriff KD, Intaglietta M, Winslow RM. Targeted O_2 delivery by low-P_{50} hemoglobin: a new basis for O_2 therapeutics. Am J Physiol Heart Circ Physiol 2003; 285: H1411-9.

[28] Sakai H, Tsuchida E. Hemoglobin-vesicles for a transfusion alternative and targeted oxygen delivery. J Liposome Res 2007; 17: 227-35.

[29] Sakai H, Cabrales P, Tsai AG, Tsuchida E, Intaglietta M. Oxygen release from low and normal P_{50} Hb vesicles in transiently occluded arterioles of the hamster window model. Am J Physiol Heart Circ Physiol 2005; 288: H2897-903.

[30] Contaldo C, Schramm S, Wettstein R, *et al.* Improved oxygenation in ischemic hamster flap tissue is correlated with increasing hemodilution with Hb vesicles and their O_2 affinity. Am J Physiol Heart Circ Physiol 2003; 285: H1140-7.

[31] Plock JA, Tromp AE, Contaldo C, *et al.* Hemoglobin vesicles reduce hypoxia-related inflammation in critically ischemic hamster flap tissue. Crit Care Med 2007; 35: 899-905.

[32] Komatsu H, Furuya T, Sato N, *et al.* Effect of hemoglobin vesicle, a cellular-type artificial oxygen carrier, on middle cerebral artery occlusion- and arachidonic acid-induced stroke models in rats. Neurosci Lett 2007; 421: 121-5.

[33] Sakai H, Sato A, Takeoka S, Tsuchida E. Rheological properties of hemoglobin vesicles (artificial oxygen carriers) suspended in a series of plasma-substitute solutions. Langmuir 2007; 23: 8121-8.

[34] Sakai H, Sato A, Takeoka S, Tsuchida E. Mechanism of flocculate formation of highly concentrated phospholipid vesicles suspended in a series of water-soluble biopolymers. Biomacromolecules 2009; 10: 2344-50.

[35] Verdu EF, Bercik P, Huang XX, *et al.* The role of luminal factors in the recovery of gastric function and behavioral changes after chronic Helicobacter pylori infection. Am J Physiol Gastrointest Liver Physiol 2008; 295: G664-70.

[36] Minoshima T. Artificial blood. Gekagijutsu-no-shimpo 1957; 5: 246-57 (in Japanese).

[37] Ajisaka K, Iwashita Y. Modification of human hemoglobin with polyethylene glycol: a new candidate for blood substitute. Biochem Biophys Res Commun 1980; 97: 1076-81.

[38] Sekiguchi S. Blood substitutes research supported by Health Sciences Research Grant. Artif Blood 1996; 4: 85-89 (in Japanese).

[39] Takaori M. Interpretation of a guidance for oxygen carrier products and their manufacturing proposed by the society of blood substitutes Japan. Artif Blood 2005; 13: 104-11 (in Japanese).

[40] Sou K, Tsuchida E. Electrostatic interactions and complement activation on the surface of phospholipid vesicle containing acidic lipids: effect of the structure of acidic groups. Biochim Biophys Acta 2008; 1778: 1035-41

[41] Takahashi D, Azuma H, Sakai H, *et al.* Phagocytosis of liposome particles by rat splenic immature monocytes makes them transiently and highly immunosuppressive. J Pharmacol Exp Ther 2011; 337: 42-9

[42] Taguchi K, Miyasato M, Ujihira H, *et al.* Hepatically-metabolized and -excreted artificial oxygen carrier, hemoglobin vesicles, can be safely used under conditions of hepatic impairment. Toxicol Appl Pharmacol 2010; 248: 234-41.

Received: December 8, 2010 Revised: January 28, 2011 Accepted: July 01, 2011

Fundamental Problems on Liquids and Related Topics
J. Phys. Soc. Jpn. **81** (2012) SA002

Protein-protein Interactions in Solution and Their Interplay with Protein Specific Functions

Takaaki Sato,*[1] Toshiko Fukasawa,[2] Togo Shimozawa,[3] Teruyuki Komatsu,[4] Hiromi Sakai,[5] and Shin'ichi Ishiwata[5,6]

[1]*International Young Researchers Empowerment Center, Shinshu University, 3-15-1 Tokida, Ueda 386-8567, Japan*
[2]*Division of Natural/Applied Sciences, Graduate School of Humanities and Sciences, Ochanomizu University, 2-1-1 Otsuka, Bunkyo-ku, Tokyo 112-8610, Japan*
[3]*Department of Physics, Graduate School of Science, University of Tokyo, 7-3-1 Hongo Bunkyou-ku, Tokyo 113-0033, Japan*
[4]*Department of Applied Chemistry, Faculty of Science and Engineering, Chuo University, 1-13-27 Kasuga, Bunkyo-ku, Tokyo 113-8551, Japan*
[5]*Waseda Bioscience Research Institute in Singapore (WABIOS), Singapore 138667, Republic of Singapore*
[6]*Department of Physics, Faculty of Science and Engineering, Waseda University, 3-4-1 Okubo, Shinjuku-ku, Tokyo 169-8555, Japan*

E-mail: takaakis@shinshu-u.ac.jp
(Received October 11, 2011)

We summarize recent developments of our small angle scattering studies on protein–protein interactions in solution. We have been focusing especially on representative proteins that function at exceptionally high concentration in human body, whose collective nature, rather than a specialized function of an isolated single molecule, plays an important role for their specific biological functions; for instance, human serum albumin (HSA), actin, and human hemoglobin (Hb). We use a static structure factor analysis, which offers information about spatial distributions of the proteins, as well as a Fourier inversion technique for the form factor, giving a real–space picture of the protein assemblies.

KEYWORDS: Small angle X-ray scattering (SAXS), Protein solutions

1. Introduction

Intermolecular interactions in globular protein solutions have attracted considerable attention because of their cardinal importance in protein crystallography, critical phenomena, and disease processes [1–12]. One representative developments in this area is a updated understanding of equilibrium cluster phase; the equilibrium cluster formation in lysozyme solutions at low to moderate ionic strength was extensively discussed based on small-angle scattering (SAS) experiments [5–7]. Stradner, *et al.* [5, 6] found two characteristic length scales in lysozyme solutions, which were manifested in the appearance of two distinct interference peaks in their small–angle neutron scattering (SANS) structure factor. The high–q and low–q peaks were respectively assigned to monomer–monomer and cluster–cluster positional correlations due to the coexisting short-range attraction and only weakly screened, thus long range electrostatic repulsion between the proteins. Despite remaining controversy [9], such a picture of the equilibrium cluster formation has broadly been accepted so far.

There appears to be two possible options when we study protein–protein interactions in solution, either to regard a protein solution as a model colloidal dispersion in which monodisperse colloidal particles interact via characteristic interaction potentials or to seek to understand the interplay of the protein–protein interactions and their specific biological functions. Nevertheless, it is important to recognize that isotropic interaction is not self–evident for any kind of protein system because proteins have irregular shape and inhomogeneously distributed patches by nature. Furthermore, for instance, owing to nonspecific ligand-binding capability of HSA, it has served many potential medical applications [2][3]. However, the fundamental problems like an influence of the ligand–binding upon protein-protein interactions still remain elusive. Better understanding of such aspects may provide a structural foundation to create functional protein-ligand complexes. It is quite apparent that without elaborately controlled site–specific attractive interactions, globular proteins cannot assemble into such functional aggregates as F-actin or microtubule [4]. The importance of these subjects recognized in the fields of soft condensed matter physics and biological physics is also applied to medical applications of bio–inspired materials [2,10].

SAS can be an eminent tool to illuminate these intriguing problems [1–15]. We will discuss protein-protein interactions in solution and their relationship with protein specific functions studied by means of small angle X-ray scattering (SAXS). We also describe a renovated analytical technique, called SQ–IFT, developed as a potential model–free route to the experimental structure factor of colloidal dispersions, involving protein solutions [1].

2. Experimental

All SAXS experiments on a series of protein solutions were carried out using a SAXSess camera (Anton Paar, Graz, Austria) equipped with a focusing multilayer optics and a block collimator providing a monochromatic, line–shaped primary beam (Cu K_α radiation, $\lambda = 0.154$ nm). The 2D scattering intensity distributions recorded by an imaging-plate (IP) detector were integrated into one-dimensional scattering intensity, $I(q)$, as a function of the magnitude of the scattering vector, q. All $I(q)$ data were corrected for transmission and background scattering from a capillary and solvent. A model-independent collimation correction procedure was made via an indirect Fourier transformation (IFT) [11,12] routine and/or based on a Lake algorithm. Absolute intensity calibration was made by using water as a secondary standard [15].

Human serum albumin (HSA) from human serum was purchased from Sigma–Aldrich (A8763, lyophilized, essentially globulin free, ≥ 99%). Two sets of several HSA samples in H_2O and in 150 mM phosphate buffer saline (PBS) solution (GIBCO®, Invitrogen) were prepared for SAXS experiments. We also investigated solutions of two types of protein hybrids; recombinant HSAs incorporating four iron–porphyrins (heme) [3] and Tris(dicarboxymethylene)–[60] fullerene C_3–isomer (CF) [2], developed by Teruyuki Komatsu (Chuo Univ., Japan) and co-workers. These hybrids, hereafter called rHSA–heme and rHSA–CF, were functionalized as an artificial oxygen carrier and a future cancer therapeutic agent, respectively. Rabbit muscle actin was prepared by the group of Shin'ichi Ishiwata (Waseda Univ., Japan) [16] and its solvent condition was elaborately controlled for polymerization experiments [4]. The hemoglobin vesicle (HbV) was prepared under sterile conditions, according to the previously reported procedures [10]. The HbVs were suspended in a physiologic saline solution at [Hb] = 100 mg ml^{-1} ([lipids] = ca. 60 mg ml^{-1}) and were deoxygenated for storage with N2 bubbling in vials.

Samples, solvents, protein concentrations, and the main targeted issues of SAXS investigations are summarized in Table I.

Table I Summary of SAXS experiments

Sample	Solvent	Concentration (mg ml^{-1})	Target of investigations
HSA	water and 150mM PBS	8.0-267	Effects of solvent ionic strength and protein concentration
rHSA-heme	water and 150mM PBS	3.0-56	Effects of heme- incorporation and solvent ionic strength
rHSA-CF	150mM PBS	5.0-56	Effects of CF- incorporation
rabbit muscle actin	G-buffer (see ref.[4])	4.0	Pursuit of actin polymerization process and identification of nucleus structure
hemoglobin vesicle (HbV)	150mM PBS	ca. 380 (inner aqueous phase)	The dissolution state of the concentrated Hbs confined into an inner aqueous phase of the vesicle

3. Results and dsicussions

3.1 Small-angle X-ray scattering

Small angle scattering (SAS) proves both intraparticle and interparticle structures of complex systems [1-15]. The scattering intensity, $I(q)$, of one-component globular particle systems is generally given by the product of the (averaged) form factor, $P(q)$, and the static structure factor, $S(q)$, as $I(q) = nP(q)S(q)$, where n is particle number density. SAS observes the structure of the particles via the convolution square (or the spatial autocorrelation function) of the scattering length density fluctuations, $\Delta\rho(\boldsymbol{r})$, i.e., electron density fluctuations for SAXS, and neutron scattering length density fluctuations for SANS. The pair-distance distribution function, $p(r)$, is an essential real-space function that contains information about size, shape, and internal structure of the particle, where $P(q)$ is the reciprocal-space coordinate associated with $p(r)$. These two functions are connected via the Fourier transformation as

$$P(q) = 4\pi\int_0^\infty p(r)\frac{\sin qr}{qr}\mathrm{d}r \quad (1)$$

Static structure factor, $S(q)$, reveals length-scale dependent density fluctuation, which offers information about interparticle potentials and spatial distributions of the particles. $S(q)$ is given by the Fourier transformation of the total correlation function, $h(r) = g(r) - 1$, or more exactly, that of $h(r)r^2$ as

$$S(q) - 1 = 4\pi n\int_0^\infty [g(r) - 1]r^2\frac{\sin qr}{qr}\mathrm{d}r \quad (2)$$

where $g(r)$ is the pair-correlation function describing spatial distributions of the colloidal particles.

3.2 Human serum albumin (HSA) at different ionic strength

Human serum albumin (HSA) (MW 66.5 kDa) is the most abundant plasma protein in our blood stream having two primary functions [17]. Nonspecific ligand binding capability of HSA enables transportation of hydrophobic molecules. This also serves as an adjuster of colloid osmotic pressure (COP) of blood. A net negative charge of ca. 18 electron charges which HSA carries at physiological condition may efficiently control its dissolution state. Using SAXS, we investigated spatial correlations of HSA at protein concentrations of $8 \le c/\mathrm{mg\ ml^{-1}} \le 267$ in water and in a 150 mM PBS solution, corresponding to the HSA volume fractions of $0.006 \le \phi_{\mathrm{HSA}} \le 0.198$. These solvent conditions were chosen to minimize ionic strength and to fulfill ionic strength and pH close to physiological conditions, respectively.

In Fig. 1, we present SAXS experiments on human serum albumin (HSA) at different ionic strength and protein concentration. The effective structure factor, $S(q)^{\mathrm{eff}}$,

shown in Fig.1(c) and (d) were obtained by dividing the normalized scattering intensity, $I(q)/c$, where c is the protein concentration, by the experimental form factor, $P(q)^{exp}$, determined at low c, where $S(q) \approx 1$. The peak in $S(q)^{eff}$ arises from protein-protein positional correlations. The mean nearest-neighbor distance d^* between the proteins is approximated as $2\pi/q^*$, where q^* is the magnitude of the scattering vector corresponding to the peak position of $S(q)^{eff}$.

Conventional SAS data analysis assumes isotropic interactions between protein molecules which depend only on the radial position. However, the actual protein-protein interactions may be more complicated, and in some cases, anisotropic. Therefore, a reliable approach that can help understand spatial distributions of proteins in solution with fewer assumptions is desired. We used the so-called SQ-IFT technique [1], developed as an extension of the well-established Indirect Fourier Transformation (IFT) technique. SQ-IFT is interaction potential model-free approach to the effective or experimental structure factor to yield the pair correlation functions (PCFs), $g(r)$, of colloidal dispersions like globular protein solutions.

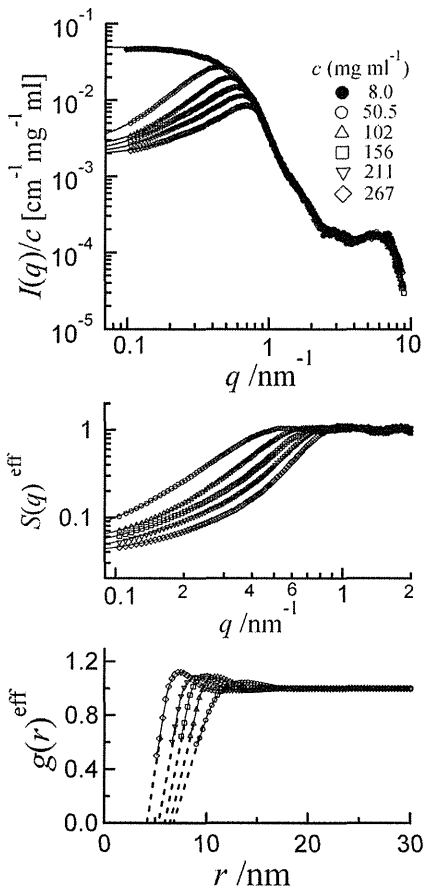

Fig.1 The normalized SAXS intensities, $I(q)/c$, the experimental (effective) structure factors, $S(q)^{eff}$, and the effective pair correlation functions, $g(r)^{eff}$, of HSA for 8.0 ≤ c/mg ml^{-1} ≤ 267at 25 °C in water. $g(r)^{eff}$ was obtained as an output of SQ-IFT.

In Fig.2, we display the extrapolated effective structure factors, $S(q \rightarrow 0)^{eff}$, and the mean nearest-neighbor distance between the proteins, d^*, read out from the first peak position in $g(r)^{eff}$, as a function of HSA volume fraction, ϕ. Theoretically, the

extrapolated structure factors to zero scattering vector, $S(q \rightarrow 0)$, is proportional to the osmotic compressibility of the system. For a monodisperse hardsphere (HS) dispersion, it is simply determined by the excluded volume effect, i.e., the packing fraction (or volume fraction) of the hardsphere, ϕ_{HS}, as

$$S(q \rightarrow 0) = \frac{(1-\phi_{HS})^4}{1+4\phi_{HS}+4\phi_{HS}^2+4\phi_{HS}^3+4\phi_{HS}^4} \quad (3)$$

When repulsive interaction between the colloidal particles is operative, $S(q \rightarrow 0)$ shows a further pronounced decrease, and if the net interaction between particles is attractive, it in turn shows an opposite trend, i.e., a greater value than that expected from the packing fraction and the predication by Equation 3. Therefore, $S(q \rightarrow 0)^{eff}$ shown in Fig.2(a) can be a good measure of the net repulsive or attractive interactions between the proteins. The more pronounced decrease of $S(q \rightarrow 0)^{eff}$ and the significantly greater d^* for aqueous HSA than that in PBS reflect stronger electric double layer repulsion forces between the HSA molecules at low ionic strength. A characteristic of electrostatic repulsion can also be recognized from the broader shape of the protein–protein correlation peak in $g(r)^{eff}$ for aqueous HSA.

In PBS, d^* decreases moderately with ϕ_{HSA}, showing a linearlike behavior. In contrast, aqueous HSA exhibits far greater d^* values especially at low ϕ, and increasing ϕ rapidly shifts d^* to lower values. A solid curve in Fig.2(b) for aqueous HSA is drawn to highlight a general relation, $d^* \propto \phi^{-1/3}$, for charged colloids at low ionic strength, where ϕ is volume fraction. At $\phi_{HSA} \sim 0.2$, d^* eventually converges to ca. 7 nm independent of ionic strength. The smaller d^* than the maximum diameter of HSA ($D_{max} \approx 8$ nm) implies the occurrence of direct contact between HSA molecules at highest ϕ_{HSA}.

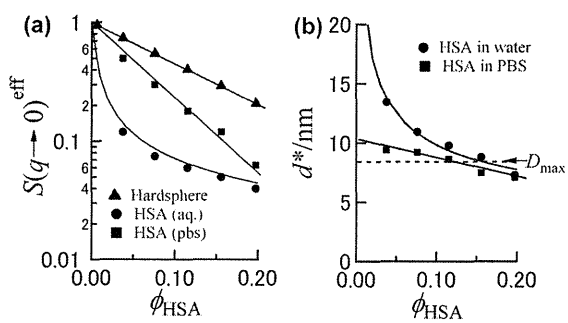

Fig.2 (a) The extrapolated effective structure factors, $S(q \rightarrow 0)^{eff}$, and (b) the mean nearest-neighbor distance between the proteins, d^*, as a function of HSA volume fraction, ϕ_{HSA}.

3.3 Recombinant HSA hybridized with iron-porphyrins and fullerene derivative

We also investigated two types of recombinant HSA hybrids incorporating four iron-porphyrins (rHSA-heme) [3] and fullerene derivative (rHSA-CF) [2]. We scrutinized spatial correlations of these proteins. In Fig. 3, we display the effective structure factors, $S(q)^{eff}$, for rHSA and rHSA-heme at c = 30 mg ml^{-1}. We found that importantly, q^* is essentially independent before and after heme binding, but it simply depends on the concentration of ions in solvent, which mainly acts as the solvent ionic strength and thus is closely related to the screening of the long-range electrostatic repulsion. The presence of hydrogen phosphate and dihydrogen phosphate ions may also affect the water structure, which may impose a secondary effect on the

protein–protein interactions.

Solutions of rHSA-heme exhibit a similar low-q upturn in $S(q)^{eff}$, independent of ionic strength. The observation suggests the emergence of a long-range attractive (LRA) interaction between the heme-incorporated rHSA molecules. The complex dielectric spectra (Data not shown) of rHSA and rHSA-heme solutions at various c were analyzed [3]. The low-frequency relaxation centered at ca. 3 MHz is assigned to the rotational diffusion of the proteins [1]. The identical relaxation times before and after heme binding, 52–58 ns, result in an identical effective molar volume for rHSA and rHSA-heme, $V^{eff} = 4.7 \times 10^4$ cm^3 mol^{-1} ($c \rightarrow 0$). This coincides nicely with the molar volume of 4.9×10^4 cm^3 mol^{-1} predicted from the molecular mass and specific volume of HSA. This demonstrates that the freedom of the rotational diffusive motion of the protein is not significantly affected by the heme-binding. The simultaneous observations of the low-q rise in $S(q)^{eff}$ and a number of pieces of evidence for monodispersity of the protein, such as d^* far exceeding the contact distance and identical molecular volume with rHSA appear to indicate that heme incorporation induces a long-range attractive (LRA) potential between the protein molecules. In the human body, heme released from methemoglobin is captured by scavenger proteins, hemopexin or HSA, and it is transported to the liver to metabolize. Therefore, heme–incorporated HSA is a naturally produced species. Our data suggest that the emergence of the collective nature of HSAs while preserving their monodispersity could be an efficient way to give the heme-bound HSA molecules a sort of marker.

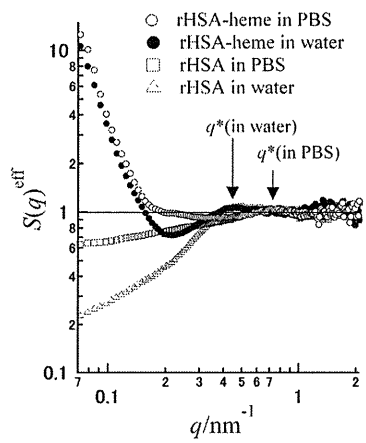

Fig.3 The effective (experimental) structure factors, $S(q)^{eff}$, of a 30 mg ml^{-1} rHSA and rHSA-heme in water (with no salt added) and in 150 mM PBS solution.

The HSA-CF complex engenders cell death owing to an easy excitation under visible light irradiation and a consequent high efficiency of the $^1O_2$ production [2]. The incorporation of a negatively charged CF molecule may influence the surface charge distribution of HSA and the net repulsive interaction between the protein molecules. However, rHSA-CF exhibits the identical isoelectric point (pI) with HSA whereas fatty acid binding induces the reduction of the pI value due to partial neutralization of the molecular charge [2]. We evaluated the protein–protein interactions of rHSA-CF (Fig.4). The normalized forward intensities, $I(q \rightarrow 0)/c$, of rHSA-CF are decreased with increasing c. $S(q \rightarrow 0)^{eff}$ of rHSA-CF and rHSA, which reflect the net repulsive forces between the protein molecules, are almost perfectly on the top of each other, suggesting the preserved net surface charges upon CF incorporation. $S(q \rightarrow 0)^{eff}$ in itself is found to be systematically lower than that predicted for hardsphere due to the remaining

electrostatic repulsion between the proteins even at physiological ionic strength.

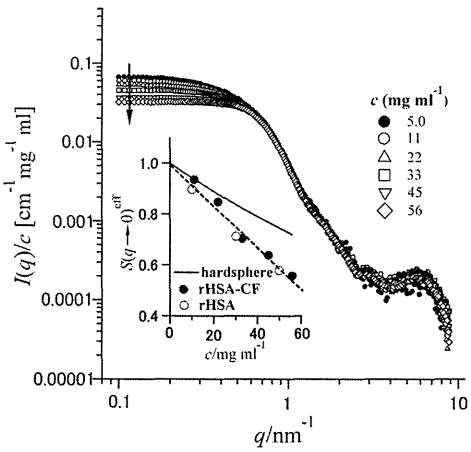

Fig.4 Normalized SAXS intensities, $I(q)/c$, of rHSA-CF complex in 150 mM PBS solutions (c 5.0–56 mg ml^{-1}). The insets show the extrapolated structure factors, $S(q \to 0)^{eff}$, of rHSA-CF and rHSA as a function of c.

3.4 A nucleation and elongation pathway of actin

Actin is a versatile protein as the major component of the muscle thin filaments as well as the cytoskeleton of eukaryotic cells [18–21]. Monomeric actin (G-actin) assembles into filament forms (F-actin) under physiological ionic conditions. It has widely been believed that based on the time course analysis of the polymerization kinetics, a polymerization nucleus is composed of 3 or 4 monomers [22]. Most experimental studies aimed at testing such a hypothesis are indirect as they are often based on either spectroscopic or microscope techniques and the interpretation remains somewhat ambiguous. The major difficulty in identifying a nucleus structure arises from the fact that it is not a stable complex, but rather, it exists only transiently. Furthermore, we have to find such a nucleus structure out of far greater number of co-existing other species like monomers.

Using SAXS, we have attempted to pursuit a nucleation and elongation pathway of rabbit muscle actin with a stepwise increase of temperature from 1 °C to 30 °C at low ionic strength (c = 4.0 mg ml^{-1} in G-buffer) [4]. Polymerization experiments have mostly been done at fixed temperature and protein concentration above critical by initiating the reaction with the added salts, such as KCl or $MgCl_2$, but instead, we observed a thermally activated polymerization process with fixed protein and salt concentrations, by varying temperature, at a relatively high actin concentration. In this case, the polymerization process can be viewed as a pseudo phase transition, and each temperature step is to be regarded as a non-equilibrium, metastable phase.

We started the SAXS experiments from the G-actin state. We observed that the forward intensity $I(q \to 0)$ starts to increase at ca. 20 °C for Ca-ATP actin, indicating the onset of actin polymerization. The critical temperature, or the onset temperature of polymerization, does not depend on the nucleotide species, i.e., ATP or ADP, under the present solvent condition. At higher temperatures, we observed the emergence of a reflection peak at q =1.15 nm^{-1}, which corresponds to the d-spacing of 5.5 nm. This can be recognized as manifestation of an orientationally averaged fiber diffraction peak reflecting the subunit axial translation, the so-called sixth– and seventh–layer lines

known as the major characteristics of the established F-actin structure.

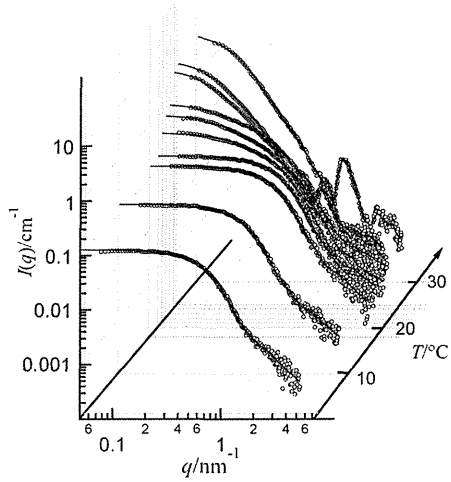

Fig.5 Temperature dependence of SAXS intensities, $I(q)$, for Ca-ATP actin at c = 4.0 mg ml^{-1} as a function of temperature.

To structurally resolve the distribution of actin oligomers especially at the initial stage of polymerization, we analyzed the data by fitting the experimentally obtained pair-distance distribution function, $p(r)$, using IFT [13,14] with the theoretically calculated $p(r)$ for the helical and linear oligomers based on the X-ray crystallography data [23]. We found that $p(r)$ at the initial stage of polymerization (at 20 °C for Ca–ATP), where the weight averaged molecular weight is still only ca. 10% greater than that of G-actin, is mostly accounted for by the superposition of monomer, linear dimer, and helical trimer. When the temperature was increased by 1°C, we observed the formation of long polymers. The finding leads us to conclude that F-actin formation is triggered by the formation of a helical trimer that essentially involves all basic intermolecular interactions between the neighboring monomers in the filament form.

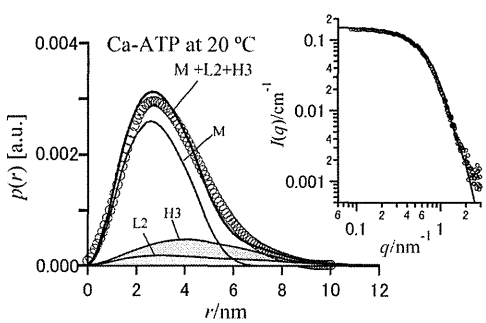

Fig.6 Structural characterization of the Ca–ATP action at the initial stage of polymerization. The experimental $p(r)$ at the critical temperature, T^* ~ 20 °C, was analyzed based on a mixture model of monomer (M), linear dimer (L2), and helical trimer (H3). In the right panel, shown is the fit in the reciprocal space.

3.5 Perspectives: human hemoglobin and poly(ethylene glycol)-conjugated proteins.

Human hemoglobin (HbA) is the protein that functions at highest concentration in the human body. Significant functional consequences of the cellular structure of the red blood cells (RBCs) are the retardation of entrapment of endogenous vasorelaxation factors (NO and CO), preservation of chemical environments, and screening of high colloid osmotic pressure (COP) of a concentrated Hb solution [24,25]. An inner aqueous phase of RBCs is filled with highly concentrated (ca. 300 mg ml^{-1}) hemoglobin solution, which makes it possible for RBCs to satisfy required oxygen-carrying capacity.

Hiromi Sakai (Waseda Univ., Japan) and co-workers have developed hemoglobin vesicle (HbV), a hierarchically structured artificial oxygen carrier that encapsulates solution of purified and highly concentrated (ca. 380 mg ml^{-1}) human hemoglobin [26–28]. An exceptionally high concentration of HbV as a liposomal product (240 nm diameter; ca. 40% volume fraction) achieves an oxygen-carrying capacity comparable to that of blood. Using SAXS, we have studied the internal structure of HbV [10]. In Fig.7, we show SAXS experiments on HbV and related systems (a concentrated Hb solution and a dispersion of counterpart empty vesicles). Consistent with the dynamic light scattering (DLS) result (data not shown), the forward intensity in $q < 0.3$ nm^{-1} is well explained by the scattering function of slightly polydisperse globular particles having an averaged diameter of 240 nm. The high–q excess component marked in $q >$ 0.3 nm^{-1} manifests internal electron density fluctuations of the HbV particle, reflecting the shorter length scale structures of the encapsulated Hb solution and the lipid bilayer. We found that the peak position as well as the height and width of static structure factor of Hb before and after encapsulation into an inner aqueous phase of HbV are almost identical, demonstrating the preserved protein-protein interactions in the confined space. We will report a detailed concentration–, temperature–, and *p*H–dependent study on Hb–Hb interactions elsewhere [29].

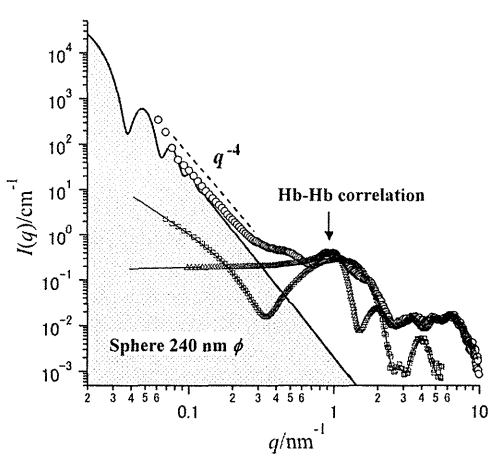

Fig.7 SAXS intensity, $I(q)$, in a log–log plot of the concentrated HbV dispersion (circles) at 25 °C in $0.06 \leq q/nm^{-1} \leq 10$ on absolute scale. Those for Hb in 150 mM PBS solution at c = 400 mg ml^{-1} (triangles) and the dispersion of empty vesicle designed for Hb encapsulation (squares) are shown together. An arrow highlights the Hb–Hb correlation peak, corresponding to $d^* \sim 7$ nm.

On the one hand, poly(ethylene glycol) (PEG) is a nontoxic, non–antigenic, and

highly water soluble polymer. The modification of biological molecules by PEG, called PEGylation, offers a number of advantages in their clinical use, like a prolonged residence in body [30]. We are now trying to elucidate effects of conjugated PEGs on the protein-protein interactions for PEG–HSA and PEG–Hb.

4. Conclusion

We have presented a series of SAXS studies on the protein–protein interactions in solution, where the collective nature of the protein molecules plays an important role for their biological functions. The results highlight the efficiency of the static structure factor analysis to obtain deep physical insights into spatial distributions and interacting schemes of globular proteins in solution. A Fourier inversion technique for the form factor is also an eminent tool to pursue a polymerization process of proteins, giving a real–space picture of protein assemblies. Our data seem to indicate that heme–filled HSAs interact via the long range attractive (LRA) potential while preserving their monodispersity. In contrast, incorporation of a fullerene derivative hardly modulates the HSA–HSA interactions. As for actin polymerization, it is likely that F-actin formation is triggered by the formation of a helical trimer that essentially involves all basic intermolecular interactions between the neighboring monomers in the filament form. We are now working on HbA and PEG–conjugated proteins, whose results will come out soon elsewhere.

Acknowledgment

T.S. would like to gratefully acknowledge all collaborators of a series of small-angle scattering studies on protein solutions reviewed and prospected in this article, especially; late Prof. Eishun Tsuchida (Waseda Univ.), Prof. Kenji Aramaki (Yokohama National Univ.), and Prof. Otto Glatter (Graz Univ.). Recent scientific activities of T.S. are supported by Special Coordination Funds for Promoting Science and Technology (International Young Researchers Empowerment project of Shinshu Univ.) from MEXT Japan.

References

[1] T. Fukasawa and T. Sato: Phys. Chem. Chem. Phys. **13** (2011) 3187.
[2] X. Qu, T. Komatsu, T. Sato, O. Glatter, H. Horinouchi, K. Kobayashi, and E. Tsuchida: Bioconjugate Chem. **19** (2008) 1556.
[3] T. Sato, T. Komatsu, A. Nakagawa, and E. Tsuchida: Phys. Rev. Lett. **98**, 208101 (2007).
[4] T. Sato, T. Shimozawa, T. Fukasawa, M. Ohtaki, K. Aramaki, K. Wakabayashi, and S. Ishiwata: BIOPHYSCS **6** (2010) 1.
[5] A. Stradner, H. Sedgwick, F. Cardinaux, W. C. K. Poon, S. U. Egelhaaf and P. Schurtenberger: Nature **432** (2004) 492.
[6] A. Stradner, F. Cardinaux, and P. Schurtenberger: J. Phys. Chem. B **110** (2006) 21222.
[7] Y. Liu, E. Fratini, P. Baglioni, W. R. Chen and S. H. Chen: Phys. Rev. Lett. **95** (2005) 118102.
[8] A. Shukla, E. Mylonas, E. Di Cola, S. Finet, P. Timmins, T. Narayanan, and D. I. Svergun: Proc. Natl. Acad. Sci. U. S. A. **105** (2008) 5075.
[9] N. Dorsaz, G. M. Thurston, A. Stradner, P. Schurtenberger and G. Foffi: J. Phys. Chem. B **113** (2009) 1693.
[10] T. Sato, H. Sakai, K. Sou, M. Medebach, O. Glatter, and E. Tsuchida: J. Phys. Chem. B **113** (2009) 8418.
[11] L. M. Rice, E. A. Montabana, and D. A. Agard: Proc. Natl. Acad. Sci. U. S. A. **105** (2008) 5378.
[12] A. I. Normana, R. Ivkov, J. G. Forbes, and S. C. Greerb: J. Chem. Phys. **108** (2005) 5599.
[13] O. Glatter: J. Appl. Crystallogr. **10** (1977) 415.
[14] O. Glatter: Acta Phys. Austriaca **47** (1977) 83.
[15] D. Orthaber, A. Bergmann, and O. Glatter: J. Appl. Crystallogr. **33** (2000) 218.
[16] H. Kondo and S. Ishiwata: J. Biochem. **79** (1976) 159.

[17] T. Peters, All About Albumin: Biochemistry, Genetics, and Medical Applications (Academic, New York, 1996).
[18] T. D. Pollard, L. Blanchoin, and R. D. Mullins: Annu. Rev. Biophys. Biomol. Struct. **29** (2000) 545.
[19] M. F. Carlier, S. Wiesner, C. L. Clainche, and D. Pantaloni: C.R. Biol. **326** (2003) 161.
[20] T. D. Pollard and G. G. Borisy: Cell **112** (2003) 453.
[21] T. D. Pollard: Annu. Rev. Biophys. Biomol. Struct. **36** (2007) 451.
[22] M. Kasai, S. Asakura, and F. Oosawa: Biochim. Biophys. Acta **57** (1962) 22.
[23] K. C. Holmes, D. Popp, W. Gebhard, and W. Kabsch: Nature **347** (1990) 44.
[24] X. Liu, M. J. Miller, M. S. Joshi, H. Sadowaska-Krowicka, D. A. Clark, J. R. Lancaster Jr.: J. Biol. Chem. **273** (1998) 18709.
[25] M. W. Vaughn, K. T. Huang, L. Kuo, J. C. Liao: J. Biol. Chem. **275** (2000) 2342.
[26] Y. Izumi, H. Sakai, K. Hamada, S. Takeoka, T. Yamahata, R. Kato, H. Nishide, E. Tsuchida, K. Kobayashi: Crit. Care Med. **24** (1996) 1869.
[27] H. Sakai, H. Horinouchi, M. Yamamoto, E. Ikeda, S. Takeoka, M. Takaori, E. Tsuchida, K. Kobayashi: Transfusion **46** (2006) 339.
[28] H. Sakai, S. Takeoka, S. I. Park, T. Kose, H. Nishide, Y. Izumi, A. Yoshizu, K. Kobayashi, E. Tsuchida: Bioconjugate Chem. **8** (1997) 23.
[29] T. Sato and H Sakai, unpublished results.
[30] F. M. Veronese and G. Pasut: Drug Discovery Today **10** (2005) 1451.