Chapter 9
Hemoglobin–Albumin Clusters as a Red Blood Cell Substitute

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Abstract Core–shell protein cluster comprising bovine hemoglobin (HbBv) in the core and human serum albumin (HSA) at the shell was created as an artificial O_2 carrier designed for use as a red blood cell (RBC) substitute. The protein cluster was prepared by covalent linkage between the Cys-34 residue of HSA and the surface Lys amino groups of HbBv using heterobifunctional cross-linker. The average HSA/HbBv ratio of one cluster was determined as 3.0 ± 0.2; therefore we indicated this hemoglobin–albumin cluster as HbBv–HSA_3. Human Hb A (HbA) can be also used for a core protein to synthesize HbA–HSA_3 cluster. The isoelectric point of HbBv–HSA_3 (pI = 5.1) was markedly lower than that of HbBv and almost identical to the value of HSA. SFM and TEM measurements revealed a triangular shape of HbBv–HSA_3. The complete 3D structure based on TEM data was reconstructed. The clusters showed moderately higher O_2 affinities than the native HbBv and HbA. Viscosity and blood cell counting measurements demonstrated that HbBv–HSA_3 has good compatibility with whole blood. Intravenous administration of HbBv–HSA_3 into anesthetized rats elicited no unfavorable increase in systemic blood pressure by vasoconstriction. The half-life of ^{125}I-labeled cluster in circulating blood is longer than that of HSA. All results indicate that HbBv–HSA_3 has sufficient preclinical safety as an alternative material for RBC transfusion. Interestingly, clusters prepared under N_2 atmosphere showed low O_2 affinity resembling human RBC. Furthermore, the exterior HSA units possess a remarkable ability to bind antioxidant agent, such as Pt nanoparticle (PtNP). The peripheral HSA–PtNP shell prevents oxidation of the core HbBv, which enables the formation of an extremely stable O_2 complex even in H_2O_2 solution. This chapter reviews the synthesis, structure, O_2-binding property, and preclinical safety of hemoglobin–albumin cluster as a promising RBC substitute for practical use.

Keywords Artificial oxygen carrier • Protein cluster • Oxygen-binding property • Blood pressure • Blood retention

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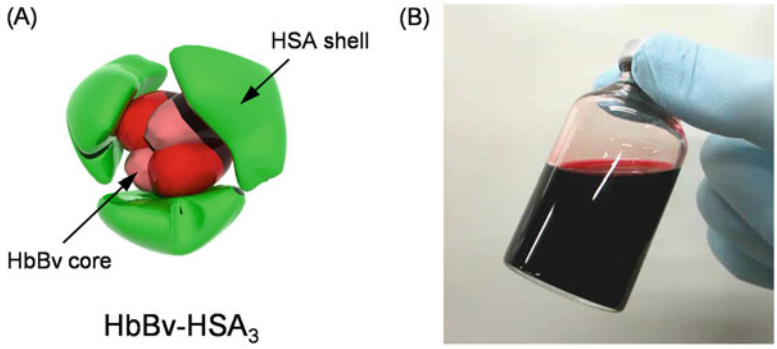

Fig. 9.1 (**A**) Illustration of molecular structure of HbBv–HSA_3 in which an HbBv core is wrapped covalently by three HSAs (Tomita et al. 2013). (**B**) HbBv–HSA_3 solution (20 g/dL) in PBS (pH 7.4)

9.1 Introduction

Over the last few decades, hemoglobin (Hb)-based O_2 carriers (HBOCs) of many kinds have been designed and developed as red blood cell (RBC) substitutes (Squires 2002; Jahr et al. 2011; Kluger and Lui 2013; Mondery-Pawlowski et al. 2013), such as intramolecularly cross-linked Hb (Snyder et al. 1987; Nagababu et al. 2002), polymerized Hb (Buehler et al. 2005; Pearce et al. 2006; Kluger and Zhang 2003; Hu and Kluger 2008), poly(ethylene glycol)-decorated Hb (Vandegriff et al. 2003; Manjula et al. 2003; Li et al. 2008, 2009), enzyme-conjugated Hb (D'Agnilloo and Chang 1998; Alagic et al. 2005), saccharide-linked Hb (Zhang et al. 2008), and nano-/microparticle-encapsulated Hb (Sakai 2012; Xiong et al. 2013). In any period, the social requests have promoted the development of the RBC substitute, for example, a need in battlefield, a concern to virus diffusion, and a primary measure for crisis management. Currently, the motive of the artificial O_2 carrier is moving to a medical measure to supplement blood transfusion treatment. The declining birthrate and aging population make it difficult to retain a stable blood transfusion system. The number of old people will continue to increase, although the population of blood donors is expected to decrease. In fact, the Japanese Red Cross Society predicts a blood shortage equivalent to 890,000 people per year in 2027 (Ministry of Health, Labor and Welfare, Japan 2014). However, no HBOC product has been assigned yet for medical use (Jahr et al. 2011; Pearce et al. 2006; Natanson et al. 2008; Kluger 2010). The major concern of the Hb derivatives is vasoconstriction, which causes a mild increase in systemic blood pressure. This pressor response is inferred to be due to quick scavenging of nitric oxide (NO), the endothelial-derived relaxing factor, by Hb leaked into the extravascular space (Shultz et al. 1993; Rohlfs et al. 1998; Doherty et al. 1998).

Recently, we prepared a covalent core–shell structured protein cluster composed of Hb in the core and human serum albumin (HSA) at the shell as a unique HBOC (Fig. 9.1) (Tomita et al. 2013; Hosaka et al. 2014; Haruki et al. 2015). The average

HSA/Hb ratio of one cluster was 3.0 ± 0.2. We indicate this hemoglobin–albumin cluster as Hb–HSA_3. It is noteworthy that intravenous transfusion of Hb–HSA_3 does not elicit the acute increase in blood pressure (Haruki et al. 2015). This is attributed to the fact that Hb–HSA_3 is not eliminated from the vasculature walls because of the electrostatic repulsion between the negative surface net charges of the cluster and the glomerular basement membrane around the endothelial cells. This chapter reviews the synthesis, structure, O_2-binding property, and preclinical safety of Hb–HSA_3 as a promising RBC substitute for practical use.

9.2 Synthesis and Structure of Hemoglobin–Albumin Cluster

HSA is a heart-shaped monomeric protein bearing one free sulfhydryl group of Cys at position 34 (Curry et al. 1998). Therefore, we used a heterobifunctional cross-linking agent, *N*-succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC), as a connector between the Cys-34 residue of HSA and the surface Lys amino groups of Hb to create a core–shell cluster of Hb and HSA (Fig. 9.2) (Tomita et al. 2013; Hosaka et al. 2014). First, SMCC was reacted with bovine Hb (HbBv) in phosphate-buffered saline (PBS, pH 7.4) solution. Then the resulting maleimide-activated HbBv was added dropwise into the HSA solution, followed by stirring at 4 °C. Size-exclusion chromatography (SEC) of the resultant mixture showed distinct three peaks at the high molecular weight region. Native PAGE also exhibited three new bands above HSA (Tomita et al. 2013; Hosaka et al. 2014). Using gel filtration chromatography (GFC), unreacted HSA was removed and the major products were collected. Based on the Hb assay and protein assay, the HSA/HbBv ratio (mol/mol) of the cluster was determined to be 3.0 ± 0.2. We indicate this product as Hb–HSA_3 with italicized subscript 3. Reaction with human adult Hb (HbA) also generated similar protein cluster, HbA–HSA_3 (Kimura et al. 2015).

The CD spectrum of HbBv–HSA_3 fit perfectly with the sum of the HbBv spectrum and a threefold-enlarged HSA spectrum (Tomita et al. 2013; Hosaka et al. 2014). This result implies that (i) the HbBv:HSA ratio in one cluster is 1:3 (mol:mol) on average and (ii) the secondary structure of the individual protein unit remains constant after the cluster formation. The isoelectric points of HbBv–HSA_3 (*p*I: 5.1) were markedly lower than the value of native HbBv (*p*I, 7.0) and resembled to that of HSA (*p*I: 4.9). These results supported that the HbBv core is covalently wrapped by HSAs (Tomita et al. 2013; Hosaka et al. 2014).

Scanning probe microscopy (SPM) images of HbBv–HSA_3 on a mica surface in PBS solution depicted clearly triangular shape of several entities (Fig. 9.3A) (Tomita et al. 2013). We were convinced of a triangular core–shell structure with HbBv in the center and three exterior HSAs are formed.

Furthermore, the 3D reconstruction of HbBv–HSA_3 based on transmission electron microscopy (TEM) images revealed a complete triangular structure (Tomita

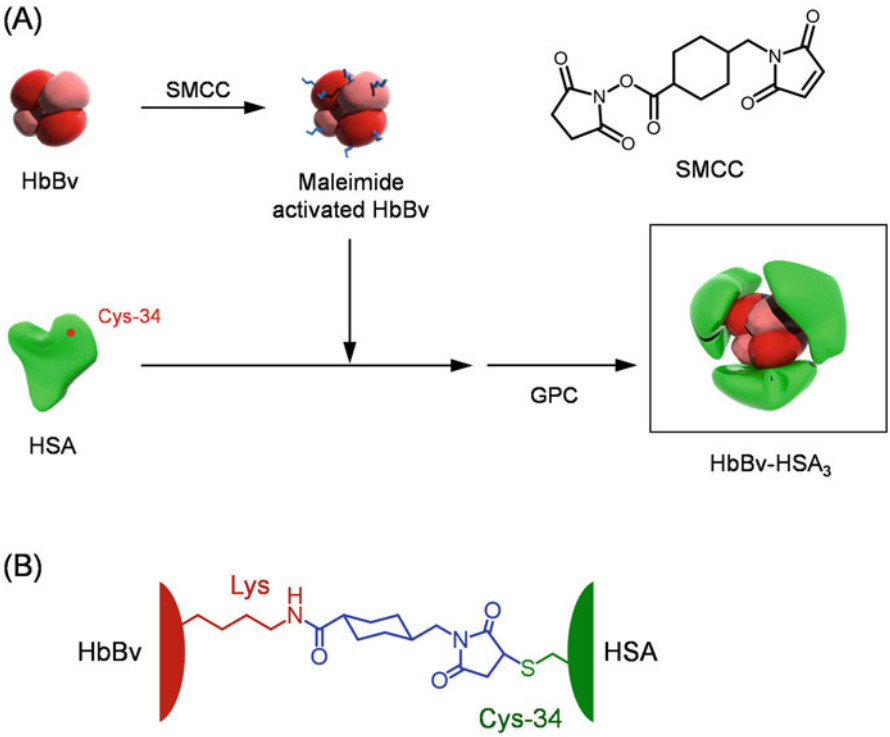

Fig. 9.2 (**A**) Schematic illustration of the synthetic route of HbBv–HSA_3 using heterobifunctional cross-linker (SMCC). (**B**) The covalent linkage structure between the Lys residue of HbBv and the Cys-34 residue of HSA

et al. 2013). The original TEM pictures of HbBv–HSA_3 showed individual particles (diameter: approximately 10 nm), but detailed structure information was unavailable owing to the low contrast. Then we used additional image processing procedure (single-particle analysis). From the obtained class sum images with an enhanced signal-to-noise ratio (Fig. 9.3B) (Tomita et al. 2013; Kimura et al. 2015), the 3D volume of HbBv–HSA_3 was reconstructed. We calculated a presentation of the protein moieties by fitting their PDB data into the reconstructed volume. The proposed geometries conferred a possible spatial arrangement of the HbBv interior and three HSA exterior. The fitting of three HSAs defined an arrangement of the Cys-34 of HSA, which suggested the potential binding Lys partners on HbBv (Fig. 9.3C). The HSA binding sites on HbA in HbA–HSA_3 are almost the same as those of HbBv–HSA_3 (Kimura et al. 2015).

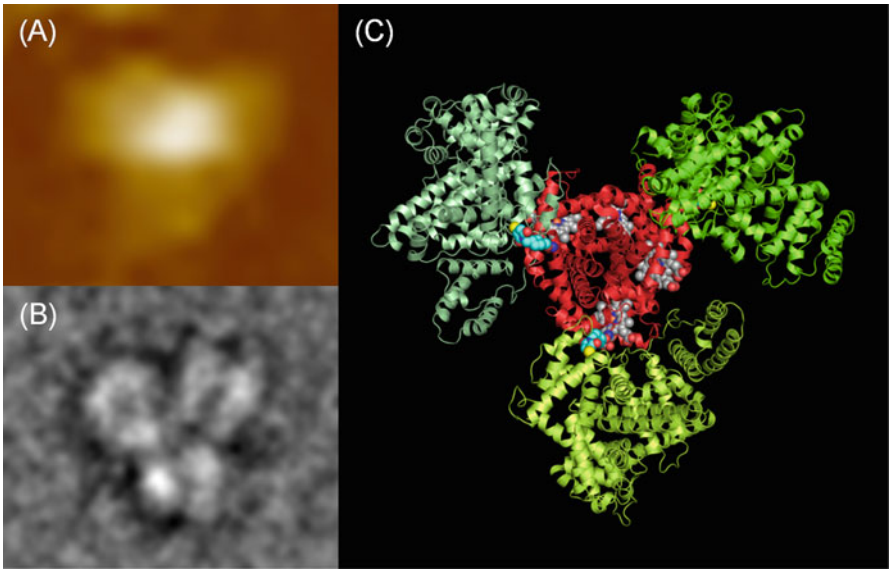

Fig. 9.3 (**A**) SPM image of HbBv–HSA_3 bound on mica surface in PBS (pH 7.4) solution at 25 °C. (**B**) A selected class sum image of HbBv–HSA_3. (**C**) Spatial molecular view of HbBv–HSA_3 derived from a 3D volume reconstruction. Color code: HbBv, *red*; HSAs, *chartreuse*, *lemon*, and *pale green*. Hemes (**C**, *gray*) in HbBv, Cys-34 (S, *yellow*) in HSA

9.3 O_2-Binding Property of Hemoglobin–Albumin Cluster

The deoxy, oxy, and carbonyl forms of HbBv–HSA_3 in PBS solution under N_2, O_2, and CO atmospheres, respectively, showed identical absorption spectra to the corresponding forms of naked HbBv (Fig. 9.4) (Hosaka et al. 2014; Antonini and Brunori 1971). We reasoned that the electronic states of the prosthetic heme groups in HbBv were unaltered by the covalent linkages of HSAs.

The O_2 affinity (P_{50}: O_2 partial pressure where Hb is half-saturated with O_2) and the cooperativity coefficient (Hill coefficient: n) of the HbBv–HSA_3 were measured using an automatic recording system for blood O_2 equilibrium curve (Hemox Analyzer). The P_{50} of native HbBv was 23 Torr at 37 °C, whereas the value of HbBv–HSA_3 was 9 Torr (Table 9.1) (Hosaka et al. 2014). The n value decreased from 2.6 to 1.5. The results imply that the HbBv–HSA_3 shows higher O_2 affinity than HbBv does. The P_{50} and n value reductions were also seen in HbA–HSA_3 (P_{50} = 8 Torr, n = 1.4) (Table 9.1) (Kimura et al. 2015). There are two possible explanations for the increase of O_2 affinity and decrease in cooperativity. The first is the binding of the maleimide terminal of SMCC to Cys-93(β) in HbBv and HbA. Modification of the sulfhydryl group of Cys-93(β), which is located nearby the proximal His-92(β) coordinated to the heme (Mueser et al. 2000), is known to enhance the O_2 affinity (Manjula et al. 2003; Li et al. 2009; Zhang et al. 2008). Furthermore, it reduces the available motion of the $\alpha_1\beta_1/\alpha_2\beta_2$ interface and induces

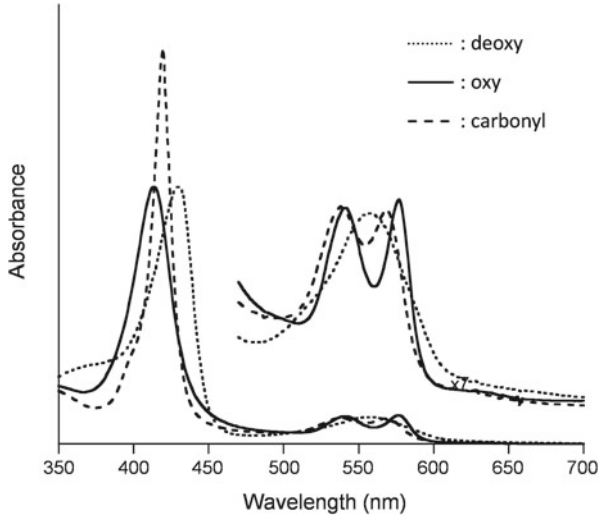

Fig. 9.4 Visible absorption spectral changes of the HbBv–HSA_3 in PBS solution (pH 7.4) at 25 °C

Table 9.1 O_2-binding parameters of HbBv–HSA_3 and HbA–HSA_3 in PBS solution (pH 7.4) at 37 °C

	P_{50} (Torr)	n	Ref.
HbBv–HSA_3	9	1.5	Hosaka et al. (2014)
HbBv–HSA_4	9	1.5	Yamada et al. (2016)
HbA–HSA_3	8	1.4	Kimura et al. (2015)
HbA(T)–HSA_3	26	1.2	Kimura et al. (2015)
$\alpha\alpha$HbA(T)–HSA_3	35	1.4	Kimura et al. (2015)
HbBv	23	2.6	Hosaka et al. (2014)
HbA	12	2.4	Kimura et al. (2015)
	12[a]	2.4[a]	Elmer et al. (2012)
RBC	25	2.5	Kimura et al. (2015)

[a]In Hemox buffer (pH 7.4), 37 °C

disturbance of the quaternary structure of Hb from the Tense (T)-state to the Relaxed (R)-state (Zhang et al. 2008). In fact, the number of cysteinyl thiols per HbBv decreased from 2.0 to 0.2 after the SMCC reaction, indicating that two Cys-93(β) of HbBv are blocked by SMCC maleimide. The second reason is the modifications of surface Lys groups of HbBv or HbA by succinimide terminal of SMCC. They are needed to create the cluster, but the chemical modifications of Lys groups on Hb influence the O_2 affinity (Kluger and Zhang 2003; Hu and Kluger 2008; Vandegriff et al. 2003). In particular, Lys-82(β) plays a key role to modulate the quaternary structural change from the T-state to R-state of Hb. Our 3D reconstruction suggested that Lys-82(β) is a binding partner of Cys-34 of HSA (Tomita et al. 2013;

Kimura et al. 2015). It can be concluded that (i) the masking of Cys-93(β) increases the O_2 affinity and (ii) modification of surface Lys groups locks the R-state configuration of the central HbBv or HbA, resulting in the decrease of O_2-binding cooperativity. Interestingly, HbBv–HSA_4, which is hemoglobin–albumin cluster bearing four HSA units (large-size variant), showed the same O_2-binding parameters as HbBv–HSA_3 (Yamada et al. 2016). It implies that the central Hb conformation is independent of the binding number of HSA.

9.4 Preclinical Safety of Hemoglobin–Albumin Cluster

The viscosity of the HbBv–HSA_3 solution (20 g/dL, [Hb] = 5.0 g/dL) is dependent on the shear rate, namely, a Newtonian fluid (Haruki et al. 2015). The viscosity at 230 s^{-1}, the shear rate in the human arterial wall, was 2.8 cP, which is lower than that of blood (3.8 cP). A mixture solution of freshly drawn whole blood and HbBv–HSA_3 (1/1, v/v) showed non-Newtonian viscosity, which obeyed a nonlinear correlation to the shear rate. The viscosity was reasonably high: 3.3 cP at 230 s^{-1}. Furthermore, we counted the number of blood cell components [RBC, white blood cell (WBC), and platelet (PLT)] of the blood/HbBv–HSA_3 mixture solution in vitro (Haruki et al. 2015). The numbers of RBC, WBC, and PLT decreased in proportion to their respective dilution ratios. These results indicate that HbBv–HSA_3 has good compatibility with whole blood.

The HbBv–HSA_3 solution (20 g/dL) was injected into anesthetized rats (6 mL/kg) and observed their mean arterial pressure (MAP) (Haruki et al. 2015). Notably, a small transient alternation in MAP was observed after administration of HbBv–HSA_3 (Fig. 9.5). The slight elevation of ΔMAP (25.3 ± 2.9 mmHg) from the basal value was followed by a decrease to 10 mmHg and retained constant during the monitoring time. The response is almost identical to that observed after infusion of HSA (20 g/dL). On the contrary, the administration of ββ-cross-linked HbBv (XLHbBv, 5 g/dL) is associated with an acute increase in ΔMAP (55.5 ± 5.9 mmHg) and urinary excretion of Hb from 10 min after the injection.

This non-vasopressor response of HbBv–HSA_3 is attributed to the negative surface net charge and high molecular weight of the cluster. HSA shows low vascular permeability of less than 1/100 Hb because of the electrostatic repulsion between the albumin surface and the glomerular basement membrane around the endothelial cells (Haraldsson et al. 2008). The isoelectric point of HbBv–HSA_3 ($pI = 5.1$) is close to that of HSA. Furthermore, the molecular weight of HbBv–HSA_3 (26.4 kDa) is much greater than that of HSA (66.5 kDa). Thus, the release of HbBv–HSA_3 into the extravascular space was attenuated. In contrast, the small XLHbBv having neutral surface charge passes through the vascular endothelium and contributes to the consumption of NO. Moreover, XLHbBv passes through the renal glomerulus, thereby inducing excretion of Hb in urine.

The ^{125}I-labeled HbBv–HSA_3 was injected into rats to evaluate blood retention (Haruki et al. 2015). The ^{125}I-labeled native HbBv was cleared rapidly from

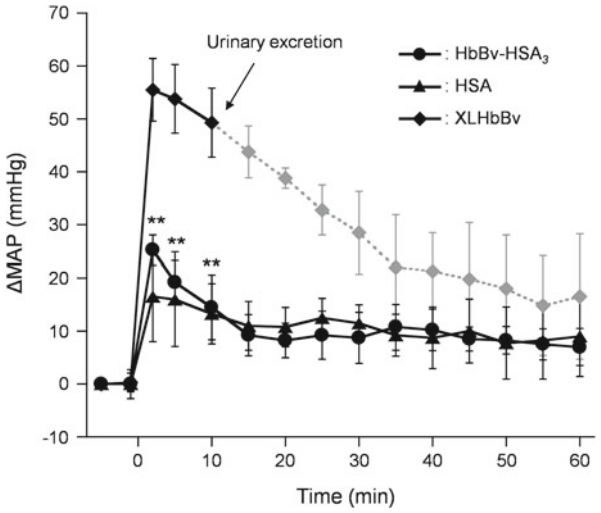

Fig. 9.5 Difference of mean arterial pressure (ΔMAP) from the basal value after intravenous administration of HbBv–HSA_3, HSA, and XLHbBv solutions to rats. Each data point represents the mean ± SD ($n=4$). $^{**}p<0.01$ vs. XLHbBv. Basal values are 84.5 ± 4.4 mmHg in the HbBv–HSA_3 group, 85.0 ± 7.3 mmHg in the HSA group, and 87.5 ± 5.1 mmHg in the XLHbBv group

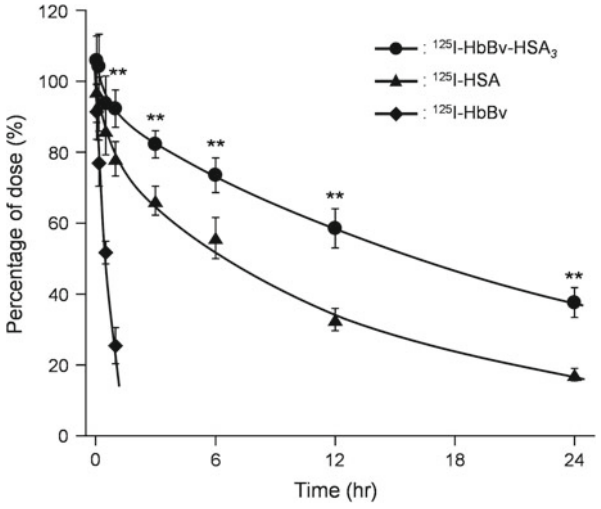

Fig. 9.6 Relative plasma concentration of ^{125}I–HbBv–HSA_3, ^{125}I–HSA, and ^{125}I–HbBv after intravenous administration to rats. Each data point represents the mean ± SD ($n=6$). $^{**}p<0.01$ vs. ^{125}I–HSA

circulation with the half-life ($T_{1/2}$) of 0.53 h (Fig. 9.6). On the one hand, the time course of HbBv–HSA_3 demonstrated very slow kinetics. The $T_{1/2}$ of HbBv–HSA_3 was significantly long (18.5 h) and 1.7-fold greater than that of HSA ($T_{1/2}$ = 11.0 h). The negative surface net charge and large molecular size of HbBv–HSA_3 prevent filtration by the renal glomerulus. We reasoned that the superior blood retention property of HbBv–HSA_3 is attributable to suppression of movement to the extravascular space and renal filtration. All parameters of HbBv–HSA_4 were comparable to those of HbBv–HSA_3 (Yamada et al. 2016). The HSA-binding number on Hb is ineffective to extend the circulation persistence.

All animals injected with HbBv–HSA_3 solution (20 g/dL, 6 mL/kg) were alive for 7 days (Haruki et al. 2015). No remarkable change was found in their appearance or behavior during the measurement time. The body weight increased gradually thereafter. The 26 analytes of the serum biochemical tests after 7 days from the administration showed almost identical data to those of the control groups (HSA injection group, sham-operated group). Microscopic observations of the stained specimens of major organs (liver, kidney, spleen, lungs, and heart) showed no histopathologic disorder in their tissues.

9.5 Various Hemoglobin–Albumin Cluster Derivatives

9.5.1 Low O_2 Affinity Model

In general, HBOCs possess high O_2 affinity compared to RBC (Nagababu et al. 2002; Kluger and Zhang 2003; Hu and Kluger 2008; Li et al. 2009). High O_2 affinity is inferred (i) to prevent the transport of a sufficient amount of O_2 to tissues under physiological conditions, but (ii) to avoid early O_2 offloading on the arterial side of circulation, which may be beneficial for targeted O_2 delivery to the hypoxic regions (Rohlfs et al. 1998; Intaglietta 2004; Winslow 2003; Zhang and Palmer 2010). One of the interesting challenges of artificial O_2 carrier is to prepare a novel HBOC with controllable O_2-binding affinity. It could become a promising RBC substitute and O_2-providing therapeutic reagent for clinical situations.

The heterobifunctional cross-linker SMCC binds not only to the amino groups of Lys on HbA but also to the sulfhydryl group of Cys-93(β). As a result, the HbA–HSA_3 showed high O_2 affinity as described before. In our synthesis, the HbA is kept in the carbonyl form to prevent the autoxidation of the hemes. It is known that the Cys-93(β) in deoxygenated T-state HbA is less accessible to cross-linking agents (Buehler et al. 2006). We found that SMCC cannot bind to the Cys-93(β) of deoxy HbA under N_2 atmosphere. As expected, the cluster prepared in N_2, HbA(T)–HSA_3, showed lower O_2 affinity (P_{50} = 26 Torr) than the native HbA (12 Torr) does (Table 9.1) (Kimura et al. 2015). The n value of HbA(T)–HSA_3 was 1.2, indicating a loss of O_2-binding cooperativity. We reasoned that the HbA center was locked in the T-state conformation by the binding of SMCC under N_2 atmosphere. Interestingly,

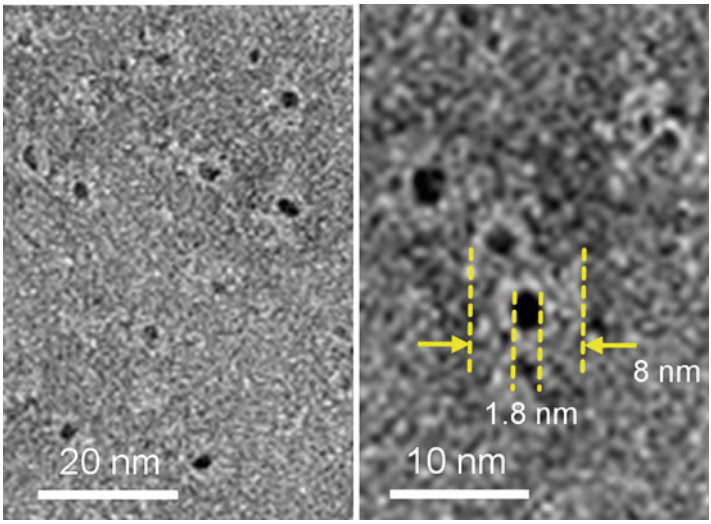

Fig. 9.7 TEM images of HSA–PtNP complexes

the cluster including an $\alpha\alpha$-cross-linked Hb with bis(3,5-dibromosalicyl)fumarate (DBBF), $\alpha\alpha$HbA(T), demonstrated markedly low O_2 affinity (P_{50} = 35 Torr): lower than that of human RBC (Table 9.1) (Kimura et al. 2015). We inferred that the T-state conformation of the $\alpha\alpha$HbA(T) core was preserved strongly by chemical modification of the surface Lys groups. These HbA–HSA_3 clusters with different O_2 affinities can support a new generation of RBC substitute that is better tuned to a role in O_2 delivery.

9.5.2 Antioxidation Model

If one can confer an additional functionality to the external HSA unit of Hb–HSA_3, it would become a promising O_2 carrier with high performance. In this context, we designed to add antioxidant property to HbBv–HSA_3. Pt nanoparticle (PtNP) is known to act as an effective catalysis for both $O_2^{\bullet-}$ and H_2O_2 dismutations (Kajita et al. 2007; Hamasaki et al. 2008; San et al. 2012) and shows almost no cytotoxicity against cells (Hamasaki et al. 2008). We found that small PtNP (diameter: approximately 1.8 nm) is incorporated into HSA. TEM images demonstrated the formation of equivalent complex of HSA and PtNP (Fig. 9.7) (Hosaka et al. 2014). Close inspections of TEM micrographs revealed that each PtNP is incorporated in the center of the protein. We reasoned that one PtNP binds to the positively charged cleft of HSA, forming a 1:1 HSA–PtNP complex. The obtained HSA–PtNP complex showed superoxide dismutase (SOD) ($O_2^{\bullet-}$ dismutation) activity and catalase (H_2O_2 dismutation) activity with high efficiency (Hosaka et al. 2014). The IC_{50}

value (the concentration of enzyme necessary to attain 50 % inhibition of the Cyt. *c* reduction) of the HSA–PtNP complex was 0.16 μM, which resembled the value of native Cu, Zn-SOD (Weser and Schubotz 1981). The HSA–PtNP complex possesses a strong capability to catalyze the dismutation of $O_2^{\bullet-}$. The catalase activity of the HSA–PtNP complex was determined by measuring the H_2O_2 decomposition. The T_{50} value (time required for quenching half of H_2O_2) of HSA–PtNP was 19 min, which is two orders of magnitude larger than that of native catalase.

The HbBv–HSA_3 also possesses the capability of binding PtNP into the HSA shells (Hosaka et al. 2014). The *K* value and binding number of PtNP with the exterior HSA unit were 1.1×10^7 M^{-1} and 1.1. The resultant HbBv–HSA_3(PtNP) cluster forms a very stable O_2 adduct, even in aqueous H_2O_2 (20 μM) solution. We can conclude that the HSA–PtNP shell acts as an efficient scavenger for external H_2O_2 and achieves protection of the core HbBv.

The similar HbBv–HSA_3 derivative with high resistance toward oxidation reactions was prepared by incorporation of Mn(II)-protoporphyrin IX into the exterior HSA units (Daijima and Komatsu 2014). These artificial O_2 carriers having triple functionalities (O_2 transport, $O_2^{\bullet-}$ dismutation, H_2O_2 dismutation) might be useful in clinical conditions with ischemia–reperfusion.

9.6 Conclusion

Covalently wrapping of HbBv or HbA with the most abundant plasma protein, HSA, generated a core–shell structured protein cluster as a promising O_2 carrier for RBC substitute. The cluster was prepared by covalent linkage between Hb's Lys and HSA's Cys-34 using heterobifunctional cross-linker. Major products were isolated using gel filtration chromatography, and the average HSA/Hb ratio of the product was 3.0 ± 0.2. We designated the clusters as HbBv–HSA_3 and HbA–HSA_3. The low isoelectric points (*p*I = 5.1) of the clusters were almost equal to that of HSA, proving the covering of Hb core by negatively charged HSA. The 3D reconstruction of HbBv–HSA_3 based on TEM images revealed a complete triangular structure. The possible spatial arrangement of the HbBv center and HSA exteriors was determined. The HbBv–HSA_3 and HbA–HSA_3 showed higher O_2 affinity (P_{50} = 9 Torr) than the native Hbs. The viscosity measurements and blood cell counting measurements of the mixture solution of whole blood and HbBv–HSA_3 revealed the high blood compatibility of this O_2-carrier protein. The administration of HbBv–HSA_3 to anesthetized rats caused a slight change in MAP, which is identical to that observed in the control group with HSA. This hemodynamic response contrasts against the acute hypertension occurred after infusion of XLHbBv. The $T_{1/2}$ of HbBv–HSA_3 was 1.7-fold longer than that of HSA. The non-vasopressor response and superior blood retention property of HbBv–HSA_3 are attributable to the negative surface net charge and larger molecular weight of the cluster. The serum biochemical parameters resembled those of the control groups. Histopathologic inspections proved that HbBv–HSA_3 gave no negative side effects in any major organ. These results support

the preclinical safety of the HbBv–HSA_3 solution. Clusters prepared under N_2 atmosphere showed low O_2 affinity (P_{50} = 26 Torr). Moreover, the cluster containing an $\alpha\alpha$-cross-linked HbA possessed markedly low O_2 affinity (P_{50} = 35 Torr). A PtNP binds within a cleft of HSA, yielding a stable HSA–PtNP complex. This platinated protein showed high $O_2^{-\bullet}$ and H_2O_2 dismutation activities. The HbBv–HSA_3 also captured PtNP into the HSA units. The obtained HbBv–HSA_3(PtNP) cluster formed very stable O_2 complex even in aqueous H_2O_2 solution. All the results indicate that a series of hemoglobin–albumin clusters can be of tremendous medical importance as an alternative material to RBCs for transfusion in many clinical situations.

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