

Review

Structural and Mutagenic Approach to Create Human Serum Albumin-Based Oxygen Carrier and Photosensitizer

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Summary: Human serum albumin (HSA) is a versatile protein found at high concentration in blood plasma and binds a range of insoluble endogenous and exogenous compounds. We have shown that complexation of functional molecules into HSA creates unique proteins never seen in nature. Complexing an iron-protoporphyrin IX into a genetically engineered heme pocket of recombinant HSA (rHSA) generates an artificial hemoprotein, which binds O₂ reversibly in much the same way as hemoglobin. A pair of site-specific mutations, (i) introduction of a proximal histidine at the Ile-142 position and (ii) substitution of Tyr-161 with Phe or Leu, allows the heme to bind O₂. Additional modification on the distal side of the heme pocket provides rHSA(triple mutant)-heme complexes with a variety of O₂ binding affinity. Complexing a carboxy-C₆₀-fullerene (CF) into HSA generates a protein photosensitizer for photodynamic cancer therapy. Energy transfer occurs from a photoexcited triplet-state of HSA-CF (HSA-³CF*) to O₂, forming singlet oxygen (¹O₂). This protein does not show dark cytotoxicity, but induces cell death under visible light irradiation.

Keywords: human serum albumin; oxygen carrier; photosensitizer; heme; site-directed mutagenesis; fullerene; singlet oxygen; photodynamic therapy

Introduction

Human serum albumin (HSA, Mw: 66.5 kDa) is the most prominent plasma protein in our bloodstream and is characterized by remarkable ability to bind a great variety of hydrophobic molecules.^{1–3)} Typical endogenous ligands for HSA are fatty acids, bilirubin, bile acids and thyroxine.^{4–8)} The protein binds a wide range of drugs. Hemin (Fe³⁺ protoporphyrin IX, **Fig. 1**) released from methemoglobin (metHb) during the enucleation of red blood cells (RBCs) is also captured by HSA. Free hemin is potentially toxic because it may catalyze hydroxyl radical formation. In 1938, Fairely verified that serum protein observed to bind the hemin was albumin and proposed the name “methemalbumin”.⁹⁾ Muller-Eberhard and Morgan reported the UV-vis absorption spectrum of the HSA-hemin complex in 1975 and supposed formation of high-spin hemoprotein with an axial coordination of amino acid residue of the protein.¹⁰⁾ The binding constant for hemin to HSA was determined to be $1.1 \times 10^8 \text{ M}^{-1}$.¹¹⁾ This strong affinity of HSA for hemin has stimulated efforts to develop HSA as an artificial hemoprotein which

mimics the diverse biological reactivities of natural hemoproteins, such as O₂ transport of Hb. If the HSA-based O₂ carrier is realized, it has the potential of acting not only as an RBC substitute, but also an O₂-providing therapeutic reagent. However, it has taken over 60 years to confer the O₂ binding capability on the HSA-hemin complex since Fairely's finding.

HSA is composed of three structurally similar domains (I–III), each containing A and B subdomains.^{12,13)} Crystallographic studies reveal that hemin is bound within a narrow cavity in subdomain IB with an axial coordination of tyrosine to the central Fe³⁺ ion and electrostatic interactions between the porphyrin propionates and a triad of basic amino acid residues (**Fig. 2**).^{14,15)} In terms of the general hydrophobicity of this α -helical pocket, the subdomain IB of HSA has similar features to the heme binding site of Hb or myoglobin (Mb), namely “heme pocket”. However, the reduced form of HSA-hemin is rapidly oxidized by O₂.¹⁶⁾

It is of current interest to prepare albumin-based fake enzymes by exploiting the ligand binding properties of HSA.^{16–19)} Casella *et al.*, demonstrated the HSA-hemin

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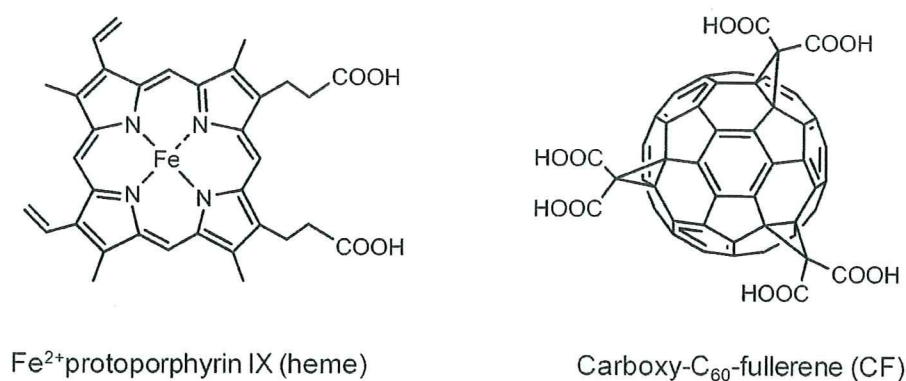
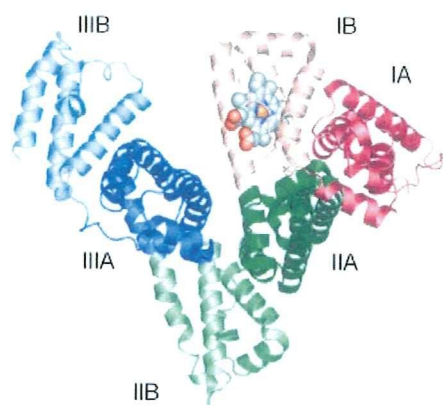
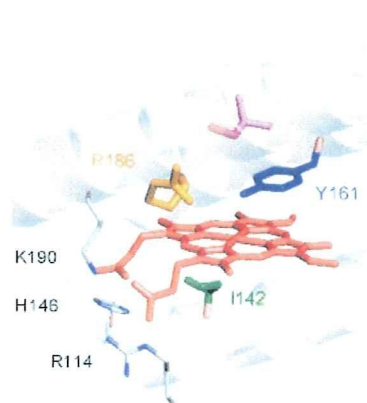


Fig. 1. Chemical formula of heme and CF

(A) HSA-hemin



(B) Heme pocket in subdomain IB



| Wild type | Positions | | | |
|-------------------|-----------|-----|---|---|
| | 142 | 161 | | |
| | I | Y | L | R |
| I142H/Y161F | H | F | L | R |
| I142H/Y161L | H | L | L | R |
| I142H/Y161F/L185N | H | F | | R |
| I142H/Y161L/L185N | H | L | | R |
| I142H/Y161F/L185Q | H | F | | R |
| I142H/Y161L/L185Q | H | L | | R |
| I142H/Y161F/L185H | H | F | | R |
| I142H/Y161L/R186L | H | L | L | |
| I142H/Y161L/R186F | H | L | L | |

Fig. 2. (A) Crystal structure of HSA-hemin complex (1O9X) from ref. 15. (B) Heme pocket structure in subdomain IB and positions of amino acids where site-specific mutations are introduced. Abbreviations of triple mutants are shown in the table.

complex exhibited weak peroxidase and catalase activity.¹⁶⁾ Gross *et al.* showed that HSA incorporating Fe^{3+} corrole or Mn^{3+} corrole is useful for enantioselective sulfoxidation of prochiral sulfides by hydrogen peroxide (up to 74% ee).¹⁷⁾ Reets *et al.* used HSA- Cu^{2+} phthalocyanine as Lewis acidic catalyst for highly enantioselective Diels-Alder reactions of azachalcones (85–98% ee).¹⁸⁾ Moreover, Gozin *et al.* prepared bovine serum albumin- C_{60} fullerene hybrid using cyclodextrin- C_{60} intermediate.¹⁹⁾ We investigated HSA incorporating synthetic Fe^{2+} porphyrin (FeP) “HSA-FeP” as an entirely synthetic O_2 transporter.^{20–39)} A saline solution of HSA-FeP is a most promising material for RBC substitute. In this review, we describe very recent results on the O_2 carrier formed by complexing natural Fe^{2+} protoporphyrin IX (heme) with genetically engineered HSA and on photosensitizer formed by complexing carboxy- C_{60} -fullerene (CF) with HSA (Fig. 1).^{40–43)}

Recombinant HSA Mutants Complexed with Heme (rHSA-Heme) as Oxygen Carrier

Naturally occurring HSA-hemin complex: Crystal structure analysis revealed that heme is bound within a narrow D-shaped hydrophobic cavity in subdomain IB of HSA where the central ferric ion is axially coordinated by Tyr-161 and the two propionate side-chains at the porphyrin periphery form salt-bridges with a triad of basic amino acid residues (Arg-114, His-146, Lys-190) (Fig. 2).^{14,15)} The UV-vis absorption spectrum of the HSA-hemin solution showed a Soret band at 405 nm and the $p\pi$ - $d\pi$ charge-transfer (CT) band at 624 nm (Fig. 3). The dominant features of the spectrum were almost the same as those of previously reported HSA-hemin^{10,16)} and H93Y recombinant Mb [rMb(H93Y)], in which the proximal His-93 was changed to tyrosine.^{44,45)} Our absorption spectral data imply that the heme is bound to Tyr-161 of HSA to form a ferric five-coordinate high-spin complex. Nevertheless, CT absorptions of the HSA-hemin appeared at a higher wavelength (λ_{max} : 624 nm) compared to

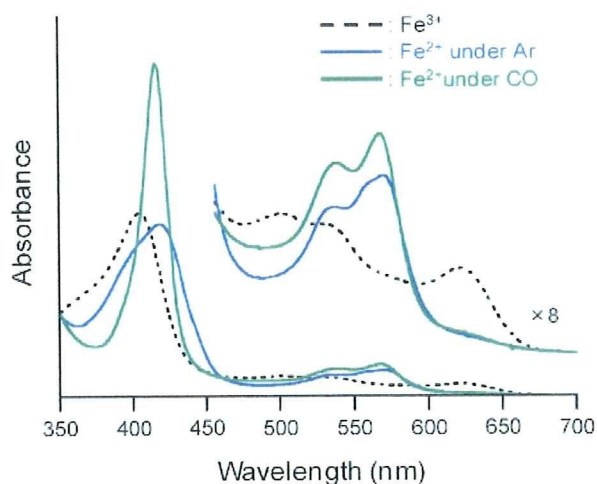


Fig. 3. UV-vis absorption spectral changes of the HSA-heme in 50 mM potassium phosphate buffered solution (pH 7.0)

rMb(H93Y) (λ_{\max} : 598–599 nm), which suggests that the axial coordination of Tyr-161 to the heme is weaker than that of rMb(H93Y). The magnetic circular dichroism (MCD) spectra also support the formation of a five-coordinate high-spin heme complex with weak Tyr-161 ligation in HSA-hemin.⁴¹⁾

Reduction of the ferric HSA-hemin by the addition of aqueous sodium dithionite under an Ar atmosphere gave a ferrous heme complex with a Soret band at 419 nm and two definite Q bands at 538 and 570 nm (Fig. 3). Based on careful inspection of UV-vis and MCD spectra, we concluded that the ferrous HSA-heme is an unusual mixture of a five-coordinate high-spin complex with Tyr-161 and a four-coordinate intermediate-spin state under an Ar atmosphere. Smulevich *et al.* recently measured resonance Raman spectroscopy of the HSA-hemin complex and strongly supported our interpretation.⁴⁶⁾

Upon the addition of O₂ gas through this solution, the central ferrous ion was rapidly oxidized even at 5 °C. This is due to the fact that HSA lacks the proximal histidine which enables the prosthetic heme group to bind O₂ in Hb and Mb.

rHSA(double mutant)-heme complexes: On the basis of the crystal structure of the HSA-hemin complex, we used site-directed mutagenesis to introduce a histidine into the heme binding site of HSA. This should provide axial coordination to the central Fe²⁺ atom of the heme and thereby promote O₂ binding. Tyr-161 was the first candidate for site-directed mutagenesis (Fig. 2), but our simulation results showed that the distance from N_ε(Y161H) to Fe(heme) would be too far (~4.0 Å). As an alternative, favorable positions for axial imidazole insertion would be Ile-142. The N_ε(I142H)-Fe distance was estimated to be 2.31 Å. We therefore designed and prepared rHSA mutants, rHSA(I142H/Y161F) and rHSA(I142H/Y161L) (Fig. 2) and evaluated the O₂ bind-

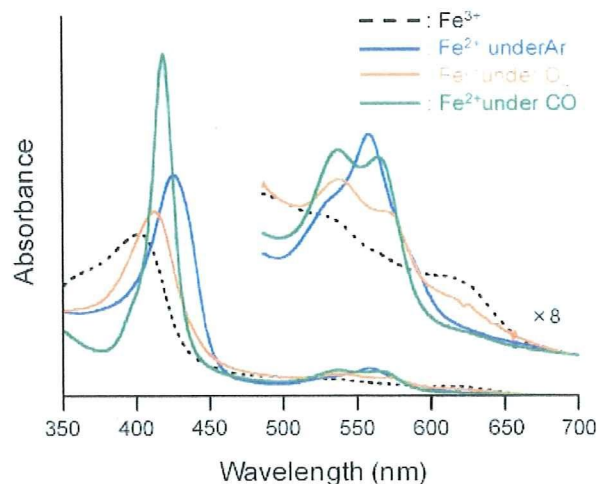


Fig. 4. UV-vis absorption spectral changes of the rHSA(I142H/Y161L)-heme in 50 mM potassium phosphate buffered solution (pH 7.0)

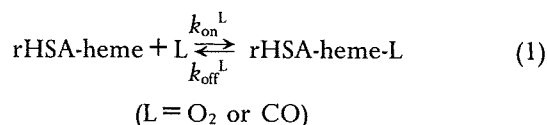
ing properties of the heme complexes.

The rHSA(double mutant)-hemin was easily reduced to the ferrous complex under an Ar atmosphere. A single broad Q band (λ_{\max} : 559 nm) in the visible absorption of rHSA(I142H/Y161F)-heme and rHSA(I142H/Y161L)-heme was similar to that of deoxy Mb⁴⁷⁾ or the synthetic chelated-heme in N,N-dimethylformamide (Fig. 4),⁴⁸⁾ indicating the formation of a five-N-coordinate high-spin complex. The heme was incorporated into the artificial heme pocket with axial His-142 coordination. Upon exposure of the rHSA(I142H/Y161F)-heme and rHSA(I142H/Y161L)-heme solutions to O₂, the UV-vis absorptions changed to that of the O₂ adduct complex at 22 °C (Fig. 4).^{47,48)} After flowing CO gas, these hemoproteins produced very stable carbonyl complexes. The single mutant rHSA(I142H)-heme which retains Y161, could not bind O₂. The polar phenolate residue at the top of the porphyrin plane probably accelerates the proton-driven oxidation of the Fe²⁺ center.

To evaluate the kinetics of O₂ and CO binding to rHSA(double mutant)-heme, laser flash photolysis experiments were carried out.^{41,42,49,50)} It is noteworthy that the absorbance decay accompanying CO recombination to rHSA(I142H/Y161F)-heme and rHSA(I142H/Y161L)-heme was composed of double-exponential profiles normally not seen in Mb. The rebinding of O₂ to the hemoproteins followed simple monophasic decay. Based on numbers from investigation on synthetic Fe²⁺ porphyrin models, it has been shown that a bending strain in the proximal histidine coordination to the central Fe²⁺ atom, “proximal-side steric effect”, increases the dissociation rate and decreases the association rate for CO, whereas it increases the O₂ dissociation rate without changing the kinetics of O₂ association.^{49,50)} Our interpretation was that there may be two different geometries for axial

His-142 coordination to the central Fe²⁺ of the heme (species I and II), each one accounting for a component of biphasic kinetics of CO rebinding. The heme molecule appears to bind to subdomain IB in two orientation (180° rotational isomers), giving two different geometries for axial His-142 coordination.

The association and dissociation rate constants for O₂ or CO ($k_{on}^{O_2}$, $k_{off}^{O_2}$, k_{on}^{CO} , k_{off}^{CO}) and binding affinities for O₂ or CO [$P_{1/2}^{O_2} = (K^{O_2})^{-1}$, $P_{1/2}^{CO} = (K^{CO})^{-1}$] for the rHSA(mutant)-heme complexes (eq. 1) are summarized in **Table 1 and 2**.



$P_{1/2}^{O_2}$ of rHSA(I142H/Y161F)-heme and rHSA(I142H/

Table 1. O₂ binding parameters of rHSA(mutant)-heme complexes in 50 mM potassium phosphate buffered solution (pH 7.0) at 22°C^a

| Hemoproteins | $k_{on}^{O_2}$ ($\mu\text{M}^{-1}\text{s}^{-1}$) | $k_{off}^{O_2}$ (ms ⁻¹) | | $P_{1/2}^{O_2}$ (Torr) | |
|------------------------------------|---|-------------------------------------|------|------------------------|-----|
| | | I | II | I | II |
| rHSA(I142H/Y161F)-Heme | 20 | 0.10 | 0.99 | 3 | 31 |
| rHSA(I142H/Y161L)-Heme | 7.5 | 0.22 | 1.70 | 18 | 134 |
| rHSA(I142H/Y161F/L185N)-Heme | 26 | 0.10 | 1.03 | 2 | 24 |
| rHSA(I142H/Y161L/L185N)-Heme | 14 | 0.02 | 0.29 | 1 | 14 |
| rHSA(I142H/Y161L/R186L)-Heme | 25 | 0.41 | 8.59 | 10 | 209 |
| rHSA(I142H/Y161L/R186F)-Heme | 21 | 0.29 | 7.01 | 9 | 203 |
| Hb α (R-state) ^b | 33 ^c | 0.013 ^d | | 0.24 | |
| Mb ^e | 14 | 0.012 | | 0.51 | |
| RBC ^f | | | | 8 | |

^aNumber I or II indicates species I or II.^{41,42} ^bHuman Hb α -subunit. ^cIn 0.1 M phosphate buffer (pH 7.0, 21.5°C).⁵¹ ^dIn 10 mM phosphate buffer (pH 7.0, 20°C).⁵² ^eSperm whale Mb. In 0.1 M potassium phosphate buffer (pH 7.0, 20°C).⁵³ ^fHuman red cell suspension. In isotonic buffer (pH 7.4, 20°C).⁶²

Y161L)-heme were determined to be 3–18 and 31–134 Torr for species I (the first phase) and species II (the second phase), respectively. Even the O₂ binding affinity of species I was 6–75-fold lower than that of native Hb α (R-state) and Mb.^{51–53} This low affinity for O₂ was kinetically due to an 8–18-fold increase in $k_{off}^{O_2}$.

In Hb and Mb, the distal His-64 stabilizes bound O₂ due to the hydrogen bonding. Rohlfs *et al.* showed that replacement of His-64 in rMb with nonpolar amino acid residues (Leu or Phe) results in loss of hydrogen bonding and increases $k_{off}^{O_2}$ (342–833-fold higher than Mb).⁵³ In rHSA(double mutant)-heme, dioxygenated heme is buried in the core of the hydrophobic cavity without any counterpart for the hydrogen bond; thus the even small $k_{off}^{O_2}$ for species I are greater than those of Hb α and Mb. In species II, the proximal-side steric effect further enhanced the dissociation rates and caused large decline in O₂ binding affinity.

We compared O₂ and CO binding properties of the rHSA(I142H/Y161F)-heme and rHSA(I142H/Y161L)-heme and found an interesting distal-side steric effect on ligand binding.⁴¹ The rHSA(I142H/Y161F)-heme complex binds O₂ and CO about 4–6 times more strongly than rHSA(I142H/Y161L)-heme, because of high association rate constants. This affect appears due to the concerted steric effects of the residues at positions 161 and 185. In the rHSA(I142H/Y161F)-heme complex, the bulky benzyl side-chain of Phe-161 (137 Å³) may prevent rotation of neighboring Leu-185, thereby providing easy access of O₂ to the heme (**Fig. 5A**). In contrast, substitution of Phe-161 by the smaller Leu-161 (102 Å³) may allow free rotation of the isopropyl group of Leu-185, which reduces the volume of the distal side (**Fig. 5B**) and hinders association of O₂ and CO with heme.

rHSA(triple mutant)-heme complexes with a distal base: HSA(I142H/Y161F)-heme and HSA(I142H/Y161L)-heme bind and release O₂, but their O₂ binding affinity is one order of magnitude lower than that of Hb α

Table 2. CO binding parameters of rHSA(mutant)-heme complexes in 50 mM potassium phosphate buffered solution (pH 7.0) at 22°C^a

| Hemoproteins | k_{on}^{CO} ($\mu\text{M}^{-1}\text{s}^{-1}$) | | k_{off}^{CO} (s ⁻¹) | | $P_{1/2}^{CO}$ (Torr) | |
|------------------------------------|---|------|-----------------------------------|-------|-----------------------|-------|
| | I | II | I | II | I | II |
| rHSA(I142H/Y161F)-Heme | 6.8 | 0.72 | 0.009 | 0.061 | 0.0011 | 0.068 |
| rHSA(I142H/Y161L)-Heme | 2.0 | 0.27 | 0.013 | 0.079 | 0.0053 | 0.240 |
| rHSA(I142H/Y161F/L185N)-Heme | 7.7 | 1.09 | 0.008 | 0.043 | 0.0008 | 0.032 |
| rHSA(I142H/Y161L/L185N)-Heme | 6.8 | 1.60 | 0.008 | 0.039 | 0.0010 | 0.020 |
| rHSA(I142H/Y161L/R186L)-Heme | 5.0 | 0.57 | 0.011 | 0.165 | 0.0018 | 0.234 |
| rHSA(I142H/Y161L/R186F)-Heme | 7.9 | 1.12 | 0.010 | 0.148 | 0.0010 | 0.107 |
| Hb α (R-state) ^b | 4.6 ^c | | 0.009 ^d | | 0.0016 ^e | |
| Mb ^f | 0.51 | | 0.019 | | 0.030 | |

^aNumber I or II indicates species I or II.^{41,42} ^bHuman Hb α -subunit. ^cIn 50 mM potassium phosphate buffer (pH 7.0, 20°C).⁶³ ^dIn 0.1 M phosphate buffer (pH 7.0, 20°C).⁶¹ ^eCalculated from $(k_{on}^{CO}/k_{off}^{CO})^{-1}$. ^fSperm whale Mb. ^gIn 0.1 M potassium phosphate buffer (pH 7.0, 20°C).⁶²

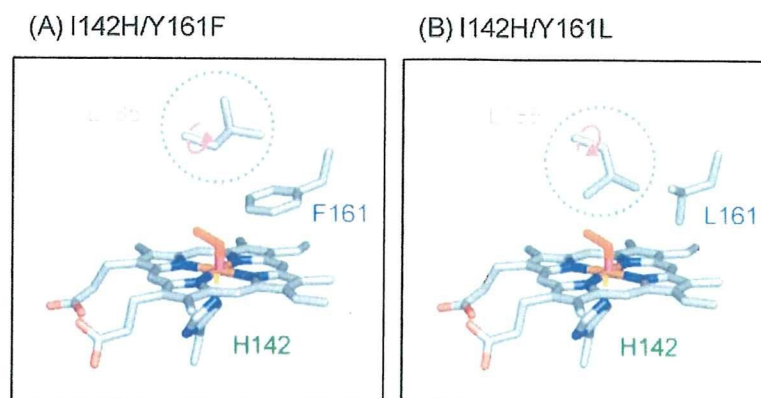


Fig. 5. Structural models of the heme pocket in dioxygenated rHSA(I142H/Y161F)-heme and rHSA(I142H/Y161L)-heme. Distal-side steric effect of Leu-185 on O₂ and CO association

(R-state). To develop this O₂ carrying albumin as a blood substitute, it is required to regulate O₂ binding affinity suitable for Hb, Mb, and human RBC. One approach to increasing O₂ binding affinity of rHSA(double mutant)-heme would be to introduce another histidine into an appropriate position on the distal side of the heme pocket. The N_ε atom of histidine may act as a proton donor to form an hydrogen bond with the coordinated O₂.

In Hb and Mb, His-64 on the distal side of the heme plays a crucial role for tuning their ligand affinity, as shown by a neutron diffraction study and high-resolution X-ray structural analysis.^{54,55} A number of systematic investigations on site-directed mutants of Hb and Mb show that the overall polarity and packing of the distal residues are key factors in regulating the equilibrium constants for ligand binding.^{53,56,57} Then, we generated new rHSA(triple mutant)-heme complexes, in which the specific third mutation was introduced near the O₂ binding site. Another important point in this design is to prevent the formation of a six-coordinate low-spin ferrous complex. Bis-histidyl hemochromes are generally autoxidized by O₂.^{58–60} Therefore, the distal amino acid must be located relatively far (> 4 Å) from the central iron. Our simulation indicated that the favorable position for the distal His insertion was Leu-185 (**Fig. 2**). Thus the polarity of the distal side of the heme in rHSA(I142H/Y161F) and rHSA(I142H/Y161L) was increased by replacing Leu-185 with asparagines, glutamine, and histidine using site-directed mutagenesis. Five triple mutants [rHSA(I142H/Y161F/L185N), rHSA(I142H/Y161L/L185N), rHSA(I142H/Y161F/L185Q), rHSA(I142H/Y161L/L185Q), and rHSA(I142H/Y161F/L185H)] (**Fig. 2**) were thus cloned and their heme complexes were prepared.

The rHSA(I142H/Y161F/L185N)-heme, rHSA(I142H/Y161L/L185N)-heme, and rHSA(I142H/Y161L/L185Q)-heme showed a visible absorption band at 558–559 nm with a small shoulder at 530 nm, that was similar to the spectrum observed for the rHSA(I142H/Y161F)-heme,

rHSA(I142H/Y161L)-heme, deoxy Mb⁴⁷) and synthetic chelated-heme.⁴⁸) The spectral patterns indicated the formation of a five-*N*-coordinate high-spin complex. In the spectra of the rHSA(I142H/Y161F/L185Q)-heme and rHSA(I142H/Y161F/L185H)-heme, the β band at 528 nm appeared slightly sharp, suggesting partial formation of a six-*N*-coordinate heme complex. On the basis of all the UV-vis absorption and MCD spectral results, we concluded that the heme is axially coordinated by His-142 in rHSA(triple mutant) and forms a five-*N*-coordinate high-spin ferrous complex under an Ar atmosphere in the case of I142H/Y161F/L185N, I142H/Y161L/L185N, and I142H/Y161L/L185Q mutants (**Fig. 6A, B, D**). In addition to the His-142 ligation, Gln-185 and His-185 partially interact with the sixth coordinate position of the central Fe²⁺ ion of the heme in I142H/Y161F/L185Q and I142H/Y161F/L185H mutants in spite of the bulky Phe-161 (**Fig. 6C, E**). We suppose that the rHSA(I142H/Y161L/L185Q)-heme may also produce a six-coordinate low-spin complex, because the small Leu-161 allows free rotation of Gln-185. However, it gave a five-coordinate high-spin ferrous complex. This suggests that the long Gln-185 may interact with neighboring amino acids (**Fig. 6D**).

With exposure of the rHSA(I142H/Y161F/L185N)-heme and rHSA(I142H/Y161L/L185N)-heme solutions to O₂, the UV-vis absorptions changed to that of the O₂ adduct complex at 22°C. However, the rHSA(I142H/Y161L/L185Q)-heme complex bound O₂ only at 5°C and was oxidized at 22°C. This rapid oxidation may suggest that the distal side of the heme has an open structure, which allows easy access of water to the heme. The rHSA(I142H/Y161F/L185Q)-heme and rHSA(I142H/Y161F/L185H)-heme complexes could not bind O₂ even at low temperature. After introducing CO gas, all hemoproteins produced stable carbonyl complexes with identical absorption spectral patterns.^{47,48})

In rHSA(double mutant)-heme complexes, there exists

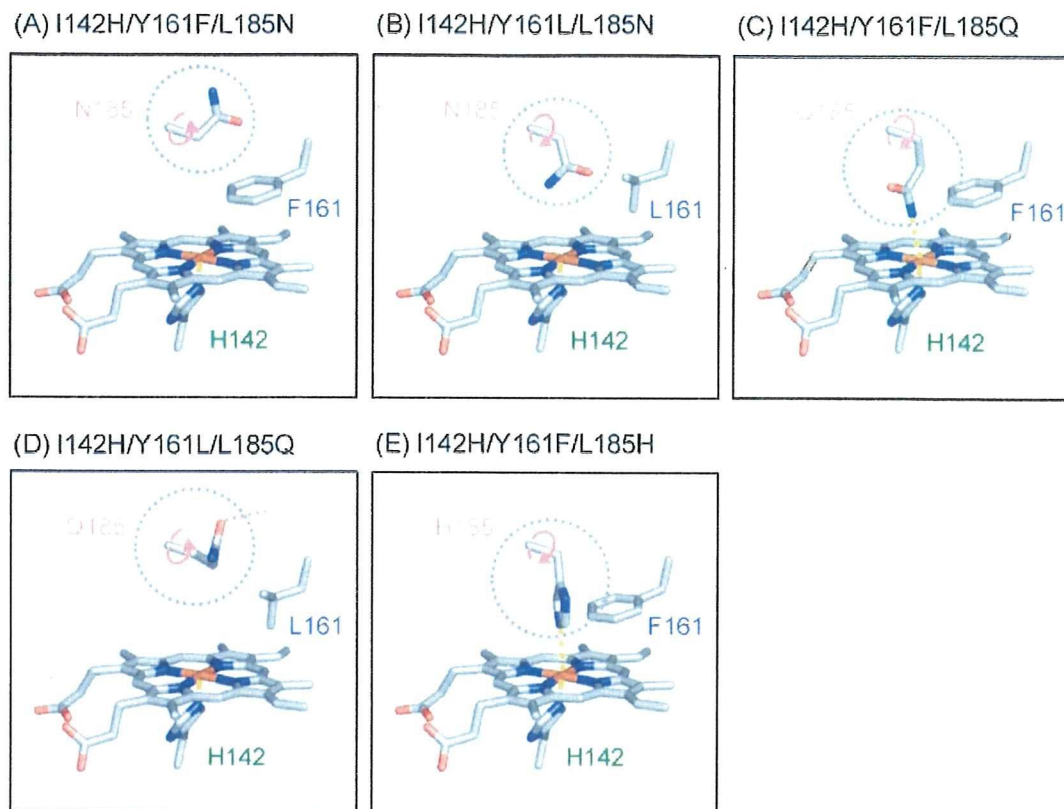


Fig. 6. Structural models of the heme pocket in rHSA(triple mutant)-heme complexes. Distal-side effect of engineered amino acid at position 185.

two geometries of axial His-142 coordination to the central Fe^{2+} ion of the heme (species I and II). In species I, the proximal His coordinates to the heme without strain, while in species II, the His-Fe ligation involves some distortion, resulting in weaker O_2 binding. In rHSA(triple mutant)-hemes, this alternative geometry of the heme plane would also arise in the same manner. As expected, the binding behavior of O_2 for rHSA(I142H/Y161F/L185N)-heme and rHSA(I142H/Y161L/L185N)-heme was almost the same as that of the original double mutants.

The $k_{\text{off}}^{\text{CO}}$ is normally an indicator of bending strain in the proximal His coordination to the central Fe^{2+} ion.^{49,50} The rHSA(I142H/Y161F)-heme, rHSA(I142H/Y161L)-heme, rHSA(I142H/Y161F/L185N)-heme, and rHSA(I142H/Y161L/L185N)-heme exhibited similar $k_{\text{off}}^{\text{CO}}$ in species I (0.008–0.013 s^{-1}) which were identical to that of Hb α (R-state) (0.009 s^{-1}) (Table 2).⁶¹ This indicates that the axial His-142 ligation to the heme in these artificial hemoproteins has the same features as that of Hb.

O_2 and CO binding parameters for the rHSA(I142H/Y161F)-heme and rHSA(I142H/Y161F/L185N)-heme complexes did not show any significant differences. The bulky benzyl side-chain of Phe-161 may retard rotation of the polar amide group of Asn-185 (Fig. 6A). In

contrast, there are significant differences in the O_2 and CO binding parameters for rHSA(I142H/Y161L)-heme and rHSA(I142H/Y161L/L185N)-heme. The Asn-185 induced 18-fold and 10-fold increases in the O_2 binding affinity for species I and II. The increase was mainly due to the 6–11-fold reduction of $k_{\text{off}}^{\text{O}_2}$. It is noteworthy that the high O_2 binding affinity ($P_{1/2}^{\text{O}_2}$: 1 Torr) for rHSA(I142H/Y161L/L185N)-heme is close to that of natural Hb α (0.24 Torr)^{51,52} and Mb (0.5 Torr)⁵³ (Table 2).

Further tuning of O_2 binding affinity of rHSA(triple mutant)-heme complexes: The O_2 binding equilibrium and kinetics of rHSA-heme complexes are significantly enhanced by site-directed mutagenesis. However, for artificial rHSA-heme solutions to provide effective O_2 transport from lungs to tissues in the body, the O_2 binding affinity of HSA(I142H/Y161L/L185N)-heme should be reduced to that of human RBC ($P_{1/2}^{\text{O}_2}$: 8 Torr).⁶² This requires an O_2 binding affinity intermediate between the values for rHSA(I142H/Y161L)-heme and rHSA(I142H/Y161L/L185N)-heme.

We thus designed new triple mutants, rHSA(I142H/Y161L/R186L) and rHSA(I142H/Y161L/R186F) (Fig. 2). An important structural factor in these mutants is Y161L, which allows rotation of the isopropyl group of Leu-185 above the O_2 coordination site. The reduced ferrous forms produced the five-*N*-coordinate high-spin

complexes under an Ar atmosphere and formed the O₂ adduct complex after bubbling O₂ gas. The distinct features of all the spectra were similar to those of the rHSA(I142H/Y161L)-heme.

Following laser flash photolysis, the absorption decay associated with O₂ recombination to the rHSA(I142H/Y161L/R186L)-heme and rHSA(I142H/Y161L/R186F)-heme was monophasic. The kinetics for CO rebinding were still composed of two single-exponentials, consistent with the existence of two different geometries of the axial His-142 coordination to the central Fe²⁺ ion of the heme.

The rHSA(I142H/Y161F)-heme binds O₂ with higher affinity than rHSA(I142H/Y161L) because of the presence of Leu at position 161, which allows a downward rotation of the L185 side chain and reduces the affinity. However, insertion of Leu or Phe at position 186 in the presence of Leu-161 yielded $k_{on}^{O_2}$ and k_{on}^{CO} 3–4-fold higher than those of rHSA(I142H/Y161L)-heme. The presence of a hydrophobic residue at position 186 may restrict the downward rotation of the isopropyl group of Leu-185 (**Fig. 7**). Overall, the O₂ and CO binding parameters of rHSA(I142H/Y161L/R186L)-heme and rHSA(I142H/Y161L/R186F)-heme were more similar to those of rHSA(I142H/Y161F)-heme, but their O₂ dissociation rate constants were 3–4-fold higher than found for rHSA(I142H/Y161F)-heme, which modestly reduced O₂ binding affinity. This may be due to increase in hydrophobicity in the distal pocket. It is noteworthy that O₂ binding affinity of the rHSA(I142H/Y161L/R186L)-heme ($P_{1/2}^{O_2}$: 10 Torr) and rHSA(I142H/Y161L/R186F)-heme ($P_{1/2}^{O_2}$: 9 Torr) is essentially the same to that of human RBC ($P_{1/2}^{O_2}$: 8 Torr).

Circulation life of rHSA complexes: It would be of great importance to study the in vivo circulation behavior of rHSA(mutant)-heme complex for practical medical applications. This investigation is currently undergoing, but we have several results on the HSA-FeP complex. In general, the ligand molecule complexed with HSA gradually dissociates from the protein when infused into the body, since the ligand is noncovalently bound into the hydrophobic cavity of HSA. We found that surface modification of HSA-FeP by poly(ethylene glycol) (PEG) significantly improved the circulation lifetime of FeP in animals and thereby retained its O₂-transporting ability for a long period.³⁴ Interestingly, the linkage form of the PEG chain dramatically affects the circulation persistence of FeP. Maleimide-PEG conjugates showed 6–8-fold longer lifetime compared to the succinimide-PEG analogue.³⁴ PEG modified rHSA(mutant)-heme would also remain in the circulatory system with long persistence, which could be a potential advantage for O₂ delivery to the tissues.

HSA Incorporating C₆₀ Fullerene as Photosensitizer for Photodynamic Therapy

Structure and photophysical properties of HSA-fullerene complex: Photodynamic therapy (PDT) is advanced cancer treatment involving a photosensitizer, visible light and tissue O₂.^{64,65} Singlet oxygen (¹O₂) formed by energy transfer from photoexcited state of sensitizer is highly cytotoxic and has been implicated as an intermediary species leading to cell death in tumors. To accelerate this ¹O₂ formation, various organic dyes, especially porphyrin derivatives, have been designed as photosensitizers.⁶⁶ The most widely used reagent in clinical PDT is Photofrin, which is a mixture of water soluble hematoporphyrin oligomers.^{63,64,69} Several porphyrin or chlorin compounds are also being tested.^{63,64,68,69} Another potential reagent is 5-aminolevulinic acid (ALA).^{70,71} ALA enters into cancer cells and induces the biosynthesis of protoporphyrin IX (PP). Buckminster[60]fullerene (C₆₀) produces ¹O₂ by energy transfer with extremely high quantum yield (Φ_A : 0.96, λ_{exc} = 532 nm)^{72,73} and shows strong resistance against laser irradiation. Consequently, various water-soluble fullerenes and fullerene-polymer hybrids have been synthesized as new photosensitizing reagents.^{73–76} However, to evaluate the biological function of fullerene, one must investigate the structure and properties of the HSA-fullerene complex, because exogenous compounds administered into the bloodstream are generally captured by HSA. We prepared HSA complexed with a tris(dicarboxymethylene)[60] fullerene C₃-isomer (HSA-CF) and characterized its photoinduced energy transfer to O₂ to produce ¹O₂ and its cytotoxicity to cancer cells under visible light.⁴³

The HSA-CF complex was prepared essentially as described previously for HSA-hemin complex.⁴¹ The gel permeation chromatogram of the orange-colored protein exhibited a single elution peak. This indicates that CF efficiently binds to HSA. The UV-vis absorption spectroscopic features of the HSA-CF solution are the sum of those from the individual HSA and CF (**Fig. 8**), which implies that CF is monomolecularly incorporated into HSA and no specific interaction occurs between the two molecules at the ground state. Gozin *et al.* reported that CF is incorporated into the subdomain IIA of HSA with a binding constant of $1.2 \times 10^7 \text{ M}^{-1}$ by fluorescent quenching experiments.⁷⁷

The HSA-CF complex was sufficiently stable to apply to HPLC measurement. In the elution profile, only a single peak was observable. The ratio of peak intensity monitored at 280 nm (based on HSA) and at 490 nm (based on CF) ($I_{280}/I_{490} = 30$) was exactly the same as the absorbance ratio at 280 nm and 490 nm in the UV-vis absorption spectrum (A_{280}/A_{490}) of HSA-CF, which suggests that all CF molecules are eluted within the HSA fraction. The ESI-TOF mass spectroscopy showed a distinct ion

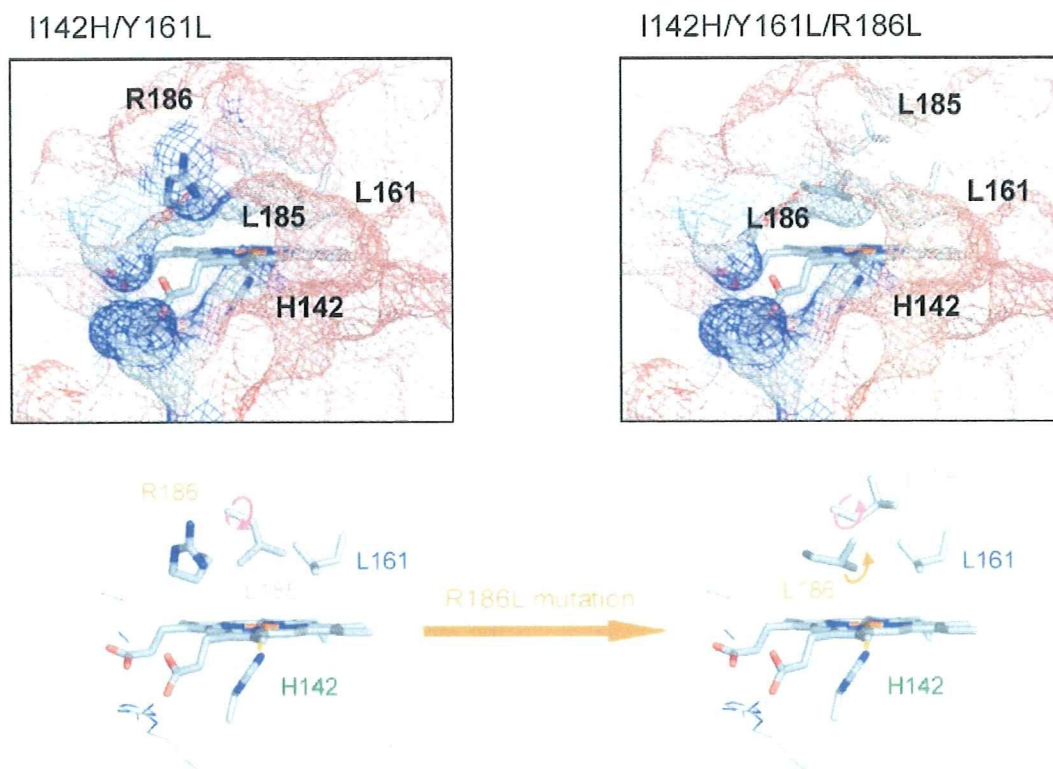


Fig. 7. Structural models of rHSA(I142H/Y161L)-heme and rHSA(I142H/Y161L/R186L)-heme complexes. Introduction of R186L mutation may induce upward rotation of the L185 residue.

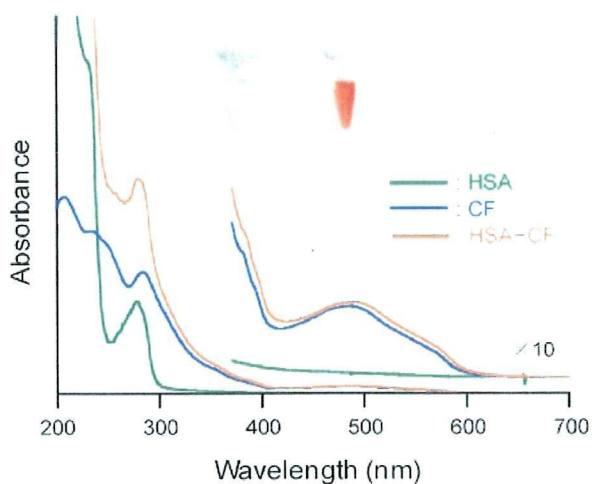


Fig. 8. UV-vis absorption spectrum of HSA-CF, CF, and HSA in 50 mM phosphate buffered solution (pH 7.0). Inset photographs are 5 g dL⁻¹ HSA (left) and HSA-CF (right) solutions.

peak at 67,587 Da, which corresponds to the mass of the equivalent complex of HSA-CF. CD spectral patterns and intensities of HSA-CF were identical to those of HSA. We inferred that fullerene binding did not change the highly ordered structure of the protein. The incorporation of negatively charged CF may influence the surface charge

distribution of albumin. However, isoelectric focusing of HSA-CF indicated the same isoelectric point with HSA.

We used small-angle X-ray scattering (SAXS) to evaluate the globular particle structure and protein-protein interactions of HSA-CF. The pair-distance distribution functions [$p(r)$] of HSA-CF and HSA were almost identical to the curve calculated from crystallographic data of HSA (**Fig. 9A**). This demonstrates that the maximum diameter (D_{\max} : ca. 8 nm) and three-dimensional particle shape of HSA are not changed by complexation of the CF molecule. The extrapolated structure factors [$S(q \rightarrow 0)$] reflect the net repulsive forces between the protein molecules. Plots of $S(q \rightarrow 0)$ for HSA-CF and HSA were lower than that predicted for hard sphere with an identical volume fraction (**Fig. 9B**), this being due to the strong electrostatic repulsion between the proteins. The perfectly same lines of HSA-CF and HSA suggest that the HSA molecule preserves its surface net charge upon CF binding. We conclude that CF is accommodated into the deep hydrophobic cavity of HSA with internal charge neutralization, and it does not induce marked change in the globular particle size or surface charge distribution of HSA.

Photoexcited triplet state of the HSA-CF complex and $^1\text{O}_2$ production: Photoexcitation of fullerenes generates the singlet state, which undergoes intersystem crossing to the triplet state in high yield.^{72,73} Laser

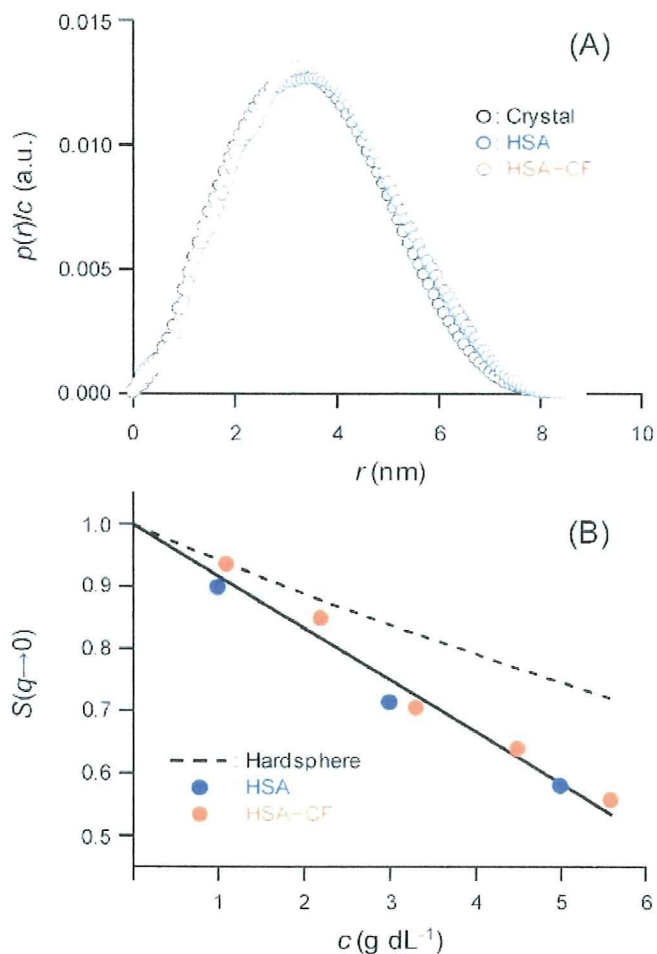


Fig. 9. (A) Pair-distance distribution functions $[p(r)]$ obtained from SAXS measurements of HSA-CF and HSA in 150 mM PBS solutions. (B) The extrapolated structure factors $[S(q \rightarrow 0)]$ of HSA-CF and HSA

flash photolysis of the HSA-CF solution under an N₂ atmosphere gave a triplet-triplet (T-T) absorption spectrum of the ³CF* chromophore ($\lambda_{\max} = 740$ nm).^{73,75,78} The time course of the absorbance decay was composed of a single exponential kinetics with a lifetime (τ_T) of 46 μ s. In the presence of O₂, the triplet lifetime of HSA-CF markedly decreased. Energy transfer took place from HSA-³CF* to the O₂ molecule to generate active ¹O₂. The Stern-Volmer plot depicts a linear correlation for O₂ concentrations (0–1.0 mM), giving the quenching rate constant [$k_q(\text{O}_2)$: $2.2 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$]. The intensity of the visible band of HSA-CF ($\lambda_{\max} = 490$ nm) did not change after 10³-times laser flash irradiation ($\lambda_{\text{ex}} = 532$ nm, 0.5 W) under air. In contrast, Soret band of HSA-PP was 6% bleached after identical flash photolysis. The light resistance of CF chromosphere is significantly higher than that of PP.

The quantum yield of ¹O₂ production (Φ_{Δ}) for HSA-CF was determined from the value of emission intensity of the ¹O₂ ($\lambda_{\max} 1270$ nm).⁷⁹ Φ_{Δ} of HSA-CF (0.46) was in

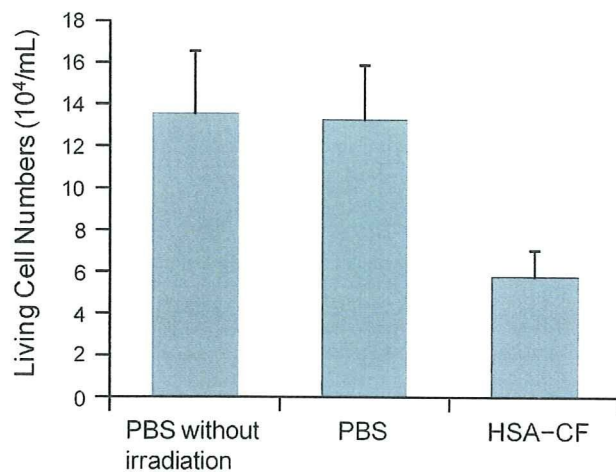


Fig. 10. Living cell numbers of LY80 with HSA-CF and PBS after visible light irradiation (20 mW cm⁻², 2 h, 36 ± 1°C). Each value represents the mean ± SD ($n = 4$).

the same range as that of monomeric CF (0.48), methylene blue (0.52), protoporphyrin IX dimethyl ester in benzene (0.59),⁷⁹ and substituted fullerenes in organic solvent,^{80,81} but somewhat lower than for rose bengal (0.75), hematoporphyrin in methanol (0.74),⁸² and pristine C₆₀ (0.96) in benzene.⁷² Substitution of the C₆₀ fullerene causes perturbation of the electronic structure of the fullerene core, thereby decreasing the quantum yield for ¹O₂ formation.^{80,81}

Cytotoxicity of HSA-CF complex: The cytotoxicity and photodynamic activity of the HSA-CF complex to LY80 tumor cells was evaluated. The cell cultures were first incubated for 24 h in the dark with HSA-CF (20 μ M) at 37°C under 5% CO₂. Cell numbers after incubation were identical to those of the control group with the phosphate buffered saline (PBS, pH 7.4) solution. The HSA-CF complex showed no dark cytotoxicity. The cell culture plate was then exposed to visible light of 350–600 nm (20 mW cm⁻²) for 2 h at 36 ± 1°C. Some cells mixed with HSA-CF showed morphological change after light irradiation, whereas the PBS group did not show any morphological change of the cells. The living cell numbers of the PBS groups with and without light were almost the same (Fig. 10), which means that light exposure did not affect the LY80 tumor cells in this experimental condition. The living cell numbers of the HSA-CF group were lower than that of the PBS group; 57% cell death occurred by visible light irradiation. This clearly implies that the HSA-CF complex acts as a photosensitizer for PDT.

Conclusions

Transport of O₂ by the rHSA(mutant)-heme complex may be of great medical importance not only as a blood alternative, but also as an O₂-therapeutic fluid. The first generation of rHSA(double mutant)-heme complexes can

be successfully engineered to bind to O₂. However, these complexes did not show optimal O₂ binding affinity. We attempted to modify the heme pocket architecture to refine O₂ binding properties. By focusing on modification on the distal side of the heme pocket, we prepared rHSA(triple mutant)-heme complexes with a broad range of O₂ binding affinity. The highest affinity mutant rHSA(I142H/Y161L/L185N) contains Asn-185, which has a short amide side-chain that enhances O₂ binding affinity. In a different approach, substitution of the polar Arg-186 with Leu or Phe caused useful reduction in O₂ binding affinity, yielding $P_{1/2}^{O_2}$ almost identical to that of human RBC.

Amphiphilic C₆₀ fullerene also be adopted as functional ligand for HSA. The HSA-CF complex is easily excited by visible light and shows a high charge transfer rate constant for O₂. The efficiency of ¹O₂ production of this artificial protein is in the same range as common dyes. The HSA-CF complex does not have dark cytotoxicity, but engenders cell death under visible light irradiation.

In both the HSA-based O₂ carrier and photosensitizer, the protein plays a crucial role for solubilizing the heme and CF in aqueous medium up to ca. 3 mM. Even at high concentration, shielding of the chromophore by negatively charged HSA inhibits oxidation of the dioxygenated heme via μ -oxo dimer formation and a bimolecular Triplet-Triplet annihilation of excited ³CF*. Furthermore, in the case of the rHSA-heme complex, a pair of site-specific mutations is essential to confer O₂ binding capability on the heme. In contrast, no mutation is required for photosensitizing HSA-CF; replacement of some amino acid around the CF binding site may enhance the photophysical properties of the CF molecule. rHSA is now manufactured on an industrial scale using yeast species *Pichia pastoris*,⁸³⁾ which allows these functional proteins for use in practical applications.

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ヒト血清アルブミンを用いた機能分子・材料の創製

Synthesis of Functional Molecules and Materials Based on Human Serum Albumin

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和文抄録

筆者らはヒト血清アルブミン (HSA) の多分子結合能を利用して、その内部に機能性分子を包接させる方法や、さらには HSA と高分子電解質の交互積層膜を多孔性膜の細孔内で作成する方法により、自然界には見ることのできないユニークな機能分子・材料を創製してきている。HSA に鉄テトラフェニルポルフィリン誘導体 (FeP) を包接させた HSA-FeP 複合体は生理条件下で酸素を可逆的に結合解離できる人工酸素運搬体となり、遺伝子組換えアルブミン (rHSA) にヘモグロビンの活性中心である鉄プロトポルフィリン (heme) を結合させた rHSA-heme 錯体も酸素吸脱着のできる人工ヘム蛋白質となる。一方、HSA-亜鉛プロトポルフィリン錯体は水の光還元による水素発生反応の増感剤として作用し、HSA-カルボキシフラーレン複合体は一重項酸素生成の光増感剤として腫瘍光線力学療法への応用が期待されている。さらに、多孔性ポリカーボネート膜をテンプレートとした鋳型内交互積層法により、HSA からなる中空シリンダー構造のナノチューブが合成できる。これらの新機能分子・材料の特徴と応用展開について、最近の話題を紹介する。

Abstract

We have synthesized unique functional molecules and materials based on human serum albumin (HSA), which have never seen in nature, by means of incorporation of functional ligands into the protein or fabrication of layer-by-layer assembly in the nanoporous membrane. HSA incorporating iron (II) tetraphenylporphyrin derivative (FeP) (HSA-FeP) is an artificial O₂ carrier which can reversibly binds and release O₂ under physiological conditions. Recombinant HSA complexed with a natural iron (II) protoporphyrin IX (heme) (rHSA-heme) also acts as O₂ transport hemoprotein. On the other hand, HSA complexed with a zinc (II) protoporphyrin IX functions as a photosensitizer for H₂ evolution from water, and HSA-carboxy fullerene hybrid produces singlet O₂ by visible light irradiation; it may be used as a sensitizer in photodynamic cancer therapy. Furthermore, HSA nanotubes are prepared by layer-by-layer deposition technique using porous polycarbonate membrane template. We highlight recent development and applications of these functional molecules and materials.

Keywords

Human serum albumin, albumin-heme, oxygen carrier, red blood cell substitute, photosensitizer, nanotubes

1. はじめに

ヒト血清アルブミン (HSA) は血清蛋白質の約 60% を占める補欠分子族を持たない単純蛋白質 (分子量: 66,500) であり、血流中ではコロイド浸透圧維持のほか、各種内因性・外因性物質 (脂肪酸, ヘミン, ビリルビン, 金属イオン, ホルモン, NO, 薬物) の運搬・貯蔵, pH 緩衝作用, エステラーゼ活性な

どの役割を担っている¹⁻³⁾。1992 年, 米国国立航空宇宙局 (NASA) の Carter らは HSA の高分解能 X 線結晶構造解析 (分解能: 2.8 Å) に成功し, その三次元構造の全容を明らかにした⁴⁾。585 個のアミノ酸からなる一本鎖ポリペプチドは 17 個のジスルフィド結合を介してハート形に折りたたまれ (長径: 約 8 nm, 厚み: 約 3 nm), 相同性の高い 3 つのドメイン I~III

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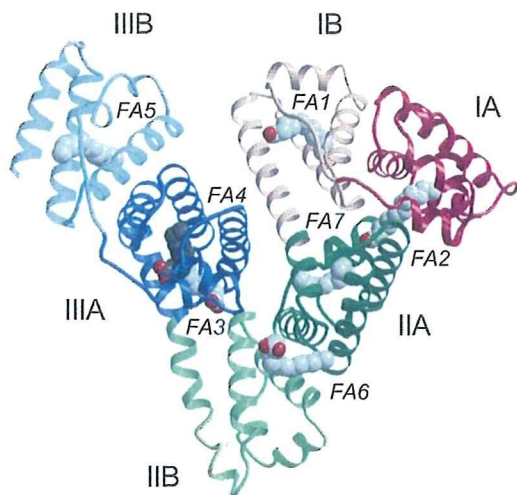


Fig. 1. Structure of HSA with seven myristic acids (PDB 1e7g). The protein secondary structure is shown schematically and the domains are colored (I; red, II; green, III; blue). The A and B subdomains are depicted in dark and light shades, respectively. The fatty acid binding sites are represented from FA1 to FA7 in italics.

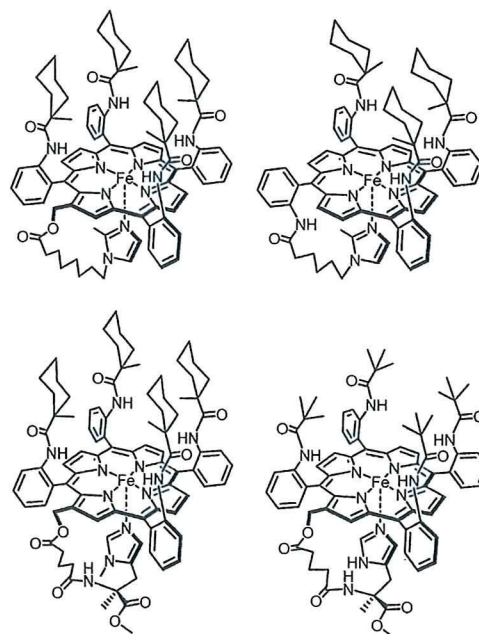


Fig. 2. Chemical formula of typical FeP molecules that can be incorporated into HSA to form HSA-FeP hybrid.

を構成している（各ドメインはさらに2つのサブドメインA, Bに分けられる。Fig. 1）。Sudlowらが提唱した古典的なHSAの薬物結合サイトI（ワルファリン、インドメタシンなど）はサブドメインIIA、薬物結合サイトII（ジアゼパム、イブプロフェンなど）はサブドメインIIIAに相当する⁹⁾。

一方、HSAは遺伝子組換え技術により大量発現が可能な蛋白質である⁶⁾。近年、その多分子結合能と高い生産性に注目が集まり、HSAに金属錯体を包接させた人工蛋白質の構築が盛んになってきている。例えば、GrossらはHSAにマンガンコロールを結合させた複合体を調製し、スルフィドの立体選択的酸化触媒として利用した⁷⁾。また、ReetzらはHSAに銅フタロシアンを結合させた複合体が、Diels-Alder反応に有用であることを明らかにした⁸⁾。

本稿では、酸素運搬体、光増感剤から、蛋白質ナノチューブまで、著者らが進めているHSAを利用した新しい機能分子・材料の創製と応用について最近の話題を紹介したい。

2. アルブミン-脂肪酸複合体の結晶構造解析

HSAの最もよく知られたリガンドに脂肪酸がある。1998年、CurryらはHSA-脂肪酸複合体のX線結晶構造解析に初めて成功し、中・長鎖脂肪酸の結合サイト（FAサイト）を特定した^{9,10)}。HSAには全ての脂肪酸に共通する7個のサイトがあり（Fig. 1）、FAサイト1, 4, 5, 7は各サブドメインの中央に、FAサイト2, 3は二つのドメインの境界面に、そしてFAサイト6は二つのサブドメインの境界面に位置する。FAサイト1-5では、脂肪酸の末端カルボキシル基が塩基性または極性アミノ酸残基と相互作用することによりしっかりと固定されている。これら5つのうちどこが最も親和性の強い部位なのか、

結晶構造からは判定できない。組換えHSAの詳細な¹³C-NMRスペクトル測定から、FAサイト2, 4, 5が親和力の強いサイトであることが明らかにされた¹¹⁾。7つのFAサイト全てに脂肪酸が結合すると、HSA全体の分子形態はダイナミックに変化する。特にドメイン間に存在するFAサイト2, 3への結合は、ドメインIIを軸として大きな形態変化を誘起した。HSAの二次構造は α -ヘリックスが67%、 β 構造が0%である。古くから脂肪酸などのリガンド結合によりHSAの二次構造が変化するという報告があるが、リガンド結合後も二次構造に変化はないことが結晶学的に示された。Curryらはその後も各種HSA-リガンド複合体の結晶構造解析を系統的に進め、多くの複合体について構造を解明している¹²⁻¹⁴⁾。現在Protein Data Bank (PDB)に登録済みのHSA-リガンド複合体のデータ47件のうち、37件はCurryらによるものである。

3. アルブミン-合成ヘム複合体（人工酸素運搬体）

我々は独自の分子設計により合成した鉄テトラフェニルポルフィリン誘導体（合成ヘム：FeP）をHSAに包接させたアルブミン-ヘム（HSA-FeP）複合体が、生理条件下でヘモグロビン（Hb）と同じように酸素を吸脱着できることを見出した¹⁵⁻¹⁸⁾。これまでに約30種類以上のFeP（Fig. 2）を合成し、その酸素結合能とヘム構造の相関を整理してきている¹⁹⁻²²⁾。酸素錯体の安定度は天然のミオグロビン（Mb）を上回り、酸素親和性（ P_{50} ）は0.1 Torrから230 Torrまで所望の値に揃えることができる。つまり、適用に応じた製剤の選択が可能となっている。HSA-FeP溶液の血液適合性は高く、血液凝固系、補体系、血小板の活性化に対しても影響を及ぼさない²³⁾。室温で2年以上の棚置保存が可能で、その間酸素結合パラメーターに変

化はない²⁴⁾。脱血交換試験（ラット、ビーグル犬）から出血ショック状態からの蘇生効果、生体内における酸素輸送能が証明されている^{25,26)}。体内投与後も修飾Hb製剤に見られるような血管内皮細胞からの漏出、一酸化窒素捕捉に伴う血管収縮、血圧亢進は全く観測されない²⁷⁾。これはHSAの表面電荷が負に帯電しているため血管から漏れ出しにくいことに起因すると考えられており、本製剤の最大の利点となっている。

一般に悪性腫瘍は放射線療法や化学療法に抵抗性を示し、その原因の一つとして腫瘍組織内低酸素細胞（hypoxic cell）の存在があげられる。細胞の異常な増殖により新生血管の生成が追いつかない湿潤性腫瘍細胞では、十分な血流および酸素化が得られず、それが治療の妨げとなっている。HSA-FeP溶液を腫瘍組織の患部近傍へ投与し、腫瘍組織内低酸素細胞の酸素化を試みたところ、患部の酸素分圧は投与前の2.5倍に増大した²⁸⁾。これは、従来報告されている修飾Hb製剤を用いた処置に比べて格段に高く²⁹⁾、粒子径の小さいHSA-FePが腫瘍内部へ容易に到達できるためと考えられる。実際に放射線療法と併用した結果、HSA-FeP投与による抗癌作用の顕著な増強効果が認められた³⁰⁾。また、HSA-FePの分子表面をポリ（エチレングリコール）で被覆すると、血中滞留時間が大幅に延長されることも明らかにされている^{31,32)}。

4. 遺伝子組換えアルブミン-プロトヘム錯体（人工酸素運搬体）

アルブミン-合成ヘム複合体の酸素結合部位は、精密に分子設計された鉄テトラフェニルポルフィリン誘導体（FeP）であり、得られたHSA-FeP複合体が酸素を可逆的に結合解離できることは、錯体化学的に見れば設計通りの機能発現といえる。HSAを用いた人工酸素運搬体開発における究極の挑戦は、やはりHbの酸素結合部位であるヘム鉄〔鉄プロトポルフィリンIX（プロトヘム，heme）〕（Fig. 3a）を用いた製剤の確立であろう。2004年、我々は遺伝子組換え技術を用いてアミノ酸の一部を改変した組換えHSA（rHSA）にhemeを包接させたrHSA-heme錯体が、水中で酸素を吸脱着できることを見出した³³⁾。

溶血により血中に放出されたメトHbから解離した鉄（III）

プロトポルフィリンIX（hemin）は、通常ヘモペキシンと呼ばれる糖蛋白質に捕捉されるが³⁴⁾、ヘモペキシンの血中濃度は約17 μ Mと低いため、遊離heminの多くは一旦HSAに結合し肝臓へと運ばれる。HeminがHSAに捕捉されることは昔から知られていた。1938年、Fairlyはheminと相互作用する血清蛋白質がHSAであることを発見し、その複合体をhemalbuminと名付けた³⁵⁾。それ以来、このHSA-hemin錯体は、何らかの生理作用があるのではないかと研究者達の関心の的となってきた。1975年、Muller-EberhardらによりHSA-hemin錯体の吸収スペクトルが報告され、heminの中心鉄にアミノ酸残基が軸配位した高スピンヘム錯体構造が推定された³⁶⁾。また、1980年にはBermanらがHSAとheminの結合定数（K：1.1 \times 10⁸ M⁻¹）を決定³⁷⁾、この値は脂肪酸に比べて約100倍高く、heminがHSAに強く結合することが明確となった。

2002年、Carterと我々のグループは独立にHSA-hemin錯体のX線結晶構造解析に成功し、heminがHSAのサブドメインIB内の疎水ポケットに結合していることを明らかにした^{38,39)}。この部位は、脂肪酸のFAサイト1に相当する。heminの中心鉄にはチロシン（Tyr）-161のフェノレート酸素が軸配位し、二つのプロピオン酸残基は、三つの塩基性アミノ酸残基（リシン、ヒスチジン、アルギニン）と相互作用していることがわかった（Fig. 3b）。よく知られているようにMbのヘムポケット内では、ヘム中心鉄にヒスチジン（His）-93が軸配位し、そのトランス側（第6配位座）に酸素分子が結合する（Fig. 3c）。さらに、配位酸素側にはもう一つのHis-64が遠位塩基として存在し、酸素錯体を安定化している。ここで両者を見比べてみると、誰もがその構造類似性に気づくであろう。しかし、残念ながらHSA-hemin錯体の中心鉄を鉄（II）に還元して酸素を吹き込んでみても、酸素錯体は得られない³⁹⁾。それは軸配位子がHisではないためである。HSA-hemin錯体が血中で何らかの役割をはたしているのではないかという期待とは裏腹に、むしろHSAはhemin分子の活性を巧みに抑え込んでいたのである。しかしながら、hemeが疎水的な分子空間に配置された構造はMbのヘムポケットと共通しているため、それならばHSAの場合でもヘム鉄の配位圏内（サブドメインIB）にHisが存在すれば、酸素錯体ができるのではないかと考えた。そこで遺伝子組

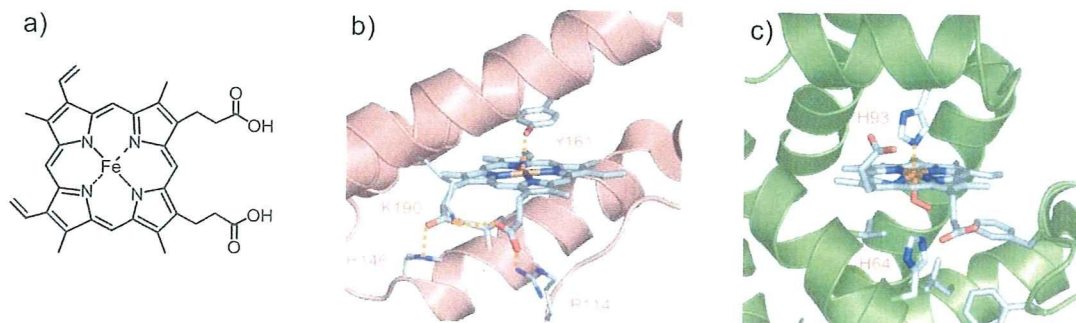


Fig. 3. (a) Chemical formula of iron (II) protoporphyrin IX (heme). Heme pocket structures of (b) HSA-hemin complex (PDB 1o9x) and (c) oxy-Mb (pdb 1mbo).

換え技術を用いて、イソロイシン (Ile)-142位置へ近位塩基として働く His を変異導入し、さらに中心鉄に軸配位している Tyr-161 を疎水性アミノ酸 (ロイシン (Leu)) に変換したところ、その組換え HSA-heme [rHSA (I142H/Y161L)-heme] 錯体は、室温で酸素を吸脱着することができた^{33,40)}。rHSA と heme から構成される酸素輸送人工ヘム蛋白質の初めての例である。そこで、酸素結合パラメーターを詳細に解析した。rHSA (I142H/Y161L)-heme 錯体の $P_{1/2}$ は 18 Torr (22°C) であり、Hb, Mb, ヒト赤血球の値に比べて高い (酸素親和性は低い)。rHSA (I142H/Y161L)-heme 錯体の低い酸素親和性は、速度論的には大きな解離速度定数 (k_{off}) に起因した^{33,40)}。rHSA の中では heme を取り巻く分子環境が疎水的であるために k_{off} が高く、酸素親和性が低く抑えられているのである。

このように酸素錯体は得られたものの、実際に rHSA (I142H/Y161L)-heme 錯体を人工酸素運搬体として利用するためには、その酸素親和性を Hb や赤血球の値に近づけなければならない。そこで、第三の変異を導入することにより、酸素親和性を上げる工夫を行った。前述したように、Hb や Mb のヘムポケット内には酸素配位座側に His-64 が遠位塩基として存在し、酸素親和性の増大に寄与している^{41,42)}。我々は rHSA の場合も、酸素配位座側の適当な位置に遠位塩基を導入すれば、酸素親和性が上昇するのではないかと考えた。分子シミュレーションの結果から配位酸素直上の Leu-185 を選定し、そこへ遠位塩基としてのアスパラギン (Asn) を導入した [rHSA (I142H/Y161L/L185N)]⁴³⁾。rHSA (I142H/Y161L/L185N)-heme 錯体 (Fig. 4) の可視吸収スペクトルは、窒素雰囲気下では鉄 (II) 5 配位高スピン錯体の形成を示し、そこへ酸素を通気すると速やかに酸素錯体型へと移行した。 $P_{1/2}$ は 1 Torr (22°C) となり、酸素親和性はもとの二重変異体に比べて 18 倍も上昇した。Asn の導入によりヘムポケットの極性が増大し k_{off} が減少した結果、酸素親和性が上がったものと考えられる。rHSA (I142H/Y161L/L185N)-heme 錯体は、Hb と同等の酸素親和性を有する人工ヘム蛋白質となった。つまり我々は、本来補欠分子族すらもたない単純蛋白質の HSA に、酸素結合能を

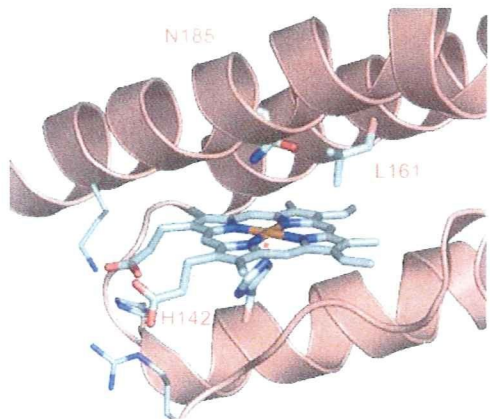


Fig. 4. Structural model of the heme pocket in rHSA (I142H/Y161L/L185N)-heme complex.

付与することに成功したばかりでなく、ヘムポケットの微小空間を部位特異的アミノ酸置換により最適化することで、酸素親和性のコントロールを実現したのである。

ごく最近、酸素配位座側にあるアルギニン (Arg)-186 を疎水性アミノ酸に変換すると、酸素親和性がヒト赤血球と同等値に調整できることを見出した⁴³⁾。rHSA-heme 錯体は天然のプロトヘムを活性中心とする、いわば“酸素輸送のできる赤色の血清蛋白質”である。臨床利用可能な赤血球代替物として、現在実用化に向けた努力が進められている。

5. アルブミン-亜鉛プロトポルフィリン錯体 (水素発生) の光増感剤)

ポルフィリン化合物の最大の魅力は、その中心金属を変えることにより多彩な機能を発揮できる点にある。特に可視光領域に大きな吸収帯を持つ特徴を生かして、光反応の増感剤として広く利用されている。しかし、鉄ポルフィリン錯体は光励起状態の寿命がきわめて短いため、一般的には光反応の増感剤には適さず、励起寿命の長い亜鉛錯体が用いられる。

水素 (H_2) は二酸化炭素排出のない近未来のクリーンエネルギーである。Grätzel らは亜鉛テトラメチルピリジニウムポルフィリンをメチルビオロゲン (MV^{2+}) /白金コロイド / EDTA 水溶液に加え可視光照射すると、水の光還元反応が進行し水素が得られることを見出した⁴⁴⁾。もしプロトポルフィリン IX (PP) が水の光還元利用できれば、天然物質を増感剤とした水素発生システムが確立できることになる。しかし、亜鉛プロトポルフィリン IX (ZnPP) は水に不溶で、そのまま使用することは難しい。そこで、ZnPP を HSA に包接させることにより HSA-ZnPP 錯体とし、水の光還元反応への応用を試みた。HSA-ZnPP 錯体水溶液に MV^{2+} を加え、アルゴン雰囲気下でレーザーフラッシュ (532 nm) を照射すると、ZnP の励起三重項状態から MV^{2+} への電子移動反応が観測された⁴⁵⁾。続いて、白金コロイド、犠牲試薬としてのトリエタノールアミンを加え可視光照射してみると、速やかに水の還元が起こり溶液中から水素が発生した (Fig. 5)。水素発生効率は亜鉛テトラメチルピリジニウムポルフィリンを用いた場合よりも高い。つまり、HSA-ZnPP 錯体は水の光還元反応における有効な増感剤として機能する。

この結果は、アルブミン-ポルフィリン錯体においてポルフィリンの中心金属を変えることにより、様々な機能を持った人工蛋白質が創製できることを示唆している。

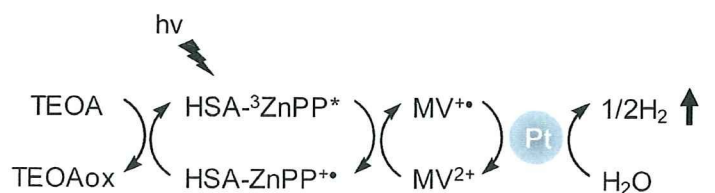


Fig. 5. Reaction scheme of photoreduction of water to hydrogen using HSA-ZnP complex.

6. アルブミン-フラレン複合体 (光線力学療法の増感剤)

金属ポルフィリン以外の機能性分子でもHSAに包接させれば、新しい人工蛋白質が合成できるはずである。そこで、HSAにフラレン誘導体を包接させたアルブミン-フラレン複合体を調製し、その構造、光物性、光線力学療法 (photodynamic therapy: PDT) における増感剤としての可能性について検討した。現在、よく知られているPDTの光増感剤には、ヘマトポルフィリンの誘導体であるフォトフリン^{46,47)}、プロトポルフィリンの前駆体である5-アミノレブリン酸⁴⁸⁾、またクロリン誘導体であるビスダイン⁴⁹⁾などがある。これらのポルフィリン誘導体に比べ、フラレンC₆₀は高い光安定性を持ち、一重項酸素生成の量子収率が高いことから、PDTの新しい増感剤として期待されている⁵⁰⁻⁵²⁾。

HSAにカルボキシフラレン (CF, Fig. 6a) を包接させたHSA-CF複合体はきわめて安定で、その水溶液は調製2年後でも沈殿・凝集など全く認められなかった (Fig. 6b)⁵³⁾。可視吸収スペクトル、HPLC、質量分析の結果から、HSAとCFが1:1で結合していること、さらに小角X線散乱測定から、CF結合後もHSAの分子径、表面電荷に変化はないことがわかった⁵³⁾。

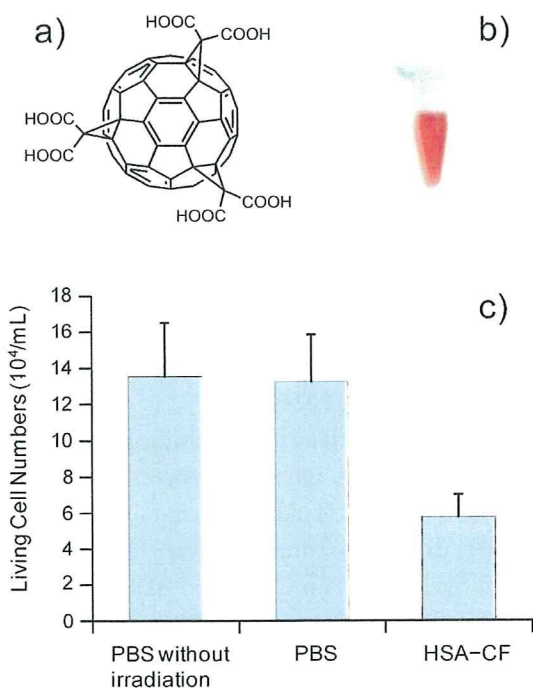


Fig. 6. (a) Chemical formula of carboxy-C₆₀-fullerene, (b) red colored solution of aqueous HSA-CF, and (c) living cell numbers of LY80 with HSA-CF and PBS after visible light irradiation (20 mW cm⁻², 2 h, 36 ± 1 °C). Each value represents the mean ± SD (n = 4).

一般に色素分子を光励起すると、まず励起一重項状態を形成し、そこから項間交差により励起三重項状態へと遷移する。一重項酸素生成の有効な光増感剤となるためには、この励起三重項状態が安定でなければならない。HSA-CF複合体の励起三重

項寿命は46 μsと長く、それはCFが蛋白質内部に固定されているため、励起種どうしによる消光が抑えられることによる。系内に酸素を共存させると、励起三重項状態から酸素分子へのエネルギー移動が観測された。生成する一重項酸素は1270 nmに蛍光を示すので、その強度から一重項酸素生成の量子収率が算出できる。HSA-CF複合体の量子収率は0.46であり、HSA-PP複合体やメチレンブルーと同程度であったことから⁵⁴⁾、本化合物が一重項酸素生成の有効な光増感剤として作用することがわかった。

続いてこの製剤の細胞毒性について検証した。腫瘍細胞 (LY80) にHSA-CF水溶液を加え、まず暗所下でそれ自身に細胞毒性がないことを確認。その後ハロゲンランプを用いて光照射 (2 hr) すると、細胞数は43%にまで減少した (Fig. 6c)⁵³⁾。この結果はHSA-CF複合体がPDTの光増感剤として有効であることを示している。

7. アルブミンナノチューブ (分子捕捉剤、薬物運搬体、ナノリアクター)

ピキア酵母を用いた遺伝子組み換え体の量産体制が確立して以来、HSAは臨床利用はもちろんバイオマテリアルの有用な素材としても注目を集めている⁶⁾。我々は最近、鋳型内交互積層法によりHSAからなる中空シリンダー構造のナノチューブを合成することに成功した⁵⁵⁻⁵⁷⁾。HSAの等電点は4.8と低く、生理条件下では分子表面が負に帯電している。上述したようにHSAがHbに比べて血管外へ逸脱し難いのは、血管内皮細胞の外側にある基底膜との静電反発による。そこでまず正電荷を有する高分子電解質 (例えば、ポリ-L-アルギニン (PLA) などのポリアミノ酸やポリエチレンジイミンなど) を多孔性ポリカーボネート (PC) 膜の細孔内に通過させ、続いてHSA水溶液を通過させる。この操作を繰り返しながら、細孔内壁にHSAの多重積層膜を作成し、最後にPC膜を溶解除去すると、HSAからなる均一で柔軟なナノチューブが得られる。孔径400 nmのPC膜にPLAとHSAを各3回ずつ通過させて作成した計6層構造からなるナノチューブの外径は約400 nm、内径は約300 nm、管壁厚は約50 nmとなる (Fig. 7)。鋳型内交互積層法 (テンプレート合成) の利点は、

- ① 電荷を有する水溶性分子 (蛋白質、生体分子、高分子電解質など) であれば、ナノチューブの素材になり得る (分子設計の自由度が高く、構成分子の選択範囲が広い)
- ② 鋳型となる多孔性膜の孔径および厚みの調節により、チューブの外径および長さを均一に制御することができる
- ③ 積層膜数の調節により、チューブの内径をナノメートルスケールで制御することができる
- ④ 複数の機能性分子を任意の順序で積層することにより、管壁構造を自由に分子設計できる
- ⑤ 他のナノチューブ合成法に比べ、調製が簡便、再現性が高く、低コスト、大型化が容易

などである。

得られたナノチューブの最大の特徴は、同重量の球状構造体

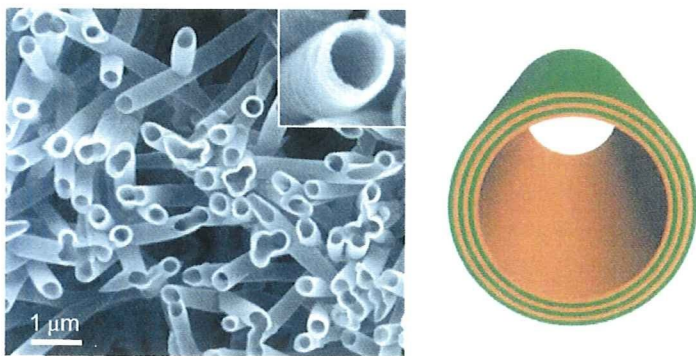


Fig. 7. SEM images of protein nanotubes comprised of (PLA/HSA)₃. Schematic illustration of the protein nanotubes prepared by template synthesis using layer-by-layer deposition technique.

に比べて比表面積が大きいことにある。HSA ナノチューブの水分散液に HSA のリガンド (薬物など) を添加すると、ナノチューブ管壁に効率よく結合し、水中から速やかに除去される。HSA に結合しない分子は全く取り込まれないので、いつまでも水中に存在する。Hb や Mb を構成成分としてナノチューブを作成することもできる⁵⁷⁾、HSA-FeP からなるナノチューブは酸素を可逆的に吸脱着する⁵⁸⁾。また、最終層成分の選定だけで内孔に合目的な特性を付与することができるので、例えば最内層に加水分解酵素を配置した HSA ナノチューブでは、内孔壁表面で基質の加水分解が進行する。逆に酵素合成により新物質を創り出すこともできる。まさにナノサイズのリアクターである。この場合、内孔以外の管壁を構成する蛋白質が HSA であることが肝要である。他にも鉄イオンの捕捉・貯蔵を担う球殻状蛋白質フェリチンからなるナノチューブも合成され、バイオナノデバイスとして磁化特性、半導体特性が検討されている。

管壁のみならず、チューブの一次元内孔空間にウイルスやリポソームなどの大きな生体超分子をサイズ選択的に捕捉することも可能である。中空シリンダーの両端開口部が自由に開閉できれば、従来のミセルやリポソームとは全く違う新しいドラッグキャリアの誕生にもつながるであろう。

8. おわりに

筆者らはヒト血清アルブミンの多分子結合能に着目し、その内部に機能性分子を取り込ませる方法により、数々の人工蛋白質を創製してきている。当初は酸素運搬体に焦点を絞った展開であったが、合成へみならず他の機能性分子も効率よく包接できることがわかり、水の還元による水素発生や一重項酸素生成の光増感剤となるアルブミンを開発した。実は多分子結合能のみならず、きわめて高い水溶性や光・熱に対する安定性といった血清蛋白質アルブミンならではの特徴が、これらの新物質系において重要な役割を果たしている。さらに、遺伝子組換え技術を駆使してアルブミンのアミノ酸配置を少しだけ変えてやると、機能制御が可能になることも明らかにした。X線結晶構造解析 (生物物理学) にもとづいた部位特異的アミノ酸置換

(分子生物学) を機能性アルブミン開発の分子設計に取り入れ、新しい方法論として定着させた。最近ではアルブミンからなる中空シリンダー構造のナノチューブを合成し、その機能展開に力を入れているが、ここでもアルブミンの高い構造安定性が大きく貢献している。ウイルス捕捉、標的薬物の結合・放出、ナノリアクター、ナノバイオデバイスなど、多くの応用展開が期待される。

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