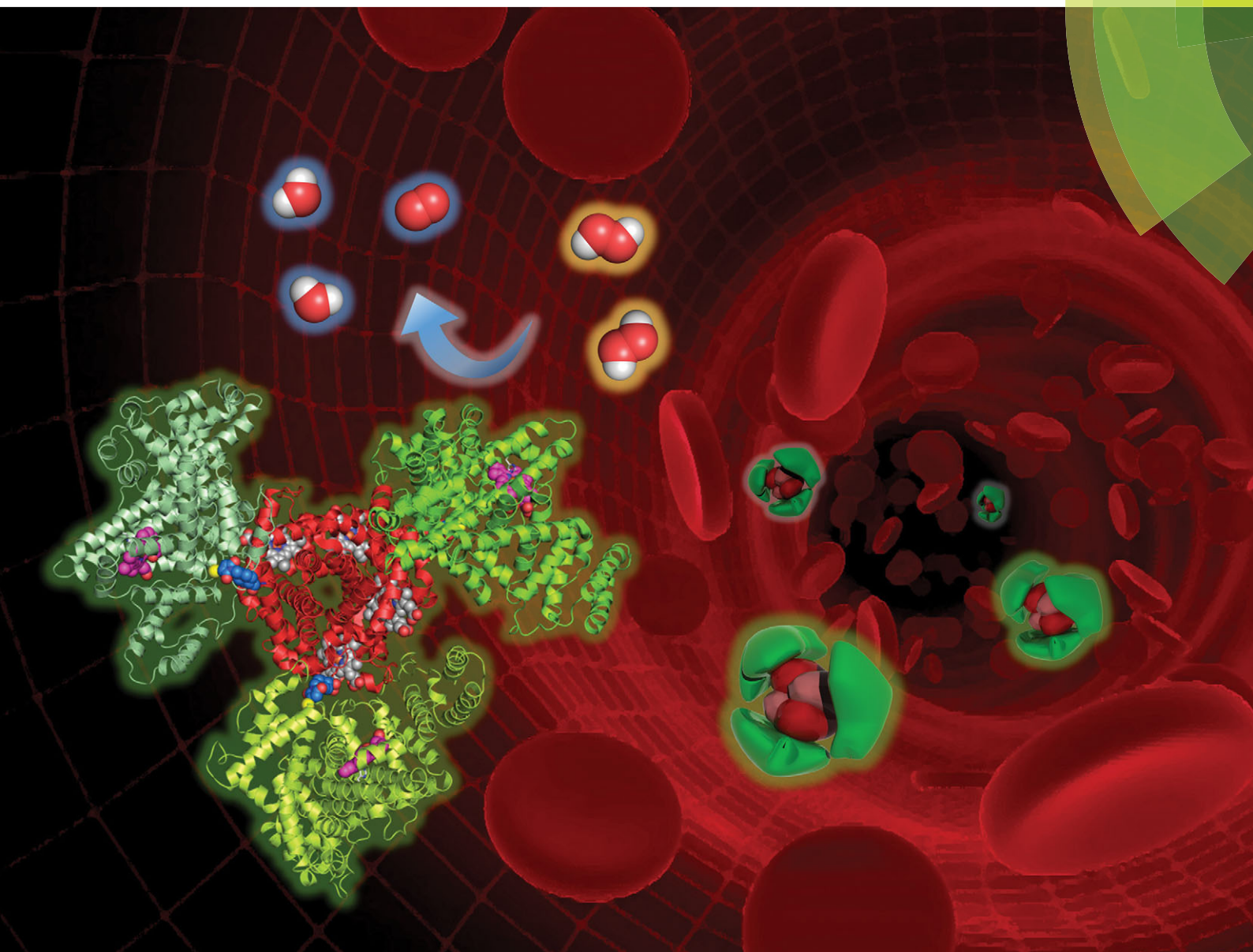


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Haemoglobin wrapped covalently by human serum albumin mutants containing Mn(III) protoporphyrin IX: an O₂ complex stable in H₂O₂ solution†

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We describe the synthesis of a haemoglobin (Hb) wrapped covalently by recombinant human serum albumin mutants [HSA(Y161H)] containing Mn(III) protoporphyrin IX (MnPP), the Hb–[HSA(Y161H)–MnPP]₃ cluster, highlighting the formation of its O₂-complex stable even in H₂O₂ solution.

Haemoglobin (Hb)-based O₂ carriers (HBOCs) of several types have been developed in the last few decades as red blood cell (RBC) substitutes and as O₂-therapeutic reagents,^{1–4} such as cross-linked Hb,^{5,6} polymerized Hb^{7,8} and (polyethylene glycol)-conjugated Hb.^{9,10} Nevertheless, the HBOCs gradually lose their O₂-transporting capability by autooxidation of Hb to the ferric haem form (metHb) with the release of a superoxide (O₂^{•−}) that is disproportionated to hydrogen peroxide (H₂O₂).¹¹ In the RBC, superoxide dismutase (SOD) converts O₂^{•−} to H₂O₂, which is then dismutated to H₂O and O₂ by catalase. Such protective enzyme systems are not present in the plasma. Therefore, these reactive oxygen species (ROS) produced by HBOCs cause not only tissue injury, but also further oxidation of Hb. In particular, H₂O₂ appears to be a potent species causing oxidative damage to Hb.¹² Consequently, HBOCs with catalase activity are expected to be tremendously useful in practical applications. Chang *et al.* prepared a poly(Hb–SOD–catalase) hybrid and showed a significantly low metHb formation rate.¹³ This Hb–(antioxidant enzymes) conjugate demonstrated both O₂-carrying and antioxidant properties.

More recently, we synthesized a covalent core–shell structured protein cluster composed of Hb at the centre and human serum albumins [wild type, HSA(wt)] at the periphery, Hb–HSA(wt)₃, which is a structurally defined HBOC.¹⁴ Because HSA possesses only one free sulfhydryl group at Cys-34, we exploited a heterobifunctional cross-linker, *N*-succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) to combine the Cys-34 of HSA(wt) and the

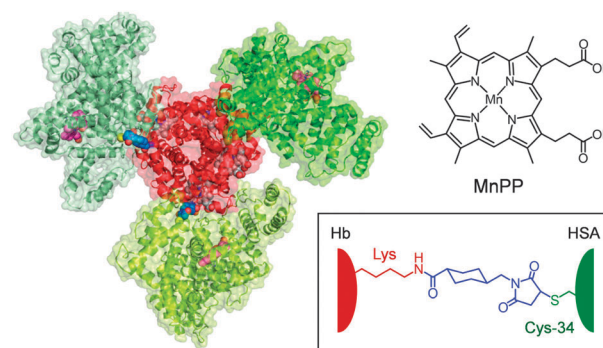


Fig. 1 Structure model of the Hb–[HSA(Y161H)–MnPP]₃ cluster (central Hb, red; peripheral HSAs, green). The haems (grey) in Hb and MnPPs (pinks) bound within subdomain 1B of the exterior HSA(Y161H) units (see Fig. 2) are shown in a space-filling representation. The Cys-34 of HSA and the surface Lys of Hb were connected with a cross-linking agent (SMCC, blue).

surface Lys ε-amino groups of Hb (Fig. 1). The major product formed is the Hb–HSA₃ heterotetramer in a triangle form. From 3D reconstruction based on transmission electron microscopy images, the binding partners in Hb were identified as Lys-90(α₁), Lys-82(β₁) and Lys-16(α₂).¹⁴

If one were able to confer antioxidant properties on the external HSA(wt) units of Hb–HSA(wt)₃, then this construct would become a promising O₂-carrier with high resistance against oxidation reactions. We chose the Mn(III) porphyrin as a potential candidate because it is an effective catalyst for H₂O₂ dismutation and because it lacks Fenton reaction-related toxicity.¹⁵ In this communication, we describe the synthesis of Hb wrapped covalently by recombinant HSA mutants containing Mn(III) protoporphyrin IX (MnPP, Fig. 1) and its markedly stable O₂-complex in H₂O₂ solution. This super-structured haemoprotein possesses the dual functionalities of O₂ transport and H₂O₂ dismutation.

The most prominent plasma protein in the bloodstream, HSA (*M*_w: 66.5 kDa), can bind widely diverse water-insoluble metabolites (fatty acids, bilirubin, thyroxine, *etc.*) and drugs (warfarin, diazepam, ibuprofen, *etc.*). This heart-shaped carrier protein consists of three similar domains (I–III), each of which

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† Electronic supplementary information (ESI) available: Experimental section, SEC curve, OEC curve, UV-Vis absorption maxima and O₂-binding parameters of the Hb–[HSA(Y161H)–MnPP]₃ cluster. See DOI: 10.1039/c4cc06076h



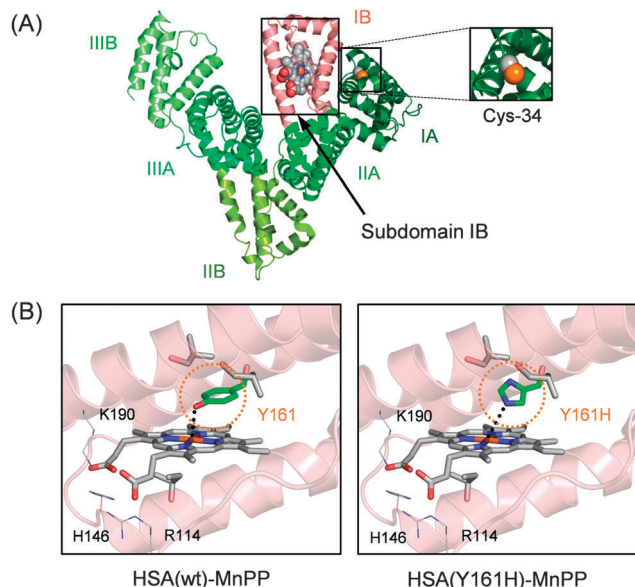


Fig. 2 (A) Structure models of HSA-MnPP complex. (B) Schematic illustration of MnPP binding site in subdomain IB of HSA (prepared from PDB ID: 1O9X). In HSA(wt), Tyr-161 axially coordinates to the central Mn ion of MnPP. In HSA(Y161H), His-161 coordinates to the central Mn of MnPP.

includes two subdomains: A and B (Fig. 2A).^{16–18} Our crystallographic and spectroscopic studies revealed that Fe(III) protoporphyrin IX (FePP) is bound within a deep hydrophobic slot in subdomain IB of HSA(wt) with (i) a weak axial coordination of Tyr-161 (phenolate oxygen) to the central Fe ion and (ii) salt-bridges between the porphyrin propionates and basic amino acid residues at the pocket entrance (Arg-114, His-146 and Lys-190).^{19,20} We demonstrated previously that MnPP is also incorporated into this pocket of HSA(wt) with Tyr-161 coordination (Fig. 2B).²¹ Moreover, the recombinant HSA(Y161H) mutant, in which Tyr-161 is replaced with His by site-directed mutagenesis, similarly captures MnPP with an axial His-161 ligation to the central Mn ion.²¹ The HSA(Y161H)-MnPP complex displays characteristic split Soret bands at 374 and 473 nm and Q-bands at 559 and 594 nm (shoulder). They resemble those of the MnPP-His complex in MnPP-reconstituted myoglobin.^{22,23} However, the catalase activities of these manganese proteins have not been elucidated. We initially examined the H₂O₂ dismutation activities of the HSA(wt)-MnPP and HSA(Y161H)-MnPP complexes in phosphate buffered saline (PBS) solution by monitoring the generated O₂ using an O₂ electrode system. Immediately after addition of H₂O₂ to the HSA-MnPP solution, an increase in dissolved O₂ was observed. With the coexistence of HSA alone, the O₂ concentration did not change. We inferred that the catalase activities of HSA-MnPP complexes were attributable to the MnPP in the protein.

Then the steady-state kinetic parameters of the HSA-MnPP complexes were determined. A hyperbolic dependence of the initial rate (v_0) on H₂O₂ concentration yielded the Michaelis constant (K_m) and catalytic constant (k_{cat}) (Table 1). It is noteworthy that the enzyme activity (k_{cat}/K_m) of HSA(Y161H)-MnPP becomes five-fold greater than that of HSA(wt)-MnPP. We can therefore conclude that the H₂O₂ dismutation activity is enhanced by replacing proximal Tyr

Table 1 Catalase activities of HSA-MnPP complexes in PBS solution (pH 7.4) at 25 °C

Catalase mimic	k_{cat} (min ⁻¹)	K_m (mM)	k_{cat}/K_m (M ⁻¹ min ⁻¹)
HSA(wt)-MnPP	8	24	341
HSA(Y161H)-MnPP	39	24	1640

with His, which has a strong electron-donating ability to the central Mn ion of MnPP.

Next, we enwrapped Hb with recombinant HSA(Y161H) mutants to prepare a Hb-HSA(Y161H)₃ cluster (see ESI†).¹⁴ The reaction mixture of SMCC-bound Hb and HSA(Y161H) includes Hb-HSA(Y161H)₃ heterotetramer as the major compound. Using gel filtration chromatography (GFC), the main fraction was harvested (Fig. S1†) (yield: 80% based on Hb). The average HSA/Hb ratio was ascertained to be 3.0 using Hb and protein assays.

Subsequently, the ethanolic MnPP was added to the PBS solution of Hb-HSA(Y161H)₃ (MnPP/cluster = 3.0, mol/mol) to create a Hb-[HSA(Y161H)-MnPP]₃ cluster. The difference absorption spectrum of Hb-[HSA(Y161H)-MnPP]₃ minus Hb-HSA(Y161H)₃ (λ_{max} : 560, 593 nm) coincided well with a three-fold-enlarged HSA(Y161H)-MnPP spectrum (Fig. 3). This observation clearly indicates that MnPP is bound within each subdomain IB of HSA(Y161H) unit by an axial His-161 coordination. In marked contrast, attempts to include MnPP into the exterior albumin units of Hb-HSA(wt)₃ clusters failed. A possible explanation is that the association of MnPP with HSA might be somewhat sterically hindered in the cluster. The Cys-34 of HSA locates in subdomain IA (Fig. 2A). Therefore, the entrance of the hydrophobic pocket for MnPP in the subdomain IB turns in the direction of the Hb centre. We inferred that a Tyr-161 coordination might be too weak to allow binding of MnPP to the HSA(wt) shell.

The isoelectric point of the Hb-HSA(Y161H)₃ cluster was satisfactorily low (pI = 5.1), which is attributed to the negative surface net charges of the exterior HSAs. The pI value of Hb-[HSA(Y161H)-MnPP]₃ cluster was also 5.1, implying that MnPP binding caused no alteration of the surface charge of the cluster. Probably, the intravenous transfusion of this hybrid haemoprotein is expected to enable long-term circulation

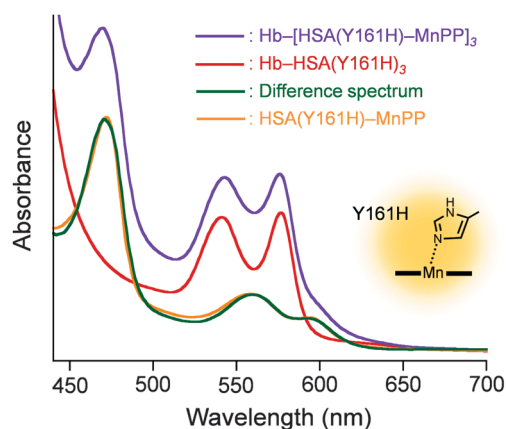


Fig. 3 Vis absorption different spectrum of Hb-[HSA(Y161H)-MnPP]₃ minus Hb-HSA(Y161H)₃, and comparison with that of HSA(Y161H)-MnPP. In PBS (pH 7.4) solution at 25 °C.



without extravasation because of the electrostatic repulsion between the negatively charged HSA surface and the glomerular basement membrane around the endothelial cells.

The visible absorption spectral maxima based on the Hb component of Hb-[HSA(Y161H)-MnPP]₃ in PBS solution were fundamentally the same as those of Hb-HSA(wt)₃ and native Hb under N₂, O₂, and CO atmosphere (deoxy, oxy, and carbonyl forms) (Table S1†).^{14,24} To evaluate the influence of MnPP on the O₂ affinity, the equilibrium between O₂ and the Hb-[HSA(Y161H)-MnPP]₃ cluster was measured. The P₅₀ value (O₂-partial pressure where Hb is half-saturated with O₂) and the co-operativity coefficient (Hill coefficient, *n*) were ascertained to be 9 Torr and 1.5, respectively (Fig. S2, Table S2†). They were identical to the values of Hb-HSA(wt)₃.¹⁴ Based on these findings, we inferred that the electronic states of the haems in Hb core were unaffected by the association of MnPP to the HSA(Y161H) shell.

Finally, the O₂-complex stability of Hb-[HSA(Y161H)-MnPP]₃ cluster in aqueous H₂O₂ solution was investigated. The H₂O₂ concentration in the normal human plasma is 4–5 μM.²⁵ It is assumed to be tens of micromolars (≤35 μM) under inflammatory conditions.²⁶ Therefore, we examined the oxidation rates of Hb-[HSA(Y161H)-MnPP]₃, Hb-HSA(wt)₃, and Hb in aqueous 10 μM H₂O₂ solution. The time courses of the absorbance increase at 630 nm (which results from metHb formation) were markedly different in these protein solutions (Fig. 4). Native Hb showed a biphasic autoxidation curve. Approximately 25% Hb is oxidized in the initial phase within 30 min, followed by a second slow oxidation process. The metHb formation level reached 35% after 120 min. The α subunits in Hb are known to be oxidized more easily than the β subunits.¹¹ Because the haem concentration was 40 μM ([Hb] = 10 μM), oxidation of the 50% α subunit occurred first. The remaining subunits were subsequently oxidized.

The rate of metHb formation was low in the Hb-HSA(wt)₃ cluster. In the initial phase, the metHb level increased to 17% within 30 min, followed by a slow oxidation reaction. The low velocity is likely to result from a wrapping effect of HSA.²⁷ As expected, the Hb-[HSA(Y161H)-MnPP]₃ cluster was remarkably stable in the H₂O₂ solution. We observed only 15% metHb after

120 min, which is 43% of the value of native Hb. This stable O₂-complex derives from the catalase activity of the HSA(Y161H)-MnPP units in the periphery. Actually, the oxidation rate of Hb in the coexistence of three-equivalent moles of HSA(Y161H)-MnPP, which are not covalently linked, was higher than that of the cluster. We therefore conclude that the HSA(Y161H)-MnPP shell acts as an efficient scavenger for external H₂O₂ and thereby achieves protection of the Hb centre.

This report is the first to describe the protein cluster comprising an Hb wrapped by genetically engineered HSA mutants containing MnPP. The HSA(wt)-MnPP complex has weak catalase activity, whereas a site-specific mutation in the subdomain IB of HSA, Tyr-161 → His, confers a five-fold-higher enzyme activity on the prosthetic MnPP group. The Hb-HSA(Y161H)₃ cluster can also accommodate MnPP into the external albumin units by axial His-161 coordination. Results show that the Hb-[HSA(Y161H)-MnPP]₃ cluster formed an extremely stable O₂-complex even in H₂O₂ solution. This unique super-structured haemoprotein with (i) negative surface net charges, (ii) high O₂-affinity, and (iii) catalase activity can be of great importance as an RBC alternative in various medical applications involving O₂ delivery to ischaemia-reperfusion tissues in which overproduction of O₂•⁻ and H₂O₂ occurs.

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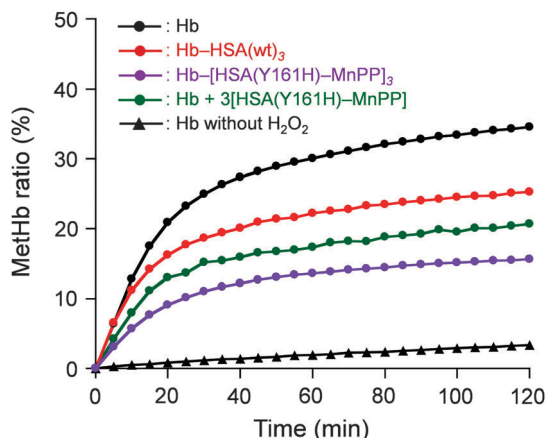


Fig. 4 MetHb formation ratio of Hb-[HSA(Y161H)-MnPP]₃ cluster in PBS (pH 7.4) solution containing 10 μM H₂O₂ at 25 °C.



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