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Poly(ethylene glycol)-Conjugated Human Serum Albumin Including Iron Porphyrins: Surface Modification Improves the O_2-Transporting Ability

Yubin Huang,[†] Teruyuki Komatsu,[†,*] Rong-Min Wang,[†,‡] Akito Nakagawa,[†] and Eishun Tsuchida[†,*]

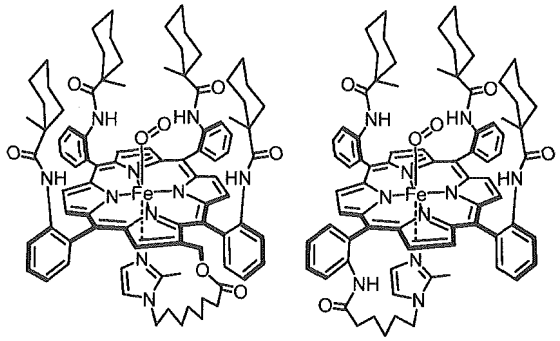
Advanced Research Institute for Science and Engineering, Waseda University, 3-4-1 Okubo, Shinjuku-ku, Tokyo 169-8555 Japan, and Key Laboratory of Polymer Materials of Gansu Province, Institute of Polymer, Northwest Normal University, Lanzhou 730070, China. Received November 3, 2005; Revised Manuscript Received February 10, 2006

Artificial O_2-carrying hemoprotein composed of human serum albumin including tetrakis(*o*-amidophenyl)-porphinatoiron(II) (Fe4P or Fe3P) [HSA−FeXP] has been modified by maleimide- or succinimide-terminated poly(ethylene glycol) (PEG), and the formed PEG bioconjugates have been physicochemically characterized. 2-Iminothiolane (IMT) reacted with the amino groups of Lys to create active thiol groups, which bind to α-maleimide-ω-methoxy PEG [Mw: 2-kDa (PEG_{M2}), 5-kDa (PEG_{M5})]. On the other hand, α-succinimidyl-ω-methoxy PEG [Mw: 2-kDa (PEG_{S2}), 5-kDa (PEG_{S5})] directly binds to Lys residues. MALDI-TOF MS of the PEG-conjugated HSA−FeXP showed distinct molecular ion peaks, which provide an accurate number of the PEG chains. In the case of PEG_{MY}(HSA−FeXP), the spectroscopic assay of the thiol groups also provided the mean of the binding numbers of the polymers, and the degree of the modification was controlled by the ratio of [IMT]/[HSA]. The viscosity and colloid osmotic pressures of the 2-kDa PEG conjugates (phosphate-buffered saline solution, [HSA] = 5 g dL^{-1}) were almost the same as that of the nonmodified one, whereas the 5-kDa PEG binding increased the rheological parameters. The presence of flexible polymers on the HSA surface retarded the association reaction of O_2 to FeXP and stabilized the oxygenated complex. Furthermore, PEG_{MY}(HSA−FeXP) exhibited a long circulation lifetime of FeXP in rats (13−16 h). On the basis of these results, it can be concluded that the surface modification of HSA−FeXP by PEG has improved its comprehensive O_2-transporting ability. In particular the PEG_{MY}(HSA−FeXP) solution could be a promising material for entirely synthetic O_2-carrying plasma expander as a red cell substitute.

INTRODUCTION

Poly(ethylene glycol) (PEG) is commonly used for the surface modification of peptides, proteins, enzymes, and liposome to confer several potential beneficial effects: not only a longer plasma half-life and nonimmunogenicity but also a solubility in organic solvents and extreme thermostability (*1*−*4*). To develop an artificial O_2 carrier, substantial efforts have been directed to the preparation of PEG-conjugated hemoglobin (Hb) over the past decades (*5*−*8*), and the optimized PEG−Hbs are currently being tested in clinical trials. Human serum albumin (HSA) is a versatile protein, which is found in our blood plasma at a high concentration (4−5 g dL^{-1}) (*9*). We have reported that HSA including tetrakis(*o*-amidophenyl)porphinatoiron(II) (Fe3P or Fe4P, Chart 1) [HSA−FeXP] can reversibly bind and release O_2 under physiological condition (pH 7.4, 37 °C) in a fashion similar to Hb (*10*). The administration of this synthetic O_2 carrier into anesthetized rats has proved its safety and O_2-transporting efficacy (*11*). Nevertheless, there is only one defect in that the FeXP molecule easily dissociates from HSA when infused into animals. This is due to the fact that FeXP is noncovalently bound to the hydrophobic cavity of albumin with binding constants (K) of 10^4−10^6 (M^{-1}). Natural heme, namely protoporphinatoiron IX, is also incorporated into HSA and shows a 10^2−10^4-fold higher K compared to FeXP (*12*); however, it is released from HSA during blood circulation with a half-life of 2.5−3.6 h (*13*, *14*). Under these circumstances,

Chart 1. Structures of O_2-Adduct Complexes of Tetrakis-(*o*-amidophenyl)porphinatoiron(II)



Fe4P Fe3P

we postulated that the surface modification of HSA−FeXP by PEG could help to prolong the circulation life of FeXP and thereby retain its O_2-transporting ability for a long period. Although HSA is a very common plasma protein, its PEG conjugation chemistry has not yet been studied in detail. It is known that a huge variety of drugs are incorporated into specific sites of HSA (*9*). The PEG modification should prevent the rapid release of these drugs from the HSA scaffold and contribute to raising their potential therapeutic efficacies.

In the present study, we have systematically prepared several PEG-conjugated HSA−FeXPs and characterized their physicochemical properties. The surface modification by PEG affects the viscosity and colloid osmotic pressure of the solution, O_2-binding behavior of the parent HSA−FeXP, and circulatory lifetime of FeXP. The PEG-conjugated HSA−FeXP could be

* Corresponding authors: (E.T.) Tel: +81-3-5286-3120, Fax: +81-3-3205-4740, E-mail: eishun@waseda.jp. (T.K.) E-mail: teruyuki@waseda.jp; eishun@waseda.jp.

[†] Waseda University.

[‡] Northwest Normal University.

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of extreme medical importance as a red cell substitute or O_2-therapeutic reagent.

EXPERIMENTAL PROCEDURES

Materials and Apparatus. All reagents were purchased from commercial sources as special grades and used without further purification. 2-Iminothiolane hydrochloride (IMT) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). α-[3-(3-Maleimido-1-oxopropyl)amino]propyl-ω-methoxy PEG [averaged Mw: 2333 (Sunbright ME-020MA, PEG_{M2}), averaged Mw: 5207 (Sunbright MEMAL-50H, PEG_{M5})] and α-succinimidyloxyglutaryl-ω-methoxy PEG [averaged Mw: 2325 (Sunbright MEGC-20HS, PEG_{S2}), averaged Mw: 5261 (Sunbright MEGC-50HS, PEG_{S5})] were purchased from NOF Corp. (Tokyo, Japan). 2-{8-(2-Methylimidazolyl-1-yl)-octanoyloxymethyl}-5,10,15,20-tetrakis{α,α,α,α-*o*-(1-methylcyclohexanamido)phenyl}porphinatoiron(III) chloride (Fe4P) and 5,10,15-tris{α,α,α-*o*-(1-methylcyclohexanamido)-phenyl}-20-mono-[β-*o*-{6-(2-methylimidazolyl) hexanamido}-phenyl]*porphinatoiron(III) chloride* (Fe3P) were synthesized using previously reported procedures (*10d,e*). Recombinant HSA was provided by the NIPRO Corp. (Osaka, Japan). The UV−vis absorption spectra were recorded using an Agilent 8453 UV−visible spectrophotometer fitted with an Agilent 89090A temperature control unit. The water was deionized using Millipore Elix and Simpli Lab-UV.

Preparation of PEG-Conjugated HSA−FeXP. The HSA−FeXP solutions (X = 3, 4, [HSA]: 5 g dL^{-1}, [FeXP]/[HSA]= 4 (mol/mol), pH 7.4) were prepared as described elsewhere (*10b,d*).

PEG_{MY}(HSA−FeXP): The modification of HSA−FeXP by α-maleimide-ω-methoxy PEG_{M2} was, for instance, carried out as follows. IMT (72 mg, 0.54 mmol) was slowly added to the HSA−FeXP solution (48 mL, [HSA]: 5 g dL^{-1}, [Fe4P] = 3 mM, pH 7.4) ([IMT]/[HSA] = 15/1, mol/mol) and gently stirred at room temperature in the dark. After 3 h, PEG_{M2} (1.44 g, [PEG_{M2}]/[HSA]:20/1, mol/mol) was added to the mixture, which was continually stirred for another 2 h. The resultant solution was ultrafiltered and washed by at least a 600 mL of phosphate-buffered saline (PBS) solution (pH 7.4) to remove any unreacted IMT and PEG_{M2} using the ADVANTEC UHP-76K holder with a Q0500 076E membrane (cutoff Mw: 50 kDa). The volume was finally condensed to 48 mL and sterilized by a DISMIC 0.45 μm filter, producing the PEG_{M2}(HSA−FeXP) solution. The FeXP concentration was determined by the assay of the iron ion by inductively coupled plasma (ICP) spectrometry using a Seiko Instruments SPS 7000A Spectrometer. The HSA concentration was calculated from the intensity of the circular dichroism spectrum at 208 nm, because the molar ellipticity of HSA (1.9×10^4 deg cm^2 $dmol^{-1}$) was unaltered after the PEG conjugation. Circular dichroism (CD) spectra were obtained using a JASCO J-725 spectropolarimeter. The concentration of the HSA sample was 0.15 μM in PBS, and quartz cuvettes with a 10-mm thickness were used for the measurements over the range of 195−250 nm. The PEG_{M2}(HSA−Fe3P) and PEG_{M5}-(HSA−Fe4P) solutions were also prepared by the same procedure. The product was sealed in a glass bottle under CO pressure and stored at 4 °C.

PEG_{SY}(HSA−Fe4P): The surface modification of HSA−Fe4P by α-succinimidyl-ω-methoxy PEG_{S2} was carried out as follows. PEG_{S2} (0.72 g, 0.36 mmol) was directly added to the HSA−Fe4P solution ([HSA]: 5 g dL^{-1}, [Fe4P] = 3 mM, pH 7.4) ([PEG_{S2}]/[HSA]:10/1, mol/mol), and the mixture was stirred at room temperature for 2 h. The resultant solution was ultrafiltered, condensed (48 mL), and sterilized as described above, producing the PEG_{S2}(HSA−Fe4P) solution. Using PEG_{S5} instead of PEG_{S2}, PEG_{S5}(HSA− Fe4P) was obtained. The Fe4P and HSA concentrations were determined by the same procedures for PEG_{MY}(HSA−FeXP).

Matrix-Associated Laser Desorption Ionization Time-of-Flight Mass Spectra (MALDI-TOF MS). The MALDI-TOF MS were obtained using a Shimadzu/Kratos AXIMA-CFR S/W Version 2, which was calibrated by BSA (Sigma A-0281) and HSA (Sigma A-3782). The specimens were prepared by mixing the aqueous sample solution (10 μM, 1 μL) and matrix (10 mg mL^{-1} sinapinic acid in 40% aqueous CH_3CN, 1 μL) on the measuring plate and air-drying.

Determination of Mean of PEG_{MY} Chains per Protein by Assay of Thiol Groups. The active thiol groups on the protein surface can be assayed by the disulfide exchange reaction with 2,2′-dithiopyridine (2,2′-DTP) to produce 2-thiopyridinone (2-TP) with an absorption at 343 nm (molar absorption coefficient (ϵ_{343}): 8.1×10^3 M^{-1} cm^{-1}) (*15*). Quantitative spectroscopic measurements conveniently provide the thiol concentration. The parent HSA−FeXP showed a small absorption band in this range, which should be subtracted from the spectrum after the disulfide exchange reaction. The difference in the thiol groups per HSA−FeXP before and after the PEG_{MY} modification corresponds to the mean of the PEG_{MY} chains on the protein surface.

Solution Properties. The viscosity and density of the PEG-conjugated HSA−FeXP solution (PBS, pH 7.4) were obtained using an Anton Paar DSC 300 capillary viscometer at 37 °C. The colloid osmotic pressures of the solutions (PBS, pH 7.4) were measured by a WESCOR 4420 Colloid Osmometer at 25 °C. A membrane filter with a 30 kDa cutoff was used.

O_2-Binding Parameters. O_2-binding to PEG-conjugated HSA−FeXP was expressed by eq 1,

$$\text{PEG(HSA−FeXP)} + O_2 \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \text{PEG(HSA−FeXPO}_2\text{)} \quad (1)$$

where $K = k_{on}/k_{off}$. The O_2-binding affinity (gaseous pressure at half O_2 binding for FeXP, $P_{1/2} = 1/K$) was determined by spectral changes at various partial pressures of O_2/N_2 as previously reported (*10b,d*). The FeXP concentrations of 10−20 μM were normally used for the UV−vis absorption spectroscopy. The spectra were recorded within the range of 350−700 nm. The half-lifetime of the O_2-adduct complex was determined by the time dependence of the absorption intensity at 550 nm (O_2-adduct species). The association and dissociation rate constants for O_2 (k_{on}, k_{off}) were measured by a competitive rebinding technique using a Unisoku TSP-1000WK laser flash photolysis as reported in a previous paper (*16*).

Circulation Lifetime in Vivo. The animal investigations were carried out using twenty male Wistar rats (297 ± 29 g). All animal handling and care were in accordance with the NIH guidelines. The protocol details were approved by the Animal Care and Use Committee of Keio University. The PEG-conjugated HSA−FeXP solution (20% volume of the circulatory blood) was intravenously injected into rats from the tail vein (1 mL/min) under an inhalation anesthesia with diethyl ether (n = 4 each). Blood was taken from the tail vein at 3, 30 min, 1, 2, 4, 8, 16 h, 1, 2, 3 days (10 time points) after the infusion and then centrifuged to isolate the serum, which was colored brown by the presence of the sample. The animals were sacrificed after the experiments by hemorrhage. The FeXP concentration was measured by an iron ion assay using ICP spectrometry as described above.

RESULTS AND DISCUSSION

Synthesis of PEG-Conjugated HSA−FeXP. The HSA−FeXP molecules were conjugated with PEG having a terminal reactive chain-end, maleimide-PEG or succinimide-PEG, at

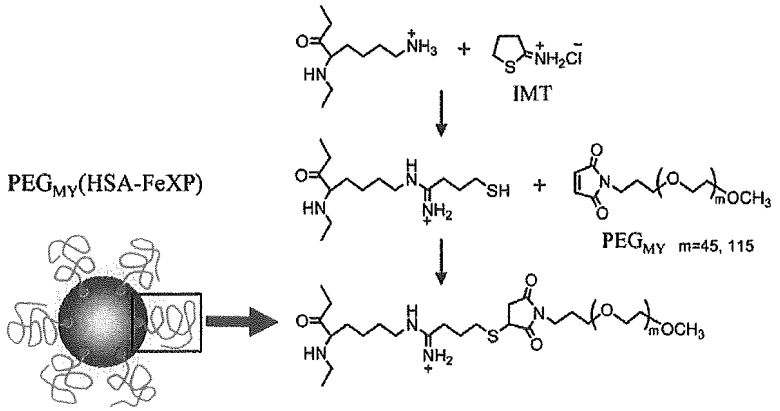

Figure 1. Two-step reaction schemes of IMT and maleimide-PEG (PEG$_{MY}$) with HSA−FeXP.

ambient temperature. Thiolation regent, IMT, quantitatively reacted with the amino groups of Lys to create active thiol groups, which bind to the α-maleimide-ω-methoxy PEG (PEG$_{M2}$ or PEG$_{M5}$) (Figure 1). The two-step reaction is reproducible and did not form any toxic side-product. On the other hand, the α-succinimidyl-ω-methoxy PEG (PEG$_{S2}$ or PEG$_{S5}$) directly binds to the amino groups of Lys. The gel permeation chromatogram (Sephacryl 200HR) of the well-washed PEG conjugate exhibited a single band, so that we did not need any further chromatographic purification.

The MALDI-TOF MS of PEG$_{M5}$(HSA−Fe4P), as prepared under the condition of [IMT]/[HSA−Fe4P] = 15/1 (mol/mol), showed five distinct ion peaks at 85, 90, 95, 101, and 106 kDa (Figure 2a). No unreacted HSA−FeXP was observed at all. The difference in each mass was 5.25 kDa, which implies that HSA−Fe4P is covalently bound to PEG$_{M5}$ and the individual peaks are attributed to PEG$_{M5}$(HSA−Fe4P) having a different number of PEG chains. Here, we have to be cautious whether these mass values involve a molecular weight of Fe4P, because our previous MALDI-TOF MS experiments of HSA−Fe4P demonstrated a single peak of HSA (Mw: 66.5 kDa); the incorporated Fe4P dissociated from the albumin during the ionization process (*10a*). In this study, we found that the mean of the surface PEG chains on HSA−FeXP is conveniently determined by a spectroscopic assay of the HSA scaffold and thiol groups. In general, the concentration of HSA is measured by the absorption at 280 nm or bromcresol green method (*17*), but they are probably obstructed by the surface modification. We then employed a CD measurement to determine the HSA concentration. The comparison of the CD spectra of HSA and PEG−HSA solutions revealed that the molecular ellipcity of albumin (ϵ_{208} = 1.9 × 10^4 deg cm^2 dmol^{-1}) is unaltered even after the PEG binding. Moreover, the presence of FeXP does not disturb the CD in the range of 190−250 nm. Therefore, the HSA concentration of PEG modified HSA−FeXP was quantitatively determined by its CD intensity at 208 nm. On the other hand, the active thiol groups on proteins are generally assayed by a disulfide exchange reaction with 2,2′-DTP (*15*). The combination of these two methodologies allows us to estimate the number of thiols on HSA−FeXP. The mean of the thiol groups was 6.7 per protein after the thiolation by IMT ([IMT]/[HSA−Fe4P] = 15 mol/mol) and decreased to 0.6 after the reaction with 20-fold excess moles of PEG$_{M5}$ (Table 1). These results suggested that the mean of 6.1 reactive thiols was conjugated with PEG$_{M5}$. The averaged molecular weight of this PEG$_{M5}$(HSA−FeXP) calculated from the intensity of the MS peak was 95 kDa. If one subtracts the total mass of the six PEG$_{M5}$ chains (5 kDa × 6 = 30 kDa) from 95 kDa, the difference of 65 kDa equals that

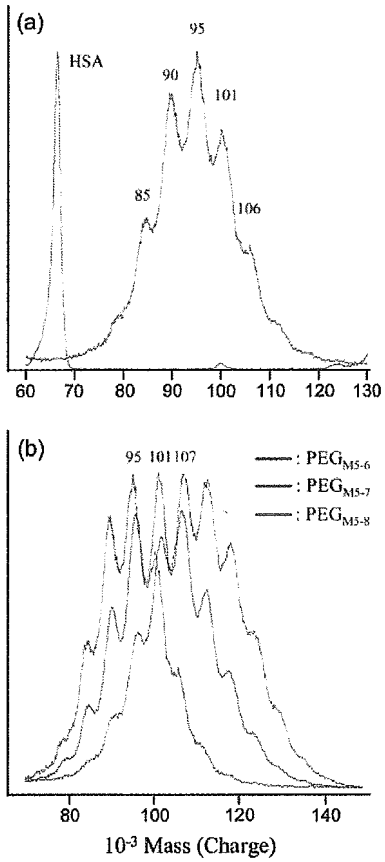

Figure 2. MALDI-TOF MS of (a) HSA and PEG$_{M5-6}$(HSA−Fe4P) and (b) PEG$_{M5}$(HSA−Fe4P) prepared in different [IMT]/[HSA−Fe4P] ratios of 15 (red), 20 (blue), and 30 (green) (mol/mol).

of HSA without Fe4P. Therefore, we concluded that all the mass ion peaks observed in the MALDI-TOF MS did not include the molecular weight of FeXP.

The number of the meleimide-PEG$_{M5}$ chains on HSA−Fe4P were modulated by the mixing ratio of [IMT]/[HSA−Fe4P] (mol/mol). The maximum peak of PEG$_{M5}$(HSA−Fe4P) in the MALDI-TOF MS significantly shifted to the higher molecular region (95 → 101 → 107 kDa) by increasing the IMT (Figure 2b). It is quite remarkable that the distributions of the entire spectral pattern were always identical. The averaged binding number of the PEG$_{M5}$ chains per HSA estimated from the intensity of the mass peak was consistent with the number determined from the assay of the thiol groups (Table 1).

Table 1. The Mean of Thiol Groups per HSA−Fe4P Molecule and Binding Number of the PEG Chains

PEG	[IMT]/[HSA−Fe4P] mol/mol	thiol groups per HSA after IMT addition (A)	thiol groups per HSA after PEG binding (B)	decreased thiol groups (B − A)[a]	averaged PEG number from MS
PEG_{M2}	10	5.6	0.5	5.1	4.6
	15	6.6	0.9	5.7	5.7
	20	8.3	1.1	7.2	6.6
PEG_{M5}	15	6.7	0.6	6.1	5.9
	20	8.0	0.9	7.1	7.2
	30	9.3	1.1	8.2	8.3

[a] This number corresponds to the binding numbers of PEG_{MY} on the protein surface.

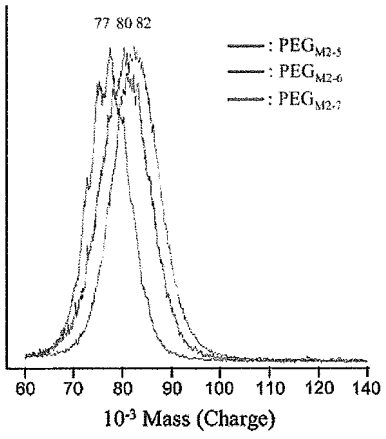

Figure 3. MALDI-TOF MS of PEG_{M2}(HSA−Fe4P) prepared in different [IMT]/[HSA−Fe4P] ratios of 10 (blue), 15 (red), and 20 (green) (mol/mol).

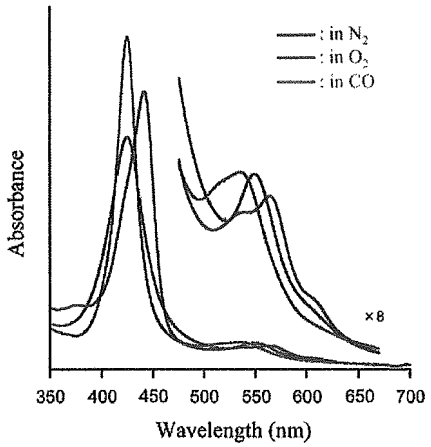

Figure 4. UV-vis absorption spectral change of PEG_{M2-6}(HSA−Fe4P) in PBS solution (pH 7.4).

Table 2. Solution Properties of PEG-Conjugated HSA−FeXP Solutions at 37 °C (pH 7.4, [FeXP] = 3 mM)

PEG	density (g/cm^3)	viscosity (cP)	COP (mmHg)
PEG_{M2-5}(HSA−Fe4P)	1.01	1.08	22
PEG_{M2-6}(HSA−Fe4P)	1.01	1.14	27
PEG_{M2-7}(HSA−Fe4P)	1.01	1.17	28
PEG_{M2-6}(HSA−Fe3P)	1.01	1.14	26
PEG_{M5-6}(HSA−Fe4P)	1.01	2.34	65
PEG_{S2-6}(HSA−Fe4P)	1.01	1.14	22
PEG_{S5-6}(HSA−Fe4P)	1.01	2.30	45
HSA−Fe4P	1.01	1.05	21
HSA	1.01	1.00	21

On the contrary, PEG_{M2}(HSA−Fe4P) demonstrated only one broad peak, because the difference in each mass is relatively close compared to the PEG_{M5} conjugate. The peak maxima of PEG_{M2}(HSA−Fe4P) also shifted to the higher region, dependent on the [IMT]/[HSA−Fe4P] ratio (Figure 3, Table 1).

The succinimide-PEG modified HSA−Fe4P showed the same MALDI-TOF MS patterns as PEG_{MY}(HSA−Fe4P) (data not shown). The number of 5-kDa PEG_{S5} chains per protein increased from 4 → 5 → 6 by elevating the ratio of [PEG_{S5}]/[HSA−Fe4P] (mol/mol): 10 → 20 → 30, respectively. However, the introduced 2-kDa PEG_{S2} number was always 6 in the range of [PEG_{S2}]/[HSA−Fe4P] 10−20. The stoichiometry of the PEG_{S2} binding could not be controlled. This is probably due to the hydrolysis of the succinimidyl end group in aqueous media. The concentration assays of [HSA] by CD and [FeXP] by ICP measurements showed that the initial FeXP/HSA ratio, 4/1 (mol/mol), were constant after the PEG conjugation.

Solution Properties. The viscosity and colloid osmotic pressure (COP) of the 2-kDa PEG-conjugates, PEG_{M2}(HSA−FeXP) and PEG_{S2}(HSA−Fe4P) (PBS solution, [HSA] = 5 g dL^{-1}, pH 7.4), were almost the same as those of the nonmodified HSA−FeXP independent of the number of the PEG chains (Table 2). In contrast, the 5-kDa PEG-conjugate solutions, PEG_{M5}(HSA−FeXP) and PEG_{S5}(HSA−Fe4P), showed a high viscosity (2.30−2.34, at a shear rate of 230 s^{-1}) and hyperoncotic property (45−65 mmHg) in comparison to those of HSA−Fe4P and HSA. From the viewpoint of the design of a blood alternative, it has to achieve a COP similar to that of human blood. However, to increase the effectiveness as a plasma expander, the COP should be higher than the physiological level (*18*). Similar approaches have been utilized for hypertonic saline−dextran formulations.

On the other hand, maintenance of the viscosity has recently been proposed as an important mechanism to preserve shear forces in the microcirculation which prevents loss of the functional capillary density (*19*). The latest PEG-Hb product has been designed to approach that of human whole blood (*7*). Anyway, the COP and viscosity of our PEG-conjugated HSA−FeXP can be adjustable to some extent based on the length of the PEG chains (2-kDa, 5-kDa) on the molecular surface.

O_2-Binding Properties. The UV−vis absorption spectrum of the PEG_{M2}(HSA−Fe4P) solution under an N_2 atmosphere showed λ_{max} at 441, 537, 563 nm (Figure 4), which indicates the formation of the ferrous five-N-coordinate high-spin complex of Fe4P with an intramolecular coordinated 2-methylimidazolyl-group (*10b,c, 20*). The other amino acid residue of HSA did not bind to the sixth-coordinate position of the central ferrous ion. Upon flowing O_2 gas through this solution, the spectral pattern shifted to that of the well-defined O_2-adduct complex of the tetrakis(phenyl)porphinatoiron(II) derivatives (λ_{max}: 424, 550 nm) (*10b,c, 20*). This oxygenation was reversibly observed, dependent on the O_2-partial pressure. After exposure of this solution to CO, PEG_{M2}(HSA−Fe4P) produced a very stable CO-adduct complex (λ_{max}: 425, 535 nm). The all maleimide- and succinimide-PEG-conjugated HSA−FeXP solutions showed similar UV−vis absorption spectra under N_2, O_2, and CO atmospheres. These spectral changes were completely the same as that observed in the nonmodified HSA−Fe4P.

Table 3. O_2-Binding Parameters of PEG-Conjugated HSA−FeXP Solution at 25 °C (pH 7.4)

system	k_{on} ($\mu M^{-1} s^{-1}$) fast	k_{on} ($\mu M^{-1} s^{-1}$) slow	k_{off} (ms^{-1}) fast	k_{off} (ms^{-1}) slow	$P_{1/2}$ (Torr)	$\tau_{1/2}$ (h) at 37 °C
PEG_{M2-5}(HSA−Fe4P)	11	5.8	0.16	0.08	38 (11)	13
PEG_{M2-6}(HSA−Fe4P)	12	4.6	0.17	0.07	32 (11)	12
PEG_{M2-7}(HSA−Fe4P)	9.3	4.7	0.16	0.08	35 (13)	12
PEG_{M2-6}(HSA−Fe3P)	15	4.2	0.52	0.14	41 (26)	8
PEG_{M5-6}(HSA−Fe4P)	12	6.2	0.17	0.09	31 (11)	16
PEG_{S2-6}(HSA−Fe4P)	10	4.3	0.14	0.06	36 (11)	13
PEG_{S5-6}(HSA−Fe4P)	12	5.5	0.25	0.11	32 (16)	18
HSA−Fe4P	31	7.3	0.53	0.13	34 (13)	9
HSA−Fe3P	29	4.4	1.1	0.16	45 (22)	4

The time course of the absorption decay after laser flash photolysis determined the association rate constants of the O_2binding to the PEG-conjugated HSA−FeXP (k_{on}). We have previously reported that the O_2-binding reaction to HSA−FeXP was significantly affected by the microenvironment around FeXP in the protein (e.g., steric hindrance of the amino acid residue and difference in polarity) (*10c−e*). As a result, the binding process of O_2 was observed as the sum of the two single-exponentials, giving fast and slow association rate constants [k_{on}(fast) and k_{on}(slow)]. This unique property of HSA−FeXP has been unaltered after the surface modification by PEG, and all kinetics accompanying the O_2 recombinations consisted of two phases (Table 3). Interestingly, their k_{on}(fast) values were 1.9−3.3-fold lower than those of the corresponding HSA−FeXP, independent of the molecular weight (2-kDa or 5-kDa) and the linkage structure of the PEG. The differences observed in the slow phase were smaller. It may be considered that the presence of flexible polymers on the protein surface retarded the diffusion of the O_2 molecule.

The O_2-binding affinities ($P_{1/2} = K(O_2)^{-1}$) of the PEG-modified HSA−FeXP series were determined by measuring the UV−vis absorption spectral changes by O_2/N_2 titration (Table 3). All the PEG conjugates showed almost the same $P_{1/2}$ values relative to that of the original HSA−FeXP, indicating that the O_2-binding equilibria were not influenced by the presence of PEGs. In contrast, the surface modification by PEG delayed the proton-driven oxidation of the $FeXPO_2$ species and prolonged the half-lifetime of the O_2-adduct complex [$\tau_{1/2}(O_2)$]. The PEG_{M5-6}(HSA−Fe4P)O_2 complex showed the longest $\tau_{1/2}(O_2)$ of 16 h at 37 °C, which is greater than those of HSA−Fe4P and natural hemoprotein, myoglobin ($\tau_{1/2}(O_2)$: 12 h at pH 7, 35 °C) (*21*). Basic polymer PEG conjugation might change the local proton concentration of the HSA interior compared to the outer aqueous solution. Actually, it was shown that the surface PEG modification of hemoproteins has modulated the redox behavior of the active heme site (*1*, *2*, *22*).

Circulation Lifetime in Bloodstream of Rats. The circulation persistence of FeXP in the bloodstream after the administration of PEG_{MY-6}(HSA−FeXP) or PEG_{SY-6}(HSA−Fe4P) solution in rats is shown in Figure 5. The concentration decays of the PEG_{MY}(HSA−FeXP) series showed single exponentials, and the half-lifetimes ($\tau_{1/2}$) were 12.9−15.8 h, independent of the molecular weight of the polymers and FeXP structures. These values are much longer than those of the corresponding nonmodified HSA−FeXP (0.6−3.2 h) (*23*). The surface modification by PEG significantly prevented the rapid clearance of the incorporated FeXP and contributed to increasing the O_2-transporting efficacy.

On the contrary, the PEG_{SY}(HSA−Fe4P) series showed biphasic kinetics and a $\tau_{1/2}$ value of 1.5−2.1 h. We have postulated two reasons for this short circulation lifetime. The first reason is the charges of the Lys residues. The maleimide-PEG connects to Lys through the ring-opened IMT, which maintains the positive charge of Lys; therefore, PEG_{MY}(HSA−FeXP) could preserve the total electrostatic potential of albumin. In contrast, the succinimide-PEG directly binds to the amino group of Lys to form the amide bond, thereby reducing the positive charge and alters the surface electrostatic potential. This changing of the molecular charge may influence the rapid clearance of the incorporated FeXP.

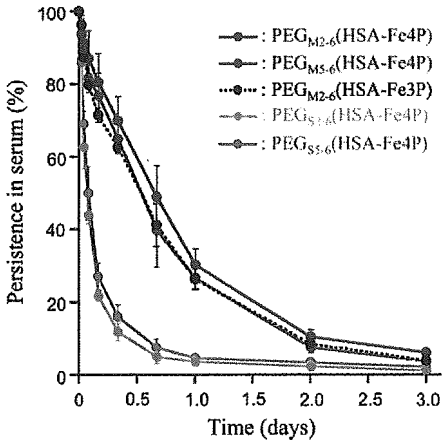

Figure 5. Persistence of FeXP in serum after administration of PEG-conjugated HSA−FeXP into Wistar rats. All values are mean ± SD (n = 4).

The second probability is the binding sites of the PEG chains. As shown in Table 1, the difference in the thiol number per protein before and after the reaction with maleimide-PEG was 0.5−1.1. This means that the binding position of PEG_{MY} is governed by the reaction place of IMT, which is small enough to statistically attach 59 Lys in HSA. Thus, the molecular surface of HSA−FeXP is uniformly covered by PEG_{MY}. On the other hand, the attaching sites of succinimide-PEG are presumably heterogeneous, because PEG_{SY} could only bind the accessible amino group of Lys due to the bulkiness of the long polymer. The incorporated Fe4P molecules might be more easily released from the PEG_{SY}-modified HSA in the circulatory system.

CONCLUSIONS

The surface modification of the albumin-based synthetic hemoprotein, HSA−FeXP, by PEG (Mw 2-kDa or 5-kDa) has improved its comprehensive O_2-transporting ability. The PEG conjugation decreased the O_2-association rate constant but retarded the irreversible oxidation of the central ferrous ion, thereby increasing the stability of the O_2-adduct complex. The 5-kDa PEG conjugation increased the viscosity and COP; however, the 2-kDa PEG conjugation did not change these rheological parameters. The linkage form of the PEG chain dramatically affects the circulation persistence of FeXP. In particular, the maleimide-PEG_{MY} conjugates showed a 6−8-fold longer lifetime compared to the succinimide-PEG_{SY} analogues. This is not dependent on the molecular weight of the polymer chains. In summary, the PEG_{MY}(HSA−FeXP) solution is the most promising candidate as an entirely synthetic O_2-carrying plasma expander for a red cell substitute.

Furthermore, we have recently found that water evaporation of the PEG_{MY}(HSA−FeXP) solution produced a red-colored thin film. Its UV−vis absorption spectrum reversibly changed from the deoxy state under an N_2 atmosphere to the oxy state by exposure to O_2 gas. This PEG_{MY}(HSA−FeXP) film was redissolved in nonaqeuous organic solvents, ethanol, chloroform, etc., and the reversible O_2 binding was again observed. The detailed study of the oxygenations of PEG_{MY}(HSA−FeXP) in a cast film and organic solvent are now underway.

ACKNOWLEDGMENT

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目で見るバイオ

ヘモグロビン小胞体は、高純度ヒトヘモグロビン(Hb)をリン脂質小胞体(リポソーム)に内包した人工赤血球である。何と、脂質成分とHbが分子間相互作用だけで形成している分子集合体である。透過型電子顕微鏡写真(図1)から、精密制御された粒子径(250nm)と、Hb(約30000個)が濃度高く内包されている様子が解る。粒子表面は約6000本のpoly(ethylene glycol)鎖で修飾してあり、室温で2年間の保存も可能な優れた安定度を有する。光学顕微鏡では粒子の形状は観察できないが、ハムスターの循環血液量の80%を交換したのちの皮下微小循環の観察では、Hb小胞体は血漿中に均一に分散し、通常は見えない毛細血管の形状までもがコントラストよく見える(図2)。愛媛大学医学部との共同で、赤血球と種々の比率で混合し、モデル血管内(28 μm径)を1mm/sの流速で流動させた実験では、赤血球(8 μm)は管の中心側を流動するのに対し(図3)、血漿層の濁りからHb小胞体は均一分散して管壁側を流動する様子が解った。慶応義塾大学医学部ほかとの共同によるHb小胞体の交換輸血試験、出血ショック蘇生試験では、赤血球と同等の酸素運搬機能が実証されている。加えて、赤血球に比較して小粒径で均一分散するので、血管狭窄部の透過、あるいは側副経路を経由した虚血性低酸素領域への酸素運搬にも有効であることが解ってきた(Univ. California, San DiegoおよびInselspital Univ. Hospital, Bernとの共同)。Hb小胞体は酸素運搬の機能を終えたのち、最終的には老化赤血球と同様に細網内皮系(主に脾、肝)に捕捉される運命をたどる。Hb小胞体をラットに投与し(20mL/kg)、1日後の脾臓を電子顕微鏡観察すると、マクロファージ食胞内にHb小胞体が多数捕捉されていたが(図4)、7日後には完全に消失した。また、抗ヒトHb抗体を用いた肝臓の免疫染色(図5)では、投与1日後にはKupffer細胞に捕捉されたHb小胞体が赤染部位として認められたが、7日後には完全消失しており、Hb小胞体は蓄積することなく、速やかに分解排泄される様相まで明らかになった。

血液型物質を含まず、感染の心配がなく、しかも長期間備蓄可能な人工赤血球の実現は、医療技術に変革をもたらすことは間違いない。現在は、担当企業に技術移転を行い、夢を膨らませて臨床試験に向けた最終作業を進めている。

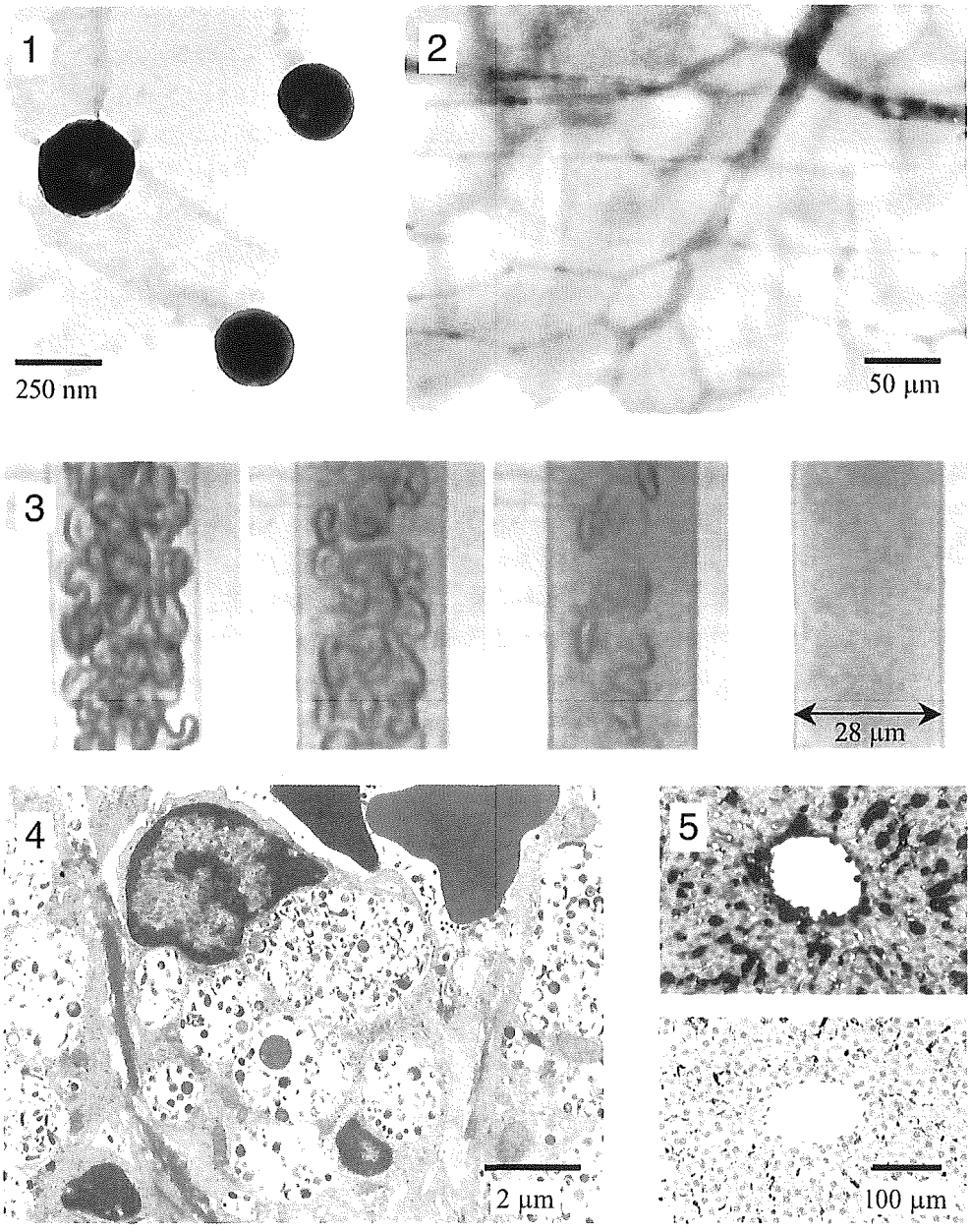
(酒井宏水、土田英俊)

キーワード：人工赤血球、微小循環、ヘモグロビン、リポソーム、輸血代替

筆者紹介：さかい・ひろみ(SAKAI, Hiromi)　早稲田大学理工学総合研究センター(Adv. Res. Inst. for Sci. and Eng., Waseda Univ.)助教授　1994年早稲田大学大学院理工学研究科博士課程修了　博士(工学)　専門：血液代替物科学　連絡先：〒169-8555 新宿区大久保3-4-1　E-mail　hiromi@waseda.jp (勤務先)

つちだ・えいしゅん(TSUCHIDA, Eishun)　同上 名誉教授　1963年早稲田大学大学院理工学研究科博士課程修了　工学博士　専門：高分子錯体　連絡先：同上

微小血管内を均一に流れる人工赤血球とその運命



＊写真左上の数字は図の番号です。

図1　人工赤血球（Hb小胞体）の透過型電子顕微鏡像

2　循環血液量の80%をHb小胞体で交換した後のハムスター背部皮下微小循環の光学顕微鏡像　波長420nm付近の光（Hbの吸収帯）を照射して撮影。

3　赤血球とHb小胞体を混合し、プラスチック製のモデル血管内を流動させたときの光学顕微鏡像　混合容積比は左から、赤血球/Hb小胞体＝100/0, 50/50, 10/90, 0/100。

4　Hb小胞体投与1日後のラット脾臓マクロファージの透過型電子顕微鏡像

5　Hb小胞体投与1日後（上）、7日後（下）の肝臓組織の抗ヒトHb抗体免疫染色像

人工赤血球・人工血小板の開発の現状

武岡真司
早稲田大学理工学術院

はじめに

筆者の所属する研究グループ（早稲田大学理工学総合研究センター）は，慶應義塾大学医学部と共同して厚生労働省科学研究，医薬品・医療機器などレギュラトリーサイエンス総合研究事業，H 16-医薬-067, 069, 071により人工赤血球と人工血小板の研究を推進している．人工血液全体の現状に関しては，厚生労働省科学研究の研究代表者小林紘一教授による本誌「講座」に詳しい[1]．本「講座」では，これらの厚生労働科学研究の成果の一部も含めて報告する．人工赤血球は，リン脂質の二分子膜小胞体（リポソーム）に酸素を酸素分圧に応じて吸・脱着する分子（ヘモグロビン）を内包させた酸素運搬体であり，これが血中に長く留まって安全，安定に酸素運搬機能を発現し続ける．それに対して人工血小板は，血管損傷部位や活性化した血小板のみを認識する分子をリポソームやアルブミン重合体に担持した微粒子であり，これが血中に長く留まって血管損傷部位に特異的に粘着して止血機能を発現する．筆者らは理工学の立場から人工赤血球や血小板の材料となる担体の設計，製造，物性評価を行ってきた．担体には，適当な血液適合性や血中滞留性が求められるが，分解性や代謝物の低毒性も重要な検討項目である．現在担体としてリン脂質分子の集合体（リポソーム）や遺伝子組み換えヒトタンパク質の複合体や重合体を選択して用いている．

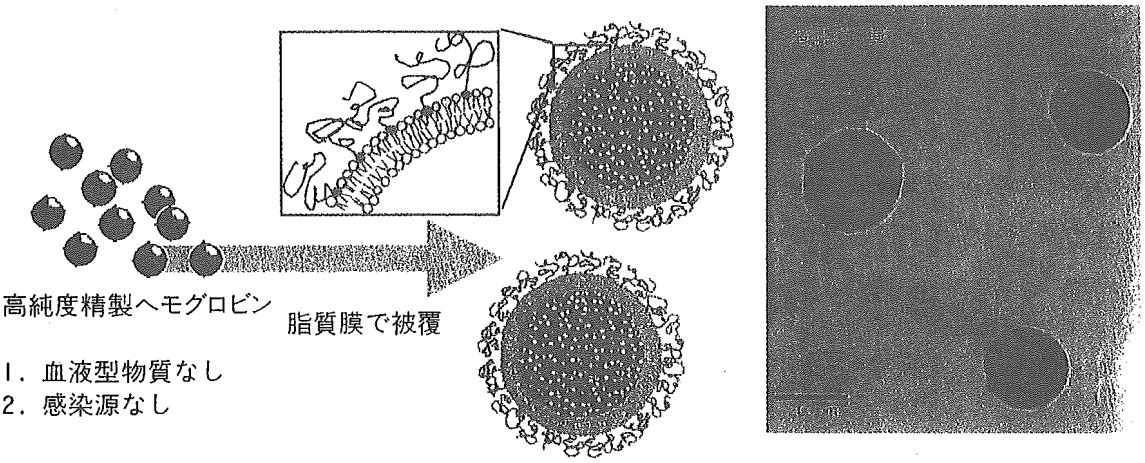
キーワード：人工赤血球，人工血小板，
ヘモグロビン

Seminar
Current Development of Artificial Red Blood Cells and Artificial Platelets

Shinji Takeoka (*Waseda University, Faculty of Science and Engineering*)
〒169-8555　東京都新宿区大久保3-4-1
早稲田大学理工学術院；教授

1. ヘモグロビン小胞体の構造

人工赤血球としてパーフルオロカーボン乳剤や修飾ヘモグロビンなどが検討され臨床使用されてきたが，機能や安全性の観点から満足できるものではなかった．筆者の所属するグループで開発を進めている，高濃度ヘモグロビンをリポソームの内水相に内包させた，赤血球と類似構造のヘモグロビン小胞体(図❶)は，最も安全度と機能が高いため早期の臨床試験着手が期待されている[2]．現段階では期限の切れた献血血液由来のヘモグロビンの有効利用が進められているが，将来的には遺伝子組み換えヒトヘモグロビンが利用されるであろう．赤血球からヘモグロビンを精製する際に，血液型を決める型物質やヘモグロビン以外のタンパク質，ウイルスや菌（もし含まれたとしても）を加熱やフィルター処理で除去されている．生理活性なヘモグロビンを安定なリン脂質膜で包むことによって，ヘモグロビンに由来する副作用（血管収縮や腎毒性，神経毒など）を回避できる．ヘモグロビン小胞体は生理食塩液に分散され，脱酸素状態で容器に密封されているため，室温で2年間の液状保存（赤血球製剤では採血後3週間の冷蔵保存）が保証されており，乾燥粉末ではさらに長期間の保存が可能である．製剤のヘモグロビン濃度



図❶　ヘモグロビン小胞体の構造と模式図ならびに透過型電子顕微鏡写真．

は10 g/dlであり，ヒト血液の値（11～15 g/dl）と比較して遜色ない．また，ヘモグロビン分子が封入されているため製剤の膠質浸透圧はほとんどゼロである．したがって，膠質浸透圧の調節が必要となる場合にはアルブミン（リコンビナント）や多糖類などのコロイド製剤と併用となる．図❶の電子顕微鏡写真では，ヘモグロビンの鉄が染色されており，数多くのヘモグロビンが脂質分子膜で包まれた小胞体構造であることと，粒子径が約250 nmに厳密に調節されていることがわかる．これは，赤血球の約1/30程度の粒子径であるので，梗塞部位の透過など赤血球にはない機能が期待できる．酸素親和度はアロステリック因子，ピリドキサール5′-リン酸の共封入により適当値に調節されている．脂質類の成分組成には，ヘモグロビンのカプセル化効率，常温で2年間液状保存できる安定性，血流中での溶血の回避と適度な血中滞留時間（人では3日程度の半減期予測），血小板や補体の活性化の回避など，に対する工夫が施されている．製造面でも分子集合技術を利用した粒子径の厳密な制御と高濃度ヘモグロビンの内包など，従来の小胞体における課題が解決できている[3]．

2. 動物試験による機能と安全性の評価

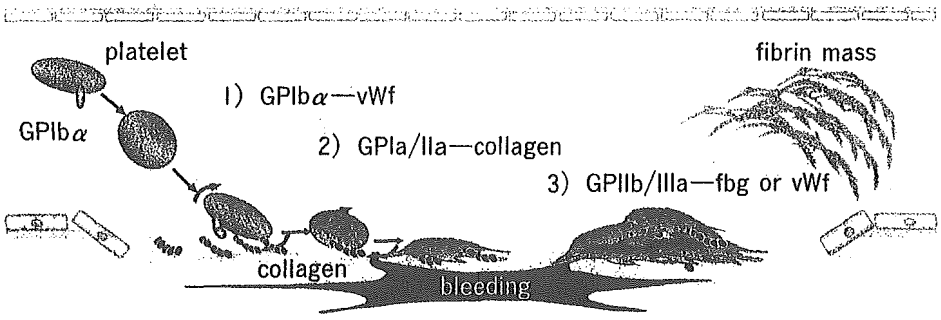
現在までに結果が得られているヘモグロビン小胞体に関する評価試験成績を簡単に紹介する．主にラットやハムスターを用いた試験であるが，基本的な安全性と酸素輸送効果は十分確認できている．また，現在霊長類を用いた安全性試験が進行している．酸素運搬効果を確認する試験として，ラット全血液量の90%をアルブミン単独で交換した場合には，70%交換あたりから血圧と腎皮質酸素分圧の低下が顕著となって死亡したが，ヘモグロビン小胞体をアルブミン溶液に分散させた系で90%交換した場合には，血圧，腎皮質酸素分圧ともに維持された[4]．ヘモグロビン小胞体のアルブミン分散液によるハムスター80%交換輸血試験では，非侵襲に測定した皮下微小循環系の組織酸素分圧は交換前の60～70%に低下するものの，対照アルブミン投与群よりも5倍以上の値が維持されていた[5]．さらにNZW兎を用いた検討では，人工呼吸下，脱血し平均血圧を30～35 mmHgに低下させた後，ヘモグロビン小胞体分散液を投与し，組織酸素分圧の多点測定を実施，とくに脳と腎臓でヘモグロビン小胞体が有意な回復効果を発揮することを明らかにしている[6]．中型動物を用いた実験としてビーグル犬（約7 kg）を用い人工呼吸下，脾臓摘出後アルブミンで75%血液希釈後さらに30%脱血し，30分経過後に人工赤血球を投与し，循環動態，血液ガス組成，組織酸素分圧，組織酸素化度，心拍出量，血中酸素濃度の回復が確認されている[7]．

安全面では，血管弛緩因子である一酸化窒素や一酸化炭素が関与してヘモグロビンに認められる抵抗血管の収縮と血圧亢進は，ヘモグロビン小胞体では認められなかった[8,9]．これは，ヘモグロビン小胞体の大きさが寄与しているものと思われる．ヘモグロビン小胞体の血中滞留時間は，ラット，ラビット，カニクイザルからヒトへ類推すると，3日間程度の半減期として見積もられ，緊急時の単回投与では十分とされる．ラットでは，血中半減期が1～1.5日間であるので，脾臓や肝臓の病理組織学的所見では，投与1日後には脾臓や肝臓に貪食されていたヘモグロビン小胞体は3日後には激減し，投与7日以内にはほとんど消失していた[10]．また，ラットでの単回交換投与（循環血液量の40%交換），反復負荷投与（10 m*l*/kg/day，14日間）による血液生化学試験（30項目）や病理試験での詳細から，ヘモグロビン小胞体成分である，脂質分解に関わるリパーゼの亢進，コレステロール値の上昇，鉄の沈着，細網内皮系の肥大が一過性に認められた以外に変動を認めていない[11]．その他免疫系，凝固系への影響も認められておらず，大量出血時の緊急対応では十分なる機能を発現するものと期待されている．

上述のような効果と安全性の高い人工酸素運搬体では，輸血の代替以外にさまざまな適応が検討されている．たとえば体外循環回路補充液としての利用の検討では，ラット体外循環モデルの作成のため小型人工心肺を試作し，ヘモグロビン小胞体分散液を充塡液として使用，血液交換率が50%以上になる条件で灌流させた後，灌流回路中のヘモグロビン小胞体を分離除去して赤血球を回収して投与し，長期生存できることを確認している[12]．虚血性疾患の治療への利用においても，虚血再灌流実験などで小粒径のヘモグロビン小胞体の効果を実証する *in vivo* 実験が進められている．*in vitro* では，微小血管モデル内を流動するヘモグロビン小胞体の酸素放出挙動の解析から，虚血領域酸素化の機序解明を進められている[13,14]．人工酸素運搬体は，腫瘍組織酸素化にも有効であることを実証し，新しい適応の可能性を提示された[15]．

3. 人工血小板の開発の考え方

血小板は出血部位に対し特異的粘着，伸展，凝集，放出，血液凝固系の活性化などの複雑な機能をもち，これらのすべてを兼備した血小板代替物の構築は事実上不可能であろう．しかし，血小板の粘着と凝集に着目して，これらの機能を付与させた担体の投与によっても，少数残存する血小板の機能補助ができるものと考えられる．筆者らは慶應義塾大学医学部内科 池田康夫教授のグループ



図❷　血小板の止血機構．
一次止血（血小板血栓）
1) 接着（tethering → rolling）GPIbα—vWf
2) 粘着（adhesion）GPIa/IIa—collagen
3) 凝集（aggregation）GPIIb/IIIa—fbg or vWf
二次止血（フィブリン血栓）

とともに，血小板膜タンパク質の一部の遺伝子組換え体や合成オリゴペプチドを担持させた微粒子を作成し，これらが血小板を巻き込んで出血部位へ集積することによって止血能が発現されることを期待して，研究を進めている[16]．

血小板による止血は，高ずり速度の血流と低ずり速度の血流では機構が異なる．図❷に示したように，高ずり速度の出血に対する血小板の止血は，出血部位に露出する血管内皮下組織であるコラーゲンに結合したフォンビルブランド因子(vWf) に対して，血小板が認識して接着して転がることから始まる．*in vitro* 観測で抗 GPIIb/IIIa 抗体を添加して GPIIb/IIIa の機能を阻害した血小板では，vWf 固定化基板上を流動方向に沿って転がる現象がみられ，この認識能は血小板表面の GPIb/V/IX 複合体の GPIbα 部が担っている[17]．次に血小板表面の GPIaIIa ($\alpha_2\beta_1$ インテグリン) や GPVI が直接コラーゲンと相互作用して血小板は粘着し，そこで活性化されると血小板は伸展して顆粒を放出するが，最も重要な過程は GPIIb/IIIa ($\alpha_{IIb}\beta_3$ インテグリン) が活性体となる現象である．フィブリノーゲンは，この活性体を認識して血小板間を架橋し凝集体を形成して一次止血を担う．引き続く凝固系の誘導によるフィブリン塊の形成 (二次止血) によって止血が完成する．そこで，高ずり速度の血流下で vWf を介してコラーゲンを認識する GPIbα，低ずり速度でコラーゲンを直接認識する GPIaIIa，活性化血小板上の GPIIb/IIIa を認識するフィブリノーゲン (Fbg) やその認識部位であるペプチドを候補とした．

4. 人工血小板の研究動向

採血液に抗 GPIIb/IIIa 抗体を添加して GPIIb/IIIa を阻害すると，血小板表面の GPIbα との相互作用によって vWf 固定化基板上を流動方向に沿って転がる現象がみられる．そして，rGPIbα を担持させたリン脂質小胞体でも血小板と同様に vWf 基板上を転がることが確認された[18]．転がる小胞体の数はずり速度が高くなるほど多くなり，rGPIbα の特性が確認できた．また，その転がり速度は小胞体を構成する膜の柔軟性と相関した[19]．すなわち“柔らかい”小胞体では転がり速度は低くなり，“硬い”小胞体では転がり速度は高くなった．他方，アルブミン重合体は，内部が充塡された無定形な綿雪のような形態をとっており，出血部位での充塡効果が期待できる．表面に rGPIbα を結合させたところ，小胞体のような vWf 基板を転がる挙動は全く認められず，高ずり速度下でも粘着する挙動が認められた．ラテックスビーズに rGPIbα を結合させた系でも粘着することから，担体が重合体である場合と膜構造をもつ場合では rGPIbα 機能の発現の仕方が異なることが示唆された[20]．

他方，主に低ずり速度の血流下でコラーゲンに直接結合する血小板膜タンパク質の遺伝子組換え体 (rGPIaIIa) を結合させた小胞体は，コラーゲン基板を特異的に認識して粘着 (停止) することが西谷ら[21]によって確認された．また，ずり速度が高くなるにつれ粘着数は減少するが，rGPIbα と rGPIaIIa ともに担持させた小胞体では，低ずり速度から高ずり速度までコラーゲン基板を粘着できる系が構築されている[22]．

さらに減少した残存血小板の凝集を補助するために，粘着して活性化した血小板同士を架橋するフィブリノーゲンを結合させたアルブミン重合体も検討した[23]．活性化血小板の固定化基板を作成し，フィブリノーゲン結合アルブミン重合体を流動させたところ基板上に一様に粘着し，抗 GPIIb/IIIa 抗体を添加した系やアルブミン重合体のみの系では粘着が抑制された．血小板数が正常値の 1/5 程度に調節された血小板減少モデル血液にフィブリノーゲン結合アルブミン重合体を添加したところ，濃度増大とともに流動血小板の粘着数が増大したことから，フィブリノーゲン結合アルブミン重合体は血小板粘着増強効果を有する微粒子であることが示唆された．しかし，フィブリノーゲンは不安定であり，しかも現状ではヒト血液由来となるため，Fbg の γ 鎖 C 末端アミノ酸序列 (H 12：HHLGGAKQAGDV) を結合さ

せた系を用いた研究を重点的に進めている[24]．H 12結合アルブミン重合体を血小板減少血液（[血小板]＝2.0×10⁴/μl）に添加しコラーゲン基板上に流動させたところ，粘着血小板の占有率が増加し，そこにH 12結合アルブミン重合体が巻き込まれていたのでH 12-polyAlbは血小板凝集を補強する効果があると考えられた．

筆者ら[25]は抗がん剤であるブスルファン投与の副作用によって血小板が減少したラットを用いて*in vivo*効果試験を行っている．血小板数が正常値の1/5程度まで減少した状態のラットに対して，セボフルラン麻酔後試料を尾静脈投与した．試料投与5分経過後，尾先端から1 cmの部位にクイックヒール（ベクトン・ディッキンソン社製）を用いて傷（長さ2.5 mm，深さ1 mm）をつけ，尾先端を生理食塩水液に浸して止血時間を計測した．また，試料投与5分前，投与30分後に採血し，各血球変動を観察した．コントロールとして生理食塩液を投与した血小板減少症モデルラット群（[血小板]＝19.8±2.8/μl）の出血時間は609±153秒であり，正常ラット群（[血小板]＝80.9±8.6/μl）の出血時間（178±56秒）と比較して約3.4倍延長した．H 12結合していないアルブミン重合体を40 mg/kg投与したところ，出血時間は短縮し(184±69秒)，投与量の減少に伴いその効果は減少した．したがって，アルブミン重合体自体でも止血効果を有することが示唆された．そこで，出血時間に影響しないアルブミン重合体の投与量（4 mg/kg）でH 12-アルブミン重合体の止血能を検討した．H 12-polyAlbの投与では，出血時間352±73秒となり出血時間は半分に短縮したが，逆配列H 12を結合させたアルブミン重合体では出血時間を短縮させないので，H 12の効果が確認された．さらに検体投与5分前，投与30分後に採血し，各血球変動を観察したところ，各検体投与前後における血球変動は生じていないことから，H 12-アルブミン重合体は血液適合性の高い微粒子系と思われた．さらにポリエチレングリコールでアルブミン重合体を表面修飾し，一部のポリエチレングリコール鎖末端にH 12を結合させた系では，投与後3時間後に同様の試験を行っても止血効果が持続していることが確認された．

他方，rGPIaIIaを担持させたアルブミン重合体ではX線照射で血小板数を正常値の1/5程度に減少させたマウスに投与したところ，コントロール群の出血時間（730±198秒）と比較して，投与量依存的に出血時間の短縮が認められた（たとえば2.4×10¹¹ particles/kgでは出血時間は337±46秒)[26]．

現在，GPIbαを結合させたアルブミン重合体やリポソームの系で*in vivo*試験が進行中であるが，予想どおりの結果が得られつつあるので，今後はこれらの混合系における最適化を目指している．

おわりに

人工赤血球は臨床試験を目指して，企業がGLP製造を行う段階に入っている．また，人工血小板の研究は，動物試験での効果と安全性を多角的に確認している段階にある．これらの製剤はいずれもわが国が最先端にあるため，有効性や安全性の試験項目や方法の設定やガイドラインの作成に対して迅速で慎重なる検討が必要である．そのためには，産官学の共同体制での研究や協議の場として，学会（たとえば日本血液代替物学会や関連学会）の果たす役割と責任も大きいと思われる．産業においては，ナノバイオロジクス領域における具体的な成果として，わが国の近未来医療への貢献はもとより，安全な血液が不足している多くの国に対しても大きな国際貢献と成り得る．まずは長期的そして全人類的な視野に立った開発を期待したい．

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CHAPTER 160

Blood Substitutes' Efficacy
Microvascular and Rheological Determinants

[1]Amy G. Tsai, [1]Pedro Cabrales, [2]Hiromi Sakai, and [1]Marcos Intaglietta
[1]*Department of Bioengineering, University of California, San Diego, La Jolla, California*
[2]*Department of Polymer Chemistry, Waseda University, Tokyo, Japan*

The development of a blood substitute, also called "artificial" blood, or more exactly an oxygen-carrying plasma expander (OCPE), is still a major goal of transfusion medicine, driven by blood shortages, problems associated with the transmission of disease by available blood, and the complex logistics of acquiring, analyzing, storing, distributing, and delivering the needed blood. To date blood is unequaled (or appears to be) in its capacity to restore circulatory volume; however, it is often remarked that if blood were proposed today as an oxygen-carrying volume restoration fluid, it would not be approved by regulatory agencies.

Blood is needed in the presence of blood losses; however, the initial anemia is usually inconsequential to the organism's survival, whereas the associated hypovolemia is tolerated only within a narrow margin. As a result, a blood substitute should ideally target both events, possibly sequentially. Therefore, the development of an effective blood substitute is also in part related to availability and detailed understanding of an effective plasma expander, which is the term used to describe a volume restoration fluid used prior to reaching the transfusion trigger, or the point in volume restitution at which the introduction of an oxygen carrier, blood, is determined to be essential.

Blood exerts its principal functions in the microcirculation, the locale of exchange of the materials that it transports. Thus any attempt to introduce a blood surrogate must ensure efficacy at this level. In contrast, most efforts aimed at developing artificial blood were made in the absence of detailed information and analysis of how oxygen is managed at the level of the microcirculation. The cornerstones in the development of artificial blood up to the present are that this fluid should restore most of the oxygen-carrying capacity of the shed blood, that it is beneficial for the resulting mixture of remaining blood and resuscitation fluid (e.g., OCPE) to have a viscosity lower than that of natural blood, and that the material should have very low oxygen affinity so that oxygen would be readily released when blood arrives to the microcirculation.

These principles have guided the development of products that are now undergoing clinical trials. It is apparent that the initial impetus for the development of artificial blood was based on the restoration of systemic functions after acute blood losses with little or no emphasis on understanding the behavior of the resuscitation fluids in the microcirculation, which is the organ system where blood performs its functions. This was in part due to the imperfect understanding of how oxygen is managed in the microcirculation in both normal and pathophysiological conditions, the role and regulation of shear stress–dependant mediators produced by the endothelium, and the lack of techniques for measuring the key transport parameters that determine efficacy in maintaining microvascular function upon the introduction of a blood surrogate.

Oxygen-carrying capacity and oncotic pressure were prescribed to be similar to that of blood, while the experience with hemodilution suggested that improvements in transport would be obtained by lowering blood viscosity to values significantly below those of whole blood. An additional

presumed beneficial modification was the use of oxygen carriers based on modified hemoglobins with high p50s, presumed to facilitate oxygen unloading and tissue oxygenation. As acellular modified molecular hemoglobin became the oxygen carrier of choice, it was found this material was vasoactive, causing hypertension, which is deleterious in resuscitation. Vasoactivity was attributed to hemoglobin scavenging NO, leading to vasoconstriction, which gave rise to a significant effort aimed at modifying the hemoglobin molecule so that its affinity for NO was reduced.

At present an optimal OCPE is still perceived to have the following properties: oxygen-carrying capacity equivalent to 10 to 14 g/dL hemoglobin, p50 greater than 30 mmHg, viscosity 1 cP, oncotic pressure approximately 25 mmHg, and low NO binding. However, studies in the microcirculation show that a fluid configured according these concepts yields problematic outcomes in terms of resuscitation from anemic hypovolemia. Furthermore, since the most favored source of hemoglobin is human, even if it were possible to obtain a one to one conversion from blood to blood substitute, the problem of blood shortages is not solved. In an effort to circumvent the human hemoglobin source there have been various attempts to obtain hemoglobin by recombinant technology. Biopure Inc. (Boston, MA) has progressed to Phase III clinical trials with a molecular hemoglobin-based fluid derived from bovine blood. However, recombinant technology has not progressed to the development of an efficacious product to date, and the bovine-based product was not developed on the basis of microcirculatory data.

The Design of an Efficacious Oxygen-Carrying Plasma Expander

To date there are virtually no rivals to hemoglobin as a transporter of oxygen from the lung to the tissue because of its ability to bind a large amount of oxygen through a chemical reaction. The discovery that fluorocarbons could dissolve a comparatively large amount of oxygen, albeit at high oxygen partial pressures, suggested using this vehicle as the oxygen transporter. However, the use of fluorocarbon-based blood replacement fluids has not materialized, in part because of the lack of definitive experimental studies on the physiology related to altered blood physical properties and changes in the distribution of oxygen partial pressure in the circulation.

Various modifications of hemoglobin have optimized its performance and mostly eliminated the vasoactivity of this molecular species. Human hemoglobin remains the most favored source because of the well-defined methodology for obtaining blood from donors, which in advanced medical systems is virtually free of parasitic, bacterial, or viral contamination. The present formulations of bovine hemoglobin appear to be vasoactive and in the long term could present unknown risks of introducing diseases that may have extraordinarily long incubation times.

If the perceived and now frequently reported blood shortage is the driving force behind the development of hemoglobin-based oxygen-carrying blood substitutes, then the use of human hemoglobin is problematic since the processing technology and formulation would require that a unit of original blood yield at least an equivalent unit of "artificial blood," a zero-sum result that does not relieve shortages. A realistic process should produce several units of equivalent hemoglobin-based oxygen-carrying blood substitute from a unit of natural blood.

The present goal in devising a human hemoglobin-based blood substitute is to circumvent the inherent toxicity of the hemoglobin molecule and to be as efficacious as an equivalent unit of natural blood but at a lower hemoglobin concentration than blood, thus introducing a multiplying factor between the original source of human hemoglobin and the final product. Advances in microvascular technology allow us to critically analyze each of the "cornerstone" precepts that have guided the development of blood substitutes to date, namely the viscosity of the material, the affinity for oxygen, the effective concentration, and the resulting colloidal osmotic pressure when the material is present in the circulation. In the following these parameters will be analyzed from a microcirculatory perspective.

The Role of Viscosity in Oxygen Transport

Blood viscosity depends on red blood cell concentration (hematocrit) and on plasma viscosity. The manipulation of these two viscosities is the basis of the clinical practice of hemodilution. Accordingly, the restitution of blood losses with conventional plasma expanders can be effectively and safely accomplished up to a 50 percent loss of the red blood cell mass, using fluids with plasma-like viscosity. The decrease in viscosity due to hemodilution causes a compensatory increase in cardiac output due to the lowered flow resistance, thus maintaining oxygen delivery.

A specific decrease in oxygen-carrying capacity is one of the parameters that defines the so-called transfusion trigger. Microcirculatory experimental studies do not support the contention that lack of oxygen-carrying capacity is the actual determining factor in the decision of transfusing blood. In the awake hamster window chamber model, neither oxygen-carrying capacity nor tissue oxygenation is in jeopardy with red blood cell losses of two-thirds of the original mass (Figure 1).

A factor that is significantly changed upon reaching the transfusion trigger is blood viscosity, which is approximately half of normal, because of the loss of red blood cells. Thus, additional losses of red blood cells will further reduce blood viscosity, which is strongly dependent on hematocrit. The reduction of blood viscosity is initially compensated by increased cardiac output. However, cardiac output seldom doubles, and blood viscosity has the potential of decreasing

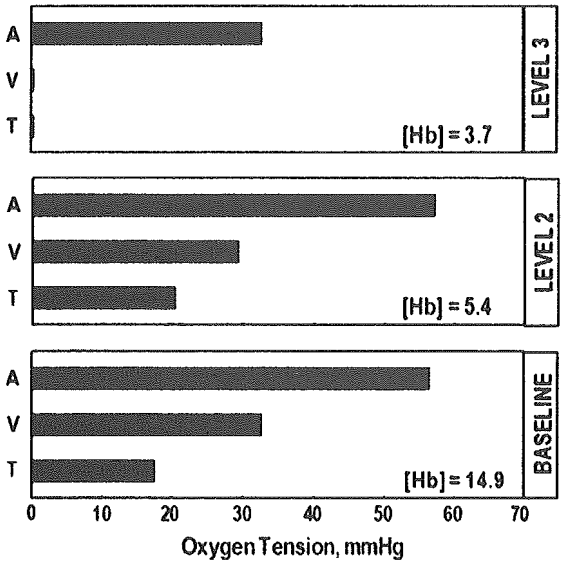

Figure 1 Distribution of oxygen in arterioles (A) and venules (V) in the tissue (T) in the awake hamster window model as a function of total hemoglobin (Hb, grams per deciliter). Normovolemic hemodilution was achieved using dextran 70 kDa. It is apparent that a loss of the order of two thirds of red blood cells has no influence on microcirculatory pO_2 of this model. (see color insert)

to 25 percent of normal upon further reductions of hematocrit, leading to the significant lowering of functional capillary density (FCD, capillaries with red blood cell transit), a condition detrimental to survival in hemorrhagic shock.

Although capillaries do not appear to be the determinant structure for the supply of oxygen in some tissues, the maintenance of FCD in shock is a critical parameter in determining outcome independently of tissue oxygen tension (pO_2), suggesting that extraction of metabolic by-products may be as critical to a capillary function as oxygenation. The relationship between FCD and survival has been demonstrated in experimental analysis of conditions during prolonged hemorrhagic shock, where the principal microvascular functional difference between survivors and nonsurvivors was that survivors maintained FCD above a threshold of about 40 percent of that present in the normal organism; there were no other significant differences between groups [1].

The blood viscosity threshold that causes the decrease in FCD appears to coincide with the decision to transfuse blood. In other words, the transfusion trigger may also be a viscosity trigger; therefore results obtained with blood transfusions may also be achieved by increasing plasma viscosity. Thus use of red blood cells solely for the purpose of increasing blood viscosity may be avoided by introducing a material that increases plasma viscosity in the circulation. In this scenario blood viscosity resulting from the balance between the diminished red blood cell concentration and the increased plasma viscosity leads to the maintenance of vascular resistance. Tsai et al. [2] explored this phenomenon by inducing extreme hemodilution with low-viscosity dextran 70 kDa. At 11 percent hematocrit, FCD and microvascular flow were significantly reduced from control. However, when plasma viscosity was maintained above 2 cP by the introduction of high-viscosity dextran 500 kDa, FCD was maintained near to control values, and microvascular flow increased significantly above control, though hematocrit was 11 percent. This effect was not found if extreme hemodilution was performed with the Biopure product Oxyglobin, even at a total blood hemoglobin content of 6.7 g Hb/dL. High-viscosity plasma also caused blood flow to increase significantly above nonhemodiluted values because of the release of shear-dependent generated endothelial relaxing factors.

Counterintuitive Rheological Findings

High-viscosity plasma restores mean arterial blood pressure (MAP) in hypotension without vasoconstriction. Systemic blood viscosity depends on hematocrit squared; thus viscous losses in major vessels of the circulation can be minimized even when plasma viscosity is increased, shifting pressure and pressure gradients from the systemic to the peripheral circulation. Reduced blood viscosity decreases shear stress and the release of vasodilators, causing vasoconstriction, which negates the benefit of reducing the rheological component of vascular resistance. Vascular resistance depends on the first power of blood viscosity and the 4th power of vessel radius. Therefore reducing blood viscosity with low viscosity plasma might decrease flow and oxygen delivery to the tissue if there is an associated vasoconstrictor stimulus. However, increased flow and/or increased viscosity augments shear stress on the endothelium since the elevation of plasma viscosity causes sustained NO-mediated dilatation in the hamster muscle microcirculation, supporting this interpretation. Enhancement of shear stress on the vessel wall results in the release of prostacyclin and NO from the vascular endothelium. Endogenous NO release reduces total peripheral resistance during moderate hemodilution. Increased wall shear stress following increased blood flow induces vasodilation [3] showing that the link between shear stress and vasodilatation is well established.

Experimental verification of the beneficial effects of high-viscosity plasma during hemodilution in the microcirculation is evidenced by effects on FCD, perfusion and vasodilatation. More recently it was demonstrated by Cabrales et al. [4] that an increase in capillary pressure is the principal mechanical event that governs the effects due to perfusion with high-viscosity plasma.

Hemodilution, Blood Viscosity, and Vasoactivity

It is now apparent that low viscosity molecular hemoglobin solutions lower FCD independently of the intrinsic

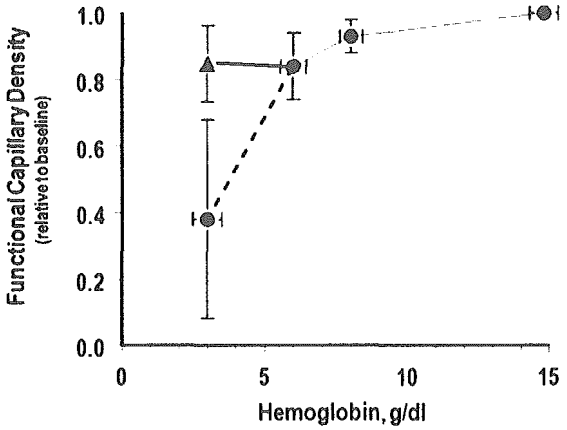

Figure 2 Changes of FCD following isovolemic hemodilution with low- and high-viscosity plasma expanders. ● Hemodilution with dextran 70 kDa maintains FCD up to a hematocrit (hemoglobin) that is 40 percent of normal. Further hemodilution with the same low-viscosity diluent causes the fall of FCD to pathologically low levels. ▲ Continuation of hemodilution with dextran 500 kDa after reaching 40 percent of normal hemoglobin with hemodilution with dextran 70. FCD is maintained to normal levels by the increased plasma viscosity. Redrawn from Tsai et al. [2].

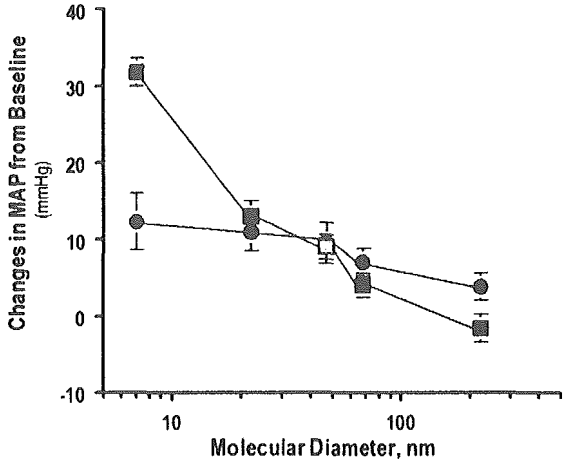

Figure 3 Changes in mean arterial blood pressure after a 5 percent by volume (5 Hb g/dL) topload infusion of free hemoglobin solutions of different molecular diameters and vesicle encapsulated hemoglobin. ■ Pressor effect after infusion. ● Pressor effect 3 hours after infusion. Redrawn from Sakai et al. [5]. (see color insert)

vasoactive properties of the hemoglobin molecule because they cause a significant decrease in blood viscosity after reaching the transfusion trigger. An additional factor attendant to the restoration of blood volume upon reaching the transfusion trigger with a plasma-like viscosity fluid is that this process brings the organism to near extreme hemodilution conditions, characterized by decreased shear stress on the endothelium, lowering the production of endothelial-derived vasodilators. Increasing plasma viscosity to about 2.0 to 2.5 cP increases shear stress and the production of vasodilators, which breaks up the vicious circle caused by extreme hemodilution, compensatory vasoconstriction and low viscosity.

Experimental results shown in Figure 2 show that the maintenance of FCD is not directly linked to oxygen delivery, but to mechanical factors related to the viscosity of the perfusion fluid and the production of vasodilators by mechanotransduction in the endothelium. Therefore an acellular oxygen carrier should maintain plasma viscosity above a specific threshold, while ensuring that overall blood viscosity does not exceed normal values.

Low blood viscosity can be compensated for by hemoglobin solutions with high viscosity. This can be achieved by mixing the hemoglobin molecule with a viscogenic material such as hydroxyethyl starch (HES) at suitable concentrations, or by modifying the hemoglobin molecule to produce an intrinsically viscous solution by increasing its molecular dimension. This latter process can be implemented by polymerization or conjugation with various molecules such as starch and polyethylene glycol, as described by Sakai et al. [5], who showed that the pressor effect is inversely related to molecular size (Figure 3). Hemoglobin molecules with these and several other beneficial features are polyethylene glycol conjugated hemoglobin molecules [6].

The Vasoconstrictive Effect of Hemoglobin

Natural hemoglobin molecules are presumed to be vasoconstrictive because of their ability to scavenge NO. However, recent experimental evidence shows that whereas NO binding is virtually identical for most hemoglobin molecules [7], the vasoconstrictive effect is not, being essentially absent in polyethylene glycol modified hemoglobins and in some very large hemoglobin polymers.

NO is produced by the endothelium as a result of shear stress and other processes. The affinity of the hemoglobin molecule for NO is due to the physical similarity between NO and O_2. Thus in general, hemoglobins with high affinity for O_2 generally also have a high affinity for NO, and vice versa. The production of genetically modified hemoglobins that appear to have little affinity for NO, while maintaining a normal affinity for O_2, may challenge this generalization; however, the fact remains that interfering with NO production with administration of L-arginine methyl ester hydrochloride (L-NAME) or scavenging NO with cell free unmodified hemoglobin causes the constriction of aortic rings, and an increase in blood pressure in experimental subjects.

The concept that hemoglobin extravasation and its location between the endothelium and smooth muscle is the principal factor causing hypertension and vasoconstriction is also questionable because the extravasated molecule will eventually saturate. In fact the presence of a NO-avid mole-

cule in plasma is sufficient to distort the diffusion field of NO from the endothelium, whereby hemoglobin does not need to extravasate to be vasoconstrictive.

NO scavenging does not provide a consistent explanation for the pressor effect of free hemoglobin in the circulation that is applicable to the different hemoglobin modifications. The lack of correlation between pressor responses and NO scavenging characteristic of hemoglobin molecules led McCarthy et al. [8] to propose that hypertension following the introduction of molecular hemoglobin in the circulation is caused by a mechanism related to the process of facilitated diffusion of oxyhemoglobin. According to this hypothesis the presence of molecular hemoglobin causes an additional flux of oxygen in the plasma layer due to the diffusion of oxyhemoglobin. Although the diffusion constant of hemoglobin is low, the amount of oxygen carried is large because hemoglobin binds a large amount of oxygen. The net result of this process is that a comparatively small concentration of molecular hemoglobin augments oxygen transfer to the vessel wall, leading to a hyperoxia signal, and consequently a vasoconstrictive response.

In vivo, peripheral vascular resistance is autoregulated at the level of the arterioles by a mechanism that senses oxygen tension, producing vasodilatory signals when blood and tissue pO_2 is low, and vice versa. This conceptualization is supported by the finding that large hemoglobin molecules are not vasoactive, although they carry oxygen. As an example, poly (ethylene glycol) (PEG) surface decorated hemoglobins (PEG-Hb) have consistently been shown to be vasoinactive. These molecules have a large volume because of the water bound by PEG. Since the diffusion constant is inversely proportional to molecular radius, it can be shown that PEG-Hb has a diffusion constant that is about one fifth that of the native hemoglobin.

Experimentation with different levels of hemoglobin surface decoration show that vasoinactivity may be partially related to the degree to which the surface of the hemoglobin molecule is shielded by the water–PEG combination [6]. This phenomenon suggests that free hemoglobin may also cause a pharmacological effect mediated at the surface of the endothelium, and that conjugation of hemoglobin with PEG may produce a shield that prevents this process.

The vasoconstrictive effects of molecular hemoglobin may have several components that sometimes reinforce each other. When blood viscosity becomes too low, there is a reflex vasoconstriction that attempts to maintain perfusion pressure, a phenomenon independent of blood oxygen-carrying capacity. Oxygen regulation plays a crucial role since the arteriolar walls and the tissue sense both the rate of oxygen delivery from the red blood cell column and local pO_2. When molecular hemoglobin is present in plasma, there is a significant additional flux of oxygen to the arteriolar wall by facilitated diffusion, a process enhanced with right-shifted oxygen dissociation hemoglobin molecules. NO scavenging can also be a factor that may be balanced by increased NO (and/or prostacyclin) production resulting from elevated shear stress caused by high-viscosity hemoglobin molecules. Furthermore, considering that modest amounts of small molecular hemoglobin can elicit a pressor response, a pharmacological effect due to "naked" small hemoglobin molecules in the circulation may also be present.

Vasoconstriction limits perfusion and decreases FCD. Although healthy organisms could probably compensate for moderate hypertensive episodes leading to corresponding decreases in FCD, these same episodes may place the organism in jeopardy if they are superposed to other vasoconstrictive stimuli, such as those inherent to hemorrhagic shock. Conversely, high plasma viscosity is critical in resuscitation, as an OCPE is administered in conditions of extreme hemodilution because there is no need for using these products prior to reaching the transfusion trigger.

Optimal Oxygen Disassociation Properties

The development of oxygen carriers has implicitly assumed that the oxygen dissociation curve should be right shifted, thus facilitating the release of oxygen. This approach does not consider the longitudinal gradient of oxygen tension in the circulation, whereby a right-shifted dissociation curve favors oxygen unloading from small arteries and arterioles. Hemodilution with hemoglobin-filled vesicles of different p50 in the hamster window chamber model has shown that improved tissue oxygenation is obtained when this parameter is 16 mmHg, instead of 34 mmHg (Department of Polymer Chemistry, Waseda University, Tokyo, Japan). PEG-conjugated hemoglobin (Hemospan, 4% Mal-PEG hemoglobin) produced by Sangart (San Diego, CA), with a p50 of 5 mmHg, used at low concentration in hemodilution maintains FCD and positive acid–base balance.

This apparent paradox may be understood by analyzing the distribution of oxygen in the microcirculation as shown in Figure 4, where oxygen tension in the microcirculation in normal conditions has a baseline tissue pO_2 level of 22 to 24 mmHg (which appears to be common for most tissues). It is notable that although oxygen is regulated to achieve this partial pressure in the tissue, anaerobic metabolism occurs when tissue pO_2 is below 2.4 to 2.9 mmHg.

A possible rationale for the high pO_2 tissue regulation is that the organism has excess oxygen-carrying capacity, not only as a requirement for extreme efforts, but also for compensation of oxygen delivery inhomogeneity in the microcirculation. The effect of this inhomogeneity becomes apparent in considering the variability of oxygen partial pressure distribution in the hamster window chamber, which is of the order of ± 4 mmHg. This variability is a consequence of the quasirandom distribution of the transport properties of the microcirculation, and therefore intrinsic to any level of tissue oxygenation. In conditions of extreme hemodilution tissue pO_2 decreases to 3 to 5 mmHg; thus, if