

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

^aThis number corresponds to the binding numbers of PEG_{MY} on the protein surface.

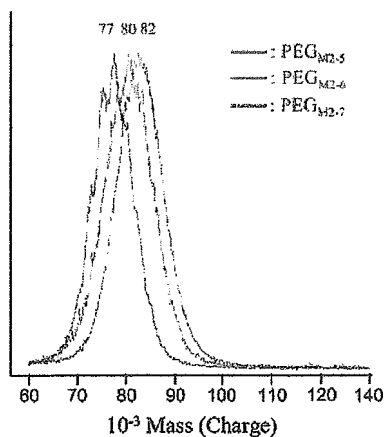


Figure 3. MALDI-TOF MS of PEG_{M2}(HSA-Fe4P) prepared in different [IMT]/[HSA-Fe4P] ratios of 10 (blue), 15 (red), and 20 (green) (mol/mol).

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

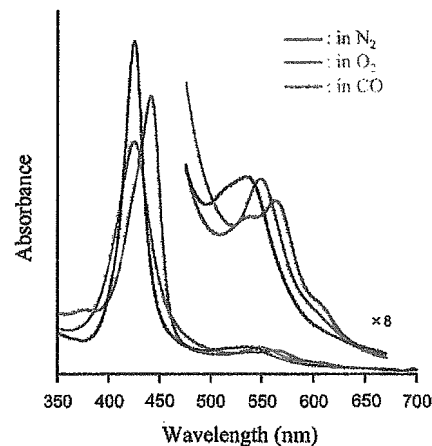


Figure 4. UV-vis absorption spectral change of PEG_{M2-6}(HSA-Fe4P) in PBS solution (pH 7.4).

(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.

O₂-Binding Properties. The UV-vis absorption spectrum of the PEG_{M2}(HSA-Fe4P) solution under an N₂ 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 O₂ gas through this solution, the spectral pattern shifted to that of the well-defined O₂-adduct complex of the tetrakis(phenyl)porphyratoiron(II) derivatives (λ_{max}: 424, 550 nm) (10b,c, 20). This oxygenation was reversibly observed, dependent on the O₂-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 N₂, O₂, and CO atmospheres. These spectral changes were completely the same as that observed in the nonmodified HSA-Fe4P.

Table 3. O₂-Binding Parameters of PEG-Conjugated HSA-FeXP Solution at 25 °C (pH 7.4)

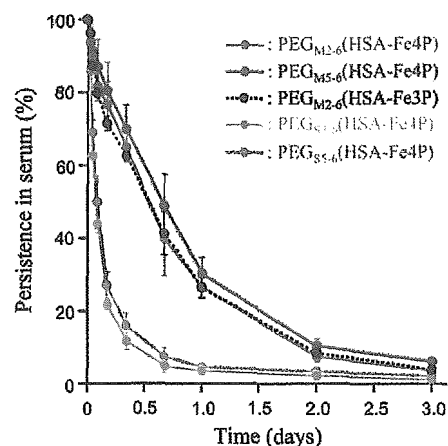
system	k_{on} ($\mu\text{M}^{-1}\text{s}^{-1}$)		k_{off} (ms^{-1})		$P_{1/2}$ (Torr)	$\tau_{1/2}$ (h) at 37 °C
	fast	slow	fast	slow		
PEG _{M2-5} (HSA-Fe4P)	11	5.8	0.16	0.08	38 (11)	13
PEG _{M2-6} (HSA-Fe4P)	12	4.6	0.17	0.07	32 (11)	12
PEG _{M2-7} (HSA-Fe4P)	9.3	4.7	0.16	0.08	35 (13)	12
PEG _{M2-6} (HSA-Fe3P)	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 O₂ binding to the PEG-conjugated HSA-FeXP (k_{on}). We have previously reported that the O₂-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 O₂ 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 O₂ 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 O₂ molecule.

The O₂-binding affinities ($P_{1/2} = K(\text{O}_2)^{-1}$) of the PEG-modified HSA-FeXP series were determined by measuring the UV-vis absorption spectral changes by O₂/N₂ 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 O₂-binding equilibria were not influenced by the presence of PEGs. In contrast, the surface modification by PEG delayed the proton-driven oxidation of the FeXPO₂ species and prolonged the half-lifetime of the O₂-adduct complex [$\tau_{1/2}(\text{O}_2)$]. The PEG_{M5-6}(HSA-Fe4P)O₂ complex showed the longest $\tau_{1/2}(\text{O}_2)$ of 16 h at 37 °C, which is greater than those of HSA-Fe4P and natural hemoprotein, myoglobin ($\tau_{1/2}(\text{O}_2)$: 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 O₂-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-

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

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.

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 O₂-transporting ability. The PEG conjugation decreased the O₂-association rate constant but retarded the irreversible oxidation of the central ferrous ion, thereby increasing the stability of the O₂-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 O₂-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 N₂ atmosphere to the oxy state by exposure to O₂ gas. This PEG_{MY}(HSA-FeXP) film was redissolved in nonaqueous organic solvents, ethanol, chloroform, etc., and the reversible O₂ 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.

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BC050315+



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 μ m 径)を 1mm/s の流速で流動させた実験では、赤血球(8 μ m)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、赤血球と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

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微小血管内を均一に流れる人工赤血球とその運命

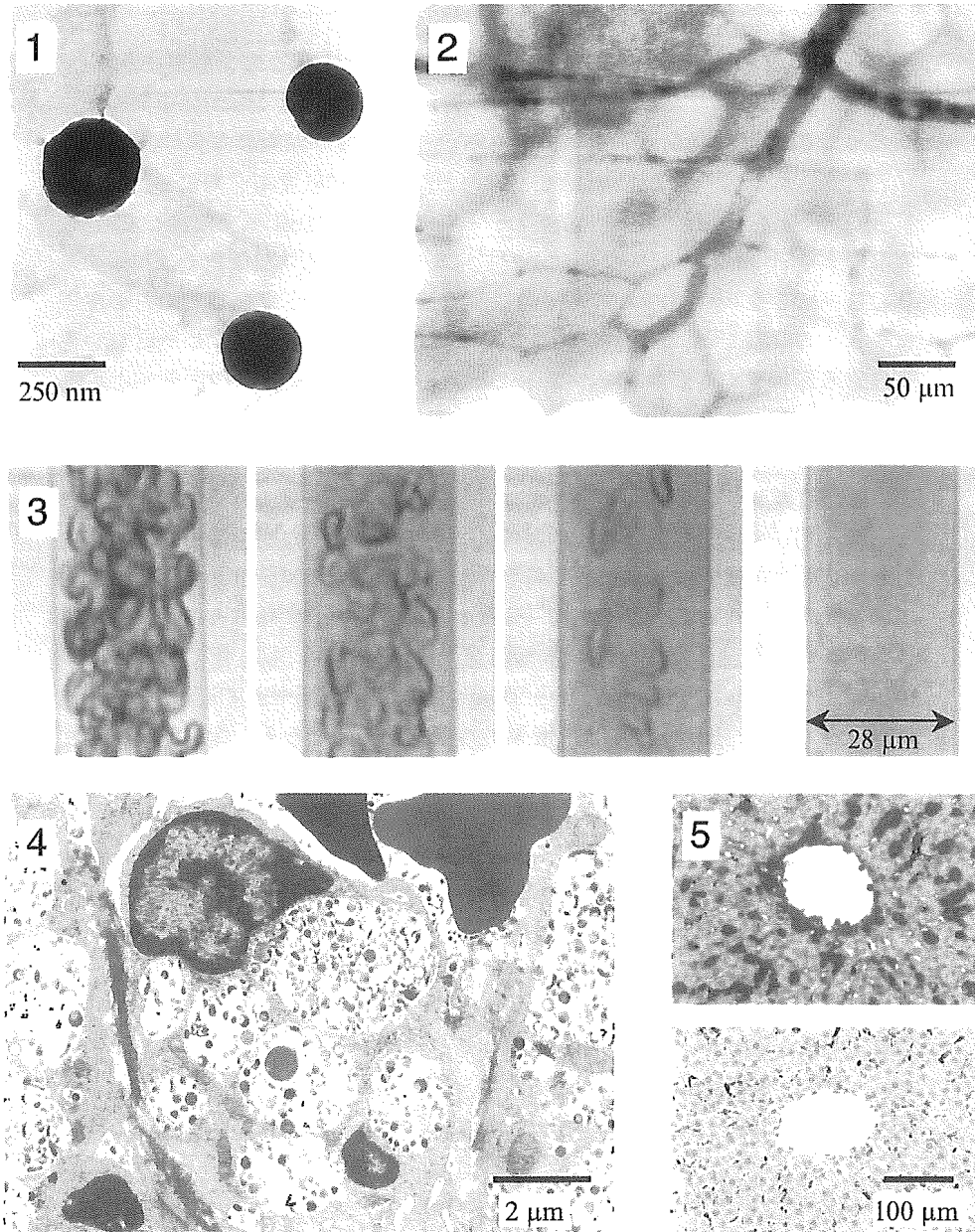


図1 人工赤血球 (Hb 小胞体) の透過型電子顕微鏡像
 2 循環血液量の 80% を Hb 小胞体で交換した後のハムスター背部皮下微小循環の光学顕微鏡像 波長 420nm 付近の光 (Hb の吸収帯) を照射して撮影。
 3 赤血球と Hb 小胞体を混合し、プラスチック製のモデル血管内を流動させたときの光学顕微鏡像 混合容積比は左から、赤血球/Hb 小胞体 = 100/0, 50/50, 10/90, 0/100。
 4 Hb 小胞体投与 1 日後のラット脾臓マクロファージの透過型電子顕微鏡像
 5 Hb 小胞体投与 1 日後 (上)、7 日後 (下) の肝臓組織の抗ヒト Hb 抗体免疫染色像

*写真左上の数字は図の番号です。

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，H16-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絃一教授による本誌「講座」に詳しい¹⁾。本「講座」では，これらの厚生労働省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

キーワード：人工赤血球，人工血小板，
ヘモグロビン

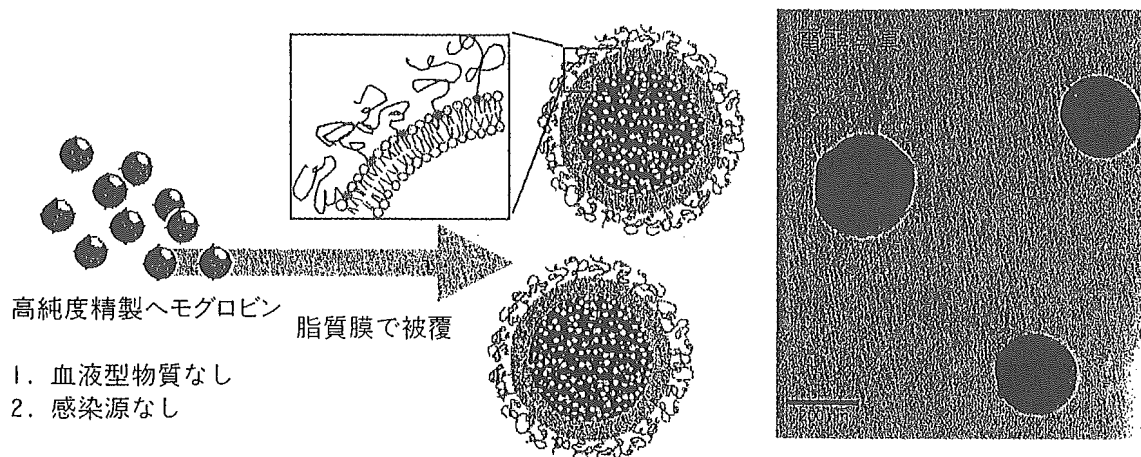
Seminar
Current Development of Artificial Red
Blood Cells and Artificial Platelets
Shinji Takeoka (Waseda University, Faculty of
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るが，分解性や代謝物の低毒性も重要な検討項目である。現在担体としてリン脂質分子の集合体（リポソーム）や遺伝子組み換えヒトタンパク質の複合体や重合体を選択して用いている。

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①）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① 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①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⁾。

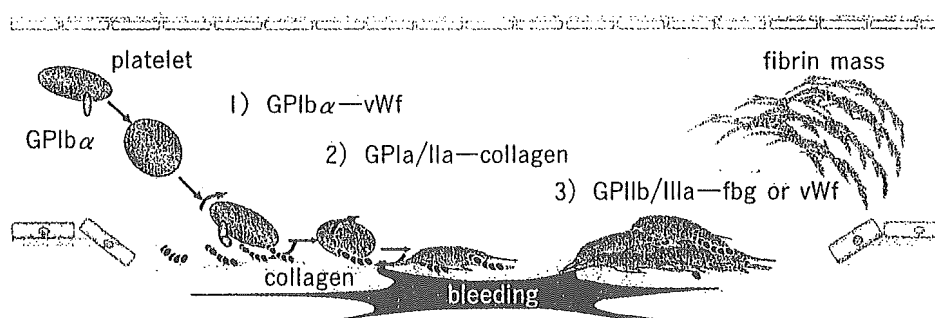
安全面では、血管弛緩因子である一酸化窒素や一酸化炭素が関与してヘモグロビンに認められる抵抗血管の収縮と血圧亢進は、ヘモグロビン小胞体では認められなかった^{8,9)}。これは、ヘモグロビン小胞体の大きさが寄与しているものと思われる。ヘモグロビン小胞体の血中滞留時間は、ラット、ラビット、カンクイザルからヒトへ類推すると、3日間程度の半減期として見積もられ、緊急時の単回投与では十分とされる。ラットでは、血中半減期が1~1.5日間であるので、脾臓や肝臓の病理組織学的所見では、投与1日後には脾臓や肝臓に貪食されていたヘモグロビン小胞体は3日後には激減し、投与7日以内にはほとんど消失していた¹⁰⁾。また、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 ml/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填液として使用、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持ち、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② 血小板の止血機構。
 一次止血（血小板血栓）
 1) 接着（tethering → rolling）GPIb α —vWf
 2) 粘着（adhesion）GPIIb/IIIa—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②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 GPIb α 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。引き続き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 GPIb α 、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 GPIb α 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、rGPIb α 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、rGPIb α 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、内部が充填された無定形な綿雪のような形態をとっており、出血部位での充填効果が期待できる。表面に rGPIb α 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 rGPIb α 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 rGPIb α 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、rGPIb α と rGPIaIIa と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]= $2.0 \times 10^4/\mu\text{l}$) 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ため 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]= $19.8 \pm 2.8/\mu\text{l}$) の出血時間は 609 ± 153 秒であり、正常ラット群 ([血小板]= $80.9 \pm 8.6/\mu\text{l}$) 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 2.4×10^{11} particles/kg では出血時間は 337 ± 46 秒)²⁶⁾。

現在、GPIb α 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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Blood Substitutes' Efficacy Microvascular and Rheological Determinants

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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 unequalled (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 man-

aged 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-dependent 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

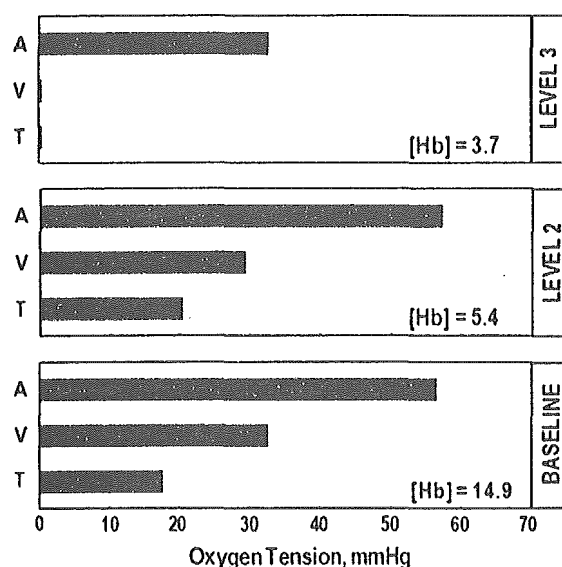


Figure 1 Distribution of oxygen in arterioles (A) and venules (V) in the tissue (T) in the awake hamster window model as a function of total hemoglobin (Hb, grams per deciliter). Normovolemic hemodilution was achieved using dextran 70 kDa. It is apparent that a loss of the order of two thirds of red 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

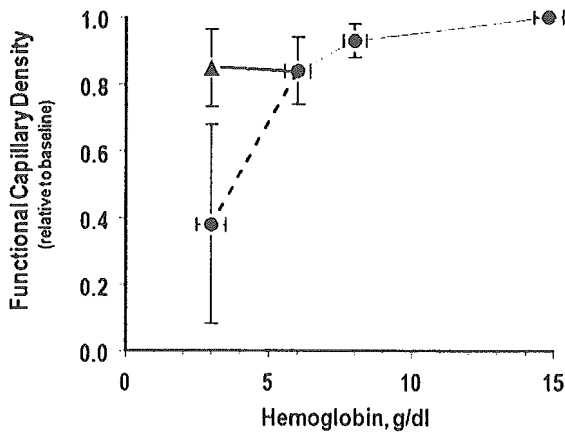


Figure 2 Changes of FCD following isovolemic hemodilution with low- and high-viscosity plasma expanders. ● Hemodilution with dextran 70 kDa maintains FCD up to a hematocrit (hemoglobin) that is 40 percent of normal. Further hemodilution with the same low-viscosity diluent causes the fall of FCD to pathologically 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].

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). Hemoglo-

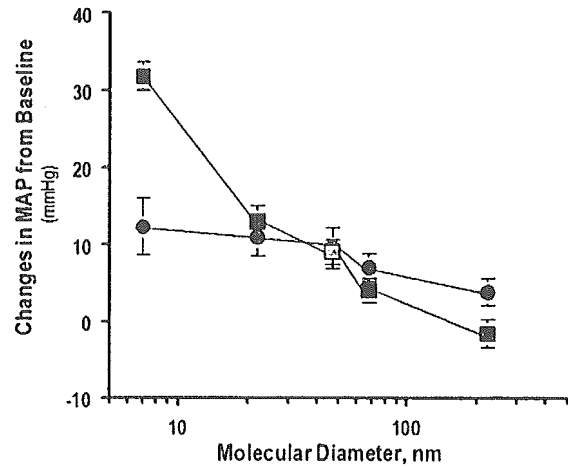


Figure 3 Changes in mean arterial blood pressure after a 5 percent by volume (5 Hb g/dL) topload infusion of free hemoglobin solutions of different molecular diameters and vesicle encapsulated hemoglobin. ■ Pressor effect after infusion. ● Pressor effect 3 hours after infusion. Redrawn from Sakai et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

bin 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 O₂. Thus in general, hemoglobins with high affinity for O₂ 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 O₂, 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 vasoactivity 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

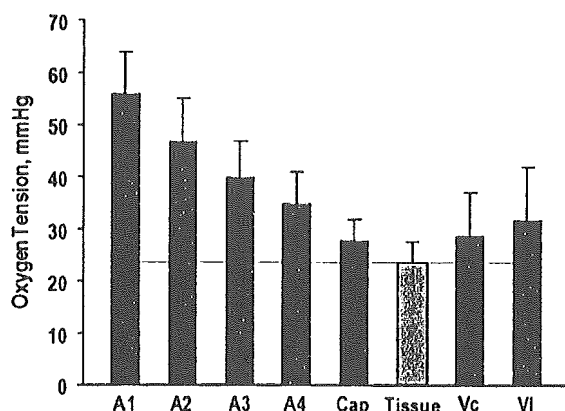


Figure 4 Distribution of pO_2 in the microvessel of the microcirculation of the hamster window preparation as a function of the arteriolar order of branching (As) and the venular order of branching (Vs). It is apparent that tissue pO_2 is narrowly regulated to a value in the range of 22 to 24 mmHg, which is significantly higher than the level associated with anaerobic metabolism.

the same variability is present, there is a significant amount of tissue that is anoxic. In the presence of a fraction of the oxygen-carrying capacity that can only be released at very low pO_2 s, a form of targeted oxygen delivery, the effects of this variability will be nullified, ensuring that all the tissue, even at low pO_2 is oxygenated above the anaerobic threshold.

Tissue pO_2 levels that may be considered harmful could, in fact, be quite safe if it were possible to eliminate the inherent variability of oxygen delivery shown by the variability of tissue pO_2 . A small quantity of a low-p50 hemoglobin oxygen carrier in the circulation accomplishes this because it delivers oxygen only to portions of the tissue where the anoxic threshold is passed, while the presence of even significant amounts of right-shifted hemoglobin would have no effect since most of the bound oxygen would be unloaded in oxygenated regions.

Cross-linked or polymerized hemoglobins developed so far have a high p50, presumed to be beneficial since it facilitates oxygen unloading. However, pO_2 in the microcirculation is regulated so that there is a significant decrease in oxygen tension from the systemic circulation to the capillaries, which typically have a pO_2 of about 30 mmHg. At this p50 half of the blood oxygen is delivered by arterioles in normal conditions; however, if the p50 of the OCPEs is above this value, as in the case of Oxyglobin (p50 = 54 mmHg), most of the oxygen in the blood should be delivered by the arterioles if this material were to replace blood. These vessels extract a significant amount of oxygen from the circulation while consuming a major portion of this oxygen flux, thus increasing their oxygen supply increases tissue oxygen inhomogeneity, which is further aggravated by the vasoconstrictor autoregulatory response already discussed.

Oxygen-Carrying Capacity

Measurements of pO_2 in the microcirculation utilizing the technique of phosphorescence oxygen quenching show that when hemodilution carried out to a total hemoglobin content in red blood cells of 5.6 g/dL, then tissue oxygen is somewhat higher than normal but not statistically significant. The required oxygen-carrying capacity can also be obtained by a simple calculation that relates the whole-body oxygen consumption and cardiac output, which yields a nearly identical number for the organism at rest. Therefore, in principle, the oxygen-carrying capacity of an OCPE does not need to reproduce the value for normal blood and can be significantly lower.

Colloid Osmotic Pressure

It is generally assumed that a blood substitute should have a colloid osmotic pressure similar to that of blood and in the range of 20 to 25 mmHg; however, several plasma expanders have zero colloid osmotic pressure (saline, Ringer's lactate) and small-volume resuscitation utilizes fluids with very high osmotic properties. To date there is no definitive answer on what is the osmotic and/or oncotic property that is most appropriate, and in all probability this is a variable that depends on the type of blood loss to be corrected. Resuscitation with noncolloidal fluids leads to tissue edema. Conversely fluids with high colloidal and osmotic pressures cause tissue fluid to come into the vascular compartment, thus decreasing the amount of fluid to be administered. Most conditions of hemorrhage are associated with endothelial edema, which has been demonstrated to be rapidly reversed upon the introduction of hyperosmotic and hyperoncotic fluids. Volume expansion fluids such as hydroxyethyl starch have relative high colloid osmotic pressures, typically in the range of 30 to 50 mmHg depending on formulation. Small molecule hemoglobin-based OCPEs have their oncotic pressure adjusted to be that of plasma, but PEG-hemoglobin modified OCPEs tend to have higher oncotic pressures.

Synthesis of an Effective Oxygen-Carrying Plasma Expander

An OCPE based on the preceding concepts is a fluid with properties fundamentally different from those of blood, since it has low oxygen-carrying capacity, p50 is low and in the neighborhood of 5 mmHg, viscoelastic properties are such that when introduced into the circulation plasma viscosity should be of the order of 2.0 to 2.5 cP, and colloidal osmotic pressure can be high. A fluid with these properties can be obtained by conjugating hemoglobin with PEG, and various formulations have been tested in both animal experiments and human trials with excellent results. Notably this

formulation is vasoinactive, and its NO-scavenging characteristics do not appear to be relevant since these fluids have the same NO binding constant as other vasoactive formulations that are vasoactive [7].

These fluids are in some cases more effective than blood because they are designed to maintain FCD, which is as necessary as restoring tissue oxygenation for the recovery from blood losses. Because in the foreseeable future OCPEs will use human hemoglobin, these fluids are practical: Their hemoglobin content is low, and more than two units of blood equivalent unit of resuscitation fluid can be obtained from one unit of blood. Finally, this low oxygen-carrying capacity is practical and safe because it yields a significant improvement of microvascular function.

Experimental Evidence

The effectiveness of different resuscitation modalities was tested experimentally in studies of extreme hemodilution and hemorrhagic shock in the microcirculation of the hamster chamber window model, which allows microcirculatory monitoring in the awake condition for a period up to 1 week, after the effects of the surgical intervention have subsided. Extreme hemodilution was chosen because in most instances, lowering systemic hematocrit to 50 percent of baseline with a suitable plasma expander does not alter microvascular hemodynamics and transport in our experimental model. Animals were hemodiluted to 60 percent of normal with dextran 70 kDa, and further hemodiluted to a final hematocrit of 11 percent using the different products simulating blood losses initially remedied with conventional plasma expanders, which upon passing the transfusion trigger are corrected with an oxygen-carrying blood substitute.

A compendium of findings in extreme hemodilution to 50 percent of normal with dextran 70kDa and further hemodilution to a final hematocrit of 11 percent with the different products is shown in Figure 5, including results obtained with PEG-Hb vesicles developed at Waseda University, Tokyo, using a somewhat different protocol where extreme hemodilution was achieved with a continuous exchange of a hemoglobin vesicle suspension. FCD is shown as a function of blood base excess, which represents systemic conditions and suggests the definition of *critical functional capillary density* as the value for this parameter at which base excess is no longer sustained and drops following modest reductions of total blood hemoglobin, that is, in the neighborhood of a 50 percent FCD reduction. The most important result is that normal base excess is obtained with total blood hemoglobin of 5 percent, if 1 percent of this is Mal-PEG-Hb—a result not found with other OCPEs.

Extreme hemodilution is not a clinically relevant procedure and serves only to study basic mechanisms. A clinically relevant test is to rescue a subject in hemorrhagic shock. Studies were therefore conducted to determine the effects of resuscitation with blood, starch, and Mal-PEG-Hb in a con-

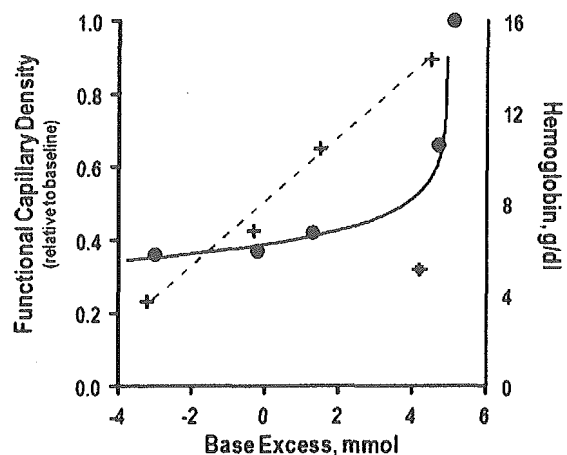


Figure 5 Relationship between total circulating hemoglobin and base excess, and FCD and base excess, for different hemoglobin modifications and concentrations, including hemoglobin vesicles, in normovolemic hemodilution experiments. The data marked ● shows the relationship between FCD and base excess, showing that MAL-Peg-Hb (▼) yields high FCD and base excess at low hemoglobin concentrations. It is apparent that base excess is a direct function of hemoglobin concentration (+) with the exception of MAL-Peg-Hb (◆), which presents normal base excess at a very low total hemoglobin content. (see color insert)

ventional 50 percent bleed shock protocol. The animals were resuscitated after 1 hour without any additional volume manipulation using shed blood, HES, and Mal-PEG-Hb with 25 percent of the blood volume. The results, shown in Figure 6, indicate that Mal-PEG-Hb is superior to both HES and blood in reestablishing microvascular function. Concurrently it was found that base excess was higher in the Mal-PEG hemoglobin-resuscitated animals than in the blood-resuscitated animals. An explanation for these findings is that low p50 hemoglobin targets oxygen delivery of oxygen to only the anoxic tissue.

An extreme hemorrhage study was performed with Mal-PEG-Hb in which rats were 50 percent exchange transfused before hemorrhage with either $\alpha\alpha$ -cross-linked hemoglobin, or 4 percent Mal-PEG hemoglobin (Figure 7). These animals were then subjected to a continuous exponential bleed (1 hour, 60 percent of blood volume) whereby at the end of the second hour after the start of bleeding 50 percent of the control animals succumbed. In these experiments it was found that at the end of one hour all animals that received Mal-PEG hemoglobin before hemorrhage survived, while all of those receiving $\alpha\alpha$ -cross-linked hemoglobin did not survive.

Summary and Conclusions

The revision of microvascular physiology related to modifying basic transport properties of blood such as plasma viscosity, p50, and hemoglobin concentration shows

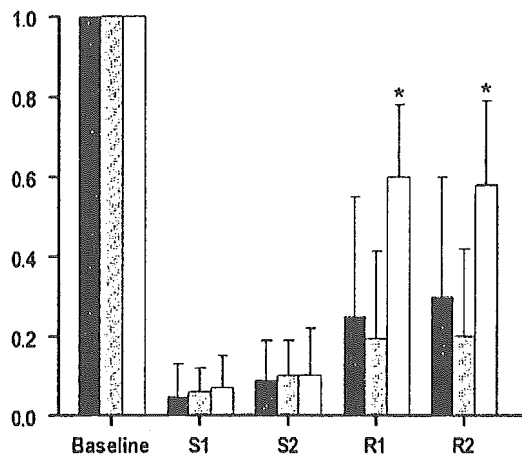


Figure 6 Recovery of FCD during resuscitation from 1 hour hemorrhagic shock with identical volumes of shed blood (black bars), 5 percent HES (shaded bars), and 4 percent MAL-Peg-Hb (white bars). S1 and S2 initial and final conditions during the shock period. R1, Recovery immediately after resuscitation; R2, 1 hour after resuscitation. $p < 0.05$ relative to shed blood and HES.

that blood or a bloodlike fluid may not be the optimal oxygen-carrying volume-restoring fluid. A critical parameter for either oxygen-carrying or noncarrying blood replacements is their viscosity, which is a factor in maintaining capillary flow.

Analysis of the microvascular consequences of changing blood rheological conditions and particularly plasma shows that low plasma viscosity is not of universal benefit. Patients following trauma, peripheral arterial occlusive disease, and acute myocardial infarction have elevated plasma viscosity, a condition presumed to be pathological. However, there are situations where increased viscosity may be a protective or beneficial mechanism.

Plasma expanders are not used after reaching the transfusion trigger because the reduction of blood oxygen-carrying capacity beyond this point is assumed to jeopardize tissue oxygenation, according to the systemic evaluation of the organism portrayed by blood gases. Conditions in the microcirculation and local microscopic tissue environment when the reduction of red blood cells is extended beyond the transfusion trigger have not been consistently explored and presently show that oxygen-carrying capacity is not the major factor in determining tissue survival.

Studies show that the transfusion trigger is also the limit for the organism to adapt to low blood viscosity in acute conditions; thus *the conventional transfusion trigger is also a viscosity trigger*. Since the administration of a molecular oxygen carrier is physically similar to continuing fluid therapy after reaching the transfusion trigger, the maintenance of FCD requires the increase of plasma viscosity which through shear stress-dependent mechanisms operating in the endothelium ensures the maintenance of optimal

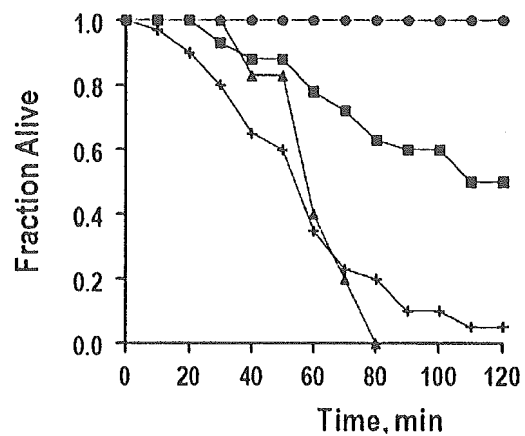


Figure 7 Controlled bleeding in rats that are 50 percent exchange transfused with MAL-Peg-Hb (●), α -cross-linked hemoglobin (▲), and a polymerized hemoglobin (+), versus controls (■) with no treatment. The study was designed so that 50 percent of the untreated (not transfused controls) would survive 120 minutes.

microvascular function. Oxygen-carrying capacity is exhausted upon red blood cell (or hemoglobin) losses that are significantly greater than those represented by the transfusion trigger. However, these losses of oxygen-carrying capacity do not need to be compensated on a one-to-one basis, if microvascular function (i.e., FCD) is maintained and an oxygen carrier is introduced only to deliver oxygen to anoxic tissue regions. This approach ensures a uniform maintenance of the whole organism above the anaerobic threshold, while limiting the amount of oxygen carrier needed to maintain metabolism. Thus the combination of maintenance of microvascular function and targeted oxygen delivery is the primary determinant of an efficacious human hemoglobin-based blood substitute that is more effective than blood in acute conditions and that also expands the available blood supply, since a unit of blood yields more than two units of surrogate blood.

Glossary

Functional capillary density: Number of capillaries in a unit volume of tissue that presents the passage of red blood cells. This parameter is experimentally determined by measuring the length of red blood cell-perfused capillaries in a microscopic field of view.

Microvascular function: A combination of parameter including flow, number of open capillaries, intact vascular permeability, and level of vessels tone that allows for the proper interaction between blood and tissue at the microscopic level.

Oxygen-carrying capacity: The amount of oxygen in milliliters at standard atmospheric conditions and temperature contained in a fluid.

p50: Partial pressure of oxygen at which hemoglobin is 50 percent saturated with oxygen.

Plasma expander: A fluid used to restore circulatory volume when oxygen-carrying capacity is adequate.

Transfusion trigger: Level of blood hemoglobin at which the decision is made to introduce red blood cells into the circulation in order to restore oxygen-carrying capacity.

Vasoactivity: Inherent property of compounds that cause vasoconstriction and the elevation of systemic blood pressure.

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Biographies

Amy G. Tsai obtained her Ph.D. in bioengineering at the University of California, San Diego, where she is currently a Senior Research Scientist. She is widely recognized for her findings on oxygen consumption by the microvasculature and the development of high-viscosity plasma expanders. She is an expert in mathematical modeling, measuring methods for the in vivo study of the microcirculation and small animal experimentation.

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Prof. Marcos Intaglietta received his Ph.D. in applied mechanics from the California Institute of Technology in Pasadena and developed his academic career at the University of California, San Diego, where he is one of the founders of the bioengineering program and department. His specialty is the study of transport phenomena in the microcirculation and the development of blood substitutes. He has developed and implemented most of the methods presently used for the study of the microcirculation.