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Please cite this article as: K. Sou, E. Tsuchida, Electrostatic interactions and complement activation on the surface of phospholipid vesicle containing acidic lipids: Effect of the structure of acidic groups, Biochim. Biophys. Acta (2008), doi:10.1016/j.bbamem.2008.01.006

「日本ヘモレオロジー学会誌」投稿論文
(印刷中)

各種代用血漿剤に分散させたヘモグロビン小胞体(人工赤血球)とその血液混合系のレオロジー特性

佐藤 敦[1]、酒井 宏水[2]、武岡 真司[1]、土田 英俊[*,2]
1) 早稲田大学大学院 理工学研究科、2) 早稲田大学理工学術院 理工学研究所

【keyword】血液代替物、代用血漿剤、レオロジー
【ランニングタイトル】人工赤血球のレオロジー特性

＊連絡先：
土田 英俊
〒169-8555 東京都 新宿区 大久保 3-4-1
早稲田大学理工学術院 理工学研究所
TEL: 03-5286-3120
FAX: 03-3205-4740
e-mail: eishun@waseda.jp

要旨

ヘモグロビン小胞体(HbV)は、高濃度ヘモグロビン(Hb)溶液をリン脂質小胞体に内包した人工赤血球(人工酸素運搬体)である。HbV 分散液の膠質浸透圧は殆どゼロなので、大量投与に際しては代用血漿剤(水溶性高分子溶液)の併用が循環血液量の維持に重要となる。従来、ヒト血清アルブミン(HSA)或いはそのリコンビナント体(rHSA)を併用して、HbV の充分な酸素運搬効果と血行動態の維持を確認してきた。しかし、臨床現場ではアルブミン以外の代用血漿剤としてデキストラン(DEX)、ヒドロキシエチルスターチ(HES)、修正ゼラチン(MFG)などが使用されており、これらが HbV の分散安定性に及ぼす影響を検討する必要がある。これまで、各種代用血漿剤に分散させた HbV のレオロジー特性を詳細に検討してきたが、実際の投与では、代用血漿剤、HbV 分散液、そして血液が互いに希釈された混合系を形成する。そこで本研究では、各種代用血漿剤に分散させた HbV と血液の混合系のレオロジー特性、およびマイクロチャネルを通過する血液の流動性に HbV 凝集体が及ぼす影響を検討した。rHSA、低分子量 HES に分散させた HbV と血液の混合系は血液と同等あるいはそれ以下の粘度を示した。一方、DEX、高分子量 HES、MFG に分散させた HbV は血液よりも高い粘度を示したが、血液との混合比の増大に伴い、粘度および貯蔵弾性率の減少を示した。各種代用血漿剤に分散させた HbV と血液の混合液はマイクロチャネルを殆ど塞栓すること無く流動し、100 μL の通過時間は単純に粘度に比例した。HbV は可逆的な凝集性を示し、血液の微小流路の塞栓因子に影響を及ぼさないものと考えられる。HbV は分散媒である代用血漿剤との組み合わせでレオロジー特性を調節できるので、輸血代替以外の用途に拡大する可能性が期待できる。

緒言

リン脂質小胞体（リポソーム）は薬剤を内包、或いは脂質膜に担持できる特徴を持つことから、ドラッグデリバリーシステムを目的とした精力的な研究が行われており、抗真菌療法や抗癌治療法として既に認可されている例もある[1]。ヘモグロビン小胞体（HbV）は、高純度・高濃度ヒトヘモグロビン（Hb）をリン脂質小胞体（粒子径 280 nm）に内包した人工酸素運搬体である[2-5]。これまでの動物投与試験から、HbV が血液と同等の酸素運搬能と十分な安全性を有することが明らかになっている。輸血代替としての投与量は非常に多く、循環血液量の 40 %以上になることも想定される。赤血球と同等の酸素運搬機能を持たせるため、HbV 分散液の固形分濃度は他のリポソーム製剤と比較して非常に高い（Hb 濃度 10 g/dL; 脂質濃度 5～6 g/dL）。従って HbV の安全性を評価する上で、ヘモレオロジーに対比させて HbV 分散液のレオロジー特性を検討することは重要である[6]。

血漿中に 5 g/dL 程存在するアルブミンは血液と間質液の膠質浸透圧（COP）を平衡に保ち、循環血液量を維持する機能を有する血漿蛋白質である。この機能を維持する為に、COP は血液代替物が備えるべき必須条件の一つである。HbV は 1 粒子あたり約 3 万個の Hb を内包しており、分散液の COP は殆どゼロである。従って、HbV の大量投与に際しては代用血漿剤の併用が循環血液量の維持に重要となる。従来、HbV を 5%アルブミン（HSA）溶液或いはリコンビナントアルブミン（rHSA）溶液に分散させ COP を 20 Torr に調節し、この分散液を用いた出血ショック蘇生試験や、循環血液量の 40～90%を置換する試験から、rHSA に分散させた HbV が血液と同等の酸素運搬効果を有することを明らかにしてきた[7]。HbV を rHSA に分散させて Hb 濃度を 8.6～10 g/dL とした場合の粘度は血液と同等であり、剪断速度依存性からニュートン流体に近い挙動を示している[8]。しかし、実際の臨床現場では HSA 以外に代用血漿剤として、デキストラン（DEX）やヒドロキシエチルスターチ（HES）が使用されており、海外ではそれ以外に、HES の高分子量体や修正ゼラチン溶液（MFG）なども使用されている[9-11]。また、HbV をこれらの代用血漿剤に分散させて投与することで、虚血領域の酸素化の改善効果も報告されている[12-14]。このような背景のもと、我々は各種代用血漿剤に分散させた HbV のレオロジー特性の詳細な解析を進めている。rHSA と低分子量 HES では HbV と混合しても顕著な粘度増大は見られないが、DEX および欧州や米国で使用されている高分子量 HES や MFG では HbV の凝集生起を認め、粘度上昇が顕著となる。しかし凝集の形成と解離は極めて可逆的で迅速であり、血液流動性測定装置 (Microchannel array flow

analyzer, MC-FAN)[15,16]のマイクロチャネルには一切の塞栓が無いことを確認している[8]。

代用血漿剤を静脈内投与した場合、血液と代用血漿剤の両方が希釈されるので、ここに HbV 分散液が投与された場合、凝集形成は低減されると考えられる。また、HbV の凝集生起が血液の流動性に与える影響を in vitro で検討する必要がある。そこで本研究では、各種代用血漿剤に分散させた HbV と血液の混合系のレオロジー特性を観測すると共に、MC-FAN を用いて HbV の凝集体が血液の流動性に及ぼす影響を明らかにすることを目的とした。

方法

1. HbV 分散液の調製

HbV 分散液は無菌条件下、既報に従って調製した [17-19]。Hb 溶液は日本赤十字社（東京）から提供を受けた期限切れ非使用赤血球から精製した。HbV の内水相には高濃度の Hb（38 g/dL）と共に、アロステリック因子としてピリドキサール 5'-リン酸（PLP、14.7 mM）がモル比で PLP/Hb = 2.5 になるように添加した。HbV の膜成分である脂質は、日本精化社製の Presome PPG-I（1,2-ジパルミトイル-*sn*-グリセロ-3-ホスファチジルコリン）、コレステロール、1,5-bis-*O*-ヘキサデシル-*N*-スクシニル-L-グルタメート = 5/5/1（モル比）に 0.3 mol%の 1,2-ジステアロイル-*sn*-グリセロ-3-ホスファチジルエタノールアミン-*N*-ポリ（エチレングリコール）（日本油脂社製）を混合して用いた。HbV は生理食塩水に分散、フィルター滅菌し（Dismic、東洋濾紙社製：0.45 µm）、窒素通気により酸素を除去して保存した [20]。HbV の粒子径は 279 ± 95 nm であった。

2. 各種代用血漿剤

遺伝子組み換えヒト血清アルブミン（rHSA, Bipha, Mw. 67 kDa, 25 wt%溶液）は生理食塩水で 5 wt%に希釈して用いた。デキストラン（DEX, 小林製薬工業 デキストラン 40 注「小林」™, Mw. 40 kDa, 10 wt%溶液）、低分子量ヒドロキシエチルスターチ（HES_{70}, 杏林製薬 サリンヘス™, Mw. 70 kDa, 6 wt %溶液）、中分子量ヒドロキシエチルスターチ（HES_{130}, HES_{200}, Fresenius 社 Voluven™, Mw. 130 kDa, 200 kDa, 6 wt%溶液）、高分子量ヒドロキシエチルスターチ（HES_{670}, Hospira 社 Hextend™, Mw. 670 kDa, 6 wt%溶液）、修正ゼラチン（MFG, B. Braun 社 Gelofusine™, Mw. 30 kDa, 4 wt%溶液）を用いた。各種代用血漿剤の膠質浸透圧（COP）は浸透圧計（model 4420, Wescor 社製、Cut-off Mw. = 10,000）を用いて測定した。物理化学的特徴は **Table 1** にまとめた。

3. 各種代用血漿剤に分散させた HbV 及び血液試料の調製

生理食塩水に分散させた HbV を超遠心分離（20 000 *g*, 30 min）で沈降させて上澄みの生理食塩水を除去後、代用血漿剤中に分散させ（[Hb] = 10 g/dL）、フィルター処理した（Dismic、東洋濾紙社製：0.45 µm）。血液試料は 5 %ヘパリン加ヒト新鮮血を用い、採血後 6 時間以内に測定を行った [21]。

4. 各種代用血漿剤に分散させた HbV とその血液混合系のレオロジー測定

粘度の剪断速度依存性はモジュラーコンパクトレオメータ（Physica MCR301：Anton Paar 社製）を用いて測定した。測定治具はコーンプレート（コーン径：50 mm、ギャップ角度：1°）を用い、25 °C で剪断速度を 10^3 から 10^{-4} s^{-1} に低下させた。貯蔵弾性率 *G'* の剪断速度依存性はキャピラリーレオメータ（DCR：Anton Paar 社製）を用いて測定した（剪断速度 5 ～ 320 s^{-1}、周波数 2 Hz、37 °C）。

5. 各種代用血漿剤に分散させた HbV とその血液混合系のマイクロチャネル流動性試験

各種代用血漿剤に分散させた HbV（[Hb] = 10 g/dL）とヘパリン加ヒト新鮮血の混合液を測定試料とした。マイクロチャネルアレイ Bloody6-7（幅 7 µm, 長さ 30 µm, 深さ 4.5 µm, 流路数 8,736 本：日立原町電子工業社製）に MC-FAN（KH-3：日立原町電子工業社製）を用いて 20 cm 水柱差で流し、試料 100 µL の通過時間を測定した。通過時間は試料測定の直前に測定された生理食塩水 100 µL の通過時間を用いて（通過時間）×12 秒/（生理食塩水通過時間）により生理食塩水通過時間が 12 秒の場合に換算した。また、流動中と停止後の試料の顕微鏡写真を撮影した。

結果

1. 各種代用血漿剤に分散させた HbV とその血液混合系のレオロジー特性

Fig. 1A に、各種代用血漿剤に分散させた HbV について、剪断速度を 10^3 s^{-1} から 10^{-4} s^{-1} に低下させたときの粘度変化を示した。rHSA に分散させた場合（HbV-rHSA）は、粘度の変化は殆ど無く、ほぼニュートン流体を示した。HbV-rHSA および HbV-HES_{70} の粘度は、ヒト血液とほぼ同じであった。使用したレオメータの検出感度では、低粘度試料は 0.5 s^{-1} が限界であった。一方、HbV-HES_{130}、HbV-HES_{200}、HbV-HES_{670}、HbV-DEX、HbV-MFG はヒト血液よりも高い粘度を示し、高剪断速度になるほど粘度が低下する Shear-thinning 流動が観測された。この非ニュートン流体の性質は HbV の凝集体の生起に起因する。これらはより低剪断速度までの測定が可能であった。HES の分子量が大きくなるにつれ粘度が高くなる傾向が認められ、高分子量であるほど HbV は凝集し易くなることが明確に示された。

Fig. 1B に、各種代用血漿剤に分散させた HbV と血液を体積比 1 : 1 で混合した溶液について、**Fig. 1A** と同様に測定した結果を示した（剪断速度 $10^3 - 10^{-4}$ s^{-1}、25 °C)。HbV-rHSA の血液混合液は血液よりも低い粘度を示した。HbV-HES_{70}、HbV-HES_{130} の血液混合液は血液とほぼ同じ粘度を示した。一方、HbV-DEX、HbV-HES_{670}、HbV-HES_{200}、HbV-MFG の血液混合液は、混合前と比較して全域で粘度の減少を示したが、血液よりも高い粘度であった。

Fig. 2 には血液と HbV 分散液の混合比を変化させたときの**(A)** 粘度、**(B)** 貯蔵弾性率 G' の変化を示す。HbV-rHSA を除く HbV 分散液に関して、血液混合比の増大に伴い粘度と G' が減少する傾向が観測された。一方、HbV-rHSA の粘度と G' は血液よりも小さいため、血液混合比の増大に伴ってこれらが増大し、血液の値に収束していく傾向が観測された。

2. 各種代用血漿剤に分散させた HbV とその血液混合系のマイクロチャネル流動性試験

各種代用血漿剤に分散させた HbV とその血液混合系の流動性試験を、体積混合比を変化させて行った。全血の通過時間（46 秒）は他の文献の報告値とほぼ一致した [22]。HbV-DEX の通過時間は 196 秒であったが、血液混合比の増大に伴い通過時間は短縮し、HbV-DEX と血液の混合比 1 : 3 においては 59 秒であった。**Fig. 3** には、各種代用血漿剤に分散させた HbV と血液を体積比 1 : 1 で混合した分散液の静止状態と流動状態の顕微鏡観察写真を示した。HbV の粒子径は 279 ± 95 nm と小さい為、均一分散系

においてはその存在を光学顕微鏡で確認できないが、静止状態で形成される凝集体はみかけのサイズが大きい為に血漿相中に確認できた。血液と同等の粘度を示した HbV-rHSA、HbV-HES_{70} は HbV の凝集体が殆ど観測されなかった。一方、高粘度を示した HbV-HES_{670}、HbV-DEX、HbV-MFG は HbV の凝集体が観測された。しかし、流動中の凝集体は解離して流路を殆ど閉塞せずに流動した。剪断速度 1000 s^{-1} における試料の粘度とマイクロチャネルの通過時間の相関を検討したところ（**Fig. 4**)、HbV 分散液の粘度と通過時間(流量の逆数)に比例関係が成立した。直線の式を最小二乗法で求めると Y = 12.1X（Y : 通過時間（sec/100 μL)、X : 粘度（mPa・s)、$R^2 = 0.9728$）であり、Poiseuille の法則に従った。

考察

リン脂質小胞体（リポソーム）が水溶性高分子と相互作用して凝集体を形成する現象については多くの報告例があるが[23-25]、その殆どが希薄溶液の濁度または光散乱の計測で観察しているに過ぎない。HbVは、表面に修飾されたポリエチレングリコール（PEG）鎖の立体反発効果によって、血漿中においても均一な分散系を形成する[26]。しかし、HbVは極めて高濃度の分散系であるため（固形分濃度: 16 g/dL, 占有体積: 40 vol%）、これが凝集した場合は、凝集体が溶液全体に三次元ネットワークを形成し、これが固体的性質を与え、粘度及び貯蔵弾性率 G' の増大として観察されると考えられる。赤血球も可逆的な凝集体を形成し、40～45 vol%の体積を占有するので、これが血液を非ニュートン流体とする主要な因子であり、また血液に水溶性高分子が混合されると赤血球が凝集し、血液粘度、血行動態、組織酸素化に影響を及ぼすことが知られている[27-30]。従って、HbV分散液についても同様のレオロジー特性の解析を行った。

HbV-rHSA分散液はほぼニュートン流体の特性を示し、高剪断速度における粘度は血液とほぼ同等であった（**Fig. 1A**）。HbVの表面に修飾されたPEG鎖がアルブミン中の分散安定度に寄与していると考えられる[3]。対照的にHES、DEX、MFGに分散させたHbVは、低剪断速度で高い粘度を示し剪断速度が高くなるに従い粘度が低下するShear-thinningを示した（**Fig. 1A**）。これは凝集分散系に特徴的な粘度挙動である。またHES(Mw. 70, 130, 200, 670 kDa)について、凝集体形成には分子量依存性が存在し、高分子量になるほど高粘度を示した。分子量が最も小さいHES_{70}に分散させた場合には血液と同等であった。現行のPEG修飾の条件は、血漿中での分散安定度を維持するには十分と思われる。しかし、本研究で代用血漿剤によってはPEG修飾が施されてもHbVの凝集体が形成してしまうことが明らかとなった。

リポソーム凝集の機序については、幾つかの理論が提唱されている。(i) 水溶性高分子鎖が粒子表面に吸着することにより粒子間を架橋する[24]、(ii) 水溶性高分子がリポソーム表面から水和水を奪い、リポソームが溶液から排除される[31]、また、(iii) リポソームの水和領域から高分子が排除されるため、リポソーム粒子間に形成される空間からも高分子が排除されることになり、バルク溶液とリポソーム粒子間の溶液との間に浸透圧差が生じ、この浸透圧差を小さくするために凝集が促進されて粒子間距離が短くなる[23,32-34]と言われている。特に(iii)の場合、粒子間に形成される空間の大きさと高分子の大きさの相対的寸法差が重要と成る。HES、DEX、MFGには分岐構造があるので、球状蛋白質のrHSAに比較し

てより低密度で排除体積の大きい構造をしていると考えられる。DEXは水中でコイル状の分子構造をとることが報告されている[35]。MFGは、修飾されたコハク酸の静電反発によって拡大した分子構造をとる[36]。この様な高分子はリポソーム表面から排除され易く、リポソーム凝集が促進されると考えられる。また、(i)の機序でも、直鎖状構造の高分子が粒子間を架橋し易いと考えられる。一方、HESの分子構造は分岐状であり[37]、その形は球状に近いので、排除体積はDEX、MFGと比較して小さいと考えられる。しかし、高分子量体になるに従い、排除体積も大きくなるので、リポソーム凝集が促進される。我々は各種代用血漿剤に含まれる水溶性高分子の慣性半径の違いから凝集度を説明できると考えており、現在その解析を進めている。また、水溶性高分子による凝集を抑制する為に、現行よりも鎖長の長いPEG鎖をHbV表面に修飾し、粒子間の立体反発力を高めることも可能と考えられる。事実、我々はリポソームの表面に修飾するPEG鎖の分子量を大きくさせると、リポソームの分散安定性が増加することを報告している[38]。

実際にHbVの投与を行う場合には、①代用血漿剤を投与した後に生理食塩水に分散されたHbVを投与、或いは②濃厚な代用血漿剤（例：25%アルブミン溶液）があればこれをHbV分散液に添加して同時に投与（[Hb] = 8.6 g/dL）するなどが想定される。血液交換率は50%程度と想定され、HbV、代用血漿剤ともに更に希釈されるので凝集は低減されると考えられる。事実、DEX、HES_{670}、MFGに分散させたHbVは血液よりも高い粘度を示したが、血液と体積比1：1で混合すると全ての剪断速度で粘度が減少した（**Fig. 1B**）。更に血液の混合比の増大に伴い、粘度および G' が減少する傾向が観測された（**Fig. 2**）。HbVと代用血漿剤の双方が血液で希釈され、凝集の低減が起きることが明らかとなった。

次に、各種代用血漿剤に分散させたHbVと血液の混合系がマイクロチャネルを流動する挙動をMC-FANを用いて検討した。血液のマイクロチャネルの通過時間は、赤血球の変形能[39]、白血球による流路の塞栓[40]および血栓による塞栓[41]などが影響すると考えられる。このうち、赤血球の変形能はレオメータでの粘度測定にも影響を及ぼすが[39]、流路の塞栓はマイクロチャネルを持つMC-FANでしか測定できない項目である。我々は各種代用血漿剤に分散させたHbVがマイクロチャネルを一切塞栓することなく流動し、100 μLの通過時間と粘度に比例関係が得られ、これがPoiseuillesの法則に従っていることを報告してきた[8]。この関係が成り立つのは流路の塞栓が無い場合に限られる。仮に流路の塞栓が顕著であれば、通過時間は大きく延長して、粘度との比例関係は成立しない筈である。結果より、各種代用血漿剤に分散させたHbVと血液の混合液の通過時間は、全例が粘度に比例した（**Fig. 4**）。よって

各種代用血漿剤に分散させたHbVは血液の流路塞栓因子に影響を及ぼさないと考えられる。事実、我々はラットを用いた60％血液交換試験において、各種代用血漿剤を投与後、HbVによる蘇生を行ったが、凝集体による影響は全く見られず、全例が生存した[42]。この理由として、代用血漿剤、血液、HbVが互いに希釈され、凝集が低減したこと、凝集体が血液の剪断速度に応じて可逆的に形成・解離したこと、そして血液中の塞栓因子に影響が無かったことなどが考えられる。

最近、幾つかの研究グループが、高粘性流体が血管壁に対し、より高い剪断応力を与えるため血管拡張分子を誘導させ結果的に末梢血流が改善されると主張している[43,44]。ErniらはHbVをHES_{200}あるいはDEX (Mw. 70 kDa)に分散させ、ハムスターの循環血液量の40%を交換し、毛細血管の塞栓が無いことを確認し、むしろ有茎皮弁の虚血領域の微小循環を改善する効果があることを見出した[12-14]。高粘性流体では末梢毛細管により均一に圧力が伝播し、血液をより均一に輸送し、有効毛細管密度(Functional Capillary Density)の向上が期待できる[45]。この点に関しては、HbVは代用血漿剤の併用によって特徴的なレオロジー特性を示し、またその調節も可能であるので、輸血代替以外の臨床応用にも利用できる可能性がある。今後は、HbVと一連の代用血漿剤を併用したときの安全性について詳細を確認するとともに、凝集の機序を明らかにして行く計画である。

結論

HbVを各種代用血漿剤に分散させた溶液と血液の混合系に関して、レオロジー特性および凝集の可逆性について検討した。$HES_{70,130}$、rHSAに分散させたHbVと血液の混合液の粘度は血液と同等もしくはそれ以下であった。DEX、高分子量HES、MFGではHbVは凝集するが、血液中においても外力に依存して瞬時に解離すると共に、血液混合比の増大に伴い凝集の形成は低減することが確認された。HbV分散液と血液の混合系のマイクロチャネルの通過時間は単純に粘度に比例し、流路の塞栓は殆ど無かったことから HbV 凝集体は血液の流路の塞栓因子に影響を与えないと考えられた。凝集体生起の生体への影響を確認する必要があるが、HbV分散液は分散媒との組み合わせでレオロジー特性を調節することができるので、輸血代替以外の用途に拡大する可能性もある。

謝辞

代用血漿剤の生理的作用に関し、高折益彦名誉教授（東宝塚さとう病院）、Dr. Amy G. Tsai（Univ. of California, San Diego）およびProf. Dominique Erni（Inselspital Hospital, Univ. of Bern）の助言を得た。また粘弾性計測に関し、大坪泰文教授（千葉大学工学部）および平野尚也氏（日本シーベルヘグナー社）より助言を得た。使用した代用血漿剤のうち、rHSA、$HES_{130, 200}$、およびMFGはそれぞれ、ニプロ社、Fresenius社、およびB. Braun社より頂戴した。記して謝意を表する。本研究は、厚生労働科学研究費補助金（医薬品・医療機器等レギュラトリーサイエンス総合研究事業）により推進された。

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図の説明

FIGURE 1. Shear rate dependence of the viscosity of **(A)** the HbV suspended in various plasma-substitute solutions, and **(B)** the mixtures of blood and HbV suspended in various plasma-substitute solutions at a volume ratio of 1/1. The shear rate decreased from 10^3 to 10^{-4} s^{-1}. [Hb] = 10 g/dL, 25°C. The blood data are inserted for comparison.

FIGURE 2. Viscoelasticity of the mixtures of blood and HbV suspended in various plasma-substitute solutions at a volume ratio of 0/1, 1/3, 1/1, 3/1, and 1/0. **(A)** The viscosity at a shear rate of 10^3 s^{-1} measured with an MCR 301 rheometer at 25°C. **(B)** The storage modulus (G') at a shear rate of 1.9 s^{-1} measured with a capillary rheometer at 37°C.

FIRURE 3. Images of microchannels during the flowing condition and the cessation of flow. The flocculate formation is apparent in the plasma phase at the cessation for HbV-DEX + blood, HbV-HES + blood, and HbV-MFG + blood. However, no flocculation was apparent for HbV-rHSA + blood. In the flowing condition, partial plugging of channels occurred due to platelets or white blood cells in blood samples (top).

FIGURE 4. Microchannel flow measurements of the mixtures of blood and HbV suspended in various plasma-substitute solutions at a volume ratio of 1/0, 3/1, 1/1, 1/3, and 0/1. The time required for the passage of 100 μL of each suspension was plotted against the viscosity at 10^3 s^{-1}. The straight line indicates a linear approximation : $Y = 12.1X$ ($R^2 = 0.9728$).

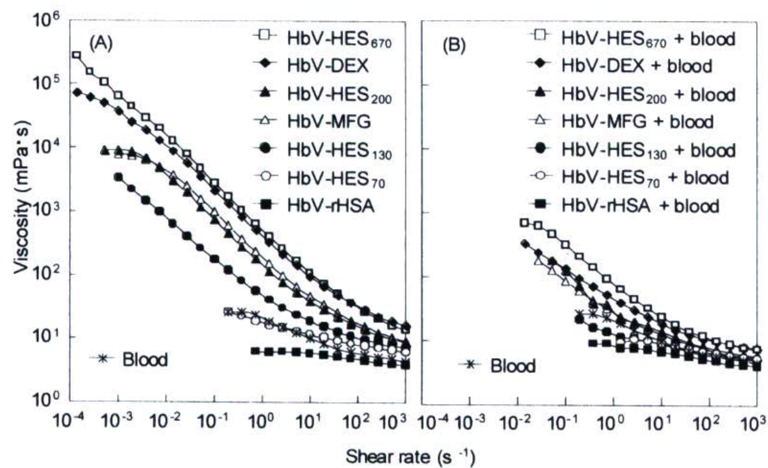

Figure 1

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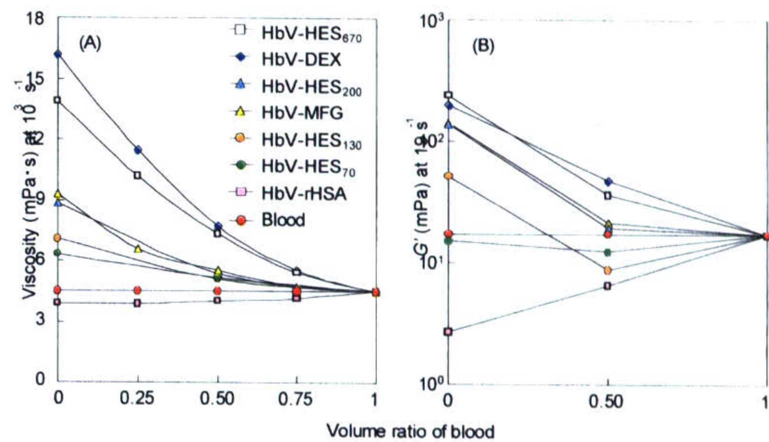


Figure 2

Sato et al.

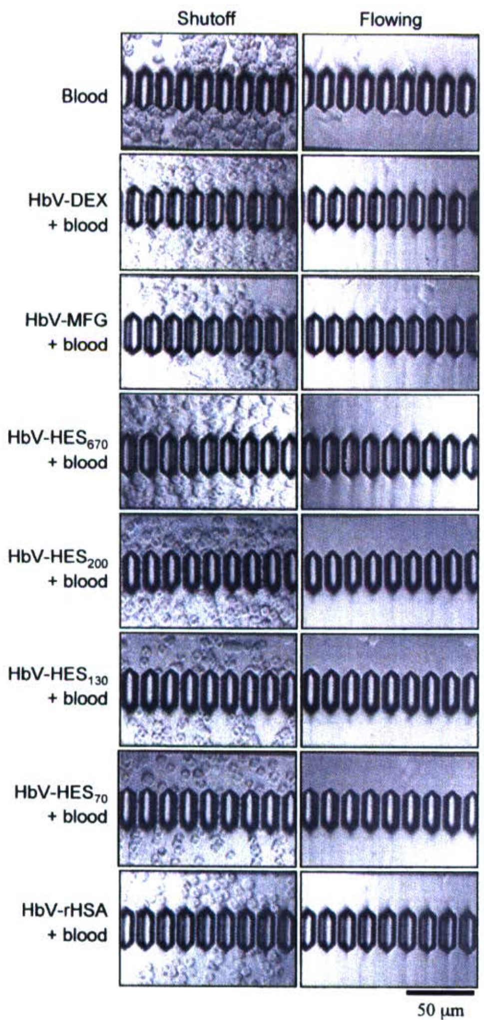


Figure 3

Sato et al.

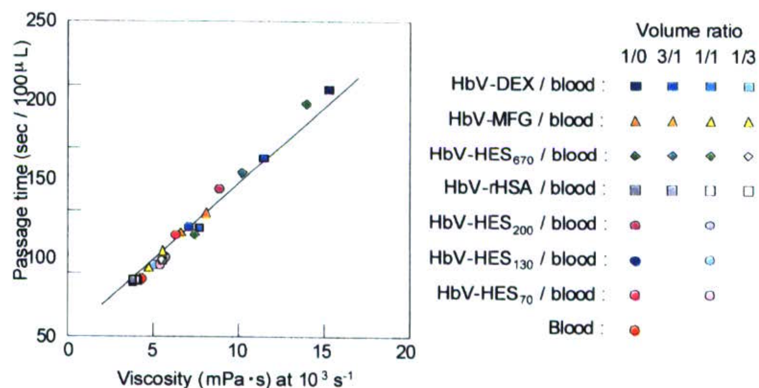


Figure 4

Sato et al.

表

Table 1. Plasma-substitute solutions and their physicochemical properties. The viscosities at 10 and 1000 s^{-1} are almost identical, indicating that these polymer solutions are Newtonian fluids.

Plasma Substitutes	Mw. (kDa)	Concentration (g/dL) in saline	COP (Torr)	Viscosity (mPa・s)	
				at 10 s^{-1}	at 1000 s^{-1}
DEX	40	10	44	4.5	4.5
MFG	30	4	44	2.2	2.3
HES_{670}	670	6	27	4.5	4.4
HES_{200}	200	6	29	2.5	2.5
HES_{130}	130	6	35	2.3	2.3
HES_{70}	70	6	34	2.0	2.0
rHSA	66.5	5	19	1.3	1.2

DEX, dextran; HES, hydroxyethyl starch; MFG, modified fluid gelatin; rHSA, recombinant human serum albumin; COP, colloid osmotic pressure.

Table 1

Sato et al.

抄録

Rheological properties of hemoglobin-vesicles (artificial red cells) suspended in a series of plasma-substitute solutions and their mixtures with blood.

Atsushi Sato[1), Hiromi Sakai[2), Shinji Takeoka[1), and Eishun Tsuchida[2)

1) Graduate School of Science and Engineering, Waseda University

2) Research Institute for Science and Engineering, Waseda University

Abstract

Hemoglobin-vesicles (HbV) are artificial oxygen carriers. The HbV suspension has an oxygen-carrying capacity that is comparable to that of blood. Since HbV suspension does not possess a colloid osmotic pressure, it should be suspended in or co-injected with an aqueous solution of a plasma substitute (water-soluble polymer), which might interact with HbV. This article describes the rheological properties of HbV suspended in a series of plasma substitute solutions and their mixtures with human blood. HbV suspended in a recombinant human serum albumin solution was nearly Newtonian and that mixtures with blood exhibited very low viscoelasticity. On the other hand, HbV suspended in a solution of dextran, modified fluid gelatin, or high-molecular-weight hydroxyethyl starch exhibited higher viscoelasticity. However, it decreased with increasing the volume ratio of blood. Microscopically, the flow pattern of the mixture of blood and flocculated HbV perfused through microchannels (4.5 μm wide, 20 cmH_2O applied pressure) showed few plugging. Furthermore, the time required for passing was simply proportional to the viscosity. This result indicates that flocculated HbVs would not affect the plugging factor of blood when perfusing through microchannels. The HbV suspension would be useful as various clinical applications in addition to its use as a transfusion alternative by manipulating the rheological property.

meso-Tetrakis($\alpha,\alpha,\alpha,\alpha$-*o*-amidophenyl)porphinatoiron(II) Bearing a Proximal Histidyl Group at the β-Pyrrolic Position via an Acyl Bond: Synthesis and O_2 Coordination in Aqueous Media

Akito Nakagawa,[1] Teruyuki Komatsu,*[1,2] and Eishun Tsuchida*[1]
[1]*Advanced Research Institute for Science and Engineering, Waseda University, 3-4-1 Okubo, Shinjuku-ku, Tokyo 169-8555*
[2]*PRESTO, Japan Science and Technology Agency (JST)*

(Received February 16, 2007; CL-070182; E-mail: teruyuki@waseda.jp, eishun@waseda.jp)

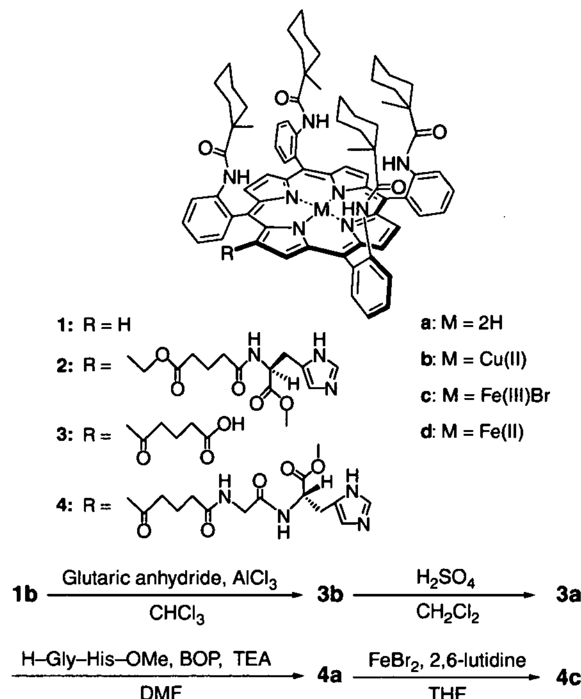
meso-Tetrakis($\alpha,\alpha,\alpha,\alpha$-*o*-(1-methylcyclohexanamido)phenyl)porphinatoiron(III) bearing a proximal histidyl group at the β-pyrrolic position via an acyl bond (**4c**) has been synthesized. Human serum albumin (HSA) incorporating the ferrous complex (**4d**) formed a stable O_2 adduct under physiological conditions (pH 7.4, 37 °C). Although an electron-withdrawing acyl group is attached to the porphyrin periphery, the O_2-binding affinity of HSA-**4d** was slightly higher than that of a similar analogue with a histidyl–alkylene group (**2d**).

In the active centers of hemoproteins, a basic amino acid residue, axially coordinated to the prosthetic heme group, namely the proximal base, plays a crucial role in controlling their biological functions, for example, histidine in hemoglobin (Hb) and cysteine in cytochrome P450. To mimic the versatile performances of the hemoproteins, numerous porphyrin derivatives have been synthesized over the past decades.[1,2] The most important factor in the molecular design of these compounds is how to confer the proximal base into the porphyrin structure by a covalent bond.

We successfully introduced a histidyl–alkylene group to the β-pyrrolic position of *meso*-tetrakis($\alpha,\alpha,\alpha,\alpha$-*o*-(1-methylcyclohexanamido)phenyl)porphine (**1a**) using the Vilsmeier reaction.[3] Human serum albumin (HSA) incorporating the ferrous complex (**2d**) can reversibly bind and release O_2 under physiological conditions (pH 7.4, 37 °C) in a fashion similar to Hb and myoglobin.[3b] The advantage of this strategy is to confer the proximal histidine to the porphyrin periphery in the last step of the synthesis.[3,4] However, the preparation processes are still labor-intensive: (1) formylation of the porphyrin, followed by (2) demetallation of copper, (3) reduction of –CHO to $-CH_2OH$, (4) connection with glutaric acid, and (5) binding of terminal histidine.[3,4] If the axial base can be introduced into the superstructured porphyrin in a few steps, it will lead to creating a new field in the hemoprotein model chemistry.

In this communication, we report for the first time, the one-step introduction of the 4-carboxybutanoyl group into the β-pyrrolic position of *meso*-tetrakis($\alpha,\alpha,\alpha,\alpha$-*o*-(1-methylcyclohexanamido)phenyl)porphyrin, which is easily converted into the hisitidine-linked porphyrin (**4a**) by other two processes. The O_2-binding property of the HSA hybrid incorporating the ferrous complex (**4d**) was then investigated in aqueous media.

The copper(II) complex of the parent porphyrin (**1b**) was synthesized according to our previously reported procedure.[3] We have found that the 4-carboxybutanoyl group is introduced by the Friedel–Crafts reaction using glutaric anhydride and aluminium chloride ($AlCl_3$) (Scheme 1). The progress of the reaction was monitored by the red shift of the absorption maxima



Scheme 1. Synthesis route of **4c**.

of the porphyrin and change in the R_f value during TLC. The brownish-red colored **3b** was purified by column chromatography and demetallated by H_2SO_4. The glycyl-*O*-methyl-L-histidine[5] was then coupled using benzotriazol-1-yl-oxytris(dimethylamino)phosphonium hexafluorophosphate (BOP). Finally, an iron insertion was carried out using $FeBr_2$ and 2,6-lutidine in anhydrous THF. The analytical data of all compounds described here were satisfactorily obtained (see Supporting Information).[6] The bathochromic shifts (3–7 nm) observed in the UV–vis absorption spectrum of **4a** compared to **2a** were due to the electron-withdrawing acyl group at the β-pyrrolic position.[6]

The ferric porphyrin (**4c**) in toluene was converted into the ferrous complex (**4d**) by reduction in a heterogeneous two-phase system (toluene/aq. $Na_2S_2O_4$) under an argon atmosphere.[4] The UV–vis absorption spectrum of the orange solution showed the formation of a five-N-coordinate high-spin complex (λ_{max}: 440, 544, 564 nm).[3,4,7] Upon exposure to O_2 or CO, the spectral pattern immediately changed to those of the O_2 adduct complex (λ_{max}: 429, 551 nm) or carbonyl complex (λ_{max}: 429, 544 nm).

The aqueous solution of the HSA-**4d** hybrid [in phosphate-buffered saline (PBS) solution (pH 7.4), [HSA]/[**4d**] = 1/4

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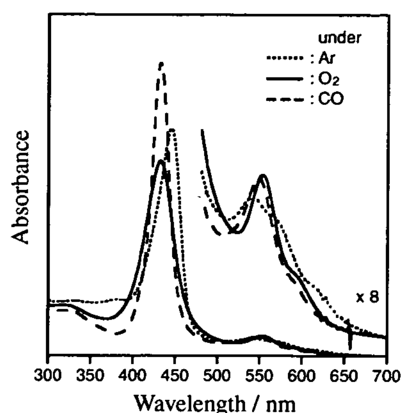

Figure 1. UV–vis absorption spectral changes of HSA-**4d** in PBS solution (pH 7.4) at 37 °C.

Table 1. O_2 binding parameters of HSA-porphinatoiron(II) in PBS solution (pH 7.4) at 25 °C[a]

Porphinatoiron(II)	$P_{1/2}$ /Torr	$k_{on}/\mu M^{-1}\,s^{-1}$		k_{off}/s^{-1}	
		fast	slow	fast	slow
2d[b]	1 (3)	54	8.8	89	14
4d	0.8 (2)	34	4.5	45	5.9

[a]The values in parenthesis are measured at 37 °C. [b]Ref 3b.

(mol/mol)] was prepared by a previously reported method.[3b] The UV–vis absorption spectrum of this aqueous solution under an argon atmosphere showed that **4d** formed a five-N-coordinate high-spin complex with an intramolecularly coordinated axial histidine (Figure 1). Upon exposure of HSA-**4d** to O_2, the absorption spectrum changed to that of the O_2 adduct complex. After reacting with the CO gas, a stable carbonyl complex was produced. The absorption maxima of HSA-**4d** showed 1–3 nm bathochromic shifts compared to those of the HSA-**2d** (Table S1).[3b,6]

The O_2-binding affinity of HSA-**4d** ($P_{1/2} = 0.8$ Torr) determined by the spectral changes at the different O_2 partial pressures was slightly higher than that of HSA-**2d** (Table 1). This is in significant contrast to the fact that the substitutions of two 3,8-vinyl groups of the imidazole-bound protoporphinatoiron(II) by acetyl groups decreased the O_2-binding affinity by 1/4–1/6 due to the reduction of the electron density in the porphyrin plane.[8,9] Our result suggested that (1) the reduced basicity of the porphyrin core by the introduction of one acyl group did not influence the O_2-binding equilibrium very much, and (2) that there is another structural factor that increases the O_2-binding affinity of the porphyrin.

To determine the association and dissociation rate constants for O_2 (k_{on}, k_{off}) to HSA-**4d**, the laser flash photolysis experiments were carried out.[3b,10,11] The absorption decay accompanying the O_2 recombination was composed of two phases of first-order kinetics, producing the fast and slow rebinding constants [k_{on}(fast) and k_{on}(slow)]. The k_{on}(fast) value was 7.6-fold higher than k_{on}(slow), and the molar concentration ratio of the two reactions was 3:1. The O_2 association to **4d** in the protein scaffold might be influenced by the microenvironment around the coordination site. This behavior was similarly observed in HSA-**2d**.[3b] The characteristics of the O_2 binding to **4d** was kinetically the low k_{off} values (approximately 1/2) compared to **2d**.

The structures of the ferrous complexes were then simulated.[12] It is remarkable that the porphyrin plane of **2d** in the five-coordinate high-spin complex was significantly domed compared to that of **4d** (Figure S1).[6] On the other hand, their O_2 adduct complexes showed similar structures having the flat porphyrin macrocycles. The difference in the five-coordinate species could be caused by the spacer moiety between the histidine and porphyrin. The rigid (histidyl-glycyl)carbonylbutanoyl group of **4d** presumably produces a favorable geometry to fix the proximal imidazole at the central iron(II) of the porphyrin, which could result in the relatively low dissociation rate constant of O_2.

In conclusion, we could successfully introduce the proximal histidyl group at the β-pyrrolic position of the *meso*-(tetrakis-*o*-amidophenyl)porphine via an acyl bond in two steps. The O_2 binding affinity was slightly higher than that of the imidazolyl–alkylene analogue, which might be due to the rigid structure of the spacer moiety between the histidine and porphyrin ring. This strategy would be useful to confer the proximal base to the superstructured porphyrin without any change in the activity, which allows us to create a new class of model heme compounds.

This work was supported by a Grant-in-Aid for Young Scientists (B) (No. 18750156) and for Scientific Research (No. 16350093) from JSPS, PRESTO from JST, and Health Science Research Grants from MHLW, Japan.

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Published on the web (Advance View) April 14, 2007; doi:10.1246/cl.2007.640

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DISCOVER SOMETHING GREAT

Influence of O_2-carrying plasma hemoprotein "albumin-heme" on complement system and platelet activation *in vitro* and physiological responses to exchange transfusion

Teruyuki Komatsu,[1] Yubin Huang,[1] Shinobu Wakamoto,[2] Hideki Abe,[2] Mitsuhiro Fujihara,[2] Hiroshi Azuma,[2] Hisami Ikeda,[2] Hisashi Yamamoto,[3,4] Hirohisa Horinouchi,[3] Koichi Kobayashi,[3] Eishun Tsuchida[1]

[1] *Advanced Research Institute for Science and Engineering, Waseda University, 3-4-1 Okubo, Shinjuku-ku, Tokyo 169-8555, Japan*
[2] *Research Department, Hokkaido Red Cross Blood Center, 2-2 Yamanote, Nishi-ku, Sapporo 063-0002, Japan*
[3] *Department of General Thoracic Surgery, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan*
[4] *Pharmaceutical Research Center, NIPRO Corp., 3023 Noji-cho, Kusatsu-shi, Shiga 525-0055, Japan*

Received 23 March 2006; revised 13 July 2006; accepted 25 July 2006
Published online 18 January 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.a.31016

Abstract: Recombinant human serum albumin (HSA) including the synthetic iron(II)-porphyrin (FeP), albumin-heme (HSA-FeP), is a unique O_2-carrying plasma hemoprotein as a red blood cell substitute. We have investigated the possible influence of HSA-FeP on the complement system and platelet activation *in vitro*. The amounts of the serum complement titer CH_{50} and terminal complement complex SC5b-9 of human blood serum, incubated with HSA-FeP (10, 20, and 40 vol %), were almost the same as those of the corresponding samples with HSA. The effect of HSA-FeP on the platelet reactivity has been demonstrated by conformational changes in the membrane glycoprotein IIb/IIIa and surface expression of an α-granule membrane protein P-selectin. Platelet activation in response to the ADP-stimulation was not influenced by the presence of HSA-FeP. It can be concluded that the albumin-heme solution does not facilitate the immunological reaction and platelet activation. Moreover, a 20% exchange transfusion with HSA-FeP into anesthetized rats has been performed to evaluate the circulation and blood parameters for 6 h. Time course changes in all parameters showed features identical to the control group (without infusion) and HSA group. © 2007 Wiley Periodicals, Inc. J Biomed Mater Res 81A: 821–826, 2007

Key words: albumin-heme; complement system; exchange transfusion; platelet activation; RBC substitute

INTRODUCTION

There has been significant progress in red blood cell (RBC) substitutes in the past decade, and several hemoglobin (Hb)-based products are currently in clinical II/III trials.[1–4] We have also developed a unique albumin-based O_2-carrier "albumin-heme," which is composed of recombinant human serum albumin (HSA) including synthetic heme (2-[8-{*N*-(2-methylimidazolyl)}-octanoyloxymethyl]-5,10,15,20-tetrakis(α,α,α,α-*o*-pivalamido)phenylporphinatoiron(II)) (FeP, Scheme 1) (HSA-FeP).[5–7] The HSA-FeP solution has a high compatibility with the blood cell components, and its O_2-transporting capability was evaluated by animal experiments.[8,9] Nevertheless, animal studies cannot predict the potential effect on human responses. Especially, immunological reactions and platelet activity need not be the same as those in animals. In this study, we report the possible influence of HSA-FeP on the human complement system and platelet activation *in vitro*.

It is known that the complement cascade is activated in trauma patients with or without hemorrhagic shock.[10] The large volume administration of HSA-FeP as a blood alternative to the human body may affect the total serum complement activity. We have measured the amounts of complement titer CH_{50} and the terminal complement complex SC5b-9 of the human blood serum after incubation with HSA-FeP.

On the other hand, the platelet activation process involves an ordered sequence of events. In particular, the

Correspondence to: E. Tsuchida; e-mail: eishun@waseda.jp
Contract grant sponsor: Health Science Research Grants, MHLW, Japan

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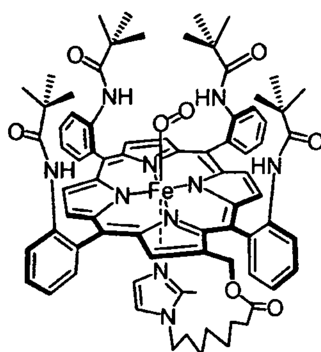

Scheme 1. Chemical structure of synthetic iron(II)-porphyrin (FeP) incorporated into HSA.

conformational changes in the membrane glycoprotein IIb/IIIa seem to be the most sensitive marker in the first event.[11–13] The activated GPIIb/IIIa creates a functional receptor for fibrinogen, which provides the link between adjacent platelets to form aggregates. The activation of this receptor can be detected by the specific monoclonal antibody PAC-1 that competes with fibrinogen.[11] The second event is the secretion of activation mediators, for example, ADP, serotonin, and thromboxane, resulting in further recruitment of platelets at the injured site.[14] The secretion process is accompanied by the rapid translocation of the α-granule membrane protein P-selectin (CD62P) to the outer membrane.[15,16] If the HSA-FeP solution activates the platelet, it may lead to amplifying the blood aggregation and inflammatory response. The PAC-1 binding and P-selectin surface expression have been assayed at the various levels of the ADP-stimulation.

Furthermore, we carried out the 20% exchange transfusion with the HSA-FeP solution into anesthetized rats and monitored the time courses of the circulation parameters (MAP, HR) and blood parameters (pH, PaO_2, PvO_2, $PaCO_2$) for 6 h.

MATERIALS AND METHODS

Materials

The recombinant human serum albumin (HSA, 25 wt %) was obtained from the NIPRO (Osaka). The HSA-FeP solution ([HSA]: 5.0 wt %, pH 7.4, [FeP]: 3.0 m*M*, COP: 21 mmHg, osmolarity: 300 mOsm, viscosity: 1.1 cP, endotoxin: <0.1 EU/mL, O_2-binding affinity ($P_{1/2}O_2$): 33 Torr) was prepared using our previously reported procedure.[8]

CH_{50} and SC5b-9

The human blood serum or plasma was well mixed with the HSA-FeP solution (the final concentration is 10, 20, and 40 vol %) and incubated for 1 h at 37°C. The CH_{50} value was determined by a 50% hemolysis assay based on Mayer's method with a commercial kit (New One point CH50 (KW), Japan BCG Supply, Tokyo). The SC5b-9 in the sample was determined using enzyme-linked immunosorbent assay kits (QUIDEL, Mountain View, CA).

PAC-1 and CD62P

The expression of PAC-1 and CD62P on platelets was measured as previously described.[17] Briefly, sodium citrate human whole blood was mixed with the HSA-FeP or HSA solution (the final concentration is 10, 20 and 40 vol %) and incubated for 10 min at 37°C. After adjusting the platelet concentration to

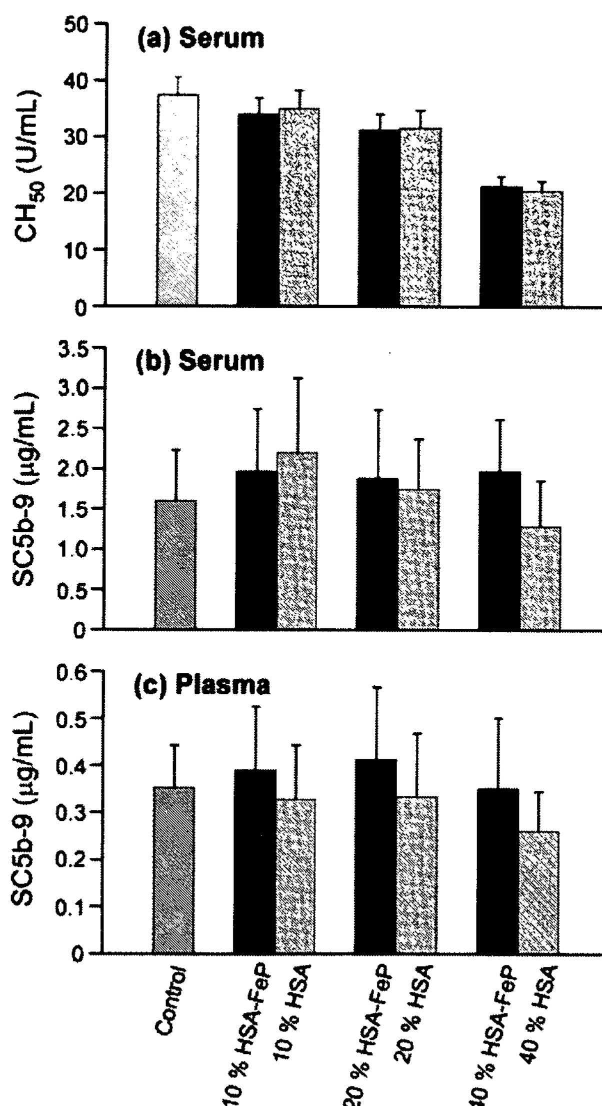

Figure 1. Influence of HSA-FeP on human serum complement after incubation for 1 h at 37°C; (a) serum complement titer (CH_{50}) in human blood serum, (b) terminal complement complex (SC5b-9) in human blood serum, and (c) SC5b-9 in human plasma. Each value represents the mean ± SD ($n = 5$).

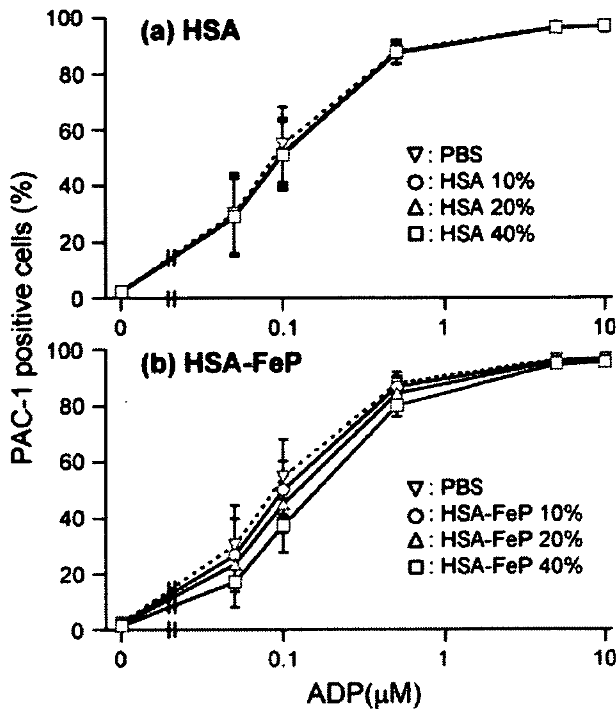

Figure 2. PAC-1 positive platelets in human whole blood mixed with (a) HSA-FeP and (b) HSA for 10 min at 37°C in response to various levels of ADP-stimulation. Each value represents the mean ± SD ($n = 5$).

$4 \times 10^5/20$ μL by Hepes Tyrode's buffer (pH 7.3), a cocktail of FITC-conjugated PAC-1, PE-conjugated anti-CD62P, and PerCP-conjugated anti-CD42a was added in equal amounts. A certain amount of ADP was added (the final ADP concentration is 0.05, 0.1, 0.5, 5, and 10 μM). The FITC-conjugated anti-mouse IgM, PE-conjugated anti-mouse IgG, and PerCP-conjugated anti-mouse IgG were used as the negative controls. All antibodies were purchased from BD Bioscience-Pharmingen (San Jose, CA). The mixture was reacted in the dark for 20 min at room temperature and fixed with 1% paraformaldehyde. The samples were analyzed by flow cytometry (LSR, BD, San Jose, CA). Fluorescence data from 10,000 platelet events were collected in the logarithmic mode. The platelet population was identified by the number of CD42a positive events. The increased activation of GPIIb/IIIa and expression of CD62P were demonstrated by the percent of PAC-1 and CD62P positive cells in the platelets, respectively.

All subjects enrolled in this research had responded to an Informed Consent which has been approved by The Committee on Human Research of Hokkaido Red Cross, and that this protocol was found acceptable by them.

Exchange transfusion

The animal experiments using Wistar rats (304 ± 7.2 g) were carried out according to our previously reported protocol.[9] After stabilization of the animal, the 20% exchange transfusion was performed by 1 mL blood withdrawal via the common carotid artery and 1 mL HSA-FeP infusion from the femoral vein (each 1 mL/min); a total of four repeating cycles ($n = 6$, HSA-FeP group). A blood sample was taken from the artery (0.3 mL) and vein (0.2 mL) at the following five times; (1) before, (2) immediately after, (3) 1 h after, (4) 3 h after, and (5) 6 h after the exchange transfusion. MAP, HR, O_2-pressure (PaO_2), CO_2-pressure ($PaCO_2$) and pH for the arterial blood, and the O_2-pressure (PvO_2) of the venous blood were measured. As a reference group, the 5 g/dL HSA solution was similarly administered to the rats ($n = 6$, HSA group).[9] Furthermore, six rats without infusion (operation only) were also used as a control group.[9]

All animal handlings were in accordance with the NIH guidelines for the care and use of laboratory animals. The protocol details were approved by the Animal Care and Use Committee of Keio University.

Data analysis

All data were represented by mean ± standard deviation (SD). Statistical analyses were performed by repeated analysis measures of variance (ANOVA) using a StatView (SAS Institute). Values of $p < 0.05$ were considered significant.

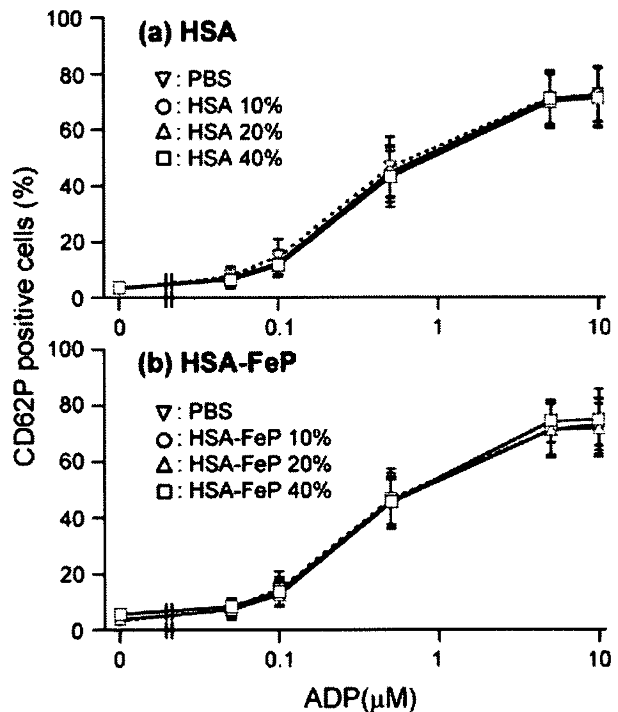

Figure 3. P-selectin expression on platelets in human whole blood mixed with (a) HSA-FeP and (b) HSA for 10 min at 37°C in response to various levels of ADP-stimulation. Each value represents the mean ± SD ($n = 5$).

RESULTS AND DISCUSSION

Complement system

Changes in the complement consumption are generally demonstrated by the complement titer CH_{50}. Ohtani et al. reported that the recombinant HSA, which is used for host albumin for HSA-FeP, shows the same immunochemical properties as the plasma HSA.[18] Thus the recombinant HSA should be a good reference in this experiment. The CH_{50} of the human blood serum incubated with 10, 20, and 40 vol % of the HSA-FeP solution were reasonably reduced in proportion to the each dilution ratio: 91, 83, and 57% of the control level [Fig. 1(a)]. The differences are almost the same as those observed in the HSA group, suggesting that the decrease in the CH_{50} with HSA-FeP did not involve any specific interaction.

On the other hand, the mean amounts of SC5b-9 in the human blood serum or plasma after the incubation with HSA-FeP were slightly higher than the control levels. However, all such differences were not significant within the experimental errors. Similar observations were found in the HSA group independent of the mixing ratio (10, 20, and 40 vol %). This implies that HSA-FeP does not enhance the production of SC5b-9.

Platelet activation

PAC-1 recognizes an epitope on the GPIIb/IIIa complex of activated platelets near the fibrinogen receptor.[19] We measured the percent of PAC-1 positive cells in the blood sample incubated with the HSA or HSA-FeP solution. When the ADP is absent, the fraction of the active cells was negligibly small, that is, 1.37–2.39%, independent of the mixing ratio of HSA or HSA-FeP (10, 20, and 40 vol %) (Fig. 2). The addition of a certain amount of ADP increased the PAC-1 positive cells, for instance, 96.9 % at 10 μ*M* in the PBS group. It is rather remarkable that the coexistence of HSA and HSA-FeP (10–40 vol %) did not disturb this concentration dependence of the ADP-stimulation (Fig. 2).

The P-selectin (CD62P) on the activated platelet interacts with vascular endothelial cells to induce hemostasis. The percent of the P-selectin positive cells in the sample with HSA or HSA-FeP solution was 3.30–5.57% independent of the mixing ratio (10, 20, and 40 vol %) (Fig. 3). The addition of ADP enhances the numbers of active cells, and the concentration dependence curves observed in the HSA and HSA-FeP groups ([ADP] = 0.05–10 μ*M*) were all identical to that of the PBS group. These results revealed that the platelet activation in response to the ADP-stimulation was not influenced by HSA-FeP. We concluded that albu-

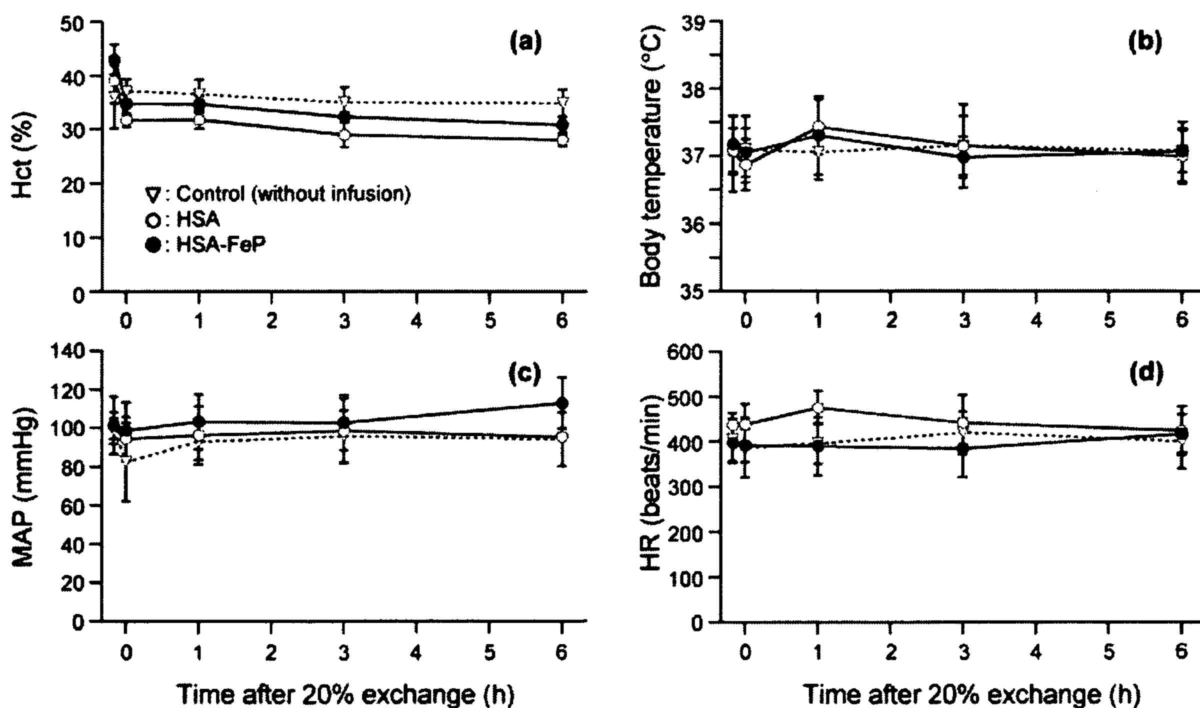

Figure 4. Time courses of (a) hematocrit (Hct), (b) body temperature, (c) mean arterial pressure (MAP), and (d) heart rate (HR) in anesthetized rats after 20% exchange transfusion with HSA-FeP or HSA solution. Each value represents the mean ± SD ($n = 6$).