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総　説

周術期／救急領域の赤血球輸血と人工酸素運搬体の展望 -Hemoglobin Vesiclesの可能性-

Transfusion Overview in Perioperative/Emergent Field and Prospect of Artificial Oxygen Carriers -A Potential of Hemoglobin Vesicles-

寺嶋克幸，杖下隆哉，坂本篤裕

Katsuyuki Terajima, Takaya Tsueshita, Atsuhiro Sakamoto

和文抄録

医療の飛躍的な発展にも関わらず，周術期や救急領域では多くの赤血球輸血の機会がある．近年の輸血療法に関する多くの報告を基に，日本赤十字社が輸血療法の実施に関する指針を発表し，改訂を重ねてきた．輸血を行うには，急性貧血時の代償機構と輸血の副作用を知らねばならない．輸血に対する安全性への努力はなされてきたが，根本的な問題の解決には至っていない．そこで，いくつもの人工酸素運搬体が開発された．しかし，安全性や効果の面で，一部のヘモグロビンを利用した人工酸素運搬体のみが本邦において前臨床応用段階で有望視されている．幸いにも，我々は最も臨床応用可能と思われるHemoglobin Vesiclesの安全性及び効果を研究する機会を得た．本稿では，麻酔や外傷治療における貧血への耐性と輸液・輸血後の予後，およびヘモグロビン利用酸素運搬体の可能性について，我々の調査結果を含め再考した．

Abstract

Despite the remarkable medical advances were made over recent years, erythrocyte transfusions are often still required during emergency care and the perioperative period. Several recent reports on blood transfusion have prompted the Japanese Red Cross Society to publish revised guidelines for implementing transfusion therapy. In order to perform this procedures, one must understand both the compensatory mechanism that occurs during acute anemia and the potential adverse reactions to transfusion. Although great efforts have been made to ensure the safety of blood transfusions, some fundamental issues have yet to be resolved. Consequently, several artificial oxygen carriers have been developed; however, in terms of their safety and effectiveness, only those that utilize hemoglobin have shown potential at the pre-clinical stage in Japan. We are privileged to have had the opportunity to study the safety and effectiveness of hemoglobin vesicles, which appear to have the greatest potential for clinical application. The present paper reviews tolerance to anemia under anesthesia and during trauma treatment, post-transfusion prognosis, and the potential application of hemoglobin-based oxygen carriers, with reference to the results of our own survey of these topics.

Keywords

Hemoglobin-based oxygen carrier, multiple organ failure, hemorrhagic shock, transfusion, red blood cell, fluid resuscitation

はじめに

麻酔中や救命救急領域では，出血や急性貧血に対する輸血の機会が非常に多い．昨年９月，「輸血療法の実施に関する指針」及び「血液製剤の使用指針」の改訂が行われた[1]．輸血方法を見直す報告が近年増加し，従来の輸血方法に対する批判も含まれている．特に，集中治療領域における輸血方法は患者予後に強く影響を及ぼす可能性がある．一方，血液代替物が開発されて75年が経過した．最近10年の進歩はめざましく，人工酸素運

日本医科大学　麻酔科学教室　〒113-8603　東京都文京区千駄木1-1-5　Department of Anesthesiology, Nippon Medical School　1-1-5, Sendagi, Bunkyo-ku, Tokyo, 113-8603, JAPAN

論文受付 2006年６月16日　論文受理 2006年７月19日

搬体は一部の国で臨床応用や臨床治験されるまでになった．現在，我が国でも数種類の人工酸素運搬体が動物試験されている．この論文では，酸素運搬体としての赤血球輸血とヘモグロビン利用の人工酸素運搬体に焦点を当て，麻酔／救急領域における赤血球輸血療法の適応と問題点，人工酸素運搬体利用による利点について我々の報告を含め概説する．

手術中の急性貧血に対する耐性

最も重要な赤血球の機能は，呼吸器（肺）から末梢組織への酸素運搬である．手術中のような院内出血は，晶質液や膠質液による循環血液量の補正後に必要な場合に対してのみ輸血が行われる．この時の輸血実施に対する判断は，生体の代償機能を超えるか否かの判断に基づく．この代償機能は，主に血行動態の変化とヘモグロビン酸素解離曲線の変化による[2]．循環血液量の保たれている急性貧血は，一回拍出量と心拍数の増加により心拍出量は増加する[3]．血液粘稠度の減少は，静脈還流増加による前負荷の増加と後負荷を軽減する[4]．そして，交感神経刺激は陽性変時・変力作用を来すことによる．従って，麻酔下の患者は，主に一回拍出量によって心拍出量を増加させる．全身的には，ヘモグロビン濃度が7 g/dLまでは酸素供給量は変化しない[3]．脳は酸素抽出率を増加することで急性貧血に対応するのに対し，心臓は酸素抽出率よりも酸素供給量の増加により代償する．微小循環に対しては血管床と血流の増大により酸素放出増加を可能にする[2]．また，貧血による2,3-DPGの増加は，酸素解離曲線を右方変位させ，末梢での酸素放出を容易にさせる．

麻酔中の冠動脈疾患患者での循環血液量の保たれている中等度急性貧血は許容されるようである．冠動脈バイパス術を予定されている患者のヘモグロビン濃度13.9 ± 1.3 g/dLから9.3 ± 1.0 g/dLへの血液希釈では，心筋虚血を示唆する心電図変化や局所壁運動異常，血行動態の変化は見られなかった[5]．このような臨床研究での対象患者は，予後を改善するために周術期もβ遮断薬による治療を継続している[6]．β遮断薬の使用の有無は，急性血液希釈による心拍出量の増加を阻害しない[7]．また，麻酔下の状況では陽性変時作用が見られないことも手術中のイベントの発生率を下げるかもしれない．さらに，オンポンプ冠動脈再建術患者のヘマトクリット値28%への急性血液希釈は，術後の血中トロポニンIやCK-MBレベルをコントロール群に比べ抑制し，カテコラミン必要量を低下させ術後不整脈発生率を低下した[8]．急性冠動脈症候群患者では，30日死亡の予測因子としてヘマトクリット値25%以上での輸血開始が挙げられた[9]．ただし，この研究は3つの急性冠動脈症候群に対する大規模研究データに基づくレトロスペクティブな研究であることを考慮しなければならない．

血液希釈によるヘモグロビン濃度6 g/dL以下の低下は，認知機能を低下させる[10-12]．ヘモグロビン濃度5 g/dLまでの低下による認知機能の低下は可逆的であり，新鮮血や保存血の違いに関わらずヘモグロビン濃度7 g/dLまで輸血することで回復する．また，吸入酸素濃度の増加によっても回復する．その障害時には，大脳誘発電位の一種である事象関連電位に見られる，刺激からの陽性波の発生までの時間，すなわちP300潜時の延長として表される可能性がある．

軽度から中等度の血液希釈は肺の換気血流不均等を改善させ[13]，肺高血圧を伴う慢性閉塞性肺疾患患者に対して肺動脈圧を減少させる[14]ことが示唆されている．血液粘度の低下や心拍出量の増加に加え，NOの増加による肺血管抵抗の減少が生じるためと考えられる．これにより，動脈血酸素分圧/吸入酸素濃度比は増加する．

腎血流量と血液分布は中等度血液希釈に影響を受けず，尿量と尿中ナトリウム排泄率は増加する可能性がある[15]．同様に，ヘマトクリット20 ± 1%までの血液希釈は肝臓や小腸，膵臓の血流を増加させる[16]．肝臓は，肝動脈の血流が86%増加し，門脈血流は28%増加した．小腸粘膜血流も増加した．ICG血管内半減期は短縮し，GPTは減少した．すなわち，肝機能を障害しないようである．

血液希釈による酸素供給量の重篤な低下は，主に乳酸値，重炭酸イオン，過剰塩基（BE），酸素抽出率，呼気二酸化炭素濃度，脈圧，心係数，収縮期血圧に影響を及ぼす[17]．臨床的に輸血を行う根拠の一つとして利用出来る可能性がある．

プレホスピタル出血性ショック

我々の関わるもう一つの分野に，救命救急や集中治療がある．救命救急の患者の多くは院外発症である．すなわち，院外発症の急性出血性ショック患者は通報により駆けつけた救急救命士により初期治療が施され，直ちに近くの救急対応病院に搬送される．このプレホスピタルの出血性ショックの特徴は，全ての血液成分が循環血液量とともに同程度に失われることである．残念なことに，我が国での救急救命士によるfluid resuscitation（輸液蘇生）は行われていない．欧米諸国では，患者搬送時間の問題もあるが，この救急救命士による初期輸液蘇生は急性出血性ショック患者の救命の一役をになっている．初期輸液蘇生の方法は，未だ発展途中である[18]．必要な止血処置とともに行われるプレホスピタルでの古典的輸液蘇生法は，大量の晶質液投与による循環維持をすることである．その後のインホスピタルで必要があれば，輸血が行われる．現在臨床治験中や開発中の人工酸素運搬体の役割の一つには，このプレホスピタルへの治療限界への挑戦が挙げられる．

人工酸素運搬体の過去の治験から学ぶ

修飾Hb 4量体のヘムアシストは，第3相臨床試験で予期せず失敗に終わった[19]．このヘモグロビン利用酸素運搬体は，臨床治験前に少量投与で循環動態に強く影響することが分かっていた[20,21]．生来のHbと同じ様に，少量投与で，肺血管抵抗や体血管抵抗を増加し心拍出量を低下させた．これは，NOのスカベンジ[22-24]とエンドセリン放出増加，アドレナリン受容体の感受性増大[25-27]，二次的な動脈壁ずり応力の減少によるものと考えられている．出血性ショック患者へのプレホスピタルでの初期蘇生として，強力な血管収縮薬の投与は死亡率を増加させる結果となった．

Hemoglobin-Vesicles（HbV）の循環動態と臓器酸素化へ及ぼす影響

HbVは我が国で開発されたHBOCである．現在，開発した早稲田大学理工学部や慶応大学医学部を中心に精力的に研究活動を行っている．昨年，我々の教室でも，動物モデルでのHbVを用いた輸液蘇生での全身循環及び臓器酸素化への影響を調査した[28]．

対象動物は，生後12 - 14週齢の雄New Zealand white rabbitとした．早稲田大学理工学部の協力を得て，ウサギ赤血球とHbV（委託製造先，㈱オキシジェニクス）の酸素解離曲線を得た（Fig. 1.）．麻酔下に小開頭と開腹，大腿部切開し，脳／肝臓／腎臓／骨格筋に酸素電極を挿入した．ベースライン測定後，平均動脈圧が30-35 mmHgとなる様に脱血を行った．その後，脱血量と等量の５％リコンビナントアルブミン溶液＋HbV溶液（HbV＋alb群），５％リコンビナントアルブミン溶液（ニプロ㈱提供）（alb群），乳酸リンゲル液（LR群），さらに脱血の３倍量の乳酸リンゲル液（3XLR群）で急速輸液蘇生を行った．測定項目は，脱血量，動脈圧，中心静脈圧，心拍出量，組織酸素分圧とした．脱血量は群間に差がなかった．血行動態の変化をTable 1.に示す．LR群は蘇生終了15分後より，3XLR群は1時間後よりベースラインに比べ血圧が低下した．心拍出量はLR群と3XLR群ともに２時間後に低下した．それに対し，HbV＋alb群とalb群の心拍出量は蘇生後に回復し，実験期間中維持された．蘇生後15分でのHbV＋alb群の計算された全身血管抵抗は，他の群と差がなかった．HbV投与による蘇生後の組織酸素分圧は，他の蘇生群と比べ低下しなかった．脳や腎臓では，蘇生後15分以降ではLR群に比べ高く維持された．BEや血中乳酸値も他群に比べより早く回復した．これらの結果は，急性出血性ショックに対するHbV溶液の投与が，生体内で血管収縮による全身や局所の血管抵抗の増大を来さず，心拍出量と組織酸素分圧を回復させることを示唆する．さらに，全身の循環と酸素化改善により乳酸値とBEを早期に回復させることが示唆された．

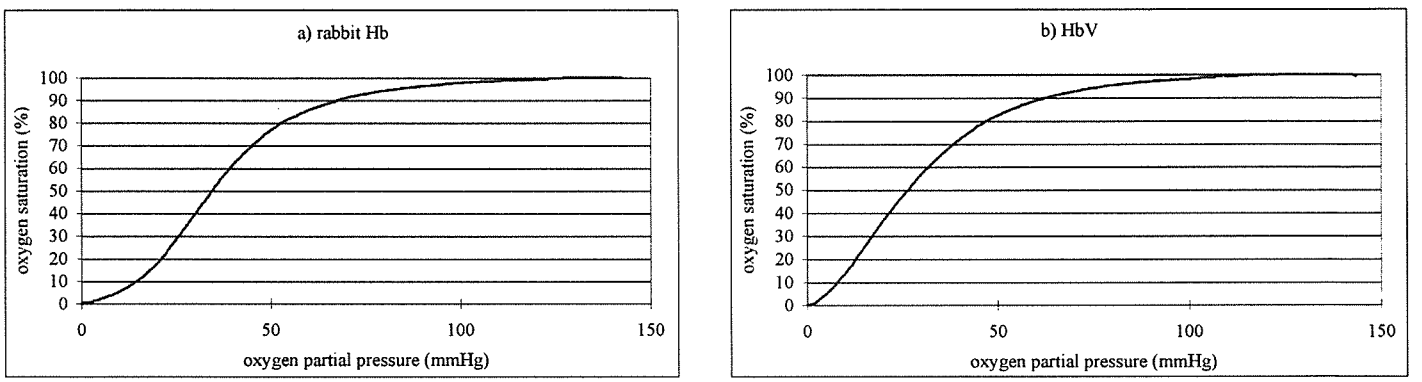

Fig. 1. The oxygen equilibrium curves for a) rabbit Hb and b) HbV.

Table 1. Hemodynamic variables in New Zealand white rabbits after inducing hemorrhagic shock (HS) by withdrawing blood and stabilization for 30 min. Animals were resuscitated using the same volume of HbV/rHSA, rHSA or RL, or using 3xRL, ove 15 min (RES). The hemodynamic variables were measured again after 15 min, 1 h and 2 h. *Significant difference from baseline ($p< 0.05$). † Significant difference from the RL group ($p< 0.05$). All values are presented as the mean ± SD (n = 6).

	Baseline	HS	30 min	RES	15 min	1 h	2 h
Mean arterial pressure (mmHg)							
HbV/rHSA	85 ± 10	33 ± 1*	43 ± 3*	88 ± 10†	90 ± 16†	90 ± 17†	90 ± 7†
rHSA	89 ± 11	32 ± 3*	36 ± 2*	68 ± 14	76 ± 9†	77 ± 9†	80 ± 9†
RL	78 ± 10	34 ± 1*	41 ± 9*	69 ± 9	56 ± 13*	51 ± 10*	54 ± 20*
3XRL	80 ± 17	31 ± 3*	38 ± 7*	67 ± 12	62 ± 17	48 ± 20*	46 ± 16*
Central venous pressure (mmHg)							
HbV/rHSA	4.5 ± 1.2	3.0 ± 0.9*	2.8 ± 1.5*	5.8 ± 1.5	5.8 ± 1.5	4.2 ± 1.2	4.7 ± 1.2
rHSA	5.2 ± 1.7	3.2 ± 1.2*	3.0 ± 1.3*	5.0 ± 1.7	4.8 ± 1.5	4.7 ± 0.5	4.2 ± 0.8
RL	6.2 ± 1.3	2.7 ± 0.8*	2.8 ± 0.4*	4.7 ± 1.4	4.0 ± 1.7*	3.8 ± 0.8*	3.5 ± 1.0*
3XRL	4.3 ± 1.0	2.8 ± 1.0*	2.8 ± 1.2*	5.5 ± 0.8	4.8 ± 1.2	3.7 ± 0.5	2.8 ± 1.0*
Cardiac Index ($l \cdot minute^{-1} \cdot m^{-2}$)							
HbV/rHSA	2.9 ± 0.7		1.2 ± 0.4*		2.9 ± 0.2†	3.2 ± 0.8†	2.8 ± 0.4†
rHSA	2.7 ± 0.8		1.0 ± 0.2*		2.7 ± 0.7	2.3 ± 0.5†	2.4 ± 0.6†
RL	2.6 ± 0.9		1.1 ± 0.4*		1.8 ± 0.4	1.3 ± 0.3	1.1 ± 0.3*
3XRL	2.9 ± 0.5		1.1 ± 0.2*		2.6 ± 0.9	1.9 ± 0.6	1.4 ± 0.4*
Systemic vascular resistance (dyne·second·cm-5)							
HbV/rHSA	1579 ± 738		1839 ± 420		1516 ± 294	1498 ± 440	1516 ± 294
rHSA	1834 ± 494		1956 ± 394		1600 ± 569	1820 ± 549	1600 ± 569
RL	1559 ± 564		2012 ± 695		1578 ± 509	1993 ± 587	1578 ± 509
3XRL	1575 ± 465		1868 ± 370		1374 ± 334	1420 ± 717	1374 ± 334

Fluid resuscitationの歴史[29]

プレホスピタルでの輸液蘇生の進歩は，残念なことに大きな戦争ごとに見られるようである．戦場では医療に対して障害がある．初期の蘇生処置から根治的治療までの時間，治療薬の保存や運送面において効果的な初期治療のための方法と薬剤が求められる．

第一次世界大戦の時は，まだ術前のfluid resuscitationが導入させておらず，多くの兵を失った．第二次世界大戦や朝鮮戦争の時は，膠質液の投与と保存血の投与による蘇生が始まった．初期生存率の改善をもたらしたが，急性腎不全のために多くの死傷者が出た．ベトナム戦争の時には，以前の経験と大量等張晶質液による初期輸液蘇生が見直され，それまでのコロイド輸液から変わった．その結果，死亡率と急性腎不全の発生率は減少したが，adult respiratory distress syndrome（ARDS）の発生率とそれによる死亡率が増加した．1970年代より当時の先端医療技術とモニタリングなどの特徴を持つ集中治療室が発達し，ARDSでの死亡率は減少した．しかし，“多臓器不全”という現在でも外傷患者の死亡原因として最も重大な問題が明白となった．

後に，初期蘇生輸液の晶質液の種類についての研究が報告された[30]．中等度出血性ショックモデルに対する輸液／輸血蘇生では，生理食塩水を用いた群は乳酸リンゲル液での蘇生群に比べ，アシドーシス（7.14 ± 0.06対7.39 ± 0.04）と死亡率の増加（50%対0%）が観察された．

Small volume fluid resuscitationと permissive hypotension

急性ショック患者の主な目的の一つには，十分な酸素供給量に回復することである．酸素供給量に関わる要素は，ヘモグロビン濃度，酸素飽和度，心拍出量を含む．そのため，心拍出量を改善するための充分な輸液量と酸素運搬体の補充が必要となる．院内蘇生の最初の24時間の酸素供給量インデックスの目標は，現在のところ500 mL/minute/m2と報告された[31]．それ以上に酸素供給量を増加させても酸素消費量を増加させず，血中乳酸値は増加したままとなることがある．これは，末梢のミトコンドリア機能障害を示し，多臓器不全に導く[32]．また，過剰な輸液や輸血は，血圧上昇や凝固能への影響による出血量の増加や，低体温，アシドーシス，脳浮腫やARDS発生率の増加のような組織浮腫の可能性がある[33]．鈍的外傷による出血源のコントロールされていない患者に対する等容量輸液蘇生は，このような危険を含んでいる．

このような患者の輸液蘇生に対し，輸液量を制限する戦略や低血圧を維持させる戦略が始まった[34]．少量高張食塩水は大量晶質液と同等の心拍出量と血漿増量効果，血管内皮の膨化を防ぐことでの末梢循環改善効果，生存率改善が示された[35]．これは，心原性ショックに対しても末梢循環改善効果をもたらす[36]．しかし，この循環改善効果は出血量の増加をもたらした．高張食塩水の研究のメタアナリシスでの結果は，標準的な晶質液での輸液蘇生後の生存率を改善しなかった[37]．この生存率改善効果は，デキストランを添加することで可能性があることが示唆された[37,38]．さらに，多施設試験での蘇生後の臓器保護効果[39]やpermissive hypotensionによる蘇生後の予後を改善する効果[40]が示された．特に，ショックを伴う閉塞性頭部外傷患者では脳浮腫を軽減し頭蓋内圧を減少し，脳還流圧を上昇することが示された[41]．さらに，この高張食塩水の蘇生は，出血性ショックや虚血再還流において炎症反応を減少させることが示唆された[42-44]．

HbVの凝固機能への影響

出血性ショックに対する輸液蘇生時には，大量出血による凝固因子の流失，凝固機能亢進による消費，輸液蘇生に用いられる溶液の影響による出血量の増大が生じる可能性がある．さらに，前述の様に，末梢循環の改善効果のある溶液は，出血量を増加させる．出血性ショックモデルに対するHbV溶液を用いた輸液蘇生は，全身末梢血管抵抗に影響を及ぼさないようである[28]．そこで我々は，HbV溶液による血液希釈が凝固機能に与える影響をin vitroで調査し，報告した[45]．

健康成人男性のボランティア７人を対象とした．採血された血液サンプルを，直ちに乳酸リンゲル液，または，生理食塩水に分散した10%HbV溶液で，それぞれ，乳酸リンゲル液，または，生理食塩水が血液サンプルに対して０%，20%，40%，60%，80%になる様に希釈した．乳酸リンゲル液で希釈した血液は，フィブリノーゲン濃度，プロトロンビン時間，活性化部分トロンボプラスチン時間を測定した．また，全ての血液サンプルまたは希釈された血液はSONOCLOT（tm）（Sienco Company，Morrison，CO）で凝固機能を測定した．SONOCLOT（tm）は，血小板機能や血漿成分凝固因子，細胞成分凝固機能を，37℃における血液の粘度抵抗の経時的な変化に基づいてクロット形成と線溶現象を測定することで評価するものである[46,47]．希釈の程度に比例して，フィブリノーゲン濃度は低下した（Fig. 2a.）．プロトロンビン時間と活性化部分トロンボプラスチン時間は，希釈の程度以上に延長する傾向を認めた（Fig. 2b, 2c.）．SONOCLOT（tm）による測定値を示す（Fig. 3.）．60%希釈まではHbV溶液は乳酸リンゲル液に比べて差はなかった．しかし，80%希釈ではHbV溶液での希釈はSonACTを延長させた．HbV溶液による高度の血液希釈は，乳酸リンゲル液に比べ抗凝固作用を示す可能性が示唆された．ただし，60%希釈までの場合，ヒドロオキシエチルスターチに比べ影響が少ない可能性がある[46,47]．我々の研究では，60%までの希釈では，乳酸リンゲル液とHbV溶液のSONOCLOT（tm）による測定値は差がなかった．すなわち，HbV溶液で高度希釈を行うような特殊な場合を除き，臨床応用される状況下では凝固異常が問題となる可能性は低いことが示唆された．

赤血球輸血による外傷後多臓器不全と肺障害

輸血を必要とする外傷患者の蘇生後のMOFの発生率を低下させることは，現在の出血性ショック治療の主な関心の一つである[48]．Injury Severity Score（ISS）15以上の513人の外傷患者でのコホート研究では，外傷後12時間以内の6単位以上の輸血はMOF発生の独立危険因子であった[49]．また，最初の外傷後

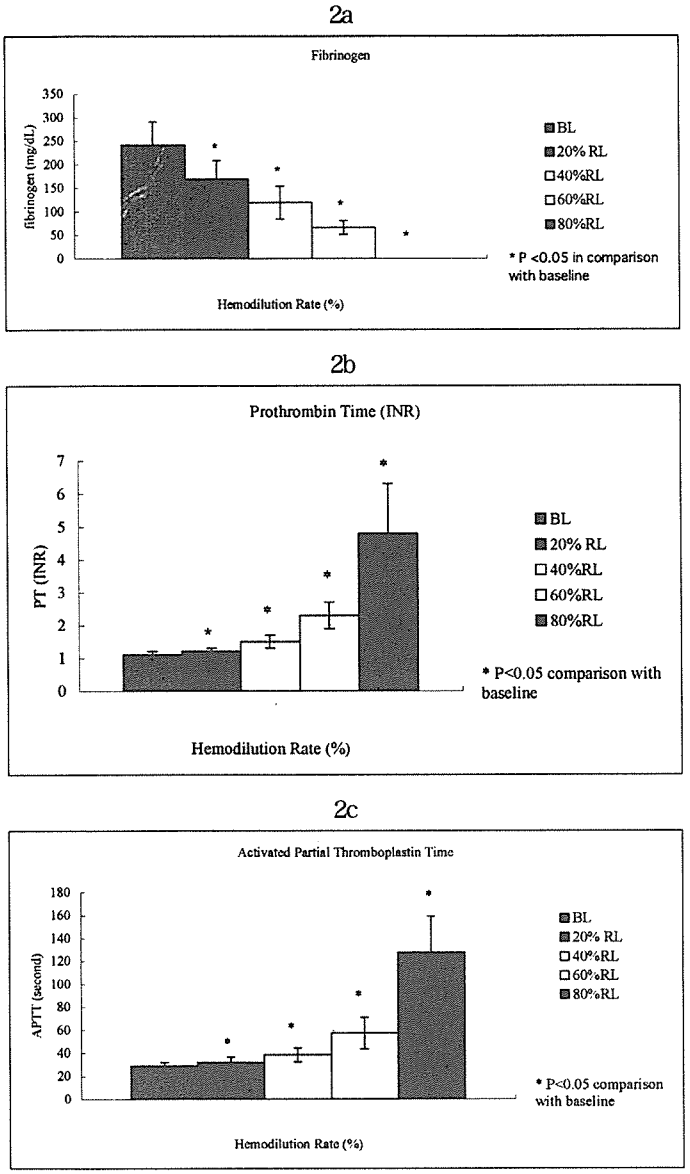

Fig. 2. Fibrinogen concentrations, prothrombin time and the activated partial thromboplastin time of diluted samples with isotonic Ringer's lactated solution (RL) or 10 g/dL Hemoglobin-Vesicles solution (HbV). Fibrinogen concentrations were decreased in inverse proportion to the dilution rate (2a). The prothrombin time and the activated partial thromboplastin time were in proportion to the square or cube of the dilution rate (2b, 2c).

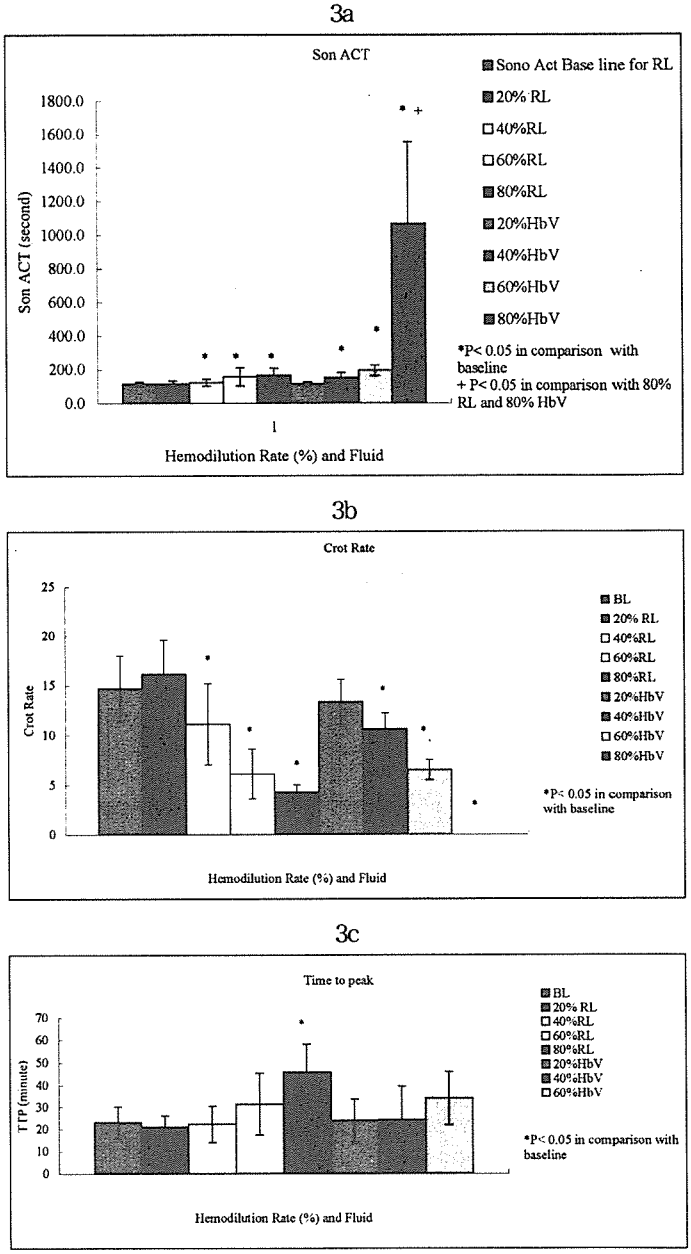

Fig. 3. SONOCLOT values of diluted samples with isotonic Ringer's lactated solution (RL) or 10 g/dL Hemoglobin-Vesicles solution (HbV). Activated coagulation time (ACT) and clot rate (CR) was prolonged in the 40% and 60% dilution with LR and HbV compared with baseline values, but there was no significance between the two groups (3a, 3b). In the 80% dilution by both solutions, ACT and time to peak was affected in comparison with baseline (3a, 3c).

6時間以内に投与された赤血球の保存期間の長さ（古さ）が外傷後MOFの発生に対する独立危険因子であった[50]．外傷後MOF発生のハイリスク患者では，循環中の好中球はプライミングされていてアポトーシスに抵抗性を示し[51]，血管内皮細胞のIntercellular adhesion molecule-1（ICAM-1）の発現が増加する．この原因の一つには，貯蔵赤血球バッグに混入する白血球があげられる[52]．そして，保存血の保存期間が長くなればなるほど，白血球や血小板由来の活性化物質は増加することが報告された[53]．さらに，貯蔵赤血球中の血漿と脂質は実験的に急性肺障害を生じることが示された[54]．

HbVの肺への影響

我々は，出血性ショックモデルに対するHbV投与後の肺への影響を調査し報告した[55]．週齢10-13週の雄Sprague-Dawleyラット72匹を対象とした．1）（単純投与）まず，36匹を自発呼

吸でセボフルレン麻酔下に，11.2 mL/kgの生理食塩水，または，生理食塩水に分散した10%HbV溶液を30分で経静脈投与した．投与後２時間，24時間，72時間に犠牲死させ，肺を摘出した．２）（出血性ショック後輸液蘇生）次に，36匹を自発呼吸でセボフルレン麻酔下に，28 mL/kgの血液を20分以上かけて脱血し，安定させた．その後，脱血量と等量の生理食塩水に分散した10%HbV溶液（HbV群），または，脱血量の３倍量の乳酸リンゲル液（RL群）で輸液蘇生を行った．ベースラインと脱血直後，蘇生後１時間，２時間後の血行動態とヘマトクリット値，BE，血中乳酸値を測定した．輸液蘇生後2時間，24時間，72時間に犠牲死させ，肺を摘出した．肺のhypoxic inducible factor 1 alpha subunit（HIF-1α），tumor necrosis factor alpha（TNF-α），interleukin-6（IL-6），heme oxygenase- 1（HO-1），inducible nitric oxide synthase（iNOS），intercellular adhesion molecule 1（ICAM-1）のmRNAの発現をRT-PCR法で測定した．脱血直後の平均動脈圧は，両群間で差はなかった（RL群33 ± 4 mmHg，HbV群30±3 mmHg）．蘇生後のヘマトクリット値は，1時間後，2時間後共に，HbV群の方が有意に高かった．蘇生後1時間後の血中乳酸値はRL群の方が有意に高かった．単純投与後は，いずれの測定時点でも，我々の測定したmRNAの発現には影響しなかった．HbV群は，RL群に比べ，投与２時間後のHIF-1αとIL-6が抑制されており，HO-1とTNF-αがより発現していた．また，72時間後のHO-1の発現が増加しており，IL-6の発現が抑制されていた．ICAM-1は単純投与でも出血性ショック後輸液蘇生でもHbV投与に影響を受けず，肺への血管内皮細胞への影響はないことが示唆された．また，Hb自体の影響として輸液蘇生直後のHIF-1αの発現抑制とHO-1発現増加が見られたと思われる．

海外でのHBOCsの研究報告と今後の展望

現在，南アフリカでは重合HbであるHemopure（Biopure Corp., Cambridge, MA）が急性出血に対して臨床利用されている．PolyHeme（Northfield Laboratory, Evanston, IL）は，大規模な第３相臨床研究が行われている[56]．基礎研究でも，外傷後MOFモデルとして２イベントモデルを作成し精力的な研究が行われている[57]．最終的な目的は，従来の赤血球輸血に比べてMOFやARDSの発生率を低下させ生存率を改善することだろう．しかし，臨床研究では対象患者の均一化や遺伝子多型性による反応の違い，フィジシャンの違い，目的の難しさなど様々な問題があり長い時間を費やす．HbVを含む人工酸素運搬体の臨床応用は，重大な副作用を来さず，緊急時の酸素運搬体としての簡便な適応に加えて，外傷直後の赤血球輸血を減らすことによるその後の急性肺障害や多臓器不全への発展を予防する可能性がある．血液成分を考えれば，酸素運搬体だけの投与は研究のプロトコルに制限があり，臨床適応するのが難しいと考えられる．このような困難にも関わらず，利用価値のある製品が十分な改良と適応を熟考された上で，臨床応用可能となることはきわめて合理的であると考えられる．我が国で開発されたHbVや海外で臨床治験中や臨床応用されているHBOCsの緊急時や手術中の患者に対する影響を理解することは，新たな種類の特徴ある人工酸素運搬体の有効な臨床応用を可能にするだろう．

謝辞

本研究は，厚労省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「救急・災害医療に利用可能な人工赤血球の開発に関する研究（研究課題番号：H17-医薬-072）の分担研究として実施した．研究を進めるにあたりHbVの供与ならびに研究の機会を与えて頂いた研究班主任研究者・慶應義塾大学医学部外科教授四津良平先生，ウサギ赤血球ならびにHbVの酸素解離曲線を提供頂きました早稲田大学理工学総合研究センター顧問研究員土田英俊先生ならびに助教授酒井宏水先生に深く感謝申し上げます．

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Hemoglobin Vesicles Containing Methemoglobin and L-Tyrosine to Suppress Methemoglobin Formation in Vitro and in Vivo

Tomoyasu Atoji,[†] Motonari Aihara,[†] Hiromi Sakai,[‡] Eishun Tsuchida,[‡] and Shinji Takeoka*[,†]

Department of Applied Chemistry, Graduate School of Science and Engineering, and Advanced Research Institute for Science and Engineering, Waseda University, Tokyo, 169-8555, Japan. Received December 8, 2005; Revised Manuscript Received July 20, 2006

Hemoglobin (Hb) vesicles have been developed as cellular-type Hb-based O_2 carriers in which a purified and concentrated Hb solution is encapsulated with a phospholipid bilayer membrane. Ferrous Hb molecules within an Hb vesicle were converted to ferric metHb by reacting with reactive oxygen species such as hydrogen peroxide (H_2O_2) generated in the living body or during the autoxidation of oxyHb in the Hb vesicle, and this leads to the loss of O_2 binding ability. The prevention of metHb formation by H_2O_2 in the Hb vesicle is required to prolong the in vivo O_2 carrying ability. We found that a mixed solution of metHb and L-tyrosine (L-Tyr) showed an effective H_2O_2 elimination ability by utilizing the reverse peroxidase activity of metHb with L-Tyr as an electron donor. The time taken for the conversion of half of oxyHb to metHb (T_{50}) was 420 min for the Hb vesicles containing 4 g/dL (620 μM) metHb and 8.5 mM L-Tyr ((metHb/L-Tyr) Hb vesicles), whereas the time of conversion for the conventional Hb vesicles was 25 min by stepwise injection of H_2O_2 (310 μM) in 10 min intervals. Furthermore, in the (metHb/L-Tyr) Hb vesicles, the metHb percentage did not reach 50% even after 48 h under a pO_2 of 40 Torr at 37 °C, whereas T_{50} of the conventional Hb vesicles was 13 h under the same conditions. Moreover, the T_{50} values of the conventional Hb vesicles and the (metHb/L-Tyr) Hb vesicles were 14 and 44 h, respectively, after injection into rats (20 mL/kg), confirming the remarkable inhibitory effect of metHb formation in vivo in the (metHb/L-Tyr) Hb vesicles.

INTRODUCTION

Hemoglobin (Hb)-based O_2 carriers (HBOCs) as red blood cell substitutes have been developed for clinical application in recent years (*1*–*3*). Their safety and usefulness have been demonstrated by many researchers in various fields such as physiology, toxicology, and biochemistry (*4*–*6*). The demand for HBOCs has been increasing year by year due to limitations of donated blood, such as infectious agents, shortage, and storage issues.

HBOCs are generally classified into two types: One is the acellular type, which comprises directly modified Hb molecules such as cross-linked Hb (*7*), polymerized Hb (*8*), and polymer-conjugated Hb (*9*). Some of the acellular-type HBOCs have advanced to phase III clinical trials (*10, 11*). Another kind is the cellular-type Hb systems such as Hb vesicles (*12*) or liposome-encapsulated Hb (*13*), in which Hb molecules are encapsulated with a phospholipid bilayer membrane. Although they are not yet in clinical trials, they have been shown to have excellent O_2 carrying ability and good safety profiles in vivo (*10, 14*–*18*).

Ferrous Hb (Hb(Fe^{2+})) molecules of HBOCs gradually lose their O_2 binding ability, because they react with reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) (*19, 20*) or nitrogen monoxide (NO*) (*21*) to become nonfunctional ferric Hb (Hb(Fe^{3+}), metHb), in addition to the autoxidation of oxyHb molecules themselves. In humans, the concentration of metHb molecules in the red blood cells is usually kept below 1% by reduction systems such as NADH-cytochrome b_5, NADPH-flavin, glutathione, and ascorbic acid. Furthermore, ROS are eliminated by several mechanisms such as superoxide dismutase (SOD), catalase, and peroxidase that help to prevent the metHb formation (*23, 24*). In contrast, metHb generated in the Hb vesicle are not reduced to the ferrous state due to the absence of the reduction systems, because constitutive enzymes and reductants necessary for metHb reduction are removed during the Hb purification process (*25*). The half-life of oxyHb of the Hb vesicles was 14 h in vivo, and more than 90% of oxyHb was converted to metHb within 48 h in the case of 20 vol % top loading to rats (*20*). In human plasma, the concentration of H_2O_2 is reported to be 4–5 μM (*26*) and to elevate to as much as 100–600 μM under inflammatory (*27*) or ischemia-reperfusion conditions (*28*). Therefore, Hb vesicles are required to have an H_2O_2 elimination system for effective prolongation of their O_2 carrying ability. We previously reported that H_2O_2 generated in the living body was the main reason for metHb formation of the Hb vesicles, and the O_2 carrying ability of the Hb vesicles in which catalase was coencapsulated was vastly prolonged in vivo by elimination of H_2O_2 (*20*). Regarding the catalase-coencapsulated vesicles, it is somewhat difficult to obtain a sufficient amount of human catalase from human donated blood. Furthermore, the catalase activity is gradually lost due to denaturation during storage at 25 °C.

Horseradish peroxidase (HRP), which also eliminates H_2O_2 in its enzymatic reaction, is converted to a ferrylHb radical Hb-(Fe^{4+}=O*) state after reaction with H_2O_2, and the radical state is returned to the ferric state by acceptance of two electrons from substrates such as *p*-hydroxyphenylacetic acid (HPA) (*29, 30*). This is a well-known reaction in an H_2O_2 assay method. L-Tyrosine (L-Tyr) dimer or polymer can also be synthesized by an HRP-catalyzed oxidation (*31, 32*). We considered from the above mechanism that if metHb, which has ferric heme like HRP does, could also function as an H_2O_2 elimination enzyme in the presence of L-Tyr as a substrate, then metHb can easily

* Corresponding author. E-mail: takeoka@waseda.jp. Tel: +813-5286-3217. Fax: +813-5286-3217.
[†] Graduate School of Science and Engineering.
[‡] Advanced Research Institute for Science and Engineering.

10.1021/bc050349h CCC: $33.50
Published on Web 09/02/2006

be prepared from purified Hb for the preparation of vesicles. Moreover, L-Tyr itself is an amino acid with high stability and good cost performance. In this study, we prepared Hb vesicles coencapsulating metHb and L-Tyr, thereby incorporating the metHb elimination system into the Hb vesicles themselves. We then evaluated the effect of this coencapsulation on the suppression of metHb formation. Furthermore, we evaluated the safety of the vesicles in vivo.

EXPERIMENTAL PROCEDURES

Purification of Hb from Red Blood Cells (*19, 33*). Hb was purified from outdated human red blood cells provided by the Japanese Red Cross. The red blood cells were washed three times with saline by centrifugation (2000*g*, 10 min) and concentrated by the removal of the supernatant. They were hemolyzed by the addition of an equal volume of water for injection, and then the stroma were removed by ultrafiltration (cutoff M_w 1000 kDa, Biomax-1000V, Millipore Co., Ltd., Bedford). The ligand exchange from O_2 to CO was carried out for the stroma-free Hb solution by CO gas flowing over the stirred solution. The proteins other than HbCO were denatured by heat treatment at 60 °C for 12 h and removed as precipitates. The HbCO solution was fractionated using ultrafiltration filters with a cutoff molecular weight between 1000 kDa and 8 kDa (Biomax-8V, Millipore), followed by concentration with an 8 kDa ultrafilter.

Reaction of Hb Samples with H_2O_2. HbCO was decarbonylated to HbO_2 by the irradiation of visible light to a liquid film of the Hb solution under O_2 atmosphere; potassium ferricyanide was added to the oxyHb solution to convert the oxyHb to metHb (*34*). The conversion of oxyHb to metHb was 99.9% as measured by the modified Evelyn−Malloy method (*35*). After the mixed solution was stirred at 25 °C for 2 h, the potassium ferricyanide was removed from the resulting metHb solution by gel filtration chromatography on Sephadex G-25 (Pharmacia AB, Uppsala). Deferoxamine mesylate (1.6 mM; DFO, Sigma, St. Louis) and L-Tyr (0 or 1 mM; free base, ICN Biomedicals, Inc., Aurora) were added to the oxyHb or metHb solutions, and these solutions were used as Hb samples. Hb samples ($|\text{heme}| = 20\ \mu M$) were reacted with 200 μM H_2O_2 ($|\text{heme}|/|H_2O_2| = 1/10$ molar ratio) in phosphate buffered saline (PBS, pH 7.4 at 37 °C), and the reaction was monitored by the repetitive scanning of a visible region from 300 to 700 nm at 2 min interval by using a UV−vis spectrometer (V-570, Jasco, Tokyo).

For the measurement of H_2O_2 concentrations, samples were reacted with H_2O_2 under the same conditions and were periodically sampled, and the concentration of H_2O_2 was determined spectrofluorometrically by measuring the amount of 6,6′-dihydroxy-[1,1′-biphenyl]-3,3′-diacetic acid (DBDA; Ex, 317 nm; Em, 405 nm), generated by the HRP-catalyzed reaction of HPA with H_2O_2. The final concentrations of HRP and HPA were 4 μM and 6 mM, respectively, and the DBDA concentration was calculated after separating the Hb samples by centrifugal filtration (cutoff 5 kDa, Ultrafree-MC, Millipore, Bedford) (*19, 29*).

Preparation of the Hb Vesicles Containing metHb and L-Tyr ((metHb/L-Tyr) Hb Vesicles) (*36*). MetHb was prepared by the reaction of HbCO (10 g/dL) with an excess amount of potassium ferricyanide. The unreacted potassium ferricyanide and ferrocyanide were removed by ultrafiltration (cutoff M_w 50 kDa, ADVANTEC, Tokyo) until the concentration of potassium ferricyanide was less than 1 μM by monitoring the absorbance with the UV−vis spectrometer. The metHb solution was concentrated to 40 g/dL using a 50 kDa cutoff filter as noted above. HbCO solutions containing 5 or 10 mol % metHb were prepared by mixing the concentrated metHb solution with a 40 g/dL HbCO solution. Pyridoxal 5′-phosphate (PLP, Sigma, St. Louis, MO) was added to the Hb solution as an allosteric effector at a 2.5 equimolar ratio of PLP to HbCO. The L-Tyr solution prepared previously was added to 0.2 N NaOH, and the resulting solution was added to the HbCO/metHb solution. The final concentrations of Hb and L-Tyr in the HbCO/metHb solution were adjusted to 40 g/dL and 1.0 and 8.5 mM, respectively. To prepare the Hb vesicles, powders of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylchorine (DPPC), cholesterol, 1,5-bis-*O*-hexadecyl-*N*-succinyl-L-glutaminate (DHSG) (Nippon Fine Chemical Co., Osaka), and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-PEG_{5000} (PEG-DSPE, NOF Co., Tokyo) were mixed at a molar ratio of 5:5:1:0.033 and added to the HbCO/metHb solution, and the mixture was stirred at 25 °C for 12 h. The resulting dispersion of the multilamellar vesicles was subsequently extruded through the nitrocellulose membrane filters with a pore size of 0.22 μm (Fuji Film Co., Tokyo) to prepare the Hb vesicles with an average diameter of 261 ± 30 nm. After the separation of unencapsulated Hb by ultracentrifugation (10000*g*, 60 min), the precipitate of the Hb vesicles was redispersed into saline in order to adjust the Hb concentration of the Hb vesicle dispersion to 10 g/dL. HbCO within the vesicles was decarbonized and oxygenated to HbO_2 by irradiation of visible light onto a liquid film under O_2 atmosphere.

Stepwise Injection of H_2O_2 to a Dispersion of (metHb/L-Tyr) Hb Vesicles. A dispersion of (metHb/L-Tyr) Hb vesicles in PBS (pH 7.4) coencapsulating metHb and L-Tyr (metHb/L-Tyr = 2 g/dL:1 mM, 4 g/dL:1 mM, or 4 g/dL:8.5 mM) was incubated with stirring, and H_2O_2 (310 μM, Ultra Pure Grade, Kanto Chemical Co., Tokyo) was injected in stepwise fashion into the solutions at 10 min intervals. Just before each injection, 20 μL of the dispersion of the (metHb/L-Tyr) Hb vesicles was sampled out, and 20 μL of a catalase solution (50 000 unit) was immediately added for the elimination of the remaining H_2O_2. The percentage of metHb in the (metHb/L-Tyr) Hb vesicles was periodically calculated by the ratio of absorbance at 405 nm (metHb) and 430 nm (deoxyHb) in the Soret band using a UV−vis spectrometer without destruction of the Hb vesicles.

Autoxidation of (metHb/L-Tyr) Hb Vesicles. A dispersion of (metHb/L-Tyr) Hb vesicles was incubated and shaken (120 times/min) under pO_2 of 40 Torr at 37 °C. The percentage of metHb in the vesicles was periodically measured with a UV−vis spectrometer.

Measurement of metHb Formation in the Hb Vesicles in Vivo. Wistar rats (body weight: 240−260 g) were anesthetized with diethyl ether, and a preparation of (metHb/L-Tyr) Hb vesicles containing 4 g/dL of metHb and 8.5 mM of L-Tyr was injected into the tail vein (20 mL/kg, $n = 6$). Blood was withdrawn from the tail vein and centrifuged (12000*g*, 5 min) to collect the vesicle fraction in the supernatant. The percentage of metHb within the vesicles was measured with a UV−vis spectrometer. The number of blood cells was measured with a blood cell counter (Sysmex, KX-21, Kobe).

RESULTS AND DISCUSSION

Reaction of the L-Tyr Containing metHb Solution with H_2O_2. We added H_2O_2 to the metHb solution (5 μM) with or without 1 mM L-Tyr. Figure 1 shows the changes of the H_2O_2 concentration in the metHb solutions with and without L-Tyr. The initial elimination rates of H_2O_2 in the metHb solutions were calculated on the basis of a pseudo-first-order rate law. The elimination rates with and without L-Tyr at 37 °C were 3.0×10^3 and $3.2 \times 10^4\ M^{-1}\ s^{-1}$, respectively. The apparent rate constant of the metHb solution containing L-Tyr was about 10 times larger than that with the metHb solution alone, and this

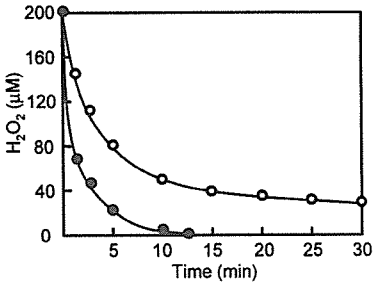

Figure 1. Time course of H_2O_2 elimination by (○) metHb (5 μM) (●) metHb (5 μM) + L-Tyr (1 mM) during the reaction with 200 μM of H_2O_2 at 37 °C.

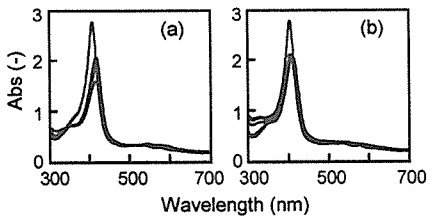

Figure 2. UV−vis spectral changes of (a) a metHb (5 μM) solution and (b) a metHb (5 μM) solution containing L-Tyr (1 mM) during the reaction with 200 μM H_2O_2 ([heme]:[H_2O_2] = = 1/10) at 37 °C. The scanning was performed at 4 min intervals, immediately after the addition of H_2O_2 solution to each Hb solution.

value was estimated to be approximately equal to the 150 units of catalase. Moreover, around 40 μM H_2O_2 remained after a 30 min reaction of 200 μM H_2O_2 in the metHb solution alone, whereas H_2O_2 was completely eliminated within 15 min in the metHb solution containing L-Tyr.

The UV−vis spectral changes during the reaction of H_2O_2 in the metHb solution (5 μM) and the metHb solution containing L-Tyr (1 mM) are shown in Figure 2a and b, respectively. In the case of metHb alone, there was a shift in the spectrum of metHb, namely, the Soret band of metHb (405 nm) was shifted to 417 nm, and the typical metHb peak at 630 nm was completely abolished, which indicated a change to a different form of metHb. There was a time-dependent decrease in peak intensities, indicating the degradation of this intermediate form of Hb during the reaction of H_2O_2. We speculated that this intermediate form of Hb was ferrylHb radical. It has been reported that the ferrylHb radical is formed by the reaction of metHb with H_2O_2 (*37*). Furthermore, the stoichiometry of metHb (5 μM) and the reacted H_2O_2 (200 μM) suggests that the metHb worked as an enzyme such as catalase, although the stability of the enzyme was low and it was degraded. Because the Fenton reaction can be prevented by the addition of DFO, which is a chelator of Fe^{3+}, this enzymatic H_2O_2 elimination was considered to be a pseudo-catalase-like reaction of metHb. Conversely, the metHb solution with L-Tyr showed a fast spectral change and then stayed constant during the reaction, as shown in Figure 2b. Also, the small spectral shift of the metHb solution with L-Tyr suggested that the metHb reacted with H_2O_2 was converted to the ferrylHb radical. The ratio of metHb to ferrylHb should be constant during reaction with H_2O_2. Comparing with the reaction mechanism of peroxidase, where the ferric state is converted to the ferrylHb radical state by the reaction of H_2O_2 to produce H_2O, and then the ferryl radical state is recovered to the ferric state by one-electron oxidation of two substrate molecules to generate another molecule of H_2O, we could compare, in this case, the peroxidase and the substrate to metHb and L-Tyr, respectively. More precisely, we could describe the enzymatic reaction as reverse peroxidation, and the substrate as H_2O_2 where L-Tyr works as an electron donor. Furthermore, we detected L-Tyr dimer (L-Tyr-L-Tyr (diTyr)), which was

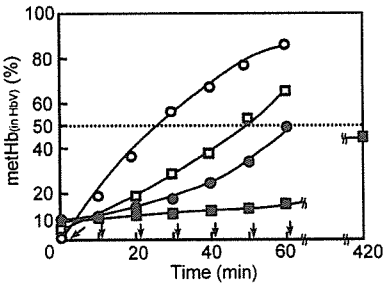

Figure 3. Time course of metHb formation in Hb vesicles at 37 °C during the stepwise addition of H_2O_2 (310 μM) to 5 g/dL Hb vesicle dispersions coencapsulating (●) 4 g/dL metHb and 1 mM L-Tyr, (□) 2 g/dL metHb and 1 mM L-Tyr, (■) 4 g/dL metHb and 8.5 mM L-Tyr, and (○) no metHb and L-Tyr.

produced from the coupling of the two L-Tyr radicals in their ortho position. Such a stable reaction of the unstable ferrylHb radical to metHb by L-Tyr would help the stable elimination reaction of H_2O_2. Therefore, we aimed to construct this system in the Hb vesicles.

H_2O_2 Stepwise Addition to the (metHb/L-Tyr) Hb Vesicles. Figure 3 shows the time course of metHb formation in the conventional Hb vesicles or the (metHb/L-Tyr) Hb vesicles by stepwise injection of H_2O_2 (310 μM) in 10 min intervals. H_2O_2 (310 M) injection to a 5 g/dL conventional Hb vesicle dispersion ([heme] = 3.1 mM) showed an elevation in the metHb percentage with increasing injections of H_2O_2, and the percentage of metHb reached 85% after 60 min (6 injections). However, the (metHb/L-Tyr) Hb vesicles (metHb/L-Tyr = 2 g/dL:1 mM, 4 g/dL:1 mM) showed a significant suppression of metHb formation in the vesicles. The metHb percentages of these Hb vesicles were 67% and 50% after 60 min (6 injections), respectively. Furthermore, by increasing the amount of L-Tyr to 8.5 mM (metHb: 4 g/dL), metHb formation was dramatically suppressed; the percentages were 17% and 45% after 60 and 420 min, respectively (6 and 42 injections, respectively).

From these results, it was confirmed that the formation of metHb from oxyHb by reaction with H_2O_2 was suppressed by the H_2O_2 elimination system of metHb and L-Tyr. Moreover, the persistence of the metHb suppression effect depends on the amount of metHb as an enzyme and L-Tyr as a substrate. The solubility of L-Tyr in pure water is 1.2 mM; however, we succeeded in preparation of an Hb solution containing 8.5 mM L-Tyr by using an alkali solution (NaOH) and the interaction of L-Tyr with highly concentrated Hb molecule (40 g/dL). We succeeded in the construction of Hb vesicles having an H_2O_2 elimination system using Hb only as protein or enzyme.

Autoxidation of the (metHb/L-Tyr) Hb Vesicles. Besides the formation of metHb by H_2O_2, oxyHb is automatically oxidized to metHb, initiated by one-electron reduction of a coordinated dioxygen molecule to generate superoxide anion radical (O_2^{-*}). MetHb formation in vesicles by such autoxidation was measured under the condition of the pO_2 of 40 Torr at 37 °C, because at this pO_2 value, the rate of metHb formation is maximal (*38*). In the conventional Hb vesicles, the percentage of metHb periodically increased by autoxidation of oxyHb in the vesicles. The T_{50} was 13 h, and the metHb percentage reached 90% after 24 h incubation (Figure 4). On the other hand, the metHb formation of the Hb vesicles containing 4 g/dL metHb and 1 mM L-Tyr was effectively suppressed; the T_{50} was 24 h. Furthermore, in the Hb vesicles containing 4 g/dL metHb and 8.5 mM L-Tyr, the percentage of metHb formed was 20% and 43% after 24 and 48 h, respectively, and did not reach 50% when the measurement ended (48 h).

Autoxidation of oxyHb is also an important factor of metHb formation. The autoxidation rate of oxyHb depends on many factors such as the O_2 affinity, P_{50}, the pO_2, temperature, and

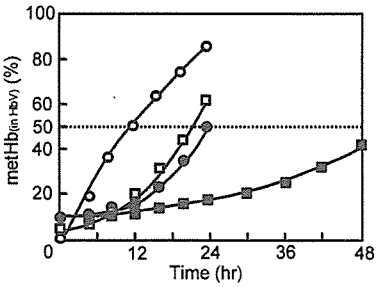

Figure 4. Time course of metHb formation in Hb vesicles under pO_2 of 40 Torr at 37 °C during autoxidation of 5 g/dL Hb vesicle dispersion coencapsulating (●) 4 g/dL metHb and 1 mM L-Tyr, (□) 2 g/dL metHb and 1 mM L-Tyr, (■) 4 g/dL metHb and 8.5 mM L-Tyr, and (○) no metHb and L-Tyr.

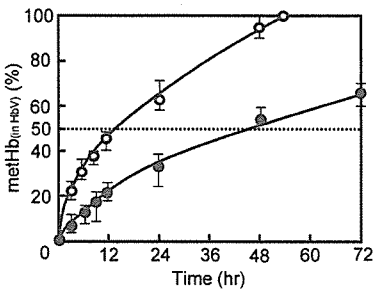

Figure 5. Time course of metHb formation in Hb vesicles injected into Wistar rats (20 mL/kg). Hb vesicles were encapsulating (●) 4 g/dL metHb and 8.5 mM L-Tyr, and (○) no metHb and L-Tyr.

pH. OxyHb molecules in the Hb vesicles are also influenced by these same factors, though the rates could be different from that of the oxyHb solution. The half-life of oxyHb to metHb (T_{50}) in the conventional Hb vesicles (P_{50} = 33 Torr) at 37 °C was about 12 h, shorter than that of the oxyHb solution (T_{50} = 24 h, PBS, pH 7.4). The autoxidation rate of the Hb vesicles containing metHb and L-Tyr was also effectively reduced in a similar way to that observed in the above experiment of H_2O_2 stepwise addition. The reason could be that the autoxidation of oxyHb is additionally influenced by H_2O_2. A superoxide anion radical (O_2^{-*}) generated by the oxyHb autoxidation is spontaneously converted to H_2O_2 in the presence of H^+. This H_2O_2 attacks the other oxyHb molecules. Therefore, the elimination of the H_2O_2 would result in the suppression of the autoxidation rate of oxyHb in the Hb vesicles.

Measurement of metHb Formation in (metHb/L-Tyr) Hb Vesicles in Vivo. Figure 5 shows the percentage of generated metHb after the intravenous injection (20 mL/kg) of the conventional Hb vesicles or the (metHb/L-Tyr) Hb vesicles into the tail vein of Wistar rats (n = 6). The metHb percentages of the conventional Hb vesicles and the (metHb/L-Tyr) Hb vesicles after 4 h were 22% and 18%, respectively, and the T_{50} values were 14 and 44 h, respectively. Furthermore, the Wistar rats were all alive for 72 h after the measurement, at which time they were sacrificed, indicating the safety of this system in vivo. These results confirmed the suppressive effect of metHb formation in vivo using the (metHb/L-Tyr) Hb vesicles.

In a previous study, we coencapsulated catalase to eliminate H_2O_2 generated in vivo, thus suppressing the formation of metHb within Hb vesicles, and we confirmed the suppression of metHb formation (*20*). The T_{50} of the catalase-coencapsulated Hb vesicles was 37 h with a catalase concentration of 4.2 × 10^4 unit/mL (0.5 g/dL catalase as a protein concentration). We estimated that the concentration of catalase was the amount at the saturation point of T_{50} prolongation, because the same T_{50} was obtained when 5.6 × 10^4 unit/mL catalase was used for coencapsulation. In comparison with these results, the (metHb/L-Tyr) system was more effective in the suppression of metHb formation by elimination of H_2O_2. It is thought that catalase activity is gradually lost at 37 °C in vivo (*20*), whereas the activity of the (metHb/L-Tyr) system should be very stable at 37 °C, and the amount of L-Tyr (8.5 mM within the Hb vesicle) was sufficient to eliminate H_2O_2 in the Hb vesicles. From these results, the increase of the metHb percentage of the (metHb/L-Tyr) Hb vesicles is likely due to the autoxidation of Hb, which is impossible to suppress in this system.

ACKNOWLEDGMENT

The authors thank Dr. K. Ohta at Oxygenix Co., Ltd., for technical advice about in vivo measurement of (metHb/L-Tyr) Hb vesicles. This work was supported in part by Health and Labor Science Research Grant, Ministry of Health, Labor and Welfare, Japan, Grant-in-Scientific Research (18500368), and 21COE "Practical Nano-Chemistry" and "Consolidated Research Institute for Advanced Science and Medical Care" from MEXT, Japan.

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BC050349H

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

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

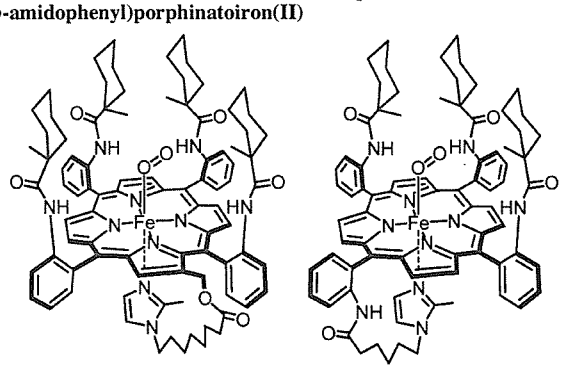
Advanced Research Institute for Science and Engineering, Waseda University, 3-4-1 Okubo, Shinjuku-ku, Tokyo 169-8555 Japan, and Key Laboratory of Polymer Materials of Gansu Province, Institute of Polymer, Northwest Normal University, Lanzhou 730070, China. Received November 3, 2005; Revised Manuscript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

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

INTRODUCTION

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

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



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

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

[†] Waseda University.

[‡] Northwest Normal University.

10.1021/bc050315+ CCC: $33.50
Published on Web 02/22/2006

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

EXPERIMENTAL PROCEDURES

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

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

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

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

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

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

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

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

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

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

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

RESULTS AND DISCUSSION

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

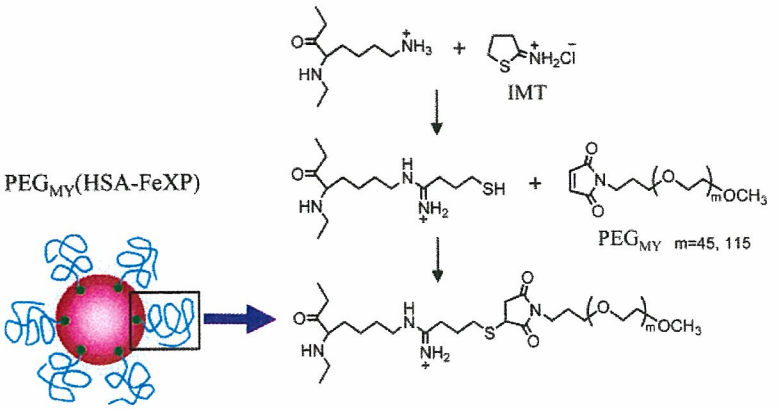


Figure 1. Two-step reaction schemes of IMT and maleimide-PEG (PEG$_{MY}$) with HSA−FeXP.

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

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

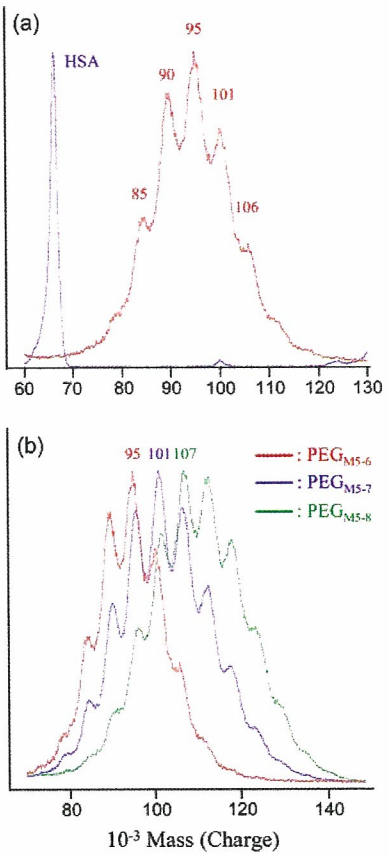


Figure 2. MALDI-TOF MS of (a) HSA and PEG$_{M5-6}$(HSA−Fe4P) and (b) PEG$_{M5}$(HSA−Fe4P) prepared in different [IMT]/[HSA−Fe4P] ratios of 15 (red), 20 (blue), and 30 (green) (mol/mol).

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

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

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

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

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

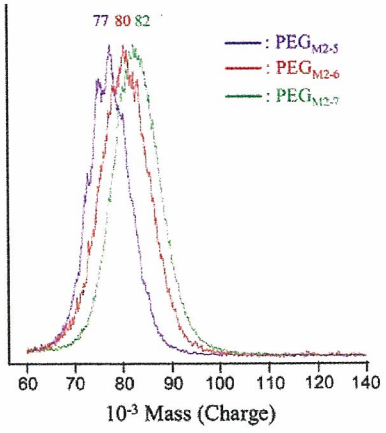


Figure 3. MALDI-TOF MS of PEG_{M2}(HSA−Fe4P) prepared in different [IMT]/[HSA−Fe4P] ratios of 10 (blue), 15 (red), and 20 (green) (mol/mol).

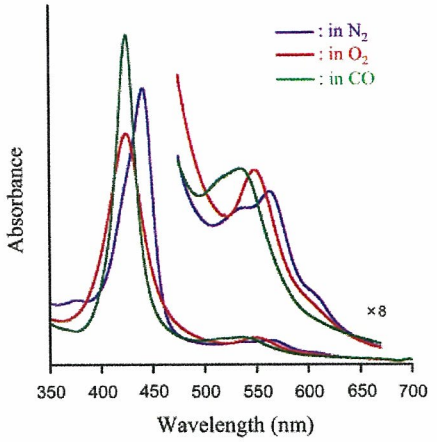


Figure 4. UV-vis absorption spectral change of PEG_{M2-6}(HSA−Fe4P) in PBS solution (pH 7.4).

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

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

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

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

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

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

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

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

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

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

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

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

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

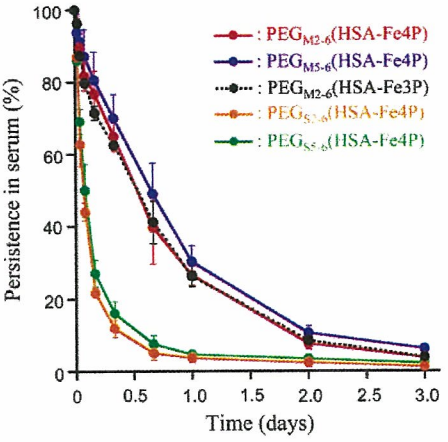


Figure 5. Persistence of FeXP in serum after administration of PEG-conjugated HSA−FeXP into Wistar rats. All values are mean ± SD (n = 4).

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

CONCLUSIONS

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

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

ACKNOWLEDGMENT

This work was partially supported by a Grant-in-Aid for Scientific Research (No. 16350093) from JSPS, a Grant-in-Aid for Exploratory Research (No. 16655049) from MEXT Japan, and Health Science Research Grants (Regulatory Science) from MHLW Japan. Prof. Koichi Kobayashi, Dr. Hirohisa Horinouchi (Keio University), and Mr. Hisashi Yamamoto (NIPRO Corp.) are greatly appreciated for their cooperation with the animal experiments.

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BC050315+