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特集／生体材料のバイオサイエンス

輸血の代替が可能な酸素輸液の実現と組織再生技術

酒井宏水*、土田英俊**

要　旨：血液型が無く、感染源を一切含まず、長期間備蓄して利用可能な酸素輸液は、輸血代替としての効能と安全性が明らかにされ実現に向け具体化してきている。この酸素輸液の利点を活用すれば、臓器移植や組織再生などの新技術にも利用でき、新しい臨床応用としても期待できると考えられる。酸素輸液の実現は、わが国の現行医療に目覚ましい変革をもたらすであろう。

1．酸素輸液とは？

わが国の献血・輸血システムは、世界的にみて最高水準にあり、安全な輸血用血液が医療機関に常備され、国民の健康福祉に大きく貢献していることは言うまでもない。肝炎やエイズなど輸血に伴う感染も嘗ては大きな社会問題となったが、献血の厳重な検査と管理が強化され、被害を最小限に留めることに成っている。特にNAT検査（核酸増幅法）採用は多大の貢献がある。しかし、検査項目から外れる病原体の存在、狂牛病や西ナイル熱、それに未知の感染源の存在の脅威に曝され、献血者の問診の徹底や献血の規制などゼロリスクを目指した新たな試みもなされている。その他の課題として、献血の赤血球保存期限は採血後僅かに3週間、それに過誤を含め血液型不適合の医療事故もある。輸血回避のため、術中輸血開始目安のHb濃度を6g/dlに低減、低温無輸血手術、自己血輸血の推奨と血液希釈、赤血球造血因子（EPO）投与による術前貯血も普及してきてはいるが、症例によっては適用できない。他方、献血1回当りの採血量は400mlになったが、人口の高齢化に伴い健康献血者の総数は低下しつづけている。わが国のように地震などの自然災害が危惧される場合は、緊急需要に対応した大量供給ができることは重要な国家的施策であり、感染の可能性を危惧することなく血液型に関係なく要請に即応して何時でも何処でも必要量を直ちに供与できる、赤血球の機能を代替する酸素輸液の出現が強く期待されているところである。血液成分のうち、血漿成分のほとんど

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は代替物が既に存在するが（表 1）、血球成分の役割の代替物の実現はまだ困難を伴っている。このうち赤血球の重要な役割である酸素運搬が可能な人工赤血球（酸素輸液）は、生体高分子を取扱う分子集合科学、バイオコンジュゲーション、錯体化学、ナノテクノロジーを基に、医工連携の投与試験を経て候補物質が特定された[1]。

赤血球にあるヘモグロビン（Hb）が酸素を可逆的に結合するので、これを利用した化学修飾 Hb（分子内・分子間架橋、高分子結合）の開発が欧米で先行した。しかし、

表 1　血液成分とその代替物

成 分	代 替 物
(血漿成分: 55 vol%)	
アルブミン	血漿増量剤 (多糖類、組換えアルブミン)
グロブリン	抗生物質、人工免疫グロブリン
フィブリノーゲン	フィブリン接着剤
各種血液凝固因子	遺伝子組換え凝固因子
電解質、アミノ酸	輸液として補充
糖類、ビタミン類	輸液として補充
(血球成分: 45 vol%)	
赤血球	人工赤血球
血小板	人工血小板
白血球	(抗生物質で対処)

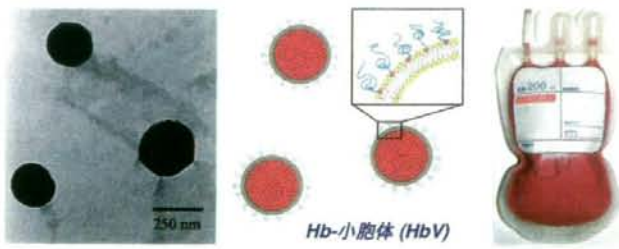


図 1　ヘモグロビン小胞体

期限切れ赤血球より精製単離した高純度 Hb をリン脂質二分子膜で被覆し、更に粒子表面にはポリエチレングリコール鎖で修飾されている。これにより分散安定度が向上する。透過型電子顕微鏡写真から、制御された粒子径（250nm）と、内水相に Hb が濃度高く内包されている様子が解る。微粒子の分散溶液であるため、外見は赤ワインと牛乳を混ぜたような色をしている。

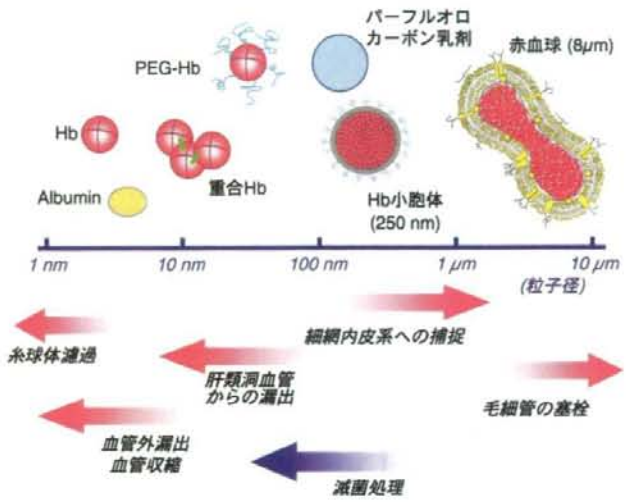
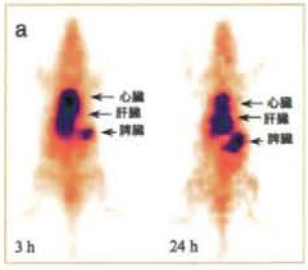
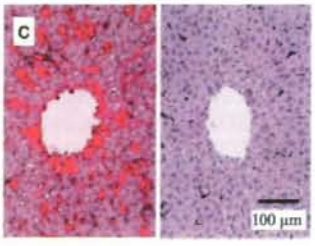


図 2　様々な人工酸素運搬体と、最適粒子径（150-300nm）の予測。

赤血球は変形能を有しているので、毛細管を変形して通過できるが、これを人工的に模倣することは困難を伴う。従って、毛細管の直径よりも小さい粒子となる。更にフィルタ滅菌が必要なので、フィルタを透過できることも要件となる。小さすぎると腎糸球体を透過してしまう。また、血管外漏出等が生起し、副作用を伴う。これらを総合すると Hb 小胞体の粒径が最適となるが、細網内皮系への捕捉の影響を良く調べる必要がある（図 3 に続く）。





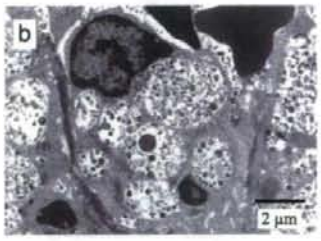


図 3　細網内皮系へのヘモグロビン小胞体の取り込みと代謝過程

(a) 放射化標識した ^{99m}Tc-Hb 小胞体投与後のガンマカメラ像。投与後は血液の容量の大きい心臓や肝臓に集中しているように見えるが、次第に肝臓や脾臓に捕捉される為、これらの臓器の強度が増大する（早大・宗慶太郎博士の実験）。(b) Hb 小胞体投与 1 日後のラット脾臓マクロファージの透過型電子顕微鏡写真。食胞中に Hb 小胞体の粒子が多数認められる。7 日後には消失する。(c) Hb 小胞体投与後のラット肝組織の顕微鏡写真。抗ヒト Hb 抗体免疫染色による赤染はヒト Hb の存在部位を示す。投与 1 日後で多量の Hb の存在を認めるが、7 日後には殆ど消失し、蓄積は全く認めない。

生体内で産生されるガス状の情報伝達分子（血管内皮由来弛緩因子：NO, CO）を捕捉して血管収縮を引起すなど、重篤な副作用が報告された。赤血球構造の生理的意義を考えれば当然の副作用であり、高純度・高濃度 Hb をリン脂質分子が形成する小胞体（リポソーム）に内包した人工赤血球（Hb 小胞体）が現在注目されている（図 1）。特徴は、①感染源なし・血液型なし、②粒子表面のポリエチレングリコール修飾と脱酸素化により 2 年間の保存ができ、蛋白質と脂質分子の集合体でも条件を選べば極めて安定な分散系になる、③赤血球と同様の細胞型構造と最適粒子径により NO, CO との反応を抑制する、④血管外への漏出が抑止される（図 2）、⑤小粒径のため、赤血球が通過できない狭窄血管を流動できる、また⑥動物試験では、優れた血液適合性、細網内皮系に捕捉された後、支障無く分解・排泄されること（図 3）、赤血球と同等の酸素運搬効果が確認された[2]。

我々はこれらの成果に自信を持ち、世界に先駆けて細胞型構造の人工赤血球を実現させるため、既に製造技術を確立して GMP 準拠の製造設備を設置し、輸血代替として Hb 小胞体の臨床試験に向けた準備を進めているところである。他方、遺伝子組換えアルブミンが間もなく国内で認可されるが、これに合成ヘムを複合させた、いわゆるアルブミン - ヘムも、Hb を用いない小粒径の人工酸素運搬体として開発が進められている[3]。

2．輸血代替としての臨床応用

Hb 小胞体が医療現場で使用できるようになれば、特に救急医療や外科的手術において、血液型不一致や感染の心配をせずに、何時でも要求に応じて投与し、医療施設までの搬送中の対処、同種血輸血の回避、または必要量の低減が可能と成る。既に Hb 小胞体の動物投与試験から、呼吸管理の主な項目：循環動態やガス交換、それに末梢組織の酸素化が、輸血をした場合と同等に正常値に保たれることが明らかになっている。

2-a）術中出血に対する投与、体外循環回路の補填液

Hb 小胞体の動物投与試験では、交換輸血法で循環血液量の実に 90% 超過の交換でも血圧が維持され、また血液ガス組成も腎皮質の酸素分圧も正常値を推移することを確認している（図 4）。臨床現場での出血に対する投与（血液交換率 40%）を想定した実験でも全例が生存し、赤血球数が 1 週間で完全に回復する。Hb 小胞体は最終的に細網内皮系に捕捉され、安全に分解、排泄される。従って、臨床では術前血液希釈、術中出血分の補給が可能となる。

更に胸部外科手術では、人工心肺（体外循環回路）の補充液としての利用が期待できる。体外循環回路の小型化も進み、成人患者の場合は補充液に晶質液を使用すれば十分である。しかし小児患者の場合は、全血液量に比較して体外循環回路の容量がまだ大きいため、無輸血充填（血漿増量剤の充填）とした場合に、術中のほんの数時間の血液希釈でも酸素欠乏により脳に障害を与え、術後の知能発達に影響を及ぼすことが報告された。そこで小林・四津ら（慶應義塾大学医学部外科）は、Hb 小胞体を充填液とすれば脳への酸素供給が維持できるのではと考え、動物実験で Hb 小胞体を用いた体外循環を実施したところ、脳の機能が保護されることを具体的に明らかにした。

2-b) 緊急時の利用：出血性ショック蘇生液

出血性ショック時の蘇生液としても検討され、赤血球と同等の酸素運搬機能を実証している。例えば、ラットの循環血液量の 50% 脱血によるショック状態にした後に Hb 小胞体を 5% アルブミンに分散させて投与したところ、循環動態も血液ガス組成も脱血液の投与と同等に推移し全例が生存する。更に、兎やビーグルを用いた出血ショック蘇生試験でも、概ね良好な成績が

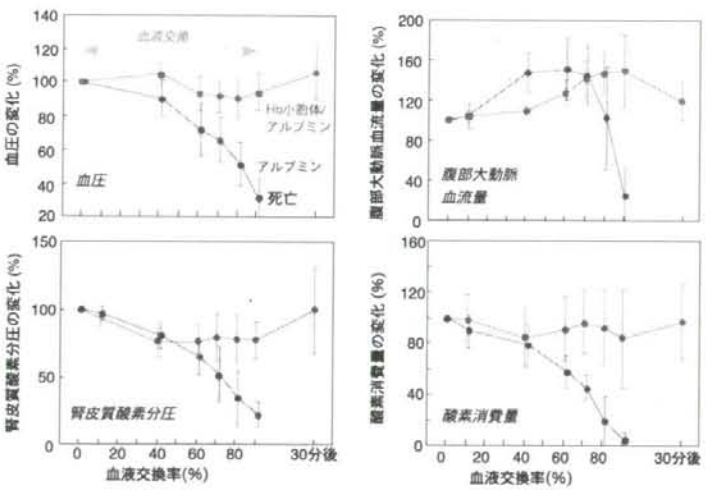


図 4　ヘモグロビン小胞体による交換輸血試験

アルブミン 5%溶液で全血液量の 90% を置換すると、血圧、腎皮質酸素分圧、心拍出量、酸素消費量も極度に低下し、30 分以内に全例が死亡した。これに対し Hb 小胞体をアルブミンに加えて 90% 置換した場合は、初期値をほぼ安定に維持し、全例が生存した。Hb 小胞体の酸素運搬機能を極めて明確に示す実験結果である。

得られている。これらの結果は、室温で備蓄可能な Hb 小胞体を、緊急時に投与して出血ショック状態の患者を蘇生させることができる可能性を意味している。各医療機関や、救急車などに常備させること、また大規模災害や有事に備える国家的な安全保障の施策として大量に備蓄すべきであることも提案をしたい[4]。

3. 臓器移植と酸素供給

2000 年の夏に Munich（ドイツ）の大学医学部附属病院を視察した時、集中治療室で臓器移植を受けたばかりの患者が何と 7 名も目の前のベッドに横たわっていた光景に驚かされた。夏はバイク事故が多く、若いドナーが多いとの裏話を聞いた。日本でも 1997 年に「臓器移植法」が制定され臓器移植が可能となったが、脳死判定に対する疑問や倫理面の不安もあるためか、症例数は海外に比較するとまだ少ないが、今後「臓器提供意思表示カード」の普及によって症例数は増加するのであろう。さて、摘出臓器移植後の生着率は、摘出してからの保存条件の向上と、移植までの経過時間との勝負で決まる。米国の臓器移植チームでご活躍された藤堂省教授（現北大医）の講演では、臓器ドナーが居る病院からレシピエントの病院まで、専用ジェット機で大陸横断を多数経験したとのことである。臓器保存液としては、UW(University of Wisconsin）液など、電解質、糖質、代用血漿剤の混合液が経験的に開発され、実際に使用されている。松本ら（京大）は、酸素溶解度の高いパーフルオロカーボン液と上述の臓器保存液の二層の間に臓器（膵臓や腸など比較的平たい臓器）を浸漬し、酸素ガスの組織内拡散によって臓器の深部組織にまで酸素を供給する Two-layer method を確立し、良好な成績を得ている。もし摘出臓器に、血液と同様の液体が血管を通して灌流されれば、酸素は組織の隅々にまで確実に運搬され、電解質や栄養分の補給、代謝産物や老廃物の除去も可能であり、臓器保存時間を飛躍的に延長することが期待できる。我々はこれまでに Hb 小胞体に代用血漿剤を混合した溶液で、動物から摘出した心臓・肝臓・小腸などの灌流試験を行い、各臓器の諸機能が数時間は保持されることを確認している。興味深いことに、採血液による灌流では、凝固系や免疫系が僅かでも活性化していると毛細管内で塞栓が形成され、均一な灌流が難しい場合があるが、Hb 小胞体では塞栓形成は無く均一な灌流が可能となっている。今後は臓器移植を想定した動物試験を行い、数日あるいは数週間の保存が可能か試す予定であるが、もし、将来的に臓器保存の時間が飛躍的に延長されれば、遠隔地への輸送も容

易になるし、また臓器移植コーディネータが余裕をもって最適のレシピエントの選定を行い、臓器移植の成功率が向上するものと期待をしているところである。

4．組織再生と酸素供給

再生医療に関する基本的概念は、ハーバード大学の Vacanti ら（2000）が発表した、生分解性高分子からなる足場材料（scaffold）に肝細胞と類洞内皮細胞を植えて培養したことであるが、組織形成と呼ぶにはほど遠い内容であった。その後、足場材料の研究が進み、細胞を用いた組織再生の概念が定着し、耳の形をした軟骨の再生に成功し、話題を呼んだ。現在、再生を目指して研究が精力的に推進されている体内組織を表 2 に記した[5]（筏義人先生の御著書より引用）。この中で、既に臨床応用レベルにまで到達し、患者に使用されているものも現れている。実用的な目覚ましい展開の一つに、岡野ら（東京女子医大）が開発した温度感応性ポリマーのポリイソプロピルアクリルアミドによる細胞培養用シートの完成がある。この培養皿は 37℃では疎水性を帯びており、患者の心筋細胞を二次元方向に培養させた後、32℃に低下させると培養皿が親水性を帯びて細胞がシート状になって回収できる。得られる筋芽細胞シート（細胞単層の厚さ）を何枚か重ねて心筋外壁から移植する治療法が試され、血管新生や壁厚の増大が確認されている。また、この培養皿を用いて患者の口腔粘膜に含まれる幹細胞をもとに培養シートを作製し、これを角膜上皮細胞疲弊症の患者に移植する臨床研究が進められている。また、森・吉岡ら（メビオール社・早大）も、

表 2　再生医工学研究の現状。

再生医工学の研究対象となっている組織と臓器
頭蓋骨、軟骨(耳、鼻)、顎骨、硬膜、歯周組織、脳細胞、角膜、網膜、毛髪、気道、食道、動脈、静脈、血管、心臓弁、心臓、膵臓、肝臓、小腸、膀胱、尿管、乳房、皮膚、指、血液細胞、腱、靭帯、半月板、関節軟骨、骨、末梢神経、中枢神経、椎間板、など
既に臨床応用の始まっている再生組織
皮膚の表皮・真皮、歯周組織、顎骨、顎堤(歯槽堤)、軟骨(肋骨・耳介・関節)、硬膜、角膜上皮、肺動脈、微小血管、心筋、四肢骨、白血球

特殊ゲル（昇温時ゲル化型熱可逆ハイドロゲル）を用い、膵ランゲルハンス島をカプセル化して培養する方法、ゲルシートを用いる創傷被覆剤、幹細胞から骨芽細胞その他への分化誘導などを明らかにしている。

言うまでもなく、細胞増殖には栄養分と酸素の供給、および老廃物など代謝産物の除去が必要であるが、培養皿での二次元培養や、微小なカプセル内の培養であれば、培養液を定期的に交換する程度で十分に必要量を賄うことができる。しかし、体外で三次元的に、しかもより大きな組織形成を目指す場合には、血管構築（血管新生）と同時に、血液に代わる成分の灌流が必要となろう。血管新生には血管内皮細胞と、血管成長因子の組み合わせが検討され、また、材料の立場から毛細血管をデザインした通りに作成し、生体に用いることも考えられている。いずれにせよ、細胞増殖を組織形成にまで繋げるには、血液に代わる代用物が必要になるのではと考え、我々の酸素輸液の出番ではないかと期待しているところである。血管生理では、血液の流れによって血管壁に生じる剪断応力の存在が極めて重要とされている。血液のヘモレオロジーに対比させた Hb 小胞体の濃厚分散液のレオロジー・デザインが求められており、既に Hb 小胞体と各種代用血漿剤の組み合わせで、溶液粘弾性の調節が可能となっている。更に、細胞増殖に必要な栄養源、電解質、成長因子など、溶液全体としてのデザインが今後必要になろう。

5. おわりに

現在開発が進められている酸素輸液（人工赤血球）は、体内投与した場合は、本物の赤血球の体内寿命（120 日）に比較して 3 日程度と短いため、短時間の使用に限られるが、血液型や感染源が無く、長期間保存できる点は赤血球の性能を凌いでいると言える。輸血代替としての利用は勿論、体外における移植臓器灌流液、組織再生の際の細胞への酸素供給液としての使用、更に赤血球よりも小粒径である利点を生かした新しい適応（虚血性領域の酸素化、抗腫瘍効果増強など）も提案され、次世代医療に著しい変革をもたらすことは間違いない[4]。

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附記

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輸液の開発の成功を具体的技術として確立するには、最近議論が高まっている組織再生領域での技術展開（人工臓器や臓器組織の再生の領域）が先ず必要と考えられる。これらの対象はわが国でも既に 30 年前から議論が開始されている。我国では臓器移植は他国よりかなり遅れている。その先を考えると、組織を人の手で作ることに移行せざるを得ない。再生医工学の現行技術に加え、遺伝子を操る技術、それに酸素輸液を駆使すれば、具体的な展開が可能になるのではないか。わが国の研究開発の過程では、技術を完成域に仕上げる時に躊躇しがちであるのは極めて残念である。我々のような研究者は技術の具体化も任務の一つである。いま機が熟している状況にあるので、是非とも結集したい。読者各位の御発意を御聞きしたい。

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Random Scope

肥満はウイルス感染による？

ルイジアナ州立大学の Baton Rouge らはアデノウイルス 36 または SMAM-1 という同様のウイルスを感染させると動物が肥満になることを示しました。人でも肥満の人の 30%がこのウイルスに対する抗体を持っているのに、肥満でない人では 10%でした。また人の脂肪組織から幹細胞を取り出し、このウイルスを感染させると、増殖し脂肪を蓄積しました。他のウイルスではそのようなことは見られませんでした。もしこれが本当に肥満の原因ならば、ワクチンを作るとか、抗ウイルス剤を投与することが肥満の予防になるはずです。でも今のところ肥満のウイルス説はまだ証拠不十分で、専門家の意見はやはり過食を防ぎ運動を十分することが肥満の予防の中心だというところにあるようです。　（Tom）

Andy Coghlan: Obesity may be catching after all. New Science 25 August 2007, p.14

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Hemoglobin-vesicles for a Transfusion Alternative and Targeted Oxygen Delivery

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Hb-vesicles (HbV) are artificial oxygen carriers that encapsulate purified Hb solution (35 g/dl) in unilamellar phospholipid vesicles (liposomes). The dispersion stability of HbV is attained using surface-modification with polyethylene glycol (PEG), so that the deoxygenated HbV can be stored at room temperature for years. Moreover, the intravenously injected HbV does not induce aggregation when contacted with blood components. Animal experiments have verified the safety and efficacy of HbV as a transfusion alternative. One advantage of HbV is that the O_2 affinity (P_{50}) of HbV can be regulated easily to that of RBC (28 torr) and to other values by manipulating the amount of the allosteric effectors, such as pyridoxal 5'-phosphate, coencapsulated in HbV. It is possible that HbV with a lower P_{50} (higher O_2 affinity) would retain O_2 in the normal tissue while unloading O_2 to a targeted hypoxic tissue. Small HbV (250–280 nm diameter) is distributed homogeneously in the plasma phase, and HbV would transport oxygen through collateral arteries in the ischemic tissues. Results of in vitro and in vivo experiments of the domestic and international collaborations have confirmed the possibility of targeted O_2 delivery by HbV.

Keywords liposome, hemoglobin, PEG, blood substitutes, ischemia

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Development of Hb-vesicles as a Transfusion Alternative

The main reasons for Hb encapsulation in red blood cells (RBCs) are prevention of Hb removal from blood circulation and preservation of the cells' chemical environment, such as the concentration of phosphates (2,3-DPG, ATP, etc.) and other electrolytes. Moreover, during the long history of the development of Hb-based O_2 carriers (HBOCs) to substitute the function of RBCs, many side effects of molecular Hb have become apparent, such as the dissociation of tetrameric Hb subunits into two dimers ($\alpha_2\beta_2 \rightarrow 2\alpha\beta$), which might induce renal toxicity, and entrapment of gaseous messenger molecules (NO and CO) inducing vasoconstriction, hypertension, reduced blood flow, and impaired tissue oxygenation at microcirculatory levels (Goda et al., 1998; Nakai et al., 1998; Sakai et al., 2000), neurological disturbances, and the malfunctioning of esophageal motor function (Murray et al., 1995). These side effects of molecular Hb also imply the importance of the cellular structure of RBC to encapsulate Hb. Unique rheological properties of blood as a non-Newtonian viscous fluid are attributed to the nature of suspension of RBCs, which constitute nearly 50% of blood volume, which is important for the homeostasis of blood circulation.

From the viewpoint of constructing 'Artificial Cells', a pioneering work of Hb encapsulation to mimic the cellular structure of RBCs was performed by Chang in 1957 (Chang, 1984), who prepared microcapsules (5 μm) made of nylon, collodion, etc. Toyoda (1965) and the Kambara-Kimoto group (Kimoto et al., 1968) also covered Hb solutions with gelatin, gum Arabic, silicone, etc. Nevertheless, it is extremely difficult to regulate the particle size, which is necessary for appropriate blood flow in the capillaries, and to obtain sufficient biocompatibility. After Bangham and Horne (1964) reported that phospholipids assemble to form vesicles in aqueous media, and that they encapsulate water-soluble materials in their inner aqueous interior, it seemed reasonable to use such vesicles for Hb encapsulation. Djordjevich and Miller (1977) prepared liposome-encapsulated Hb (LEH) composed of phospholipids, cholesterol, fatty acids, etc. Many groups have attempted LEH since then (Beissinger et al., 1986; Brandl and Gregoriadis, 1994; Hayward et al., 1985; Hunt and Burnette, 1983; Kato et al., 1985; Li et al., 2005; Liu and Yonetani, 1994; Usuba et al., 1991). In the US, Naval Research Laboratories have reported remarkable progress using LEH (Rudolph et al., 1991).

However, some intrinsic issues of encapsulated Hbs remain, mainly related to molecular assembly and particle dispersion. What we call Hb-vesicles (HbV) with high-efficiency production processes and their improved properties, were established by Tsuchida's group (Sakai et al., 1996; Takeoka et al., 1996; Sou et al., 2003) based on technologies of molecular assembly and precise analyses of pharmacological and physiological aspects (Fig. 1). The salient characteristics of HbV are the following:

i. Human Hb is purified completely via pasteurization at 60°C and ultrafiltration; no viruses are included in it (Sakai et al., 1993; Abe et al., 2001; Naito et al., 2002).
ii. A concentrated Hb solution, nearly 35 g/dl, is encapsulated within a thin bilayer membrane (Sakai et al., 1996; Sou et al., 2003; Takeoka et al., 1996).
iii. A new synthetic lipid is used to prevent platelet activation (Abe et al., 2006; Wakamoto et al., 2005).
iv. PEG-modification guarantees long-term storage of over two years at room temperature, blood compatibility, and extended circulation half-life (Phillips et al., 1999; Sakai et al., 1997, 1998, 2000; Sou et al., 2000, 2005; Wakamoto et al., 2001; Yoshioka et al., 1991).

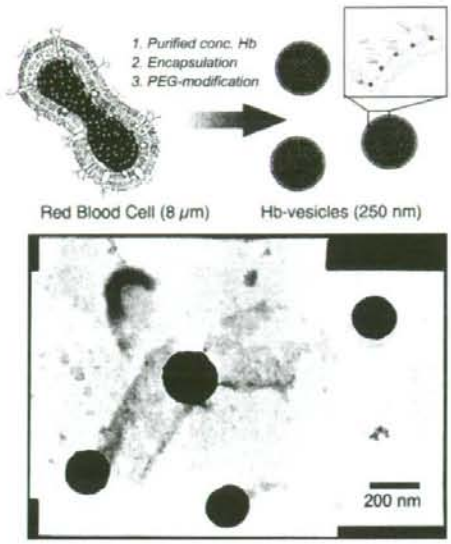

Figure 1. Schematic representation and transmittance electron micrograph of Hb-vesicles as artificial oxygen carriers. The purified and concentrated Hb solution (35 g/dl) is encapsulated in phospholipid vesicles and the surface is modified using PEG chains. The particle size is well regulated at 250 nm.

v. The cellular structure, which resembles that of red blood cells, shields all side effects of molecular Hb, such as scavenging NO and CO (Goda et al., 1998; Nakai et al., 1998; Sakai et al., 2000).
vi. The particle size (250 nm) is appropriate for sterilization, circulation persistence, and biodistribution (Sou et al., 2003; 2005).
vii. Hb-vesicles do not show colloid osmotic pressure. Addition of a plasma substitute solution such as recombinant albumin is effective to regulate colloid osmotic pressure (Izumi et al., 1997; Sakai et al., 2004; 2006; 2007). We confirmed sufficient O_2-transport comparable with RBC in the animal experiments of extreme hemodilution up to 90% blood exchange and resuscitation from hemorrhagic shock, and priming solution for cardiopulmonary bypass (ECMO) (Yamazaki et al., 2006).

Regulation of Oxygen Affinity

The O_2 affinity of purified Hb (expressed as P_{50}, O_2 tension at which Hb is half-saturated with O_2) in phosphate-buffered saline solution is about 14 torr; Hb strongly binds O_2 and does not release O_2 at 40 torr, as a partial pressure of mixed venous blood. Historically, it has been widely believed that the O_2 affinity should be regulated similarly to RBC, at about 25–30 torr, using an allosteric effector or by a direct chemical modification of the Hb molecules. Theoretically, this allows sufficient O_2 unloading during blood microcirculation, as can be inferred according to the arterio-venous difference in the levels of O_2 saturation in accordance to an O_2 equilibrium curve (Fig. 2). It has been expected that decreasing the O_2 affinity (increasing P_{50}) increases O_2 unloading, which is supported by

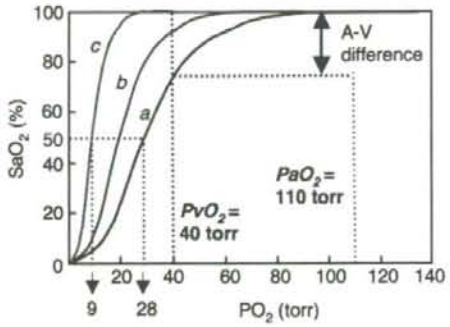

Figure 2. Oxygen dissociation curves of RBC or HBOCs such as Hb-vesicles. (a) P_{50} = 28 torr, (b), 15 torr, (c) 9 torr. (a) Oxygen dissociation curve of human RBC (P_{50} = 28 torr). In the normal condition, the amount of O_2 released during one circulation is calculated as the arterio-venous (A-V) difference of about 25%. In a hypoxic tissue, such as 5 torr, oxygen saturation (SaO_2) of RBC is only 2%, and most of the O_2 is already released before the blood enters the hypoxic tissue. (c) For a high O_2-affinity HBOC, it does not release at a normal tissue because SaO_2 at 40 torr is nearly 100%. On the other hand, SaO_2 at 5 torr is estimated as about 26%, which is higher than that for RBCs; consequently, HBOC remains a major source of O_2.

the result that the RBC with a high P_{50} shows an enhanced O_2 release for improved exercise capacity in a mice model (Shirasawa et al., 2003).

If this theory is correct, the P_{50} of Hb in HbV should be equivalent to that of human RBCs, i.e., 28 torr, or higher. Pyridoxal 5′-phosphate (PLP) is coencapsulated in HbV as an allosteric effector to regulate P_{50} (Sakai et al., 2000; Wang et al., 1992). The main binding site of PLP is the N-terminal of the α-chains and β-chains and β-82 lysine within the β-cleft, which is part of the binding site of natural allosteric effector, 2,3-diphosphoglyceric acid (2,3-DPG) (Benesch et al., 1973). The bound PLP retards the dissociation of the ionic linkage between the β-chains of Hb during conversion of deoxy to oxyHb in the same manner as does 2,3-DPG. Consequently, the O_2 affinity of Hb decreases in the presence of PLP. The P_{50} of HbV can be regulated to 5–150 torr by coencapsulating the appropriate amount of PLP or inositol hexaphosphate (IHP) as an allosteric effector and other electrolytes (Wang et al., 1992). Equimolar PLP to Hb (PLP/Hb = 1/1 by mol) was coencapsulated, and P_{50} was regulated to 18 torr. When the molar ratio PLP/Hb was 3/1, P_{50} was regulated to 32 torr. The present HbV contains PLP at PLP/Hb = 2.5 by mol; the resulting P_{50} is about 25–28 torr, which shows sufficient O_2 transporting capacity as a transfusion alternative for extreme hemodilution, resuscitative fluid for hemorrhagic shock, and prime solution for extracorporeal circulation. Much higher P_{50}, such as 50 torr, can be attained using the combination of a stronger allosteric effector, IHP. The resulting HBOC shows incomplete oxygenation at FiO_2 = 20%, and a larger arterio-venous difference requires respiration at a higher FiO_2, which might engender hyperoxia-induced lung injury. Whatever the case might be, it should be emphasized that the O_2 affinities of HbV can be regulated quite easily without changing the other physical parameters. However, in the case of the other modified Hb solutions, their chemical structures determine their O_2 affinities. For that reason, regulation is difficult. The appropriate O_2 affinities for O_2 carriers have not been completely determined. However, the simple regulation of the O_2 affinity might

be useful to meet the requirements of clinical indications such as oxygenation of ischemic tissues.

Oxygenation of Ischemic Tissues Using Hb-vesicles

In an ischemic tissue, the blood flow is extremely reduced. As a result, O_2 tension is very low: such as 5 torr. Normal RBCs should have already released O_2 before they reach the ischemic tissue. The left-shifted curve (lower P_{50}) indicates that Hb does not release O_2, even in the venous side in a normal condition. However, RBC or an HBOC with a lower P_{50} than usual can carry O_2 to an ischemic tissue (Linberg et al., 1998; Stein and Ellsoworth, 1993). Dr. Erni and colleagues at Inselspital Hospital of University of Berne developed a hamster skin flap model in which the blood flow of one branch is blocked completely and the tissue becomes completely ischemic (Erni et al., 1999). Exchange transfusion was performed using low and high P_{50} Hb-vesicles, which revealed the improved oxygenation of the ischemic part, especially with the low P_{50}-Hb-vesicles (Contaldo et al., 2003, 2005; Erni et al., 2003; Plock et al., 2005, 2007). Collateral blood flow should occur even to the ischemic part, and the Hb-vesicles retain O_2 to the ischemic part via the collateral arteries. This is the first example of the effectiveness of HbV for an ischemic tissue; it implies its applicability for other ischemic diseases. We observed O_2-releasing behavior in both in-vitro and in-vivo conditions for detailed clarification of this mechanism.

The O_2 release from flowing HbVs was examined using an O_2-permeable, fluorinated ethylenepropylene copolymer tube (28 μm inner diameter) exposed to a deoxygenated environment (Sakai et al., 2003). Measurement of O_2 release was performed using an apparatus that consisted of an inverted microscope and a scanning-grating spectrophotometer with a photon-count detector; the O_2 release rate was determined based on the visible absorption spectrum in the *Q* band of Hb. The HbVs and fresh human RBCs were mixed in various volume ratios at the Hb concentration of 10 g/dl in isotonic saline, which contained 5 g/dl albumin. The suspension was perfused at the centerline flow velocity of 1 mm/s through the narrow tube. The mixtures of acellular Hb solution and RBCs were also tested. The values of P_{50} of HbV, Hb, and RBC were fixed as almost identical (28 torr). Irrespective of the mixing ratio, the rate of O_2 release from the HbV/RBC mixtures was similar to that of RBCs alone. On the other hand, the addition of 50 vol% of acellular Hb solution to RBCs markedly enhanced the rate of deoxygenation. This outstanding difference in the rate of O_2 release between the HbV suspension and the acellular Hb solution is probably attributable mainly to the difference in the particle size (250 vs. 7 nm), which affects their diffusion for the facilitated O_2 transport. It is reported that faster O_2 release of acellular Hb would induce autoregulatory vasoconstriction. Consequently, it is advantageous that the O_2-release behavior of HbV is similar with that of RBC at the same P_{50}, and would retain O_2 in blood circulation to the peripheral tissues.

We prepared two HbVs with different P_{50} values (8 and 29 torr, respectively designated as HbV_8 and HbV_{29}) and observed their O_2-releasing behavior from an occluded arteriole in a hamster skinfold window model in collaboration with Prof. Intaglietta of UCSD (Sakai et al., 2005). Conscious hamsters received HbV_8 or HbV_{29} at a dose rate of 7 ml/kg. In the microscopic view, an arteriole (diameter: 53.0 ± 6.6 μm) was occluded transcutaneously by a glass pipette on a manipulator; the reduction of the intra-arteriolar PO_2 100 μm down from the occlusion was measured using the phosphorescence quenching of preinfused Pd-porphyrin. The baseline arteriolar PO_2 (50–52 torr) decreased to about 5 torr for all groups. Occlusion after HbV_8 infusion showed a slightly slower rate of

PO_2 reduction than that after HbV_{29} infusion. The arteriolar O_2 content was calculated at each reducing PO_2 in combination with the O_2 equilibrium curves of HbVs; those calculations clarified that HbV_8 showed a significantly slower rate of O_2 release compared to that of HbV_{29} and was a primary source of O_2 (maximum fraction, 0.55) overwhelming RBCs when the PO_2 was reduced (e.g., <10 torr) despite a small dosage of HbV. This result supports the possible utilization of Hb-based O_2 carriers with lower P_{50} for oxygenation of ischemic tissues.

The O_2 transport capacity of HbV_8 and HbV_{29} was investigated in the hamster chamber window model. Extreme hemodilution was performed using 5% recombinant albumin (rHSA) until Hct became 35% of the baseline value (Cabrales et al., 2005). At this condition, all tissues are totally hypoxic. Isovolemic exchange was continued using HbV suspended in rHSA solution to a total [Hb] of 5.7 g/dl in blood. Final Hct was 11% for the HbV groups, with a plasma [Hb] of 2.1 ± 0.1 g/dl after exchange with HbV_8 or HbV_{29}. A reference group was hemodiluted to Hct 11% using only rHSA. All groups showed stable blood pressure and heart rate. Arterial oxygen tensions were significantly higher than baseline for the HbV groups and the rHSA group and significantly lower for the HbV groups compared to those of the rHSA group. Blood pressure was significantly higher for the HbV_8 group compared to those of the HbV_{29} group. Arteriolar and venular blood flows were significantly higher than baseline for the HbV groups. Microvascular O_2 delivery and extraction were similar for the HbV groups, but lower for the rHSA group. Venular and tissue O_2 tensions were significantly higher for the HbV_8 versus the HbV_{29} and rHSA groups. In this systemically ischemic model after extreme hemodilution, an improved tissue O_2 tension is obtained when a high, rather than low, affinity HbV was injected. Results show that the high-affinity HbV can deliver O_2 more efficiently and more homogeneously to the peripheral tissues.

Recent studies have shown the effectiveness of HBOCs with a lower P_{50} (higher O_2 affinity) as a means of implementing O_2 delivery targeted to ischemic tissues (Banes et al., 1998, 2003; Tsai et al., 2003; Winslow et al., 2005; Nemoto et al., 2006). Our experimental data support those previous observations and ensure the possible utilization of HBOCs for remedying ischemic conditions in addition to its utilization as a transfusion alternative. The salient difference between HbV and other HBOCs such as chemically-modified Hb solutions is that the physicochemical properties of HbV are adjustable, not only P_{50}, but also the particle size, surface properties, Hb concentration inside the vesicles, and rheological properties. Accordingly, the use of HbV provides unique opportunities for versatile therapeutic approaches.

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Solution to the problems of acellular hemoglobins by encapsulation and the intrinsic issues of hemoglobin vesicles as a molecular assembly

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SUMMARY

Hemoglobin vesicles (HbV), or liposome-encapsulated hemoglobins, are developed as artificial oxygen carriers for the use as a transfusion alternative. The safety and efficacy of HbV have been clarified in detail: HbV can overcome the side effects of hemoglobin (Hb) molecules (stroma-free, and intra- or intermolecularly crosslinked) such as vasoconstriction, hypertension and possible vascular damage induced by direct contact of the vascular surface with Hb. On the other hand, intrinsic issues related to the suspension of HbV as a molecular assembly have to be considered: blood compatibility, structural and dispersion stabilities of the vesicles, and the requirement of prompt degradation in the reticuloendothelial system. Having overcome these issues, the results make us confident in advancing further development of HbV. Easy manipulation of physicochemical parameters of HbV provides possibilities for various clinical applications in addition to their use as a transfusion alternative.

PHYSIOLOGICAL IMPORTANCE OF THE RED BLOOD CELL CELLULAR STRUCTURE FOR ENCAPSULATED HEMOGLOBIN DESIGN

It has been well documented during the long history of the development of hemoglobin (Hb)-based oxygen carriers (HBOCs, Figure 1) that many side effects of stroma-free Hb and chemically modified Hbs exist: renal toxicity; entrapment of gaseous messenger molecules [nitric oxide (NO) and carbon monoxide (CO)] inducing vasoconstriction, hypertension, reduced blood flow, and reduced tissue oxygenation at microcirculatory levels[1–4]; neurological disturbances; malfunction of esophageal motor function[5]; and myocardial lesions.[6,7] These side effects of Hb molecules imply the importance of the cellular structure of red blood cells (RBCs). From the retrospective and recent observations, the main justifications for Hb encapsulation in RBCs are: (i) a decreased high colloidal osmotic pressure[8]; (ii) prevention of the removal of Hb from blood circulation; (iii) prevention of direct contact of toxic Hb molecules and endothelial lining[9]; (iv) preservation of the chemical environment in

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