5. アルブミン-ヘム二量体

rHSA の Cys-34 を 1,6-ビスマレイミドヘキサンで選択的に架橋した rHSA 二量体を調製し、それに 16 分子の heme を包接させた rHSA-heme 二量体も合成している[13]。rHSA-heme 二量体水溶液は、コロイド浸透圧を生理的条件に保ちながら、ヒト血液を上回る量の酸素を結合できる酸素輸液である。rHSA 二量体の MALDI-TOF 質量分析スペクトルには、明瞭な分子イオンピーク (m/z 132,741) が観測され、二量体の形成が確認された。rHSA の二次構造、表面電荷は架橋後も変化せず、rHSA 5.0 wt%溶液のコロイド浸透圧 (19 Torr) と同じ値を有する rHSA 二量体溶液の濃度は、8.5 wt%と決定された。さらに、rHSA 二量体の血中半減期 (16 hr) は rHSA に比べ 2 倍に延長することも明らかにしている。

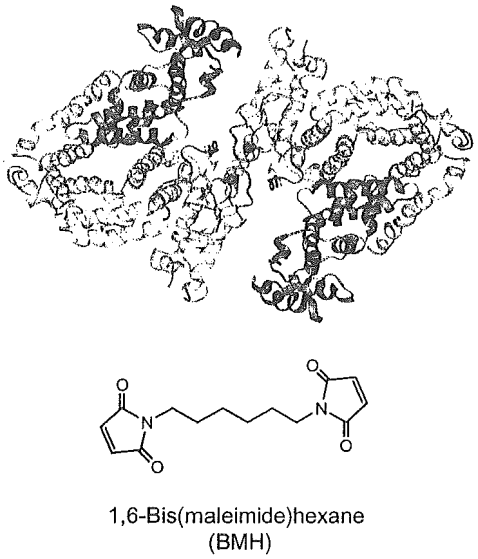

Figure 4 Simulated structure of cross-linked rHSA dimer with BMH.

6. まとめ

アルブミン-ヘム製剤は、生体内で安全に酸素を運搬できる完全合成系酸素輸液である。原料を生体物質に依存していないため、感染の心配は全くない。もちろん血液型フリーなので、いつでもどこでも交差試験なしに直ちに体内へ投与することのできる赤血球代替物となる。

現在、我々はさらなるアルブミン新物質群の開発を展開している。アルブミン二量体で得られた基礎知見を拡張し、構造明確なアルブミン多量体を合成。ヘムとの複合化により超高濃度酸素輸送系が具体化できる。また、ヘムをアルブミンに共有結合で強固に固定したヘム結合アルブミンや、遺伝子組換え技術により産生したヘムポケットを持つアルブミンに天然のヘムを結合させたアルブミン-ヘム錯体も創製し、その機能評価を進めている。

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[輸血医療のトピックス]

人工血液（人工赤血球）の開発動向

武岡真司*

キーワード◉人工血液　人工赤血球　ヘモグロビン小胞体　機能と安全性評価

はじめに

われわれは常にウイルスの猛威に曝されており，血液事業も大きな影響を受けている．わが国では，平成９年度から厚生科学研究 高度先端医療研究事業「人工血液開発分野」が設置され，人工赤血球，人工血小板，人工抗体の３部門に分かれた本格的な研究展開が進められてきた．人工血液は，現行の輸血用血液製剤を補完し，安全な製剤の安定供給の観点から，21世紀の医療の進歩に大きな影響を与えうるものと期待されている．わが国においても，人工血液の製品化に向けた研究開発の促進が国の基本方針（平成14年７月24日衆議院厚生労働委員会決議，医薬品・医療機器の安全対策の推進に関する件）となっている．

本稿では，実現が見えてきた人工赤血球に焦点を絞るが，この実現により，①緊急時に血液型を選ばずに輸血ができ，②HIV，肝炎やその他，未知のウイルスも含めたウイルスや細菌の感染症の心配がなく，③大量備蓄可能であるので大震災などの災害時などにも即応が可能，などが期待される．

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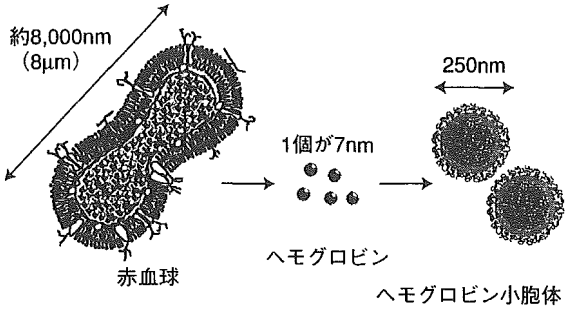

図１　赤血球から精製したヘモグロビンによるヘモグロビン小胞体の構築

I. 人工赤血球開発の現状

人工赤血球として，パーフルオロカーボン乳剤や修飾ヘモグロビンなどが検討され，臨床使用されてきたが，機能や安全性の観点から満足できるものではなかった．現在わが国を中心に研究が進められている，高濃度ヘモグロビンをリン脂質の二分子膜で包み込んだ，赤血球と類似のヘモグロビン小胞体（hemoglobin vesicles；HbV）（図１）が最も安全度が高く，実用が期待されている[1,2]．現段階では期限の切れた献血血液由来のヘモグロビンの有効利用が進められているが，将来的にはヘモグロビンは遺伝子組換え体が利用されるであろう．

赤血球からのヘモグロビン精製時に血液型を決める型物質やヘモグロビン以外の蛋白質，ウイルス（もし含まれたとしても）が，加熱やフィルター処理で完全に除去される．安定なリン脂質膜で包み直すため，室温で２年間の液状保存

表1　ヘモグロビン小胞体の規格

項目	規格値
粒径（nm）	240～280
P_{50}（torr）	27～34
Hb（g/dl）	10.0±0.4（8.6±0.4*）
総脂質（g/dl）	4.6～5.4（5.3～5.9*）
Hb/総脂質（g/g）	1.6～2.1
PEG-脂質（mol%）	0.3
metHb（%）	＜3
HbCO（%）	＜2
粘度（cP at 230s^{-1}）	2～3（3～4*）
晶質浸透圧（mOsm）	300
膠質浸透圧（torr）	0（20*）
pH（37℃）	7.4
エンドトキシン（EU/ml）	＜0.1
無菌試験	検出なし

*20%遺伝子組換えヒト血清アルブミン製剤と混合後
PEG：ポリエチレングリコール

（赤血球製剤では採血後3週間の冷蔵保存）が保証されており，乾燥粉末ではさらに長期間の保存が可能であるため，人工物の大きな長所とされている．

ヘモグロビン小胞体は，筆者の所属する早稲田大学理工学総合研究センター土田英俊名誉教授の研究グループと慶應義塾大学医学部小林紘一教授，末松 誠教授の研究グループを中心とした共同研究（厚生労働科学研究費補助金による）が中心となって進められており，民間会社との連携によって，製剤化と早期の臨床試験着手を目指している．

II. ヘモグロビン小胞体の機能と安全性評価

ヘモグロビン小胞体製剤の物性規格を表1にまとめた．ヘモグロビンがカプセル化されているため膠質浸透圧はほとんどゼロである．赤血球が生食に分散した状態と考えていただきたい．したがって，膠質浸透圧の調節が必要となる場合には（遺伝子組換え）ヒト血清アルブミンなどと併用となる．粒径は250nmに厳密に調節されており，赤血球の約1/30程度であるので，梗塞部位の透過など赤血球にはない機能が期待できる．

酸素親和度は，アロステリック因子，ピリドキサール5'-リン酸（PLP）の共封入によって適当値に調節可能である．脂質類の成分組成や含量には工夫が施されており，常温で2年間液状保存[3]，血流中での溶血の回避と適度な血中滞留時間（ヒトでは3日程度の予測），血小板や補体の活性化の回避など，従来の小胞体における課題が解決できている．

以下に，現在までに結果が得られているヘモグロビン小胞体に関する評価試験成績を簡単に紹介する．主にラットやハムスターを用いた試験であるが，基本的な安全性と酸素輸送効果は十分確認できている．また，現在，霊長類を用いた安全性試験が進行している．

酸素運搬効果をみるためのラット全血液量の90%をアルブミン単独で交換した場合には，70%交換辺りから血圧と腎皮質酸素分圧の低下が顕著となって死亡したが，ヘモグロビン小胞体をアルブミン溶液に分散させた系で90%交換した場合には，血圧，腎皮質酸素分圧共に維持された[4]．ヘモグロビン小胞体のアルブミン分散液によるハムスター80%交換輸血試験では，非侵襲に測定した皮下微小循環系の組織酸素分圧は，交換前の60～70%に低下するものの，対照アルブミン投与群よりも5倍以上の値が維持されていた[5]．さらには，修飾ヘモグロビンに認められる抵抗血管の収縮と血圧亢進は全く認めなかった．これは，ヘモグロビン小胞体は血管を透過しない大きさであるので，血管内皮由来弛緩因子である一酸化窒素への影響はほとんどないと考えられている[6]．

ラット25%^{99m}Tc標識ヘモグロビン小胞体負荷試験では，血中半減期はほぼ35時間程度であった．ヘモグロビン小胞体は，主として肝臓のクッパー細胞と脾臓のマクロファージに捕捉

され，老廃赤血球などの代謝と同様の経路をたどるものと考えられる．

ラット20ml/kg負荷投与試験により，細網内皮系での代謝過程，血液生化学検査を実施したところ，肝臓と脾臓の重量は一過性に増大し，貪食細胞に取り込まれたヘモグロビン小胞体は，1週間後にはほとんど消失した．肝機能・腎機能には特に異常を認めず，リパーゼは有意な一過性の亢進を示したが，アミラーゼには変動は認められなかった[7]．代謝過程で，脂質成分，特にコレステロールが血清中に出現し，7日後には正常値に戻った．

さらに，ラット（10ml/kg/日）での14日間の反復投与ならびにその後の14日間生存試験では，全例（14例）生存し，体重も増加し続け，生化学検査では脂質成分とリパーゼの一過性の亢進以外には変動を認めず，14日後には正常値に戻った[8]．

代謝臓器である肝臓では，ヘモグロビンでは内因性一酸化炭素を消去し，ビリルビンの過剰生成と胆汁分泌機能の低下を招来したが，ヘモグロビン小胞体ではそのような作用を認めなかった[9,10]．

ヘモグロビンは，自動酸化や活性酸素との反応によってヘム鉄が2価から3価に酸化（メト化）すると，酸素を結合できなくなる．メト体はFe^{3+}イオンを遊離し，これがフェントン反応を誘導してヒドロキシラジカルの発生を触媒する．ところが，ヘモグロビン小胞体の場合，ヘモグロビンは脂質二分子膜で被覆されているため，活性酸素が関連する反応はヘモグロビン小胞体の外へ影響を及ぼさないことが*in vitro*試験で確認されている[11]．細網内皮系にて代謝されたヘモグロビン小胞体由来の鉄の動態に関しては，今後の研究が待たれるところであろう．

まとめ

人工赤血球は，救急救命における輸血療法の補完を当面の目標としており，役目が終われば比較的すみやかに代謝臓器で代謝され，生合成される自身の赤血球と置き換わる．また，遺伝子組換えアルブミンにヘム誘導体を複合させたアルブミン—ヘムは，膠質浸透圧ももった酸素運搬体であり，ヘモグロビン小胞体よりも小さな粒子径などの特徴を活かした新しい酸素療法などへの適用も期待されている[2]．また，人工血小板も慶應義塾大学医学部池田康夫教授の研究グループで最先端の研究が行われており，*in vivo*成績が得られつつある[12]

このように，人工赤血球，人工血小板は，わが国では主に厚生労働科学研究補助金にて研究が進められているが，これはわが国の近未来医療への貢献はもとより，安全な血液が不足している多くの国に対しても大きな国際貢献となりうる．民間で行うには採算性も重要であるが，まずは長期的そして全人類的な視野に立った開発を期待したい．

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酸素輸液の展望

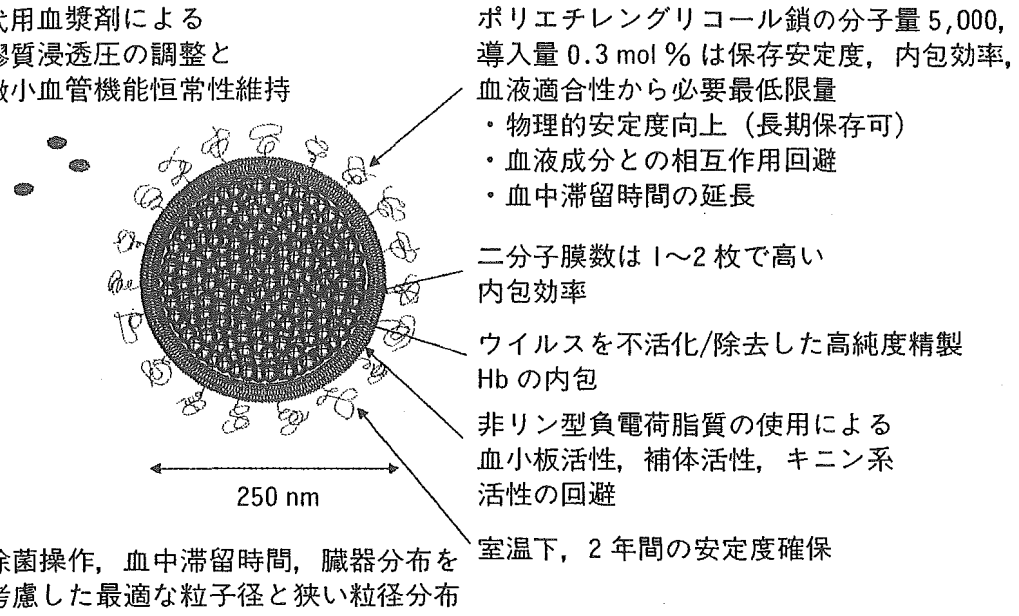
1) 小胞体型人工赤血球の開発動向

(1) 人工赤血球の必要性

事故や手術時に大量の血液が消失した場合には，輸液の補充によって循環血液量を維持するか，輸血によって酸素も補充する措置がその出血量や状況に応じて選択される．しかし，献血血液による同種血輸血では感染のリスクが否定できず，自己血輸血では利用できる状況が制限されている．したがって，酸素輸液（人工赤血球）の開発は必要であり，最新の科学技術の進歩と共にその製剤化技術，有効性や安全性の評価技術および医療上の適応などに関する研究が加速的に進展している．人工赤血球は，① 血液型を選ばずに輸血ができ，② HIV，肝炎やその他未知のウイルスなどによる感染症の心配がなく，③ 備蓄が可能であるため即時に大量供給が可能である．さらに④ 品質が保証された安全な製剤供給の観点から，現行の輸血用血液製剤を補完し，21 世紀医療の進歩に大きな影響を与え得るものと期待されている．

(2) 人工赤血球の開発の現状

酸素輸液としては，パーフルオロカーボン乳剤や修飾ヘモグロビン（Hb）などさまざまな種類が検討されてきたが，赤血球と類似の構造をもつ高濃度



図❶　Hb小胞体の特徴．

Hbをリン脂質二分子膜で包み込んだHb小胞体（図❶）が，最も安全度の高い製剤として開発が進められている[1,2]．現段階では期限の切れた献血血液由来のHbの有効利用が進められているが，将来的にはHbは遺伝子組換え体が利用されるであろう．Hb精製時に血液型を決める型物質やHb以外のタンパク質，ウイルス（もし含まれたとしても）が除去され，安定なリン脂質膜で包み直すため室温で2年間の保存が可能であり，人工物の大きな長所となっている．

③ ヘモグロビン小胞体の性状と評価

Hb小胞体の製造については，膜構成脂質とHb分子の精密な集合制御技術と膜処理やガス交換技術の組合せによって，現実的な量産体制が構築されつつある．製剤の物性規格を表❶にまとめた．赤血球と同様，膠質浸透圧はほとんどゼロである．膠質浸透圧の調節が必要な場合にはアルブミン製剤

表❶　Hb 小胞体の規格

項　目	規格値
粒径（nm）	240～280
P_{50}（torr）	27～34
[Hb]（g/dl）	10.0±0.4　(8.6±0.4[a])
[総脂質]（g/dl）	4.6～5.4　(5.3～5.9[a])
[Hb]/ [総脂質]（g/g）	1.6～2.1
[PEG-脂質]（mol %）	0.3
metHb（%）	＜3
HbCO（%）	＜2
粘度（cP at 230 s^{-1}）	2～3（3～4[a]）
晶質浸透圧（mOsm）	300
膠質浸透圧（Torr）	0（20[a]）
pH（37℃）	7.4
エンドトキシン（EU/ml）	＜0.1
無菌試験	検出なし

[a]：20% アルブミン製剤と混合後.

（将来的には遺伝子組換えヒト血清アルブミン）などの代用血漿剤を併用する．粒径は 250 nm と赤血球の約 1/30 であるので，虚血部位の酸素化など赤血球にはない機能が期待できる．通常の遠心分離（4,000 g，6 min）では赤血球は沈殿するが，Hb 小胞体は沈殿しない．赤血球と分離できる利点の反面，血液分析に影響を及ぼすので高分子の添加や超遠心分離操作（50,000 g，20 min）で沈殿させる[3]．また，酸素親和度はアロステリック因子，ピリドキサール 5′-リン酸の共封入により赤血球と同様の値に調節されている．脂質類の成分組成には特別な工夫が施されており，常温で 2 年間液状保存を可能とし[4]，血流中での溶血の回避と適当な血中滞留時間，補体・血小板・白血球への影響がほとんどない[5]など，従来のリポソーム製剤で指摘さ

れてきた課題が解決されている．当然のことながら，ウイルスの不活化と除去が製造工程に組込まれ，無菌試験，エンドトキシンやパイロジェンなどの試験にも合格した製剤となっている．

高折[6]はHb小胞体の安全性と有効性を検討するための動物試験項目を細かく提示しており，それに従った試験が厚生労働科学研究で進められている．現在までに得られている評価試験の概要を示す．主にラットやハムスターを用いた試験であるが，基本的な安全性と酸素輸送効果は十分確認できている．また，現在霊長類を用いた安全性試験が進行している．

ラット25% ^{99m}Tc標識Hb小胞体負荷試験では，血中半減期は35時間程度であった．Hb小胞体は主として肝臓のクッパー細胞と脾臓のマクロファージに捕捉され，老廃赤血球などの代謝と同様の経路をたどるものと考えられる．ラット20 m*l*/kg負荷投与試験により細網内皮系での代謝過程，血液生化学検査を実施したところ，肝臓と脾臓の重量は一過性に増大し，貪食細胞に取り込まれたHb小胞体は1週間後にはほとんど消失した．また，血液生化学検査を詳細に行ったが，リパーゼやコレステロール値の一過性の有意な亢進以外の変動は認められなかった[7]．ラット40%血液交換・長期生存試験では，ヘマトクリット値は7日後に前値に復し[8]，ラット（10 m*l*/kg/day）で14日間の反復投与ならびにその後の14日間生存試験では，全例（14例）生存し体重も増加し続けた．生化学検査では脂質成分とリパーゼの亢進以外には変動を認めず，14日後には正常値に戻った[9]ことから，きわめて安全度の高い製剤であることが明らかとなった．活性酸素によってHbはフェリル体やメト体となって酸素結合能を失い鉄イオンが遊離するが，Hb小胞体はこれらを閉じ込めたまま，赤血球と同様に主として脾臓で代謝されるため安全性が高いものと考察される[10]．肝臓の微小循環動態観察では，Hbをそのまま投与すると内因性COの消去による類洞血管の収縮，ビリルビンの過剰生成と胆汁分泌機能の低下が認められたが，Hb小胞体ではそのような作用は観測されなかった[11,12]．しかし，類洞内のクッパー細胞

がHb小胞体を捕捉すると肥大して一過性に類洞血管の狭小化をきたす，また，貪食細胞の機能飽和による生体防御機構の低下が懸念されるため，適正投与量が存在することが示唆された．

酸素運搬効果をみる動物実験の結果を以下に紹介する．ラット40%脱血ショック・同量投与による回復試験では，生理食塩液，メトヘモグロビン小胞体分散液と比較して有意な酸素運搬効果を確認し，これは同Hb濃度の赤血球分散液と同等であった[13]．また，ラット全血液量の90%をHb小胞体のアルブミン分散液で交換した場合には，血行動態，血液ガスパラメータ，組織酸素分圧ともに維持され，アルブミン溶液で交換した場合と比較して顕著な有効性を示した[14]．ハムスターの血液量の80%をHb小胞体のアルブミン分散液で交換して皮下微小循環動態を非侵襲に観測したところ，組織酸素分圧は脱血前の60～70%まで低下したものの，対照アルブミン投与群での5倍以上の値が維持されていた[15]．また，修飾Hbに認められる抵抗血管の収縮と血圧亢進の現象は全く認めなかった[16]．現在，試料の安全性とともに有効性も証明し得る動物モデルとして，ビーグル犬の脾摘後50%の脱血によりショックを作成し，1時間経過した後に蘇生液としてHb小胞体のアルブミン分散液を投与するモデルを検討している．

(4) 人工赤血球の適用と展望

Hb小胞体は現在，実施企業でGLP (good laboratory practice) による非臨床試験，GMP (good manufacturing practice) 製造体制の準備が進められている[17]．また，本邦に適した臨床試験のためのプロトコールの検討も過去の人工酸素運搬体の臨床例や課題を参考に進められている[18]．当面，希釈式自己血輸血，希少血液型患者での一般輸血の代替，あるいは予測せざる手術時出血への代用血漿剤と併用した単回での投与などを目標としており，救急用途への拡大が考えられている．その際には，現行の赤血球濃厚液の使

用がガイドライン[18]に即した人工赤血球の使用ガイドラインが必要となるであろう．さらには腫瘍の酸素化による放射線治療効果の促進，赤血球が通過できない狭窄部も通過できるサイズをもつ酸素運搬体による組織の酸素化，人工心肺を用いた体外循環回路の充塡液としての輸血の代替，人工呼吸器でも改善しない重い肺障害に適用する液体換気用の酸素富化膜などの適応が検討されている．また，パルスオキシメータなどのモニターや血液生化学検査では項目によっては影響がでると推測されるため，装置やプログラムの改良も必要であろう．安全で有効な小胞体型人工赤血球が臨床現場で使用され，救命に役立つ日が1日も早くくることを願う次第である．

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（武 岡 真 司）

Reprint from

H. Ishii, M. Suematsu, K. Tanishita, H. Suzuki (Eds.)

Organ Microcirculation
A Gateway to Diagnostic and Therapeutic Interventions

Springer

Design and Modification of Nanoparticles for Blood Substitutes

Shinji Takeoka

Key words. Liposome, Nanoparticle, Hemoglobin, Red-blood-cell substitute, Platelet substitute

Introduction

We have been developing the technology of stabilized and functionalized nanoparticles such as liposomes for 20 years. When phospholipids and cholesterol are dispersed into an aqueous solution, they spontaneously assemble to form vesicles with a bimolecular (bilayer) membrane. There are many parameters, such as size, size distribution, lamellarity (the number of bilayer membranes), membrane fluidity, surface charge, surface modification, membrane permeability, that characterize liposomes. They can be adjusted as need dictates to allow for changing encapsulation of functional molecules, release triggered by external stimuli, conjugation of functional sites on the surface, rolling or adhesion properties of liposomes, and control of blood circulation time. On the other hand, we have to consider their physical and chemical stability during storage or blood circulation. Surface modification with polyoxyethylene (POE) chains is one of the most effective ways to impart such stabilization.

In this chapter, I introduce two examples of nanoparticle application; one is a liposome encapsulating concentrated hemoglobin (Hb-vesicle) for a red-blood-cell substitute, and the other is a liposome bearing recognition proteins or peptides on the surface and used as a platelet substitute. The microcirculation, pharmacokinetics, and histopathological change were studied in relation to the characteristics of the particles as well as their oxygen-binding and releasing properties. In the case of the platelet substitutes, nanoparticles

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bearing receptor proteins of the platelet surface recognize the collagen surface under shear rates. The rolling and adhesion properties will be discussed depending on the stiffness or membrane fluidity of the particles.

Present Status of the Development of Red-Blood-Cell Substitutes [1,2]

Hb-vesicles that encapsulate concentrated hemoglobin with a phospholipids bilayer membrane have a similar structure to red blood cells, and are expected to be used soon in clinical tests because the degree of safety and efficacy are considered to be high. Although effective use of the hemoglobin from donated and expired blood should be promoted at the present stage, recombinant human hemoglobin will be used in the future. During hemoglobin purification from red blood cells, stroma including the glycoproteins which determine a blood type, proteins other than hemoglobin, and the viruses are removed by heating or filter processing. By encapsulating hemoglobin with a stabilized phospholipids membrane with POE-lipid, liquid-state preservation for 2 years is guaranteed at room temperature under nitrogen atmosphere [3], and with dry powder, further prolonged preservation is possible. These points are advantages for an artificial oxygen carrier.

The design of the red-blood-cell substitutes (POE-modified hemoglobin vesicles) are summarized in Fig. 1. Hb-vesicles are dispersed into a saline solution and enclosed with the bottle in a state of deoxidization. The hemoglobin concentration is 10 g/dl and is close to that of human blood. Moreover, because hemoglobin molecules are encapsulated, the colloid osmotic pressure of the solution is zero. Therefore, when regulation of colloid osmotic pressure

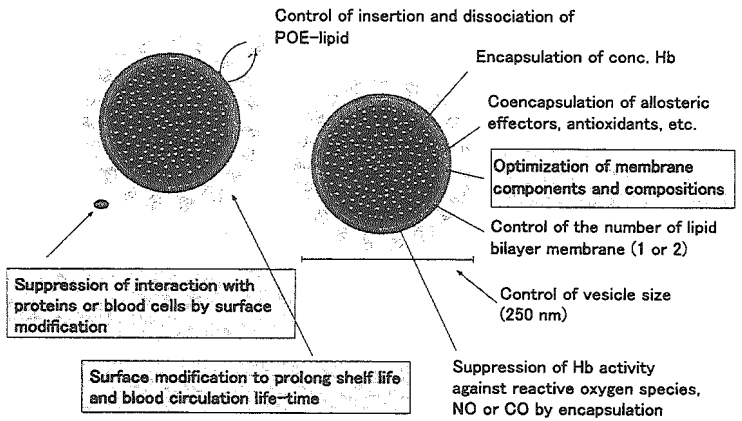

FIG. 1. Design of POE-modified hemoglobin vesicles (Hb-vesicles) as red-blood-cell substitutes

is needed, a solution of colloids such as albumin and polysaccharide will be used with the Hb-vesicle dispersion. The particle diameter is strictly adjusted to 250 nm. The degree of oxygen affinity is adjusted to a suitable value by coencapsulating an allosteric effector such as pyridoxal 5′-phosphate. The optimization of the composition of the lipid components resulted in high encapsulation efficiency of hemoglobin in the Hb-vesicle, a stability of 2 years in a liquid state, the prevention of hemolysis, an appropriate lifetime in blood circulation, and avoidance of platelet and complement activation. Furthermore, large-scale manufacturing has been improved by the introduction of freeze-thawing and freeze-drying operations which can control a molecular assembling state before encapsulating hemoglobin molecules.

Present Results of Safety and Efficacy Tests

Although in vivo testing was carried out using rats or hamsters, we confirmed the fundamental safety and oxygen transporting ability. Safety tests using primates is in progress. When 90% of the volume of rat blood was exchanged by the albumin dispersion of the Hb-vesicles, the oxygen partial pressure of the renal cortex was maintained as was blood pressure [4]. On the other hand, when the blood was exchanged by an albumin solution in the same concentration, the fall of blood pressure and oxygen partial pressure of the renal cortex became noticeable at 70% exchange, and all rats died just after 90% exchange.

In the hamster 80% exchange transfusion examination with the albumin dispersion of the Hb-vesicles, the noninvasively measured oxygen partial pressure of the subcutaneous tissue microcirculatory system was maintained at 5 times or more than that of the control albumin group although it fell to 60%–70% before exchange [5]. The contraction of a resistance blood vessel and the rise of blood pressure was not confirmed at all, but it was confirmed with modified hemoglobin products. Because the Hb-vesicle has a size that does not penetrate a blood vessel, there is no influence on the activity of nitrogen oxide as an endothelium-derived relaxation factor [6]. Furthermore, the Hb-vesicles cannot penetrate the sinusoidal vessels of liver (several holes 10–200 nm in size are open in the blood vessels) like old red blood cells, but are metabolized by Kupfer cells of liver and macrophages in reticuloendothelial systems. On the other hand, acellular hemoglobin molecules in the liver influenced liver microcirculation by eliminating carbon monoxide as a gaseous vasodilator, caused overgeneration of bilirubin, and suppressed bile secretion [7,8]. The half-life of Hb-vesicles in human blood circulation was estimated to be about three days on the basis of the results in rats, rabbits, and monkey. Moreover, from details of the blood biochemistry examination and pathology examination in the single and repetitive administration, we confirmed the transitional rise of lipase in connection with lipid decomposition, the transitional rise of a cho-

lesterol value, iron content, and the transitional hypertrophy of a reticuloendothelial system [9]. It was concluded that Hb-vesicles are expected to function adequately in cases of extensive bleeding.

Development of Platelet Substitutes

The history of platelet substitutes is short compared with that of red blood cells, with few examples of research. A platelet has complicated functions, such as adhesion specific to the bleeding site, expansion, aggregation, secretion, and the activation of a blood coagulation system. Needless to say, we cannot make platelet substitutes that have these all. However, a bleeding tendency is strongly apparent in such bleeding diseases as Bernard-Soulier syndrome and thrombasthenia, in which adhesion and aggregation ability are lacking. In these conditions, a hemostatic effect can be expected by the infusion of particles having functions such as adhesion and the aggregation of platelets due to the assistance of the function of the remaining platelets. Although clinical tests were carried out with human red blood cells [10] or albumin microcapsules [11] conjugating fibrinogen, and with the dried powder of human platelets [12], all clinical tests were suspended due to problems of safety and efficacy. Moreover, since the blood components of human origin were used, the risk of infection cannot be avoided completely. The platelet substitutes created by our group use liposomes and recombinant human albumin as biocompatible particles. They also use recombinant proteins of the part of platelet membrane or synthetic oligopeptides by conjugating to those particles for the purpose of accumulation to the bleeding site involving native platelets, expecting to achieve hemostasis.

The mechanisms of platelet adhesion differ between the blood flow of high shear rate and that of low shear rate. As shown in Fig. 2, the hemostasis of the platelets to bleeding in a high shear rate begins from a platelet recognition of the von Willebrand factor (vWf) bound to the collagen in the subcutaneous tissue of a blood vessel exposed to the bleeding site, followed by platelet adhesion and rolling. This recognition ability comes from the GPIb/V/IX complex containing GPIbα on the surface of a platelet [13]. Next, the platelets will progress and a granule will be secreted if GPIaIIa ($\alpha_2\beta_1$ integrin) or GPVI on the surface of the platelet directly interacts with the collagen and is activated. Fibrinogen recognizes the activated GPIIb/IIIa ($\alpha_{IIb}\beta_3$ integrin), constructs a crosslink between blood platelets, forms an aggregate, and serves as primary hemostasis. Hemostasis is completed by formation (secondary hemostasis) of the fibrin clot by induction of the coagulation system.

The target platelet substitutes bear the water-soluble part of receptor proteins such as GPIbα which recognizes collagen through vWf under the blood

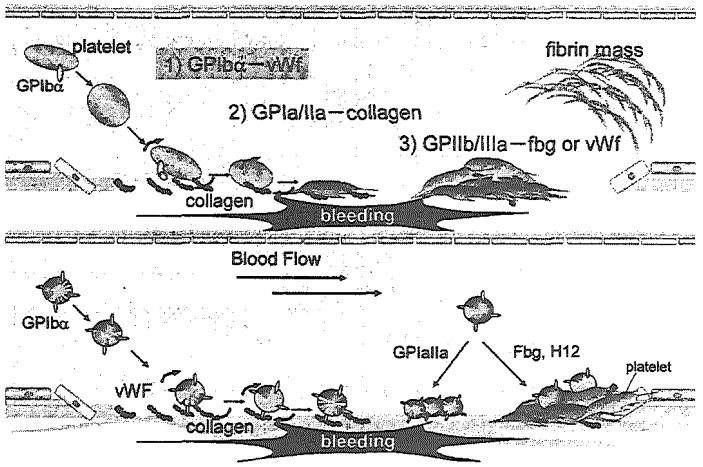

FIG. 2. Design of platelet substitutes studied from functions of natural platelets

flow of high shear rates and GPIaIIa which recognizes collagen directly at low shear rates. Instead of GPIIb/IIIa on the surface of an activated platelet, they bear the fibrinogen or its oligopeptide to assist the platelet aggregation as ligands of the GPIIb/IIIa.

If an anti-GPIIb/IIIa antibody is added to a platelet dispersion to inhibit GPIIb/IIIa activity, the platelets roll on the vWf-immobilized plate along the flow direction by the interaction of rGPIbα on the platelet and the vWf. Interestingly, phospholipid liposomes conjugating rGPIbα roll on the vWf-immobilized plate as well [14]. The number of the rolling liposomes increased with the shear rate, indicating the characteristic of rGPIbα. Moreover, the rolling speed was correlated with the membrane fluidity of the liposomes. That is, the rolling speed of "soft" liposomes was low, whereas that of the "hard" liposomes was high [15]. However, the rGPIbα-liposomes did not continue rolling but departed from the plate after rolling some length. This was remarkable as the "soft" liposomes. When the amount of rGPIbα on the surface of the liposome after the experiment was measured, it was suggested that rGPIbα-lipid should dissociate from the bilayer membrane during the rolling on the vWf-plate. Now rGPIbα-lipid which cannot dissociate serves as a point of a molecular design.

On the other hand, the rGPIaIIa-liposomes directly recognize collagen under the blood flow of low shear rates and adhered to (stopped at) the collagen-immobilized plate [16]. In this case, the number of the adhering liposomes decreased as the shear rate rose. However, liposomes conjugating both rGPIbα and GPIaIIa adhered on the collagen plate under the blood flow from low to high shear rates [17]. If the liposomes having platelet activation factors

or coagulation factors in the internal aqueous phase accumulate at the bleeding site, they will be able to contribute effectively to hemostasis by releasing their contents. We also focused on using polymerized albumin particles as effective platelet substitutes and obtained some unique in vitro and in vivo results [18,19].

Conclusions

For red-blood-cell substitutes the present target is the supportive treatment of transfusion therapy in emergency, and nonclinical and clinical studies will be scheduled within 2 years.

On the other hand, the research of platelet substitutes has just started. There is a conflict between the carrier design for the extension of circulation lifetime and the carrier design to show the hemostatic activity by recognizing the bleeding site; therefore, we need to resolve this conflict and to design platelet substitutes for prophylactic or chronic treatments. And we also need a method to confirm that the candidate does not create a thrombus in blood circulation.

Although profitability is important in the development of blood substitutes, one that is based on a long-term view with consideration for human beings is expected first. At present, as many discoveries about the dynamic function of platelets have accumulated in a short period of time with the progress of biotechnology and opto-electronics, and the manufacturing technology of recombinant proteins or carriers is progressing, a usable product is sure to be invented in the near future.

Acknowledgments. The author thanks Drs. E. Tsuchida, H. Sakai, K. Sou, Y. Teramura, and Y. Okamura at Waseda University, and Drs. K. Kobayashi, Y. Ikeda, M. Suematsu, H. Horinouchi, M. Handa, and M. Murata at Keio University for useful discussions and suggestions. This work was supported in part by Health and Labor Sciences Research Grants (Research on Pharmaceutical and Medical Safety); the Ministry of Health, Labor, and Welfare, Japan; and Grants-in-Aid from the JSPS, Japan, and 21COE "Practical Nano-Chemistry" from MEXT, Japan.

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Artificial Oxygen Carrier

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Safety and Efficacy of Hemoglobin-Vesicles and Albumin-Hemes

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Summary. Keio University and Waseda University have worked together on artificial O_2 carrier research for 20 years in close cooperation. Two candidate materials have been selected from the viewpoints of safety, efficacy, and cost performance. One is Hemoglobin-vesicles (HbV) and the other is albumin-heme (rHSA-heme). This chapter summarizes our video presentation that introduced the recent results of our research into HbV and rHSA-heme.

Key words. Blood substitutes, Oxygen carriers, Hemoglobin-vesicles, Albumin-heme, Oxygen therapeutics

Introduction: Keio-Waseda Joint Research Project

For human beings to survive, it is necessary to continuously deliver oxygen that is needed for the respiration of all tissue cells. Red blood cell, a so-called moving internal-organ, reversibly binds and releases O_2 under physiological conditions. From this point of view, red blood cell substitutes, or O_2-Infusions, are very important. In order to promote this research, we have emphasized that the establishment of basic science for macromolecular complexes and molecular assemblies is essential. We have systematically studied the Metal Complexes (synthetic heme derivatives) embedded into a hydrophobic cluster, and clarified that the electronic processes of the active sites are controlled by the surrounding molecular environment. Therefore, the reaction activity and its rate constant are observed as cooperative phenomena with the

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properties of the molecular atmosphere. In other words, the development of our O_2-Infusion has been based on "the Regulation of the Electronic Process on Macromolecular Complexes and Synthesis of Functional Materials" [1,2].

Reproducing the O_2-binding ability of red blood cells (RBC), that is, the development of a synthetic O_2 carrier that does not need hemoglobin (Hb), was the starting point of our study. In general, central ferric iron of a heme is immediately oxidized by O_2 in water, preventing the O_2 coordination process from being observed. Therefore, the electron transfer must be prevented. We were able to detect the formation of the O_2-adduct complex, but for only several nano seconds, by utilizing the molecular atmosphere and controlling the electron density in the iron center. Based on this finding, we succeeded in 1983 with reversible and stable O_2 coordination and preparation of phospholipid vesicles embedded amphiphilic-heme, known as lipidheme/phospholipids vesicles (Fig. 1) [3–6]. This was the world's first example of reversible O_2-binding taking place under physiological conditions. For example, human blood can dissolve about 27 ml of O_2 per dl, however a 10 mM lipidheme-phospholipid vesicle solution can dissolve 29 ml of O_2 per dl. This material is suitable for O_2-Infusion.

Soon after this discovery, Professor Kobayashi of Keio University asked Professor Tsuchida for a chance to evaluate the lipidheme solution with in vivo experiments. Since then the joint research and collaboration has continued since that time. We have synthesized over one hundred types of heme, and recently synthesized new lipidheme–bearing phospholipid groups, which complete self-organization in water to form stable vesicles. In 1985 Dr. Sekiguchi at Hokkaido Red Cross Blood Center proposed that Professor Tsuchida consider the utilization of outdated red blood cells and Hbs because, while the totally synthetic system is definitely promising it appeared that it

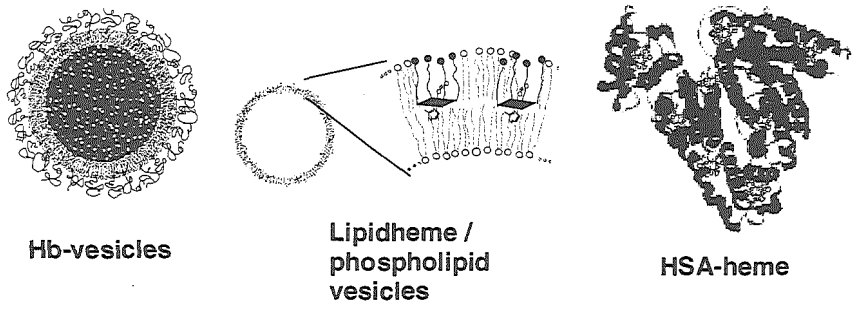

FIG. 1. Schematic representation of lipidheme-vesicle, hemoglobin-vesicle, and albumin-heme

would take considerable time to arrive at a social consensus for its use. We started to produce Hb-vesicles (HbV) using purified Hbs and molecular assembly technologies. In the late 1990's, a mass-production system for recombinant human serum albumin (rHSA) was established and we then prepared albumin-heme hybrids (rHSA-heme) using its non-specific binding ability, which is now considered to be a promising synthetic material.

Based on our effective integration of molecular science and technologies for functional materials developed by Waseda University, and the outstanding evaluation system of safety and efficacy developed by Keio University using animal experiments, we have made strong progress in our research on the O_2-Infusion Project. During this period, we have received substantial funding support from the Japanese government. In the near future, mass production and clinical tests of O_2-Infusion will be started by a certain pharmaceutical industry.

Background and the Significance of HbV

Historically, the first attempt of Hb-based O_2 carrier in this area was to simply use stroma-free Hb. However, several problems became apparent, including dissociation into dimers that have a short circulation time, renal toxicity, high oncotic pressure and high O_2 affinity. Since the 1970s, various approaches were developed to overcome these problems [7,8]. This includes intramolecular crosslinking, polymerization and polymer-conjugation. However, in some cases the significantly different structure in comparison with red blood cells resulted in side effects such as vasoconstriction [9].

Another idea is to encapsulate Hb with a lipid bilayer membrane to produce HbV that solves all the problems of molecular Hb [10]. Red blood cells have a biconcave structure with a diameter of about 8000 nm. Red blood cells can deform to a parachute-like configuration to pass through narrow capillaries. The possibility of infection and blood-type mismatching, and short shelf life are the main problems. Purified Hb does not contain blood-type antigen and pathogen, thus serves as a safe raw material for HbV.

HbV, with a diameter of 250 nm, do not have deformability but are small enough to penetrate capillaries or constrict vessels that RBC cannot penetrate. The surface of the vesicles is modified with polyethylene glycol (PEG) to ensure homogeneous dispersion when circulated in the blood and a shelf life of two years. The idea of Hb encapsulation with a polymer membrane mimicking the structure of RBC originated from Dr. Chang at McGill University [7]. After that, the encapsulation of Hb within a phospholipid vesicle was studied by Dr. Djordjevici at the University of Illinois in the 1970s [11]. However, it was not so easy to make HbV with a regulated diameter and ade-

quate O_2 transport capacity. We made a breakthrough in routinely producing HbV by using fundamental knowledge of macromolecular and supramolecular sciences. Some of the related technologies have already been published in academic journals [12–19]. Several liters of HbV are routinely prepared in a completely sterile condition. Hb is purified from outdated red blood cells, and concentrated to 40 g/dl. Virus removal is performed using a combination of pasteurization at 60°C and filtration with a virus removal filter. The Hb encapsulation with phospholipids bilayer membrane and size regulation was performed with an extrusion method. The vesicular surface is modified with PEG chains. The suspension of Hb-vesicles is dated at the final stage.

The particle diameter of HbV is regulated to about 250 nm, therefore, the bottle of HbV is turbid, and is a suspension. One vesicle contains about 30,000 Hb molecules, and it does not show oncotic pressure. There is no chemical modification of Hb. Table 1 summarizes the physicochemical characteristics of HbV. O_2 affinity is controllable with an appropriate amount of allosteric effectors, pyridoxal 5-phosphate. Hb concentration is regulated to 10 g/dl, and the weight ratio of Hb to total lipid approaches 2.0 by using an ultra pure and concentrated Hb solution of 40 g/dl, which is covered with a thin lipid bilayer membrane. The surface is modified with 0.3 mol% of PEG-lipid. Viscosity, osmolarity, and oncotic pressure are regulated according to the physiological conditions.

HbV can be stored for over two years in a liquid state at room temperature [17]. There is little change in turbidity, diameter, and P_{50}. MetHb content decreases due to the presence of reductant inside the HbV, which reduces the

TABLE 1. Physicochemical characteristics of HbV suspended in 5% albumin (HSA)

Parameters	HbV/HSA	Human blood (RBC)	Analytical method
diameter (nm)	220–280	8000	Light scattering method
P_{50} (Torr)	27–34[1]	26–28	Hemox Analyzer
[Hb] (g/dl)	10 ± 0.5	12–17	CyanometHb method
[Lipid] (g/dl)	5.3–5.9	1.8–2.5[2]	Molibuden-blue method
[Hb]/[Lipid] (g/g)	1.6–2.0	6.7[3]	—
[PEG-lipid] (mol%)	0.3	—	^{1}H-NMR
metHb (%)	<3	<0.5	CyanometHb method
viscosity (cP)[4]	3.7	3–4	Capillary rheometer
osmolarity (mOsm)	300	ca. 300	(suspended in saline)
oncotic press. (Torr)	20	20–25	Wescor colloid osmometer
pH at 37°C	7.4	7.2–7.4	pH meter
Endotoxin (EU/mL)	<0.1	—	LAL assay
Pyrogen	Free	—	rabbit pyrogen test

[1] Adjustable, [2] Total cell membrane components, [3] Weight ratio of Hb to total cell membrane components, [4] At 230 s^{-1}.

trace amount of metHb during storage. This excellent stability is obtained by deoxygenation and PEG-modification. Deoxygenation prevents metHb formation. The surface modification of HbV, with PEG chains prevents vesicular aggregation and leakage of Hb and other reagents inside the vesicles. Liquid state storage is convenient for emergency infusion compared to freeze-dried powder or the frozen state.

In Vivo Efficacy of HbV

The efficacy of HbV has been confirmed with isovolemic hemodilution and resuscitation from hemorrhagic shock. Some of the results have already been published in academic journals in the fields of emergency medicine and physiology [20–28]. In this chapter two important facts are described. One is isovolemic hemodilution with 90% blood exchange in a rat model. The other is resuscitation from hemorrhagic shock in a hamster model.

To confirm the O_2 transporting ability of HbV, extreme hemodilution was performed with HbV suspended in human serum albumin (HSA) [21,23] (Fig. 2). The final level of blood exchange reached 90%. Needle-type O_2 electrodes were inserted into the renal cortex and skeletal muscle, and the blood flow rate in the abdominal aorta was measured with the pulsed Doppler method. Hemodilution with albumin alone resulted in significant reductions in mean

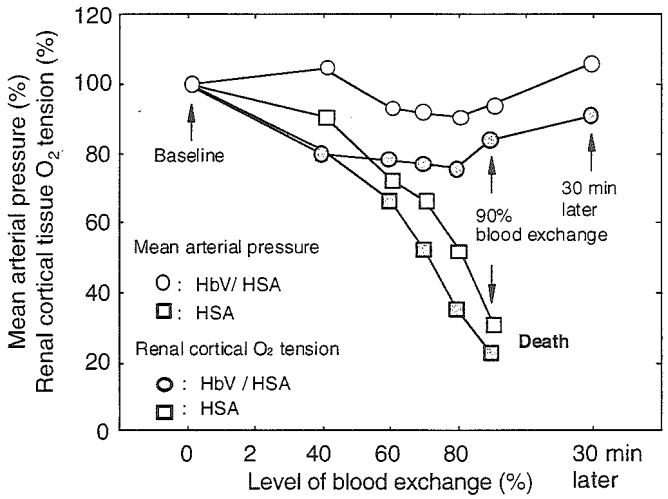

FIG. 2. 90% exchange-transfusion with HbV suspended in HSA (HbV/HSA), or HSA alone. Mean arterial pressure and renal cortical oxygen tension were monitored