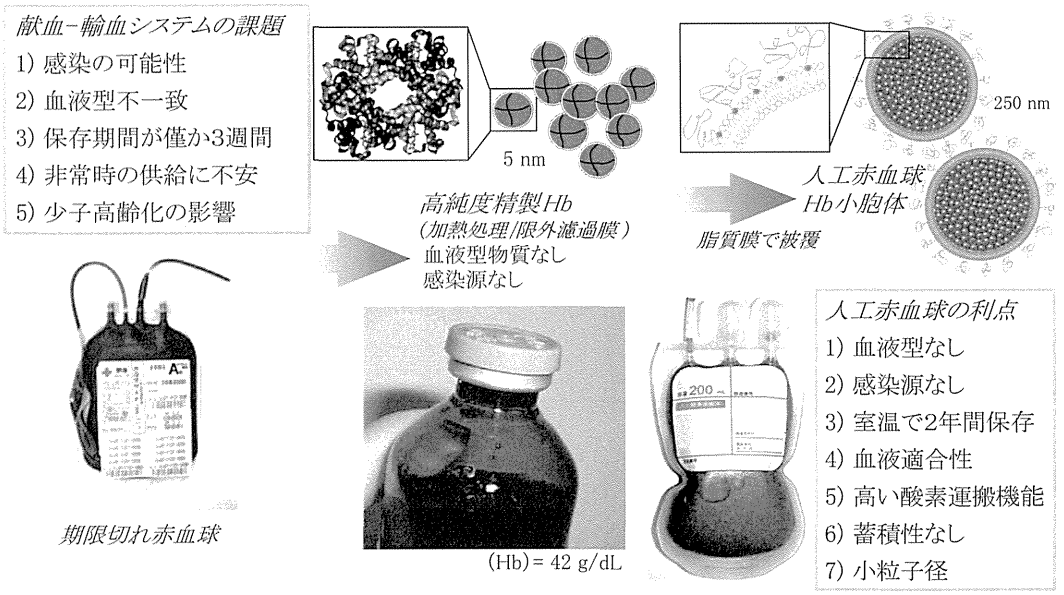

図-1　人工赤血球の調製法の概略

の人工赤血球：Hb小胞体を研究している（**図-1**）[2]。

高純度・高濃度Hb溶液（濃度35%以上、約3万個のHb分子）を、脂質分子二層膜（厚さ5nm）からなる小胞体（いわゆるリポソーム）に内包したHb小胞体（平均粒子径250nm）は、脂質成分とHbが分子間相互作用（二次的相互作用：疎水的相互作用、静電的相互作用、水素結合など）だけで形成している分子集合体である（**図-2**）。原料のHbは日本赤十字社から提供される検査済みの献血由来の期限切れ赤血球由来であるが、精製に際しHbに一酸化炭素（CO）を結合させて安定化し、60℃、10時間の加熱処理とウィルス除去膜処理の組合せで、感染に対する安全性を確保できる。あるいは、ブタやウシなど家畜の血液から採取したHbも同様に利用できる。COは光解離によって除去する。約1,500本のポリエチレングリコール（PEG）鎖を粒子表面に配置することにより、小胞体粒子間の凝集抑制と分散安定度の向上の効果が得られ、さらに脱酸素化して容器に封入することにより溶液のまま室温にて長期保存が可能になった。「ナマモノ」の血液から高純度Hb溶液を単離し、これを人工赤血球という安定な「物質」に再生したといえる。微粒子表面の性質は、生体適合性を決定する要素である。PEG修飾と負電荷脂質の導入により補体活性や凝固系の活性、あるいは阻害を誘導しない微粒子を構成している。

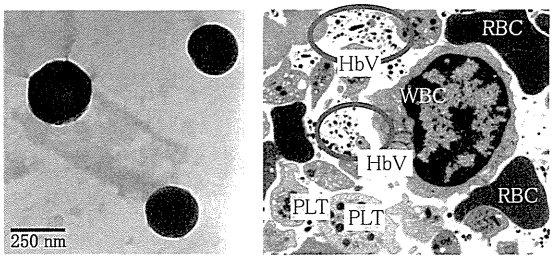

図-2　人工赤血球の電子顕微鏡写真

3. 人工赤血球濃厚分散液の特徴と体内酸素輸送

人工赤血球の分散液（[Hb]＝10g/dL）は、粒子占有体積が40%である。血液において赤血球の占有体積（ヘマトクリット値）が40〜55%であることとほぼ同等である。微粒子分散液の粘弾性特性として、せん断速度の上昇に応じて粘度が僅かに上昇する非Newton性を呈する。しかし、粒子径が赤血球より小さいので、血液よりはNewton性に近い流体となる。血漿は電解質由来の晶質浸透圧と、蛋白質（主にアルブミンとグロブリン）由来の膠質浸透圧の両方を持っている。対して、蛋白質Hbを内包した人工赤血球は赤血球と同様に、膠質浸透圧を示さない。従って、血管内に大量に投与する場合には、水溶性高分子（アルブミンや、ヒドロキシエチルスターチなど）からなる代用血漿剤溶液など、膠質浸透圧を持つ溶液を併用すればよい。高分子量のヒドロキシエチルスターチに微粒子を分散させると、枯渇性相互作用（depletion interaction）により微粒子が極めて弱い相互作用によって凝集することが知られている。人工赤血球もそのような現象が観察されることがあるが、微弱なせん断応力によって凝集は容易に

解離することが解っている。

血液の微小血管内における流動様式を観察すると、赤血球は管の中央を流れようとする。結果として内壁近傍には、赤血球の存在しない血漿層が形成される（**図-3**）。下流に分岐がある場合、赤血球は流れの早い方に沢山流れ、流れの遅い方には血漿が多く流れることがある。これを血漿分離（plasma-skimming）とよぶ。人工赤血球は赤血球の大きさの1/30程度で、血漿層に均一に分散して流れる。従って、赤血球が流れ難い血管にも流入し、酸素を供給すると予想されている。実際に、毛細管内に均一に分散して流動することも確認されている[3]。このことは、血管性の虚血性疾患において、組織酸素化に威力を発揮すると考えられ、実際に効果が得られている。

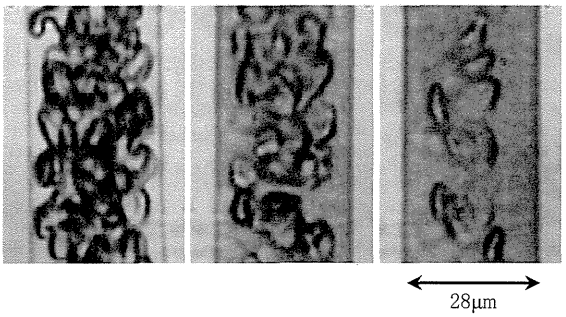

図-3　微小細管内の流動様式

人工赤血球分散液については、これまでに多くの動物投与試験を実施し、安全性と有効性について実証して来た（**表-1**）。出血性ショックにおける蘇生液、人工心肺など体外循環回路の補填液、臓器灌流液、腫瘍組織の酸素化による放射線感受性の向上、脳梗塞モデル、有茎皮弁における低酸素領域の酸素化、などが挙げられる。また、最近では、酸素のみならず、COを結合した状態で投与することも試みている。勿論、COは毒ガスである。しかし、生体内でごく微量産生されている。COには細胞保護効果があり、生体内で活性酸素の産生を制御する機序が提案されている。その他、さまざまな応用が研究され、全て論文として公表されている[4]。また、安全性試験についてもかなりの知見の蓄積があるので、総説などをご覧いただきたい[5]。

4. 人工赤血球の造粒技術に関する話題

リポソームは、水系媒体に分散されたリン脂質分子が自発的に集合形成するカプセル状の構造体である。1960年代に英国のBanghamらによって初めて発見された。今やリポソーム製剤は既に抗癌剤、抗真菌剤のデリバリーシステムとして実用

表-1　人工赤血球（ヘモグロビン小胞体）製剤の有効性試験（結果は全て学術誌に公表済み）[3]

No.	動物試験	主な効能
1	ラット、循環血液量の90%交換輸血	血圧維持、赤血球と同等の酸素を運搬
2	ラット、循環血液量の50%出血性ショックからの蘇生	血圧維持、赤血球と同等の蘇生効果あり
3	ウサギ、循環血液量の40%出血性ショック（反復出血）からの蘇生	サイトカインTNFα過剰産生の抑制
4	ビーグル犬、循環血液量の50%出血性ショックからの蘇生	中型動物での大量・急速蘇生効果を確認
5	ラット、体外循環（人工心肺）充填液としての利用	高次脳機能を改善
6	ラット、制御不能出血モデル蘇生	生存時間の延長
7	ラット、出血性ショック蘇生（CO結合体の投与）	再灌流時における細胞保護効果の確認
8	ハムスター、循環血液量の50%出血性ショックからの蘇生、無麻酔	出血性ショック蘇生に有効、微小循環の回復
9	ラット、脳梗塞モデルに対する投与	梗塞巣の拡大を抑制
10	マウス、摘出小腸の体外灌流	37℃、2hr灌流で組織構造を維持
11	ハムスター、有茎皮弁虚血部位の酸素化	側副経路を経由した酸素輸送を確認
12	マウス、皮弁モデル虚血領域酸素化	創傷治癒効果を確認
13	マウス、担がんモデルに対する投与	放射線感受性の向上、腫瘍重量の減少
14	ラット、^{15}O-酸素結合Hb小胞体の脳梗塞モデルへの投与とPET診断	脳梗塞領域のイメージングが可能

化されている。リポソームの製造方法として専門書に記載されている方法としては[6]、超音波照射法、有機溶媒を用いる逆相法、界面活性剤を用いて分散させた後、これを透析で除去する方法などがある（表-2）。人工赤血球の場合、Hbのような蛋白質を扱い、かつ、血管内投与を前提とした製剤の製造においては、工程中の蛋白質の変性や、残存物質の懸念があり、これらの方法は向いていない。また、一般的なリポソーム製剤と比較して大量投与を前提とする人工赤血球製剤の製造法としては、効率が極めて低い。

人工赤血球の粒子ひとつの性能を表すパラメータとして、単位脂質重量に対するHb重量の比が使われる。この値が高いほど、Hbに結合した酸素を効率よく運搬できることになる。そのためには、粒子の内水相のHb濃度をできるだけ高くすることが必要であり、要するに高濃度（赤血球内部と同等の35～45g/dL）のHb溶液中に複合脂質を分散させて、小胞体が形成される時にHbを濃度が高い状態で内包させることが要件となる。高濃度Hb溶液は粘度が高く、そこに嵩高い脂質粉末を分散させるとさらに粘度が高くなる。これをいわゆる押出し法（Extrusion Method）によって孔径の異なるフィルタを段階的に例えば、孔径3.0μm、0.8μm、0.6μm、0.45μm、0.3μm、0.22μmの順で透過させて粒子径を調節する方法をこれまで採用してきた（図-4）。しかしこの場合は、フィルタの交換が煩雑である上に、フィルタの目詰まりが起こり易い。それを回避するために、脂質を予め水溶液中で、リポソームを形成させて凍結乾燥して得られた粉末を使用する方法が知られている。しかし、水を凍結乾燥で除去する操作は極めて長時間を要し、またコストもかかり、産業化を考えた場合には効率が良いとはいえなかった。粘稠な濃厚Hb溶液に添加できる乾燥脂質の重量も攪拌効率や押出し法の効率の面で制約を受け、せいぜい6g/dLが上限であった（6gの脂質を1dLの濃厚Hb溶液に分散させること）。攪拌後に大量に発生する泡を消去するのに時間を要すること、また泡が蛋白質の変性を助長すること、脂質粉末が完全に分散せずに塊になって残存することも課題であった。

また、乾燥した複合脂質粉末を粘稠な濃厚Hb溶液に分散させる方法として、プロペラ式攪拌器を用いる方法は、脂質塊が形成されることがあり結果として長時間を要すること、また脂質粉末が

表-2　主なリポソーム調製法

方法名	操作
超音波処理法（プローブ法、浴槽法）	脂質粉末を機能物質の溶解した水溶液に超音波で強制的に分散させて小粒子径リポソームを形成させる。
有機溶媒注入法	予め脂質を有機溶媒に溶解させ、これを少しずつ機能物質の溶解した水溶液中に注入してリポソームを形成し、有機溶媒を最終的に排除させる。
界面活性剤除去法	界面活性剤を使って脂質分子を水中に分散させてリポソームを形成させたあと、透析などによって界面活性剤のミセルを除去する。
凍結融解法	凍結融解を繰り返すことによって、脂質二分子膜に亀裂を生じさせ、粒子径を小さくしたり、あるいは逆に融合させて大きくしたりする。この形態変化の際に、内水相に機能物質を内包させる。
逆相蒸発法	エーテルなど揮発性有機溶媒に脂質を溶解させ、水と混濁させたまま、徐々にエーテルを除去し、脂質を水中に分散させてリポソームを形成する。
押出し法	何らかの方法で脂質を機能物質の溶解した水溶液中に分散させてリポソームを形成させたあと、加圧して孔径の異なるフィルタに段階的に透過させることにより、粒子径を厳密に制御する方法。
乾燥リポソーム粉末－水和法	上述の何らかの方法でリポソーム分散液を調製したあと、何らかの保護剤を添加して凍結乾燥粉末とする。これを使用前に、機能物質の溶解した水溶液に分散させることによって、リポソーム内に機能物質を封入させる。
高圧乳化分散法	何らかの方法で機能物質の溶解した水溶液中に脂質を分散させたあと、高圧高速で対面に噴出させて衝突させてせん断応力を発生させ、それにより粒子径を小さくする。
混錬法	混合したい成分を充填した容器が自転しながら公転することにより、混合物に極めて高いせん断応力が生じ、混合を迅速に行う。粉末状脂質濃度が極めて高い状況で機能物質の溶解した水溶液と混合させて、リポソームを効率よく作成する。

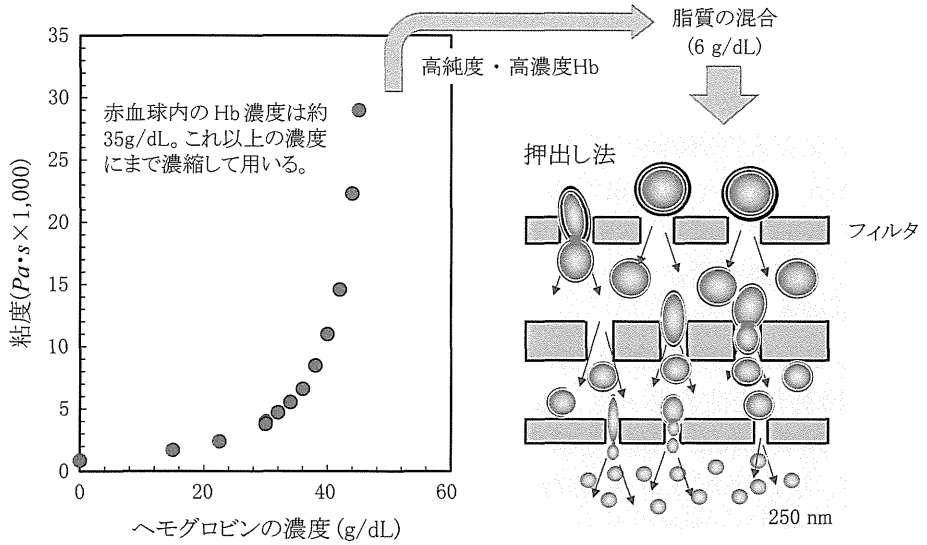

図-4 精製濃縮ヘモグロビン溶液の粘度と、押出し法による造粒のメカニズム

水和するときに発生する気泡は粘稠溶液中ではなかなか消えず、これが押出し法におけるフィルタの通過性を低下させることや、分散しきれなかった脂質塊がフィルタ上に残り損失となることも問題であった。Hbの回収率はせいぜい20%となり、内包されなかったHbは、再度回収して再濃縮して再利用するか、あるいは廃棄せざるを得ず、極めて効率の悪いものであった。

粘稠なHb溶液－複合脂質分散液を、高圧乳化分散法によって、高圧高速で対面に噴出させて衝突させてせん断応力を発生させ、それにより粒子径を小さくする方法が知られている。しかしこの方法では、せん断応力の調節が難しいこと、また、Hb脂質分散液を回路に通すためにある程度の流動性が必要であり、従って脂質の濃度を高めることができず、結果としてHbの回収率は低いものであった。

そこで我々は「混錬法」による人工赤血球の新しい製造方法を検討した。成分を収納した密閉容器が自転しながら公転もすることにより、容器内に極めて強いせん断力を生じ、粘稠な物質でも攪拌することができる。驚いたことに、攪拌されることで水系でもせん断応力により熱を発生する。この方法により濃厚系での混合攪拌が可能となり、結果としてヘモグロビンの回収率を従来よりも格段と高め、工程を簡略化し、操作時間を短縮でき、また、生体適合性を高めることもできるリン脂質小胞体（リポソーム）製剤の製造方法を提供することができた。効率よく「多量の嵩高い乾燥状態の複合脂質粉末」と「粘稠な濃厚ヘモグロビン溶液」を混合し、濃厚ヘモグロビン溶液を小胞体に内包し、かつ粒子径を調節し、かつヘモグロビンの回収率を高めることができる。人工赤血球の調製法として、乾燥脂質粉末を濃度高く濃厚Hb溶液に均一に分散させ、かつ粒子径を小さくして調節し、かつHbの回収率を高め、かつ操作中のHbの変性を抑制することのできる、混錬操作の原理を採用する方法を考案し、国際特許出願を完了した[7]。詳細については割愛するが、本方法は、リポソーム製剤の新しい製造法として期待できる。粒子ひとつひとつの酸素運搬機能を上げるには、やはり乾燥した複合脂質粉末を濃厚ヘモグロビン溶液と直接的に混合することが重要である。

5. おわりに（実用化への課題と期待）

表-1に記載した使用例のほか、人工赤血球は、移植臓器の保存灌流液としての利用、輸血拒否患者の対応、獣医領域への利用、レーザー治療のターゲットとしての利用など、さまざまな可能性が芽生えてきている。問題なのは、現段階で実用化に向けて製薬会社からの支援をなかなかいただけないことである。本製剤が従来にない範疇の製剤であること、投与量が数リットルに及ぶ大量投与を伴うが安全性が担保されるのか、また現在輸血で間に合っているのに何故その代用物が必要か、など消極的な意見を聞くこともある。今回は安全性評価試験については割愛させていただいたが、安全性を実証する実に多くの非臨床 *in vivo* 試験結果が得られており[4),5)]、次の段階に進むべき対象であると考えている。期限切れとなった献血液や、検査で不合格になった献血液は現在廃棄され

ているが、これらを有効利用することは重要な課題ではないだろうか。さらに大規模災害や有事の際の大量需要にどう対応するかを考えれば、やはり血液型がなく備蓄可能な人工赤血球が必要ではないか。また、今回紹介したように輸血では為し得ない疾患の治療や外科的手法にも用いることができる可能性があるし、献血技術が十分でない諸国への国際貢献も期待できる。

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5) 酒井宏水、堀之内宏久、東寛、小田切優樹、小林紘一：“輸血代替としての人工赤血球（ヘモグロビン小胞体）製剤の安全性試験”、人工血液、21、p. 36－48 (2013)
6) 野島庄七、砂本順三、井上圭三：“リポソーム”、南江堂 (1988)
7) 酒井宏水：“小胞体の製造法”、WO2012137834A1

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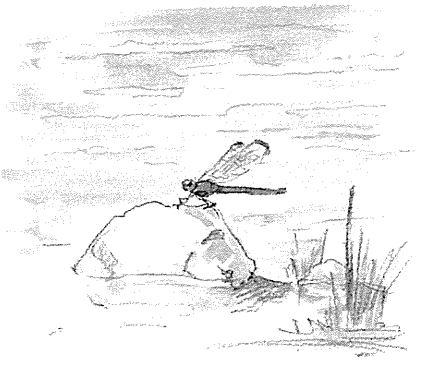


先生からのMessage

私は学生時代から、「高分子化学」を基盤とする人工赤血球製剤の研究に取り組んでいます。

奈良県立医科大学の化学教室では、化学の手法を駆使して医学・医療に役立つ研究を、さらに発展させていきたいと考えています。私の研究室では、自分で考え、設計したものを実際に合成、作製し、物理化学的な試験や動物投与試験を通して自ら検証・評価することができます。私たちの生活に深く関わる化学の視点で身の回りを見ることの面白さ、ものづくりの楽しさや興奮、奥深さを一緒に探究していきましょう。

日本が誇るMADE IN JAPAN

夢ナビ講義 No.06440

人工赤血球

人工赤血球で血液不足を解決する日がやってくる

奈良県立医科大学 医学部 化学教室 教授
酒井 宏水 先生

心配される将来の血液不足

日本は少子高齢化などにより、2027年には101万人分の輸血用血液が不足すると言われています。また献血により集められた血液は、冷蔵保存しても赤血球の変質や細菌の繁殖の可能性があるため、長く蓄えてはおけません。輸血を介したウイルス感染の可能性も完全には克服されていません。血液不足や災害など有事に備えて、安全な血液の確保が求められているのです。そこで、血液を人工的に作る研究が注目されています。

ヘモグロビンから赤血球を再生

アメリカ、ヨーロッパなど世界で人工血液の研究は行われていますが、日本はその研究をリードする国のひとつです。「人工赤血球」の研究では、ラットの全血液の90％を人工赤血球に置き換えることにも成功しています。

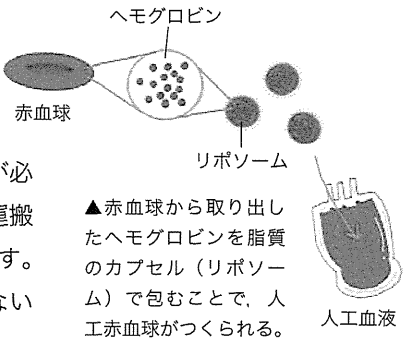
赤血球はヘモグロビンを濃度高く封入した細胞であり、肺で酸素を取り込んで全身の隅々にまで運搬する働きを担っています。赤血球の細胞膜表面は、糖の化合物でできた「糖鎖」で覆われており、糖鎖には血液型の違いを生み出す物質も含まれています。

人工赤血球を作る方法として確立されたのが、血液からヘモグロビンのみを精製し、細胞膜の代わりに脂質の合成化合物で作った化学的に安定なカプセルでヘモグロビンを覆うというやり方です。ヘモグロビンは保存期限が切れた献血血液から精製します。精製の段階で加熱と濾過により細菌やウイルスは除外されます。さらに、糖鎖のある細胞膜も完全に排除するので、血液型を問わずにいつでもどこでも投与できます。つまり、廃棄される血液を有効利用し、感染の心配がなく、長期保存ができ、誰にでも投与できる赤血球に「再生」することができるのです。

脳梗塞や心筋梗塞の治療にも活用

人工赤血球は輸血の代わりとしての利用だけではなく、迅速な酸素供給が鍵となる脳梗塞や心筋梗塞の初期段階の治療、移植用の臓器の保存などへの活用も期待されています。まだいくつかの壁はありますが、将来は医療機関に人工赤血球製剤を常備する日がやってくるかもしれません。

ヘモグロビンの再利用で、赤血球の寿命を延ばす



▲赤血球から取り出したヘモグロビンを脂質のカプセル（リポソーム）で包むことで，人工赤血球がつくられる。

事故でケガをしたときや，大きな手術をするときなど，大量の血液が必要になるときには「輸血」が行われます。血液成分のなかでも，酸素運搬という重要な機能を果たしている「赤血球」は，補うべき重要な成分です。しかし，輸血用の赤血球は，日本ではたったの３週間しか保存できないのです。

からだ中で働く酸素の配達員

赤血球は，骨髄などでつくられた造血幹細胞から生まれます。少しずつ性質やかたちを変えながら成熟していき，最後に細胞の中心ともいえる核を放出して赤血球へと変身するのです。その中に含まれるたくさんのヘモグロビン分子が，酸素と結合したり離れたりすることで，私たちのからだの隅々にまで酸素を送り届けています。

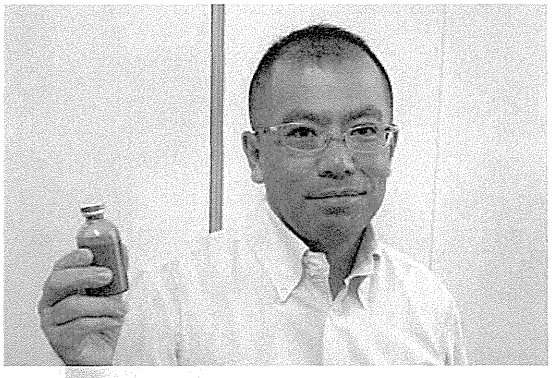
からだの中では赤血球の寿命は120日ですが，献血用に取り出したものはたったの21日間しか保存できません。それ以上は，赤血球が分解されて溶け出すヘモグロビンによって毒性が出てしまったり，細菌が増殖したりするため，献血液として使うことができなくなるのです。

脂質の膜でヘモグロビンを包む

そこで注目されているのが，より扱いやすく長期保存ができる「人工赤血球」の研究です。その中でも奈良県立医科大学の酒井宏水さんが取り組んでいるのは，ヘモグロビンの「再利用」。使用期限が切れてしまった血液から，ヘモグロビンだけをきれいに取り出して，リポソームと呼ばれる人工的につくった小さな脂質の膜に閉じ込めるのです。「赤血球という『生モノ』を人工の『モノ』に変換するので，ウイルスや細菌がなく，血液型もなく，室温で２年以上保存でき，安全性も扱いやすさも格段に高まりました」。すでに，ラットの血液の90%を，この人工赤血球に置き換えても生存可能なことを確認済みです。

条件の厳しさは期待の裏返し

大掛かりな手術では，１回で数Ｌもの量の血液が必要になります。大量に使うことを想定して，安全性は必要以上に確認しなければなりません。複数回の投与を行うと何が起こるのか，脳内出血や肝硬変，免疫異常など病気の個体に投与しても問題ないか……あらゆる場面を想定し，マウスやラットを使った安全性の検証が進められています。社会的なインパクトも大きいからこそ，求められる条件も高くなってきます。でも，近い未来，私たちのからだの中を人工赤血球が駆けめぐる日が来るかもしれません。　　（文・石澤 敏洋）



協力：酒井　宏水（さかい　ひろみ）
奈良県立医科大学医学部医学科教授。1994年，早稲田大学理工学研究科博士課程修了。博士（工学），博士（医学）。早稲田大学理工学総合研究センターや慶應義塾大学総合医科学研究センター，早稲田バイオサイエンスシンガポール研究所などでの研究経験を経て，2013年４月より現職。

移植・人工臓器

人工赤血球(ヘモグロビンベシクル)の実現に向けて

Artificial red cells(hemoglobin vesicles)for future use

日本の献血・輸血システムの安全性は世界最高水準にあり，現行の医療に不可欠である．しかし，高齢化社会により献血液の需給バランスが崩れることが懸念されているし，緊急時や大規模災害時の救命医療においては，血液型検査，感染の可能性，短い保存期限などが危機管理体制の不安要素になりうる．人工赤血球(ヘモグロビンベシクル；Hb-V)は感染源を含まず，血液型がなく，長期間備蓄でき，輸血治療を補完する技術として開発されてきた(図1)．受傷現場で即座に投与できれば，救命率を格段に向上させることにつながるものと期待される．さらに，脆弱な赤血球と比べ化学的に安定で，物性値の調節が可能なので，輸血では対応のできない疾患の治療や外科的治療への可能性も検討されている．

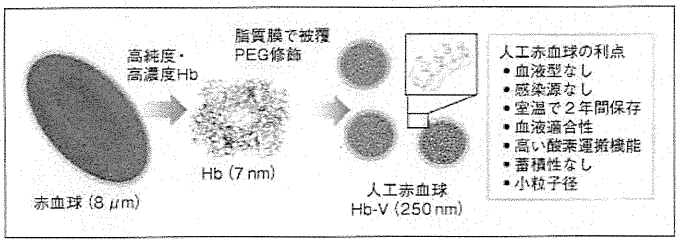


図 1　人工赤血球(ヘモグロビンベシクル)の概略と利点

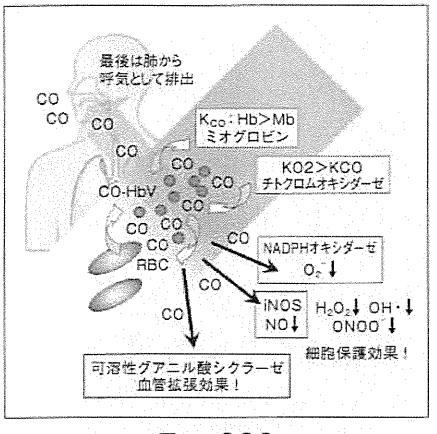


図 2　●●●

血管内において，CO-HbVから放出されたCOは赤血球とも交換する．ミオグロビンなどのヘム蛋白質にも結合する．活性酸素産生に関連するヘム蛋白にも作用することで細胞保護効果が得られるのではと考えている．肝では血管拡張効果を示す．最終的に呼気として体外に排出される．

O_2運搬効果，CO運搬効果

高純度高濃度ヒトHb溶液を脂質膜で被覆して小胞体(liposome)とし，粒子表面のPEG修飾と脱酸素化により長期間の室温備蓄が可能である．また，脂質膜で被覆することでHbの毒性を遮蔽できる．出血性ショック，あるいは制御不能出血モデルに対する投与では，赤血球輸血と同等の酸素運搬(蘇生)効果を示すことを明らかにしてきた．微小循環系では血管の分岐点で血流速に違いがあるとPlasma skimming(血漿分離)が起き，軸集中して流動する赤血球の配分に著しい偏りが生じる(ヘマトクリット値が異なる)ことがある．しかし，Hb-Vは微粒子(250 nm)のため血漿層に均一に分散して流動し，血漿の流れさえあれば着実に酸素を運搬できる．この現象は，虚血性疾患など，血液循環の悪化した組織の酸素化に有効である．また，臓器保存液としての効能も明らかになった．マウス摘出小腸の動脈からHb-Vを灌流させることにより蠕動運動が2時間以上継続する．ラット下肢を切断してHb-Vを6時間灌流させたあと再接着すると，壊死することなく生着し，機能の復元も認められた[1]．他方，一酸化炭素(CO)を結合したHb-Vの投与ではCOの徐放により細胞保護効果を示し，出血性ショック蘇生モデルにおける再灌流傷害の低減[2]，また突発性肺線維化症モデルで緩和効果が認められた[3]．これらはCOが活性酸素産生に関与するヘム蛋白質と相互作用するためと考えている．COを徐放した後，Hb-Vは肺を通過するときに酸素を結合し，酸素運搬体として機能し，またCOは最終的には肺を通って呼気として排出される(図2)．このように，新しい使用法がいくつも明らかになった．

血中機能寿命の延長

血管内においてHb-VはHbO_2の自動酸化によりmetHbが漸増し，酸素運搬機能が低下することが課題とされていた．最近，脂質膜透過性の電子メディエータを併用することにより赤血球解糖系が産生する電子エネルギーをもとにmetHbを還元できることがわかった[4]．赤血球内のNAD(P)Hが電子供与体であり，酸化型$NAD(P)^+$は解糖系により繰り返し充電され，還元型になる．Hb-VにはmetHb還元酵素はないが，赤血球内の解糖酵素系を活用する，いわば間接的酵素的metHb還元系が構築できたといえる．これによって血中における機能寿命が大幅に延長できた．

おわりに

Hb-V製剤の動物投与試験の結果は，ほぼすべてが学術誌に公表されている．最近，カプセル化工程が混錬法によって簡略化され，効率よく製造できるようになった．Hb-Vは完成度の高い医薬品の候補物質であると考えているが，本製剤の必要性が十分に認識されず，製薬企業の協力がなかなか得られない状況が続いている．しかし，危機管理対策として人工赤血球が有用であることは明白であり，アカデミアを中心に他関連機関の協力も得て実用化に向けた取組みを続けている．

1) Araki, J. et al.：*Transplantation*. (in press)
2) Sakai, H. et al.：*Shock*, **31**：507-514, 2009.
3) Nagao, S. et al.：*Biomaterials*, **35**：6553-6562, 2014.
4) Sakai, H. et al.：*Bioconju. Chem.*, **25**：1301-1310, 2014.

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(12) **Patent Application Publication**

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(43) **Pub. Date: Feb. 6, 2014**

(54) **METHOD FOR PRODUCING VESICLES**

(75) Inventor: **Hiromi Sakai**, Tokyo (JP)

(73) Assignee: **WASEDA UNIVERSITY**, Tokyo (JP)

(21) Appl. No.: **14/009,736**

(22) PCT Filed: **Apr. 4, 2012**

(86) PCT No.: **PCT/JP2012/059233**

§ 371 (c)(1),
(2), (4) Date: **Oct. 3, 2013**

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Publication Classification

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A61J 3/00 (2006.01)

(52) **U.S. Cl.**
CPC ***A61J 3/00*** (2013.01)
USPC **514/786**; 264/4.3

(57) **ABSTRACT**

Provided is a method for producing vesicles which comprise a lipid as a main component and which encapsulate a functional substance therein. The method includes the steps of (a) putting the functional substance, lipid and water in a cylindrical container; and (b) producing the vesicles encapsulating the functional substance in lipid vesicles which comprise the lipid as a major component and which encapsulate the functional substance therein, by kneading the contents of the container with simultaneous rotational movement of the container around its center axis together with revolutionary movement of the container about a predetermined axis of revolution.

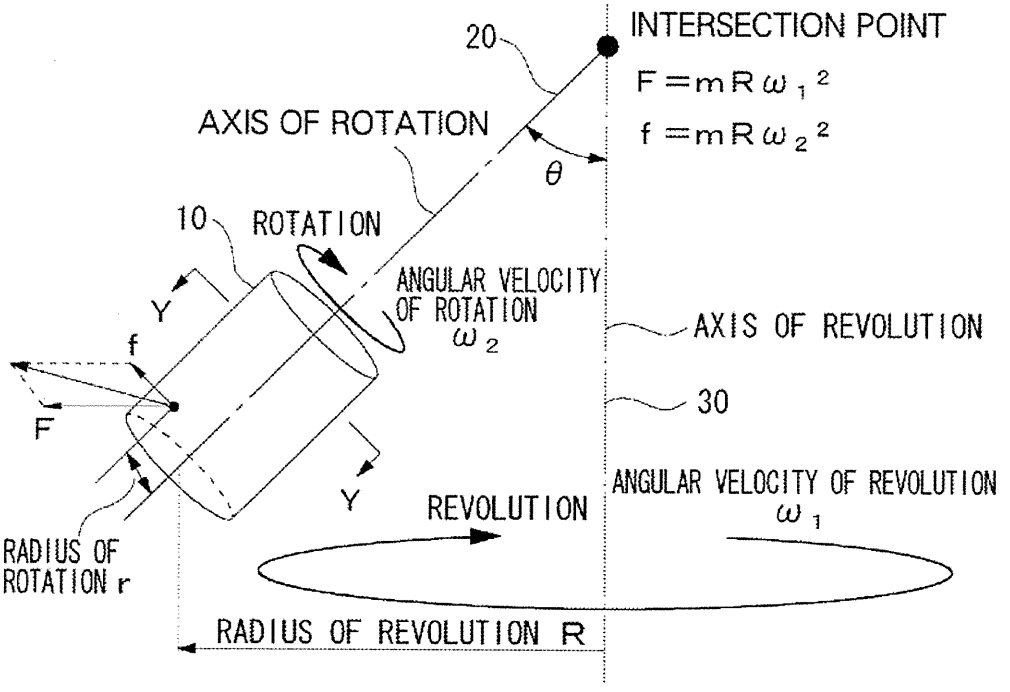
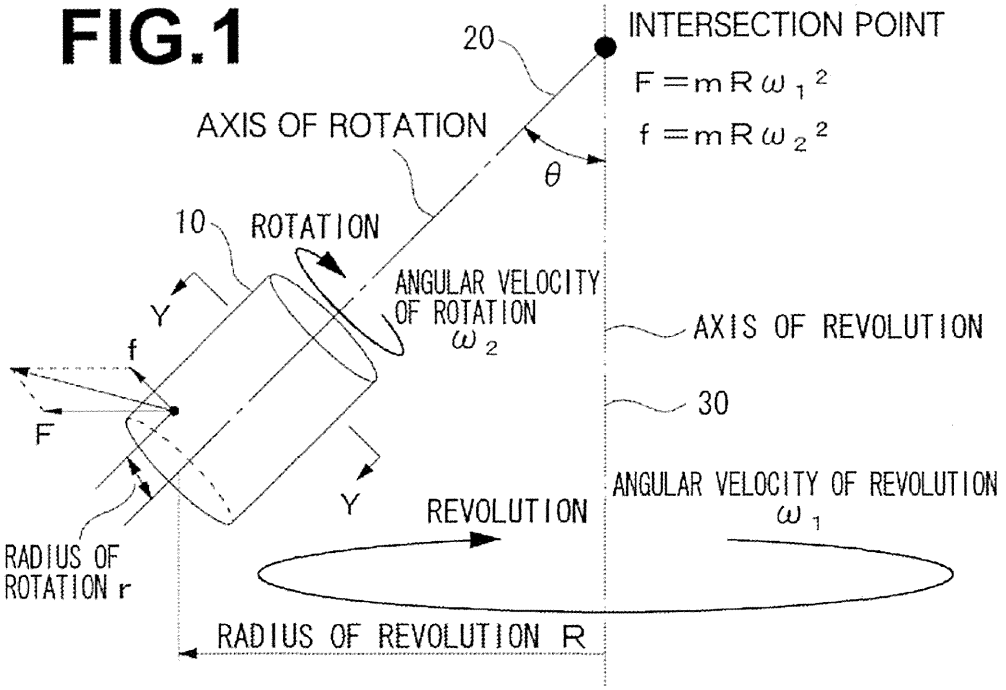


FIG.1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INTERSECTION POINT
$F = mR\omega_1^2$
$f = mR\omega_2^2$
AXIS OF ROTATION
θ
10
ROTATION
ANGULAR VELOCITY OF ROTATION ω_2
AXIS OF REVOLUTION
Y
f
F
30
Y
REVOLUTION
ANGULAR VELOCITY OF REVOLUTION ω_1
RADIUS OF ROTATION r
RADIUS OF REVOLUTION R

FIG.2

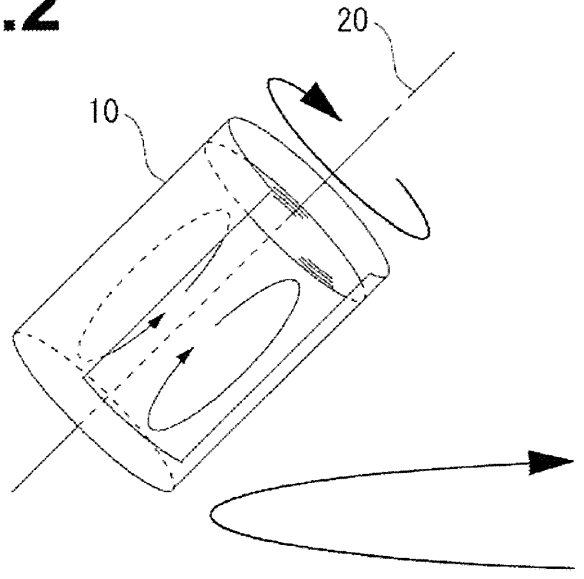
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FIG.3

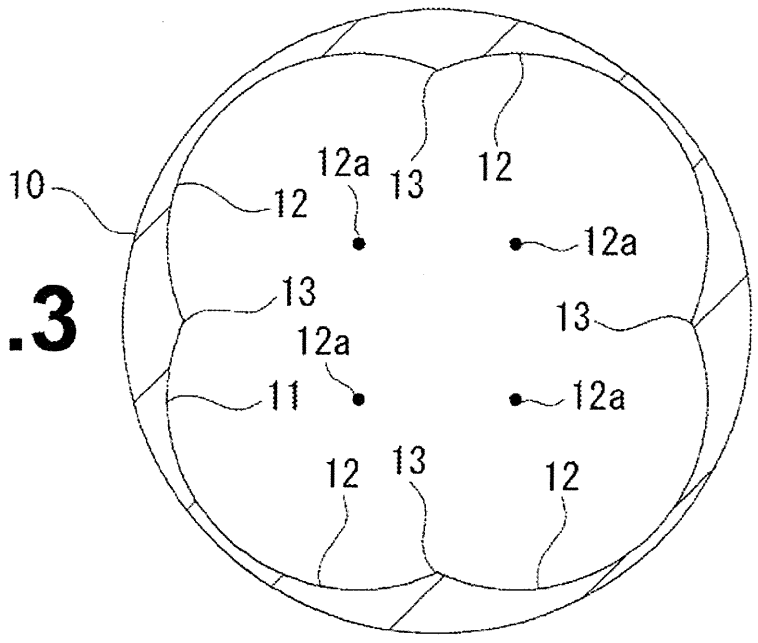
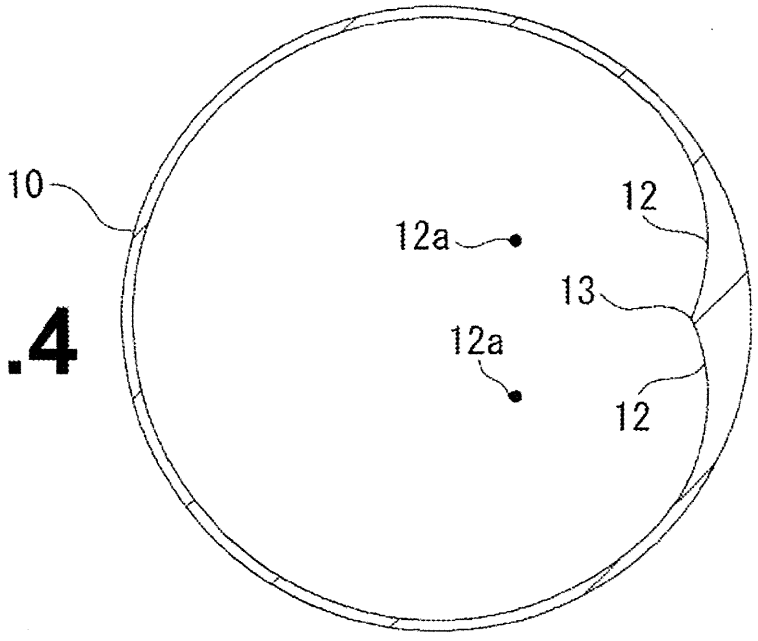


FIG.4



METHOD FOR PRODUCING VESICLES

FIELD OF THE INVENTION

[0001] The present invention relates to a method for producing a phospholipid vesicle (liposome) preparation. Specifically, the present invention relates to a method for producing a dense suspension of an artificial oxygen carrier (hemoglobin vesicle) with greater efficiency than had been conventionally possible.

DESCRIPTION OF THE RELATED ART

[0002] The current system for blood donation and transfusion has been established as an indispensable technique for clinical medicine. With many problems (infection, shelf life, decrease in the number of blood donors, and so on), however, it is an urgent challenge to find an alternative to the donated blood. Especially needed is the development of an alternative to red blood cells, which have an oxygen-carrying function. As hemoglobin (Hb), which is contained in red blood cells at a high concentration, is an oxygen-binding protein, an artificial red blood cell using hemoglobin has been developed. It is possible to completely remove pathogens and blood group antigens from hemoglobin by rigorous purification from red blood cells. Versions of chemically modified hemoglobins (those bound to water-soluble polymers, those with intramolecular crosslinks, and those which are polymerized) as well as hemoglobin vesicles and the like which encapsulate highly purified hemoglobin in the inner aqueous phase of phospholipid vesicles or polymer capsules have been known as artificial oxygen carriers that utilize hemoglobin. Reviewing global trends, the development of modified hemoglobin solutions took a lead, and some of them reached the final phase of clinical trials. Series of development projects have been abandoned due to unexpected adverse effects and selection is still in progress. This is because they lack a physiologically significant structure of the red blood cell. The red blood cell is a discoid and biconcave particle of approximately 8 micrometers in diameter with a depressions at the center thereof, which has highly concentrated (about 35%) hemoglobin (molecular weight: 64,500), which is toxic in nature, encapsulated within the red blood cell membrane. Chemical modification of hemoglobin alone cannot eliminate hemoglobin toxicity completely (Non-patent Document 1; Sakai H. and Tsuchida H. (2009) Pharmacia, 45: 23-28 (in Japanese)).

[0003] Historically, it was reported in the late 1960's that self-assembly of an amphiphilic molecule, phospholipid, in water results in a bilayer membrane, which composes a vesicle structure (phospholipid vesicle, liposome). Since the late 1970's, attempts have been made to encapsulate hemoglobin in the phospholipid vesicle. Such encapsulation was not realized, however, because preparation was difficult such as control of vesicle size, and because there were not sufficient means to prevent aggregation caused by interaction with blood plasma proteins (Non-patent Document 2; Djordjevich L. and Miller I. F. (1977) Fed. Proc., 36: 567). Later, however, a technique to cover a highly concentrated solution of hemoglobin with phospholipid bilayer membrane employing an extrusion method, and control of vesicle size such that vesicles could flow, especially through blood capillaries, as well as improvements in dispersion stability were achieved, and a hemoglobin vesicle was reported as being produced (Non-patent Document 3; Sakai H, Hamada K, Takeoka S, Nishide H, Tsuchida E. (1996) Biotechnol. Prog., 12: 119-125). It was possible to suppress aggregation among vesicular particles and to increase the dispersion stability by disposing polyethylene glycol on particle surfaces. It was also possible to preserve the vesicle in a liquid state for a long period of time at room temperature by removing oxygen completely from the suspension (Patent Document 1; Japanese Patent No.: 3466516). Animal testing were conducted and the safety of the hemoglobin vesicle as an alternative to blood transfusion and the details of oxygen-carrier functions were clarified (Non-patent Document 4; Sakai H, Sou K, Horinouchi H, Kobayashi K, Tsuchida E. (2008) J. Intern. Med., 263: 4-15). So far, its use as a resuscitation fluid for hemorrhagic shock, as a supplementary administration (dilution of blood) to patients with repetitive bleedings during surgical operations, as a prime fluid for extracorporeal circulation circuits (artificial heart-lung machines) and the like have been examined and the hemoglobin vesicle has been demonstrated to have a sufficient efficacy as an oxygen carrier. For diseases which cannot be treated by transfusions, prevention of expansion of infarct zone by administering to models of cardiac and cerebral infarction, supply of oxygen in ischemic region of pedicled flap, improvement of the efficacy of radiation therapy by oxygenation, and use as an oxygen source for an organ to be transplanted and for cultured cells, have been examined (Non-patent Document 5; Tsuchida E, Sou K, Nakagawa A, Sakai H, Komatsu T, Kobayashi K. (2009) Bioconjug. Chem., 20: 1419-40).

DISCLOSURE OF THE INVENTION

[0004] Known methods for preparing phospholipid vesicles include ultrasonic irradiation, reverse-phase preparation with organic solvents, dispersion with a surfactant followed by subsequent removal with dialysis, and the like (Non-patent Document 6; *"Liposomes,"* ed. by Nojima S., Sunamoto J., Inoue K. (1988) Nankodo, Tokyo Japan). For the manufacture of a preparation comprising a functional protein such as hemoglobin of which administration into blood vessels is presumed, however, these methods are not suitable because of concerns regarding protein denaturation during the manufacturing process and regarding the presence of residual substances. In addition, in view of the large dose to be administered compared to liposome preparations in general, these methods are extremely inefficient in producing an artificial red blood cell preparation. The ratio of hemoglobin weight per unit lipid weight is employed as a parameter representing the performance of an individual particle of the artificial red blood cell. The higher the value of the parameter, the more efficiently the hemoglobin-bound oxygen can be delivered. To this effect, it is necessary to set the hemoglobin concentration in the inner aqueous phase of the particle as high as possible. More specifically, it is required to disperse complex lipid in a solution of highly concentrated hemoglobin (e.g. 35-45 g/dL) and to encapsulate the highly concentrated hemoglobin while the vesicles are formed. The solution of highly concentrated hemoglobin is viscous and as lipid powder is dispersed in it, it will become more viscous.

[0005] In case the particle size is controlled according to the so-called Extrusion Method by passing through filters with different pore sizes in a stepwise manner (for example, passing through MF filters of EMD Millipore Corporation with pore sizes of 3.0 micrometers, 0.8 micrometers, 0.6 micrometers, 0.45 micrometers, 0.3 micrometers, and 0.22 micrometers in this order), changing the filters is tedious and the filters tend to clog. To circumvent these problems, it has

been known to use freeze-dried powder of vesicles which have been formed with the lipid in the aqueous solution (Non-patent Document 7: Sou K., Naito Y., Endo T., Takeoka S., Tsuchida E. (2003) Biotechnol. Prog., 19: 1547-1552; Patent Document 2; Japanese Patent Application No. 2000-344459 (WO2002/038128)). However, removing water by freeze drying takes an extremely long time and costs are high. Thus, with industrial implementation in mind, the low efficiency has been a problem. Furthermore, dried vesicles of small particle sizes may be included and, after dispersion in the hemoglobin solution, may still persist without sufficiently encapsulating hemoglobin. Considering the efficiency of mixing and extrusion, there was a limitation in the weight of dry lipid that can be supplemented in the viscous and dense solution of hemoglobin. The upper limit was 6 g/dL at most (meaning that 6 g of lipid is dispersed in 1 dL of dense hemoglobin solution). There were also problems that, after the mixing, it takes a long time to remove a large amount of foam which is formed, that the foam contributes to denature proteins, and that the lipid powder remains as lumps without being dispersed completely.

[0006] As a method for dispersing dried powder of complex lipid into the viscous and dense solution of hemoglobin using a blade agitator, it has been an additional problem that it takes a long time to implement the method because lipid lumps may sometimes be formed, that the foam formed during hydration of the lipid powder hardly disappears in the viscous solution, reducing the filtration rate in the extrusion procedure, and that the lipid lumps, which could not be dispersed, remain on the filter and result in loss. Recovery yield for the hemoglobin was 20% at best. The hemoglobin which was not included in the vesicles had to be recycled by recollection and re-concentration, or had to be discarded. Thus, this method was extremely inefficient.

[0007] Another known method comprises a step of reducing particle size by generating shear stress by causing collision of opposing flows of viscous solutions of hemoglobin dispersed with complex lipid at high velocity and pressure (Non-patent Document 8; Beissinger R. L., Farmer M. C., Gossage J. L. (1986) ASAIO Trans., 32: 58-63). In this method, however, it was difficult to control the shear stress. In order to pass the solutions of hemoglobin dispersed with lipid through a circuit, the solutions were required to have some degree of fluidity, and therefore, the lipid concentration had to be reduced to about 6 g/dL. As a result, the recovery yield of hemoglobin was as low as about 20%.

[0008] Still another known method comprises the steps of forming a paste by emulsifying lipid powder with a small volume of water to become hydrated and swell, and encapsulating hemoglobin by mixing the paste with a solution of hemoglobin at a high speed to form an emulsion (Patent Document 3; Japanese Published Patent Application No.: 2009-035517). When the dried lipid was hydrated with the small volume of water, it was possible that vesicles were formed. After mixing with the hemoglobin, the vesicles may remain empty (only water inside) without encapsulating the hemoglobin. As a result, the hemoglobin could not be encapsulated efficiently. Thus, reduced encapsulation efficiency is expected.

[0009] The present invention was developed in view of the above-mentioned problems. An object of the present invention is to provide a novel method for producing a preparation of phospholipid vesicles (liposomes).

[0010] Another object of the present invention is to provide a method for producing efficiently a dense solution of a dispersed artificial oxygen carrier (hemoglobin vesicle) which is used, especially in the medical field, as an alternative to blood transfusions.

[0011] A further object of the present invention is to provide a method for producing a preparation of phospholipid vesicles (liposomes) which increase the recovery yield of hemoglobin remarkably better than had been conventionally possible, with more simplified processes, shorter operation time, and higher biocompatibility.

[0012] In order to enhance oxygen carrier performance of an individual particle, it is also important to mix dried complex lipid powder directly with a dense hemoglobin solution. Thus it is also an object of the present invention to provide an efficient method for mixing "a large amount of bulky powder of dried complex lipid" with a "viscous and dense solution of hemoglobin," encapsulating the dense solution of hemoglobin inside the vesicles, controlling the particle size and increasing the recovery yield of hemoglobin.

[0013] The phospholipid vesicle first contacts blood components after administration into a blood vessel. It has been known that serious complement activations are triggered depending on the lipid composition of the phospholipid vesicle, affecting hemodynamics (Non-patent Document 9; Szebeni J. (2005) Toxicology, 216: 106-121). It has been understood that a small amount of negatively-charged lipid is effective in encapsulating hemoglobin into phospholipid vesicles efficiently. It was reported, however, that some of the negatively-charged lipids can trigger a complement activation and that whether the complement reaction is triggered depends on the molecular species of the negatively-charged lipids. Therefore, it is important to select a particular species of the negatively-charged lipids. In addition, as the major lipid component of phosphatidylcholine-type phospholipids, 1,2-dipalmitoyl-sn-glycero-3-phosphoatidylcholine (DPPC) and hydrogenated lecithin derived from soybeans (HSPC) are frequently used. Their gel-liquid phase transition temperatures are as high as 41° C. and 50-56° C., respectively. During the course of completion of the present invention, it was realized that higher energy (shear stress, agitation speed) is required to control the particle size by dispersing in the dense hemoglobin solution complex lipid comprising lipid with a higher phase transition temperature. It was required to select a lipid with a lower phase transition temperature (below 30° C.). The lipid membrane composition of phospholipid vesicles is heterogeneous with multiple components. Thus, it is an object of the present invention to select each of the components and to determine their compound ratio.

[0014] Considering the above-mentioned background and problems and studying diligently, the inventors of the present invention has conceived of a method for preparing an artificial red blood cell by adopting the principle of kneading operations, to disperse dried lipid powder homogeneously in a dense hemoglobin solution, to form a small vesicle, to control the particle size, to increase recovery yield of hemoglobin, and to suppress denaturation of hemoglobin during the operation. For example, although a mixer/deaerator (Patent Document 4; Japanese Patent No.: 3860770; Patent Document 5; Japanese Patent No.:2711964) is being used to prepare a kneaded material (a paste-like material in which a small amount of liquid is mixed with a large amount of powder), the present inventors discovered that the mixer/deaerator has a function other than simple mixing. The present inventors also

focused on the point that the machine can carry out the operation efficiently. Basically, the machine carries out planetary movement of simultaneously revolving while rotating a cylindrical container in which a sample to be kneaded is included, so that the sample can be homogeneously mixed and foam can be destroyed by the centrifugal force of revolution. By kneading in a closed container, it is possible to maintain a sterile atmosphere. In one embodiment of the present invention, a larger amount of lipid powder than before can be added and dispersed in a hemoglobin solution, and a solution of dispersed hemoglobin vesicle can be prepared efficiently. In addition, it is possible not only to disperse the lipid but also to control the particle size of the vesicles which are formed by the self-assembly of lipid molecules. As a strong shear stress is generated, it is important to pretreat the hemoglobin to be bound to carbon monoxide or to be transformed as deoxyhemoglobin in order to stabilize the hemoglobin at divalent iron state. In the prior art, it has been known that binding of carbon monoxide to hemoglobin enables viral inactivation by heating treatment (low temperature pasteurization) during the purification process of hemoglobin (Patent Document 6: Japanese Patent No.: 3331433), that a transfusion preparation has a cytoprotective effect by administering hemoglobin vesicle which is bound to carbon monoxide (Patent Document 7: Japanese Published Patent Application No.:2007-269665), and that a transfusion preparation can be stored for a long time by transforming to deoxyhemoglobin (Patent Document 8: Japanese Patent No.: 3466516). The present invention is the first to use this technique to prevent denaturation during the kneading operation.

[0015] Conventionally, examples of application of kneading machines (mixer/deaerator) include: gold/silver/carbon paste and the like for electrical conducting/resistor materials; glass/ceramic paste and the like for sealing/insulating materials; epoxy resin and the like for sealing materials/adhesives; LED fluorescent agent silicone resin and the like for molding materials; ink/paint/pigment and the like for mixing different coloring materials; dental materials/pharmaceutical ointment materials/cosmetic base materials and the like for manufacturing; precision parts and the like for abrasive purposes; and for emulsifying oil and water such as in mayonnaise which are immiscible in nature, in the food industry. There has been no example, like the present invention, of dispersing lipid molecules to form molecular assembly or using a kneading machine to prepare a liposome for i.v. injection. The reason is presumably because, in the conventional so-called liposome preparation, there has been no need to encapsulate such a high concentration of solution, or at such a high efficiency, or to administer such a high dose. It was first reported in 1977 (Non-patent Document 2: Djordjevich L. and Miller I. F. (1977) Fed. Proc., 36: 567) that hemoglobin is encapsulated in phospholipid vesicles (liposomes). Thirty-four years have passed since then. Development of kneading machines based on the planetary movement of containers dates back to earlier than 1992 (Patent Document 5: Japanese Patent No.: 2711964), from which nineteen years have passed. During this period, as there has been no example of the kneading technique being used for the preparation of hemoglobin vesicles, it is apparent that the present invention could not be readily conceived of by those skilled in the art.

[0016] Besides the above-mentioned mixer/deaerator apparatus (e.g. manufactured by THINKY CORPORATION, KURABO INDUSTRIES LTD., etc.), apparatuses are known that have an kneading effect by a rotation and revolution movement (planetary movement) with two twisted open frame type blades as well as an action of impact and shear by a turbine blade (PD Mixer, Planetary Mixer, etc. manufactured by INOUE MFG., INC.), thus these apparatuses may be used as well with an effort to control the pressure for destroying the foam, an effort to suppress foaming, and an effort to generate enough shear stress.

[0017] As for the phosphatidylcholine-type phospholipids, which are the major component, it is easy to disperse complex lipid powder, to promote the forming of vesicles, and to control particle size by using one which has the lowest possible phase transition temperature. Conventionally, a negatively charged lipid had been required in order to increase encapsulation efficiency. However, it is possible to increase the biocompatibility with 1,5-O-dihexadecyl-N-succinyl-L-glutamate (Non-patent Document 10: Sou K., Tsuchida E. (2008) Biochim. Biophys. Acta, 1778: 1035-1041). As such, it is extremely critical to select the components and composition of the phospholipid vesicle as multicomponent complex.

[0018] In case the particle size of the hemoglobin vesicle is about 200-300 nm, it may be necessary to apply some pressure for the hemoglobin vesicle to pass through a sterile filter with a pore size of 0.22 micrometers, so that the sterility could not be guaranteed. In such a case, beta-propiolactone is to be supplemented in the final step of the process (Non-patent Document 11: LoGrippo G. A., Wolfran B. R., Rupe C. E. (1964) J.A.M.A., 187: 722-766). As this reagent may induce protein denaturation according to the principle of sterility, it is important to stabilize hemoglobin beforehand by the above-mentioned methods. As beta-propiolactone is carcinogenic in nature, it is necessary to confirm that the residual compound is completely inactivated by hydrolysis.

[0019] To achieve the above-mentioned objects, according to other major aspects of the present invention, a method is provided for producing vesicles encapsulating a functional substance comprising the steps of: (a) containing the functional substance, lipid and water in a cylindrical container; and (b) producing lipid vesicles which comprise the lipid as a main component and which encapsulate the functional substance therein, by kneading the contents of the container with simultaneous movements of rotating the container around its center axis together with revolving the container about a predetermined axis of revolution.

[0020] In addition, according to an embodiment of the present invention, a method is provided for producing vesicles, wherein the contents contained in the cylindrical container in step (a) of the above-mentioned method are prepared by adding the lipid in an aqueous solution comprising the functional substance. Furthermore, a method is provided for producing vesicles, wherein the content contained in the cylindrical container in step (a) of the above-mentioned method is prepared by dispersing a lipid powder in the water, the lipid powder being obtained by drying the lipid comprising the functional substance. Also, a method is provided for producing vesicles, wherein the functional substance is hemoglobin and the aqueous solution of step (a) is prepared by adding 15 g or more of a complex lipid powder as the lipid per 1 dL of an aqueous solution of hemoglobin in which 30-50 g/dL of hemoglobin is dissolved. Also, a method is provided for producing vesicles, wherein in step (b) of the above-mentioned method the aqueous solution is kneaded by revolving and rotating the cylindrical container at a rate of revolution for the cylindrical container of 200-300 rpm and at a rate

of rotation for the cylindrical container of 100-3000 rpm. Also, a method is provided for producing vesicles further comprising the steps of: (c), after step (b), adding water or saline to a liquid or paste in the cylindrical container; and (d), after step (c), reducing the viscosity of the liquid or paste in the cylindrical container by further rotating the container around the center axis together with revolving the container about the predetermined axis of revolution. Also, a method is provided for producing vesicles further comprising a step (e), after step (d), removing the functional substance which is not encapsulated in the lipid by applying an ultrafiltration membrane technique or ultracentrifugation technique to the fluid or paste in the cylindrical container.

[0021] In addition, according to an embodiment of the present invention, a method is provided for producing vesicles, wherein in step (b) of the above-mentioned method, the step of kneading the aqueous solution by rotating the cylindrical container around the center axis together with revolving about the predetermined revolution axis is implemented multiple times, and during the interval between two consecutive kneading steps, a cooling treatment is performed to cool down the fluid or paste by stopping at least one of rotation and revolution of the cylindrical container or by reducing the rate of at least one of rotation and revolution of the cylindrical container. Also, a method is provided for producing vesicles, wherein the cylindrical container of the above-mentioned method has multiple concave-curved surfaces on the inner periphery of its sidewall and the centers of curvature of the adjacent concave-curved surfaces are at different positions, whereby a convex-shaped crest that protrudes toward the interior of the cylindrical container is formed between the adjacent concave-curved surfaces.

[0022] To achieve the above-mentioned objects, according to other major aspects of the present invention, a method is provided for producing vesicles in which a functional substance is encapsulated by lipid, comprising the steps of: (a) adding lipid to an aqueous solution of the functional substance in which the functional substance is dissolved in water, wherein the viscosity of the aqueous solution of the functional substance is 4 cP or higher as measured at 23° C. under a condition of shear velocity at 1000 s^{-1}; and (b) encapsulating the functional substance with the lipid by kneading a mixture prepared in step (a), wherein the viscosity of the kneaded mixture is 1000 cP or higher as measured at 23° C. under a condition of shear velocity at 1000 s^{-1}.

[0023] In addition, according to an embodiment of the present invention, a method is provided for producing vesicles, wherein the hemoglobin is carbonyl hemoglobin with its heme at a divalent iron state or deoxyhemoglobin with its heme at a divalent iron state. Also, a method is provided for producing vesicles further comprising a step of removing contaminating unstable proteins by denaturation with a treatment that heats the aqueous solution of hemoglobin to 50° C. or higher for five hours or longer before adding the lipid to the aqueous solution of hemoglobin, and removing the contaminating unstable proteins with an ultrafiltration membrane or centrifugation, wherein the removing step is implemented in order to reduce the occurrence of insoluble matter of the denatured protein in the course of the kneading treatment in step (b). Also, a method is provided for producing vesicles, wherein the lipid in the above-mentioned method is comprised of a phosphatidylcholine-type phospholipid, cholesterol, a negatively-charged lipid and a lipid bound with polyethylene glycol. Also, a method is provided for producing vesicles, wherein the lipid is comprised of a phosphatidylcholine-type phospholipid of 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine, cholesterol, a negatively-charged lipid of 1,5-O-dihexadecyl-N-succinyl-glutamate, and a lipid bound with polyethylene glycol of 1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine-N-poly(oxyethylene)5000 (molecular weight of the polyethylene glycol chain: 5,000). Also, a method is provided for producing vesicles, wherein the gel-liquid phase transition temperature of the phosphatidylcholine-type phospholipid is 30° C. or lower. Also, a method is provided for producing vesicles, comprising a step of producing dried lipid powder from a lipid lamellar gel as the lipid of the above-mentioned method, wherein the step of producing the dried lipid powder has steps of producing an aqueous solution of the lipid by adding 15 g/dL or more of the lipid powder in pure water which is comprised of substantially no solute; kneading the aqueous solution of lipid within a cylindrical container, rotating the container around its center axis together with revolving the container about a predetermined axis of revolution, and obtaining the dried lipid powder from the lipid lamellar gel by freeze-drying the kneaded aqueous solution of lipid.

[0024] To achieve the above-mentioned objects, according to other major aspects of the present invention, a method is provided for producing vesicles, wherein lipid molecules are dispersed in an aqueous solution by kneading a 15 g/dL or higher concentration of dried complex lipid powder into the aqueous solution, wherein the aqueous solution is comprised of a functional substance which is intended to be encapsulated in the vesicle, wherein the lipid molecules spontaneously self-assemble to compose the vesicle and simultaneously to encapsulate the functional substance, and wherein the particle size of the vesicle is controlled by shear stress generated by the kneading.

[0025] In addition, according to other major aspects of the present invention, a method is provided for producing hemoglobin vesicles, wherein the efficiency of encapsulating hemoglobin is 60% or higher by dispersing lipid molecules in a solution of hemoglobin with a dried complex lipid powder (15 g/dL or more) kneaded into a dense solution of hemoglobin (30-50 g/dL), and composing the lipid molecules by spontaneous self-assembly, wherein the dry weight ratio of hemoglobin and the complex lipid, which compose the hemoglobin vesicle, is over 1.2, and wherein the average particle size of the vesicle is controlled at 400 nm or smaller.

[0026] In addition, according to an embodiment of the present invention, a method is provided for producing vesicles or a hemoglobin vesicle, employing planetary movement that moves the cylindrical container with a sealed sample in the above-mentioned method simultaneously in rotating and revolving directions, and kneading the sample homogeneously while foam formed during hydration of dried complex lipid powder is removed by centrifugal force.

[0027] In addition, according to an embodiment of the present invention, a method is provided for producing vesicles and hemoglobin vesicles, wherein the number of revolutions and rotations in the above-mentioned method is 200-3000 rpm and 100-3000 rpm, respectively, and wherein the method comprises an operation of removing the functional substance or hemoglobin which has not been encapsulated, by an ultrafiltration membrane of ultracentrifugation, after adding water or saline buffer solution to the viscous vesicle paste and dispersing the paste to make fluid liquid by moving simultaneously in the rotating and revolving direc-

tions one more time, as a treatment for the viscous vesicle paste obtained by the kneading operation.

[0028] In addition, according to an embodiment of the present invention, a method is provided for producing hemoglobin vesicles, wherein the above-mentioned method is operated in a state that suppresses denaturation of hemoglobin due to shear stress and heat generated during the kneading operation, by transforming the hemoglobin of the dense hemoglobin solution into carbonyl hemoglobin which is bound with carbon monoxide as a gas molecule, or deoxyhemoglobin which is not bound with any gas molecule, so that the heme of the hemoglobin is kept at a divalent iron state.

[0029] In addition, according to an embodiment of the present invention, a method is provided for producing hemoglobin vesicles wherein, by a treatment of heating the dense hemoglobin solution used in the above-mentioned method at 60° C. for ten hours or longer beforehand, and removing contaminated unstable proteins in denatured state by ultrafiltration or centrifugation, the occurrence of insoluble matter of denatured protein under shear stress is reduced during the subsequent operation of kneading with the complex lipid powder.

[0030] In addition, according to an embodiment of the present invention, a method is provided for producing vesicles and hemoglobin vesicles, wherein the lipid composition of the dried powder of the complex lipid in the above-mentioned method is composed of a phosphatidylcholine-type phospholipid, cholesterol, a negatively-charged lipid, and a lipid bound with polyethylene glycol.

[0031] In addition, according to an embodiment of the present invention, a method is provided for producing vesicles and hemoglobin vesicles, wherein the dried powder of the complex lipid used in the above-mentioned method is composed of a phosphatidylcholine-type phospholipid of 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine, cholesterol, a negatively-charged lipid of 1,5-O-dihexadecyl-N-succinyl-glutamate, and a lipid bound with polyethylene glycol of 1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine-N-poly(oxyethylene) 5000 (molecular weight of the polyethylene glycol chain: 5,000).

[0032] In addition, according to an embodiment of the present invention, a method is provided for facilitating dispersion of complex lipid and control of particle size in the production of vesicles and hemoglobin vesicles, by substituting a phosphatidylcholine-type phospholipid in the above-mentioned method with a phosphatidylcholine-type phospholipid of which the gel-liquid phase transition temperature is 30° C. or lower.

[0033] In addition, according to other major aspects of the present invention, a method is provided for producing a lipid lamellar gel comprising the steps of adding 15 g/dL of a dried powder of complex lipid to pure water which is comprised of substantially no solute. A method is also provided for producing a dried powder of the lipid lamellar gel by freeze-drying the lipid lamellar gel derived from the above-mentioned method for producing the lipid lamellar gel.

[0034] According to the present invention, hemoglobin vesicles can be produced in a shorter time with higher yield than had been conventionally possible. It is shown that the operation of the present invention not only kneads lipid powder and dense hemoglobin solution homogeneously, but also disperses lipid molecules in the hemoglobin solution, encapsulates hemoglobin in the inner aqueous phase of the higher order structure of the lipid bilayer membrane (liposome capsule) which is spontaneously reorganized, and is capable of controlling the particle size of the hemoglobin vesicle at an optimum value. Thus, the present invention enables greater simplification of operation processes than expected as a whole. In addition, it is possible to increase production efficiency in a method for encapsulating a functional protein other than hemoglobin and low molecular weight compounds, a method for producing vesicles, and a method for producing a lipid gel.

[0035] The features of the present invention and remarkable actions and effects other than those described above will be apparent to those skilled in the art by referring to the following description of embodiments and figures that exemplify the principle of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0036] FIG. **1** is a schematic representation showing a kneading machine of one embodiment of the present invention.

[0037] FIG. **2** is a magnified schematic representation showing a part of the kneading machine.

[0038] FIG. **3** is a cross-section view of an exemplary cylindrical container.

[0039] FIG. **4** is a cross-section view of an exemplary cylindrical container.

BEST MODE FOR CARRYING OUT THE INVENTION

[0040] Embodiments of the present invention will be described below.

[0041] Hemoglobin vesicles of the present embodiment comprise phospholipid vesicles (liposomes) composed of lipid bilayer membranes, of which phospholipid is the major component, and in which hemoglobin is included. The hemoglobin vesicle of this embodiment also comprises a capsule formed by a surfactant which is co-polymerized with a hydrophilic polymer and a hydrophobic polymer (polymersome) in which the hemoglobin is included. The hemoglobin solution may be derived from donated human blood, domestic animals, or recombinant hemoglobin. Although hemoglobin, a globular protein, is relatively stable among proteins, oxyhemoglobin will be converted into methemoglobin by auto-oxidation over time. Heat may be generated by shear stress during kneading with a kneading machine of this embodiment (mixer/deaerator: an apparatus which rotates and revolves at the same time). It is better to pretreat the hemoglobin with carbon monoxide to convert the hemoglobin to carbonyl hemoglobin (HbCO), or to remove oxygen completely to convert the hemoglobin to deoxyhemoglobin (deoxyHb), in order to give heat tolerance to the hemoglobin with the center iron of the heme in a ferrous state. By keeping the hemoglobin concentration as high as 35-45 g/dL, the concentration of hemoglobin encapsulated in the vesicle may also be kept high. A dense solution of hemoglobin, the raw material, is concentrated following repeated dialysis with ultrafiltration membrane, and it is necessary to prevent clogging of the ultrafiltration membrane. It may be difficult to separate contaminated proteins which are prone to denaturation (residual glycolytic enzymes, enzymes for reducing metHb, carbonic anhydrase and the like) as they come to be denaturated and insoluble during the course of kneading. It is, therefore, preferable to use hemoglobin which has been heat treated (at 60° C., for 10-12 hours) to remove the denatured

contaminated proteins. The hemoglobin may be supplemented with a previously determined amount of pyridoxal 5'-phosphate or chlorine ions as an allosteric effector. For targeting oxygen to an ischemic region, the hemoglobin may also be encapsulated in liposome without supplementing any allosteric effectors.

[0042] The weight ratio of hemoglobin to lipid is a parameter representing the oxygen carrier efficiency of hemoglobin vesicle. Supposing that the ratio is about 2.0 and recovery yield is aimed at 100%, for example, it is a guide to supplement with 20 g of lipid powder, as 40 g of hemoglobin is resolved in 1 dL of hemoglobin solution at 40 g/dL. According to our study, good results are obtained when 15 g or more (preferably 20-25 g) of dried lipid powder is supplemented to ldL of dense solution of hemoglobin at 35-45 g/dL, and the mixture is kneaded.

[0043] As a method for kneading, it is good to use a kneading machine based on planetary movement with revolution and rotation. For example, a kneading procedure (revolution: 500-1500 rpm; rotation: 200-600 rpm) for 3-6 minutes while the mixture is sealed in a sterile cylindrical container made of stainless steel, polyethylene, Teflon® and the like, is repeated 30 times using a kneading machine manufactured by THINKY CORPORATION (mixer/deaerator, ARE-310). As shown in the schematic representations of FIGS. **1** and **2**, the kneading machine has a cylindrical container **10** with a structure in which the cylindrical container **10** rotates around a center axis **20** (axis of rotation) and revolves about a center axis **30** (axis of revolution) at the same time. The axis of rotation **20** is not on parallel with the axis of revolution **30**. Preferably the axis of rotation **20** is at an angle of θ with respect to the axis of revolution **30**. Preferably θ is 30° or more and 60° or less. In each of the examples described below, the axis of rotation **20** is at an angle of about 45°, a median value of the above-mentioned angle range, with respect to the axis of revolution **30**. By setting the angular orientation in this manner, good results were obtained as will be described below. In addition, in case the angle of the axis of rotation **20** with respect to the axis of revolution **30** is set in the above-mentioned rage, results are obtained which are similar to those described below. Therefore, as for the shape of the cylindrical container and kneading conditions, it is preferable but not limited to set the angle θ within the above range of angles. The angle value θ may be set at an angle near 0° or over 60°, for example, using a cylindrical container with barrel-shaped bulging sides. Thus, it is possible, and the present invention does not exclude, setting the angle value θ out of the above-mentioned range of angles. It is possible to promote the kneading by using a cylindrical container which does not have a smooth inner wall but an inner wall with an obstacle on the inner wall or the bottom surface that impedes fluid movement. As illustrated in FIG. **3**, for example, the kneading efficiency is increased by using a cylindrical container with irregularities on the inner sidewall (a container with a cloverleaf cross section viewed from above). FIG. **3** is a Y-Y cross section view of FIG. **1**. The cylindrical container **10** shown in FIG. **3** has a plurality of concave surfaces **12** on the inner periphery of its sidewall **11** and the centers of curvature **12***a* of the adjacent concave-curved surfaces are at different positions, whereby a convex-shaped crest **13** that protrudes toward the interior of the cylindrical container **10** is formed between the adjacent concave-curved surfaces **12**. As shown in FIGS. **3** and **4**, the number of the convex-shaped crests **13** on the inner periphery of its sidewall may be one or more. The cylindrical container **10** may have a similar crest. Increases in the temperature of the container are monitored, and if necessary, the entire container may be cooled down and then the kneading may be resumed thereafter. This operation results in a paste such as a tooth paste in which the hemoglobin solution and the complex lipid are kneaded. Usually, when lipid powder is dispersed in an aqueous solution, it takes time to remove foam. With the method of the present invention, however, the resulting paste has hardly any foam because the foam is removed by the centrifugal force of revolution. The paste is processed with the same machine. When pure water or saline is added to the paste, sealed in and kneaded by the above-mentioned machine for about another minute under similar conditions, the paste is dispersed to become a fluid dispersion of hemoglobin vesicles, which simplifies the subsequent operations. In case lipid which is not completely dispersed by the kneading or protein which is denatured and become insoluble remain, they are removed at this step by light centrifugation (about 2000-5000×g) or by filtration. To remove hemoglobin which is not encapsulated, the fluid dispersion of hemoglobin vesicles is treated with an ultrafiltration membrane (molecular weight cut-off 1000 kDa) or ultracentrifugation (about 50000×g) to remove the supernatant followed by re-dispersion of the precipitate in saline. The resulting hemoglobin vesicle is characterized regarding recovery yield of hemoglobin, average particle size and measurement of concentrations of components. Depending on the kneading conditions, the resulting hemoglobin has a recovery yield of hemoglobin at 50-70%, an average particle size of 200-400 mm and a weight ratio of hemoglobin to complex lipid of 1.2 or more.

[0044] A kneading machine based on planetary movement with revolution and rotation is commercially available from many companies as various models, which differ in the rates of revolution and rotation and their ratio. As the dimensions of the machine vary depending on the kneading capacity (from about 10 mL to 10 L), the larger the machine is, the longer the radius is, and the greater the kneading efficiency is with the same rate of movement. Therefore, the optimum rate for kneading differs for different models. In general, machines which run in the range of a rate of revolution of 200-300 rpm and at a rate of rotation of 100-3000 rpm are commercially available, and thus kneading operations suitable for vesicle preparation may be performed under these conditions. It is preferable to set the rate of revolution below 2000 rpm to minimize the denaturation of components by heat and to complete the operation in as soon as possible. Also, the higher the concentration of the hemoglobin solution is, the higher the viscosity is (6, 11 and 29 cP at 35, 40, 45 and 45 g/dL, respectively). As for the control of particle size, it is important to have a strong shear stress. In order to control particle size, it is advantageous to use a hemoglobin solution with high viscosity. The strong shear stress, on the contrary, tends to invite a large amount of heat and denatured protein. When the concentration of the hemoglobin solution is too low, the hemoglobin content encapsulated in the resulting particle is reduced. In view of these considerations, it is preferable to set the concentration of the hemoglobin solution at 35-45 g/dL. It is desirable to set the viscosity of the hemoglobin solution at 5 cP or higher as measured at 23° C. under a condition of shear velocity at 1000 s^{-1}. The fluid dispersion of hemoglobin vesicles is preserved after sealing in a container which does not permeate gas (glass container) as a formulation in a CO-bound form, subsequent to adjusting the concentration, or

alternatively, after sealing as a formulation converted in deoxy-form with a nitrogen gas aeration or a gas exchange unit such as an artificial lung, subsequent to carrying out CO photo dissociation and removal of oxygen and adjusting the concentration. When the particle size is sufficiently small, it is possible to filtrate through a sterilized filter with a pore size of 0.22 micrometers. In case it is impossible to carry out filter sterilization, the entire process may be kept in a sterile atmosphere, or a predetermined amount of beta-propiolactone may be supplemented in the final step of process. The principle of sterility for this reagent is to denature DNA by direct binding. As the reagent may also denature protein by direct binding, it is possible to suppress denaturation of hemoglobin (conversion to methemoglobin) to some degree by supplementing beta-propiolactone in a state where oxygen dissolved inside the hemoglobin vesicles is completely excluded (in a carbonyl hemoglobin or deoxyhemoglobin state) in order to protect the hemoglobin.

[0045] As for complex lipid components of the phospholipid vesicle, four ingredients, a phosphatidylcholine-type phospholipid, cholesterol, a negatively-charged lipid and a lipid bound with polyethylene glycol, are appropriate as major components. However, the phospholipids vesicle is not limited to that having these components. The complex lipids are prepared with unlimited combinations and composition ratios. As for the phosphatidylcholine-type phospholipid, fatty acids, which are bound to hydroxyl groups at 1- and 2-positions of glycerol backbones by ether bonds, are preferably saturated in order to suppress lipid peroxidation. It is preferable to use 1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine (DMPC), 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphatidylcholine (DSPC), 1-palmitoyl-2-myristoyl-sn-glycero-3-phosphatidylcholine (PMPC), 1-stearoyl-2-myristoyl-sn-glycero-3-phosphatidylcholine (SMPC), or hydrogenated lecithin derived from soybean (HSPC). With unsaturated type phospholipids, it is preferable to use a phospholipid bound with oleic acid, a relatively stable fatty acid, such as 1-stearoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (SOPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC), and the like. Of these phospholipids, DPPC is supposed to be preferable, considering the stability of the resulting vesicles, as well as accumulation subsequent to capture by macrophages, which should be avoided. In view of the ease of production, it is desirable to use a phosphatidylcholine-type lipid with phase transition temperature below that of DPPC (Tc=41° C.) such as SMPC (Tc=30° C.), PMPC (28° C.), DMPC (23° C.), SOPC (6° C.) and POPC (−3° C.), because of their favorable characteristics regarding dispersion and control of particle size. Regarding the negatively-charged lipid, it is preferable to use 1,5-O-dihexadecyl-N-succinyl-L-glutamate. Conventionally, with a phosphatidylcholine-type lipid with low phase transition temperature, it is regarded that the resulting vesicle structure is unstable. It is necessary, however, to judge whether the structure is sufficiently stable, from the viewpoint of actual intended use.

[0046] Different procedures can be employed to prepare the dried powder of complex lipid to be kneaded with the dense hemoglobin solution as follows: (1) a dried powder obtained by first dissolving all the components in a predetermined organic solvent, followed by rapid removal of the organic solvent by the CRUX method; (2) a dried powder obtained by first dissolving all the components in t-butanol or benzene, followed by freeze-drying; (3) a dried vesicle powder obtained by dissolving the components in an organic solvent such as t-butanol or ether, contacted with water, forming vesicles in water by removing the organic solvent and freeze-drying the vesicles; (4) a dried vesicle powder obtained by dispersing the complex lipid powder obtained by procedure (1) or (2) in water to form vesicles and freeze-drying the vesicles; and (5) a freeze-dried powder of lipid gel obtained by hydrating the complex lipid powder obtained by procedure (1) or (2) with the above-mentioned kneading machine. However, it takes a long time to freeze-dry an aqueous solution, and it also costs an enormous amount of labor and electric power to run a freeze drying apparatus. In view of efficiency, it is desirable to use the complex lipid powder obtained by procedure (1) or (2) as it is. In the embodiments of the present invention, sufficient efficiency in encapsulating hemoglobin and controlling average particle size is achieved by kneading the dried complex lipid powder obtained by procedure (1) or (2) directly with the dense hemoglobin solution.

EXAMPLES

Example 1

[0047] 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC), cholesterol,1,5-O-dihexadecyl-N-succinyl-glutamate (DHSG) and 1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine-N-poly(oxyethylene)5000 (DSPE-PEG5000, molecular weight of PEG chain: 5000) with molar ratio of 5:4:0.9:0.03, respectively, and total weight of 10 g were dissolved as complex lipids in 1 dL of t-butanol in a 0.5 L eggplant shaped flask. The solution was frozen with a dry ice/methanol mixed refrigerant and applied to a freeze-drying machine (TOKYO RIKAKIKAI CO., LTD., FD-1000) for 24 hours to obtain a complex lipid powder. Ten grams of the powder was put in a cylindrical container (outer diameter: 76 mm; height: about 93 mm, with irregularities on the inner wall and a cloverleaf cross section viewed from above) made of Teflon®, and supplemented with highly purified human hemoglobin solution (HbCO form bound with carbon monoxide, 45 g/dL, 0.4 dL, pH 7.4). The weight ratio of hemoglobin (18 g) and lipid (10 g) was 1.8:1. The container was sealed with an inner cap and applied to a kneading machine (rotation and revolution mixer, THINKY CORPORATION, ARE-310) to be kneaded with revolution at 1500 rpm and rotation at 600 rpm for 3 minutes. After cooling for 3 minutes, the gas phase in the container was completely replaced with carbon monoxide gas and the container was sealed. The operation of kneading with revolution at 1500 rpm and rotation at 600 rpm for 3 minutes and cooling for 3 minutes was repeated a total of 30 times (90 minutes) to complete kneading treatment. At that time, the temperature of the paste was as high as about 40° C. by the shear stress during the kneading and the heat transferred from the machine. Thus, it was judged as important to cause the paste to react with carbon monoxide to suppress denaturation of hemoglobin. A dense and homogeneous paste with a dark red color was obtained. No lipid lumps or foam was observed. Then, 0.50 dL of cooled saline was added and run on the kneading machine for 15 seconds to reduce the viscosity of the paste and to make a fluid dispersion. The fluid dispersion was further diluted two-fold with saline and centrifuged (3000 rpm, 30 minutes, Hitachi Koki Co., Ltd., CF12RX) to obtain a precipitate of a fraction with large particle size and a precipitate of partially

denatured hemoglobin. The supernatant phase was collected into an ultracentrifuge tube (a container of 230 mL), filled with saline and spun at 50,000 rpm for 30 minutes with an ultracentrifuge (Hitachi Koki Co., Ltd., CP90WX). Measuring the hemoglobin concentrations and volumes of the fluid dispersion before spinning and the supernatant after spinning, the encapsulating efficiency was calculated to be about 65%. The particle size was measured with a nanoparticle analyzer by HORIBA, Ltd. to be about 270 nm. The percentage of methemoglobin content was calculated based on the soret band spectrum and confirmed to be less than 3%. The precipitate obtained by the ultracentrifugation was dispersed in saline again, the hemoglobin concentration was adjusted to 2.5 g/dL, and put into a 2 L eggplant shaped flask in 2 dL increments to light dissociate CO gas and to convert the precipitate to oxygen-bound hemoglobin by running with a rotary evaporator and being illuminated from outside with a halogen lamp for 45 minutes. The obtained solution was separated again with an ultracentrifuge. The precipitate was dispersed in saline, and the hemoglobin concentration was adjusted to 10 g/dL. The lipid concentration of the dispersion was 6.8 g/dL and the weight ratio of hemoglobin to lipid was 1.5. Aliquots were prepared in vials and sealed with caps made of butyl rubber. The hemoglobin was converted to deoxy-form by nitrogen gas aeration to exclude oxygen. Then, beta-propiolactone was supplemented at a concentration of 0.5% or lower to perform sterilization.

Comparative Example 1

[0048] Four lipid components were dissolved in t-butanol to be a homogeneous solution, and freeze-dried to obtain 10 g of complex lipid powder as described in connection with Example 1. The powder was added to 0.4 dL of 45 g/dL hemoglobin solution and mixed for 24 hours with a stirrer and a stirrer tip (length: 2 cm). A large amount of lipid lumps which was not dispersed was found and the solution was too dense to control the particle size using the conventional Extrusion Method.

Comparative Example 2

[0049] As described in connection with Example 1, four lipid components were dissolved in t-butanol to make a homogeneous solution, and freeze-dried to obtain complex lipid powder. Five grams of the powder was supplemented to 1 dL of 45 g/dL hemoglobin solution, and mixed for 12 hours with a stirrer and a stirrer tip (length: 2 cm). Some lipid lumps were found. The mixture was treated with the Extrusion method using an extruder of Lipex Biomembranes, Inc. and the particle size was controlled by step wise filtration with filters having pore sizes 3.0, 1.0, 0.8, 0.65, 0.45, and 0.22 micrometers in this order. At this time, the viscosity at 25° C. was measured using a rheometer (MCR-300, Anton Paar GmbH) to be 62 cP (shear velocity at 1000 s^{-1}), 70 cP (shear velocity at 100 s^{-1}) and 98 cP (shear velocity at 10 s^{-1}). The amount recovered was 0.8 dL and 0.2 dL was lost during the processes. The encapsulation efficiency was as low as 20%. Adjusting hemoglobin concentration at 10 g/dL, 70 mL of hemoglobin vesicle dispersion was obtained.

Comparative Example 3

[0050] In the procedures described in connection with Example 1, oxyhemoglobin, hemoglobin bound with oxygen, was used to prepare a complex lipid powder which was kneaded by similar procedures. When the paste was diluted and centrifuged (200 rpm, 60 minutes), a large amount of insoluble precipitate with medium brown color was found. The percentage of methemoglobin in supernatant hemoglobin increased to about 10%. Thus, it was judged that stabilization of hemoglobin by converting into carbon monoxide-bound hemoglobin or deoxyhemoglobin is an indispensable step.

Example 2

[0051] Using the same species of lipids as Example 1, DPPC, cholesterol, DHSG and DSPE-PEG5000 at a molar ratio of 5:4:0.9:0.03 and a total weight of 10 g were dissolved in t-butanol in an eggplant shaped flask. Freezing with liquid nitrogen and freeze-drying were carried out to obtain a complex lipid powder. Then, the complex lipid was put into the same cylindrical container made of Teflon® as that used in Example 1, and supplemented with 0.5 dL of ultrapure water. As the weight of the negatively-charged lipid DHSG was 1.13 g, corresponding to 1.48 millimol, 0.074 mL of 1N NaOH solution was added to neutralize the negatively charged lipid DHSG with the same amount of NaOH. Then, the container was sealed with the inner cap and applied to a kneading machine (rotation and revolution mixer, THINKY CORPORATION, ARE-310) to perform kneading with revolution at 2000 rpm and rotation at 800 rpm for 5 minutes. After cooling for 5 minutes, kneading was carried out again for 5 minutes so that kneading was carried out for 10 minutes in total. An obtained lipid lamellar gel was frozen with liquid nitrogen and freeze dried to obtain freeze-dried powder of the lipid gel. 0.4 dL of HbCO solution was added to 10 g of the complex lipid similar to the procedures described in connection with Example 1, and the container was sealed with CO and kneading was carried out using the kneading machine (rotation and revolution mixer, revolution at 1500 rpm and rotation at 600 rpm) to obtain a kneaded paste of hemoglobin solution and freeze dried lipid powder. Saline was added to the paste and diluted, the paste was dispersed in the same manner, and aggregates and the like were separated and removed by centrifugation. The upper layer solution was further separated by ultracentrifugation and the concentration and volume of hemoglobin which was not encapsulated was measured, to calculate the encapsulation efficiency as about 60%. The precipitated hemoglobin vesicles were dispersed in saline again and hemoglobin concentration was adjusted to 10 g/dL, and aliquots of 0.50 dL were dispensed in 1 dL vials and sealed. Aeration was carried out with carbon monoxide gas to exclude dissolved oxygen, and the hemoglobin was completely converted to that which is bound to carbon monoxide. Then, beta-propiolactone was added at a concentration of 0.5% or lower to perform sterilization.

Example 3

[0052] Complex lipid powder was prepared by Nippon Fine Chemical Co., Ltd. with DPPC, cholesterol, DHSG and DSPE-PEG 5000 dissolved in an organic solvent at a molar ratio of 5:4:0.9:0.03, and the organic solvent being rapidly evaporated by the CRUX method. 10 g of the complex lipid powder was added to 0.4 dL of a dense hemoglobin solution in a manner similar to that of Example 1, and kneading with a kneading machine (rotation and revolution mixer, THINKY CORPORATION, ARE-310, revolution at 1000 rpm and rotation at 400 rpm) for 6 minutes and cooling for 3 minutes