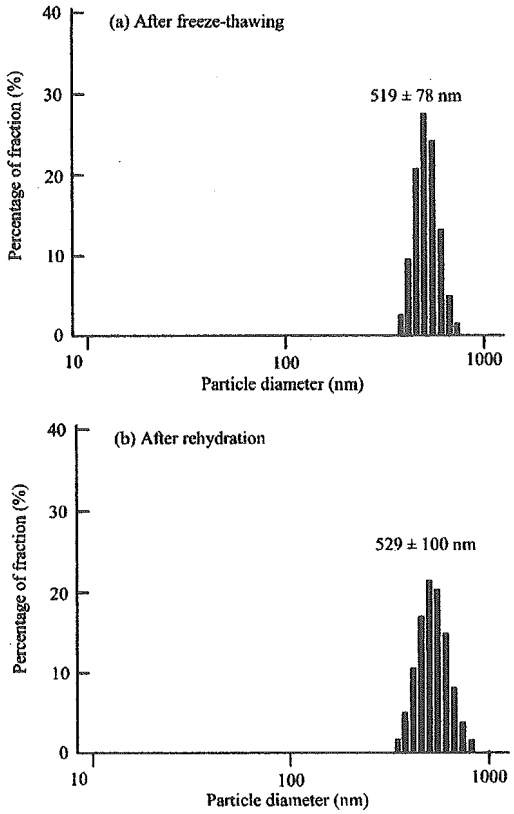

Figure 4. Size distribution of the vesicles measured with a COULTER submicron particle analyzer. The size of the vesicles is represented as the average diameter ± standard deviation: (a) vesicle dispersion just after freeze–thawing and (b) vesicle dispersion rehydrated in a Hb solution.

Extrusion of Rehydrated Vesicles. Further size control is necessary for the dehydrated vesicles (529 ± 100 nm mean diameter) to obtain the final targeting size of HbV (250 ± 20 nm mean diameter). We performed the extrusion for the rehydrated vesicles to regulate the size and encapsulate Hb into the rehydrated vesicles. In addition, the effect of the presizing of the vesicles on the filter permeability was compared with that of the conventional method. In a conventional extrusion method of polydispersed HbV, we used filters with six different pore sizes (FM_3, $FM_{0.8}$, $FM_{0.65}$, $FM_{0.45}$, $FM_{0.3}$, and $FM_{0.22}$) (*24*). One advantage of the present method is the omission of two filters ($FM_{0.8}$ and $FM_{0.65}$) because the size of the vesicles has already been controlled to 529 ± 100 nm. The filter permeability of the dispersion is compared in Figure 5 with the various concentrations of the lipids during the freeze–thawing. The concentration of the lipids during the extrusion was adjusted to 5 g dL^{-1} in all of the experiments. The solution viscosity of the Hb (40 g dL^{-1}) was 50 cP at 140 s^{-1} (25 °C). When the lipid mixture was dispersed into a Hb solution at 5 g dL^{-1}, the viscosity increased to 75 cP at 140 s^{-1} (25 °C). The rate of the filter permeation of HbV was remarkably improved for all filters using the lyophilized powder of the freeze–thawed vesicles compared with the conventional method. The average time required for extrusion was 30 times shorter when the freeze–thawing was carried out at the lipid concentration of 1 and 2 g dL^{-1}. The freeze–thawing at a higher lipid concentration decreased the filter permeability. The rate was twice as low when the lipid concentration was above 3 g dL^{-1} because of the higher content of large vesicles (>3μm).

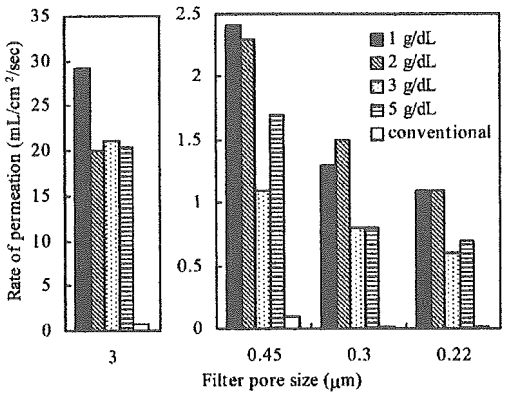

Figure 5. Effect of freeze–thawing on the rate of filter permeation of the HbV with concentration of the lipids during the freeze–thawing. The lyophilized powder of the freeze–thawed vesicles was rehydrated with a Hb solution (40 g dL^{-1}) at 5 g dL^{-1} and the dispersion was extruded through a membrane filter (4.9 cm^2) at a N_2 pressure (20 kg fcm^{-2}) at 14 °C.

The mechanism of the sizing with the filter extrusion is the disruption of the vesicles and the stripping of the bilayer membrane during the passage through the smaller pore size than that of vesicles (*41, 42*). The large multilamellar vesicles having an onion-like structure are unfavorable for filter extrusion because of the low deformability of the vesicles. The high filter permeability of the freeze–thawed vesicles would be due to the low number of bilayer membranes (the lamellarity is ca. 2) and the precontrolled size. The diameter of the vesicles was finalized to 250 ± 20 nm after passing through the $FM_{0.22}$, and Hb was encapsulated into the vesicles with a high efficiency (1.7–1.8 as the value of [Hb]/[lipids]). The reconstruction of the freeze–thawed vesicles by extrusion is necessary to produce HbV with a high encapsulation efficiency. The optimal size of the freeze–thawed vesicles is determined from the size of the final vesicles. If the freeze–thawed vesicles had a size similar to the final pore size during the filter extrusion, the vesicles could pass through the filter membrane without reconstruction of the vesicles for encapsulation. If the freeze–thawed vesicles were very large in comparison to the filter pore size at extrusion, the merit of this pretreatment would be lost. In this sense, the reasonable size of the freeze–thawed vesicles should be two or three times larger than that of the final size. Since the targeting size of HbV is a 250 nm diameter, the size of the freeze–thawed vesicles (ca. 500 nm) is reasonable to encapsulate Hb by extrusion. The metHb content of the final HbV was equal to that of the starting Hb solution (below 2%), meaning that the denaturation of the Hb did not occur during the extrusion process.

In conclusion, we have made great progress in the extrusion using the freeze–thawed vesicles. The desirable cooling rate and concentration of the lipids were −140 °C min^{-1} and 2 g dL^{-1}, respectively. The time required for extrusion of the HbV was 30 times shorter using the lyophilized powder of the freeze–thawed vesicles under these conditions. This finding would be available to manufacture size-controlled vesicles encapsulating concentrated proteins or unstable drugs.

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原著

酸素輸液ヘモグロビン小胞体に混在するリポポリサッカライドの定量法

Detection of Lipopolysaccharide Contaminating Hemoglobin-Vesicles as Artificial Oxygen Carriers

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和文抄録

菌体由来のリポポリサッカライド（LPS）は両親媒性分子であるため，疎水性相互作用によりリン脂質小胞体或いはリポソームの脂質二分子膜に取込まれ易く，一般的なリムルステスト（*Limulus* Amebocyte Lysate: LALゲル化反応試験）では正確に定量されないことが問題となる．リン脂質小胞体の内水相に高濃度ヘモグロビン（Hb）を内包したHb小胞体（HbV）の品質管理の面でも正確なLPS定量の確立は重要課題である．そこで本研究では，界面活性剤deca (oxyethylene) dodecyl ether ($C_{12}E_{10}$) でHbVを溶解してLPSを遊離させた後，LAL試薬と混合し，ゲル化反応を比濁時間分析法によって解析する方法を検討した（検出波長660 nm). 界面活性剤$C_{12}E_{10}$はゲル化反応を濃度依存的に阻害するので，$C_{12}E_{10}$濃度とHbV可溶化および阻害作用の相関を解析し，LPSの検出限界が0.1 EU/mLとなる最適測定法を確立した．

Abstract

A method to quantitatively measure the bacterial endotoxin content (lipopolysaccharide, LPS) in phospholipid vesicles or liposomes has been required because the conventional Limulus amebocyte lysate (LAL) test does not provide an accurate measurement due to the hydrophobic interaction of LPS and vesicles that shields the activity of LPS to clot the LAL coagulant. Hemoglobin-vesicles (HbV) are artificial oxygen carriers encapsulating a conc. Hb solution in phospholipid vesicles. To accurately measure the LPS content in the HbV for the quality control, we tested the solubilization of HbV with deca(oxyethylene) dodecyl ether ($C_{12}E_{10}$) to release the LPS entrapped in the vesicles as a pretreatment for the succeeding LAL assay of the kinetic-turbidimetric gel clotting analysis (detecting wavelength, 660 nm). The $C_{12}E_{10}$ surfactant interferes with the gel clotting in a concentration dependent manner, and the optimal condition was determined in terms of minimizing the dilution factor and $C_{12}E_{10}$ concentration. We clarified the condition that allowed the measurement of LPS higher than 0.1 EU/mL in the HbV suspension.

Keywords

endotoxin, hemoglobin, lipopolysaccharide, oxygen carriers, *limulus* amebocyte lysate.

1．緒言

ヘモグロビン（Hb）を濃度高くリン脂質小胞体の内水相に内包したヘモグロビン小胞体（HbV）が人工酸素運搬体として開発され，赤血球と同等の酸素運搬機能と安全性が動物投与試験から明らかにされてきた[1-5]．生物製剤に分類されるHbVの製造工程はGMP（good manufacturing practice）の基準に沿って，不純物やウィルス／菌体の混入防御に関して厳格に対処しなければならない．エンドトキシンとして知られるグラム陰性菌体由来のリポポリサッカライド（LPS）は極く微量でも様々な毒性を示す[6]．例えばLPSの致死量（LD_{50}）は，ラットで3 mg/kg，犬で1 mg/kgである[7,8]．米国FDAはヒトに対する非経口的投与薬剤の場合，LPS許容投与量を5 EU/kgと規定している[9]．この規定はHbV静注液にも該当すると考えられる．LPS濃度はエンドトキシン活性　(Endotoxin Unit: EU; 1EU=100

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pg）として表され，カブトガニの血球抽出物（*Limulus* amebocyte lysate;LAL試薬）との反応によって測定する（リムルステスト）．LALはLPSに対し濃度依存的に凝固しゲルを形成する[10]．ウサギ発熱試験に比較して，リムルステストでは検体が少量でも繰返し測定できる利点を有する[11]．人工酸素運搬体の適応は主として出血ショック蘇生や血液希釈であり，投与量は20 mL/kg或いはそれ以上が想定され，この場合LPSの許容量は，0.25 EU/mL（= 5/20）となる．これは注射用水の許容量0.25 EU/mLと同じである．

菌体由来LPSは両親媒性の巨大分子であり，蛋白質や生体膜と疎水性相互作用をする[12]．HbもLPSと強く結合し様々な神経毒性を示すことが報告されている[13]．LAL試薬のゲル化を惹起するのはLPS分子中のLipid-Aとよばれる糖結合リン脂質の部分である[14]．数本の脂肪酸を有するLipid-Aは，リン脂質小胞体の二分子膜に容易に挿入されるので，LPS特有のLAL試薬のゲル化反応や，その他の反応性が阻害される[15,16]．従って，HbVの品質管理においてLPSの正確な定量法の確立が極めて重要と成る[17-20]．本研究では，界面活性剤を利用してHbVを容易に溶解しLPSを遊離する条件を見出すとともに（Fig. 1），トキシノメータ®によりLAL試薬のゲル化反応を比濁時間分析法によって解析し，阻害反応をできるだけ抑制する前処理条件を決定することを目的とした．

2．方法

ヘモグロビン小胞体（HbV）の調製

PEG修飾HbVは既報[21-23]に従い無菌雰囲気下にて調製した．高純度Hbは北海道赤十字血液センター（札幌）および日本赤十字社（東京）から提供された期限切れ献血血液から精製した．限外濾過膜によるストローマ除去と，加熱処理（60℃, 10時間）で変性沈澱した夾雑蛋白質の除去により，高純度Hbを得た（>99.9%）[24]．濃厚Hb溶液（38 g/dL）にアロステリック因子として14.7mMのpyridoxal-5`-phosphate（PLP，メルク社製，Darmsdart, Germany）を，モル比でPLP/Hb = 2.5となるように添加し小胞体に内包した．小胞体の脂質二分子膜の構成成分は，1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine（DPPC），コレステロール，1,5-di-*O*-hexadecyl-*N*-succinyl-L-glutamate（DPEA）（日本精化製，大阪）および，1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-PEG_{5000}（PEG-DSPE，日本油脂社製，東京）である[25]．DPPC/コレステロール/DPEA/PEG-DSPEの混合比は5/5/1/0.033（モル比）とした．HbV粒子は生理食塩水中にHb濃度10 g/dLになるように分散した．HbVの物性パラメータは以下の通り:粒径，251±80 nm; [Hb], 10 g/dL; [metHb], <3%; [lipids], 5.6 g/dL;酸素親和度（P_{50}），31Torr．分析法は全て既報[26]に従った．

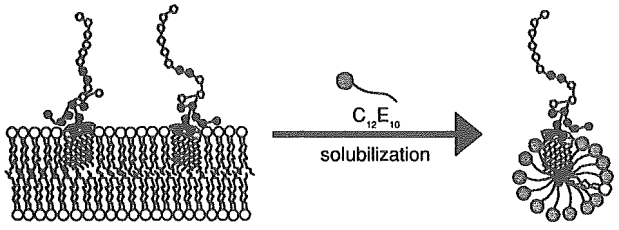

Figure 1. Solubilization of LPS-contaminated vesicles with a detergent to form mixed micelles.

界面活性剤によるHbVの可溶化条件の決定

HbV分散液を純水で2又は5倍希釈し（[Hb]=2 or 5 g/dL, 200 μL），deca(oxyehylene) dodecyl ether（$C_{12}E_{10}$, Sigma社製，純水中に0～10vol%で溶解，800 μL）と混合して石英キュベット（2 mm）に入れた．混合液を42℃，2分加温して可溶化を促進させた．紫外可視分光光度計（V-560，東京電子，東京）により，660 nmの濁度を測定した．比較として，リポソームや生体膜の可溶化によく用いられるドデシル硫酸ナトリウム（SDS，関東化学（株）製，東京），Triton-X100，nona(oxyethylene) dodecyl ether（$C_{12}E_9$）(Sigma社製）についても比較検討した．

リムルステストにおける検量線と界面活性剤の影響

日本薬局方で規定された標準LPS粉末（*E. coli* UKT-B由来，マンニトールとグリシンを含む，和光純薬工業社製，東京）を注射用蒸留水（大塚製薬社製，大阪）に溶解して原液（[LPS] =500 EU/mL）を調製した．LPS標準溶液（[LPS]=0.01-20 EU/mL）は原液を注射用蒸留水で希釈して調製した．LPS標準溶液（200 μL）と$C_{12}E_{10}$溶液（0～5 vol%, 800mL）を混合し，注射用蒸留水で8倍希釈し，等容量のLAL試薬（リムルスES-Ⅱテストワコー，和光純薬工業社製）と混合した．LAL試薬には，カブトガニ*Limulus Polyphemus*血球抽出物，トリス塩酸緩衝液（pH = 7.1），β-1,3-グルカン誘導体を含む．注射用蒸留水中のLPS含量は検出範囲外（<0.0001 EU/mL）であった．LPS含量は，LAL試薬とLPSのゲル化反応による濁度変化をトトキシノメータ®（ET-201，和光純薬工業社製）を用いた比濁時間分析法により定量した．ゲル化時間はマニュアルに従い660 nmの透過率が95%まで減少した時間と定義した．Hb溶液およびHbVの場合，Hbの強い光吸収が400～600 nmにあるため，キシノメータ®の測定波長660 nmは，Hbの吸収を避けているので適当と考える．

HbV分散液中のLPS定量法とその検証

Fig. 2のスキームに沿って，HbV分散液（[lipid] = 6.0 g/dL, [Hb] = 10 g/dL）をLPSフリーの試験管に分注して注射用蒸留水で希釈し，$C_{12}E_{10}$溶液と混合，42℃で2分加温した．次いで混合液を注射用蒸留水により8倍希釈しその後，溶液をLAL試薬に添加し，ゲル化による濁度変化をトキシノメーターET-201を用いた比濁時間分析法により解析した．

本法の妥当性を検証するため，添加LPSの回収率を測定した．

LPS標準溶液（[LPS] = 0.4～10.0 EU/mL）を脂質粉末に添加し，脂質濃度を6.0 g/dLに調整した．脂質分散液を攪拌し，注射用蒸留水により5倍希釈し，凍結融解を3回行ってLPS担持小胞体を調製した．回収率は，上述の方法によって定量したLPS濃度と添加LPS濃度の比較から算出した．

更にHbVでも同様の実験を行った．HbV分散液（[Hb]= 4 g/dL）を等容量のLPS標準液（[LPS] = 0.04～4.0 EU/mL）と混合してLPS担持HbV分散液（[Hb] = 2 g/dL）を調製し，$C_{12}E_{10}$溶液で溶解し上述の方法によりリムルステストを行った．Hb溶液については，同上のLPS標準溶液（[LPS] = 0.002～2.0 EU/mL）を等容量のHb溶液（[Hb] = 4 g/dL）と混合して，LPS含有Hb溶液（[Hb] = 2 g/dL）を調製し，直接LAL試薬と混合後LPS濃度を定量した．回収率は，定量したLPS濃度と添加LPS濃度の比較から算出した．

$C_{12}E_{10}$処理の有効性を確認するために，LPS担持小胞体を上述の方法に従って，$C_{12}E_{10}$を使う系と使わない系で比較検討した．先ず，LPS溶液（1.0 EU/mL）を脂質粉末に添加（1.2 g/dL）して小胞体を調製．脂質分散液に凍結融解を3度行って分散を高め，粒径を約500 nmに制御した．上述のように，$C_{12}E_{10}$を使う系と使わない系でLPS濃度を定量した．

3．結果

$C_{12}E_{10}$界面活性剤によるHbVの可溶化

波長660 nmにおけるHbV分散液の吸光度は1.13であり，HbV粒子による光散乱（濁度）のためHb溶液の吸光度（0.08）よりも極めて高い値となった．しかし$C_{12}E_{10}$溶液の添加につれ吸光度は減少し，HbVの可溶化が示唆された．完全溶解には2 vol%の$C_{12}E_{10}$溶液の添加を必要とした．この時の吸光度は小胞体が無いHb溶液のみの吸光度と等しくなった．可溶化処理後，溶液は赤色から褐色に変化し，metHbへの変化が示唆されたが，沈澱形成は認められなかった．結果的に溶液中の$C_{12}E_{10}$濃度とHbVの脂質濃度は各々1.6 g/dL，0.24 g/dLであった．$C_{12}E_{10}$は混合ミセルの87 wt%（86 mol%）を占めている．他の界面活性剤については，$C_{12}E_9$が$C_{12}E_{10}$と同様のHbV可溶化能を示した．一方，Triton-X100では吸光度は0.4までの低下，SDSでは0.6までの低下であり，HbVを充分に可溶化できなかった．

リムルステストに対する$C_{12}E_{10}$の影響

ゲル化時間とLPS終濃度の関係を対数プロットとして示し，これを検量線とした（Fig. 3）．幅広いLPS濃度範囲（LPS終濃度：0.000125～0.25 EU/mL）で直線関係が得られた．$C_{12}E_{10}$濃度を増加させるにつれ，ゲル化が妨げられる傾向（ゲル化時間の延長）が見られた．しかし，$C_{12}E_{10}$終濃度が0.1 vol%の時はゲル化の著しい阻害は見られなかった．LPS濃度0.01 EU/mLの場合，$C_{12}E_{10}$無添加のゲル化時間は約32分であったが，$C_{12}E_{10}$濃度0.1 vol%では39分，2.0 vol%では約107分に延長した．エチレンオキサイド鎖が1ユニット短い$C_{12}E_9$の場合，濃度0.1 vol%の場合はゲル化時間が50分に延長し，$C_{12}E_9$のゲル化阻害効果は$C_{12}E_{10}$よりも高かった．

HbV分散液中のLPS定量

LPS検出限界の向上のため，可溶化処理におけるHbV濃度の増加，又は希釈率の低減によって希釈倍率を変えてみた（Table1）．$C_{12}E_{10}$濃度0.1 vol%の条件（Entry-1）に，最小の検出限界（0.1 EU/mL）が得られた．Entry-2，3，4の条件では希釈倍率を減らすことができたが，Fig. 3に示すように，$C_{12}E_{10}$濃度が0.4 vol%以上では著しいゲル化の阻害起こるため，検出限界が悪くなった（0.23 EU/mL）．

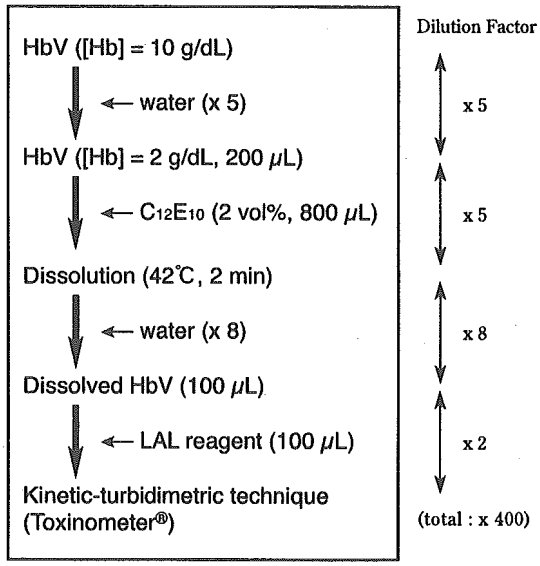

Figure 2. Procedures to quantitatively measure LPS in the HbV suspension using $C_{12}E_{10}$ for HbV solubilization and LAL assay, and dilution factors at every mixing of solutions. The experimental condition is for Entry 1 in Table 1.

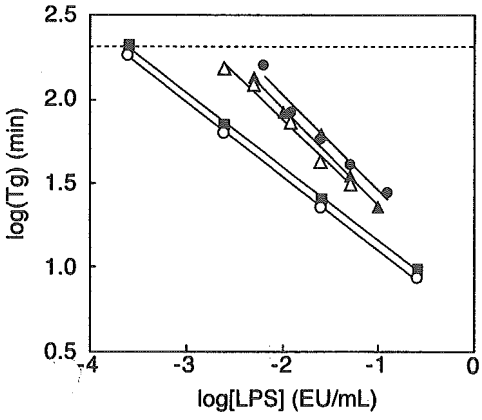

Figure 3. Calibration curves for the quantitative measurement of LPS in the presence of $C_{12}E_{10}$ at various concentrations (vol%); 2.0 (●), 0.8 (▲), 0.4 (△), 0.1 (■), 0 (○). [LPS] is the final concentration in the test tube for turbidimetry. Dilution factors should be multiplied to obtain [LPS] in the HbV suspension at [Hb] = 10 g/dL. For example, [LPS] should be multiplied with 400 at [$C_{12}E_{10}$] = 0.1 vol%. The broken line indicates the gelation time limit for Toxinometer® (200 min).

Table 1. Solubilization conditions of HbV with $C_{12}E_{10}$ and detection limit of LPS. The calibration curves are shown in Fig. 3.

Entry	Condition of HbV solubilization: HbV / 200 μL ([Hb] g/dL)	Condition of HbV solubilization: $C_{12}E_{10}$ / 800 μL ([$C_{12}E_{10}$] vol%)	dilution factor[a]	Final [$C_{12}E_{10}$] (vol%)	Detection limit of LPS (EU/mL HbV)[b]
1[c]	2	2	400	0.1	>0.1
2	2	2	100	0.4	>0.25
3	2	2	50	0.8	>0.25
4	5	5	20	2.0	>0.23

[a] see Fig. 2. from HbV([Hb]= 10 g/dL) to gel clotting assay.
[b] at [Hb]=10 g/dL.
[c] The optimal condition in this study

Table 2. Inhibition/enhancement testing to monitor the recovery of spiked LPS. LPS was spiked in the $C_{12}E_{10}$ -solubilized vesicles and HbV, and Hb solution without $C_{12}E_{10}$. *n = 3. All the data are converted to the conditions of [Hb]=10 g/dL, or [lipid]=6 g/dL.

Sample	Spiked LPS (EU/mL)	Recovery (%)
Vesicles (6 g/dL) with $C_{12}E_{10}$	0.4	108.6±4.6*
	1.25	92
	2.5	116
	5	103
	10	120
HbV (Hb, 10 g/dL; lipid, 6 g/dL) with $C_{12}E_{10}$	0.1	107
	0.4	111.3±3.6*
	1.0	114
	10.0	112
Hb solution (10 g/dL) without $C_{12}E_{10}$	0.005	121
	0.02	103.9±4.4*
	0.05	101
	0.5	124
	5	97

添加LPSの回収率は，低濃度から高濃度まで幅広いLPS濃度範囲で92%と124%の間におさまった（Table 2）．Hb溶液のLPS定量では，界面活性剤を必要としないためより低いLPS濃度（0.005 EU/mL）の検出が可能であり，$C_{12}E_{10}$を必要とするHbVの検出限界（0.1 EU/mL）と比べて検出感度が向上した．1987年に発表されたFDAガイドラインによると，検出限界濃度の4倍濃度でのLPS添加回収試験の実施を必要とし，回収率は100±25%以内でなければならない．そこでリン脂質小胞体に0.4 EU/mL（=4×0.1 EU/mL），Hb溶液に0.02 EU/mL（=4×0.005 EU/mL）のLPSを添加し回収率を測定したところ，全て100±25%以内であることが確認できた．

LPS担持小胞体中のLPS定量では，$C_{12}E_{10}$を用いた上記リムルステストにより1.03 EU/mLと判明した．一方，$C_{12}E_{10}$を添加せず小胞体を破壊しない状態でリムルステストをしたところ0.6 EU/mLとなった．この場合，回収率は60%に留まった．

4．考察

両親媒性LPSとリン脂質小胞体の疎水性相互作用がLAL試薬の活性に影響すること，またその他LPSの生理活性が抑制されることは多々報告されている[15,16,27,28]．他方，HbとLPSの相互作用についてはJurgensらが最近，Hb分子1個当たり3～5個のLPSが結合することを報告している[29]．従ってリムルステストによるHbVのLPS定量の前段階として，何らかのLPS遊離法が必要なことは明らかである．これまでにリン脂質小胞体の前処理として有機溶媒や界面活性剤を使用する方法が報告されているが[18,19,30]，HbVに多量に含まれる蛋白質Hbは有機溶媒に不溶なので，我々は界面活性剤を利用する方法を検討し，Hb濃度10 g/dLのときLPS濃度0.1 EU/mLまでの測定を可能とした．HbVの分散状態と形態の安定度は極めて高いが，界面活性剤として$C_{12}E_{10}$を添加し42℃で2分間インキュベーションすればHbVは完全に溶解でき，前処理法として利用できることが明らかとなった．

Fig. 3に示すように，$C_{12}E_{10}$は特に高濃度でLAL試薬のゲル化時間を延長した．しかし$C_{12}E_{10}$最終濃度0.1 vol%では阻害作用は低減される．他の界面活性剤を検討したところ，nona (oxyethylene) dodecyl ether（$C_{12}E_9$）はHbVの溶解能には優れるがより強いゲル化阻害作用を示し，Triton-X100はHbVの溶解能が不十分となった．イオン性界面活性剤SDSも十分に溶解できないし，更に文献によればSDSは僅か0.005 wt%以上でLAL試薬のゲル化を完全に抑制する[19]．対照的に非イオン性$C_{12}E_{10}$は，濃度依存的に穏和なゲル化阻害作用を示す．つまり，$C_{12}E_{10}$はHbVの溶解能に優れ，且つ，リムルステストにおいて十分に低いゲル化阻害作用を示すに留まる．HbVを$C_{12}E_{10}$で溶解すると混合ミセルが形成されるが（Fig. 1），このとき$C_{12}E_{10}$は構成成分全体の86 mol%を占め，PEG-DSPEの含量（0.04 mol%）を遥かに凌ぐ．$C_{12}E_{10}$ミセルの会合数は約100なので[31]，LAL試薬に含まれるLPS認識蛋白質（Factor C）はPEG-DSPEの立体障害の影響を殆ど受けずに容易に混合ミセルの親疎水界面付近に存在するLPSのLipid-A部位と結合することができると考えられる．

LPS検出限界の向上には，希釈率の低減と$C_{12}E_{10}$濃度の低下が要件と成る．しかし希釈率低減は逆に$C_{12}E_{10}$最終濃度の上昇をもたらす．検討の結果，$C_{12}E_{10}$濃度が0.1 vol%のとき，Fig. 3に示す通り阻害作用は極めて僅かであり，Table1のEntry-1に示す最適の前処理条件を決定できた．このとき総希釈倍率は400となった．FDAの指針には，最大投与量とLAL試薬の感度に応じた最大希釈倍率（Maximum Valid Dilution, MVD）が定義されている．HbVの許容LPS濃度 0.25 EU/mLと，Fig. 3のLPS検量線上の検出限界0.00025 EU/mL（log（0.00025）EU/mL=-3.60）より，HbVのMVDは1000（= 0.25/0.00025）と算出される．従って希釈率400はMVD範囲内にある．

小胞体に含有するLPSの定量を$C_{12}E_{10}$の有無で比較したところ，$C_{12}E_{10}$で溶解した方が高いLPS濃度を示したことから，脂質二分子膜内に挿入していたLPS或いは小胞体の内水相に取込まれていたLPSが遊離して検出されたことが明らかである．LPS添加回収試験の結果，Table 2に示す通り広範なLPS濃度において回収率は92～124%の範囲で再現性があった．FDA指針に従えば，回収率が100±25%のときに溶質の影響が無いと判断できる[9]．この基準値は1991年に100±50%に変更された[32]．Hb分子1個当たり3～5個のLPSが結合すると報告されているが[29]，我々のHb溶液での添加回収試験ではHbに拠る阻害作用を認めていない．LevinらはHbに結合したLPSはLAL試薬のゲル化を促進すると報告しているが[13,33]，逆にArchambaultらはLPSがHbに結合することでLAL活性が低下するとしている[34]．JurgensらはLAL活性はLPSとHbの濃度に依存することを観測している[29]．このような見解の相違は恐らく，LPSの濃度設定に関係すると考えられる．我々の実験条件では，LPS濃度を0.2 EU/mL（約20 pg/mL）以下に設定したが，他の研究グループは著しく高いLPS濃度（100 pg/mL～800 μg/mL）で測定を実施しており，場合によってはLPSの臨界凝集濃度（10～38 μg/mL）を越えている[35]．LPSが凝集するとLAL活性が著しく低下することが知られているので[36]，Hbが存在するとLPSは凝集せずにHbに結合して分散性が向上し，見かけ上LAL活性が上昇したと考えられる．他方，我々の測定条件では極めて希薄なLPS濃度に設定しているためLPSの凝集は無く，より自由にHb溶液中に分散し，Hb溶液の阻害作用は無いものと推測できる．

5．結論

本研究では，Hb小胞体を界面活性剤（$C_{12}E_{10}$）で前処理することによりHb小胞体に結合したLPSを遊離させた後に，LAL法にてLPS定量する方法を検討し，0.1 EU/mLまでの検出限界を得ることができた．また，添加回収法によりその妥当性を検証することができた．本法は現在，Hb小胞体の製造工程で日常的に使用されている．

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Detection of Lipopolysaccharide in Hemoglobin-Vesicles by *Limulus* Amebocyte Lysate Test with Kinetic–Turbidimetric Gel Clotting Analysis and Pretreatment of Surfactant

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ABSTRACT: A method to quantitatively measure the bacterial endotoxin content (lipopolysaccharide, LPS) in phospholipid vesicles or liposomes is necessary because the conventional *Limulus* amebocyte lysate (LAL) test does not provide an accurate measurement due to the hydrophobic interaction of LPS and vesicles that shields the activity of LPS to clot the LAL coagulant. This interference was evident from isothermal titration calorimetry results in our study that clearly demonstrated the insertion of the LPS molecule into the phospholipid bilayer membrane. Hemoglobin-vesicles (HbVs; particle diameter = 251 ± 80 nm; [Hb] = 10 g/dL) are artificial oxygen carriers encapsulating a conc. Hb solution in phospholipid vesicles, and their oxygen transporting ability has been extensively studied. To accurately measure the LPS content in the HbV suspension, we tested the solubilization of HbV with deca(oxyethylene) dodecyl ether ($C_{12}E_{10}$), used to release the LPS entrapped in the vesicles, as a pretreatment for the succeeding LAL assay of the kinetic–turbidimetric gel clotting (detecting wavelength, 660 nm). The $C_{12}E_{10}$ surfactant interferes with the gel clotting in a concentration-dependent manner, and the optimal condition was determined in terms of minimizing the dilution factor and $C_{12}E_{10}$ concentration. We clarified the condition that allowed the measurement of LPS at >0.1 endotoxin units (EU)/mL in the HbV suspension. Moreover, the utilization of histidine-immobilized agarose gel effectively concentrated the trace amount of LPS from the $C_{12}E_{10}$-solubilized HbV solution and washed out $C_{12}E_{10}$ as an inhibitory element. The LAL assay with the LPS-adsorbed gel resulted in the detection limit of 0.0025 EU/mL. Pretreatment with $C_{12}E_{10}$ would be applicable not only to HbVs but also to other drug delivery systems using phospholipid vesicles encapsulating or incorporating functional molecules. J Pharm Sci 93:310–321, 2004

Keywords: liposomes; calorimetry (ITC); surfactants; phospholipids; nanotechnology

INTRODUCTION

Phospholipid vesicles or liposomes have been extensively studied as a drug delivery system since the formation of a vesicular structure was discovered in the suspension of egg yolk phosphatidylcholine,[1] and some are now approved for clinical use as antifungal or anticancer therapies.[2] Vesicles encapsulating concentrated hemoglobin (Hb), so-called Hb-vesicles (HbVs) or liposome-encapsulated Hb (LEH), have been developed as oxygen carriers, and their sufficient ability to transport oxygen that is comparable to the ability of blood has been well clarified.[3–7] In comparison with other Hb-based oxygen carriers, such as polymerized Hb or crosslinked Hb, HbVs most closely reproduce the characteristics of natural red blood cells, such as the cell membrane function of physically preventing the direct contact of Hb

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with the components of blood and vasculature during circulation.[8–11] The advantages of artificial oxygen carriers are the absence of blood-type antigens and infectious viruses and stability for long-term storage.[12] In this sense, the infusion of oxygen carriers becomes superior to the conventional blood transfusion, which still has the potential of mismatching, the risk of infections secondary to infusion of contaminated blood, and the problem of only a few weeks storage life.[13]

The process of production of a HbV has to be guaranteed with a good manufacturing practice (GMP) standard so that the HbV is a biological product that adheres to the strict regulations of impurity and viral and bacterial contamination. It is strictly required to monitor the content of the lipopolysaccharide (LPS), known as an endotoxin, which is a component of the outer membrane of gram-negative bacteria that possesses a large variety of biological influences on numerous mammalian cells and tissues.[14] An endotoxin is an extremely potent toxin with lethal doses (LD_{50}) of 3 and 1 mg/kg in rats and dogs, respectively.[15,16] The U.S. Food and Drug Administration (FDA) has established a guideline for human maximal permissible endotoxin dose for parenteral products [5 endotoxin units (EU)/kg][17] that may include Hb-based oxygen carriers. This limit is based on the endotoxin activity (1 EU = 100 pg) and can be measured by the *Limulus* amebocyte lysate (LAL) assay, in which LAL clots and forms a gel in the presence of LPS.[18] In general, the LAL method has advantages over the rabbit pyrogen testing because the LAL method requires a lower amount of sample and the assays can easily be repeated.[19] Because the volume of oxygen carriers to be infused for shock resuscitation or acute hemodilution is estimated to be <20 mL/kg, the specific endotoxin limits should be 0.25 EU/mL (= 5/20), which is similar to that for water for injection (0.25 EU/mL).

Bacterial LPS is an amphiphilic gigantic macromolecule, therefore, it hydrophobically interacts with protein and biomembranes.[20] Hb strongly interacts with LPS, showing synergistic toxicity.[21–23] The constituent of endotoxin that causes LAL gelation is a glycophospholipid that is designated lipid-A.[24] Lipid-A possesses several fatty acid constituents that are readily inserted into the bilayer membrane of the phospholipid vesicles. The inclusion of lipid-A in the phospholipid vesicles markedly reduces several functions of lipid-A, such as its LAL gelation activity.[25,26] As a consequence, the researchers who study HbVs or other phospholipid vesicles for delivering other functional molecules encountered a problem in measuring the LPS content for the quality control of these materials.[27–30] Considering this background information, we aimed to find the optimal condition for the pretreatment of HbVs using a surfactant to release LPS[28,29] with a minimal interference effect for the subsequent kinetic-turbidimetric LAL assay using a Toxinometer®. For a better detection limit, we tested the histidine-immobilized agarose gel that effectively adsorbs LPS to concentrate the trace amount of LPS and to eliminate the solutes that interfere with the LAL assay.[30–33]

EXPERIMENTAL

Preparation of Poly(ethylene glycol) (PEG)-Modified Hb-Vesicles (HbVs)

The PEG-modified HbVs were prepared under sterile conditions as previously reported.[8,34–36] Hb was purified from outdated donated blood provided by the Hokkaido Red Cross Blood Center (Sapporo, Japan) and Japanese Red Cross (Tokyo, Japan). The purification process included ultrafiltration to remove the stromal components and pasteurization at 60°C for 10 h to denature the concomitant proteins. This process results in extremely high purity of Hb (>99.9%).[35,37] The encapsulated Hb (38 g/dL) contained 14.7 mM pyridoxal 5′-phosphate (PLP, Merck Company, Darmsdart, Germany) as an allosteric effector at a PLP/Hb molar ratio of 2.5. The lipid bilayer was composed of a mixture of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), cholesterol, and 1,5-bis-*O*-hexadecyl-*N*-succinyl-L-glutamate (DPEA) at a molar ratio of 5:5:1 (Nippon Fine Chemical Company, Osaka, Japan), and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-PEG_{5000} (PEG–DSPE; NOF Company, Tokyo, Japan).[38] Thus, the vesicular surface is covered with PEG chains. The molar composition of DPPC/cholesterol/DPEA/PEG–DSPE was 5:5:1:0.033. The HbV particles were suspended in saline at an Hb concentration of 10 g/dL. The physicochemical parameters of the HbV were as follows: particle diameter, 251 ± 80 nm; [Hb], 10 g/dL; [metHb], <3%; [carbonylhemoglobin (HbCO)], <2%; lipids, 5.6 g/dL; and oxygen affinity (P_{50}), 31 Torr. All the analytical methods are described elsewhere.[12]

Thermodynamic Analysis of Insertion of LPS into Vesicles by Isothermal Titration Calorimetry (ITC)

An OMEGA titration microcalorimeter (MCS ITC, Microcal Inc., Northampton, MA) was used to analyze the interaction of LPS with the phospholipid bilayer membrane.[38,39] The model phospholipid vesicles of DPPC/cholesterol/DPEA/PEG–DSPE (5:5:1:0.033 by mol) were prepared under sterile conditions by simply dispersing the lipid powders in pure water (0.05 g/dL). The resulting suspension was freeze-thawed to enhance the dispersion and to regulate the particle diameter to 519 ± 78 nm. Twenty-five cumulative injections of an LPS solution (10 μL, 334 μM in pure water, from *Salmonella Minnesota* wild type, MW = ~20 kDa[40]; Sigma Chemical Company, St. Louis, MO) into the vesicle suspension (1.35 mL, [lipid] = 835 μM) were performed using a computer-controlled microsyringe while the suspension was stirred at 400 rpm and the temperature was strictly controlled at 37°C. Changes in the calorific values and the total calorific values were automatically measured. An identical injection of LPS into pure water in the absence of vesicles and injection of pure water into the vesicle suspension were performed as references.

Determination of the Solubilization Condition of HbV with Surfactants

An HbV suspension, diluted twice or five times with pure water ([Hb] = 2 or 5 g/dL, 200 μL), and deca(oxyethylene) dodecyl ether ($C_{12}E_{10}$, 0–10 vol% dissolved in pure water, 800 μL; Sigma Chemical Company) were mixed in a quartz cuvette (2 mm thickness). The resulting suspension was heated at 42°C for 2 min to enhance the solubilization. The turbidity was measured at 660 nm with an ultraviolet–visible (UV–vis) spectrophotometer (V-560, Jasco, Tokyo). For comparison, other surfactants that are often used for the dissolution of liposomes or biomembranes,[28,29,41] [sodium dodecyl sulfate (SDS), Kanto Chemical Company, Tokyo, Japan; Triton-X100 and nona(oxyethylene) dodecyl ether ($C_{12}E_9$), Sigma Chemical Company] were also tested for the solubilization of HbV.

Calibration Curves for LAL Assay and Influence of a Surfactant

A powdered LPS (LPS purified from *Escherichia coli* UKT-B containing mannitol and glycine, Wako Pure Chemical Industries, Tokyo, Japan), a control standard LPS defined in Japanese Pharmacopoeia, was dissolved in pure water (Water for Injection, Otsuka Pharmaceutical Company, Tokyo, Japan) to prepare the stock solution ([LPS] = 500 EU/mL). The LPS standard solutions ([LPS] = 0.01~20 EU/mL) were prepared by dilution of the stock solution with pure water. The LPS solutions (200 μL) were mixed with a $C_{12}E_{10}$ solution (0–5 vol%, 800 μL), diluted eight times with pure water, and then mixed with an equal amount of LAL solution (*Limulus* ES-II Test Wako, Wako Pure Chemical Industries) containing a lysate from *Limulus polyphemus*, Tris-HCl buffer (pH = 7.1), and a derivative of β-1,3-glucan. The LPS content in the water for injection is below the detection limit (<0.0001 EU/mL). The LPS content was measured as the turbidity change during the gel clotting in the reaction of the LAL reagent and endotoxin reaction with a parallel turbidimetric time assay using a Toxinometer® (ET-201, Wako Pure Chemical Industries).[42,43] The gelation time was defined by the reduction of the transmittance at 660 nm to 95% of the initial value according to the instruction manual.

Measurement of LPS in an HbV Suspension (Method 1) and Confirmation of its Validity

The HbV suspension ([lipid] = 6.0 g/dL, [Hb] = 10 g/dL) was put in LPS-free glass tubes, diluted with water for injection, mixed with a $C_{12}E_{10}$ solution, and then heated at 42°C for 2 min, as shown in Figure 1. The solution was then diluted eight times with water for injection. The LAL reagent (Wako Pure Chemicals Industries) was then added to the solution, and the LPS content was measured as the turbidity change in gel-clotting in the LAL–endotoxin reaction with a parallel turbidimetric time assay using a Toxinometer® ET-201.

To evaluate the validity of the Method 1, the recovery of spiked LPS was measured. An LPS standard solution ([LPS] = 0.4, .25, 2.5, 5.0, and 10.0 EU/mL) was added to the powdered lipid to adjust the lipid concentration to 6.0 g/dL. The suspension was agitated, diluted five times with the water for injection, and then freeze-thawed three times to prepare the LPS-contaminated vesicles. The recovery ratio was calculated by comparing the LPS concentration measured by the method already described and the spiked LPS concentration. A similar experiment was performed with HbV. An HbV suspension ([Hb] = 4 g/dL)

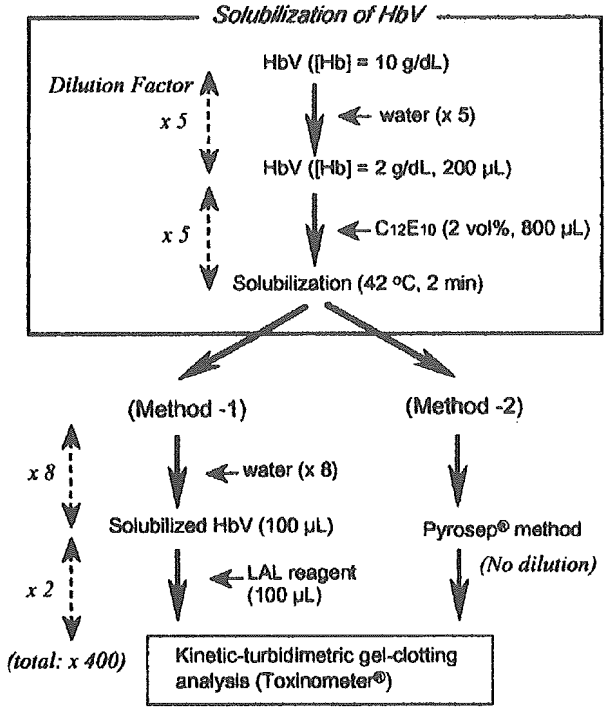

Figure 1. Procedures to quantitatively measure LPS in the HbV suspension using $C_{12}E_{10}$ for HbV solubilization and LAL assay, with dilution factors at every mixing of solutions. Method 1 is the condition for Entry 1 in Table 1. The Pyrosep® method (Method 2) does not have a dilution after solubilization of HbV.

was mixed with an equal volume of the LPS standard solution ([LPS] = 0.04, 0.16, 0.4, and 4.0 EU/mL) to prepare the LPS-spiked HbV ([Hb] = 2 g/dL), and solubilized by $C_{12}E_{10}$ for LAL assay as already described. As for the Hb solution, the same LPS standard solution ([LPS] = 0.002, 0.008, 0.02, 0.2, and 2.0 EU/mL) was mixed with an equal volume of an Hb solution ([Hb] = 4 g/dL) to prepare the LPS-spiked Hb solution ([Hb] = 2 g/dL), which was directly mixed with the LAL reagent to measure the LPS concentration. The recovery ratio was calculated by comparing it to the spiked LPS.

To confirm the effectiveness of the $C_{12}E_{10}$ treatment, LPS-contaminated vesicles were tested by Method 1 with or without $C_{12}E_{10}$. The vesicles were prepared by the addition of an LPS solution (1.0 EU/mL) to the powdered lipids (1.2 g/dL). The resulting suspension was freeze-thawed three times to enhance the dispersion of the lipids and to regulate the particle diameter to ~500 nm. The LPS concentration was measured, as already mentioned, using $C_{12}E_{10}$, and also without the addition of the $C_{12}E_{10}$ solution.

LPS Measurement in $C_{12}E_{10}$-Solubilized HbV using Histidine-Immobilized Agarose Gel (Pyrosep®, Method 2)

The *Limulus* PS Single Test Wako (Wako Pure Chemicals Industries) was used. A 5-mL aliquot of the $C_{12}E_{10}$-solubilized HbV solution ([Hb] = 0.4 g/dL, $[C_{12}E_{10}]$ = 1.6 vol%) was injected into a glass capillary column that contained 0.7 mL of histidine-immobilized agarose gel (Pyrosep®) to adsorb the LPS into the gel. The glass capillary column has a filter at the connected end of a silicone tube to retain the gel but allows permeation of the solution by aspirating air through the silicone tube. The gel was washed with 2 mL of LPS-free water to remove all the solutes except LPS, and then 0.3 mL of LAL-ES reagent (*Limulus polypherus* amebocyte lysate lyophilized, containing Tris-HCl buffer and β-1,3-glucan derivative) was injected into the capillary. All of the suspension was immediately pushed back into a glass vial, and the gelation time of the suspension was measured with a Toxinometer® (ET-201or ET-301 BL) with an extended MT-358 analysis module (Wako Pure Chemicals Industries). The time course of the transmittance change was detected at 660 nm, as already described. The inhibition/enhancement testing was performed to confirm the recovery ratio of the spiked LPS to the HbV in the same manner as already described.

RESULTS

Interaction of LPS with Phospholipid Vesicles Measured by Isothermal Titration Calorimetry (ITC)

ITC of the LPS injection showed exothermic peaks for every injection into the vesicles suspension, and the enthalpy change (ΔH) was calculated to be ~−80 kcal/mol (Fig. 2). As a reference experiment, the LPS solution was injected into pure water, and it was confirmed that the thermodynamic change was negligibly small (data not shown). The maximum amount of incorporation was 7.6 mol% into the outer surface of the vesicles under the assumption that the lamellarity of the vesicles was 2.

Solubilization of HbV with $C_{12}E_{10}$ Surfactant

The absorbance of the HbV suspension at 660 nm was 1.13, which is significantly higher than that of the Hb solution (0.08) due to the light scattering effect of the HbV particles. However,

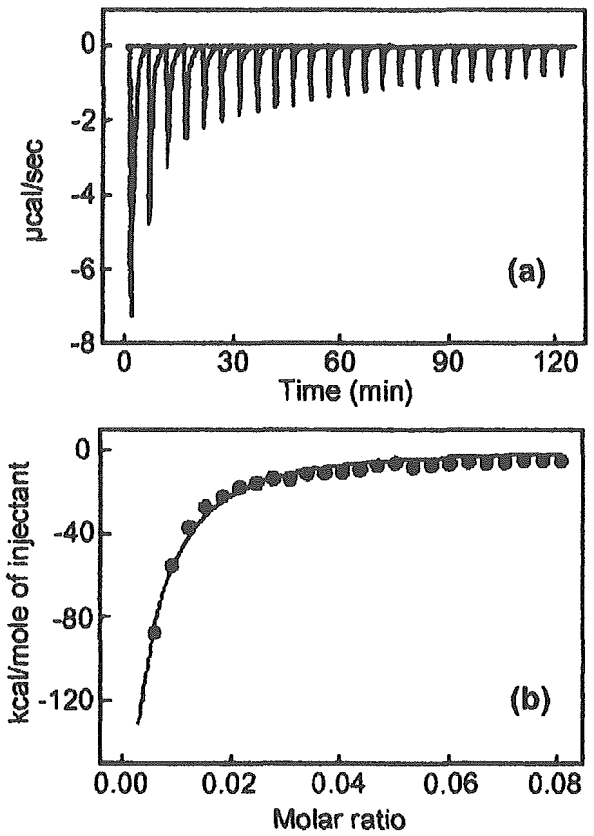

Figure 2. (a) Raw and (b) integrated data of the isothermal titration calorimetry for the incorporation of LPS (*Salmonella Minnesota* wild type) into phospholipid vesicles: [LPS] = 334 μM in a syringe (10 μL/one injection), [lipid] = 835 μM in a cell.

this absorbance decreased with the addition of the $C_{12}E_{10}$ solution, indicating the solubilization of HbV with the surfactant (Fig. 3). The complete solubilization required the addition of 2 vol% of $C_{12}E_{10}$ solution. At this concentration, the absorbance at 660 nm was identical with the pure Hb solution without vesicles. After the solubilization procedure, the color of the solution changed from red to brown, indicating the formation of methemoglobin. The concentration of $C_{12}E_{10}$ and the lipids of HbV in the resulting solution are 1.6 and 0.24 g/dL, respectively. $C_{12}E_{10}$ shares 87 wt% (86 mol%) of the mixed micelles. As for the other surfactants, $C_{12}E_9$ showed a similar ability to dissolve HbV that was evident from the profiles of the reduction of the light scattering of HbV (Fig. 3). On the other hand, Triton-X and sodium dodecyl sulfate (SDS) showed incomplete reduction of the absorbance and they could not sufficiently dissolve HbV.

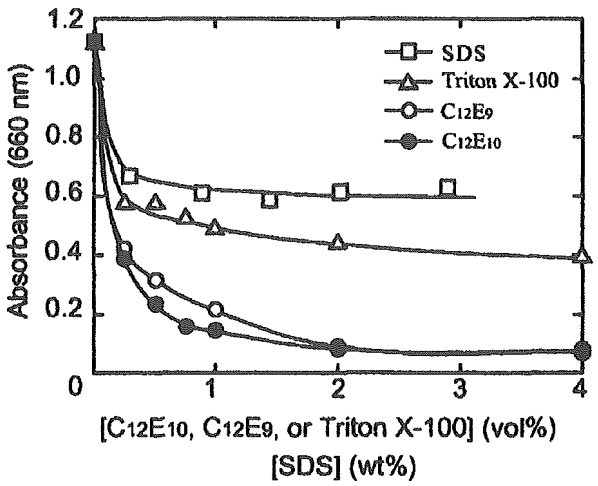

Figure 3. Absorption changes at 660 nm of HbV after solubilization of HbV by the addition of surfactants ($C_{12}E_{10}$, $C_{12}E_9$, Triton-X100, and SDS). Various concentration of a surfactant (800 μL) was added to the HbV suspension ([Hb] = 2 g/dL, 200 μL) and incubated at 42°C for 2 min.

Effect of $C_{12}E_{10}$ on Calibration Curves for LPS in LAL Assay (Method 1)

The calibration curves for Method 1 were drawn as logarithm plots of the gelation time versus the final LPS concentration (Fig. 4). Actually, the

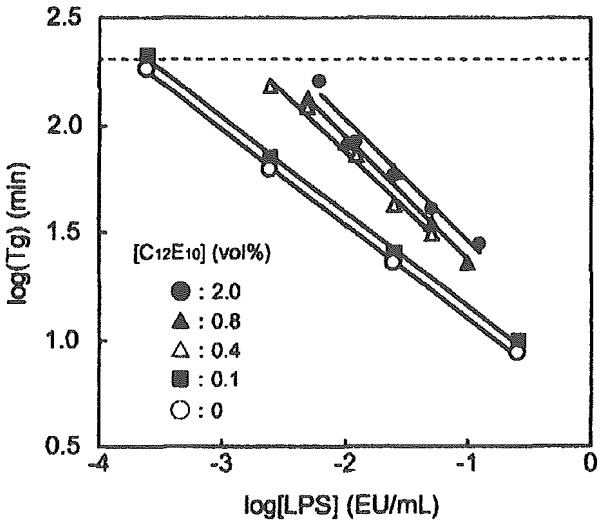

Figure 4. Calibration curves for the quantitative measurement of LPS in the presence of $C_{12}E_{10}$ at various concentrations. [LPS] is the final concentration in the test tube for turbidimetry. Dilution factors should be multiplied to obtain [LPS] in the HbV suspension at [Hb] = 10 g/dL. For example, [LPS] should be multiplied with 400 at $[C_{12}E_{10}] = 0.1$ wt%. The broken line indicates the gelation time limit for Toxinometer® (200 min).

plots show a linear relationship over a wide range of LPS concentrations (final concentration: 0.000125–0.25 EU/mL). The gelation time limit for the Toxinometer® is 200 min (log 200 = 2.3) as shown by the dashed line. Increasing the $C_{12}E_{10}$ concentration tended to retard the gelation. However, 0.1 vol% of the final $C_{12}E_{10}$ concentration did not show a significant retardation. At the LPS concentration of 0.01 EU/mL, the gelation time of ~32 min without $C_{12}E_{10}$ was prolonged to 39 min with 0.1 vol% of $C_{12}E_{10}$ and to ~107 min with 2.0 vol% of $C_{12}E_{10}$. In the case of the other surfactant, the presence of 0.1 vol% $C_{12}E_9$ prolonged the gelation time to 50 min (data not shown). Therefore, the inhibitory effect of $C_{12}E_9$ is stronger than that of $C_{12}E_{10}$.

LPS Measurement in HbV (Method 1)

To improve the LPS detection limit, the dilution factor could be minimized by increasing the HbV concentration at the solubilization process or by decreasing the amount of diluent (Table 1). The lowest detection limit (0.1 EU/mL) was obtained for entry No.1, with 0.1 vol% $C_{12}E_{10}$. The conditions of entries 2, 3, and 4 could reduce the dilution factor, however, [$C_{12}E_{10}$] at >0.4 vol% resulted in a significant inhibition of the gelation, as shown in Figure 4 and by the worsened detection limit (0.23 EU/mL).

The recoveries of the spiked LPS ranged between 92 and 124% for a wide range of LPS concentrations (Table 2, next page). The LPS measurement in an Hb solution did not require a surfactant so it was possible to detect a lower concentration (0.005 EU/mL), which was significantly better than the detection limit for HbV (0.1 EU/mL) that required $C_{12}E_{10}$. The FDA Guideline, published in 1987, requires that the inhibition/enhancement testing of spiked LPS at four times the amount of the detection limit and that the recovery should be within 100 ± 25%.[17] We tested the addition of LPS at 0.4 EU/mL (= 4 × 0.1 EU/mL) to the phospholipid vesicles and HbVs and at 0.02 EU/mL (= 4 × 0.005 EU/mL) to the Hb solution, and all the recoveries were within 100 ± 25%.

The LPS content in the LPS-contaminated vesicles, measured with the aforementioned LAL assay using $C_{12}E_{10}$, was 1.03 EU/mL (Table 3). On the other hand, the LAL assay without $C_{12}E_{10}$ resulted in 0.6 EU/mL. The recovery was calculated to be 60%.

The Pyrosep® Method to Detect LPS in $C_{12}E_{10}$-Solubilized HbV (Method 2)

The calibration curves of the Pyrosep® method in the presence of $C_{12}E_{10}$ showed a detection limit of 0.0001 EU/mL or lower (Fig. 5). The dilution factor for the HbV measurement was 25. Therefore, the LPS detection limit for an HbV suspension at [Hb] = 10 g/dL is 0.0025 EU/mL. One HbV suspension showing an LPS content of <0.1 EU/mL as measured by Method 1 was shown to have an LPS content of 0.011 EU/mL by the Pyrosep® method; this HbV was used for the inhibition/enhancement testing. The recovery of spiked LPS (Fig. 5, inset; 0.001, 0.01, and 0.1 EU/mL) from an HbV suspension was within 100 ± 25% over a wide range of LPS concentrations.

DISCUSSION

The interaction between an amphiphilic LPS molecule and a phospholipid vesicle should influence the accuracy of the LAL assay. It has been

Table 1. Solubilization Condition of HbV with $C_{12}E_{10}$ and Detection Limit of LPS[a]

Entry	Condition of HbV Solubilization		Dilution Factor[b]	Final vol% [$C_{12}E_{10}$]	Detection Limit of LPS (EU/mL HbV)[c]
	HbV/200 μL ([Hb] g/dL)	$C_{12}E_{10}$/800 μL ([$C_{12}E_{10}$] vol%)			
1[d]	2	2	400	0.1	>0.1
2	2	2	100	0.4	>0.25
3	2	2	50	0.8	>0.25
4	5	5	20	2.0	>0.23

[a]The calibration curves are shown in Figure 4.
[b]See Figure 1, from HbV ([Hb] = 10 g/dL) to gel clotting assay.
[c]At [Hb] = 10 g/dL.
[d]The optimal condition in this study.

Table 2. Inhibition/Enhancement Testing to Monitor the Recovery of Spiked LPS

Sample	Spiked LPS (EU/mL)[a]	Recovery (%)
Vesicles (6 g/dL) with $C_{12}E_{10}$	0.4	108.6 ± 4.6[b]
	1.25	92
	2.5	116
	5	103
	10	120
HbV (Hb, 10 g/dL; lipid, 6 g/dL) with $C_{12}E_{10}$	0.1	107
	0.4	111.3 ± 3.6[b]
	1.0	114
	10.0	112
Hb solution (10 g/dL) without $C_{12}E_{10}$	0.005	121
	0.02	103.9 ± 4.4[b]
	0.05	101
	0.5	124
	5	97

[a]LPS was spiked in the $C_{12}E_{10}$-solubilized vesicles and HbV, and Hb solution without $C_{12}E_{10}$. All the spiked LPS concentrations are converted to the conditions of [Hb] = 10 g/dL, or [lipid] = 6 g/dL. All the recoveries were within 100 ± 25%.
[b]$n = 3$.

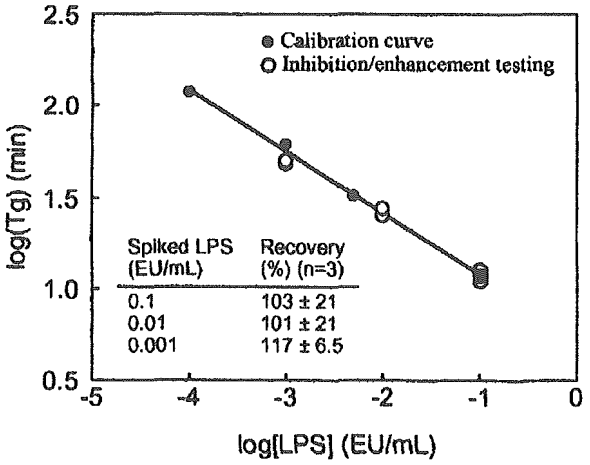

Figure 5. A calibration curve for the Pyrosep® method including the treatment of $C_{12}E_{10}$, and the results of the LPS-spiking test to the HbV suspension. Inset: The recovery of the spiked LPS in the HbV suspension. During the procedure, the HbV suspension was diluted 25 times. Therefore, the spiked LPS concentrations of 0.1, 0.01, and 0.001 EU/mL correspond to 2.5, 0.25, and 0.025 EU/mL, respectively, at [Hb] = 10 g/dL. The original LPS content in the HbV suspension was 0.011 EU/mL at [Hb] = 10 g/dL.

well documented that the biological activity of the LPS is significantly suppressed when an LPS molecule is incorporated into phospholipid vesicles.[25,26,44,45] However, there has been no report on the quantitative observation of the LPS–phospholipid vesicle interaction. Using the ITC method, we quantitatively clarified for the first time that LPS from the *Salmonella Minnesota* wild type (smooth form) was inserted into the phospholipid vesicles with an enthalpy change (ΔH) of ~−80 kcal/mol and the maximum incorporation of ~7.6 mol% on the outer surface of the vesicles. To our knowledge, the ΔH value of PEG_{5000}–DSPE (MW of PEG = 5 kDa) for the same phospholipid vesicles is only −13 kcal/mol.[38]

Table 3. Detection of LPS in the LPS-Contaminated Vesicles with or Without the $C_{12}E_{10}$ Treatment[a]

Treatment	LPS (EU/mL)	Recovery (%)
Without $C_{12}E_{10}$	0.6	60
With $C_{12}E_{10}$	1.03	103

[a][lipid] = 1.2 g/dL, [spiked LPS] = 1.0 EU/mL. The higher recovery of the $C_{12}E_{10}$-solubilized vesicles indicates the necessity of the pretreatment.

This comparison indicates that LPS inserted into the bilayer membrane is thermodynamically more stabilized than PEG_{5000}–DSPE. The large difference in ΔH is probably due not only to the hydrophobic interactions by the eight alkyl chains of LPS but also to the hydrogen bonding of the amide bonds into the interface of the hydrophobic and hydrophilic regions that contribute to the interaction of LPS with the phospholipid bilayer membrane. As for the interaction between Hb and LPS, the ITC analysis was recently reported by Jurgens et al.,[46] who clarified that 3–5 LPS molecules bind to one Hb molecule.

These ITC results clearly emphasize the necessity of the pretreatment of HbV for the LAL quantitative measurement of LPS; that is, to release LPS from the phospholipid vesicles using an organic solvent[47] or surfactant.[28,29] We tested the solubilization of HbV with a surfactant because of the presence of a large amount of Hb that is insoluble in an organic solvent. We determined the optimal condition as Method 1 to detect the LPS content, with the best detection limit at 0.1 EU/mL for HbV at [Hb] = 10 g/dL. The lipid bilayer of HbV is composed of DPPC/cholesterol/DPEA/PEG_{5000}–DSPE at a molar ratio of 5:5:1:0.033, and the vesicles are quite stable due to the

high phase transition temperature (T_c) of DPPC (41°C), the improved molecular packing using cholesterol, the repulsive force between the bilayer membrane due to the presence of a negatively charged DPEA, and the steric hindrance of the PEG chains of PEG–DSPE. However, the procedure of the $C_{12}E_{10}$ addition and incubation at 42°C (near T_c) for 2 min completely diminished the light scattering of HbV, and this condition can effectively solubilize the HbV.

We confirmed that $C_{12}E_{10}$ showed a significant inhibitory effect at a higher concentration that is probably due to the inhibition of gel formation or solubilization of the gelated coagulin (Fig. 3). However, the final concentration of $C_{12}E_{10}$ at 0.1 vol% showed a moderate inhibitory effect. As for the other surfactants, nona(oxyethylene) dodecyl ether ($C_{12}E_9$) showed a more significant inhibitory effect for the LAL assay even though it showed a sufficient solubilization of HbV, and Triton-X 100 showed a lower solubilization of HbV. An anionic surfactant, SDS, did not show complete HbV solubilization. Moreover, according to the literature, SDS completely inhibits gelation only at >0.005 wt%.[29] On the contrary, nonionic surfactants, especially $C_{12}E_{10}$, moderately decrease the LAL activity in relation to the surfactant concentration. $C_{12}E_{10}$ has a sufficient ability to dissolve vesicles and a moderate inhibitory effect on the LAL assay. The solubilization of HbV with $C_{12}E_{10}$ should lead to the formation of micellar structures containing $C_{12}E_{10}$, LPS, and the lipid components. $C_{12}E_{10}$ shares 86 mol% of the components of the micelles, which is significantly higher than PEG–DSPE (0.04 mol%), and the aggregation number of the $C_{12}E_{10}$ micelle is estimated to be ~100.[41] Therefore, the LAL proteins could interact with the lipid-A moiety at the interface of the aqueous and hydrophobic phases of the micelles without the excluded volume effect of the PEG–DSPE.

Improvement of the detection limit requires a lower dilution factor and lower $C_{12}E_{10}$ concentration. However, a reduction in the dilution factor is accompanied by an increase in the concentration of $C_{12}E_{10}$. When the [$C_{12}E_{10}$] was 0.1 vol%, the inhibitory effect was minimal (see Fig. 4), and we obtained the optimal pretreatment condition as entry No. 1 in Table 1. The total dilution factor of this condition is 400. The FDA Guideline defines the maximum valid dilution (MVD) depending on the maximum dose (mL/kg) and lysate sensitivity.[17] The MVD for HbV should be 1000 (= 0.25/0.00025) under the condition that the LPS limit for HbV is 0.25 EU/mL, and the detection limit in the standard curve is 0.00025 EU/mL [log(0.00025) EU/mL = −3.60; (see Fig. 4). Therefore, the dilution factor of 400 is within the MVD.

We compared the measurement of the LPS content in the vesicles with or without $C_{12}E_{10}$ and found that the solubilization of vesicles with $C_{12}E_{10}$ showed a higher LPS concentration. This result clearly demonstrates that LPS molecules in the bilayer membrane or in the inner aqueous phase of the vesicles are released and detected after the solubilization. The recovery of spiked LPS was reproducible within the range 92–120% over a wide range of LPS concentrations. The FDA Guideline requires LPS recovery in the range of 100 ± 25% to indicate no influence of solutes in a specimen.[17] This value was revised to 100 ± 50% in 1991.[48] In spite of the ITC analysis of Hb that indicated 3–5 LPS molecules bind to one Hb,[46] we did not see any interference effect by the presence of Hb. Our result is in contrast to previous reports. Levin et al.[22,49] reported that LPS bound to Hb showed activation of LAL, whereas Archambault et al.[50] reported a significant reduction of LAL activity when the LPS was bound to Hb. Jurgens et al.[46] reported that LAL activity depended on the concentrations of LPS and Hb in the assay. The reason for these discrepancies is not clear, however, there is a large difference in the LPS concentrations for the LAL assay between the reports. In our assay conditions, we measured the LPS concentration at <0.2 EU/mL (~20 pg/mL). On the other hand, other groups conducted the experiments at much higher LPS concentrations (100 pg/mL–800 μg/mL). In some cases, the LPS concentrations were above the critical aggregation concentrations (10–38 μg/mL).[40] It was reported that the LAL activity was significantly suppressed by the LPS aggregation.[51] In the presence of Hb, LPS should bind to Hb rather than form aggregates, resulting in an apparent enhancement of the LAL activity. On the contrary, in our significantly diluted assay condition, it can be speculated that LPS should not be aggregated and be more freely dispersed from Hb, and the presence of Hb should not induce any inhibition or enhancement.

The LPS measurement is often performed to monitor patients with septic shock. Because blood plasma contains some unknown elements that inhibit or accelerate the LAL assay, Obayashi et al.[52] proposed perchroic acid (PCA) treatment to inactivate or remove the interfering plasma components and to obtain a sufficient recovery of

the spiked LPS. In this method, the proteins denatured by PCA are removed as a precipitate. However, this method leads to underestimation of the LPS content because LPS strongly binds to plasma proteins, such as albumin, lipoprotein, and the LPS-binding protein,[53] which should denature and precipitate after the PCA. The new PCA method[54] includes the addition of an alkaline solution to solubilize the denatured protein so that the recovery of LPS is much improved. The dilution of plasma with water and subsequent heating at 100°C for 10 min in the presence of a weak surfactant such as Triton-X100 also releases LPS from the plasma protein.[42,45] On the other hand, our HbV does not contain plasma-derived interfering elements because it is made from ultrapurified Hb solution. During the solubilization of HbV with $C_{12}E_{10}$ at 42°C for 2 min, Hb is partly oxidized to form metHb. However, there is no further denaturation of metHb and its precipitate. Moreover, a derivative of β-1,3-glucan, which is well-known as a significant interfering element for the LAL assay,[55,56] is intentionally added to the LAL reagent from Wako Pure Chemicals Industries, so the influence of contaminated β-1,3-glucan in a specimen is eliminated.[57] For all the pretreatments, the kinetic–turbidimetric assay system can be used for the LAL clotting assay using the Toxinometer®.[43,58] In the case of Hb and HbV, the strong absorption band of the Hb molecule between 400 and 600 nm may affect not only the turbidimetric measurement but also the chromogenic measurement with detection wavelengths of 405 or 545 nm (e.g., Endospecy, Seikagaku Kogyo, Ltd., Japan). In this sense, the detection wavelength of 660 nm using the Toxinometer® should be appropriate.[58]

The Pyrosep® method (Method 2) was very effective in detecting trace amounts of LPS in the $C_{12}E_{10}$-solubilized HbV samples. The solubilization of HbV requires a dilution factor of 25 as in Method 1 (see Fig. 1). There is no additional dilution afterward because the solubilized HbV was treated with the Pyrosep® column and the LPS-bound agarose gel was directly mixed with the LAL reagent. As a result, the lowest LPS concentration for the calibration curve was 0.0001 EU/mL, with a dilution factor of only 25; therefore, the detection limit was calculated to be 0.0025 EU/mL in HbV at [Hb] = 10 g/dL. The LPS content of one HbV suspension was determined to be <0.1 EU/mL, by Method 1, whereas it was 0.011 EU/mL as measured by the Pyrosep® method. This latter method also showed a sufficient recovery of the spiked LPS for a wide range of LPS concentrations. Not only the electrostatic interaction between the cationic region of the histidine residue and the anionic region of LPS, but also the hydrophobic interaction between the spacer region of the histidine–agarose conjugate and alkyl chains of LPS should contribute to the specific adsorption of LPS on the agarose gel.[30] After the adsorption of LPS, the agarose gel was washed with LPS-free water so that all the interfering elements, such as a surfactant, were removed before reacting with LAL reagent. Because Hb was also washed out, the detection wavelength was not limited to 660 nm to avoid the absorption band of Hb. As far as we know, this is the first attempt to measure the LPS content by the combination of Pyrosep® and surfactant pretreatment.

This modified LAL assay using $C_{12}E_{10}$ and the Toxinometer® is routinely used in our production system of HbVs. Significant attention is paid to the quality control of HbVs for preclinical studies, and all the HbVs prepared under sterile conditions showed an LPS content of <0.1 EU/mL at [Hb] = 10 g/dL. Moreover, utilization of the Pyrosep® improved the detection limit to 0.0025 EU/mL. Our method enables an accurate measurement of trace amounts of LPS in HbVs as an oxygen carrier of which the dose rate should be significantly large in comparison with the conventional drugs. Of course, this method is applicable to the quality control of other phospholipid vesicles and also protein drugs such as albumin and their recombinant types that strongly bind LPS.

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