Immobil. Biotechnol. **25**, 335–346
46. Chen, X., Nishide, H., and Tsuchida, E. (1996) *Bull Chem. Soc. Jpn.* **69**, 255–259
47. Bouwer, S. T., Hoofd, L., and Kreuzer, F. (1997) *Biochim. Biophys. Acta* **1338**, 127–136
48. Minton, A. P., and Ross, P. D. (1978) *J. Phys. Chem.* **82**, 1934–1938
49. Sakai, H., Tomiyama, K., Masada, Y., Takeoka, S., Horinouchi, H., Kobayashi, K., and Tsuchida, E. (2003) *Clin. Chem. Lab. Med.* **41**, 222–231
50. Azarov, I., Huang, K. T., Basu, S., Gladwin, M. T., Hogg, N., and Kim-Shapiro, D. B. (2005) *J. Biol. Chem.* **280**, 39024–39032
51. Gow, A. J., and Stamler, J. S. (1998) *Nature* **391**, 169–173
52. Rose, E. J., and Hoffman, B. M. (1983) *J. Am. Chem. Soc.* **105**, 2866–2873
53. Olson, J. S., Foley, E. W., Maillett, D. H., and Paster, E. V. (2003) *Methods Mol. Med.* **82**, 65–91
54. Herold, S., Exner, M., and Nauser, T. (2001) *Biochemistry* **40**, 3385–3395
55. Carlsen, E., and Comroe, J. H. (1958) *J. Gen. Physiol.* **42**, 83–107
56. Kavdia, M., Tsoukias, N. M., and Popel, A. S. (2002) *Am. J. Physiol.* **282**, H2245–H2253
57. Jeffers, A., Gladwin, M. T., and Kim-Shapiro, D. B. (2006) *Free Radic. Biol. Med.* **41**, 1557–1565
58. Vandegriff, K. D., McCarthy, M., Rohlfs, R. J., and Winslow, R. M. (1997) *Biophys. Chem.* **69**, 23–30
59. Tsai, A. G., Cabrales, P., Manjula, B. N., Acharya, S. A., Winslow, R. M., and Intaglietta, M. (2006) *Blood* **108**, 3603–3610
60. Nakai, K., Sakuma, I., Ohta, T., Ando, J., Kitabatake, A., Nakazato, Y., and Takahashi, T. A. (1998) *J. Lab. Clin. Med.* **132**, 313–319
61. Matheson, B., Kwansa, H. E., Bucci, E., Rebel, A., and Koehler, R. C. (2002) *J. Appl. Physiol.* **93**, 1479–1486
62. Doherty, D. H., Doyle, M. P., Curry, S. R., Vali, R. J., Fattor, T. J., Olson, J. S., and Lemon, D. D. (1998) *Nat. Biotechnol.* **16**, 672–676

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Electrostatic interactions and complement activation on the surface of phospholipid vesicle containing acidic lipids: Effect of the structure of acidic groups

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Abstract

Anionic vesicles containing acidic phospholipids are known complement activators. To clarify which negative physicochemical electrostatic charges on vesicles and structural specificities of acidic lipids are critical to complement activation, the electrostatic properties and activity to complement of two anionic vesicles modified with a carboxylic acid derivative or a conventional acidic phospholipid were compared. Electrophoretic mobility measurements indicated that the negative zeta potential and the electrostatic interactivity of these two anionic vesicles were equal at pH 7.4. However, the infusion of vesicles containing acidic phospholipid induced significant complement activation, while vesicles containing the carboxylic acid derivative failed to activate complement. These results indicate that the negative charge on the surface of vesicles is not critical for the activation complement, suggesting that complement activation is specific to the structure of acidic groups. This finding is likely to be important to the design of anionic biointerfaces and may support the promising medical applications of this anionic vesicle modified with a carboxylic acid derivative.
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Keywords: Liposome; Acidic lipid; Anionic surface; Electrostatic interaction; Complement activation

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1. Introduction

Anionic vesicles (liposomes), commonly formulated by mixing acidic phospholipids such as phosphoglycerol, phosphoserin, phosphoinositol, phosphatidic acid, cardiolipin, and poly (ethylene glycol) (PEG) conjugated phosphoethanolamine in lipid components are known to mediate complement activation [1–5]. Complement activation, which is followed by systemic immune activation and anaphylaxis shock, is regarded as a critical problem in the clinical setting of various biomaterials. The C1q subcomponent of C1 has a highly cationic region in residues 14–26 of the C1qA polypeptide chain, and this specific region of the collagenous stalk of C1q has been identified as being involved in interactions with negatively charged activators [5–8]. It is believed that the antibody-independent binding of C1q to the negatively charged surface of vesicles initiates the activation of the complement cascade via the classical complement pathway [2]. To prevent complement activation, the acidic phospholipids can be removed or their negative charge can be protected by chemical modification [2,4]. It has also been reported that surface modification with a dense PEG layer is effective for preventing complement activation by covering the surface charge [9].

Our group has developed phospholipid vesicles called hemoglobin-vesicles that encapsulate human hemoglobin and that can be used as a substitute for red blood cells and as an alternative to conventional transfusion [10–14]. To achieve this challenging application of vesicles, we had to develop anionic vesicles capable of encapsulating hemoglobin using a minimum amount of lipids, retaining the negative charge on the membrane which reduces the lamellarity of vesicles and is required for improving encapsulation capacity [11–13]. Several acidic lipids have been tested and a carboxylic acid derivative, L-glutamic

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acid, *N*-(3-carboxy-1-oxopropyl)-, 1,5-dihexadecyl ester (SA), has been used as an anionic component. Vesicles containing SA have been demonstrated to efficiently encapsulated hemoglobin, and given their stability, can be infused in considerably large doses as a red blood cell substitute [15,16]. In addition, administration of this formulation is not associated with considerable rejection by the blood immune system and complement in animals [17,18]. Recent collaboration involving our group found that vesicles containing SA are selectively captured by bone marrow macrophages at small injection doses in rabbits [19]. This targeting of bone marrow has not yet been reported for conventional anionic vesicles. These findings suggest that the surface characteristics of the anionic vesicles have a marked effect on their associated biological events and that this is highly dependent upon the nature of the acidic groups.

Based on the hypothesis that the negative electrostatic charge of acidic phospholipids on the surface of vesicles is one of the factors responsible for inducing complement activation, the electrostatics of vesicles containing SA may differ from those of vesicles containing acidic phospholipids. Conversely, when the electrostatic interactivity of these acidic lipids is equal, biological events such as complement activation are likely to be mediated by the molecular specificity of acidic lipids. To elucidate what aspects are critical to the biocompatibility and performance of anionic vesicles, a comparative analysis of the electrostatic interfacial properties of vesicles containing either SA or acidic phospholipids is essential. In this study, we elucidated the electrostatic interfacial properties of vesicles containing SA or an acidic phospholipid by electrophoretic mobility measurement and observed complement activation after the infusion of these vesicles in animals. The results clearly demonstrated that the negatively charged group on the surface of anionic vesicles is not critical to the activation of complement.

2. Materials and methods

2.1. Materials

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (PC), cholesterol (CH), and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (PG), and L-glutamic acid, *N*-(3-carboxy-1-oxopropyl)-, 1,5-dihexadecyl ester (SA), were purchased from Nippon Fine Chemical Co. Ltd. (Osaka, Japan). 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[monomethoxy poly(ethylene glycol) (5000)] (PEG-DSPE) was purchased from NOF Co. (Tokyo, Japan). Pentalysine and poly-L-lysine (Mw. 15–30 kDa) were purchased from SIGMA (St. Louis, MO).

2.2. Preparation of vesicles

Vesicle samples were produced using PC and CH (1/1, m/m, PC-vesicles), and varying amounts of SA (SA-vesicles) or PG (PG-vesicles). The mixed lipids were added to 10 mM phosphate buffer (NaCl; 20 mM, pH 7.4) and the dispersion was introduced into an extruder (Lipex Biomembrane, Canada) and extruded through the membrane filters (final pore size: 0.2 μm, Isopore®, Millipore, Tokyo, Japan) under pressure using nitrogen gas. For animal experiments, vesicles were composed of PC, CH, and SA or PG (1:1:0.2 molar ratio), with 0.3 mol% of PEG-DSPE incorporated to prevent the aggregation of vesicles [20]. Samples for animal experiments were prepared under sterile conditions.

2.3. Characterization of vesicles

The diameters of the resulting vesicles were determined with a COULTER submicron particle analyzer (N4SD, Coulter, Hialeah, FL), and the average diameter ± standard deviation (SD) was calculated. The phospholipid concentration was determined using a cholineoxidase method (Phospholipid C Test Wako; Wako Pure Chem., Tokyo). Endotoxin contamination in the samples prepared for the animal experiment was determined to be less than 0.1 EU/mL by the Limulus assay test [21].

2.4. Determination of zeta potential

A 10 μL aliquot of vesicles (lipid concentration: 2 g/dL) was diluted in 2 mL of 10 mM phosphate buffer (pH 3–9, 37 °C) containing 20 mM NaCl and incubated for 1 h at 37 °C. The pH was determined using a pH meter with a pH electrode (F-52, HORIBA, Kyoto, Japan) at 37 °C and the electrophoretic mobility of vesicles (lipid concentration: 0.01 g/dL) were determined by Laser Doppler Velocimetry (Zeta-Sizer Nano ZS, Malvern Instruments, Malvern, Worcestershire, UK). Measurement conditions and parameters were as follows: 37 °C, dielectric constant 74.4 (for dilute water solution), viscosity 0.6864 cP (for dilute water solution), and the applied voltage was 20 V/cm. The zeta potential was determined by measuring the electrophoretic mobility (U_E), and the zeta potential (ζ) and applied to the Henry equation:

$$U_E = \frac{2\varepsilon\zeta f(\kappa R)}{3\eta} \quad (1)$$

where ε is the dielectric constant, η is the viscosity of the solvent, $f(\kappa R)$ is the Henry function, κ is the Debye–Hückel parameter and the R is the radius of the particle. The Smoluchowski equation was used with $f(\kappa R)=1.5$ employed in the zeta potential calculations. Measurements were performed four times for each sample and statistical analysis was conducted using the average ± SD of the four measurements.

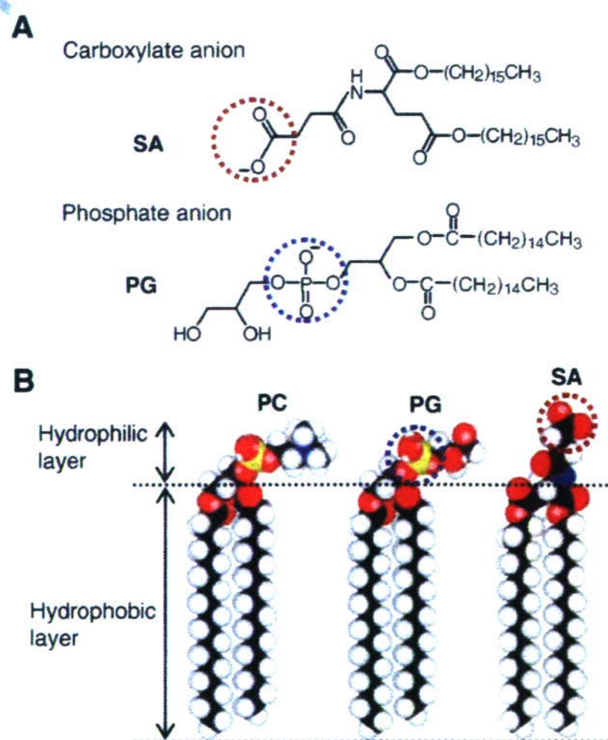

Fig. 1. Structure of anionic compounds for surface modification of vesicles. (A) Chemical structure of L-glutamic acid, *N*-(3-carboxy-1-oxopropyl)-, 1,5-dihexadecyl ester (SA) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (PG). The focus of this study is the different acidic groups shown in the dotted circle. (B) The CPK model of SA and PG with 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (PC) to estimate the distance of acidic groups from the membrane surface of PC. CPK models show the structure with minimized energy in molecular mechanistic calculation.

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133 *2.5. Electrostatic interactivity*

134 The electrostatic interactivity of the anionic vesicles was evaluated using the
135 change in the zeta potential in presence of Ca^{2+}, pentalysine, and poly-L-lysine
136 (Mw. 15–30 kDa) as an index. A 10 µL aliquot of vesicles (lipid concentration:
137 2 g/dL) was diluted in 2 mL of 10 mM 2-[4-(2-Hydroxyethyl)-1-piperazinyl]
138 ethanesulfonic acid (HEPES) buffer (pH 7.4, 37 °C) containing 0–3 mM $CaCl_2$
139 and 17–20 mM NaCl (total 20 mM), or containing varying amounts of pen-
140 talysine or poly-L-lysine (Mw. 15–30 kDa) with 20 mM NaCl. The dispersions
141 were incubated for 1 h at 37 °C before mobility measurement of the vesicles was
142 performed by electrophoresis as described in Section 2.4.

143 *2.6. Animal experiments*

144 Animal experiments were conducted under the guidelines recommended by
145 the National Institutes of Health, Animal Use and Care and the protocol was
146 approved by the Steering Committee for Animal Experimentation at Waseda
147 University. Male Wistar rats (250±20 g) were anesthetized with ether. The
148 vesicular dispersion (5 g/dL) was introduced into rats through the tail veins
149 at 1 mL/min (n=5 for each sample). Each rat received 5.6 mL/kg of body
150 weight of vesicle dispersion (lipids: 280 mg/kg of body weight). At 1 or 24 h
151 after injection, the blood was collected and centrifuged to separate the serum
152 (1×10^3 g, 10 min). The collected serum was further ultracentrifuged to remove
153 the vesicles (3×10^5 g, 30 min). The 50% hemolytic unit of complement serum
154 (CH50) was determined in accordance with general procedures for clinical
155 laboratory tests by a commercial company (BML, Japan).

156 *2.7. Statistical methods*

157 The data from the animal experiments are reported as means±standard
158 error of the mean. Statistical analysis was performed using Microsoft Excel for
159 Windows and CH50 values were compared using Student's unpaired t test.

160 **3. Results and discussion**

161 *3.1. Samples*

162 Two characteristic acidic lipids used in this study are shown
163 in Fig. 1. SA and PG each have carboxylic acid and phosphoric
164 acid as ionized groups, respectively. Both lipids have a strongly
165 hydrophobic dialkyl structure to fix the ionized groups on ve-
166 sicle surface. The molecular length indicated by the CPK model
167 showed that the carboxylate anion of SA and the phosphate
168 anion of PG would be located at the surface of PC-vesicles
169 (Fig. 1B). Various amounts of SA and PG were incorporated into
170 the PC/CH membrane (1:1, molar ratio) of the anionic vesicles.
171 The size of vesicles was controlled by extrusion methods (final
172 pore size: 0.2 µm), with final mean diameters of approximately
173 200 nm (Table 1). The vesicles prepared without acidic lipids

t1.1 Table 1
t1.2 Diameter of prepared vesicles containing various amounts of acidic lipid

	Acidic lipid (mol%)	Mean diameter±SD (nm)	
		SA-vesicles	PG-vesicles
t1.5	0 (PC-vesicles)	232±60	232±60
t1.6	1	224±58	230±60
t1.7	5	193±50	196±58
t1.8	9	205±40	204±49
t1.9	15	194±45	199±55
t1.10	20	198±52	194±64

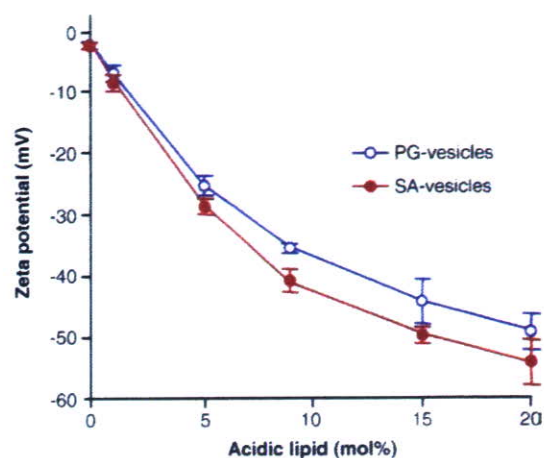

Fig. 2. Zeta potential of vesicles modified with SA (SA-vesicles) and PG (PG-vesicles) as a function of acidic lipid content. Zeta potentials were measured in 10 mM phosphate buffer (pH 7.4, NaCl: 20 mM) at 37 °C.

or with 1 mol% of acidic lipids tended to be slightly larger 174
(ca 30 nm) than vesicles containing more acidic lipids. This 175
effect of acidic lipids on the size of vesicles could be due to the 176
improved dispersion stability of vesicles and electrostatic re- 177
pulsion of the anionic surfaces. The vesicles without acidic lipids 178
were observed to precipitate in a day, indicating poor dispersion 179
stability. When the vesicles without acidic lipids contain a small 180
amount of PEG-DSPE to prevent the aggregation of vesicles, the 181
diameter of vesicles without acidic lipids was 202±49 nm. 182
Therefore, we guess that the slightly large diameter of vesicles 183
without acidic lipids or with 1 mol% of acidic lipids would be 184
caused by the high aggregability of vesicles. The low ag- 185
gregability of vesicles having a large zeta potential due to the 186
electrostatic repulsive interaction between vesicles is an ad- 187
vantage of anionic vesicles as stable dispersions. 188

3.2. Zeta potential of vesicles 189

The zeta potential is the electrostatic potential at the hydro- 190
dynamic slip plane, and is characterized as having an electrical 191
double-layer consisting of the Stern layer and the diffuse layer. 192
Fig. 2 shows the zeta potential of prepared vesicles as a func- 193
tion of acidic lipid content at pH 7.4. Vesicles containing PC/CH 194
(1:1, molar ratio) have an almost neutral surface (zeta potential: 195
−2.22±0.62 mV), indicating that the surface is inactive for elec- 196
trostatic events. The magnitude of the negative charge on the 197
surface increased with the incorporation of SA or PG, indicating 198
that the ionized groups of SA and PG act to characterize the vesicle 199
surface depending on their content. The zeta potentials of SA- 200
vesicles and PG-vesicles reached −54.2±3.68 mV and −49.0± 201
2.89 mV for acidic lipids of 20 mol%, respectively, with the 202
negative zeta potential of SA-vesicles being relatively higher 203
compared to that of PG-vesicles at any concentration. In theory, 204
the electrostatic potential is dependent upon distance from the 205
membrane surface as well as surface charge density [22]. The 206
slightly extended negative charge of SA from the surface shown 207
in Fig. 1B, would reduce the distance between the change to slip 208
plane, resulting in the higher negative zeta potential of SA-vesicles. 209

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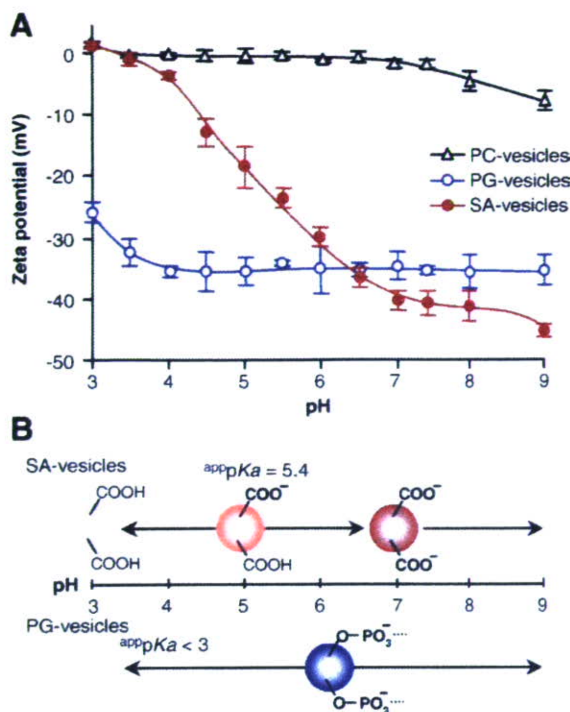

Fig. 3. Ionization state of acidic groups as a function of pH. (A) Zeta potential of vesicles (PC/CH, 1:1, molar ratio) (PC-vesicles), and vesicles containing 9 mol% of SA (SA-vesicles) or PG (PG-vesicles) at various pH. Zeta potentials were measured in 10 mM phosphate buffer (NaCl: 20 mM) at various pH (37 °C). (B) Schematic representation of the ionization state of acidic groups with pH. The apparent pK_as of SA-vesicles and PG-vesicles were calculated to be 5.4 and <3.

This result demonstrated that the capacity of SA as an anionic component of vesicles is equal to acidic phospholipids at pH 7.4.

3.3. Ionization properties of acidic lipids

Stability of the ionization state conferred by acidity is an important characteristic of acidic compounds. We examined the ionization properties of acidic lipids as a function of pH using SA-vesicles and PG-vesicles containing acidic lipids of 9 mol% with zeta potentials of -40.7 ± 2.09 mV and -35.4 ± 0.61 mV (at pH 7.4), respectively. As shown in Fig. 3A, the zeta potential of SA-vesicles varied markedly depending on the environmental pH (pH 3–7). The change in the zeta potential of SA-vesicles is thought to reflect the ionization state of SA, as control vesicles without SA, namely PC-vesicles, maintained almost neutral surfaces irrespective of pH. The relationship between pH and the pK_a of the acid is expressed using the well-known Henderson–Hasselbalch equation as follows:

$$pH = pK_a + \log \frac{[-COO^-]}{[-COOH]}. \quad (2)$$

When we analyzed the data shown in Fig. 3A using Eq. (2) and the assumption that the zeta potential was linearly correlated with the ionization acid, the pK_a of the carboxyl group of SA was estimated as 5.4 (Fig. 3B). Above pH 7, the zeta potential of SA-vesicles was almost constant, indicating that the carboxyl group of SA would mostly be ionized above pH 7. The zeta potentials of PG-vesicles were almost constant in the range pH 4–9, indicating that the ionized form of the phosphoric acid moiety is stable in this range. The change in the zeta potential observed at a pH lower than pH 4, and its pK_a would be lower than pH 3 [23,24]. Thus, we confirmed that the surface of SA-vesicles and PG-vesicles exhibited the characteristics of a weak acid with SA and a strong acid with PG, respectively, indicating that the individual characteristics of acidic groups are expressed on the surface of vesicles. We also observed that the magnitude of the negative electrostatic charges in SA-vesicles was equal to that observed in PG-vesicles at approximately neutral pH.

3.4. Electrostatic interactivity

Ca^{2+} is found in biological fluids (normally 2–3 mM in plasma) and is known to mediate biological processes by binding to the anionic domains such as those involved in the specific binding of proteins to membranes [25,26]. Acidic phospholipids, such as PG and PS, are also known to bind Ca^{2+} [27–29]. As shown in Fig. 4, the negative charge on anionic vesicles was suppressed by increasing the concentration of Ca^{2+}. When the concentration of Ca^{2+} was increased to 3 mM, the zeta potentials of SA-vesicles and PG-vesicles were -16.4 ± 1.9 mV and -13.8 ± 1.4 mV, respectively. Recently, Hautala et al. reported that vesicles containing phosphatidic acid possess a specifically strong affinity for Ca^{2+}, and that the zeta potential of these vesicles changed from being strongly negative to positive after binding Ca^{2+} [29]. These authors also showed that other acidic phospholipids, including PG, do not exhibit a strong affinity towards becoming cationic. Consequently, one aim of this experiment was to determine whether the binding of Ca^{2+} is a specificity factor between SA-vesicles and PG-vesicles, and also whether the surface of SA-vesicles remained negative in the presence of Ca^{2+}. Our experiment showed that the surface of the SA-vesicles remained

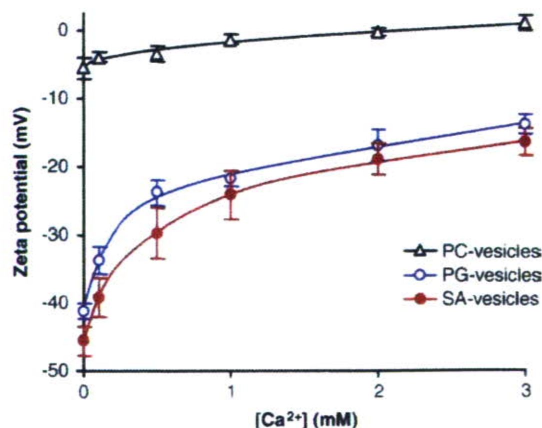

Fig. 4. Change in zeta potential of vesicles as a function of Ca^{2+} concentration. Vesicles were dispersed at 100 μg/mL in 10 mM HEPES buffer (pH 7.4, at 37 °C) containing NaCl and $CaCl_2$ (total: 20 mM). PC-vesicles: PC/ CH (1:1, molar ratio), SA-vesicles: PC/CH/SA (1:1:0.2, molar ratio), and PG-vesicles: PC/CH/PG (1:1:0.2, molar ratio).

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negative in the presence of Ca^{2+}. In addition, comparisons of SA-vesicles and PG-vesicles also showed that the specificity of binding Ca^{2+} was not observed.

Additional model-based studies of electrostatic interactivity, pentalysine and poly-L-lysine (Mw. 15–30 kDa with a repeating primary lysine amine) were also conducted. Oligomers or polymers of lysine are often used to model basic peptides or macromolecules and their electrostatic interactions on membranes [30,31]. As shown in Fig. 5, the zeta potential is a linear function of the concentration of pentalysine. The lines, which represent the least-squares best fit, have slopes of 9.3 and 7.4 mV per decade for the pentalysine concentrations in SA-vesicles and PG-vesicles. The slope for PC-vesicles was as little as 0.26 mV per decade of pentalysine concentration (data not shown), indicating that the negative charge of acidic lipids mediate the interaction with basic pentalysine. A change in the zeta potential is due to binding of basic peptide [31] and a similar decay slope of the zeta potential would indicate that the binding constant of a basic peptide to SA-vesicles and PG-vesicles was similar. As shown in Fig. 6, the change in the zeta potential of these vesicles due to the interaction with poly-L-lysine increased drastically, changing from a negative to a positive in presence of 1.5–2 μg/mL poly-L-lysine. Conversely, the change in the surface potential of PC-vesicles was negligible, indicating that the acidic lipids mediate the interaction with basic macromolecules. This experiment also demonstrates that the interactivity of SA-vesicles and PG-vesicles to basic macromolecules is equal at pH 7.4, and that within an electrostatic context, SA-vesicles and PG-vesicles interact similarly with basic compounds at physiological pH. It has been shown that electrostatic interactions are involved in the binding of C1q to the surface of anionic vesicles containing acidic phospholipid [6]. In the event that the negative charge on the surface of vesicles is critical for complement activation, both the SA-vesicles and PG-vesicles should be capable of activating the complement system to similar degree. We therefore conducted animal experiments to clarify the issue of complement activation by the anionic electrostatic charge of vesicles.

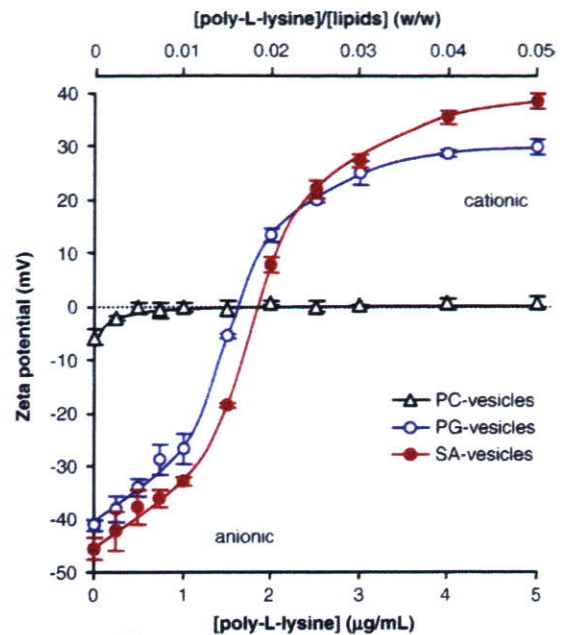

Fig. 6. Change in zeta potential of vesicles as a function of the concentration of basic macromolecule (poly-L-lysine, Mw. 15–30 kDa). Vesicles were dispersed at 100 μg/mL in 10 mM HEPES buffer (pH 7.4, at 37 °C, NaCl, 20 mM) containing various amount of poly-L-lysine. PC-vesicles: PC/CH (1:1, molar ratio), SA-vesicles: PC/CH/SA (1:1:0.2, molar ratio), and PG-vesicles: PC/CH/PG (1:1:0.2, molar ratio).

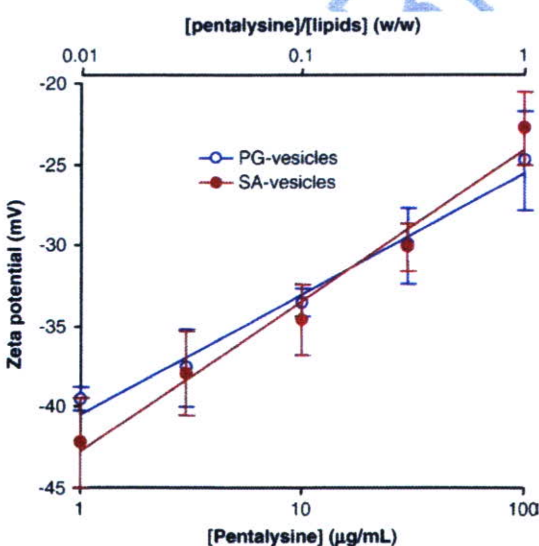

Fig. 5. Change in zeta potential of vesicles as a function of the concentration of basic oligomer (pentalysine). Vesicles were dispersed at 100 μg/mL in 10 mM HEPES buffer (pH 7.4, at 37 °C, NaCl, 20 mM) containing various amount of pentalysine. The lines have slopes of 9.3 and 7.4 mV per decade for the pentalysine concentrations in SA-vesicles and PG-vesicles. SA-vesicles: PC/CH/SA (1:1:0.2, molar ratio) and PG-vesicles: PC/CH/PG (1:1:0.2, molar ratio).

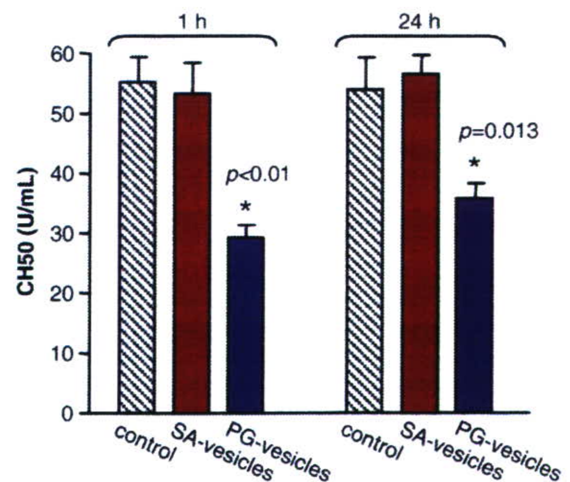

Fig. 7. Comparison of the 50% hemolytic unit of complement (CH50) in rat serum at 1 or 24 h after infusion of saline (control), SA-vesicles, or PG-vesicles. CH50 value for PG-vesicles was significantly lower than that of the control ($p<0.01$ at 1 h, $p=0.013$ at 24 h), indicating the complement consumption in serum after activation. Equal CH50 value for SA-vesicles with the control indicates that SA-vesicles failed to activate complement.

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3.5. Complement activation

Serum was collected at 1 and 24 h after the infusion of SA-vesicles or PG-vesicles in experimental rats to determine CH50 levels. The control group received saline as a vehicle. The values of CH50 in control rats, and SA-vesicles- and PG-vesicles-administered rats, were 55.1±4.1 U/mL, 53.2±4.8 U/mL, and 29.3±1.6 U/mL at 1 h after infusion of sample, respectively. At 24 h, the values of CH50 in control rats, and SA-vesicles- and PG-vesicles-administered rats, were 54.0±4.7 U/mL, 56.5±3.3 U/mL, and 35.8±2.3 U/mL, respectively (Fig. 7). The lower CH50 levels observed in the PG-vesicles-administered group in comparison with the control group indicate that complement consumption occurred after activation. These findings imply that significant complement activation is induced in rats receiving PG-vesicles compared to the control group ($p<0.01$ at 1 h, $p=0.013$ at 24 h). Complement consumption was not observed in rats administered SA-vesicles.

Since the negative charge and electrostatic interactivity of SA-vesicles were the same as in PG-vesicles (Figs. 2–6), the data obtained from the animal experiments indicates that the negative charge on the anionic vesicle is not a critical factor underlying the activation of complement. The first step in the activation of the classical complement pathway involves the binding of an activator to C1q, resulting in the activation of serine proteases C1r and C1s. It has been suggested that the negative charge of an activator such as PG-vesicles is involved in some way with the binding of the activator to C1q [1–8]. Assuming that the electrostatic interaction is non-specific, SA-vesicles should interact with C1q electrostatically. Since the action of complement proteases, which follows the binding of the activator to the C1q, is known to be highly specific [32,33], it seems likely that complement activation on anionic surface is limited to an activation step rather than a binding step. Such specific activation of complement by the anionic vesicles in the present study may be involved in the physiological regulation of complement activation on anionic biomembranes.

4. Conclusions

The carboxylic acid of SA and phosphoric acid of PG have equal capacity as anionic components of vesicles at neutral pH. The results presented in this investigation demonstrated that the negative electrostatic charge of anionic vesicles is not a critical factor in the activation of complement. Rather, the induction of complement activation by anionic vesicles is dependent on the structure of acidic lipids. This finding may facilitate development and various biological applications of anionic vesicles.

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References

[1] H.C. Loughrey, M.B. Bally, L.M. Reinish, P.R. Cullis, The binding of phosphatidylglycerol liposomes to rat platelets is mediated by complement, Thromb. Haemost. 64 (1990) 172–176.

[2] A. Chonn, P.R. Cullis, D.V. Devine, The role of surface charge in the activation of the classical and alternative pathways of complement by liposomes, J. Immunol. 146 (1991) 4234–4241.

[3] J. Szebeni, The interaction of liposomes with the complement system, Crit. Rev. Ther. Drug Carr. Syst. 15 (1998) 57–88.

[4] S.M. Moghimi, I. Hamad, T.L. Andresen, K. Jorgensen, J. Szebeni, Methylation of the phosphate oxygen moiety of phospholipid-methoxy (polyethylene glycol) conjugate prevents PEGylated liposome-mediated complement activation and anaphylatoxin production, FASEB J. 20 (2006) 2591–2593.

[5] A.J. Bradley, D.E. Brooks, R. Norris-Jones, D.V. Devine, C1q binding to liposomes is surface charge dependent and is inhibited by peptides consisting of residues 14–26 of the human C1qA chain in a sequence independent manner, Biochim. Biophys. Acta 1418 (1999) 19–30.

[6] A.J. Bradley, E. Maurer-Spurej, D.E. Brooks, D.V. Devine, Unusual electrostatic effects on binding of C1q to anionic liposomes: role of anionic phospholipid domains and their line tension, Biochemistry 38 (1999) 8112.

[7] H. Jiang, B. Cooper, F.A. Robey, H. Gewurz, DNA binds and activates complement via residues 14–26 of the human C1q A chain, J. Biol. Chem. 267 (1992) 25597–25601.

[8] H. Jiang, D. Burdick, C.G. Glabe, C.W. Cotman, A.J. Tenner, beta-Amyloid activates complement by binding to a specific region of the collagen-like domain of the C1q A chain, J. Immunol. 152 (1994) 5050–5059.

[9] A.J. Bradley, D.V. Devine, S.M. Ansell, J. Janzen, D.E. Brooks, Inhibition of liposome-induced complement activation by incorporated poly(ethylene glycol)-lipids, Arch. Biochem. Biophys. 357 (1998) 185–194.

[10] E. Tsuchida (Ed.), Substitute: Present and Future Perspective, Elsevier Science, Amsterdam, 1998.

[11] S. Takeoka, T. Ohgushi, K. Terase, T. Ohmori, E. Tsuchida, Layer-controlled hemoglobin vesicles by interaction of hemoglobin with a phospholipid assembly, Langmuir 12 (1996) 1755–1759.

[12] H. Sakai, K. Hamada, S. Takeoka, H. Nishide, E. Tsuchida, Physical properties of hemoglobin vesicles as red cell substitutes, Biotechnol Prog. 12 (1996) 119–125.

[13] K. Sou, Y. Naito, T. Endo, S. Takeoka, E. Tsuchida, Effective encapsulation of proteins into size-controlled phospholipid vesicles using freeze-thawing and extrusion, Biotechnol Prog. 19 (2003) 1547–1552.

[14] H. Sakai, H. Horinouchi, M. Yamamoto, E. Ikeda, S. Takeoka, M. Takaori, E. Tsuchida, K. Kobayashi, Acute 40 percent exchange-transfusion with hemoglobin-vesicles (HbV) suspended in recombinant human serum albumin solution: degradation of HbV and erythropoiesis in a rat spleen for 2 weeks, Transfusion 46 (2006) 339–347.

[15] H. Saka i, Y. Masada, H. Horinouchi, E. Ikada, K. Sou, S. Takeoka, M. Suematsu, M. Takaori, K. Kobayashi, E. Tsuchida, Physiological capacity of the reticuloendothelial system for the degradation of hemoglobin vesicles (artificial oxygen carriers) after massive intravenous doses by daily repeated infusions for 14 days, J. Pharmacol. Exp. Ther. 311 (2004) 874–884.

[16] K. Sou, R. Klipper, B. Goins, E. Tsuchida, W.T. Phillips, Circulation kinetics and organ distribution of Hb-vesicles developed as a red blood cell substitute, J. Pharmacol. Exp. Ther. 312 (2005) 702–709.

[17] H. Abe, M. Fujihara, H. Azuma, H. Ikeda, K. Ikebuchi, S. Takeoka, E. Tsuchida, H. Harashima, Interaction of hemoglobin vesicles, a cellular-type artificial oxygen carrier, with human plasma: effects on coagulation, kallikrein-kinin, and complement systems, Artif. Cells Blood Substit. Biotechnol. 34 (2006) 1–10.

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[18] H. Abe, H. Azuma, M. Yamaguchi, M. Fujihara, H. Ikeda, H. Sakai, S. Takeoka, E. Tsuchida, Effects of hemoglobin vesicles, a liposomal artificial oxygen carrier, on hematological responses, complement and anaphylactic reactions in rats, Artif. Cells Blood Substit. Biotechnol. 35 (2007) 157–172.
[19] K. Sou, B. Goins, S. Takeoka, E. Tsuchida, W.T. Phillips, Selective uptake of surface-modified phospholipid vesicles by bone marrow macrophages in vivo, Biomaterials 28 (2007) 2655–2666.
[20] K. Sou, T. Endo, S. Takeoka, E. Tsuchida, Poly(ethylene glycol)-modification of the phospholipid vesicles by using the spontaneous incorporation of poly (ethylene glycol)-lipid into the vesicles, Bioconjug. Chem. 11 (2000) 372–379.
[21] H. Saka i, S. Hisamoto, I. Fukutomi, K. Sou, S. Takeoka, E. Tsuchida, Detection of lipopolysaccharide in hemoglobin-vesicles by Limulus amebocyte lysate test with kinetic-turbidimetric gel clotting analysis and pretreatment of surfactant, J. Pharm. Sci. 93 (2004) 310–321.
[22] S. McLaughlin, The electrostatic properties of membranes, Annu. Rev. Biophys. Biophys. Chem. 18 (1989) 113–136.
[23] A. Watts, K. Harlos, W. Maschke, D. Marsh, Control of the structure and fluidity of phosphatidylglycerol bilayers by pH titration, Biochim. Biophys. Acta 510 (1978) 63–74.
[24] J.F. Tocanne, J. Teissié, Ionization of phospholipids and phospholipid-supported interfacial lateral diffusion of protons in membrane model systems, Biochim. Biophys. Acta 1031 (1990) 111–142.
[25] J. Rizo, T.C. Sudhof, C2-domains, structure and function of a universal Ca^{2+}-binding domain, J. Biol. Chem. 273 (1998) 15879–15882.
[26] J.E. Murphy, D. Tacon, P.R. Tedbury, J.M. Hadden, S. Knowling, T. Sawamura, M. Peckham, S.E. Phillips, J.H. Walker, S. Ponnambalam, LOX-1 scavenger receptor mediates calcium-dependent recognition of phosphatidylserine and apoptotic cells, Biochem. J. 393 (2006) 107–115.
[27] A. Lau, A. McLaughlin, S. McLaughlin, The adsorption of divalent cations to phosphatidylglycerol bilayer membranes, Biochim. Biophys. Acta 645 (1981) 279–292.
[28] C.G. Sinn, M. Antonietti, R. Dimova, Binding of calcium to phosphatidylcholine-phosphatidylserine membranes, Colloids and Surfaces A: Physicochem. Eng. Aspects 282–283 (2006) 410–419.
[29] J.T. Hautala, M.L. Riekkola, S.K. Wiedmer, Anionic phospholipid coatings in capillary electrochromatography. Binding of Ca^{2+} to phospholipid phosphate group, J. Chromatogr. A 1150 (2007) 339–347.
[30] D. Volodkin, V. Ball, P. Schaaf, J.C. Voegel, H. Mohwald, Complexation of phosphocholine liposomes with polylysine. Stabilization by surface coverage versus aggregation, Biochim. Biophys. Acta 1768 (2007) 280–290.
[31] D. Murray, A. Arbuzova, G. Hangyás-Mihályné, A. Gambhir, N. Ben-Tal, B. Honig, S. MaLaughlin, Electrostatic properties of membranes containing acidic lipids and adsorbed basic peptides: theory and experiment, Biophys. J. 77 (1999) 3176–3188.
[32] G.J. Arlaud, C. Gaboriaud, N.M. Thielens, M. Budayova-Spano, V. Rossi, J.C. Fontecilla-Camps, Structural biology of the C1 complex of complement unveils the mechanisms of its activation and proteolytic activity, Mol. Immunol. 39 (2002) 383–394.
[33] G. O'Brien, N.S. Quinsey, J.C. Whisstock, R.N. Pike, Importance of the prime subsites of the C1s protease of the classical complement pathway for recognition of substrates, Biochemistry 42 (2003) 14939–14945.

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「日本ヘモレオロジー学会誌」投稿論文

(印刷中)

各種代用血漿剤に分散させたヘモグロビン小胞体(人工赤血球)とその血液混合系のレオロジー特性

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【keyword】血液代替物、代用血漿剤、レオロジー

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要旨

ヘモグロビン小胞体(HbV)は、高濃度ヘモグロビン(Hb)溶液をリン脂質小胞体に内包した人工赤血球(人工酸素運搬体)である。HbV 分散液の膠質浸透圧は殆どゼロなので、大量投与に際しては代用血漿剤(水溶性高分子溶液)の併用が循環血液量の維持に重要となる。従来、ヒト血清アルブミン(HSA)或いはそのリコンビナント体(rHSA)を併用して、HbV の充分な酸素運搬効果と血行動態の維持を確認してきた。しかし、臨床現場ではアルブミン以外の代用血漿剤としてデキストラン(DEX)、ヒドロキシエチルスターチ(HES)、修正ゼラチン(MFG)などが使用されており、これらが HbV の分散安定性に及ぼす影響を検討する必要がある。これまで、各種代用血漿剤に分散させた HbV のレオロジー特性を詳細に検討してきたが、実際の投与では、代用血漿剤、HbV 分散液、そして血液が互いに希釈された混合系を形成する。そこで本研究では、各種代用血漿剤に分散させた HbV と血液の混合系のレオロジー特性、およびマイクロチャネルを通過する血液の流動性に HbV 凝集体が及ぼす影響を検討した。rHSA、低分子量 HES に分散させた HbV と血液の混合系は血液と同等あるいはそれ以下の粘度を示した。一方、DEX、高分子量 HES、MFG に分散させた HbV は血液よりも高い粘度を示したが、血液との混合比の増大に伴い、粘度および貯蔵弾性率の減少を示した。各種代用血漿剤に分散させた HbV と血液の混合液はマイクロチャネルを殆ど塞栓すること無く流動し、100 μL の通過時間は単純に粘度に比例した。HbV は可逆的な凝集性を示し、血液の微小流路の塞栓因子に影響を及ぼさないものと考えられる。HbV は分散媒である代用血漿剤との組み合わせでレオロジー特性を調節できるので、輸血代替以外の用途に拡大する可能性が期待できる。

緒言

リン脂質小胞体（リポソーム）は薬剤を内包、或いは脂質膜に担持できる特徴を持つことから、ドラッグデリバリーシステムを目的とした精力的な研究が行われており、抗真菌療法や抗癌治療法として既に認可されている例もある[1]。ヘモグロビン小胞体（HbV）は、高純度・高濃度ヒトヘモグロビン（Hb）をリン脂質小胞体（粒子径 280 nm）に内包した人工酸素運搬体である[2-5]。これまでの動物投与試験から、HbV が血液と同等の酸素運搬能と十分な安全性を有することが明らかになっている。輸血代替としての投与量は非常に多く、循環血液量の 40 %以上になることも想定される。赤血球と同等の酸素運搬機能を持たせるため、HbV 分散液の固形分濃度は他のリポソーム製剤と比較して非常に高い（Hb 濃度 10 g/dL; 脂質濃度 5～6 g/dL)。従って HbV の安全性を評価する上で、ヘモレオロジーに対比させて HbV 分散液のレオロジー特性を検討することは重要である[6]。

血漿中に 5 g/dL 程存在するアルブミンは血液と間質液の膠質浸透圧（COP）を平衡に保ち、循環血液量を維持する機能を有する血漿蛋白質である。この機能を維持する為に、COP は血液代替物が備えるべき必須条件の一つである。HbV は 1 粒子あたり約 3 万個の Hb を内包しており、分散液の COP は殆どゼロである。従って、HbV の大量投与に際しては代用血漿剤の併用が循環血液量の維持に重要となる。従来、HbV を 5%アルブミン（HSA）溶液或いはリコンビナントアルブミン（rHSA）溶液に分散させ COP を 20 Torr に調節し、この分散液を用いた出血ショック蘇生試験や、循環血液量の 40～90%を置換する試験から、rHSA に分散させた HbV が血液と同等の酸素運搬効果を有することを明らかにしてきた[7]。HbV を rHSA に分散させて Hb 濃度を 8.6～10 g/dL とした場合の粘度は血液と同等であり、剪断速度依存性からニュートン流体に近い挙動を示している[8]。しかし、実際の臨床現場では HSA 以外に代用血漿剤として、デキストラン（DEX）やヒドロキシエチルスターチ（HES）が使用されており、海外ではそれ以外に、HES の高分子量体や修正ゼラチン溶液（MFG）なども使用されている[9-11]。また、HbV をこれらの代用血漿剤に分散させて投与することで、虚血領域の酸素化の改善効果も報告されている[12-14]。このような背景のもと、我々は各種代用血漿剤に分散させた HbV のレオロジー特性の詳細な解析を進めている。rHSA と低分子量 HES では HbV と混合しても顕著な粘度増大は見られないが、DEX および欧州や米国で使用されている高分子量 HES や MFG では HbV の凝集生起を認め、粘度上昇が顕著となる。しかし凝集の形成と解離は極めて可逆的で迅速であり、血液流動性測定装置 (Microchannel array flow

analyzer, MC-FAN）[15,16]のマイクロチャネルには一切の寒栓が無いことを確認している[8]。

代用血漿剤を静脈内投与した場合、血液と代用血漿剤の両方が希釈されるので、ここに HbV 分散液が投与された場合、凝集形成は低減されると考えられる。また、HbV の凝集生起が血液の流動性に与える影響を in vitro で検討する必要がある。そこで本研究では、各種代用血漿剤に分散させた HbV と血液の混合系のレオロジー特性を観測すると共に、MC-FAN を用いて HbV の凝集体が血液の流動性に及ぼす影響を明らかにすることを目的とした。

方法

1. HbV 分散液の調製

HbV 分散液は無菌条件下、既報に従って調製した[17-19]。Hb 溶液は日本赤十字社（東京）から提供を受けた期限切れ非使用赤血球から精製した。HbV の内水相には高濃度の Hb（38 g/dL）と共に、アロステリック因子としてピリドキサール 5'-リン酸（PLP、14.7 mM）がモル比で PLP/Hb = 2.5 になるように添加した。HbV の膜成分である脂質は、日本精化社製の Presome PPG-I（1,2-ジパルミトイル-*sn*-グリセロ-3-ホスファチジルコリン）、コレステロール、1,5-bis-*O*-ヘキサデシル-*N*-スクシニル-L-グルタメート = 5/5/1（モル比）に 0.3 mol%の 1,2-ジステアロイル-*sn*-グリセロ-3-ホスファチジルエタノールアミン-*N*-ポリ（エチレングリコール）（日本油脂社製）を混合して用いた。HbV は生理食塩水に分散、フィルター滅菌し（Dismic、東洋濾紙社製：0.45 μm）、窒素通気により酸素を除去して保存した[20]。HbV の粒子径は 279 ± 95 nm であった。

2. 各種代用血漿剤

遺伝子組み換えヒト血清アルブミン（rHSA, Bipha, Mw. 67 kDa, 25 wt%溶液）は生理食塩水で 5 wt%に希釈して用いた。デキストラン（DEX, 小林製薬工業 デキストラン 40 注 $_{「小林」}$ ^{TM}, Mw. 40 kDa, 10 wt%溶液）、低分子量ヒドロキシエチルスターチ（HES_{70}, 杏林製薬 サリンヘス^{TM}, Mw. 70 kDa, 6 wt %溶液）、中分子量ヒドロキシエチルスターチ（HES_{130}, HES_{200}, Fresenius 社 Voluven^{TM}, Mw. 130 kDa, 200 kDa, 6 wt%溶液）、高分子量ヒドロキシエチルスターチ（HES_{670}, Hospira 社 Hextend^{TM}, Mw. 670 kDa, 6 wt%溶液）、修正ゼラチン（MFG, B. Braun 社 Gelofusine^{TM}, Mw. 30 kDa, 4 wt%溶液）を用いた。各種代用血漿剤の膠質浸透圧（COP）は浸透圧計（model 4420, Wescor 社製、Cut-off Mw. = 10,000）を用いて測定した。物理化学的特徴は **Table 1** にまとめた。

3. 各種代用血漿剤に分散させた HbV 及び血液試料の調製

生理食塩水に分散させた HbV を超遠心分離（20 000 *g*, 30 min）で沈降させて上澄みの生理食塩水を除去後、代用血漿剤中に分散させ（[Hb] = 10 g/dL）、フィルター処理した（Dismic、東洋濾紙社製：0.45 μm）。血液試料は 5 %ヘパリン加ヒト新鮮血を用い、採血後 6 時間以内に測定を行った[21]。

4. 各種代用血漿剤に分散させた HbV とその血液混合系のレオロジー測定

粘度の剪断速度依存性はモジュラーコンパクトレオメータ（Physica MCR301：Anton Paar 社製）を用いて測定した。測定治具はコーンプレート（コーン径：50 mm、ギャップ角度：1°）を用い、25 °C で剪断速度を 10^3 から 10^{-4} s^{-1} に低下させた。貯蔵弾性率 *G'* の剪断速度依存性はキャピラリーレオメータ（DCR：Anton Paar 社製）を用いて測定した（剪断速度 5 〜 320 s^{-1}、周波数 2 Hz、37 °C）。

5. 各種代用血漿剤に分散させた HbV とその血液混合系のマイクロチャネル流動性試験

各種代用血漿剤に分散させた HbV（[Hb] = 10 g/dL）とヘパリン加ヒト新鮮血の混合液を測定試料とした。マイクロチャネルアレイ Bloody6-7（幅 7 μm, 長さ 30 μm, 深さ 4.5 μm, 流路数 8,736 本：日立原町電子工業社製）に MC-FAN（KH-3：日立原町電子工業社製）を用いて 20 cm 水柱差で流し、試料 100 μL の通過時間を測定した。通過時間は試料測定の直前に測定された生理食塩水 100 μL の通過時間を用いて（通過時間）×12 秒/（生理食塩水通過時間）により生理食塩水通過時間が 12 秒の場合に換算した。また、流動中と停止後の試料の顕微鏡写真を撮影した。

結果

1. 各種代用血漿剤に分散させた HbV とその血液混合系のレオロジー特性

Fig. 1A に、各種代用血漿剤に分散させた HbV について、剪断速度を $10^3\ s^{-1}$ から $10^{-4}\ s^{-1}$ に低下させたときの粘度変化を示した。rHSA に分散させた場合（HbV-rHSA）は、粘度の変化は殆ど無く、ほぼニュートン流体を示した。HbV-rHSA および HbV-HES_{70} の粘度は、ヒト血液とほぼ同じであった。使用したレオメータの検出感度では、低粘度試料は 0.5 s^{-1} が限界であった。一方、HbV-HES_{130}、HbV-HES_{200}、HbV-HES_{670}、HbV-DEX、HbV-MFG はヒト血液よりも高い粘度を示し、高剪断速度になるほど粘度が低下する Shear-thinning 流動が観測された。この非ニュートン流体の性質は HbV の凝集体の生起に起因する。これらはより低剪断速度までの測定が可能であった。HES の分子量が大きくなるにつれ粘度が高くなる傾向が認められ、高分子量であるほど HbV は凝集し易くなることが明確に示された。

Fig. 1B に、各種代用血漿剤に分散させた HbV と血液を体積比 1 : 1 で混合した溶液について、**Fig. 1A** と同様に測定した結果を示した（剪断速度 $10^3 - 10^{-4}\ s^{-1}$、25 °C）。HbV-rHSA の血液混合液は血液よりも低い粘度を示した。HbV-HES_{70}、HbV-HES_{130} の血液混合液は血液とほぼ同じ粘度を示した。一方、HbV-DEX、HbV-HES_{670}、HbV-HES_{200}、HbV-MFG の血液混合液は、混合前と比較して全域で粘度の減少を示したが、血液よりも高い粘度であった。

Fig. 2 には血液と HbV 分散液の混合比を変化させたときの**(A)** 粘度、**(B)** 貯蔵弾性率 G' の変化を示す。HbV-rHSA を除く HbV 分散液に関して、血液混合比の増大に伴い粘度と G' が減少する傾向が観測された。一方、HbV-rHSA の粘度と G' は血液よりも小さいため、血液混合比の増大に伴ってこれらが増大し、血液の値に収束していく傾向が観測された。

2. 各種代用血漿剤に分散させた HbV とその血液混合系のマイクロチャネル流動性試験

各種代用血漿剤に分散させた HbV とその血液混合系の流動性試験を、体積混合比を変化させて行った。全血の通過時間（46 秒）は他の文献の報告値とほぼ一致した [22]。HbV-DEX の通過時間は 196 秒であったが、血液混合比の増大に伴い通過時間は短縮し、HbV-DEX と血液の混合比 1 : 3 においては 59 秒であった。**Fig. 3** には、各種代用血漿剤に分散させた HbV と血液を体積比 1 : 1 で混合した分散液の静止状態と流動状態の顕微鏡観察写真を示した。HbV の粒子径は 279 ± 95 nm と小さい為、均一分散系

においてはその存在を光学顕微鏡で確認できないが、静止状態で形成される凝集体はみかけのサイズが大きい為に血漿相中に確認できた。血液と同等の粘度を示した HbV-rHSA、HbV-HES_{70} は HbV の凝集体が殆ど観測されなかった。一方、高粘度を示した HbV-HES_{670}、HbV-DEX、HbV-MFG は HbV の凝集体が観測された。しかし、流動中の凝集体は解離して流路を殆ど閉塞せずに流動した。剪断速度 1000 s^{-1} における試料の粘度とマイクロチャネルの通過時間の相関を検討したところ（**Fig. 4**）、HbV 分散液の粘度と通過時間(流量の逆数)に比例関係が成立した。直線の式を最小二乗法で求めると Y = 12.1X（Y : 通過時間（sec/100 µL）、X : 粘度（mPa・s）、$R^2 = 0.9728$）であり、Poiseuille の法則に従った。

考察

リン脂質小胞体（リポソーム）が水溶性高分子と相互作用して凝集体を形成する現象については多くの報告例があるが[23-25]、その殆どが希薄溶液の濁度または光散乱の計測で観察しているに過ぎない。HbVは、表面に修飾されたポリエチレングリコール（PEG）鎖の立体反発効果によって、血漿中においても均一な分散系を形成する[26]。しかし、HbVは極めて高濃度の分散系であるため（固形分濃度: 16 g/dL, 占有体積: 40 vol%）、これが凝集した場合は、凝集体が溶液全体に三次元ネットワークを形成し、これが固体的性質を与え、粘度及び貯蔵弾性率 G' の増大として観察されると考えられる。赤血球も可逆的な凝集体を形成し、40～45 vol%の体積を占有するので、これが血液を非ニュートン流体とする主要な因子であり、また血液に水溶性高分子が混合されると赤血球が凝集し、血液粘度、血行動態、組織酸素化に影響を及ぼすことが知られている[27-30]。従って、HbV分散液についても同様のレオロジー特性の解析を行った。

HbV-rHSA分散液はほぼニュートン流体の特性を示し、高剪断速度における粘度は血液とほぼ同等であった（**Fig. 1A**）。HbVの表面に修飾されたPEG鎖がアルブミン中の分散安定度に寄与していると考えられる[3]。対照的にHES、DEX、MFGに分散させたHbVは、低剪断速度で高い粘度を示し剪断速度が高くなるに従い粘度が低下するShear-thinningを示した（**Fig. 1A**）。これは凝集分散系に特徴的な粘度挙動である。またHES(Mw. 70, 130, 200, 670 kDa)について、凝集体形成には分子量依存性が存在し、高分子量になるほど高粘度を示した。分子量が最も小さい HES_{70} に分散させた場合には血液と同等であった。現行のPEG修飾の条件は、血漿中での分散安定度を維持するには十分と思われる。しかし、本研究で代用血漿剤によってはPEG修飾が施されてもHbVの凝集体が形成してしまうことが明らかとなった。

リポソーム凝集の機序については、幾つかの理論が提唱されている。(i) 水溶性高分子鎖が粒子表面に吸着することにより粒子間を架橋する[24]、(ii) 水溶性高分子がリポソーム表面から水和水を奪い、リポソームが溶液から排除される[31]、また、(iii) リポソームの水和領域から高分子が排除されるため、リポソーム粒子間に形成される空間からも高分子が排除されることになり、バルク溶液とリポソーム粒子間の溶液との間に浸透圧差が生じ、この浸透圧差を小さくするために凝集が促進されて粒子間距離が短くなる[23, 32-34]と言われている。特に(iii)の場合、粒子間に形成される空間の大きさと高分子の大きさの相対的寸法差が重要と成る。HES、DEX、MFGには分岐構造があるので、球状蛋白質のrHSAに比較し

てより低密度で排除体積の大きい構造をしていると考えられる。DEXは水中でコイル状の分子構造をとることが報告されている[35]。MFGは、修飾されたコハク酸の静電反発によって拡大した分子構造をとる[36]。この様な高分子はリポソーム表面から排除され易く、リポソーム凝集が促進されると考えられる。また、(i)の機序でも、直鎖状構造の高分子が粒子間を架橋し易いと考えられる。一方、HESの分子構造は分岐状であり[37]、その形は球状に近いので、排除体積はDEX、MFGと比較して小さいと考えられる。しかし、高分子量体になるに従い、排除体積も大きくなるので、リポソーム凝集が促進される。我々は各種代用血漿剤に含まれる水溶性高分子の慣性半径の違いから凝集度を説明できると考えており、現在その解析を進めている。また、水溶性高分子による凝集を抑制する為に、現行よりも鎖長の長いPEG鎖をHbV表面に修飾し、粒子間の立体反発力を高めることも可能と考えられる。事実、我々はリポソームの表面に修飾するPEG鎖の分子量を大きくさせると、リポソームの分散安定性が増加することを報告している[38]。

実際にHbVの投与を行う場合には、①代用血漿剤を投与した後に生理食塩水に分散されたHbVを投与、或いは②濃厚な代用血漿剤（例：25%アルブミン溶液）があればこれをHbV分散液に添加して同時に投与（[Hb] = 8.6 g/dL）するなどが想定される。血液交換率は50%程度と想定され、HbV、代用血漿剤ともに更に希釈されるので凝集は低減されると考えられる。事実、DEX、HES_{670}、MFGに分散させたHbVは血液よりも高い粘度を示したが、血液と体積比1：1で混合すると全ての剪断速度で粘度が減少した（**Fig. 1B**）。更に血液の混合比の増大に伴い、粘度および G' が減少する傾向が観測された（**Fig. 2**）。HbVと代用血漿剤の双方が血液で希釈され、凝集の低減が起きることが明らかとなった。

次に、各種代用血漿剤に分散させたHbVと血液の混合系がマイクロチャネルを流動する挙動をMC-FANを用いて検討した。血液のマイクロチャネルの通過時間は、赤血球の変形能[39]、白血球による流路の塞栓[40]および血栓による塞栓[41]などが影響すると考えられる。このうち、赤血球の変形能はレオメータでの粘度測定にも影響を及ぼすが[39]、流路の塞栓はマイクロチャネルを持つMC-FANでしか測定できない項目である。我々は各種代用血漿剤に分散させたHbVがマイクロチャネルを一切塞栓することなく流動し、100 μLの通過時間と粘度に比例関係が得られ、これがPoiseuillesの法則に従っていることを報告してきた[8]。この関係が成り立つのは流路の塞栓が無い場合に限られる。仮に流路の塞栓が顕著であれば、通過時間は大きく延長して、粘度との比例関係は成立しない筈である。結果より、各種代用血漿剤に分散させたHbVと血液の混合液の通過時間は、全例が粘度に比例した（**Fig. 4**）。よって

各種代用血漿剤に分散させたHbVは血液の流路塞栓因子に影響を及ぼさないと考えられる。事実、我々はラットを用いた60%血液交換試験において、各種代用血漿剤を投与後、HbVによる蘇生を行ったが、凝集体による影響は全く見られず、全例が生存した[42]。この理由として、代用血漿剤、血液、HbVが互いに希釈され、凝集が低減したこと、凝集体が血液の剪断速度に応じて可逆的に形成・解離したこと、そして血液中の塞栓因子に影響が無かったことなどが考えられる。

最近、幾つかの研究グループが、高粘性流体が血管壁に対し、より高い剪断応力を与えるため血管拡張分子を誘導させ結果的に末梢血流が改善されると主張している[43,44]。Emiらは HbV を HES_{200} あるいはDEX (Mw. 70 kDa)に分散させ、ハムスターの循環血液量の40%を交換し、毛細血管の塞栓が無いことを確認し、むしろ有茎皮弁の虚血領域の微小循環を改善する効果があることを見出した[12-14]。高粘性流体では末梢毛細管により均一に圧力が伝播し、血液をより均一に輸送し、有効毛細管密度(Functional Capillary Density)の向上が期待できる[45]。この点に関しては、HbVは代用血漿剤の併用によって特徴的なレオロジー特性を示し、またその調節も可能であるので、輸血代替以外の臨床応用にも利用できる可能性がある。今後は、HbVと一連の代用血漿剤を併用したときの安全性について詳細を確認するとともに、凝集の機序を明らかにして行く計画である。

結論

HbVを各種代用血漿剤に分散させた溶液と血液の混合系に関して、レオロジー特性および凝集の可逆性について検討した。$HES_{70,130}$、rHSAに分散させたHbVと血液の混合液の粘度は血液と同等もしくはそれ以下であった。DEX、高分子量HES、MFGではHbVは凝集するが、血液中においても外力に依存して瞬時に解離すると共に、血液混合比の増大に伴い凝集の形成は低減することが確認された。HbV分散液と血液の混合系のマイクロチャネルの通過時間は単純に粘度に比例し、流路の塞栓は殆ど無かったことから HbV 凝集体は血液の流路の塞栓因子に影響を与えないと考えられた。凝集体生起の生体への影響を確認する必要があるが、HbV分散液は分散媒との組み合わせでレオロジー特性を調節することができるので、輸血代替以外の用途に拡大する可能性もある。

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参考文献

1) Torchilin VP. Recent advances with liposomes as pharmaceutical carriers. *Nat. Rev. Drug Discovery*, 2005; 4: 145-160.

2) Izumi Y, Sakai H, Hamada K, Takeoka S, Yamahata T, Kato R, Nishide H, Tsuchida E, and Kobayashi K. Physiologic responses to exchange transfusion with hemoglobin vesicles as an artificial oxygen carrier in anesthetized rats: Changes in mean arterial pressure and renal cortical tissue oxygen tension. *Crit. Care Med.*, 1996; 24: 1869-1873.

3) Sakai H, Takeoka S, Park SI, Kose T, Nishide H, Izumi Y, Yoshizu A, Kobayashi K, and Tsuchida E. Surface modification of hemoglobin vesicles with poly(ethylene glycol) and effects on aggregation, viscosity, and blood flow during 90 % exchange transfusion in anesthetized rats. *Bioconjugate Chem.*, 1997; 8: 23-30.

4) Chang TM, Hemoglobin-based red blood cell substitutes. *Artif. Organs*, 2004; 28: 789-794.

5) Phillips WT, Klipper, RW, Awasthi VD, Rudolph AS, Cliff R, Kwasiborski V, Goins BA. Polyethylene glycol-modified liposome-encapsulated hemoglobin: A long circulating red cell substitute *J. Pharmacol. Exp. Ther.*, 1999; 288: 665-670.

6) Sakai H, Hamada K, Takeoka S, Nishide H, and Tsuchida E. Physical properties of hemoglobin vesicles as red cell substitutes. *Biotechnol. Prog.*, 1996; 12: 119-125.

7) Sakai H, Horinouchi H, Yamamoto M, Ikeda E, Takeoka S, Takaori M, Tsuchida E, and Kobayashi K. Acute 40 percent exchange-transfusion with hemoglobin-vesicles (HbV) suspended in recombinant human serum albumin solution: degradation of HbV and erythropoiesis in a rat spleen for 2 weeks. *Transfusion*, 2006; 46: 339-347.

8) Sakai H, Sato A, Takeoka S, and Tsuchida E. Rheologial properties of hemoglobin vesicles (artificial oxygen carriers) suspended in a series of plasma-substitute solutions. *Langmuir,* 2007; 23: 8121-8128.

9) Webb AR, Nash GB, Dormandy JA, and Bennett ED. A comparison of the effects of artificial plasma substitutes, albumin and saline solutions on in vitro apparent blood viscosity. *Clin. Hemorheol.,* 1990; 10: 287-296

10) Webb AR, Barclay SA, and Bennett ED. In vitro colloid osmotic pressure of commonly used plasma expanders and substitutes: a study of the diffusibility of colloid molecules. *Intensive Care Med.,* 1989; 15: 116-120.

11) Traylor RJ and Pearl RG. Crystalloid versus colloid versus colloid: all colloids are not created equal. *Anesth. Analg.,* 1996; 83: 209-212.

12) Plock JA., Contaldo C, Sakai H, Tsuchida E, Leunig M, Banic A, Menger MD, and Erni D. Is hemoglobin in hemoglobin vesicles infused for isovolemic hemodilution necessary to improve oxygenation in critically ischemic hamster skin? *Am. J. Physiol. Heart Circ. Physiol.,* 2005; 289: H2624-H2631.

13) Contaldo C, Schramm S, Wettstein R, Sakai H, Takeoka S, Tsuchida E, Leunig M, Banic A, and Erni D. Improved oxygenation in ischemic hamster flap tissue is correlated with increasing hemodilution with Hb vesicles and their O_2 affinity. *Am. J. Physiol. Heart Circ. Physiol.,* 2003; 285: H1140-H1147.

14) Contaldo C, Plock J, Sakai H, Takeoka S, Tsuchida E, Leunig M, Banic A, and Erni D. New generation of hemoglobin-based oxygen carriers evaluated for oxygenation of critically ischemic hamster flap tissue. *Crit. Care Med.,* 2005; 33: 806-812.

15) Kikuchi Y, Sato K, Ohki H, and Kaneko T. Optically accessible microchannels formed in a single-crystal silicon substrate for studies of blood rheology. *Microvasc. Res.,* 1992; 44: 226-240.

16) Kikuchi Y, Sato K, and Mizuguchi Y. Modified cell-flow microchannels in a single-crystal silicon substrate and flow behavior of blood cells. *Microvasc. Res.,* 1994; 47: 222-231.

17) Sakai H, Takeoka S, Yokohama H, Seino Y, Nishide H, and Tsuchida E. Purification of concentrated Hb using organic solvent and heat treatment. *Protein Expression Purif.,* 1993; 4: 563-569.

18) Sakai H, Yuasa M, Onuma H, Takeoka S, and Tsuchida E. Synthesis and physicochemical characterization of a series of hemoglobin-based oxygen carriers: objective comparison between cellular and acellular types. *Bioconjugate Chem.,* 2000; 11: 56-64.

19) Sou K, Naito Y, Endo T, Takeoka S, and Tsuchida E. Effective encapsulation of proteins into size-controlled phospholipid vesicles using freeze-thawing and extrusion. *Biotechnol. Progr.,* 2003; 19: 1547-1552.

20) Sakai H, Tomiyama K, Sou K, Takeoka S, and Tsuchida E. Poly(ethylene glycol)-conjugation and deoxygenation enable long-term preservation of hemoglobin-vesicles as oxygen carriers in a liquid state. *Bioconjugate Chem.,* 2000; 11: 425-432.

21) 関 耕二, 角野 博之, 村上 正巳. MC-FAN を用いて測定した血液流動性について. *臨床病理,* 2003; 51: 770-775.

22) 菊池 佑二, 高橋 千栄子, 磯野 厚子. 健常者の部分母集団における MC-FAN 全血通過時間の分布. *ヘモレオロジー研究会誌,* 2001; 4: 7-14.

23) Meyuhas D, Nir S, and Lichtenberg D. Aggregation of phospholipid vesicles by water-soluble polymers.

Biophys. J., 1996; 71: 2602-2612.

24) Sunamoto J, Iwamoto K, and Kondo H. Liposomal membranes. VII. Fusion and aggregation of egg lecithin liposomes as promoted by polysaccharides. *Biochem. Biophys. Res. Commun.*, 1980; 94: 1367-1373.

25) Sou K, Endo T, Takeoka S, and Tsuchida E. Poly(ethylene glycol)-modification of the phospholipid vesicles by using the spontaneous incorporation of poly(ethylene glycol)-lipid into the vesicles. *Bioconjugate Chem.*, 2000; 11: 372-379.

26) Sakai H, Tsai AG, Kerger H, Park SI, Takeoka S, Nishide H, Tsuchida E, and Intagfietta M. Subcutaneous microvascular responses to hemodilution with a red cell substitute consisting of polyethyleneglycol-modified vesicles encapsulating hemoglobin. *J. Biomed. Mater. Res.*, 1998; 40: 66-78.

27) Freyburger G, Dubreuil M, Boisseau MR, and Janvier G. Rheological properties of commonly used plasma substitutes during preoperative normovolaemic acute haemodilution. *Br. J. Anaesth.*, 1996; 76: 519-525.

28) Eckmann DM, Bowers S, Stecker M, and Cheung AT. Hematocrit, volume expander, temperature, and shear rate effects on blood viscosity. *Anesth. Analg.*, 2000; 91: 539-545.

29) Bishop JJ, Nance PR, Popel AS, Intaglietta M, and Johnson PC. Effect of erythrocyte aggregation on velocity profiles in venules. *Am. J. Physiol. Heart Circ. Physiol.*, 2001; 280: H222-H236.

30) Tateishi N, Suzuki Y, Cicha I, and Maeda N. O_2 release from erythrocytes flowing in a narrow O_2-permeable tube: effects of erythrocyte aggregation. *Am. J. Physiol. Heart Circ. Physiol.*, 2001; 281: H448-H456.

31) Tilcock CP and Fisher D. The interaction of phospholipid membranes with poly(ethylene glycol). Vesicle aggregation and lipid exchange. *Biochim. Biophys. Acta*, 1982; 688: 645-652.

32) Neu B and Meiselman HJ. Depletion-mediated red blood cell aggregation in polymer solutions. *Biophys. J.*, 2002; 83: 2482-2490.

33) Neu B and Meiselman HJ. Depletion interactions in polymer solutions promote red blood cell adhesion to albumin-coated surfaces. *Biochim. Biophys. Acta*, 2006; 1760: 1772-1779.

34) Vincent B, Edwards J, Emmett S, and Jones A. Depletion flocculation in dispersions of sterically-stabilised particles ("soft spheres"). *Colloids Surf.*, 1986; 18: 261-281.

35) Hirata Y, Sano Y, Aoki M, Shohji H, Katoh S, Abe J, Hitsukuri S, and Yamamoto H. Small-angle X-ray scattering studies of moderately concentrated dextran solution. *Carbohydr. Polym.*, 2003; 53: 331-335.

36) Saddler JM, and Horsey PJ. The new generation gelatins. *Anaesthesia*, 1987; 42: 998-1004.

37) Gosch CI, Haase T, Wolf BA, and Kulicke WM. Molar mass distribution and size of hydroxyethyl starch fractions obtained by continuous polymer fractionation. *Starch*, 2002; 54: 375-384.

38) Takeoka S, Mori K, Ohkawa H, Sou K, and Tsuchida E. Synthesis and assembly of poly (ethylene glycol)-lipids with mono-, di-, and tetraacyl chains and a poly (ethylene glycol) chain of various molecular weights. *J. Am. Chem. Soc.*, 2000; 122: 7927-7935.

39) Lee CYJ, Kim KC, Park HW, Song JH, and Lee CH. Rheological properties of erythrocytes from male hypercholesterolemia. *Microvasc. Res.*, 2004; 67: 133-138.

40) Miyamoto K, Ogura Y, Kenmochi S, and Honda Y. Role of leukocytes in diabetic microcirculatory disturbances. *Microvasc. Res.*, 1997; 54: 43-48.

41) Kamada H, Hattori K, Hayashi T, Suzuki K. In vitro evaluation of blood coagulation activation and microthrombus formation by a microchannel array flow analyzer. *Thromb. Res.*, 2004; 114: 195-203.

42) 酒井 宏水, 佐藤 敦, 宮川 賀仁, 武岡 真司, 堀之内 宏久, 高折 益彦, 小林 紘一, 土田 英俊. ヘモグロビン小胞体と各種代用血漿剤の併用に関する検討. *人工血液*, 2007; 15: 23.

43) Cabrales P, Tsai AG, and Intaglietta M. Alginate plasma expander maintains perfusion and plasma viscosity during extreme hemodilution. *Am. J. Physiol. Heart Cric. Physiol.*, 2005; 288: H1708-H1716.

44) Cabrales P, Intaglietta M. and Tsai AG. Increase plasma viscosity sustains microcirculation after resuscitation from hemorrhagic shock and continuous bleeding. *Shock*, 2005; 23: 549-555.

45) Tsai AG, Friesenecker B, McCarthy M, Sakai H, and Intaglietta M. Plasma viscosity regulates capillary perfusion during extreme hemodilution in hamster skin fold model. *Am. J. Physiol. Heart Circ. Physiol.*, 1998; 275: H2170-2180.

図の説明

FIGURE 1. Shear rate dependence of the viscosity of **(A)** the HbV suspended in various plasma-substitute solutions, and **(B)** the mixtures of blood and HbV suspended in various plasma-substitute solutions at a volume ratio of 1/1. The shear rate decreased from 10^3 to 10^{-4} s^{-1}. [Hb] = 10 g/dL, 25°C. The blood data are inserted for comparison.

FIGURE 2. Viscoelasticity of the mixtures of blood and HbV suspended in various plasma-substitute solutions at a volume ratio of 0/1, 1/3, 1/1, 3/1, and 1/0. **(A)** The viscosity at a shear rate of 10^3 s^{-1} measured with an MCR 301 rheometer at 25°C. **(B)** The storage modulus (G') at a shear rate of 1.9 s^{-1} measured with a capillary rheometer at 37°C.

FIRURE 3. Images of microchannels during the flowing condition and the cessation of flow. The flocculate formation is apparent in the plasma phase at the cessation for HbV-DEX + blood, HbV-HES + blood, and HbV-MFG + blood. However, no flocculation was apparent for HbV-rHSA + blood. In the flowing condition, partial plugging of channels occurred due to platelets or white blood cells in blood samples (top).

FIGURE 4. Microchannel flow measurements of the mixtures of blood and HbV suspended in various plasma-substitute solutions at a volume ratio of 1/0, 3/1, 1/1, 1/3, and 0/1. The time required for the passage of 100 μL of each suspension was plotted against the viscosity at 10^3 s^{-1}. The straight line indicates a linear approximation : Y = 12.1X (R^2 = 0.9728).

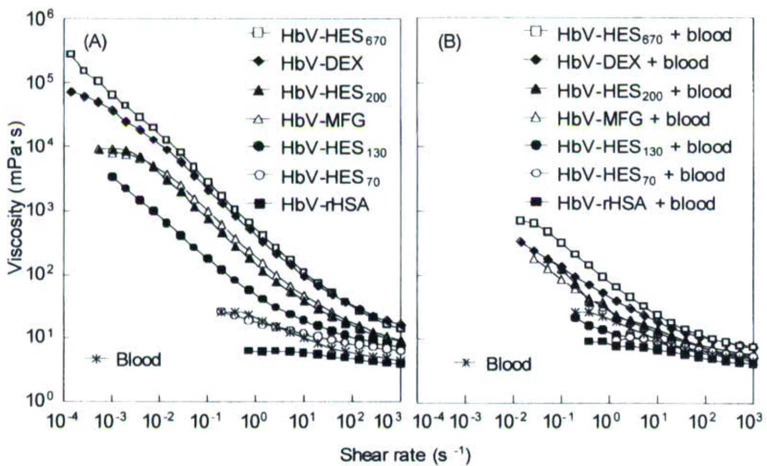

Figure 1

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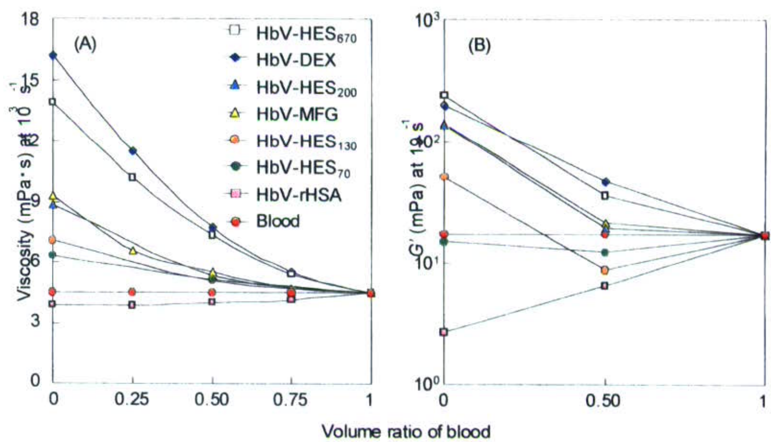

Figure 2

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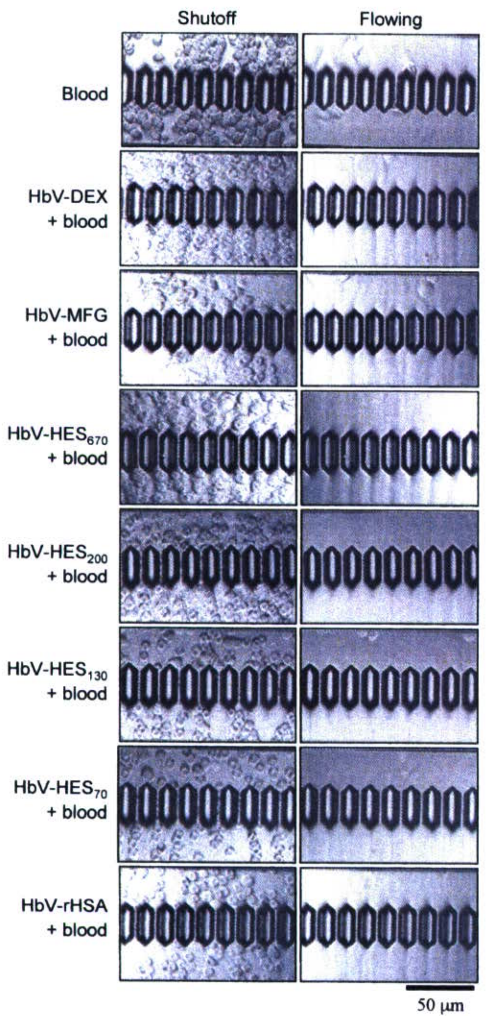

Figure 3

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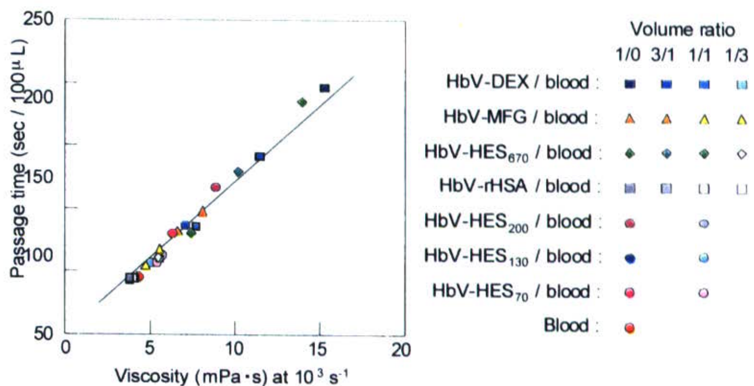

Figure 4

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