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Fragmentation of Human Erythrocyte Ghosts and Their Application as a Carrier of Heme Complex

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Human erythrocyte ghosts were taken into fragments by sonication. The average radius of the fragmented ghosts gradually decreased with the sonication time. The fragmented ghosts were obtained as spheres and reached to about 0.1 μm. In spite of the change in radius, their membrane fluidity was the same as that of the unsonicated ghost membrane. The [*meso*-α,α,α,α-tetrakis(*o*-pivalamidophenyl)porphinato]iron(III)-*mono* (1-dodecyl-2-methylimidazole) complex was incorporated into the hydrophobic region of the fragmented ghost membrane. This hybrid showed a semistable oxygen adduct in pH 7.0 phosphate buffer solution.

Human erythrocyte ghost membranes have been widely used in many fields due to their interesting character and brief preparation method.[1] The purified ghosts could be obtained easily in large quantities by direct hemolysis. They have also been studied extensively as a typical model for cell membranes. Ghost membranes are turned into liposomes by treating them in hypotonic buffer solution. These liposomes become inside-out when they were prepared in hypotonic alkaline buffer solution, but right side out liposomes were generally obtained by treating them in hypotonic Tris buffer solution.[2,3]

It is known that bilayer membranes can be used as vehicles for biological-active materials. The authors succeeded in oxygen-binding by incorporating the [*meso*-α,α,α,α-tetrakis(*o*-pivalamidophenyl)porphinato]-iron(III)-*mono*(1-dodecyl-2-methylimidazole) complex (abbreviated as "heme") into phosphatidylcholine liposomes.[4] Human erythrocyte ghost membranes were expected to carry the heme complex instead of synthetic lipid liposomes, because the fluidity of ghost membranes was relatively low. This paper describes the fragmentation of human erythrocyte ghost and the oxygen-binding ability of the heme embedded in the fragmented ghost membrane.

Experimental

Preparation of Ghost Membranes. Human erythrocytes were isolated from whole blood of healthy donors. Erythrocytes were centrifuged (3000 min^{-1} for 5 min at 4 °C) three times with 10 volumes of 0.9% NaCl solution to remove buff coating. The erythrocytes were lysed with hypotonic Tris buffer solution (pH 7.4).[1] Erythrocyte white ghosts were collected by centrifugation (13000 min^{-1} for 20 min at 4 °C). Then this pellet was dispersed in isotonic Tris buffer solution and was incubated at 37 °C for 1 h before use.

Incorporation of Glycoprotein in Liposome. Bovine glycoprotein (fraction IV) and dimyristoyl phosphatidylcholine (DMPC) were purchased from Sigma. The phospholipid liposomes with glycoprotein incorporated could be prepared by the dialysis with cholic acid.[5] A suitable amount of purified glycoprotein was added to 50 mg DMPC in isotonic Tris buffer solution (pH 7.0). The detergent solution containing the glycoprotein and phospholipid was then extensively dialyzed against a Tris buffer solution at 37 °C for 48 h. The final concentration of DMPC was set to 0.1 wt% in the buffer solution. Glycoprotein was set to 1—25% (wt/wt DMPC).

Sonication. Fragmented white ghosts were prepared by sonication (Tomy Seiko Co., Ltd., UR-200P) with different sonication times from 0 to 30 min at 25 W in an ice bath.

Scanning Electron Microscopy. Fragmented ghosts were fixed with 1% glutaraldehyde. Radius of each of the fragmented ghosts was measured directly by Hitachi S-800 scanning electron microscope without spattering.

Fluorescence Depolarization Measurement. Fluorescence probes sodium 8-anilino-1-naphthalenesulfonate (ANS) and 1,6-diphenyl-1,3,5-hexatriene (DPH) were purchased from Tokyo Kasei Co., Ltd. Erythrocyte ghosts were labeled with ANS or DPH by incubation with gentle shaking at 37 °C for 12 h. The stained erythrocyte ghosts were washed once with isotonic Tris buffer solution. The ghosts were centrifuged at 10000 min^{-1} for 30 min at 4 °C, and then were resuspended in the same buffer. The labeled ghosts were sonicated for a suitable period of time and the molecular motion of component lipids were measured by means of fluorescence depolarization measurements (JASCO FP-550 fluorescence spectrophotometer). Lights with wavelengths of 375 nm and 366 nm were used for excitation of membrane-incorporated ANS and DPH respectively. The emission intensities from ANS and DPH were detected at 480 nm and 432 nm. The fluorescence polarization was evaluated from averaging the fluorescence intensities polarized parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the direction of the polarized excitation beam. The degree of polarization (P) is defined in the following equation:

$$P = (I_{\parallel} - I_{\perp})/(I_{\parallel} + I_{\perp})$$

The $1/P$-value represents the membrane fluidity.

Incorporation of Heme. Dichloromethane solution of the iron(III) derivative of heme ([Fe]=0.05 mM (1 M=1 mol dm^{-3}), [1-dodecyl-2-methylimidazole]=2.25 mM) was evaporated to prepare a thin film on a glass flask under nitrogen gas flow. A phosphate buffer solution containing 10 vol% of the white ghost (pH 7.0) was mixed with the thin film, then it was sonicated for a suitable period of time under nitrogen atmosphere. In degassed solution, the incorporated iron(III) derivative of heme was reduced by L-ascorbic acid. The resulting solution was incubated at 37 °C for 1 h. Oxygen gas was bubbled through the solution for 15 s to induce the oxygen-binding of heme. The reduction and oxygen-binding were detected by visible absorption spectrometry (Hitachi-320 UV. *vis.* spectrophotometer).

Results and Discussion

Fragmentation of Human Erythrocyte Ghosts. Human erythrocyte ghosts turned into fragments by the sonication, and they formed smaller liposomes than the initial ghosts. The shape and radius of fragmented

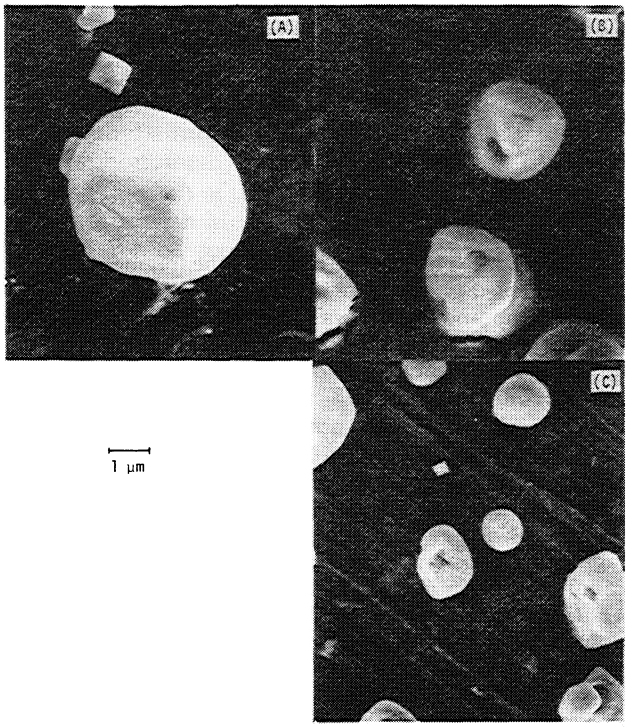

Fig. 1. Scanning electron micrographs of fragmented ghost.
Sonication time was 10 s (A), 60 s (B), 5 min (C) respectively.

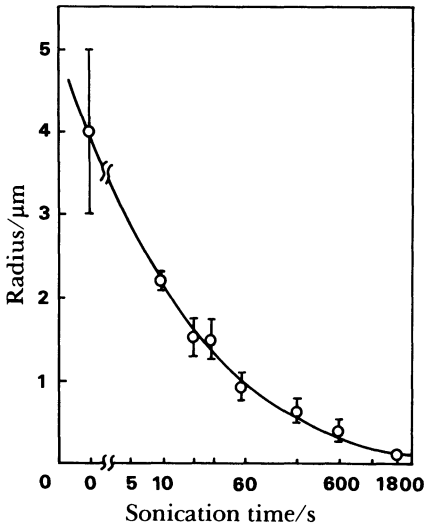

Fig. 2. Effect of sonication time on the average radius of fragmented ghost.
Average radius was directly measured by SEM. Sonication (25 W, in ice bath)

ghosts were detected directly by means of high resolution scanning electron microscopy. The fragmented ghost was generally observed as a sphere, as shown in Fig. 1. The average radius greatly depended on the sonication time. Figure 2 shows the effect of sonication time on the radius of the fragmented ghosts. The average radius of the intact white ghost was about 4 μm, which decreased with increasing sonication time and turned about 1 μm after 60 s sonication. Finally it reached to about 0.1 μm by 30 min sonication.

The motion of membrane-constituted molecules

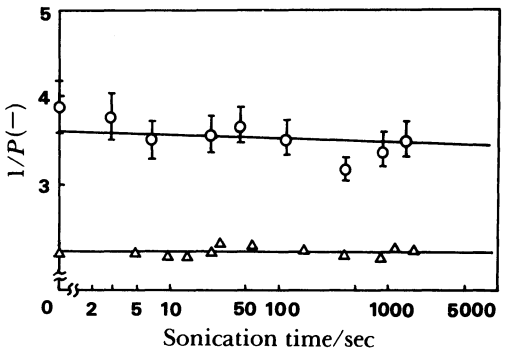

Fig. 3. Effect of sonication time on the membrane fluidity of fragmented ghosts.
Sonication (25 W, in ice bath), ○: ANS, △: DPH, [ANS]=[DPH]=10^{-6} M.

Table 1. Membrane fluidity of fragmented ghost and DMPC liposome with or without glycoprotein

Liposome	Membrane fluidity (1/P)	
	ANS	DPH
DMPC	8.24	3.75
Glycoprotein incorporated	2.85	2.11
Fragmented ghost	3.68	2.20

[DMPC]=0.1 wt%; [Glycoprotein]=10% (wt/wt DMPC); [ghost]=1 vol%; [DPH]=1.0×10^{-6} M; at 37°C

could be detected by the fluorescence depolarization measurements.[6,7] Fluorescence measurements with different types of probe molecules gave us important information for membrane fluidity at molecular level, because the location of the incorporated probe molecules depends on their molecular structure.[6] As an ANS molecule dissociates to give an anion, ANS can be incorporated into the hydrophobic region closest to the surface of the membrane.[8] On the other hand, as DPH is a conjugated aromatic hydrocarbon, it is located at the center of the hydrophobic hydrocarbon of cell membranes.[9] It was observed that the membrane fluidity of the fragmented ghosts was independent of their radius and was almost the same as that of unsonicated ghost membrane, as shown in Fig. 3. This shows that the network structure of the ghost membrane was taken off by sonication but the composition of membrane-constituted molecules was almost unchanged.

The membrane fluidity of the fragmented ghosts was compared with that of the DMPC liposome and DMPC liposome with glycoprotein incorporated. Results are summarized in Table 1. When the liposomes are labeled with ANS, the membrane fluidity of the fragmented ghost was higher than that of DMPC liposome with glycoprotein incorporated. But it was suppressed more strongly than that of DMPC liposome only. On the other hand, the membrane fluidity of fragmented ghost was almost the same as that of DMPC liposome with 10% (wt/wt DMPC) of glycoprotein when labeled by DPH. These results show that the effect of sonication in fragmented ghost membrane was more remarkable in the surface region than in the hydro-

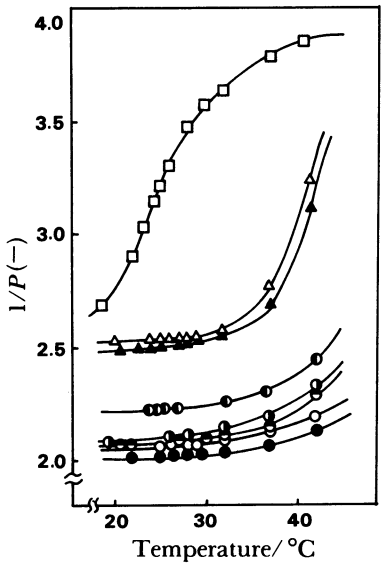

Fig. 4. Temperature dependence of the membrane fluidity of the DMPC liposome with the glycoprotein incorporated.
[DMPC]=0.1 wt%, [DPH]=10^{-6} M, Sonication (25 W, for 30 sec), [glycoprotein]=0.0 (□), 1.0 (△), 2.5 (▲), 5.0 (◐), 10 (◑), 15 (◓), 20 (○), 25 (●) % (wt/wt DMPC)

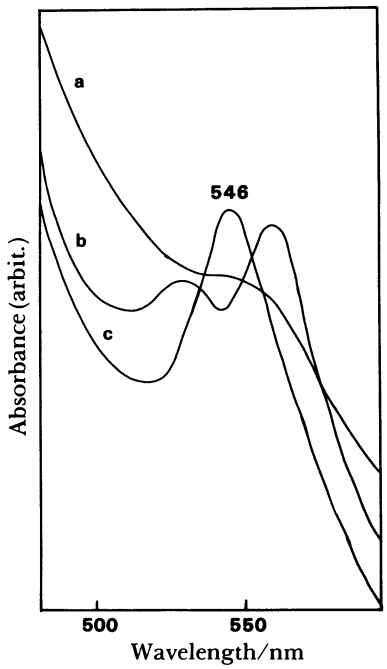

Fig. 5. Visible absorption spectra of ghost-embedded heme in fragmented ghost.
[Fe]=0.05 mM, [Ligand]/[Fe]=50, [ghost]=10 vol%.

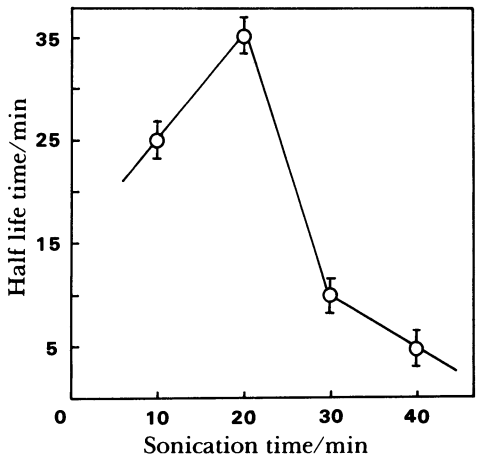

Fig. 6. Relation between the dispersion state and the life time of oxygen adduct on ghost-embedded heme. Sonication (25 W, in ice bath)

phobic region. The membrane fluidity of the initial ghost membranes was considered to be higher than that of human erythrocytes. The membrane fluidity of erythrocytes was reduced by the existence of the network structure. Though the network structure of spectrin in the fragmented ghost was destroyed by the sonication, the membrane fluidity in the hydrophobic region was kept lower because of the existence of the membrane proteins. Figure 4 shows the temperature dependence of the fluidity of the DMPC liposome with or without glycoprotein. The membrane fluidity of the DMPC liposome increased remarkably at 24°C. This temperature corresponded to the gel to liquid-crystalline phase transition temperature (T_c). On the other hand, the membrane fluidity of DMPC liposome with glycoprotein incorporated was gradually increased at temperature higher than T_c, and the transition point became unclear with increasing concentration of the incorporated glycoprotein, as shown in Fig. 4. So, it is clear that the membrane fluidity of DMPC liposome with glycoprotein incorporated remarkably depended on the concentration of the incorporated glycoprotein. The effect of the concentration was particularly, dramatic when the concentration of incorporated glycoprotein was less than 5.0% (wt/wt DMPC). The molecular motion of lipids was considered to be suppressed through the interaction with the incorporated glycoprotein. When 10% (wt/wt DMPC) of glycoprotein was incorporated to DMPC liposome, the membrane fluidity in the hydrophobic region was almost the same as that of fragmented ghost membrane, as shown in Table 1. It is expected that these fragmented ghosts could be applicable as a membrane model when synthetic pospholipid hybrid liposomes are prepared. This is because fragmented ghost liposomes could be prepared easily and their membrane fluidity was adequately suppressed, in almost the same way as that of ghost membranes or phospholipid liposomes containing glycoprotein.

Application as a Carrier of Heme Complex. The fragmented ghost containing heme in the hydrophobic region (ghost-embedded heme) was prepared by a sonication method. The stability and oxygen-binding ability of the ghost-embedded heme suspension was measured by means of visible absorption spectrometry, as shown in Fig. 5. Fig. 5 (a) shows the visible spectra of the ghost-embedded heme before reduction. They were incubated at 37°C for 1 h after adding a reducing agent. The absorption bands of the ghost-embedded heme were observed at 535 nm and 555 nm, corresponding to the penta-coordinated heme, as shown in Fig. 5 (b). The oxygen adducts were obtained after bubbling oxygen gas for 15 sec through that solution. The formation of oxygen adducts was

detected by the appearance of a single peak at 546 nm, as shown in Fig. 5 (c). This peak coincided with that of semistable oxygen adducts of the liposome-embedded heme.[4] In this system, it is important to analyze the effect of sonication time of the dispersion state of ghost-embedded heme and of the life time of its oxygen adduct. These results are summarized in Fig. 6. It was found that ghost-embedded heme could be dispersed relatively homogeneously by a suitable period of sonication time. The life time of the semistable oxygen adducts turns longer (35 min) when they were sonicated for 20 min at 25 W. But most of ghost-embedded heme phase-separated (aggregated) when incubated overnight. Contrary to this, the life time of the semistable oxygen adducts was only for 20 min when ghosts were fragmented by 30 min sonication. But, they were quite stable and the dispersion state was maintained over several days. As the fluidity was completely independent of the sonication time, it was better for the vehicle size to be as small as possible. The reason of the effect of sonication time on the life time of oxygen adducts has not been clarified yet. It is expected that more stable oxygen adducts with longer life time should be obtained by the improvement of the composition of iron heme/complex/the membrane-constituted molecules as well as the physicochemical conditions.

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