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INTERACTION BETWEEN PHOSPHOLIPID ASSEMBLIES AND HEMOGLOBIN (Hb)

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ABSTRACT

In order to prepare Hb-vesicles having a high oxygen-transporting ability, it is important to encapsulate concentrated Hb with a mixed lipid membrane having low lamellarity (n). The concentration of Hb in the Hb-vesicles ($[Hb]_{in}$) increased with the Hb concentration used for the preparation of Hb-vesicles. Hb-vesicles with smaller lamellarity could be obtained by lowering the preparation temperature. The encapsulation of Hb was significantly influenced by the ionic strength and the solution pH because lipids and Hb are electrolytes. The lamellarity decreases with increasing solution pH because of electrostatic repulsion between the surfaces of lipid membranes. On the other hand, $[Hb]_{in}$ increases when the solution pH is lowered below the pI of Hb (7.0 at 25°C) due to electrostatic interaction between Hb and the surface of the membrane. This interaction decreases with the ionic strength of the Hb solution, leading to a lower $[Hb]_{in}$.

INTRODUCTION

Phospholipids assemble spontaneously in aqueous solutions to form multilamellar vesicles. A vesicle encapsulating hemoglobin (Hb), Hb-vesicle, was studied in detail for the purpose of developing artificial red cells [1–4]. The amount of oxygen transported by this system can be determined by increasing the concentra-

tion of Hb ($[Hb]_{in}$) in an aqueous phase of a vesicle with a low number of bilayers (lamellarity) as well as by optimizing the oxygen-transporting efficiency (OTE) of Hb from lung to terminal tissues. The value of OTE is determined from the P_{50} and Hill coefficient, controlled by the addition of an appropriate composition of allosteric effectors (IHP, PLP, or 2,3-DPG).

On the other hand, $[Hb]_{in}$ and lamellarity (n) should be adjusted by introducing the concept of molecular assemblies, namely, interaction between Hb and bilayer constituents, interaction between the surface of lipid membranes, and the influence of the membrane fluidity on the encapsulation of Hb. Since vesicles are prepared in concentrated Hb solutions in order to increase the $[Hb]_{in}$, the study about the interaction between Hb and phospholipids in assembling becomes important [4]. Both Hb and phospholipid vesicles can be regarded as kinds of hard and globular polyelectrolytes. Hb has an isoelectric point (pI) of 7.0 at 25°C, and phospholipid assemblies containing a fatty acid have pK_as around 7.4 [5]. Therefore, the interaction between phospholipid assemblies and Hb should change at the pI of Hb and also be influenced by the ionic strength. In this paper, the properties of polyelectrolytes or polyelectrolyte complexes are introduced for the preparation of Hb-vesicles with a high encapsulation efficiency of Hb.

EXPERIMENTAL

Materials

Diacylphosphatidylcholine (PC): 1,2-bis(2,4-octadecadienoyl)-*sn*-glycero-3-phosphocholine (DODPC) [6] was purchased from Nippon Oil & Fats Co. Japan. Cholesterol was used as a membrane stabilizer. Negative charges were introduced using stearic acid (SA) in order to enhance the Hb encapsulation efficiency and stabilize the vesicles. These lipids were dissolved in benzene at a 7/7/2 composition (by moles) of PC/cholesterol/SA, and then freeze-dried.

Purification of Hb [7]

Outdated red cells (RC) from the Hokkaido Red Cross Blood Center (Japan) was diluted with an equivalent amount of saline. It was shaken under a CO atmosphere to convert HbO_2 to HbCO. After washing the RC twice with saline by centrifugation (1000g, 10 minutes), the concentrated RC solution (25 g/dL) was mixed with CH_2Cl_2 (20 vol%), shaken for 3 minutes, and then centrifuged (1900g, 15 minutes). An Hb solution was separated as a top layer. The residual CH_2Cl_2 in the Hb solution was removed by a rotary evaporator at 40°C for 20 minutes in the dark. The HbCO solution was heated at 60°C for 1 hour in the dark. Denaturated proteins other than HbCO were removed by centrifugation (1900g, 20 minutes), and the ultrapure concentrated Hb solution was obtained with a yield of 80% from the washed and concentrated RC solution.

Thus, purified Hb solution was dialyzed for several hours against pure water to remove ions with low molecular weights with a seamless cellulose tube membrane. After the adjustment of pH and the ionic strength by added solutions of Na_2CO_3 and NaCl, the Hb solution was ultrafiltrated by using UF-LMF (TOSOH Co.) with

an ultrafiltration filter (UF-30PS, cutoff MW:30,000), yielding a highly concentrated Hb solution ([Hb]: ~45 g/dL).

Preparation of Hb Vesicles [4]

Large multilamellar vesicles, prepared simply by dispersing a lipid mixture into a Hb solution, were extruded through polycarbonate membrane filters. Vesicles which had a smaller size and fewer lamellarity, were prepared by extrusion by reducing the pore size of the filter to 0.2 μm ϕ. Free Hb was removed by gel permeation chromatography (Sepharose CL-4B).

Evaluation of Hb Encapsulation [8]

The volume of an inner aqueous phase of one vesicle (V_{in}) and the number of molecules in one vesicle (N) are represented in Eqs. (1) and (2), respectively, as a function of lamellarity (n). The structural parameters in those equations and the values used for calculation are listed in Table 1.

$$V_{in}\ (\mathrm{cm}^3) = \frac{4}{3}\pi\{r - n(d + d_w) + d_w\}^3 \times 10^{-21} \tag{1}$$

$$N = \frac{4\pi}{S}\sum_{i=1}^{n}[\{r - (i-1)(d + d_w)\}^2 + \{r - i(d + d_w) + d_w\}^2] \tag{2}$$

From Eq. (2), the volume of vesicles of unit weight of lipids ($V_{calc.}$ (cm^3/g)) is represented by Eq. (3), where M is the average molecular weight of mixed lipids (546) and N_A is Avogadro's number.

$$V_{calc.}\ (\mathrm{cm}^3/\mathrm{g}) = \frac{\frac{4}{3}\pi r^3 \times 10^{-21}}{\mathrm{M} \times (N/N_A)} \tag{3}$$

On the other hand, when the weight of added lipid mixture is m (g) and the total volume of vesicles is V (cm^3), the volume of vesicles of the unit weight of lipids ($V_{meas.}$) is V/m, which was measured by the following method. When m' (g) of glucose is added to V' (mL) of an Hb-vesicle solution, the apparent concentration of glucose ($C_{app.}$) is represented as m'/V' (g/mL). After the separation of Hb-vesicle by centrifugation (50,000g, 30 minutes), the glucose concentration of a supernatant, C (g/mL), is measured by a phenol sulfuric acid method. $V_{meas.}$ (cm^3/g) is obtained from Eq. (4), where m (g) is the weight of mixed lipids.

TABLE 1. Structural Parameters of Hb-Vesicles

Radius of vesicles (r)	100 nm
Thickness of anhydrous layer (d)	5 nm
Thickness of aqueous phase between bilayers (d_w)	1.3 nm
Average area per lipid (S)	0.36 nm^2
Lamellarity (n)	Variable

$$V_{meas.}\ (cm^3/g) = \frac{V}{m} = \frac{V'(1 - C_{app.}/C)}{m} \quad (4)$$

The weight ratio of Hb to mixed lipid, which is the parameter for Hb encapsulation, is represented as Eq. (5), where $[Hb]_{in}$ is the concentration of Hb in an inner aqueous phase of a vesicle.

$$\frac{[Hb]}{[Lipid]} = \frac{[Hb]_{in} \times V_{in}}{M \times (N/N_A)} \times 100 \quad (5)$$

The [Hb]/[Lipid] ratio was obtained by a cyanomet Hb method to measure the Hb concentration, and an Allen's method was used to measure the phospholipid concentration. Supposing $V_{meas.} = V_{calc.}$, N was obtained from Eqs. (3) and (5), and the lamellarity (n) was calculated from Eq. (4). The Hb concentration of the inner aqueous phase was calculated by introducing the measured [Hb]/[Lipid] ratio into Eq. (5).

Measurement

Viscometric Analysis [10]

Mixed lipid vesicles (200 nm ϕ): PC/cholesterol/2,4-octadecadienoic acid (ODA) (7/7/2 by moles) in saline were prepared by the extrusion method. After regulating the lipid concentration (1.0 g/dL) and pH (7.4) by NaOH and bubbling with N_2, the suspension was irradiated with γ-ray (0.75 Mrad/h × 1 h, 4°C) to polymerize the diene groups in lipids [6, 9]. Polymerization conversion was 71%, calculated by the decrease in absorbance at 255 nm. The vesicle suspension was concentrated by ultracentrifugation (50,000g, 90 minutes), yielding 14.5 g/dL of suspension. This was diluted with 77 mM phosphate buffer solutions or Hb solutions (44 g/dL, [NaCl] = 0.9 g/dL) for viscometric analysis.

The viscometric measurements were performed with a cone-plate-rotation viscometer (Vismetron VS-AK, Shibaura System Co.) at shear rates of 1.15–230 s^{-1} at 25°C. The concentrations of Hb and lipid in the samples are 20 and 8 g/dL, respectively.

Microviscosity of Membrane

The fluidity of the lipid membrane of the vesicles was analyzed by steady-state anisotropy of incorporated 1,6-diphenyl-1,3,5-hexatriene (DPH) by a spectrofluorometer (JASCO FP-770). DPH was excited at 357 nm through a sharp cut filter (HOYA B-370, transparent region: 320–380 nm; HOYA Glass Works), and its fluorescence (430 nm) was detected through a filter (HOYA L-37, transparent region: >390 nm).

Diacylphosphatidylcholine (PC)/cholesterol/myristic acid (MA) (7/7/2 by moles) vesicles in a N/30-phosphate buffer (pH 7.4) were prepared by the extrusion method (100 nm ϕ, PC: egg yolk phosphatidylcholine (EYL), DODPC, hydrogenated EYL (H-EYL), [PC] = 500 μM). DPH in a THF solution (5 mM) was added to the vesicle suspension at a concentration of 5 μM (PC/DPH = 1000/1) and incubated at 40°C for 1 hour before measurement. The Perrins equation relating to

membrane fluidity [11] represents the microviscosity of the medium when DPH is present:

$$\eta = C(r)T\tau S \tag{7}$$

$$S = r/(r - r_0) \tag{8}$$

where η is the microviscosity, $C(r)$ is a parameter relating to the fluorophore, T is the absolute temperature, τ is the excited state lifetime, r is the measured fluorescence anisotropy, and r_0 is the limiting fluorescence anisotropy (0.362 at 25°C) [11]. Since S is proportional to η, this value represents the fluidity of a membrane at a specific temperature.

RESULTS AND DISCUSSION

Influence of Hb Concentration on Encapsulation

Vesicles were prepared in Hb solutions with various concentrations at pH 7.0. The total volume of vesicles is constant against the concentration of Hb. This means that the lamellarity of vesicles does not depend on the Hb concentration because values of the other parameters, which influence the total volume of vesicles, such as lipid concentration and vesicle diameter, are the same. Figure 1 shows that the concentration of Hb in vesicles ($[Hb]_{in}$) increases with the concentration of Hb at preparation ([Hb]). It should be noted that $[Hb]_{in}$ is not proportional to [Hb], that is, a deviation ([Hb] − $[Hb]_{in}$) increases with [Hb]. Hb molecules tend to form

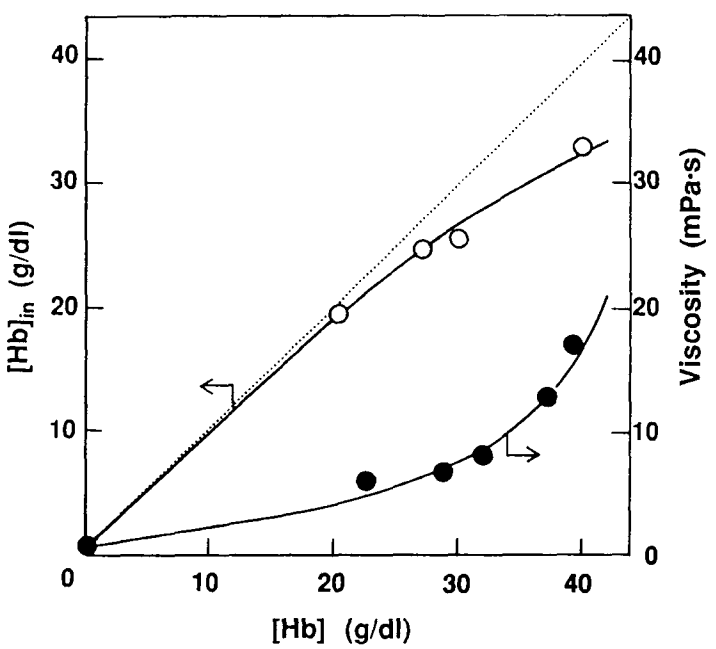

FIG. 1. Encapsulation profiles of Hb into vesicles with increasing fed Hb concentration (○) and the solution viscosity (●). Shear rate = 11.5 S^{-1}).

larger flocculates at higher Hb concentration, leading to a nonlinear increase in the solution viscosity as shown in Fig. 1. Therefore, it is more difficult to encapsulate such larger flocculates into vesicles. However, when the concentration of Hb at preparation was 40 g/dL, $[Hb]_{in}$ was 33 g/dL, which is a relatively high encapsulation efficiency in spite of the high molecular weight (65,000) of the encapsulated molecule (Hb).

Influence of Temperature at Preparation

Since HbCO is stable against temperature up to 70°C [7], it is possible to study the dependence on preparation temperature, i.e., extrusion temperature, on Hb encapsulation efficiency. The [Hb]/[Lipid] ratio of 1.5 at 4°C decreases almost linearly with increasing temperature (Fig. 2). This behavior was analyzed from lamellarity (n) and the concentration of Hb in vesicles (Fig. 3). $[Hb]_{in}$ goes up slightly from 25 to 27 g/dL when the preparation temperature is increased from 4 to 11°C, which reflects the decrease in solution viscosity by the dissociation of Hb flocculation. On the other hand, the lamellarity increases with the preparation temperature. This was the main contribution to low encapsulation efficiency ([Hb]/Lipid] ratio) at higher temperature, as shown in Fig. 2.

The size of vesicles prepared at different temperatures was constant when measured at room temperature. The increase in lamellarity can be discussed from two points of view: changes of the solution pH and membrane fluidity at different temperatures.

(1) The pH of the Hb solution (40 g/dL) increases from 7.0 to 7.4 when the temperature is reduced from 22 to 4°C. This can be explained by protonation of the dissociated moieties of Hb. On the other hand, there was no temperature depen-

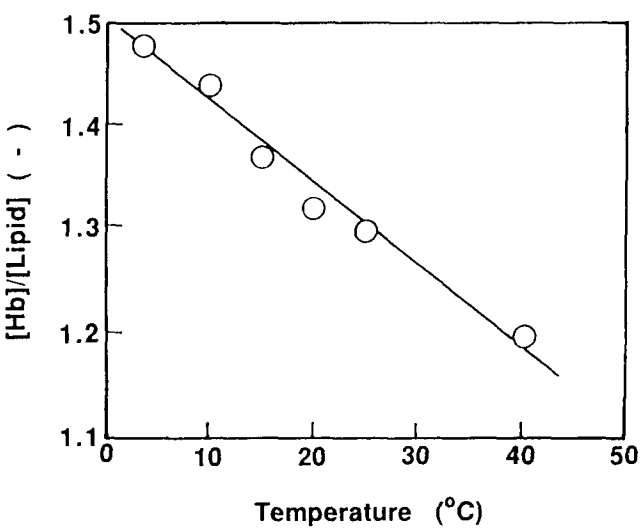

FIG. 2. Influence of the preparation temperature on the [Hb]/[Lipid] ratio. [Hb] = 40 g/dL, [NaCl] = 0.57 g/dL, pH 6.9.

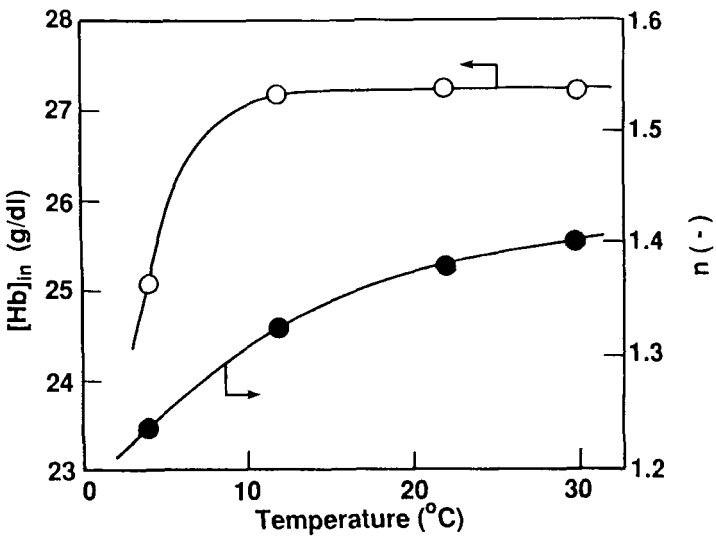

FIG. 3. Influence of the preparation temperature on n (●) and $[Hb]_{in}$ (○). [Hb] = 31 g/dL, [NaCl] = 0.6 g/dL, pH 7.0.

dence of the pH of the phospholipid vesicle solution in the absence of Hb (data not shown here). The dissociation of fatty acid in the negatively charged bilayer membrane would be enhanced by such a pH increase, resulting in a smaller number of lamellarity by electrostatic repulsion between the negatively charged surface of a lipid membrane.

(2) In the preparation of Hb-vesicles, multilamellar vesicles are converted to vesicles of smaller size and lower lamellarity by shear stress at extrusion. When the membrane fluidity becomes high, the deformation of vesicles by shear stress would occur more easily even for a lipid membrane with higher lamellarity. This idea is supported by the following results. Multilamellar vesicles, with lipid membranes which had different fluidities at the same temperature, were prepared. The membranes were PCs/cholesterol/MA at a molar ratio of 7/7/2. PCs were EYL, DODPC, and H-EYL with gel-to-liquid crystalline phase transition temperatures of −15, 18, and 49°C, respectively. The parameter S, which relates to the degree of membrane fluidity, can be estimated by measuring the fluorescence anisotropy of DPH incorporated into each bilayer membrane [11]. A high S value means a low microviscosity around the DPH molecule incorporated into the hydrophobic center of a bilayer membrane. As depicted in Table 2, the mixed membrane containing H-EYL shows the highest S value and the highest [Hb]/[Lipid] ratio.

Furthermore, in order to eliminate the influence of Hb on lamellarity, vesicles were prepared at different temperatures in the absence of Hb, and the lamellarity was measured. The lamellarity of DMPC/cholesterol/MA (7/7/2 by moles) vesicle (in 150 mM Tris-HCl buffer, pH 7.4) decreased from 1.70 to 1.51 with a lowering of the preparation temperature from 40 to 4°C. These results indicate that multilamellar vesicles with lower membrane fluidity result in vesicles with lower lamellarity after extrusion.

TABLE 2. Influence of Molecular Mobility of Lipids on Hb Encapsulation

Lipids (7/7/2)	T_c of PC, °C	S	[Hb]/[Lipid]
EYL/chol/MA	−15	2.0	0.8
DODPC/chol/MA	18	3.1	1.1
H-EYL/chol/MA	49	3.6	1.3

Influence of Solution pH

Hb encapsulation should be influenced by solution pH. The dependence of the [Hb]/[Lipid] ratio on the pH of Hb solutions used for the preparation of Hb-vesicles is shown in Fig. 4. A maximum in the [Hb]/[Lipid] ratio was observed

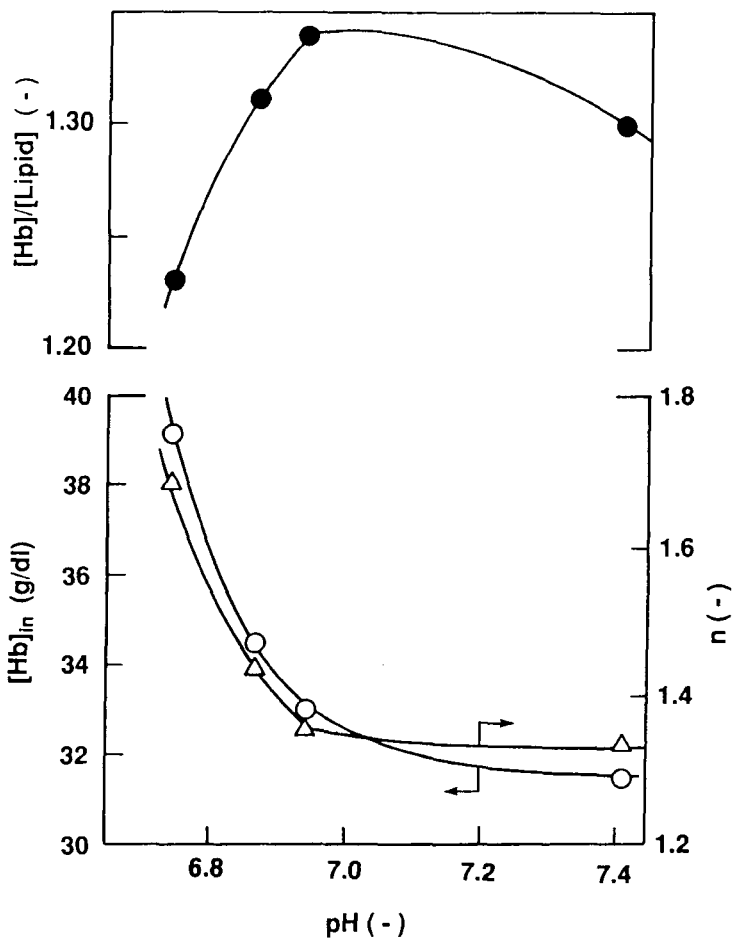

FIG. 4. Influence of pH in Hb solutions on the [Hb]/[Lipid] ratio (●), n (△), and $[Hb]_{in}$ (○). [Hb] = 40 g/dL, [NaCl] = 0.6 g/dL, 4°C.

at around pH 6.9, which was almost the same as an isoelectric point of Hb (pI = 7.0) at 25°C. Since the preparation temperature was 4°C, the pI of Hb would shift to the higher side of 7.0. The surface of the lipid membrane is negatively charged in an observed pH range of 6.4–7.8 by the incorporation of fatty acid of which pK_a was estimated as 7.4 in a mixed lipid membrane at a composition of PC/cholesterol/fatty acid = 7/7/2 by moles. Therefore, the polarity change of Hb at the pI or the change of charge density on the surface of the membrane accompanied by a pH change should influence the interaction between Hb and the bilayer membrane. The influences of pH on the lamellarity (n) and $[Hb]_{in}$ are summarized in Fig. 4. In the calculation of n and $[Hb]_{in}$, the pH dependence of the average molecular area should be considered. The molecular area of stearic acid without dissociation and with complete dissociation were reported to be 19 Å^2 [12] and 70 Å^2 [13], respectively. However, in a pH change from 6.7 to 7.5, the average molecular area of mixed lipid changes only 1.8% as calculated from the Henderson–Hasselbach equation.

It can be easily understood that the lamellarity of vesicles decreases when the electrostatic repulsion between the negatively charged surfaces of a lipid membrane becomes high [14], i.e., at high pH. On the other hand, negatively charged Hb above the pI should receive an electrostatic repulsion from the negatively charged surface of the bilayer membrane. This causes the lower encapsulation of Hb into vesicles, i.e., lower $[Hb]_{in}$. The electrostatic repulsion between negatively charged Hb and the negatively charged surface of the membrane decreases with lowering pH. When the solution pH becomes lower than pI, electrostatic interaction between positively charged Hb and the negatively charged surface occurs. This change should explain the significant increase in $[Hb]_{in}$ with lowering pH.

The above discussions can be supported by solution viscosity. Unsaturated membrane components (DODPC) were polymerized with γ-rays to prevent the deformation of vesicles induced by interaction with Hb. Figure 5 illustrates the pH dependence of solution viscosity after mixing Hb solutions and polymerized vesicle dispersions. Those of Hb solutions and polymerized vesicle dispersions are also depicted as references. A maximum of the viscosity of Hb solutions was observed at the pI because the least electrostatic repulsion between Hb molecules occurs there. A slow decrease in the viscosity of vesicle dispersion with pH is due to increased electrostatic repulsion between vesicles. The mixture of solutions shows a remarkable increase of viscosity below the pI, indicating a higher interaction between positively charged Hb and the negatively charged surface of vesicles.

Influence of the Salt Concentration

The effect of NaCl concentration in a Hb solution on Hb encapsulation was studied, and the results are shown in Fig. 6: The [Hb]/[Lipid] ratio decreases with NaCl concentration. This can be explained in terms of the larger number of lamellarity and the lower $[Hb]_{in}$ with increased salt concentration. The viscosity of vesicle dispersions (8 g/dL) increases from 14 to 16 cP with increasing NaCl concentration from 150 to 450 mM because of the shielding effect of electrostatic repulsion between negatively charged vesicles [10]. Therefore, the shielding effect of negative charges among bilayers should explain the increase of lamellarity of the resulting vesicles with a high NaCl concentration.

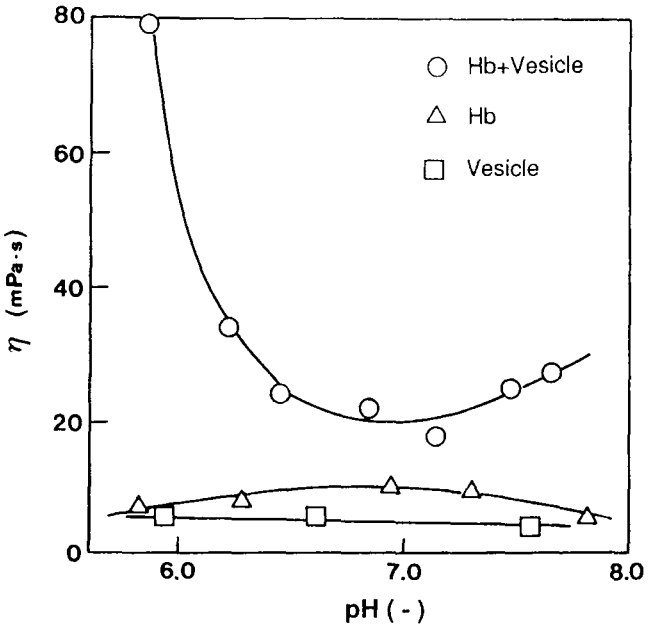

FIG. 5. Change of the solution viscosity of the mixture of Hb solutions (20 g/dL) and polymeric vesicle dispersions (8 g/dL) at various pH values. Shear rate = 11.5 S^{-1}.

On the other hand, the viscosity of Hb solutions (25 g/100 mL) decreases a little from 5.0 to 3.9 cP with increasing NaCl concentration from 22 to 290 mM. In this concentration region, interaction between Hb molecules is lowered by adding salt, i.e., "salt in" effect [15]. This cannot explain the decrease in $[Hb]_{in}$ with NaCl concentration. However, it can be explained as follows. Though the pH of the Hb solution used for the preparation of Hb-vesicles was around p*I*, the local pH between the Hb solution and lamellar membrane containing fatty acid would become lower than the p*I* because the dissociation of the fatty acid occurs at hydration of the lipid membrane by the Hb solution. Therefore, interaction between positively charged Hb and the negatively charged surface of the membrane would exist at the encapsulation of Hb into vesicles. This interaction is high at low ionic strength in the system, and it becomes low at the high ionic strength due to the shielding effect by microions.

Interaction of Hb with Phospholipid Assemblies

The above described results show that a high electrostatic interaction between Hb and negatively charged phospholipid assemblies (bilayer membrane) is necessary in order to obtain high $[Hb]_{in}$. This can be realized if the pH and the salt concentration of a Hb solution are low. However, high electrostatic interaction between Hb and a negatively charged phospholipid membrane, such as phosphatidylserine membrane, are known to cause several denaturation processes of Hb at specific conditions such as 5 mM phosphate and pH 5.6 [16, 17]. The processes are Hb-lipid

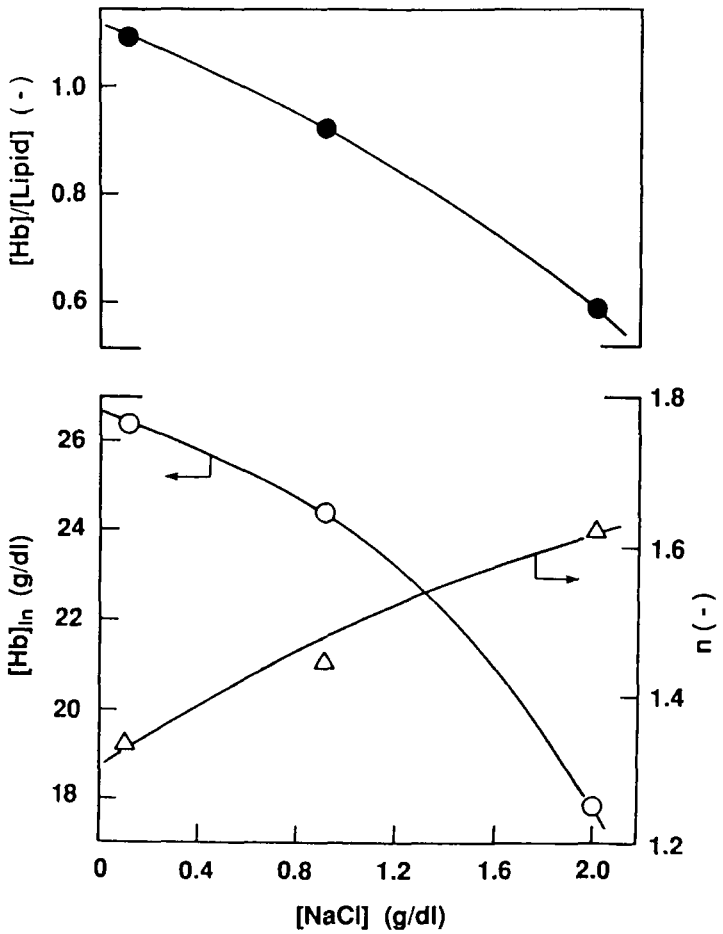

FIG. 6. Influence of NaCl concentration in Hb solutions on the [Hb]/[Lipid] ratio (●), n (△), and $[Hb]_{in}$ (○). [Hb] = 27 g/dL, pH 7.1, 4°C.

complex formation, the penetration of Hb into a bilayer, the displacement of heme in Hb, unfolding of Hb in the hydrophobic region of a bilayer, detachment of the heme from globin, etc. In addition, if unsaturated lipids were used, the oxidation of Hb was also enhanced, coupled with oxidation of the unsaturated lipids [18–20]. However, these processes were significantly inhibited by incorporation of cholesterol [16]. The influence of such an interaction of positively charged Hb with the negatively charged surface of a phospholipid membrane should be considered carefully, especially when the solution pH and ionic strength are low because the interaction is high. In our experimental conditions (pH > 6.7, [NaCl] > 100 mM, 45 mol% cholesterol content), such a denaturation does not occur.

Furthermore, the oxygen affinity of Hb is controlled by incorporating such allosteric effectors as 2,3-DPG, IHP, or PLP [21]. Salt concentration and pH are also important effectors. Appropriate regions exist for the composition of those

allosteric effectors. Within the region, the composition should be determined by considering the factors that influence Hb encapsulation efficiency.

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