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Superoxide Dismutase-like Activity of Liposomes Modified with Dodecanoyl His and Metal Ions

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The liposome modified with simple ligand and metal ions shows the superoxide dismutase-like activity. The membrane fluidity of various liposomes modified with the functional ligand (Dodecanoyl-His; Dodec-His) and the clustering of the ligand on the liposome surface were first characterized, showing that the clustering of Dodec-His could be induced on the liposome surface at gel-phase. The capacity of adsorption of Cu and Zn was found to be increased dependently on the type of liposome, resulting in the maximal adsorption in liposome prepared by 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) at gel state and with higher ligand clustering state. As a result on the SOD-like activity of the metal/ligand-modified liposome, the SOD-like activity was found to be induced by using the above liposomes although its activity level is not so high.

Key words : membrane stress biotechnology／liposome／LIPOzyme／ligand／superoxide dismutase

1. Introduction

Oxidative stresses caused by the reactive oxygen species (ROS) (e.g. superoxide, hydrogen peroxide and hydroxyl radical) are inevitable stresses of biological systems on earth [1]. It is known that, in biological cells, superoxide dismutase (SOD) and catalase (CAT) eliminate the ROS to protect the cell from oxidative stresses [2]. The natural antioxidative enzymes are, however, known to be unstable inside the biological environment and are mostly exposed to various kinds of oxidative stresses (i.e. ROS). The SOD structure is, for example, destroyed and furthermore was fragmented into pieces in the presence of hydrogen peroxide, which could be formed by the conversion of superoxide with SOD itself [3, 4]. The artificial enzymes that mimic the above antioxidative enzymes and show high stability need to be designed to efficiently eliminate the ROS, such as superoxide and hydrogen peroxide.

Much effort has been devoted to the design and development of artificial antioxidative enzymes [5, 6] although several efforts have been made for the control of their activities through the modulation of the structure of the metal-ligand complex by the suitably-designed chemical structures of the ligand [7, 8]. A model biomembrane, liposome, is a possible candidate to provide a common platform for different catalytic centers of the natural enzymes. Such a liposome herewith possesses several benefits in the regulation of catalytic activity, where it can provide (i) a nano hydrophobic environment, (ii) a stress-responsible character, (iii) a microdomain structure, and (iv) membrane-membrane interactions. Some researchers have reported the effectiveness of the use of a model biomembrane (liposome) as a platform to immobilize the functional catalytic center [9～11]. Enzyme-like activity, such as that of SOD [9, 12～14] and cholesterol oxidase [15], has already been regulated by the membrane properties of the liposome, as well as affording functional elements on the liposome surface. The above enzyme-like function of liposome, which can herewith be defined as "LIPOzyme" [16], can be utilized for the design of the artificial enzymes.

The final purpose is to design and develop the liposome catalysis, which can be defined as LIPOzyme [16], demonstrating the activity of the SOD. As shown in

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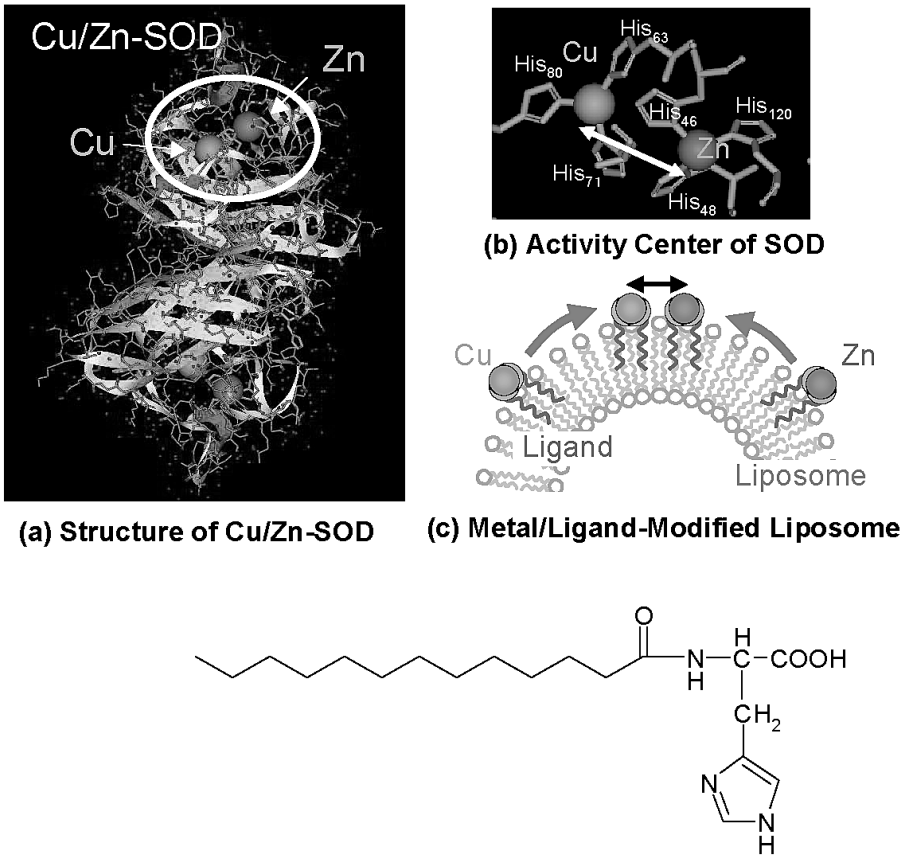

Fig. 1 Schematic Illustration of SOD LIPOzyme Using Metal/Ligand-Modified Liposome. (a) and (b) show the structure of Cu/Zn-SOD and that of its active center (Data from Protein Data Bank (ID = 1CBJ, http://www.rcsb.org/pdb/) were visualized by using ViewerLite (http://www.accerlrys.com). (c) The schematic illustration of metal/ligand modified liposome which could be utilized for the design of the SOD LIPOzyme. (d) The chemical structure of N-dodecanoyl-Histidine (Dodec-His) used as a ligand to be modified.

Fig. 1(a) and (b), the active center of SOD is known to consist of the Cu and Zn coordinating with six His residues and one Asp residue. In this study, it has been investigated whether the molecular assembly of simple and minimal elements (His and Cu/Zn) on the liposome could induce the SOD-like activity (Fig. 1(c)). After the liposome modified with dodecanoyl-His (Dodec-His, Fig. 1(d)) was prepared, the basic characteristics of the Dodec-His modified liposome and the adsorption behaviors of metal ions (Cu and Zn) on it were systematically investigated. Based on these results, the SOD-like activity of the metal/ligand-modified liposome was finally investigated.

2. Materials and Methods

2.1 Materials

1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC: $T_m = 42\,^\circ\mathrm{C}$), 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC : $T_m = 23\,^\circ\mathrm{C}$), and 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC: $T_m = 0\,^\circ\mathrm{C}$) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Dodecanoyl-Histidine (Dodec-His) (Fig. 1(d)) and Pyrene-dodecanoyl-histidine (Py-Dodec-His) was synthesized and prepared according to the modified method described in the previous literature [17]. Other chemicals of commercially guaranteed reagent grade were purchased from Wako and were used without further purification.

2.2 Preparations of Dodec-His modified Liposome

The liposomes modified with Dodec-His were prepared by using the following procedure according to the previous work [12～15, 17]. Phospholipid and Dodec-His (with a molar ratio 10 : 1) were dissolved in chloro-

form/methanol (lipid concentration: 2 mM). After the solvent was evaporated, the resulting thin film was dried for at least 2 hours under a vacuum. The lipid film was hydrated by 50 mM potassium phosphate buffer to form the multilamellar vesicles. The solution of the multilamellar vesicle was treated by ultrasonication (15 min, 80 W, 5 ml) on ice (4 ℃) to obtain the liposome with the size of 30 nm.

2.3 Characterization of Membrane Fluidity

The membrane fluidity of liposomes was determined by using a hydrophobic fluorescence probe, diphenyl hexatriene (DPH) [18]. DPH was added to the solution of liposome-copolymers or liposome-proteins at a final concentration of 1 μM. The fluorescence polarization of the DPH probe in the liposome membrane was measured at a wavelength of 360 nm for the excitation and 430 nm for the emission. The fluorescence intensity of the DPH was measured by using the fluorescent spectrophotometer (JASCO FP-777, Japan). The degree of fluorescence polarization (P) was calculated using the following equation:

$$\frac{1}{P} = \frac{(I_{//} + I_{\perp})}{(I_{//} - I_{\perp})}$$

where $I_{//}$ and $I_{\perp}$ are the intensities of the light emitted with its polarization plane parallel (//) and perpendicular ($\perp$) to that of the exciting beam, respectively. The term "fluidity" is inversely proportional to the degree of fluorescence polarization of the probe; that is, the 'membrane fluidity' of the interior of the membrane was defined by ($1/P$) of DPH.

2.4 Characterization of Ligands Cluster on the Liposome Surface

It has been reported that the pyrene molecules with different clustering state (monomer and dimer) shows the different fluorescence spectra. The clustering state of the Dodec-His molecules on the liposome membrane was evaluated by using the Pyrence-conjugated acylated-Histidine (Py-Dodec-His). The liposome was modified with Py-Dodec-His in replace of Dodec-His at molar ratio of 10%. The fluorescence spectra of the Py-Dodec-His modified liposome were measured by using the fluorescent spectrophotometer (JASCO FP-777, Japan) at the excitation wavelength of 350 nm. The spectra of fluorescence emission were recorded. The relative clustered state of the ligands was assessed as a ratio of the fluorescence at excimer and that at monomer (E/M ratio) [12].

2.5 Metal Adsorption on Dodec-His Modified Liposome

The metal adsorption experiment was carried out by shaking the liposome suspension in the presence of metal ion at 25 ℃ for 24 h. After the above operation, the liposome and metal ion were separated by ultralfiltration (Ultra free MC; Millipore) at the specific centrifugal condition (5000 × g for 10 min). The concentration of metal ion in the filtered solution was measured by using fluorescent indicators for metal ion, such as Phen Green or Bio.

2.6 SOD-like Activity

A NBT (nitro blue tetrazorium) method was employed for the measurement of SOD-like activity. In this assay, super oxide anion ($\cdot O^{2-}$) was produced by 1 mM xanthine / 2.5 μM xanthine oxidase. The SOD-like activity was determined at 25 ℃ as the inhibition rate of the production of the NBT formazan caused by the oxidation through the superoxide anion [19]. The half maximal inhibitory concentration (IC_{50}) was determined in the presence of Mn-HPyP-modified liposome at the Mn-HPyP concentration of 0.05～10 μM. It has been confirmed that there is no significant error caused by the unexpected side reaction caused by the components (xanthine, xanthine oxidase and NBT) in the reaction mixtures.

3. Results and Discussion

3.1 Characterization of Properties of Ligand-Modified Liposome

It has been reported that the clustered state of the ligands with hydrophobic tails was dependent on the phase of liposome [12, 13]. The membrane properties of Dodec-His modified liposome, such as membrane fluidity and ligand clustering, were first characterized.

Fig. 2(a) shows the temperature dependence of the membrane fluidity, analyzed by DPH, of different type of liposomes. In the case of DLPC liposome, the value of membrane fluidity was gradually increased with the increase of the temperature and was reached to the saturated value at more than 40 ℃. A distinct phase transition behavior was observed in the case of DMPC and

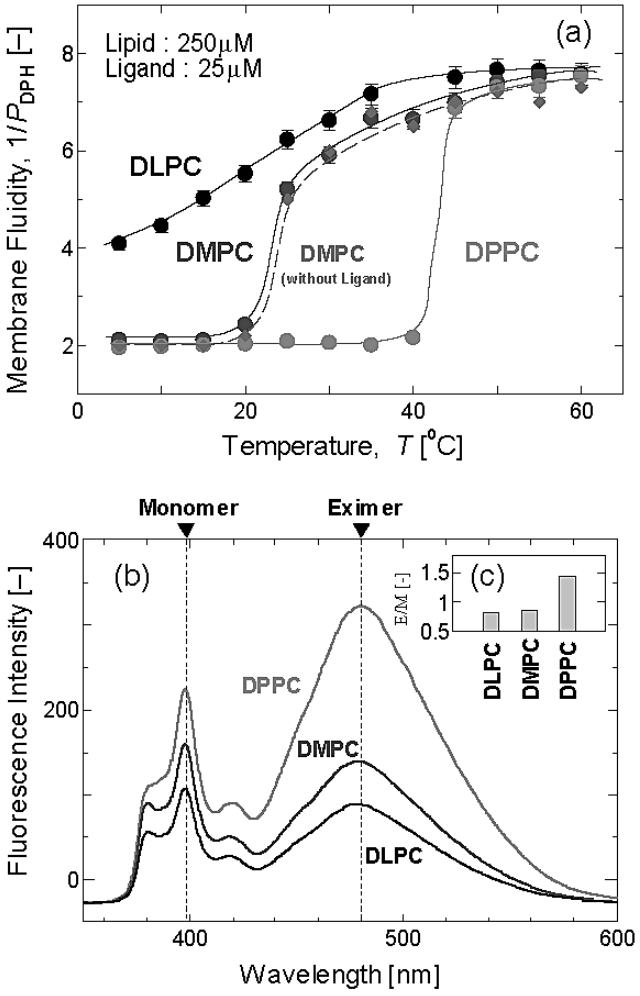

Fig. 2 Characterization of Membrane Properties of Dodec-His Modified Liposome. (a) Temperature dependence of the membrane fluidity of the Dodec-His modified liposome prepared with different lipids (DLPC, DMPC, and DPPC). DPH was added at the molar ratio of 1 : 250 against lipid concentration and was used as a fluorescence probe of the membrane fluidity. (b) Fluorescence spectra of the Py-Dodec-His in different types of liposome at 25 °C. (c) The relative fluorescence of eximer and monomer (E/M ratio) in different liposomes (DLPC, DMPC, and DPPC).

DPPC liposomes, where the value sharply increased at the temperature of 22 °C and 43 °C, respectively. It has been reported that the phase transition temperatures of the DLPC, DMPC, and DPPC liposomes are 0 °C, 23 °C, and 45 °C, respectively [13]. The observed temperature for the phase transition of liposome from gel phase to liquid-crystalline phase was well corresponding with that of the previous report although a slight difference in the value of the membrane fluidity between the liposome and ligand modified-liposome was observed. It was thus found that the phase transition behaviors of the liposome was not significantly affected by the modification of the liposome with the 10 mol% Dodec-His.

The clustered state of the Dodec-His molecules on the liposome membrane was further studied by using the Py-Dodec-His as its probe molecule. Fig. 2(b) shows the fluorescence spectra of the Py-Dodec-His-modified liposomes at 25 °C, where two major peaks were observed at 398 nm and 480 nm. It has been reported that the Pyrene molecule shows the different fluorescence values depending on the clustering state of the molecules [12, 20]. The fluorescence at 398 nm and 480 nm corresponds with the extents of the monomer and dimer (excimer), respectively. The relative fluorescence of the excimer and monomer (E/M) of the probe molecules in different liposomes was shown in Fig. 2(c). Although the E/M ratio in DLPC and DMPC liposomes at liquid-crystalline state was small, the value was increased in the case of DPPC liposomes because of the increase of the excimer extent.

It has been previously shown that the Hexadecyl-porphyrin (HPyP) molecules could undergo their clustering on the liposome surface, depending on the physical state of the lipid membrane, where the clustering of the molecules was distinctly observed in the case of liposome at gel phase [13, 14]. The clustering of acylated-tryptophan, which has similar structure as Dodec-His, has reported to be observed on the lipid membrane surface at the gel phase [17]. It has been reported that the fatty acid, which has a hydrogen donor and accepter, also show the clustering behaviors on the lipid membrane at the gel phase because of the hydrogen bond formation between the molecules in the hydrophobic environment of the liposome membrane [21]. The present ligand has the hydrogen bond donor and accepter in the amide region of the molecule as shown in Fig. 1(d). According to the previous report on the molecular arrangement of the lipid membrane with different physical states [22], the lipid bilayer membrane at liquid crystalline phase could intake more water molecules inside the lipid membrane as compared with that at gel phase, suggesting that the former has the less hydrophobic or hydrated surface.

It is considered that the Dodec-His molecules could induce their clustering because of the hydrogen bond formation of the amide group in the lipid membrane which can provide a relatively dehydrated environment.

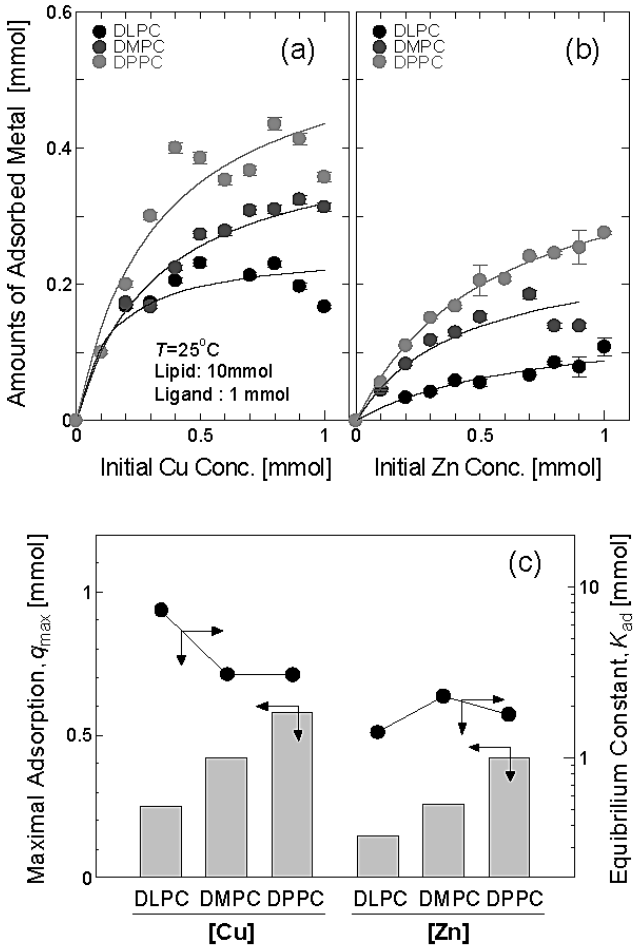

Fig. 3 Adsorption Behaviors of Cupper and Zinc on Dodec-His modified Liposomes. Adsorption was performed at 25 ℃ for 12 hours in the solution of liposome composed of 10 mM lipid and 1mM Dodec-His with 0.1-1 mM copper or zinc. (a) and (b), respectively, show the adsorption isotherm of Cu and Zn on Dodec-His modified liposomes. The curve was calculated based on the Langmuir-type adsorption isotherm equation. (c) shows the maximal adsorption (q_{max}) and equibrium constant (K_{ad}), which determined from the fitting calculation of (a) and (b).

3.2 Metal Adsorption Behaviors on Ligand-Modified Liposome

A minimal requirement to elucidate the metalloenzyme-like activity is the metal adsorption on the surface of the ligand-modified liposome. The adsorption of metal ions, such as Cupper (Cu) and Zinc (Zn), on the Dodec-His modified liposome was further investigated at 25 ℃ by varying the type of liposomes (i.e. DLPC, DMPC, and DPPC).

Fig. 3(a) and (b) show the dependence of the Cu and Zn concentration on the amounts of adsorbed metal ions on the Dodec-His-modified liposome at 25 ℃. The adsorbed amounts of the metal ions increased with the increase of the metal concentration and were varied depending on the type of liposomes, where the maximal adsorption was observed in the case of DPPC liposomes. The adsorbed amounts of Cu were, in general, higher than those of Zn when the same type of liposome was used. A Langmuir-type isotherm was herewith applied for the analysis of the adsorption behaviors and the theoretical curve was also shown in Fig. 3(a) and (b). The parameters obtained from the fitting analysis, such as q_{max} and K_{ad}, were summarized in Fig. 3(c). The results show that the adsorption constant (K_{ad}) of Cu was 2～4 folds higher than that of Zn. In both cases of the Cu and Zn adsorption, the q_{max} value was found to increase with the increase of the membrane fluidity ($1/P$) at 25 ℃, where the q_{max} values of Cu and Zn on Dodec-His-modified DPPC liposomes were, respectively, 0.56 and 0.41 mmol against 1.0 mmol ligand.

The adsorption of transition metal ions on the liposomes modified with the metal affinity ligands harboring carboxyl group and thiol group in the structure, where similar adsorption behavior on the ligand modified liposome was obtained in both cases of Cu and Zn and the adsorption was governed by the hydrogen-bond network of the ligand and the phosphate group of the lipid membrane [23]. Different from the extraction behaviors in organic/water two-phase systems, the higher selectivity of the Cu or Zn adsorption was then observed [23]. In the present experimental system, the ligand used, Dodec-His, has some possible chelating groups in the hydrophilic region of the ligand, such as carboxyl group and imidazole group (Fig. 1(d)). Among them, the pKa values of the carboxyl group and imidazole group were, respectively, determined as 9.1 and 5.6 through the pH titration experiment if the Dodec-His was modified into the lipid membrane (data not shown), implying that the metal ion could preferably coordinate with imidazole group. At the neutral pH region, it was also confirmed that the UV absorbance at 260 nm, derived from imidazole group, was strongly affected by adding the metal ions to the Dodec-His modified liposome, showing that the imidazole group of the ligand could mainly contribute to the metal adsorption on the Dodec-His-modified liposome.

It seems that the difference in the adsorption behaviors of metal ions could be closely related to the environmental condition neighboring the coordination

Table 1 Comparison of IC_{50} Values of SOD and Liposome-Recruited SOD

Enzymes / LIPOzyme			Metal / Ligand Ratio [-] (*1)	IC_{50} [μM] (*2)	
[SOD LIPOzyme] (This Study) (*3)					
<Ligand>	<Lipid>	<Metal>			
Dodec-His	DLPC	Cu	0.19	25	[4.8]
Dodec-His	DMPC	Cu	0.24	56	[13.4]
Dodec-His	DPPC	Cu	0.35	9.0	[3.2]
Dodec-His	DLPC	Zn	0.06	n.d.	
Dodec-His	DMPC	Zn	0.13	n.d.	
Dodec-His	DPPC	Zn	0.19	45	[8.7]
[Others for Comparison]					
Dodec-His	DPPC	Cu/Zn	0.36 (Cu: 0.24, Zn: 0.12)	18	[6.5] (*4)
His (*5)	POPC	Cu	-	n.d.	
His (*5)	POPC	Zn	-	n.d.	
His (*5)	POPC	Cu/Zn	-	3200	(*6)
HPyP	DMPC	Mn	1.0	0.35	[0.35] (*7)
[Enzyme]					
Cu/Zn-SOD (Native)			n.d.	0.034	

*1 Relative Ratio of Adsorbed Metal on Ligand. The value was calculated based on Langmuir isotherm equation with parameters calculated in Figure 3(c).
*2 IC_{50} values were evaluated from the SOD inhibitory activity at different concentration by using the NBT method at 25°C. The values inside the square brackets show the IC_{50} values recalculated based on the ratio of the adsorbed metal on the ligands.
*3 Initial concentration of metal was adjusted to 0.5 mM
*4 Cu/Zn mixture was applied at the initial concentration of Cu (0.25 mM) and Zn (0.25 mM)
*5 SOD-like activity was measured just after 10 mM Histidine was mixed with 1 mM Cu and/or 1 mM Zn in the presence of POPC liposomes.
*6 The enzymatic activity of SOD was lost within 12 hours although it was kept for at least two hours.
*7 Previous data (H. Umakoshi *et al.*, *Langmuir*, **24**, 4451 (2008))

n.d.: The effective SOD activity was not detected owing to the lower value in inhibitory activity (less than 20%)
-: No data

group of the ligand molecule. Especially, in the case of the DPPC liposomes which shows the gel phase and higher clustering nature of the Dodec-His, the adsorption capacity of both Cu and Zn was maximal. In the case of the natural Cu/Zn-SOD, the Cu and Zn were coordinated with imidazole groups of six His residues and carboxyl group of an Asp [24] and its activity center was well wrapped by the relatively hydrophobic amino acid residues [25]. It is generally known that the hydrogen bond or metal-ligand coordination could be stabilized in the dehydrated (hydrophobic) environment [26]. Considering its hydrophobic (or non-hydrating) environment of the surface of DPPC liposomes [22], the cluster-formation of the ligands and, furthermore, the metal-ligand complex could be stabilized on the surface of the liposome.

It is thus concluded that a similar coordination structure, by the imidazole group and carboxyl group of the Dodec-His, was formed on the surface of the liposomes and could be utilized for the construction of the mimics

of the active center of SOD.

3.3 SOD-like Activity of Metal/Ligand-Modified Liposome

Based on the above results of the basic characteristics of membrane and the metal adsorption on it, the SOD-like activity of the metal/ligand-modified liposome was finally investigated.

The SOD activity of the liposomes modified with Dodec-His and metal ion (Cu or Zn) was determined as the IC_{50} value and the value was summarized in Table 1. In the case of Zn/Dodec-His modified liposome, the SOD-like activity was not observed in the case of DLPC and DMPC liposomes and that for DPPC was low ($IC_{50} = 45$ μM). The relatively higher value in SOD-like activity was observed in the case of Cu/Dodec-His modified liposome as compared with that of Zn/Dodec-His modified liposome. Although the IC_{50} value of DLPC and DMPC was similar with those of Zn /Dodec-His modified DPPC (25 μM and 56 μM), the SOD activity was maximal in the case of the DPPC ($IC_{50} = 9$ μM). The obtained IC_{50} values were furthermore corrected based on the net amounts of the adsorbed metal on the Dodec-His modified liposome (Fig. 3) and were shown inside the square brackets. It was found that the DMPC liposomes with mixed phase of gel and liquid crystalline showed the lowest activity and that was the highest in the case of DPPC liposomes modified with Cu and Dodec-His.

The obtained SOD-like activity of Cu/Dodec-His modified DPPC liposomes was compared with those of other type of liposome catalysis and natural SOD. The addition of both Cu and Zn has previously reported to increase the SOD-like activity of the oxidized and fragmented SOD on the surface of liposome [14]. Although the addition of both Cu and Zn was investigated, the activity level was not significantly affected ($IC_{50} = 8.7$ μM). In order to compare the role of acyl group modification, the SOD-like activity of His and Cu/Zn with liposome was measured similarly in the case of previous study [27], resulting that the enzymatic activity level was approximately one-thousand times lower than the present results and the SOD-like activity was not stable for time incubation. The above results show that the effectiveness of the His-metal complex on the surface of lipid membrane. However, the SOD-like activity of Cu/Dodec-His modified DPPC liposomes was 10 times lower than that of hydrophobically-modified Mn-porphyrin [14]. The value was also much lower (5% of native) than that of native SOD. It has been reported that the molecular recognition function of the liposome itself could be achieved by a combination of the non-specific interactions of the lipid surface [28]. Although the level of the SOD-like activity of the present liposome is retained to the lower level, its tuning could be performed through the modulation of the structure of active center complex and the regulation of the substrate recognition on the structure based on the basic characteristics of liposome surface induced under the stress condition.

It was thus shown that the simple ligand-modified liposome can create a metal complex similar to the active center of SOD although further investigation is needed.

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