The partitioning behavior of horseradish peroxidase (HRP) to phospholipid- and surfactant vesicles has been analyzed. Peripheral binding of the zwitterionic phospholipid vesicle membranes is proposed with insertion into the membrane interior of negatively charged surfactant vesicles, resulting from non-electrostatic interaction. The insertion of HRP is interpreted by considering the interaction between headgroups of phospholipid and between headgroups of surfactant.

1. Introduction

Phospholipid- and surfactant vesicles are promising materials for artificial cells [1], reactors [1-3], biosensor units [4-6], or vehicles in drug-delivery [7]. These applications originate from the compartmental effects [3] and self-assembly of structures [8]. In addition, the vesicle system can be regarded as a type of W/O/W emulsion system [9]. As such the vesicle system can achieve a simple chemical process with consumption of less energy and material. As a consequence, the vesicle system can operate in the fields of (bio)separation [4,10,11] and of designable reactions [9].

For efficient use of vesicles, the clarification of the biophysical and engineering aspects of the partition mechanism of target materials including small (bio)molecules and proteins to vesicle membranes is important. Investigations regarding the partitioning behavior of small (bio)molecules have been widely studied as compared with proteins [9,12]. Proteins are generally categorized into soluble and membrane proteins. The partition behavior of a membrane protein originates from the amino acid sequence [13]. Hydrophobic regions on the secondary structure favor the hydrophobic region of lipid membranes to be partitioned. Alternatively, the partition behavior of soluble proteins depends on their conformation and property of lipid membranes according to the reports by using small-sized proteins (at most 30 kDa) [14,15] and antimicrobial / cell-penetrating peptides [16]. The headgroup mobility on the surface of vesicle membranes generates a hydrophobic crevice/pothole [17], which is a platform for the binding of small-sized proteins [14]. The possibility that this scenario mentioned above can be applied to soluble proteins with high molecular weight is still unclear.

In line with this, horseradish peroxidase (HRP) is a good candidate for a mid-sized protein as a model system other than serum albumin from human or bovine (66.5 kDa), from the viewpoints of its...
functionality and possible partitioning properties. HRP has the molecular weight of about 40 kDa and its polypeptide chain of 308 amino acids folds to 13 α-helices and three β-sheets [18]. The HRP conformation is stabilized by four disulfide bonds [19]. In addition, the catalytic properties of HRP have been well defined in several reviews [19-21]. In particular, the catalytic action of HRP on the vesicle surface is considered as a key step for a reaction selectivity of polyaniline polymerization [22].

A clarification of the partition behavior of a mid-sized protein HRP would result in two important possibilities: (a) whether a scenario obtained by using small-sized proteins can be applied to mid-sized proteins and (b) whether a deeper understanding of the HRP-catalyzed polymerization reaction of polyaniline to control its selectivity can be obtained.

In this study, we evaluated the binding characteristics of the target protein HRP to vesicle membranes. In previous studies, negatively charged surfactant vesicles composed of sodium di-2-ethylhexylsulfosuccinate (AOT) and sodium dodecylbenzene sulfonate (SDBS) / decanoic acid (DA) (mixing ratio 1 : 1) are used because these surfactant vesicles indicated the strong interaction with HRP [22]. In this paper two zwitterionic phospholipids 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dimirystoyl-sn-glycero-3-phosphocholine (DMPC) were used as a control system for surfactant vesicles. The interaction between HRP and vesicles was probed using two approaches: calcein leakage experiments [4,5,23] and quartz crystal microbalance (QCM) methods [24]. The calcein leakage can be a quantitative index for the extent of HRP-vesicle interaction and indicate the change in membrane structure caused by HRP. The QCM method can give the adsorbed mass of HRP as the quantitative index for the HRP-vesicle interaction. The partition property of HRP, resulting in the HRP-vesicle interaction, was analyzed with a thermodynamic model, surface pressure isotherms and dielectric measurements. The possible partitioning mechanism of HRP to vesicle membranes is discussed.

2. Experimental

2.1 Reagents

DOPC, DA, and HRP from horseradish were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan) and DMPC, SDBS, and AOT from the Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Calcein was obtained from Dojindo (Kumamoto, Japan). Other chemical reagents were of analytical grade.

2.2 Preparation of vesicles

A vesicle with a particle size of 100 nm was prepared according to the conventional method [5,17]. The lipid mixture was dissolved in chloroform in an eggplant flask, and chloroform was removed using an evaporator to form a lipid thin film. Drying was performed overnight under vacuum to remove all remaining solvent. The dried lipid film was then hydrated with 50 mM Tris HCl solution (pH 7.5) containing 100 mM NaCl to obtain a total lipid concentration of 10 mM and a final volume of 3 mL. Multilayer vesicles were prepared by the freeze-thaw method, resulting in a uniform particle size of 100 nm after preparation by the extrusion method. These vesicles were examined by a cryo-TEM to confirm their morphology.

2.3 Calcein leakage assay

A calcein leakage experiment was performed using previously reported procedures [4,5,23]. The
vesicles entrapping calcein were prepared by a hydration method. In outline, lipid films were hydrated with a calcein solution (100 mM, pH 7.5). Size exclusion chromatography was performed to remove calcein not entrapped. Calcein-encapsulated vesicles and an HRP solution (Tris-HCl 50mM, NaCl 100mM, pH7.5) were mixed to obtain a solution containing 250 μM and 10 μM of each, and the fluorescence intensity was measured at an excitation wavelength of 490 nm and a emission wavelength of 520 nm with a fluorescence spectrophotometer (FP6500, Nihon Bunko, Osaka). Finally, Triton-X 100 was added to disrupt the vesicles, and the maximum fluorescence intensity was measured to obtain the calcein leak rate RF value.

Scheme 1. The chemical structure of the lipids used: (a) DOPC, (b) DMPC, (c) SDBS, (d) DA, (e) AOT.

2.4 Quartz crystal microbalance (QCM) experiments.

Based on previous methodology [24], the amount of HRP adsorbed on lipid membranes was measured with the QCM combined with the immobilization technique of lipid membrane. The QCM (Au) electrode was immersed in an ethanol solution (1 mM) containing 11-mercaptopaneanoic acid (MUA) for 12 hours to form a thiol self-assembled membrane (SAM) on the gold surface. After drying and washing, the SAM-based electrode was exposed for 4 hours to 17 mM each of N-hydroxysuccinimide (NHS) and N-(3-dimethylaminopropyl)-N-ethylcarboimide hydrochloride to activate the terminal carboxy group. The NHS-activated SAM on electrode was dried an coupled to an ALS / CHI electrical microbalance quartz crystal (400, BAS Inc.). The amino group-doped (PE: 2 mol% content) vesicle suspension (5 mL) was loaded for 1 hour to the electrode for its immobilization.

According to Sauerbrey's equation (Eq. (1)), there is a linear relationship between the decrease in frequency (−Δf) and the increase in mass per unit area (Δm/A).
\[
\Delta f = -\frac{2f_0^2 \Delta m}{\sqrt{\mu \rho} A}
\]

where \(\Delta m\), \(f_0\), \(A\), \(\mu\), and \(\rho\) represents mass change, fundamental frequency, electrode area Shear stress of quartz, and Amber quartz density, respectively.

2.5 Surface pressure analysis

Surface pressure analysis was undertaken to determine the \(\pi\)-\(A\) isotherm, and protein orientation analysis was performed using a small LB film-making device FSD-220C (U.S.I Corp.) and LB LIFT CONTROLLER FSD-23 (U.S.I Corp.) as in an earlier report [5]. First, 60 \(\mu\)L of lipid (1 mM) dissolved in chloroform was dispersed in a water tank filled with ultrapure water. After allowing the lipid to stand at the gas-liquid interface for 15 minutes, pressure was applied to the surface to investigate the change in occupied area \((A)\) due to the change in the surface pressure \((\pi)\). The lipid was dispersed and allowed to stand for 15 min, then 60 \(\mu\)L of protein solution (HRP: 5 mg / mL) was added to the water bath, after 5 min passed, Changes in \(A\) due to differences in \(\pi\) were investigated similarly, and the orientation pattern of proteins was interpreted from changes in the form of the curves due to the presence or absence of protein.

2.6 Dielectric dispersion analysis

In order to analyze the relaxation behavior derived from the microscopic motion of the lipid molecule assembly interface, we used a coaxial cell with an RF impedance analyzer or a network analyzer, following previously reported method [17]. The calibration was performed by using open, short, and load modes. Thereafter, the cell constant \(C_0\) was determined by using water, methanol, and ethanol. The sample was packed into the electrode, and the capacitance \(C_p\) and conductivity \(G\) were measured at a frequency of 1 MHz to 1 GHz, and the relative permittivity \(\varepsilon'\) and dielectric loss \(\varepsilon''\) were calculated from the following equations:

\[
\varepsilon' = \frac{C_p}{C_0}\quad (2)
\]

\[
\varepsilon'' = \frac{G - G_0}{2\pi f C_0}\quad (3)
\]

where \(f\) represent frequency [Hz] and \(G_0\) conductivity at frequency \(f = 0\) [S].

2.7 Thermodynamic analysis

The theory underpinning the investigations was obtained from the literatures [15,16]. In summary, the binding process was divided into the electrostatic and non-electrostatic interactions. The former and latter were defined as \(\Delta G_{el}\) and \(\Delta G_{non-el}\). The total free energy, \(\Delta G = \Delta G_{el} + \Delta G_{non-el}\). The \(\Delta G = -RT\ln K_{app,1}\) and \(\Delta G_{non-el} = -RT\ln K_{app,2}\) values were obtained by an estimating the apparent partitioning coefficient of HRP to vesicle, \(K_{app,1}\), defined in the complex formation model and \(K_{app,2}\) in the distribution model, respectively. An important point is that the \(\Delta G\) and \(\Delta G_{non-el}\) values can be evaluated from the concentration-dependency of the experimental data regarding the HRP-vesicle interaction.

2.8 Visualization of lysine residues on HRP

Information concerning the structure of HRP was acquired from Protein Data Base (PDB: https://www.rcsb.org/). Its ID was 1hch. The file of 1hch was recorded as 1hch.pdb to be restored in the software for visualization. The UCSF Chimera 1.14 (http://www.cgl.ucsf.edu/chimera/) that was free
software was used.

3. Results and Discussion

3.1 Cryo-TEM observation of vesicles

In the first series of experiments, a cryo-TEM observation was performed to confirm the morphology of vesicles prepared by using phospholipids and surfactants. Figure 1 shows the representative cryo-TEM images for individual vesicles. DOPC and DMPC vesicles were spherical. SDBS/DA (1:1 molar ratio) and AOT vesicles had not only spherical but also non-spherical shapes such as prolate and stomatocyte (a part of the sphere is dented). The mean diameters of DOPC, DMPC, AOT and SDBS/DA vesicles estimated from cryo-TEM images were 105.2, 110.8, 98.3 and 89.2 nm, respectively. In general, a deformation of spherical vesicles into prolate and stomatocyte reduces their volume, resulting in a reduction of mean diameter. These results are in agreement with those obtained from a dynamic light scattering method (data not shown).

3.2 Interaction between HRP and vesicles

The interaction between HRP and vesicles was examined from using two approaches: (i) calcein leakage assays [4,5,23] and (ii) quartz crystal microbalance (QCM) measurements [24].

Figure 1. Cryo-TEM images for (a) DMPC, (b) DOPC, (c) SDBS/DA (1:1 mol%), and (d) AOT vesicles.

Figure 2. (a) Time dependence of HRP-induced leakage of calcein from DOPC and SDBS/DA vesicles. Gray triangles record the calcein leakage from SDBS/DA (1:1 mol%) vesicles in the absence of HRP. The HRP concentration dependencies on $RF_{\text{max}}$ for SDBS/DA (1:1 mol%) and DOPC vesicles are shown in (b) and (c). Arrows in Figures 2(b) and (c) represent the concentration limit of HRP to calcein leakage.
Figure 2(a) shows the HRP-induced calcein leakage from vesicles \((RF)\). No increase in \(RF\) values was observed in the absence of HRP (gray triangles). The addition of HRP elevated the \(RF\) value in both cases (circles and squares). The \(RF\) values track the HRP-vesicle interactions. All the vesicles show first-order kinetics of calcein leakage. The maximum value of \(RF\) was defined as \(RF_{\text{max}}\) (see Figure 2(a)). The surfactant vesicles show higher \(RF_{\text{max}}\) values than phospholipid vesicles. The order of \(RF_{\text{max}}\) values at \(C_{\text{pro}} = 2.5 \, \mu\text{M}\) was SDBS/DA ~ AOT > DOPC > DMPC. In addition, the concentration dependence of HRP on \(RF_{\text{max}}\) values was investigated in the HRP concentration range 0.01 to 10 \(\mu\text{M}\). Figure 2(b) shows the increase in \(RF_{\text{max}}\) value starts around 0.1 ~1 \(\mu\text{M}\), which is a detection limit concentration of HRP to the calcein leakage. The vesicle strongly interacting with HRP favors a low limit concentration. As a trend, surfactant vesicles favor a low limit concentration in contrast to phospholipid vesicles. Consequently, surfactant vesicles interact with HRP more readily than phospholipid vesicles.

In another approach (ii), the amount of protein adsorbed to vesicles can be measured by QCM combined with an immobilization technique of the lipid membrane. This system is a powerful tool for the measurement of the mass of adsorbed protein from the change in frequency of a crystal oscillator that vibrates at a fixed frequency (resonance frequency) [24]. As shown in Figure 3(a), the frequency decrease between 180 and 300 seconds \((f_{\text{pro}})\) corresponds to the mass of HRP adsorbed on vesicles. To compare the amount of adsorbed HRP on vesicles, the \(f_{\text{pro}}\) value was normalized by the immobilized amount of lipid membranes on the QCM electrode \((f_{\text{mem}})\), which corresponds to the adsorbed mass of HRP per one vesicle. The \(f_{\text{pro}} / f_{\text{mem}}\) values obtained for individual vesicles are shown in Figure 3(b). The adsorbed amounts of HRP on the AOT and SDBS/DA vesicles were larger than on other phospholipid vesicles. This may be due to the negative charge of AOT and SDBS.

3.3 Thermodynamic analysis of HRP binding

The driving force for the HRP-vesicle interactions above was then interpreted using a thermodynamic approach [15,16]. This requires information on the concentration dependence of HRP for the HRP-vesicle interactions like those in Figures 2(b) and (c). The experimental data were then analyzed as outlined in section 2.7 to obtain the electrostatic contribution \(\Delta G_{\text{el}}\). The values of \(\Delta G_{\text{el}}\) = -2.4 kJ/mol (25.0%) for DOPC and -3.2 kJ/mol (23.2%) for DMPC were obtained. SDBS/DA and AOT gave values of
$\Delta G_{el} = -2.9 \text{ kJ/mol (31.1%)}$ and $-3.1 \text{ kJ/mol (29.0%)$. The figures in parentheses are the contributions of $\Delta G_{el}$ to the total free energy $\Delta G$. The electrostatic contribution to HRP-vesicle interaction was similar for the zwitterionic phospholipid systems and the negatively charged surfactant systems. These results, it was suggested that the non-electrostatic interactions (approx. 70%) is the main driving force for the HRP-vesicle interactions.

### 3.4 Binding properties of HRP to lipid membranes

The orientation of the protein relative to the lipid membrane can be assessed by measuring the $\pi$-$A$ isotherm [5]. The $\pi$-$A$ isotherm is shifted to a higher $A$ value when the protein is inserted into the vesicle membrane. This is because the occupied area of the lipid increases with protein insertion. In contrast, no significant shift of the isotherm arises from either peripheral binding of HRP or from no interaction between HRP and lipid membranes. With this in mind, the impact of HRP addition to the $\pi$-$A$ isotherm is assessed.

![Figure 4](image1.png)

**Figure 4.** Effect of HRP addition to surface pressure isotherm for (a) DOPC and (b) SDBS/DA monolayer membranes.

![Figure 5](image2.png)

**Figure 5.** (a) Typical dielectric spectra for DOPC. (b) The relaxation time at the rotational motion.
shown in Figure 4. Figure 4(a) shows no significant shift of the $\pi$-$A$ isotherm by HRP addition, possibly suggesting either only peripheral binding of HRP or no interaction between HRP and the DOPC membranes. Taking into consideration both the definite leakage of calcein from DOPC vesicles (Figure 2(b)) and the adsorbed amount of HRP bound to them (Figure 3(b)), HRP was most likely to be peripherally bound to DOPC membranes. The same is true for DMPC membranes. On the other hand, Figure 4(b) obviously demonstrates a shift of $\pi$-$A$ isotherm to the a higher $A$ value, strongly suggesting the insertion of HRP into SDBS/DA membranes. The same was true for AOT membranes (data not shown).

The dynamic property of the surface of lipid membranes is related to the rotational Brownian motion of headgroup of lipids [14,17]. The dielectric dispersion analysis was used to estimate the mobility of headgroups [17]. Figure 5(a) shows typical dielectric spectra for DOPC. This mobility includes lateral diffusion of DOPC molecules, the rotational Brownian motion of headgroups and of water molecules bound to DOPC. The characteristic frequency of the rotational Brownian motion $f_c$ was 50 MHz corresponding to approx. 2.0 ns. Relaxation times for other lipids are shown in Figure 5(b). The order of relaxation time was DMPC > DOPC > SDBS/DA > AOT. DOPC and DMPC bear phosphocholine units, PO$_2^-$-N$^+$-(CH$_3$)$_3$, headgroups. In contrast, AOT and SDBS have a sulfonate group, SO$_3^-$-Na$^+$, as headgroup. The SO$_3^-$Na$^+$ unit is smaller than PO$_2^-$-N$^+$-(CH$_3$)$_3$. Interaction between headgroups are likely to depend on their sizes. Likewise, the acyl-acyl interactions will also affect the interaction between headgroups of lipids: i.e. the branched C$_8$ in AOT will result in weaker interactions than those arising from the linear C$_{14}$ and C$_{16}$ units in DMPC and DOPC (see Scheme 1). Consequently the order of relaxation times suggests that the interactions between headgroups for surfactant vesicles are weak when compared with those in phospholipid vesicles.

Figures 4 and 5 indicate that HRP could easily displace AOT and SDBS due to the weak interactions between surfactants. This is likely to relate to HRP insertion into the lipid membrane phase.

3.5 Possible binding mechanisms

Two scenarios describing the partitioning behavior of proteins, including their insertion, are worthy of consideration: a free volume [12] or a crevice/pothole [17] system. A simulation study regarding the free volume in lipid membranes has predicted the presence of voids in the membrane interior and interface [12]. The probability of formation of voids of more than 1 nm in size for is quite low for HRP binding (its size is 2.4 nm in diameter [26]). As a consequence, an interpretation based on free volume is not possible. The plausibility of a crevice/pothole-based scenario should be considered instead. The headgroup mobility at the surface of vesicle membranes generates a crevice/pothole which has a hydrophobic (low permittivity) environment [17]. Small proteins bind in the crevice/pothole via electrostatic interactions and subsequently the subsequent non-electrostatic interactions occur in the low dielectric environment to reinforce the unstable hydrogen bonds [14]. Whether the binding of protein to the vesicle membrane is driven similarly is discussed below.

The electrostatic interaction between vesicle membrane and HRP depends on the net charge of HRP. This is slightly positive under conditions used in this work because the isoelectric point of HRP is pI = 7.2 [5] ~ 9 [19]. This arise from the presence of six lysine residues in the HRP molecule (Lys65, 84, 149, 174, 232, and 241 [25]). According to the literature [26], the susceptibility of Lys to chemical modification depends on its position: Lys232 is the most reactive; Lys241 and Lys174 are less reactive; Lys65, Lys84,
and Lys149 are hardly modified. The typical agents for chemical modifications of Lys are maleic anhydride derivatives or bis(N-hydroxysuccinimidyl succinate) derivatives [27] that are comparable to the size of PO$_2^-$N(CH$_3$)$_3$ and SO$_3^-$Na$^-$. It is therefore possible that HRP can interact with lipid membranes via electrostatic interaction between Lys232 (Lys241 and Lys174) and negative charge of headgroup in lipid molecules (PO$_2^-$N(CH$_3$)$_3$ or SO$_3^-$Na$^-$). The location of Lys232, Lys241, and Lys174 are depicted using the UCSF Chimera 1.14 in Figure 6(a). The six Lys residues are shown in red. Lys 174, Lys 232, and Lys 241 are located closer to the surface of HRP than the other Lys residues. Consequently, the electrostatic interactions between Lys 232 (Lys241 and Lys174) and negatively charged groups of lipid molecules would be favourable. Furthermore, the calcein leakage in negatively charged vesicles is more readily induced than in zwitterionic phospholipid vesicles (Figure 2). Therefore, the interaction between HRP and vesicle membranes initially will be an electrostatic rather than non-electrostatic (Figure 6(b)). This interpretation is in agreement with the previous study regarding the insertion of a $\beta$-peptide to phospholipid vesicles [15].

The possibility that HRP is bound to the crevice/pothole on lipid membranes needs to be considered discussed. The present work concerning the HRP-vesicle interactions is consistent with previous reports on small proteins [14], which implied that the binding of HRP involves crevices/potholes in lipid membranes. These were estimated to have a similar size to the hydrophobic fluorescence probe, sodium 8-anilino-1-naphthalenesulfonate (ANS: 0.93 nm in diameter) [17]. Therefore, HRP (2.4 nm in diameter [26]) is presumed to be too large to use a crevice/pothole for the direct insertion into HRP. Also, the conformation of HRP is fairly stable (the index for the intramolecular hydrogen bonding stability $\rho_{pr}$ is estimated to be 7.4 ± 0.4) [28]. It is noteworthy that protein molecules with stable conformations have high $\rho_{pr}$ values [10]. It has also been reported that the binding of HRP into the vesicle membrane interior has only a small impact on the conformation around the heme iron reaction center [29]. These findings probably relate to the stabilization of the HRP conformation by four disulfide bonds within the HRP molecule [18]. As a consequence, the binding of HRP to a crevice/pothole will occur without a large conformational change.

The possibility of HRP aggregation on vesicle membranes needs to be addressed. Generally, a protein with a low $\rho_{pr}$ value favors formation of dimers/oligomers, aggregation, or fibrillation [30]. As stated in the previous paragraph, the conformation of HRP that is fairly stable due to the high $\rho_{pr}$ value is stable over the binding process of protein to vesicle membranes. It is therefore concluded that HRP

Figure 6. (a) Topology of lysines in HRP (1hch.pdb). (b) Schematic illustration of HRP-vesicle interactions.
interacts with vesicle membranes without any association or aggregation (Figure 6(b)).

Finally, the insertion step of HRP is discussed. The electrostatic interaction between Lys in HRP and the headgroups is modulated by their mobility at the membrane surface. The weak interaction between headgroups like surfactants is most unlikely to act as a barrier to the HRP insertion (Figure 5(b)). Furthermore, the interaction process coupled with non-electrostatic interactions (section 3.3), which results in favorable insertion of HRP into vesicle membranes. Conversely, the strong interaction between headgroups like phospholipids act as a barrier to the HRP insertion. This will result in the peripheral binding of HRP to vesicle membranes. In addition, the long acyl chain of the phospholipids could act as a steric barrier to the insertion of HRP molecules, as compared with the short acyl chains as in AOT. These mechanisms reduce the low limit concentration of calcein leakage discussed in the section 3.2.

The HRP-vesicle interactions depicted in Figure 6(b) can be explained by the same scenario as the small-sized proteins reported before [14].

4. Conclusion

HRP interacts with vesicle membranes by a consecutive process. First, HRP is likely to bind to the vesicle membrane based on the electrostatic interaction between accessible Lys on HRP and negatively charged parts of headgroups, followed by the insertion of HRP into membranes using the weak interactions between headgroups of lipids. This step is driven by non-electrostatic forces. Otherwise, HRP is peripherally located at the membrane surface of vesicles. As a consequence, the weak interaction between headgroups of lipids enhances the HRP-vesicle interaction based on calcein leakage and HRP adsorption. The present work provides a deeper understanding of the translocation or insertion of positively charged polypeptide (protein) to negatively charged phospholipid vesicles [16] and heat-induced translocation of partially denatured proteins [31,32].

Acknowledgement

This study was supported by a JSPS KAKENHI Grant Number JP 19J14000.

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