Interaction of (+)-Catechin with a Lipid Bilayer Studied by the Spin Probe Method

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The interaction of (+)-catechin with a lipid bilayer was examined by the spin probe method. The spin probe, 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO), was dissolved in an aqueous dipalmitoylphosphatidylcholine (DPPC) dispersion containing (+)-catechin. The temperature dependence of the TEMPO parameter was measured. The increase of this parameter due to pretransition was eliminated by the addition of (+)-catechin, suggesting that it was adsorbed to the lipid membrane surface in the gel state, which hindered the change of the membrane from a flat to wavy structure. In the temperature region of the main transition, the TEMPO parameter increased rapidly, then gradually with increasing temperature, which could be explained by the eutectic phase diagram. The rotational correlation time of a spin probe 16-doxylstearic acid and the order parameter of 5-doxylstearic acid in the aqueous dispersion system of egg yolk phosphatidylcholine revealed that the motion of the alkyl chain in the liquid crystal state was hindered in the center of the membrane as well as near the surface by the adsorption of (+)-catechin.

Key words: spin probe; (+)-catechin; lipid bilayer; 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) parameter; order parameter

Among the variations of the spin probe method, the TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl) parameter has been used to characterize the phase transition of the lipid membrane and the effect of additives on it.5,7 On the other hand, the order parameter (S) of 5NS (5-doxylstearic acid) and the rotational correlation time (τc) of 16NS (16-doxylstearic acid) were both found useful to examine the dynamics of alkyl chains in the membrane.30 We applied these two methods to study the interaction of (+)-catechin with the lipid membrane.

Experimental

Materials. TEMPO, 5NS and 16NS were obtained from Sigma-Aldrich Japan Co. Dipalmitoylphosphatidylcholine (DPPC) and egg yolk phosphatidylcholine (egg PC) were purchased from Nihon Seika Co. and used without further purification. (+)-Catechin (Tokyo Kasei), stearic acid (Tokyo Kasei) and cholesterol (Wako) were each of guaranteed grade. The structures of these compounds are shown in Fig. 1.

Method.

Evaluation of the TEMPO parameter. DPPC (150 mg) was dispersed in water (3 ml) and sonicated until the suspension had become translucent, unilamellar liposome usually being formed in such a state. The suspension (400 μl) and an aqueous solution of TEMPO (5/C2H10C4M, 40 μl) were mixed, and then various amounts of the additives, (+)-catechin, stearic acid and cholesterol, were added. In the cases of water-insoluble stearic acid and cholesterol, each was first spread as a mixture with chloroform on the container wall, before the suspension was added and the mixture was incubated at 42°C. The additive was transferred into the liposome by this operation. All the samples were sonicated again at a temperature above the main transition point. It was ascertained by the reproducibility of repeated measurements whether or not a sample was...
in the equilibrium state. Spectra were measured at intervals of 2°C from 28°C to 50°C. After reaching each temperature, the sample was stood for more than ten minutes to attain the equilibrium state, and the ESR spectrum was measured twice to ascertain consistency.

Evaluation of temperature. Egg PC (25 mg), 5NS or 16NS (0.25 mg), and various amounts of cholesterol or stearic acid were mixed by dissolving in chloroform and then evaporating the solvent. Water (0.5 ml) was added to the mixture, before it was sonicated to prepare a liposome suspension. In the case of (+)-catechin, this was added after preparing the suspension. The ESR spectrum was also measured at least twice for each sample.

Measurement of the ESR spectrum. A sample was sucked into a flat cell and fixed in the variable-temperature device. The ESR spectrum was measured with a JEOL JES-RE3X spectrometer.

Results and Discussion

**TEMPO parameter**

TEMPO is a small spin probe that shows both hydrophilicity and lipophilicity, so it can be dissolved in both water and in the interior of the lipid membrane. Figure 2 shows ESR spectra of TEMPO dissolved in an aqueous DPPC dispersion (DPPC–H₂O system). In the trace at 30°C, there is a single peak on the high-field side. A small subsidiary peak emerges at 36°C, and the peak becomes larger at 44°C. As peaks h and p in the figure are attributed to TEMPO dissolved in the membrane and in water, the TEMPO parameter defined as h/(h + p) is considered to be the value corresponding to the distribution ratio of TEMPO in the membrane.6)

Figure 3A shows the temperature dependence of the TEMPO parameter for the DPPC–H₂O system. A slight increase was observed at around 33°C which corresponds to the pretransition point.6) The structure of the lipid membrane is in the rigid gel state at a temperature below the main transition point (41°C) and the motion of alkyl chains of the lipid is hindered. As the membrane is a kind of solid, it is difficult to incorporate an additive like TEMPO possessing a structure dissimilar to that of the lipid. However, the gel state of the membrane changes from a flat to a wavy structure at the pretransition point.9) This causes an increase in the number of defects in the membrane capable of incorporating TEMPO, so the TEMPO parameter increases slightly after pretransition. The main transition occurs at 41°C, which corresponds to the melting point of the alkyl chain of the lipid, and the degree of motion of the alkyl chains suddenly increases. The membrane changes from a rigid gel to a soft, liquid-crystal state and

![Fig. 2. ESR Spectra of TEMPO in the DPPC–H₂O System at Each Temperature. Peaks h and p are respectively due to TEMPO in the lipid membrane and in water.](image)
becomes able to dissolve various additives. The TEMPO parameter therefore increases abruptly at this point. As can be easily seen from Fig. 3, the TEMPO parameter gives information about the phase transition.

The effect of (+)-catechin and the other additives, stearic acid and cholesterol, was examined with the results also shown in Fig. 3. Stearic acid and cholesterol were selected as reference compounds because they are hydrophobic and easily incorporated into the interior of the lipid membrane. Their position in the membrane is also well known. Cholesterol is a rigid flat molecule and can be incorporated in both the gel and liquid-crystal state. In the gel state, cholesterol acts as an impurity in the crystal of DPPC and breaks the structure, so, the TEMPO parameter in the DPPC–cholesterol–H$_2$O system (Fig. 3C) is larger than that in the DPPC–H$_2$O system at room temperature. However, the value at temperatures above the main transition point is smaller. This is because the flat, large cholesterol molecule suppresses the motion of alkyl chains of the lipid in the liquid-crystal state and lowers the solubility of TEMPO in it, leading to a decrease in the TEMPO parameter. The increase due to main transition is still apparent, although the temperature is lower. This represents a kind of melting point depression. On the other hand, the pretransition point is eliminated by the addition of cholesterol. An analysis of the TEMPO parameter for the DPPC–steaeric acid–H$_2$O system was difficult, because stearic acid has a long alkyl chain like DPPC in which TEMPO can dissolve, so, it was impossible to determine the position of TEMPO in the membrane.

**Adsorption of (+)-catechin to the lipid membrane**

The TEMPO parameter for the DPPC–(+)-catechin–H$_2$O system (Fig. 3B) is apparently different from that of DPPC–H$_2$O system, showing that (+)-catechin interacts with the DPPC membrane and affects the structure. We consider that (+)-catechin could be adsorbed to the surface of the membrane, despite it being strongly hydrophilic. The TEMPO parameter at 30 °C was almost the same as that for the DPPC–H$_2$O system, but no increase due to pretransition was apparent. This result means that (+)-catechin was indeed adsorbed to the surface in the gel state and inhibited the change from a flat to wavy structure. The TEMPO parameter increased abruptly at 39 °C like the case of the main transition with DPPC. However, it increased gradually after that over a wide temperature range.

We can explain these results by using the phase diagram for a binary system having an eutectic point. In the liquid-crystal state above 48 °C, it is assumed that (+)-catechin exists in two states. One is strongly adsorbed to the lipid and forms a kind of complex, while the other weakly interacts with the lipids and is distributed within the complex (tentatively termed “free (+)-catechin”). In other words, the liquid crystal state is a binary system comprising of the complex and free (+)-catechin. A single liquid crystal phase is formed above 48 °C, but the solidification of the pure complex begins by lowering the temperature. The quantity of the solid gel phase formed by the complex increases as the temperature is lowered further, which causes a slow decrease in the TEMPO parameter because TEMPO cannot dissolve in the solid. Finally, all the liquid crystal freezes simultaneously at the eutectic point of 39 °C, and the TEMPO parameter suddenly decreases at this temperature.

It can be concluded from this analysis that the membrane formed from the complex, namely the (+)-catechin-adsorbed DPPC membrane in the gel state, has a main transition temperature higher than that of DPPC. This is not strange because the main transition temperature of a DPPC membrane has been reported to depend
on the degree of hydration of the headgroup, suggesting that the rigidity of the surface affects the main transition temperature. It is therefore considered that the adsorbed (+)-catechin interacting with DPPC stabilizes the surface structure, resulting in an increase of the main transition temperature.

**Order parameter ($S$)**

The spin probes, 5NS and 16NS, are derivatives of stearic acid and have long alkyl chains like DPPC, so it is considered that these probes would be incorporated in the lipid bilayer, arranging their alkyl chains in parallel with those of the lipids. Therefore, it is usually assumed that the motion of the alkyl chains of the spin probe represents that of the lipid molecule. The hyperfine splitting constant ($A$) and $g$ factor of the spin probe are largely anisotropic, so the line shape depends on the mobility of the probe. If the probe rotates rapidly, its magnetic properties are averaged and the three-line spectrum is measured. However, if the motion is hindered, three separate lines are not apparent and a complicated line shape results. This line shape depends on the mobility of the probe and, therefore, information on the motion can be obtained by an analysis of the line shape.

Figure 4 shows the ESR spectrum of 5NS incorporated in the membrane prepared from egg PC. The mobility of the probe in such a spectrum is represented by $S$, which can be calculated by the following equation:

$$S = \frac{(A_{||} - A_{\perp})/2}{(A_{XX} + A_{YY})/2}$$

Here, $A_{XX}$, $A_{YY}$ and $A_{ZZ}$ are the principal values of $A$, and have been reported as 6.3, 5.8 and 33.6 G, respectively. $A_{\perp}$ and $A_{||}$ are the perpendicular and parallel components of the observed anisotropy of the hyperfine splitting constant ($A$) and can be measured from the separation between the peaks and troughs, as shown in the figure. $S$ varies between 0 and 1: as the mobility of the probe becomes lower, $S$ becomes larger.

Figure 5 shows the dependence of $S$ for 5NS on the molar ratio of each additive. The addition of stearic acid did not affect $S$, suggesting that the motion of the alkyl chains of the lipid was not altered substantially by the incorporation of the long alkyl chains of stearic acid. In contrast, the effect of cholesterol is evident: the value increases with increasing quantity of cholesterol and seems to reach a constant value. This can be explained by the above-mentioned idea that the incorporation of a large flat molecule in the array of alkyl chains would hinder the motion of these chains.

The effect of (+)-catechin was larger than that of cholesterol, although it did not penetrate into the membrane. The mechanism must therefore be different...
from that of cholesterol. Lipid molecules can move freely in the liquid crystal membrane, which is known as lateral diffusion. This means that the attractive force between headgroups of the lipid molecules is not large. If (+)-catechin is strongly adsorbed to the surface, it might connect the lipid molecules and hinder lateral diffusion. This would result in tightening of the surface, which would lead to a decrease in the mobility of the alkyl chains of the lipid and, as a result, to an increase in $S$.

**Rotational correlation time ($\tau_C$)**

Figure 6 shows the ESR spectrum of 16NS incorporated to the membrane prepared from egg PC. The three lines are separated perfectly, and each line is almost symmetrical about the center, in contrast to the case of 5NS. This is because 16NS has more single bonds than 5NS between the headgroup and the radical moiety, which ensures sufficient mobility for the radical moiety despite the headgroup being fixed to the membrane surface. In such case, it is possible to calculate $\tau_C$ by using equation (2):\(^{(13)}\)

$$\tau_C = B \cdot \Delta H_{(m=+1)}(I_{(m=+1)}/I_{(m=-1)})^{1/2} - 1 \quad \text{(2)}$$

Here, $B$ is a constant determined from the principal values of $A$ and the $g$ factor; the value of $6.6 \times 10^{-10} \text{ s Gauss}^{-1}$ being used.\(^{(12)}\) $\Delta H_{(m=+1)}$ is the peak-to-peak width in gauss of the low-field line. $I_{(m=+1)}$ and $I_{(m=-1)}$ are the peak-to-peak heights in arbitrary units of the low- and high-field lines, respectively. The value of $\tau_C$ represents the time the probe needs to rotate one radian as an average, which reflects the mobility of the probe and the rigidity of the surroundings of the probe. An increase in $\tau_C$ means that the motion of the probe is suppressed.

Figure 7 shows the dependence of $\tau_C$ for 16NS on the molar ratio of each additive. The results are similar to those in Fig. 5. The incorporation of stearic acid did not affect the motion of the alkyl chains, but cholesterol hindered this motion. The adsorption of (+)-catechin also hindered the motion. The difference between Figs. 5 and 7 is that the effect of (+)-catechin is far larger than that of cholesterol in Fig. 5 but almost same in Fig. 7. This is because the cholesterol molecule is buried within the lipid membrane paralleling the plane with alkyl chains of the lipid, so that the degree of hindrance to the motion of the lipid might be same near the surface and deep within the membrane. In contrast, (+)-catechin is adsorbed to the surface and inhibits the motion of the headgroup, but does not act directly on the alkyl chains. Fixing the headgroup of the lipid suppresses the motion of the chains near the surface, but the end still retains mobility because there are many single

\[\Delta H_{(m=+1)}\]

\[I_{(m=+1)}\]

\[I_{(m=-1)}\]

\[1 \text{ mT}\]

**Fig. 6.** ESR Spectrum of 16NS in the DPPC–H$_2$O System, and the Peak-to-Peak Height and Width of Each Line.

**Fig. 7.** Dependence of the Rotational Correlation Time of 16NS in the DPPC–H$_2$O System Containing an Additive on the Molar Ratio of the Additive to DPPC.

A. (+)-catechin; B. cholesterol; C. stearic acid
bonds between them. Therefore, the effect of adsorbed (+)-catechin is larger near the surface than within the membrane. This reflects the difference between Figs. 5 and 7, while Fig. 7 shows that the effect was transmitted even deep within the membrane.

This study has revealed that (+)-catechin interacted with the lipid membrane and affected the structure of the membrane.

References