Technical report


DSC and NMR Spectroscopic Studies of the Interaction between Camphorated Phenol and Phospholipid Liposomes

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To clarify the interaction mechanism of biological activities induced by camphorated phenol (CP), the interactions between CP and phospholipid liposomes [dipalmitoyl phosphatidylcholine (DPPC) liposomes, dimyristoyl phosphatidylcholine (DMPC) liposomes and DMPC/dilauroyl phosphatidylethanolamine (DLEA) liposomes] were studied by DSC and NMR spectroscopy. CP exhibited a larger DSC phase transition properties [shift of phase transition temperature to a lower temperature and decrease in Height/Half-Height Width (H/HHW) of DSC peak] than phenol in the various liposome systems. It was concluded from the NMR studies that CP is highly incorporated into the DPPC bilayer, the ¹H and ¹³C signals of phenol in a complex between phenol and camphor being markedly broadened but shielded in the presence of DPPC liposomes. It was clear that CP is incorporated as a complex into the lipid bilayers.

Key words: Camphorated phenol, Phospholipid liposomes, Interaction

INTRODUCTION

Camphorous compounds have antiseptics, analgesic, anti-pruritics, and counterirritant properties. Camphorated phenol (CP) has been used in conservative dentistry as a sedative and/or disinfectant. Since a complex between camphor and phenol is stable, phenol has been reported to release slowly from the camphorated phenol complex. The caustic potency of phenol released from CP is thus reduced¹. Recently, Soekanto et al. have investigated the toxicity of this compound in dental pulp cell culture². We recently reported that phenol derivatives such as eugenol and thymol are strongly incorporated into the lipid bilayers of phospholipid liposomes³⁴. To clarify whether CP is able to act as a complex in lipid bilayers as a solution, we investigated the interaction between CP and various phospholipid liposomes such as dipalmitoyl phosphatidylcholine (DPPC) liposomes, dimyristoyl phosphatidylcholine (DMPC) liposomes and DMPC/dilauroyl phosphatidylethanolamine (DLEA) liposomes as models of biological membranes, using differential scanning calorimetry (DSC) and ¹H-and ¹³C NMR spectroscopy.
MATERIALS AND METHODS

Materials
The following chemicals and reagents were obtained from the indicated companies: L-a-dipalmitoyl phosphatidylcholine (DPPC), L-a-dimyristoyl phosphatidylcholine (DMPC) and dilauroyl phosphatidylethanolamine (DLEA) (Sigma Chem. Co. USA); deuterium oxide (D$_2$O) and 3-(trimethylsilyl) propionic acid sodium salt-d$_4$ (TMSPA) (Merck Chem. Co., Darmstadt, Germany); and a-camphor and phenol (Wako Pure Chem Ind. Ltd., Osaka). Camphorated phenol (CP) was prepared by mixing camphor: phenol: ethanol=6:3:1 (w/w/w).

Preparation of camphorated phenol/DPPC liposomes and phenol/DPPC liposomes for NMR study.
The mixture of CP/DPPC (5.0 mg/73.4 mg) with ethanol was dissolved in chloroform, then evaporated in vacuo to dryness in an NMR sample tube. To the dried mixture, 0.5 mL D$_2$O was added. The mixture on the surface of the tube was shaken in a vortex mixer and then sonicated under nitrogen for 10 min by means of a bath-type sonicator. Two samples were measured by NMR.

The preparation of phenol/DPPC liposomes (1:2 molar ratio) was performed in the same manner as that described above but ethanol was not added.

Preparation of DPPC, DMPC and DMPC/DLEA (2:1 molar ratio) liposomes.
An appropriate amount of each of DPPC, DMPC and DMPC/DLEA was dissolved in chloroform. Each was subjected to vacuum and then dispersed by vortexing in a 70 m mol/L sodium phosphate buffer at pH 6.8. They were sonicated under a nitrogen atmosphere for 30 min. Approx. 5% w/w phospholipid in buffer solution and the appropriate concentration of camphorated phenol (CP) dissolved in ethanol were prepared as samples for DSC scanning. After mixing, the samples were allowed to equilibrate for 24 h at 5°C before they were scanned on DSC. The DSC measurements were carried out in the same manner as those previously described.

Nuclear magnetic resonance spectroscopy study
$^1$H and $^{13}$C-NMR spectra were measured with high resolution nuclear magnetic resonance spectrometers (270 MHz, JEOL GX-270 and 500 MHz, JEOL ALPHA500, Tokyo Japan) at room temperature, and all of the chemical shifts in D$_2$O were expressed as δ ppm down from the internal trimethylsilylpropionic acid sodium salt signal. Assignment of signals was performed by combination analyses of NOE differential technique, homospin decoupling technique, heterocosy and HMBC spectra.

The chemical structure of camphor for NMR assignment is shown in Fig. 1. The chemical shifts of camphor in 7% DMSO-d$_6$/D$_2$O were as follows: $^1$H: 0.87 (s, 3H, 7α-CH3), 0.94 (s, 3H, 1-CH3), 1.02 (s, 3H, 7b-CH3), 1.41 (dd+ddd, 2H, J=10.4, 9.2, 2.4 Hz, H-5end and 6end), 1.84 (ddd, 1H, J=10.4, 9.2 Hz, H-6exo), 1.98 (d, 1H, J=18.9 Hz, H-3end), 2.02 (m, 1H, J=10.4, 4.6, 3.0, 2.4 Hz, H-5exo), 2.21 (dd, 1H, J=4.6, 4.6 Hz, H-4), 2.49 (ddd, 1H, J=18.9, 4.6, 3.0 Hz, H-3exo).
**RESULTS AND DISCUSSION**

**DSC studies**

The DSC curves of DMPC liposomes are shown in Fig. 2A. As the concentration of CP increased, the DSC peak broadened. The phase transition temperature (Tm) and H/HHW of DMPC and DMPC/DLEA liposomes induced by CP are shown in Table 1. Changes in Tm and H/HHW appeared at dilution 1/250 (CP 12.8 mM). At dilution less than 1/100, changes in the phase transition properties (Tm, H/HHW) of DMPC/DLEA liposomes were larger than those at of DMPC liposomes. For a dilution of 1/10, the shifting of Tm to a lower temperature and H/HHW of DMPC liposomes were 3°C and 3, respectively, whereas those of DMPC/DLEA liposomes were 7°C and 2. This indicates that the effects of CP on the DLEA bilayer were larger than on the DMPC bilayer. Chepharines like DLEA are known to be present in greater quantities in bacterial membranes than in mammalian cells. Thus, the relatively large bactericidal effect of CP may be caused by its interactions with phosphatidyl ethanolamines in membrane lipids.

The DSC curves of DPPC liposomes induced by CP are shown in Fig. 2B. Three peaks appeared in the DSC charts at the dilution 1/100 (see Fig. 2 Be). At dilutions
Fig. 2 Thermogram of DMPC liposomes (A) and DPPC liposomes with increasing concentration of CP, camphorated phenol (B) and phenol (C). Aa: 1/250 dilution of CP (phase transition temperature determined by the center point of DSC peak: 23.0°C), Ab: 1/100 dilution, Ac: 1/20 dilution, Ad: 1/10 dilution (broad DSC peak); Be: 1/100 dilution (phase transition temperature determined by centric DSC peak: 1: 32.5°C; 2: 37.5°C and 3: 41.5°C), Bf: 1/30 (4: 25.0°C, 5: 30.0°C), Bg: 1/10 (6: 25.0°C, 7: 29.5°C), Bh: 1/2 dilution (no DSC peak); Ci: DPPC liposomes without additives, control (8: 31.5°C and 9: 41.0°C), Cj: 100 mM phenol and Ck: 500 mM phenol (pretransition abolished and main transition at 39.5°C). All scans were plotted at 5°C/min.

of 1/30 and 1/10, a broadened twin DSC peak (approx. 25.0 and 30.0°C) appeared (Fig. 2 Bf). Since the main transition temperature of DPPC liposomes without additives is 41.0°C and the pretransition temperature is 31.5°C (see Fig. 2 Ci), the phase transition temperature of Bf and Bg was shifted down by 6-11°C, together with broadening DSC peak. Also, the DSC peak at the dilution 1/2 (Fig. 2 Bh) was absent, demonstrating a strong interaction between CP and DPPC liposomes. It was concluded from this that CP impregnated strongly into the acyl chains of DPPC phospholipids, and that at high concentrations, mixed CP-DPPC micells and/or aggregation seemed to be formed because of the disappearance of DSC peak. The turning point of the phase transition properties of DMPC, DMPC/DLEA and DPPC liposomes induced by CP appeared to be at the dilution level of 1/100 (see Table 1).

On the other hand, the interaction between phenol and DPPC liposomes was smaller than that of CP. At 500 mM of phenol, an approximate dilution of 1/20, the
Table 1 Changes in phase transition temperature (Tm) and phase transition profile value (Height/Height Width of DSC peak, H/HHW) of dimyristoyl phosphatidylcholine liposomes, DMPC liposomes and DMPC/dilauroylphosphatidylethanolamine, DLEA (2:1 molar ratio) liposomes induced by camphorated phenol (CP) in a phosphate buffer solution with 12.5% ethanol at pH 6.8

<table>
<thead>
<tr>
<th>Dilution rate w/w</th>
<th>DMPC liposomes</th>
<th>DMPC/DLEA liposomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tm (°C)</td>
<td>H/HHW</td>
</tr>
<tr>
<td>Control</td>
<td>22.0</td>
<td>100</td>
</tr>
<tr>
<td>1/1000</td>
<td>22.0</td>
<td>98</td>
</tr>
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<td>16</td>
</tr>
<tr>
<td>1/10</td>
<td>19.0</td>
<td>3</td>
</tr>
</tbody>
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H/HHW of control: calculated as 100

DSC curves of DPPC liposomes showed a slightly broadened main transition and abolishment of pretransition (Fig. 2 Ck). DSC peaks of 100 mol/L phenol were almost comparable with the control (Fig. 2 Cj). It was concluded from this that the phase transition properties of phenol were lower than the camphorated counterpart, as evaluated from the molar concentration of added phenol.

NMR studies

1H and 13C-NMR spectra of camphorated phenol (CP) and CP/DPPC liposomes in D2O are shown in Fig. 3. The proton signals of camphor and phenol were markedly broadened in the liposomes but large signals of water soluble ethanol, which was used to disperse CP in the liposome system, appeared in the spectra due to their being unable to impregnate the liposomes (see Fig. 3C-F). The 1H signals of phenol were markedly broadened in the liposomes, indicating that phenol interacted strongly with the DPPC bilayers. Also, signals of choline methyl and acyl chains of DPPC were observed in the CP/DPPC liposomes, indicating the mobility of acyl chains of DPPC. This was quite different from results of phenol/DPPC liposomes, in that signals of phenol were clearly detected and no signals were shielded (see Fig. 3B and 3F).

The 13C-NMR spectra of CP/DPPC liposomes were similar to those of 1H. However, the 13C NMR signals of CP/DPPC liposomes were observed by 40,000 accumulation (Fig. 3F), indicating that signals of camphor, phenol, and choline methyl and acyl chains of DPPC were observed and that, in particular, those of phenol were broadened. It was concluded that phenol exhibits a lower interaction with DPPC bilayers than its camphorated counterpart. This indicates that phenol may incorporate more probably into biological membranes than original phenol by forming a CP complex.

We used a 1:1 complex of camphorated phenol in this experiment. In a camphor-phenol system, the complex between phenol and camphor can not be explained adequately only in terms of a 1:1 complex but formation of complex species containing two molecules of phenol is shown to be stable. Therefore, camphorated
phenol in our experiment is likely to have a more unstable camphor without forming a complex. Our results suggest that the biological activity of phenol increases on formation of a complex between camphor and phenol. This means that the addition of camphor to phenol increases the toxicity of phenol\(^2\) and also increases its pharmacological activity such as the sedative effect\(^3\). It has been previously reported that camphor is a relatively weak sensory irritant\(^4\). Our results demonstrated the stability of a CP complex. If a CP 1:2 complex was put to clinical use, it might have a greater pharmacological effect.

REFERENCES


4) Fujisawa, S. and Kadoma, Y.: \(^1\)H NMR spectroscopic studies of the interaction of


