¹H NMR Spectroscopic Studies of the Interaction of Thymol with Phospholipid Liposomes

Seiichiro FUJISAWA and Yoshinori KADOMA¹

School of Dentistry, Meikai University,
1-1 Keyakidai, Sakado, Saitama 350-0283, Japan

¹Institute for Medical and Dental Engineering,
Tokyo Medical and Dental University, 2-3-10,
Surugadai, Kanda, Chiyoda-ku, Tokyo 101-0062, Japan

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The interaction of thymol with dipalmitoylphosphatidylcholine (DPPC) liposomes was studied by ¹H-NMR spectroscopy. It was found that thymol was easily interpregnated into DPPC liposomes and then caused movements of acyl chains of DPPC liposomes when thymol was added directly to DPPC liposomes from the outside, resulting from the fact that proton signals of thymol shifted to a higher field and those of the phosphodiester head group and acyl chains of DPPC were observed.

No proton signals of thymol were not observed in the liposome system prepared by directly thymol acting to DPPC; Thymol/DPPC (1:1 molar ratio) liposomes and Thymol/DPPC/Cholesterol (2:1:1) liposomes, indicating the complete disruption of the phospholipid bilayer with formation of mixed thymol lipid micelles or other aggregates.

Key words: Interaction, Thymol, DPPC liposomes

INTRODUCTION

Thymol is applied to fissure caries¹⁾ and orthodontic devices²⁾ as a varnish to prevent caries. Also, it is used as a disinfectant for root canal therapies and for root canal sealers³⁾. Thymol (2-isopropyl-5-methylphenol) is a good bactericide¹⁻³⁾ but is known to have adverse effects^{4,5)}. The turbidity caused by the reaction of thymol with the protein and lipid constituents of serum was widely applied to a thymol turbidity test and the mechanisms were discussed⁶⁾. However, the mechanism of the reaction of thymol with lipid bilayers in biological membranes has not yet been clarified.

Recently, there has been an acceleration of interest in the use of artificial liposomes for modelling various biological membranes such as erythrocytes⁷⁾. Lipid soluble compounds such as drugs, monomers and detergents act predominantly on lipid bilayers in biological membranes due to impregnation into the bilayer resulting from a hydrophobic interaction⁸⁾. Partitioning of lipid soluble compounds into DPPC (dipalmitoylphosphatidylcholine) liposomes causes changes in the phase transition temperature (*T*_m), and enthalpy (ΔH) and *H*/*HHW* of DPPC liposomes, as a model of biological membranes⁸⁻¹¹⁾. We have previously investigated the interaction of phenolic compounds with DPPC liposomes using differential scanning calorimetry (DSC), and discussed the relationship between DSC phase transition properties and

cytotoxicities⁸⁾. However, DSC studies were not always useful in exploring the mechanism whereby thymol acts on liposomes⁹⁾. Nuclear magnetic resonance (NMR) spectroscopy is one of the most powerful methods for studying this interaction¹⁰⁾. Thus, in the present studies, we investigated the interaction between thymol and DPPC liposomes, Thymol/DPPC liposomes and Thymol/DPPC/Cholesterol liposomes using NMR spectroscopy, together with DSC studies. Further, biological activities of thymol were discussed in DSC and NMR spectroscopy of the various liposome system.

MATERIALS AND METHODS

Materials

The following chemicals and reagents were obtained from the indicated companies. L- α -dipalmitoyl phosphatidylcholine (DPPC), cholesterol (CS, Sigma Chemical Co., St. Louis, MO, USA.); 3-(trimethylsilyl) propionic acid sodium salt-d4 (TMSPA), deuterium oxide (D₂O, Merck Chemical Co. Darmstadt, Germany); thymol (>99%, 2-isopropyl-5-methylphenol) and eugenol (>98%, Tokyo Chemical Industry Co.), phenol (98+%, Wako Pure Chemical Industry Ltd).

Preparation of the liposomes

Eugenol/DPPC (1 : 2, molar ratio), Phenol/DPPC (1 : 2), Thymol/DPPC (1 : 2 and 1 : 1) and Thymol/DPPC/CS (1 : 2 : 1) liposomes were prepared in the manner previously described¹⁰⁾. Liposomes for NMR studies were prepared in D₂O and those for DSC studies in 10 mM sodium phosphate buffer solution at pH 7.0. DPPC was 10% w/v. DPPC liposomes with thymol were prepared by adding 10 mM thymol in CD₃OD (10 μ L) to DPPC liposomes in D₂O (0.5 mL).

DSC studies

Ten μ L of liposomes was pipetted into a DSC container. The samples were allowed to equilibrate for 24 h before they were scanned on DSC-Rigaku (Rigakudenkikogyo Co., Tokyo, Japan) at a heating rate of 5 $^{\circ}$ C/min with a range setting of 0.5mcal/sec. DSC measurements were carried out in the same manner as those previously described⁹⁾.

NMR spectroscopic studies

A JEOL JNM-GX 270 spectrometer (JEOL Tokyo Japan) was used to measure ¹H-NMR spectra at 270 MHz. The chemical shift of thymol and DPPC resonance are reported in ppm downfield from external TMSPA in D₂O.

Hemolysis studies.

The present experimental method is similar to that described in the previous paper⁹⁾. Fifty percent hemolysis of eugenol and thymol was determined in 1.5% w/v human erythrocytes in 70 mM sodium phosphate buffer solution at pH 7.0 after the incubation for 30 min at 37 $^{\circ}$ C.

RESULTS AND DISCUSSION

DSC Studies

Phase transition temperature (T_m) and Height/Half-Height Width (H/HHW) of the DSC peak of Phenol/DPPC (1:2), Eugenol/DPPC (1:2) and Thymol/DPPC (1:2) liposomes are shown in Table 1. T_m and H/HHW of the control were 41.0°C and 100, respectively. The T_m shift of eugenol to a lower temperature (19.5°C) was the largest, whereas that of phenol (1.0°C) was the smallest. Changes in T_m of thymol (13.5°C) were smaller than those of eugenol. Also, the H/HHW of phenol was 75.0, whereas those of eugenol and thymol were 6.3 and 9.0, respectively.

We have previously studied the DSC phase transition properties (T_m , ΔH , and H/HHW) of DPPC liposomes induced by phenolic compounds, indicating that those of phenolic compounds were dependent on their hydrophobicity, that the relationship between DSC phase transition properties and hydrophobicity was parabolic, and furthermore, that thymol had a larger hydrophobicity than eugenol while showing lower DSC phase transition properties⁹. We also concluded from the present experiment that the shift of T_m and decrease in H/HHW of thymol were smaller than those of eugenol. Further, we suggested that the DPPC liposomes induced by eugenol increased with the enthalpy (ΔS), as calculated from the formula: $\Delta S = \Delta H / T_m$. Therefore, eugenol enhances the increase in movements of acyl chains of lipid bilayers. On the other hand, the hemolytic activity and cytotoxicity (caused by thymol) were larger than those of eugenol, as shown in Table 1. Regrettably, from these DSC studies, we were not able to clarify the reasons why thymol showed a large hemolysis and large cytotoxicity compared to eugenol in view of a DSC measurement. Therefore, we studied the interaction of thymol with DPPC using ¹H-NMR spectroscopy.

¹H-NMR studies of the interaction between DPPC liposomes and thymol

The chemical structure of thymol and DPPC and their numbering system are shown in Fig. 1. Changes in ¹H-NMR chemical shifts (δ) of both DPPC liposomes and thymol induced by the interaction between DPPC liposomes and thymol at 52°C are

Table 1 Changes in phase transition temperature (T_m)* and transition profile value (H/HHW)* of Phenol/DPPC (1:2, molar ratio), Eugenol/DPPC (1:2) and Thymol/DPPC (1:2) liposomes in sodium phosphate buffer solution at pH 7.0, 50% hemolysis (H_{50} , mmol/L) and ID_{50} (mmol/L)

	Control	Phe/DPPC Liposomes	Eug/DPPC Liposomes	Thym/DPPC Liposomes	Phe	Eug	Thy
T_m (°C)	41.0±0.5	40.0±0.0	21.5±0.5	28.7±0.2			
H/HHW	100	75.0±0.1	6.3±1.7	9.0±0.0			
H_{50} **					—	14.5±1.2	5.8±0.5
ID_{50} #					6.1	1.1	0.49

*n=3, mean±SD** n=5; Phe: phenol; Eug: eugenol; Thy: thymol, #from reference 14.

shown in Table 2. ^1H -NMR spectra of thymol, DPPC liposomes and DPPC liposomes with thymol at 52°C are shown in Fig. 2.

As shown in Fig. 2 and in Table 2, the exogenous thymol molecules caused an increase in fluidity and mobility of the lipid bilayer of DPPC liposomes, resulting from the appearance of the six proton signals (I to VI) of DPPC that were observed when DPPC liposomes exist in a liquid phase. Also, such an NMR behavior was observed at 30°C , below the T_m (41.0°C) of DPPC liposomes (data not shown due to almost same δ as at 52°C). Results obtained from the chemical shift difference ($\Delta\delta$) between thymol and thymol with DPPC liposomes appear to demonstrate that thymol is located anisotropically in the DPPC bilayer. That is, the 4-H and 3-H moieties are located in a region opposite to the surface due to strong 3-H and 4-H shifting to a higher field. The moiety of 6-H near the OH group and 2-isopropyl moiety are also located close to the surface, resulting in less shifting of 6-H and $\text{CH}_3\text{-CH-CH}_3$. Bulky moiety of $\text{CH}_3\text{-CH-CH}_3$ of the isopropyl group appeared not to be able to penetrate into the liposomes. The NMR data suggested that thymol, in part, migrated directly into the lipid bilayer, with the driving force for incorporation coming from its hydro-

Fig.1 The chemical structure of thymol and L- α -dipalmitoylphosphatidylcholine (DPPC) and their numbering for NMR chemical shifts.

Fig. 2 ^1H -NMR spectra of thymol, DPPC liposomes and DPPC liposomes with thymol at 52°C . 10% w/v DPPC liposomes. 10 mM thymol. Alphabetical and roman figures see Fig. 1.
 a, 2- $(\text{CH}_3)_2$; b, 5- CH_3 ; c, $-\text{CH}<$; d, 6-H; e, 4-H and f, 3-H

Table 2 ^1H NMR chemical shifts (δ) of DPPC liposomes with thymol and chemical shift difference ($\Delta\delta$) in D_2O at 52°C

Assignment	Thymol A	DPPC liposomes with Thymol B	DPPC liposomes C	$\Delta\delta$ A-B
2-(CH_3) ₂	1.19 (6H d J=6.6 Hz)	1.17	—	0.02
—CH<	3.20 (1 Hm)	—	—	—
5- CH_3	2.26 (3 Hs)	2.26	—	0.00
6-H	6.77 (1 Hs)	6.69	—	0.08
4-H	6.84 (1 H d J=7.9 Hz)	6.53	—	0.31
3-H	7.23 (1 H d J=7.9 Hz)	6.91	—	0.32
I		0.93	0.92	
II		1.32	1.34	
III		3.19	3.28*(3.38**)	
IV		3.61	NS	
V		4.16	NS	
VI		5.25	NS	

The δ values are in ppm downfield from TMSPA used as an external reference in D_2O , Thymol (10 mM) in CD_3OD was added to DPPC liposomes (DPPC: 16.7 mM) in D_2O (0.5 mL), d doublet, m multiplet, s singlet, NS no signal. * inner, ** outer. $\Delta\delta$ chemical shift difference, signals (I – VI) due to DPPC

phobic interaction.

NMR studies of Thymol/DPPC liposomes and Thymol/DPPC/CS liposomes

^1H -NMR spectra of DPPC liposomes, Thymol/DPPC (1:1, molar ratio) liposomes, and Thymol/DPPC (1:2) liposomes at 30 and 54°C are shown in Fig. 3. Since the phase transition temperature of DPPC liposomes is 41.0°C , DPPC liposomes at 30°C exist in a solid phase, whereas those at 54°C are in a liquid phase. Only a sharp signal of III, $\text{N}^+(\text{CH}_3)_3$ was detected in DPPC liposomes at 30°C (Fig. 3.A), whereas broad signals of I; terminal methyl, II; acyl chains and III were detected at 52°C (Fig. 3.E). However, in the Thymol/DPPC liposomes, no signals of thymol were observed at either 30 or 52°C , indicating strong impregnation of thymol into the lipid bilayers. On the other hand, broadened signals (II, III and VI) in Thymol/DPPC (1:2) liposomes were detected at 30°C (Fig. 3.B), indicating that the fluidity of DPPC liposomes was enhanced at the 1:2 molar ratio but reduced at the 1:1 molar ratio, resulting from the fact that only a markedly broadened signal of III was observed at either 30 or 52°C (Fig. 3.C and G). It was considered from this that the complete disruption of the phospholipid bilayer with formation of mixed thymol lipid micelles or other aggregates occurred in Thymol/DPPC (1:1) liposomes, which is probably characterized by enthalpy, ΔH and/or H/HHW reduced to an undetectable level. It was clear that an aggregation of Thymol/DPPC (1:1) liposomes was observed in NMR sample tubes after the measurement in this study.

In Thmol/DPPC/CS liposomes, no proton signals due to thymol and DPPC were observed at either 30 or 52°C (Fig. 3.D and H), indicating the strong interaction of

Fig. 3 ^1H -NMR spectra of DPPC liposomes (A and E), Thymol/DPPC (1:2) liposomes (B and F), Thymol/DPPC (1:1) Liposomes (C and G) and Thymol/DPPC/CS (1:2:1) Liposomes (D and H). A, B, C and D; at 30°C. E, F, G and H; at 52°C.

thymol and DPPC. This is comparable with Thymol/DPPC (1 : 1) liposomes. It is known that cholesterol plays a very important role in biological membranes characterized by the so-called dual effect (stabilization and labilization) of membrane fluidity¹²⁾. Cholesterol shows a stabilizing effect, which is characterized by a decrease in membrane permeability, on lipid bilayers of biological membranes. We have previously reported that NMR chemical shifts of triethylene glycol dimethacrylate (TEGDMA) are distinguished in TEGDMA/DPPC/CS liposomes, whereas they are broadened in TEGDMA/DPPC liposomes¹³⁾ and that those of Bis-GMA are markedly broadened in both Bis-GMA/DPPC liposomes and Bis-GMA/DPPC/CS liposomes¹³⁾. It seemed that the NMR behavior of thymol was comparable with Bis-GMA. Also, we found that Tm of DPPC liposomes induced by TEGDMA shifted to a markedly lower temperature than that of Bis-GMA, suggesting that if anything, the interaction of TEGDMA was likely to be in the style of eugenol.

Furthermore, we have previously reported that in ¹H-NMR spectra of both Eugenol/CS/DPPC (1 : 1 : 2, molar ratio) and Eugenol/DPPC (1 : 1) liposomes, all proton signals due to eugenol and proton signals (I, II, III, V and VI) of DPPC at 37°C were observed¹⁰⁾, and were markedly broadened. Comparing the present our results to the data described above, it was clear that the interaction of eugenol was less than thymol. Abolishment of the proton signal of N⁺(CH₃)₃ of DPPC induced by thymol suggested that the strong interaction of thymol with DPPC arose from hydrogen bonding involving the OH group of thymol and the phosphodiester head group of DPPC. This was not the case with eugenol. On the other hand, we confirmed in this study (Table 1) that the Tm of DPPC liposomes induced by eugenol shifted to a lower temperature than that of thymol. This shifting of the Tm to a lower temperature is known to enhance the movement of the acyl chain of DPPC or the fluidity of liposome membranes. Thus, eugenol was considered to enhance the movement of the acyl chains of DPPC, and thymol to enhance a hydrophobic interaction with liposomes due to its high hydrophobicity. An investigation of the liposome model-system using an NMR spectroscopy appeared to indicate the rationale for chemically determining the degree and nature of interaction of thymol with biological systems. NMR studies supported the DSC studies. The predictors are considered to be quite accurate.

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