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HEMOLYTIC ACTIVITY OF A DENTAL ADHESIVE MONOMER AND ITS INTERACTION WITH DIPALMITOYL PHOSPHATIDYLCHOLINE LIPOSOMES NMR and DSC measurements

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Abstract

To clarify the mechanism of the hemolytic activity of 4-META* (4-methacryloyloxyethyl trimellitate anhydride), an evaluation of the interaction of 4-META and its related compounds (4-MET; phthalic anhydride, PAN; and phthalic acid, PA) with DPPC liposomes using a DSC and NMR spectroscopy is presented.

Changes in NMR chemical shifts, phase transition temperatures and broadening of the main transition induced by 4-META and PAN were greater than those induced by 4-MET and PA. The order of decreasing interactions is as follows: 4-META>PAN>4-MET>PA.

Ionized compounds such as 4-MET and PA did not interact with erythrocyte membranes.

Keywords: DSC, heat capacities, hemolytic activity, interaction, 4-META, NMR, phospholipid liposomes

Introduction

Recently, the use of artificial liposomes as a model for various biological membranes such as erythrocytes and lisosomes has become of interest, because the liposomes have proved to be useful in exploring the mechanism whereby drugs, detergents, and toxins act on the phospholipid bilayer [1]. We previously employed phospholipid liposomes as a model system in order to study the interaction of various lipid soluble compounds used in dentistry, with biological membranes [2]. In particular, we investigated the molecular mechanism of the interaction of methacrylates with liposomes by the application of DSC, and monitored the transition properties (phase transition temperature, T_m; enthalpy, ΔH; and Height·Half-Height Width^{-1}, H·HHW^{-1} of the DSC peak) of liposomes induced by methacrylates in a biological system, indicating that changes in T_m, ΔH and H·HHW^{-1} are correlated with the biological activities of these compounds [3].

* 4-methacryloyloxyethoxycarbonylphthalic anhydride

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Methacrylates with carboxyl, phosphate and other functional groups are widely used in dentistry as bonding agents, since they are effective in promoting adsorption and adhesion to cationic tooth surfaces of enamel and dentin composed of hydroxyapatite [4]. 4-META, having both polymerizable methacrylate and a functionable phthalic anhydride group, is currently used as a bonding agent of the 4-META-MMA-TBB resin system [5, 6].

There have been numerous studies of cytotoxicities and pulp responses. Since high concentrations of 4-META are not obtained in water due to its high hydrophobicity, some reports have shown an adverse effect of 4-META on pulp, and cytotoxicity when solubilized at high concentration in biological systems by methyl methacrylate and dimethyl sulfoxide [7–10]. Despite numerous studies on 4-META, it remains unclear how 4-META impregnates biological membranes, because the biological activity depends on the concentration of this compounds, its pH or pKa.

In the present study, we examined the effects of the dental adhesive monomer 4-META and its related compounds (4-MET, hydrolysis product of the anhydride moiety of 4-META; PA; and PAN) on human erythrocytes, and further investigated the differences in NMR spectra and DSC phase transition properties of the DPPC:4-META and DPPC:4-MET liposome system, DMPC liposomes with 4-META, DMPC:DLEA liposomes with 4-META, DPPC liposomes with PA, and DPPC liposomes with PAN, in order to monitor the impregnation mechanism of 4-META in biological system such as pulp, gingiva and periodontal tissues.

Experiments

L-α-dimyristoylphosphatidylcholine(DMPC), *L*-α-dipalmitoylphosphatidylcholine (DPPC), dilauroylphosphatidylethanoleamine (DLEA), phthalic acid (PA) and phthalic anhydride (PAN) were used without further purification. Methyl methacrylate (MMA) was used after purification. 4-META and 4-MET(4-methacryloyloxyetoxycarbonylphthalic acid) were prepared according to the method reported previously [11, 12].

^{1}H-NMR of 4-META yielded (The δ values are in ppm downfield from TMS used as external reference in $CDCL_3$): δ=1.95(3H,m)2-CH_3, δ=4.54(2H,m)H4, δ=4.68(2H,m)H5, δ=5.61(1H,m)H1a, δ=6.15(1H,m)H1b, δ=8.11(1H double d, J =0.7, 7.9 Hz)H5′, δ=8.56(1H, double d, J=1.3,7.9 Hz)H6′ and δ=8.64(1H double d, J=0.7, 1.3 Hz)H2′; *m.p.* 96.0–96.5°C. ^{1}H-NMR of 4-MET in $CDCL_3$ yielded: δ=1.96(3H,m)2-CH_3, δ=4.54(2H,m)H4, δ=4.63 (2H,m)H5, δ= 5.61(1H,m)H1a, δ=6.16(1H,m)H1b, δ=7.90(1H,d, J=7.9 Hz)H5′, δ=8.29(1H, double d, J=1.6, 7.9 Hz)H6′ and δ=8.54(1H d, J=1.6 Hz)H2′; *m.p.* 119.8–120.9°C.

Hemolysis studies. The present experimental method used is similar to that described in a previous paper [13]. The percentage of hemolysis (H%) for each

sample was calculated using the following equation. $H\%=(A_{sample}-A_o)(A_{100}-A_0)^{-1}$ X100, where A_{100} and A_0 are the absorbances (540 nm) of 100% and of non-hemolyzed cells, respectively.

Preparation of liposomes. An appropriate amount of DPPC was dissolved in chloroform and dried under vacuum. PA or PAN was added to the dried DPPC film and dispersed in D_2O or a D_2O buffer solution by vortexing on a Vortex shaker at 45°C for 2–3 min and then sonicated under a nitrogen atmosphere for 3–5 min at 45°C.

The preparations of DPPC:4-META, DPPC:4-MET, DMPC and DMPC:DLEA liposomes were similar to those described above, provided that mixtures of DPPC:4-META, DPPC:4-MET or DPPC:DLEA have been dissolved in chloroform.

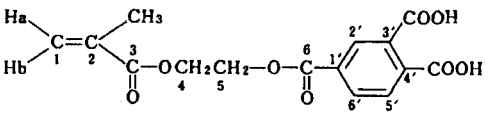
D_2O buffer solution was prepared as follows. A solution of 0.1*M* KD_2PO_4–D_2O was adjusted to pH 2.2, 7.0 and 7.4 by addition of 40%NAOD–D_2O solution.

DSC studies. An aliquot of sample (20 μl) was transferred from a NMR tube into a DSC sample container. The sample was scanned in a sealed calorimetric container on a DSC-Rigaku calorimeter (Rigaku Denki Co. Ltd., Tokyo, Japan) at a heating rate of 5°C min^{-1} with a range setting of 0.5 mcal s^{-1}. The instrument was calibrated with indium as standard. ΔH was calculated from the area under the curve determined by cutting out the DSC-curves and weighing the chart paper, in a manner similar to that previously reported [3]. In the case of DMPC:DLEA liposomes (2:1 molar ratio) with 4-META, and DMPC liposomes with 4-META, first 4-META was placed directly into the bottom of DSC sample containers and then the liposomes were added to it. The sample was then allowed to equilibrate for 14 h at 5°C and then finally shaken again for 1 min by hand at 25°C before scanning.

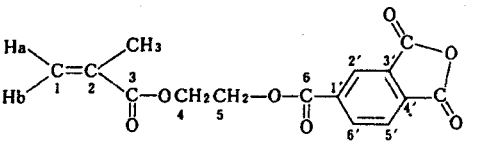
NMR studies. ^{1}H-NMR spectra were measured at 30, 37 and 52°C under JEOL JNM-GX270 spectrometer(JEOL, Tokyo, Japan) at 270 MHz. The chemical shifts (δ_H) of PA, PAN, 4-META and 4-MET and DPPC are reported in ppm downfield from the external standard, TMSPA in a manner similar to that previously reported [14]. The accuracy of δ_H was 0.01 ppm in this experiment.

Results and discussion

Structures of 4-META, 4-MET and their numbering system are shown in Fig. 1. The percentages of hemolysis caused by 4-META, 4-MET, PA, PAN, 4-META+MMA and 4-MET +MMA are shown in Table 1. The hemolysis percentages for 4-META, 4-META+MMA and PAN were each approximately 20%, whereas those for 4-MET, 4-MET+MMA and PA were less than 4%. 4-META and PAN showed greater hemolytic activity than 4-MET and PA. In contrast, for both 4-META and PAN, layer of the suspension on non-coagulated erythrocytes was observed close to the bottom of test tubes where coagulated erythrocytes were present, after centrifuge separation. The non-coagulated erythrocytes were



4-MET



4-META

Fig. 1 Chemical structure of 4-META 4-MET, phthalic anhydride (PAN) and *o*-phthalic acid (PA)

extracted into test tubes and a chloroform-methanol solution (2:1 v/v) was added. After centrifugation (1000×*g* for 5 min), an absorbance at 254 nm caused by the benzene ring was observed in this solution. This indicates that 4-META or PAN are likely to impregnate the lipids in erythrocyte membranes.

Presumably, this impregnation effect of 4-META on erythrocyte membranes is associated with the structure of either proteins or phospholipids containing cholesterol, or both [14]. We have shown that 4-META interacts with serum albumin at low concentration [15]. However, there have been no detailed reports showing that 4-META will impregnate the phospholipid bilayer in biological membranes. Thus, we studied the interaction of 4-META and its related compounds with various liposome systems.

DSC is a relatively inexpensive thermoanalytical technique that allows the study of the thermotropic properties of membranes in the presence of biologically active molecules. Changes in T_m, and H·HHW^{-1} of DPPC liposomes with

Table 1 Effects of 4-META and its related compounds (4-MET, phthalic acid and phthalic anhydride) on human erythrocytes

Compound	Concentration/ mmol l^{-1}	Hemolysis percentage/% mean (SD)
4-META	2.5	17.1(5.9)
4-MET	2.5	1.9(1.1)
o-Phthalic acid	5	3.7(2.8)
Phthalic anhydride	5	18.9(1.3)
4-META plus 125 mM MMA	4	21.3(5.6)
4-MET plus 125 mM MMA	4	2.0(0.5)

Reaction time 40 min at 37°C, 4% human erthrocytes in a phosphate buffer solution at pH 7.4, MMA: methyl methacrylate, SD: standard deviation of three sample

Table 2 Changes in the phase transition temperature (T_m) and Height·Half-Height Width^{-1}(H·HHW^{-1}) of the DSC peak of dipalmitoylphosphatidylcholine (DPPC) liposomes with *o*-phthalic acid(PA), DPPC liposomes with phthalic anhydride (PAN), DPPC:4-META (5:1, 3:1 molar ratio) liposomes and DPPC:4-MET liposomes (3:1 molar ratio) in D_2O and a D_2O buffer solution

System of liposomes*	DPPC:Compound molar ratio	T_m/°C	H·HHW^{-1}
DPPC(Control)		41	100
DPPC plus PA	1:1	40.5	65
DPPC plus PA	1:1	40.5	70
DPPC plus PA**	1:1	37.5	31
DPPC plus PA**	1:1	37	32
DPPC plus PAN	1:1	33	106
DPPC plus PAN	1:1	33.5	115
DPPC:4-META	5:1	39.5	74
DPPC:4-META	3:1	32.5	9
DPPC:4-META	3:1	33	4
DPPC:4-MET#	3:1	39	80
DPPC:4-MET##	3:1	28	two peaks

* pH 7.4, ** pH 2.2, # pH 7.0, ## pH 2.5

PA and PAN, DPPC:4-META liposomes and DPPC:4-MET liposomes are shown in Table 2. In the case of PA, T_m and H·HHW^{-1} of the main transition at pH 7.4 were 40.5°C and 65–70, respectively. Those at pH 2.2 were 35–37 °C and 22–31, respectively. PA at pH 7.4 had a small pretransition at 33.5°C that appears in the control, whereas its pretransition at pH 2.2 was abolished.

Clearly, PA caused broadening of the DSC curve and shifted T_m to a lower temperature through a decrease in pH from 7.4 to 2.2. The interaction of PA with DPPC liposomes at pH 2.2 is likely to result from the hydrogen bonding between non-ionized carboxyl molecules of PA and the phosphodiester head group of DPPC. PAN yielded a T_m of 33–33.5°C and H·HHW^{-1} of 106–115. PAN shifted T_m by 7–7.5°C to a lower temperature as compared with that of PA at pH 7.4, due to its hydrophobicity. The H·HHW^{-1} value for PAN was above 100, indicating that PAN caused a sharp DSC transition.

The DSC curves in Fig. 2 show the results obtained for samples of the DPPC:4-META and the DPPC:4-MET liposome system. T_m and H·HHW^{-1} for the DPPC:4-META (5:1 molar ratio) system were 39.5°C and 74, respectively. Those for the DPPC:4-META(3:1)liposome system were 32.5–33°C and 4–9, respectively. Changes in T_m and H·HHW^{-1} were found to depend on the concentration of 4-META to DPPC. A shift of T_m to a lower temperature and broadening of

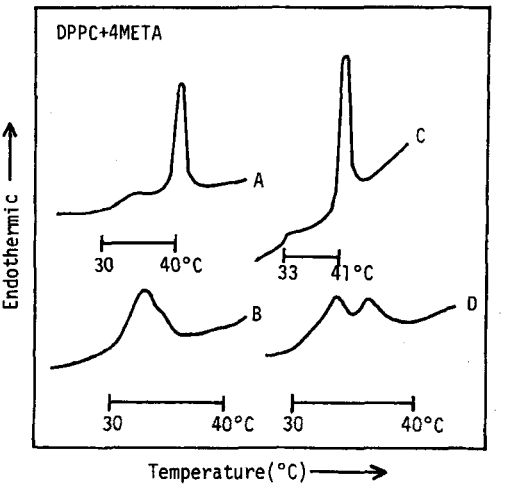


Fig. 2 DSC curves for various DPPC liposome systems. A: DPPC:4-META(5:1 molar ratio) liposome system; B: DPPC:4-META(3:1 molar ratio) liposome system; C:DPPC liposomes(control); D:DPPC:4-MET(3:1 molar ratio) liposome system at pH 2.5

the main transition showed that the interaction of 4-META with the DPPC bilayer increased as the concentration increased. The large interaction of 4-META at high concentration is considered to be due to its high hydrophobicity. In the DPPC:4-MET (3:1) liposomes system, changes in phase transition profiles depend on the pH. Its T_m and H·HHW^{-1} at pH 7.0 in a D_2O buffer solution were 39°C and 80, respectively. Namely, 4-MET produced a slight broadening of the main transition and a shift of T_m to a lower temperature by 2°C, indicating that the interaction of 4-MET with DPPC bilayer was as small as that of PA at pH 7.4. The most striking trend was characterized by two transitions (peak A and peak B) at pH 2.5. T_m of peak A became intensified at 28°C. This was associated with a large shift to a lower temperature. It showed the strong interaction of 4-MET with the DPPC bilayer.

The appearance of peak A is interpreted as being due to the formation of 4-META:DPPC complex [16]. T_c of peak A was 31°C and that of peak B was 34°C, where T_c is the temperature at which excess specific heat reaches maximum. T_c is scan rate-dependent, therefore, T_m was exhibited in our experiment as described in previous reports [3].

The ΔH of DPPC liposomes induced by PAN and 4-META was almost the same as that of the original DPPC liposomes (36.4 kJ mol^{-1}) in the present experiments. However, their T_m shifted to a low temperature as compared to that of the original liposomes (41°C). This indicates that the entropy (ΔS) increased in the system, as calculated from the formula: $\Delta S = \Delta H T_m^{-1}$. Therefore, the effect of PAN and 4-META on DPPC liposomes may be associated with changes in the

Table 3 Changes in the phase transition temperature (T_m) and Height·Half-Height Width^{-1} (H·HHW^{-1}) of the DSC peak of the dimyristoylphosphatidylcholine (DMPC)-dilauroylphosphatidylethanolamine (DLEA)(2:1 molar ratio) liposomes with 4-META and DMPC liposomes with 4-META in sodium phosphate buffer solution at pH 6.8

System of liposomes	Molar ratio DMPC:DLEA:4-META	T_m/°C	H·HHW^{-1}
DMPC:DLEA	2:1:0	22.0	100
DMPC:DLEA	2:1:0.5	22.0	89
DMPC:DLEA	2:1:1	21.0	64
DMPC:DLEA	2:1:2	no DSC peak	
DMPC	2:0:0	22.5	100
DMPC	2:0:2	21.5	83

H·HHW^{-1} is calculated as control is to 100. DMPC, 70 mmol

membrane structure (an increase in the fluidity of the phospholipid bilayer). It has been previously reported that the lowering of T_m produces an increase in fluidity, disorder and mobility of the lipid bilayer and that a decrease in ΔH indicates an interaction of compounds with acyl chains of lipid bilayer in biological membranes [17]. 4-META that is highly hemolytic showed marked changes in transition properties in our experiments.

The results obtained for samples of DMPC liposomes with 4-META and DMPC:DLEA liposomes with 4-META are shown in Table 3.

4-META shifted T_m to a lower temperature by 1°C and produced a broadening of the main transition of DMPC:DLEA liposomes. The DSC peak of DMPC:DLEA liposomes with 4-META at molar ratio of DMPC:DLEA:4-META=2:1:2 did not appear in the curve in the temperature range of 5–45°C, suggesting that the complete disruption of the phospholipid bilayer with formation of mixed 4-META lipid micelles or other aggregates occurred and that ΔH was reduced to zero. On the other hand, T_m and H·HHW^{-1} of DMPC liposomes with 4-META at a corresponding molar ratio of DMPC:4-META =2:2 were 21.5°C and 83, respectively. This indicates that changes in the transition properties of original DMPC liposomes caused by 4-META were markedly smaller than that of DMPC:DLEA liposomes, demonstrating that the action of 4-META on the DLEA bilayer was larger than that on the DMPC bilayer, suggesting an interaction between 4-META and the NH_2 group of DLEA. 4-META, which is highly hemolytic, may migrate directly across the lipid bilayer with the driving force for incorporation derived from hydrophobic interaction and may, in part, interact with the NH_2 or OH groups of proteins and lipids in erythrocyte membranes.

The ^{1}H-NMR spectra of the DPPC:4-MET liposome system are shown in Fig. 3. ^{1}H signals, chemical shifts (δ_H), for H1a, H1b, H4, H5 and H6′) were not detected and those for 2-CH_3 decreased markedly and broadened in the liposomes

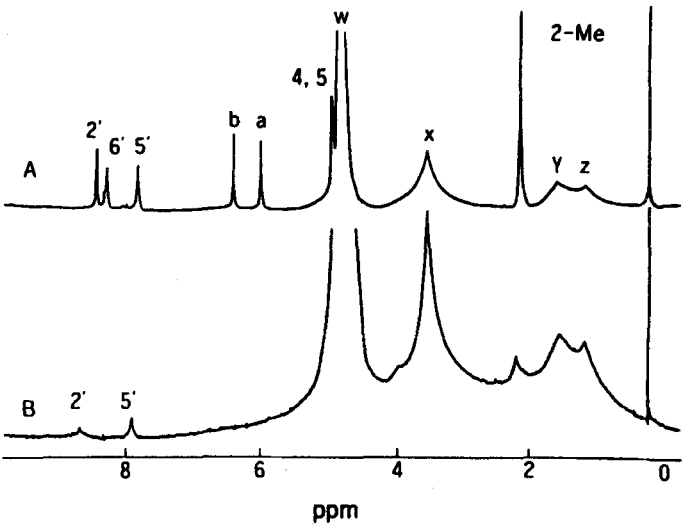


Fig. 3 ^{1}H-NMR spectra of DPPC:4-MET (3:1 molar ratio) liposomes. A: in a D_2O buffer solution at pH 7.0; 2′, 6′, 5′,b,a,4,5 and 2-ME: signals of 4-MET; w:DHO, X:choline $N^+(CH_3)_3$ of DPPC, Y:acyl chains$(CH_2)_{14}$ of DPPC, Z:terminal CH_3 of DPPC, B: in D_2O at pH 2.5

at pH 2.5. Also, the proton signals of H2′ and H5′ were broadened as shown in Fig. 3. The chemical shift difference ($\Delta\delta_H$) values, $\Delta\delta_H=\delta_H$ of 4-MET–δ_H of the DPPC:4-MET) liposome system, for H2′, H5′ and 2-CH_3 were 0.11, –0.10 and 0.11, respectively. The δ_H of H5′ was strongly shielded because of shifting to a higher field. The profile of the NMR spectra of the DPPC:4-META liposome system appeared to be similar to that of the DPPC:4-MET liposome system (Data not shown). The time-course for the hydrolysis of 4-META to 4-MET was determined by comparing an integral value of the isolated H5′ of 4-META with that of H6′ of 4-MET, resulting in the rapid hydrolysis rate of 4-META in D_2O solution. The chemical-shift measurements indicate that 4-MET is located anisotropically in the DPPC liposomes at pH 2.5. That is, the H2′ and COOH moiety are located in a region close to the surface, whereas the methacryloyloxy group, and H6′ of the ring penetrate deeply to some distance between the acyl chains of DPPC. In contrast, all signals due to 4-MET in the DPPC:4-MET (3:1) liposome system appeared clearly in the NMR spectrum at pH 7.4 without shielding, indicating that 4-MET has a small interaction with DPPC bilayer characterized by an adsorption or weak insertion into the lipid bilayer. In our experiment, 4-MET showed small hemolytic activity in a buffer solution at pH 7.4. However, it showed strong interaction with DPPC bilayer at low pH, such a behavior was the same as that of PA. This is due to the hydrophobic interaction caused by formation of hydrogen bond between carboxyl groups and phosphodiester head groups

of DPPC. Thus, when a large amount of 4-MET is applied directly to a dentin surface with vital pulp, this compound may cause damage to the pulp.

It should be mentioned that the interaction of 4-META with biological membranes is a more complicated process than the model system of liposomes, since the membranes contain proteins, and their lipid might be organized in nonrandomized fusion [18], however there were many similarities between the results obtained for hemolysis and those for the liposome system in our experiments. Our studies indicate that the liposomes are most useful in exploring the mechanism of biological action using lipid soluble small compounds, and that they can be used in a model system for evaluation of these compounds using DSC and NMR.

Conclusion

From our findings and the above discussion several conclusions can be drawn.

1. 4-META and PAN showed stronger hemolytic activity than 4-MET and PA, indicating that ionized compounds do not penetrate erythrocyte membranes.

2. In DSC studies, 4-META and PAN with DPPC bilayer produced a large shift of T_m to a lower temperature and a broadening of the main transition ($H \cdot HHW^{-1}$).

High concentration of 4-META with DMPC:DLEA liposomes produced the abolishing of the main transition due to the strong interaction.

3. In NMR studies, the NMR spectra of the DPPC:4-META system were similar to those of the DPPC:4-MET liposome system, resulting from the hydrolysis of 4-META in D_2O. The δ_H of 4-MET in DPPC bilayer was affected by the pH in the medium. ^{1}H-signals of 4-MET in the DPPC:4-MET liposome system markedly broadened at pH 2.5, resulting from an increase in the hydrophobicity of 4-MET due to the formation of a hydrogen bond.

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