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pH–Dependent Complexation of Methacryloyloxydecyl Dihydrogen Phosphate (MDP) with Dipalmitoylphosphatidylcholine (DPPC) Liposomes: DSC and NMR Measurements

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pH–Dependent Complexation of Methacryloyloxydecyl Dihydrogen Phosphate (MDP) with Dipalmitoylphosphatidylcholine (DPPC) Liposomes: DSC and NMR Measurements

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Abstract

Motivation. Chelatable methacrylates with phosphate groups, such as MDP, have been used as tooth–bonding agents in dentistry. MDP has shown acceptable pulp response and clinical performance. However, the effect of MDP on biological membranes remains unknown. Liposomes have been employed in model systems at the membrane level to study the interaction of lipid–soluble drugs with biological membranes, and DSC and NMR spectroscopy have been used as powerful, yet relatively rapid and inexpensive, techniques for these studies. In the present study, we investigated the DSC phase–transition properties of DPPC liposomes induced by MDP and the changes in NMR chemical shifts of MDP associated with DPPC liposomes at various pH values.

Method. DPPC/MDP (2:1 molar ratio) liposomes were prepared. Phase transition temperature (Tm) and enthalpy of DPPC/MDP liposomes were determined by DSC. ^{1}H–NMR chemical shifts and coupling constants of DPPC/MDP liposomes were investigated in D_2O at pH 2.4 and pH 7.0 at 25, 35 and 50 °C. HOMO, LUMO, partial charges, dipole moments, hydrophobicities (octanol–water partition coefficient, log P) for COSMO (water) or non–COSMO (vacuum) were calculated by the PM3 semiempirical method.

Results. The DSC main peak for DPPC/MDP at pH 2.4 was markedly broadened with a considerably decreased enthalpy, whereas a peak with a shoulder at 35 °C appeared at pH 7.0. At pH 2.4, ^{1}H–NMR signals of MDP associated with DPPC liposomes were not found because of shielding, but those of the phosphodiester groups of DPPC were clearly apparent above or below the Tm. In contrast, at pH 7.0, signals of MDP together with those of DPPC appeared at 35 °C, above the Tm. The appearance of signals of MDP associated with DPPC liposomes was dependent on pH and Tm.

Conclusions. The DSC and NMR measurements suggest that DPPC–MDP complexation under acidic conditions probably occurs by the formation of hydrogen bonds between un–ionized dihydroxy phosphate groups of MDP and the phosphodiester functions of the DPPC surface. The log P for un–ionized MDP was an order of magnitude greater than that for ionized one, suggesting that the strong DPPC–MDP complexation possibly arises from hydrophobic interactions between acyl chains of DPPC and un–ionized MDP. The PM3 calculation for HOMO and/or LUMO energy in COSMO suggested that the HOMO for DPPC probably interacts preferentially

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with the LUMO for un–ionized MDP. Charges–dipole, dipole–dipole interactions are probably of restricted importance for a minor driving force for DPPC–MDP interactions. The PM3 calculations contributed to interpretation of the NMR aspects of the interaction between MDP and liposomes.

Keywords. DPPC liposomes; tooth adhesive material; interaction; NMR chemical shifts; DSC; PM3.

Abbreviations and notations

DPPC, L–α–dipalmitoylphosphatidylcholine	Q_{max}, maximum partial charge in molecule
DSC, differential scanning calorimetry	Q_{min}, minimum partial charge in molecule
D_2O, deuterium oxide	NMR, nuclear magnetic resonance
Log P, octanol-water partition coefficient	HOMO, the highest occupied molecular orbital
ε_{max}, the highest electron density	LUMO, the lowest unoccupied molecular orbital

1 INTRODUCTION

MDP has been widely used as a tooth bonding agent in dental adhesive resin restorative systems [1,2]. The functional properties of this compound include its ability to interact with calcium in the hydroxyapatite around tooth collagen, together with a methacryloyl function for intermolecular polymerization. However, the biological activity of this compound is incompletely understood. In studies of the mechanisms of biological activity, liposomes formed from natural or synthetic phospholipids are widely used as models for biological membranes [3,4]. We have previously used DSC and NMR spectroscopy to phase–transition properties and NMR chemical shifts are valid indicators of interactions between biological membranes and dental materials such as methacrylates. Specifically, we have previously investigated the interaction of MDP with DPPC liposomes as assessed by DSC and NMR spectroscopy, but the mechanism of the interaction remained unknown [6]. The application of computational chemistry to dental materials and bioactive agents is an exciting and rapidly expanding area [7–10]. We recently used the PM3 semiempirical calculation method to interpret the mechanism of interaction between methacrylates and liposomes, suggesting that descriptors such as LUMO and HOMO energies and partial charges are useful tools for the interpretation of experimental results [11]. In the light of these recent developments, we have further investigated by ^{1}H–NMR spectroscopy the interaction between MDP and DPPC liposomes, relating the descriptors calculated by PM3 methods to the ^{1}H NMR chemical shifts of MDP associated with DPPC liposomes at various pH values.

2 MATERIALS AND METHODS

2.1 Chemicals

The following chemicals and reagents were obtained from the indicated companies: DPPC, Sigma Chemical Co., St. Louis, Mo., USA; D_2O, NaOD, KD_2PO_4, $CDCl_3$, and 3–(trimethylsilyl)propionic acid sodium salt–d_4 (TMSPA), Merck Chemical Co., Darmstadt, Germany.

2.1.1 Synthesis of MDP

10–Hydroxydecyl methacrylate was prepared from 1,10–decanediol and methacryloyl chloride. The product was isolated by column chromatography on silica gel. The purified alcohol and triethylamine were slowly added to phosphorous oxychloride. After the termination of the reaction, the mixture was extracted with ether and the solvent was evaporated. The resulting viscous MDP was washed several times with *n*–hexane and dried (yield about 74%). The purity of the MDP was verified by NMR and infrared spectroscopy [12]. The chemical structure of MDP, with significant atoms or groups identified by letters, is shown in Figure 1A. ^{1}H–NMR in D_2O buffer at pH 7.0 (δ, ppm): 1.31 (f; 12H, br s), 1.64 (e and g; 4H, m), 1.92 (c; 3H, s), 3.84 (h; 2H, double t, J = 6.60, 6.60 Hz), 4.17 (d; 2H, t, J = 6.6 Hz), 5.67 (a; 1H, br s), 6.10 (b; 1H, br s). ^{1}H–NMR in $CDCl_3$ (δ, ppm): 1.29 (12H, br s), 1.67 (4H, m), 1.94 (3H, m), 4.03 (2H, double t, J = 6.59, 6.60 Hz), 4.14 (2H, t, J = 6.60 Hz), 5.55 (1H, m), 6.10 (1H, m), 8.61 (OH).

2.1.2 Preparation of multilamellar liposomes

An appropriate amount of DPPC was dissolved in chloroform and dried under vacuum. MDP was added to the dried lipid film, and the mixture was dispersed in D_2O by vortex–mixing on a Vortex shaker at 45 °C for 2–3 min followed by sonication under a nitrogen atmosphere for 10 min at 45 °C. The molar ratio of DPPC to MDP was 0.5. Suspensions containing approximately 10% DPPC liposomes were prepared.

2.1.3 NMR spectroscopy

^{1}H–NMR spectra were measured at 25, 30, 35 and 50°C with JEOL (Tokyo, Japan) JNM–GX270 or ALPHA 500 instruments at resolutions of 0.01 ppm and 0.0013 ppm, respectively.

2.1.4 pH measurement

pH was measured with a COM–8 pH meter (Denki Kagaku Keiki Co., Ltd, Tokyo, Japan). A solution of 0.1M KD_2PO_4/D_2O was adjusted to pH 7.0.

2.1.5 DSC studies

Samples for NMR measurements were used. Each 10–microL sample was sealed in a DSC specimen container. The samples were scanned in a sealed container on a DSC–Rigaku calorimeter (Rigaku Denki Co., Ltd, Tokyo, Japan) operating at a heating rate of 5 °C /min with a range setting of 0.5 mcal/s. Measurement of enthalpy was performed as described previously [5].

2.1.6 PM3 calculation

Theoretical calculations were carried out by the restricted Hartree–Fock level (RHF) PM3 semiempirical method, as implemented in the MOPAC program on a Tektronix Cache Workstation (version 4.9). Solvent effects for MDP were calculated using the COSMO (Conductor–like

Screening Model) methodology. For the dielectric constant of the medium, we assumed water (e = 78.4) as solvent. HOMO, LUMO, and partial charges for COSMO and non–COSMO (vacuum) were calculated by the PM3 semiempirical method.

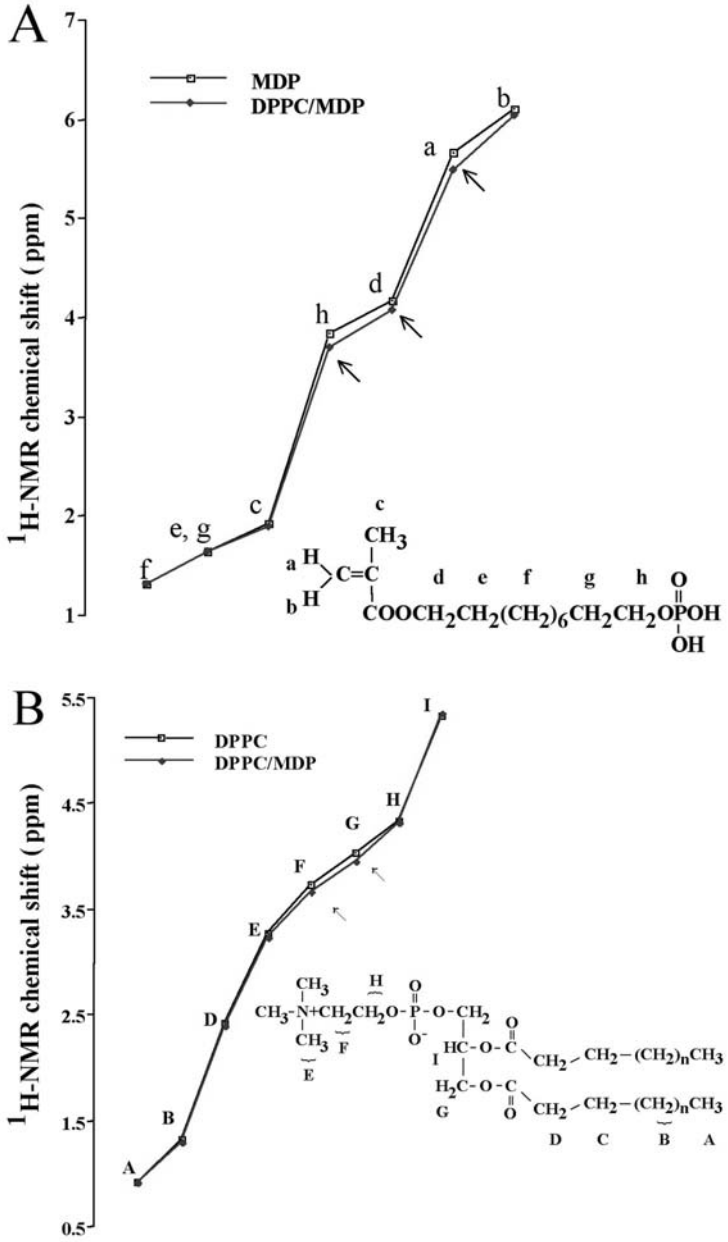


Figure 1. ^{1}H–NMR chemical shifts of MDP alone versus DPPC/MDP (2:1) liposomes at pH 7.0, 35**°C** (A), and of DPPC liposomes versus DPPC/MDP (2:1) liposomes at pH 2.4, 50°C (B). The chemical shifts are in ppm downfield from 3–(trimethylsilyl)propionic acid sodium salts (TMSPA). NMR spectra for A and B are shown in Figures 3(II) and (IV), respectively.

2.2 Chemical Data

2.2.1 Chemical shift difference

The chemical–shifts of MDP, MDP associated with DPPC/MDP liposomes, DPPC liposomes

and DPPC/MDP liposomes are shown in Figure 1. Letters correspond to significant atom or groups, as shown on the chemical structure in the insets. →indicates a significant difference. (*A*) The chemical shift difference (ppm) between MDP and DPPC/MDP liposomes for h, d, and e is 0.13, 0.09 and 0.17, respectively. (*B*) The chemical shift difference (ppm) between DPPC liposomes and DPPC/MDP liposomes for F and G is 0.06 and 0.08, respectively.

2.2.2 DSC curves

The phase–transition temperature (Tm) and enthalpy of DPPC liposomes and DPPC/MDP liposomes can be determined by analyzing the phase transition data of DSC curves. Tm does not represent the beginning of the transition (Ts) for each peak but a temperature on the cross point between the base line and the access line of DSC peak (running from Ts to a temperature (Tc) at which the specific heat reaches a maximum). DSC curves for DPPC/MDP liposomes are shown in Figure 2.

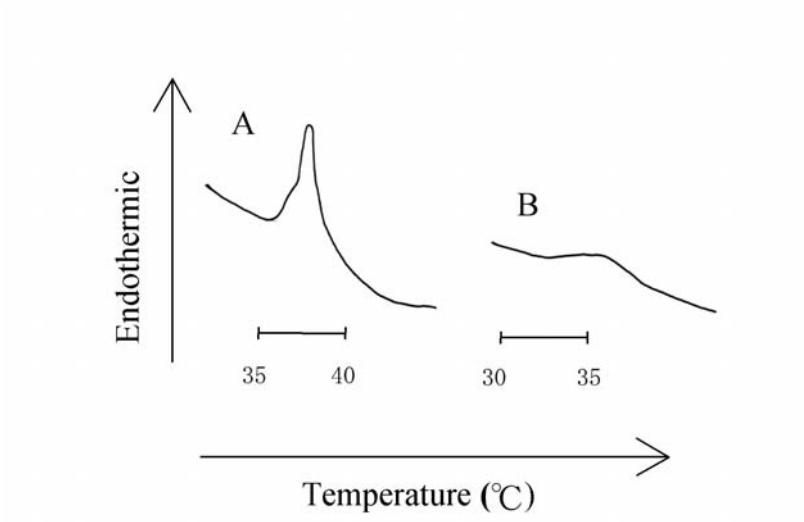


Figure 2. DSC curves for DPPC/MDP (2:1) liposomes at pH 7.0 (A) and pH 2.4 (B) in D_2O or D_2O buffer solution. The procedure of measurement is described in the Materials and Methods.

2.2.3 NMR spectra

NMR is one of the most powerful methods in studying the DPPC–MDP interactions. Effects of the temperature and pH on the ^{1}H–NMR chemical shifts of DPPC/MDP liposomes are shown in Figure 3.

2.2.4 Computational data

A biological membrane is composed by lipid bilayer. Phospholipid liposomes are often employed as a model system in order to study the interaction of drugs with biological membranes. The phase–transition properties and changes in NMR–chemical shifts of phospholipid liposomes

induced by MDP may be related to biological activities of this compound. Information available from computational methods may be useful for interpreting the molecular mechanism of interactions of MDP with liposomes. PC (phosphatidylcholine with β–acetyl–γ–acetyl groups) was used as a model compound. HOMO, LUMO and partial charge for PC, un–ionized MDP and ionized MDP were calculated in COSMO by the PM3 method (Figure 4). COSMO was used, since biological systems are in a water–phase.

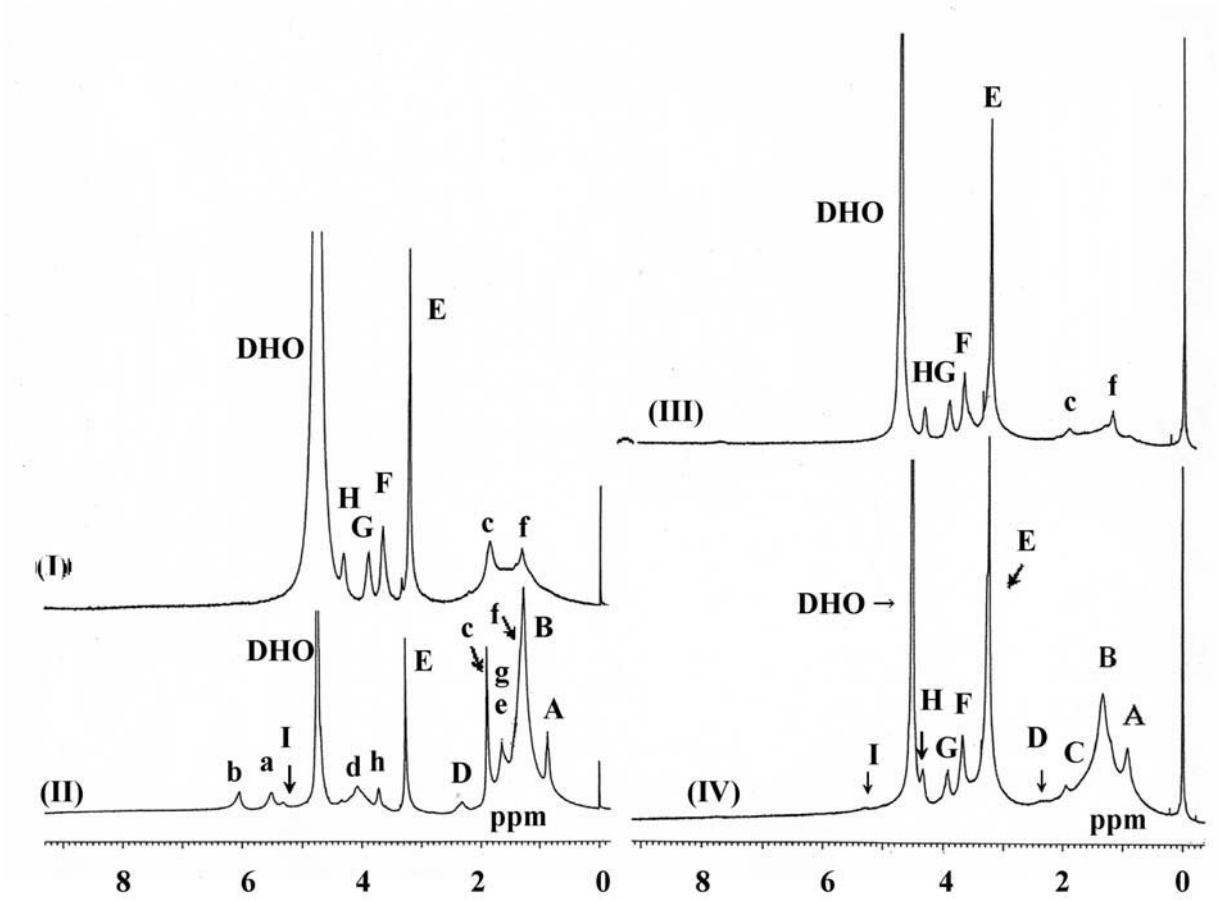


Figure 3. ^{1}H–NMR spectra of DPPC/MDP (2:1) liposomes at pH 7.0 at 25 °C (I) and at 35°C (II), and at pH 2.4 at 30°C (III) and at 50°C (IV) in D_2O buffer solutions (NaOD+KH_2PO_4). The chemical shifts are in ppm downfield from TMSPA. For identification of signals, see Figure 1. Signals for terminal CH_3 (A) and acyl chains (B) of DPPC that appear in the liquid–crystalline phase of liposomes are observed in the panels, II and IV. Signals, E, F and H due to the protons of phosphatidylcholine polar groups (O–C–C–N) are observed in the panels, I, III, and IV. All signals due to the proton of MDP are observed in the panel of II.

3 RESULTS AND DISCUSSION

The samples of DPPC/MDP (2:1, molar ratio) liposomes in D_2O or D_2O buffer (pH 7.0) that were assayed by NMR spectroscopy were also scanned by DSC. The DSC curves are shown in Figure 2. The pH (pD) of DPPC/MDP liposomes in D_2O was approximately 2.4, and the DSC curve of this sample was markedly broad, suggesting that disruption of the bilayers occurs at low pH

values with consequent reduction of enthalpy to zero. In contrast, in D_2O buffer at pH 7.0, the DPPC/MDP liposome sample showed an endothermic peak with a shoulder. The main Tm for DPPC/MDP at pH 7.0 was 35.0 °C and its enthalpy was approximately 8.20 kcal/mol, which was about 10% less than that of the control (Tm 41.0 °C and enthalpy approximately 8.9 kcal/mol) [5]. It is clear from this finding of a markedly broadened DSC peak that DPPC–MDP complexation occurs at low pH values.

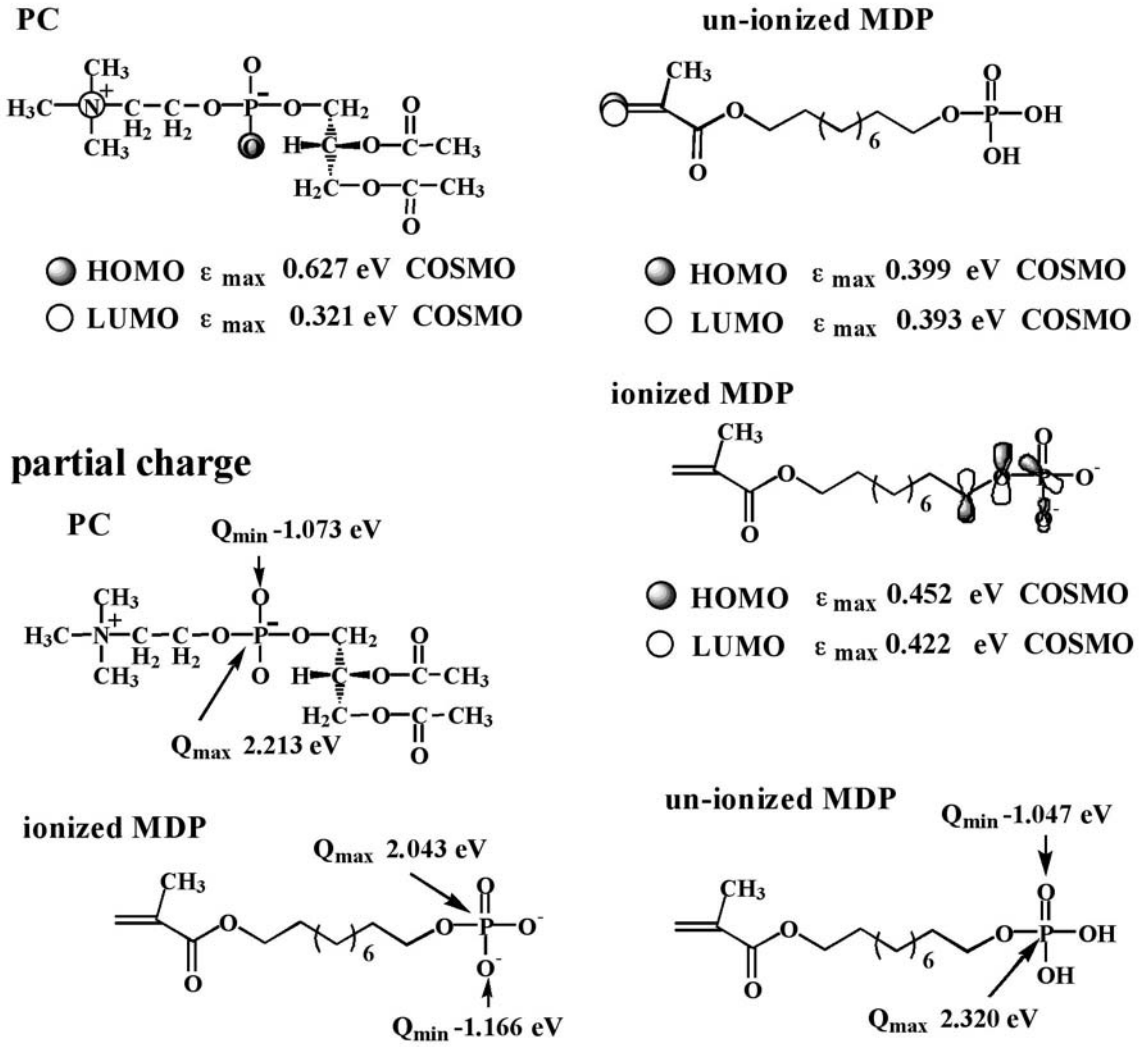


Figure 4. HOMO, LUMO, and partial charge for PC, for ionized MDP and un–ionized MDP. The values for each compound have been calculated by the PM3 method after geometry optimization was performed using CONFLEX5 (Conflex Co., Tokyo, Japan).

^{1}H–NMR spectra of MDP associated with DPPC liposomes in D_2O or D_2O buffer (pH 7.0) at 25, 30, 35 and 50 °C are shown in Figure 3. Chemical shift differences between MDP or DPPC liposomes and DPPC/MDP liposomes are summarized in Figures 1A and B.

In the NMR spectrum of DPPC/MDP liposomes at pH 7.0 at 25 °C (I), only broad signals for c and e of MDP molecules were apparent, and other proton signals for MDP were not found. In contrast, the signals for E, F, G and H of the phosphodiester groups of DPPC molecules appeared clearly at 25 °C, but signals H, G and F were abolished at 35 °C (II). At this temperature, the signals of acyl chains (B) and terminal methyls (A) appeared together with signals for MDP (II). As shown in Figure 2, DPPC/MDP liposomes exist in the liquid phase above 35 °C. At this temperature, MDP incorporated into lipid bilayers could appear at the surface of liposome membranes as a result of their enhanced fluidity and mobility. Figure 1A shows considerably greater up–field shifting in DPPC/MDP liposomes of the proton signals (a, b, d) of the methacryloyloxy group of MDP, **H_2C=C(**CH_3**)**–$COOCH_2$–, compared with other MDP signals (c, e, g and f), which suggests an extensive interaction between the methacryloyloxy groups of MDP and the phosphodiester groups of DPPC. The signal of h was also significantly shifted up–field, possibly due to the effect of the adjacent dihydrogen phosphate group. On the other hand, in D_2O at 30 °C at pH 2.4 (III), only high–intensity signals for c and f of MDP were found whereas, conversely, signals for the phosphodiester group of DPPC (F, G, H and E) were clearly apparent. At 50 °C (IV), no signals for MDP were found, whereas signals for DPPC (A, B, C, D, E, F, G, H and I) were observed together with a clear increase in the fluidity of acyl chains (signal B). Figure 1B shows the chemical shift differences for DPPC molecules between DPPC liposomes and DPPC/MDP liposomes in D_2O at pH 2.4, with a particular increase in up–field shifting of the F and G signals in DPPC/MDP liposomes compared with the corresponding signals in DPPC liposomes.

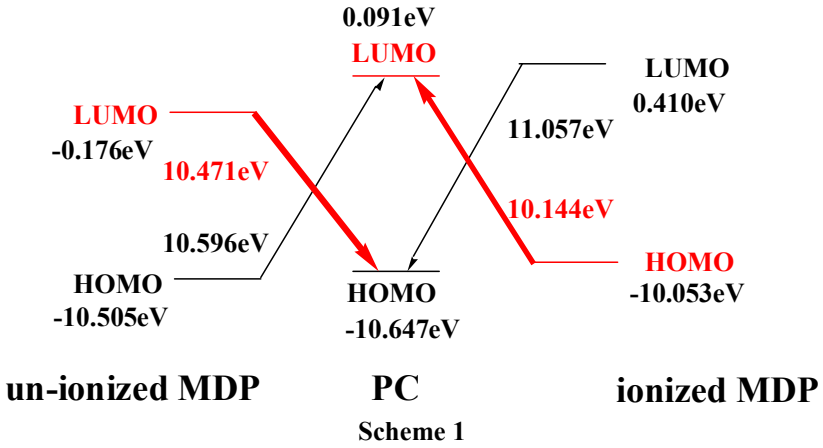
It is clear from the present findings that MDP associated with DPPC liposomes is extensively incorporated into DPPC lipid bilayers under acidic conditions. The broad DSC peak of DPPC/MDP liposomes at pH 2.4 suggests the formation of mixed DPPC–MDP micelles or other aggregates. In general, it has been suggested that the attractive force for DPPC–MDP interaction may arise from (1) hydrogen bonding involving un–ionized phosphate groups of MDP and from (2) consequently increased hydrophobic interactions between the hydrocarbon portion (decyl methylenes) of MDP and the acyl chains of DPPC. Charge–dipole interactions are probably of restricted importance for a minor driving force for DPPC–MDP interactions. Note: As is known, the C = O group in DPPC or MDP molecules has a permanent electric dipole. The dipole moment for DPPC and MDP was computed by the PM3 method, indicating that the moment for corresponding compounds is 18.5 D and 6.41 D, respectively. Hence, the C=O groups in DPPC may create some additional dipole–dipole interaction between adjacent phospholipids or MDP.

Together with findings shown in Figures 1–3, MDP appeared to form a hydrogen bond at a low

pH and therefore, its hydrophobicity increased. To clarify the point, we investigated the hydrophobicity for un–ionized MDP and ionized MDP. The hydrophobicity (octanol–water partition coefficient, log P) of MDPs was calculated using the PM3 method, indicating that the log P for un–ionized MDP was 4.089, whereas that for ionized one was 3.066. This suggested that the strong interaction between un–ionized MDP and DPPC liposomes at a low pH might have been derived from the high hydrophobic activity of un–ionized MDP.

The appearance of a shoulder on the DSC curve for DPPC/MDP at pH 7.0 shifted the Tm to a lower temperature. The formation of such a shoulder for 2,4–dinitrophenols was previously reported to be due to an interaction occurring at the surface between hydrophobic and hydrophilic domains in the glycerol backbone of lipids [4].

We could not calculate the HOMO and LUMO energies for DPPC with large acyl chains by the PM3 method, because the DPPC molecule is too large, and therefore we performed the calculation for phosphatidylcholine (PC) with β-acetyl-γ-acetyl groups instead of DPPC, as shown in Figure 4. The following scheme for HOMO–LUMO interactions may be proposed:



Scheme 1

The PM3 calculation in COSMO suggested that the HOMO for DPPC probably interacts preferentially with the LUMO for un–ionized MDP; the difference of energy for [PCε_{HOMO} – un–ionized MDPε_{LUMO}] is 10.471 eV, whereas that for [PCε_{LUMO} – un–ionized MDPε_{HOMO}] is 10.596 eV. The relationships between HOMO and LUMO for the DPPC/MDP interaction were similar in both COSMO and non–COSMO. In contrast, the LUMO of PC probably interacts preferentially with the HOMO for ionized MDP; the difference of energy for [PCε_{HOMO} – ionized MDPε_{LUMO}] is 10.144 eV, whereas that for [PCε_{LUMO} – ionized MDPε_{HOMO}] is 11.057 eV. In general, the most highest density for methacrylates exists in the beta–carbon, $H_2\underline{\mathbf{C}}$=C($CH_3$)COOR both for the HOMO and the LUMO, and therefore radicals derived from decomposition of peroxides in

polymerization systems attack this beta–carbons and polymerization of methacrylates is initiated by radical addition to their beta–carbons. However, the beta–carbons in ionized MDP do not possess the higest density (Figure 4). This suggested that polymerization of MDP does not occur when MDP is ionized.

Additionally, partial charges for the MDP molecule were calculated in COSMO. The charges for the most negative atom and for the most positive atom (Q_{min} and Q_{max}, respectively) in the MDP and PC molecules in COSMO are associated with the P atoms, and their values in both compounds are similar. On the basis of the relationships between HOMO and LUMO for each molecule, the beta–carbon in the methacryloyloxy group in MDP preferentially interacts with the phosphodiester function on the DPPC bilayer surface, as shown in Figure 3. Therefore, signals, a, b and c were strongly shifted up–field or were shielded in DPPC/MDP liposomes. Also, the chemical shift differences for DPPC molecules between DPPC and DPPC/MDP liposomes in D_2O at pH 2.4 showed strong up–field shifting of F (N–CH_2) and G (glycerol CH_2), which are located in the phosphodiester head function, compared with the corresponding signals in DPPC liposomes. This agrees with the findings of the HOMO–LUMO calculations.

Computational chemistry has contributed to many aspects of chemical research and has now begun to be applied to biomedical research. The application of computational chemistry might provide information used in rational design of new monomer in dental materials.

4 CONCLUSIONS

The complexation of DPPC–MDP is strongly dependent on the pH of medium. The strong complexation occurred at pH 2.4, resulting from the broadening of DSC peak of DPPC/MDP liposomes and the shielding of proton signals for MDP. A driving force for complexation is suggested to be the formation of hydrogen bonds between un–ionized MDP and the phosphodiester functions of DPPC surface at a low pH. The NMR chemical shifts of MDP associated with DPPC liposomes could be interpreted in terms of molecular descriptors obtained from PM3 calculations.

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Biographies

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