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Diene-containing 1,3-diacylglycero-2-phosphoric acid

A pH-sensitive ultraviolet probe and polymerization

Etsuo Hasegawa, Eiichi Shouji, and Eishun Tsuchida*

Department of Polymer Chemistry, Waseda University, Tokyo, 169 Japan

Summary

A polymerizable 1,3-diacylglycero-2-phosphoric acid, 1,3-bis(octadeca-trans-2,trans-4-dienoyl)-rac-glycero-2-phosphoric acid 1, was synthesized. The lipid bilayer formation and its pH-dependence were elucidated by ultraviolet, fluorescence, DSC and ^{1}H- and ^{31}P-NMR measurements. The rate of photopolymerization as bilayer assemblies was sensitive to pH, i.e. the the degree of dissociation of the phosphoric group of 1.

Introduction

Charged phospholipids, i.e. phosphatidic acids, phosphatidylserines and phosphatidylglycerols, take an important role in keeping the charged states of biological cell surfaces negative. They are sensitive to pH, because the degree of dissociation of charged groups depends on the hydrogen concentration of medium (1). Generally phospholipids with a net neutral charge are studied in detail, while they are almost silent to the pH change of medium.

Not only azobenzene chromophore-containing synthetic amphiphiles (2) but also polymerizable phospholipids (3) have been found to be useful as uv/vis probes to clarify the states of molecular assemblies. Typical compounds are neutral phospholipids having diene (4-7) or diyne (8) groups in acyl chains, but to our knowledge few charged glycerophospholipids have been used as a probe.

In this communication, we report the synthesis of a new acidic and polymerizable phospholipid, 1,3-bis(octadeca-trans-2,trans-4-dienoyl)-rac-glycero-2-phosphoric acid 1, having two chemically-equivalent polymerizable groups (diene groups), the pH-dependent change of the state of aggregation and the pH-dependent photopolymerization behavior.

Results and Discussion

Synthesis of Polymerizable Phosphatidic Acid

A series of our studies on the polymerization behavior of asymmmetrical (1,2-diacylglycerol-type) and symmetrical (1,3-diacylglycerol-type)

*To whom offprint requests should be sent

glycerophosphocholines, where diene groups are used as ultraviolet probes, have proved that the latter compound is superior to avoid complication observed in the former phospholipids (5-7). Here, a 1,3-diacyl derivative 1 was then to be synthesized .

1,3-Bis(octadeca-trans-2,trans-4-dienoyl)-rac-glycerol (9) was phosphorylated in dichloromethane by phosphorous oxytrichloride and hydrolyzed with a sodium hydrogen carbonate/EDTA mixture in tetrahydrofuran-water. After purification described in the experimental section, 1 was obtained (yield: 41%). The structure of 1 was determined by ^{13}C-NMR (Figure 1), IR, and fast atom bonbardment (FAB) mass measurements and elementary analyses.

Characterization of Molecular Aggregates of 1

To the thin films of 1 prepared on the glass wall of a round bottomed flask by evaporating the methanol solution on a rotatory evaporator under reduced pressure was added water, D_2O or 25mM Tris buffer (5mM EDTA). It was ultrasonicated under Ar to give a transparent lipid dispersion. The average diameters of the aggregates determined by a Coulter N4SD submicron particle analyzer were 32nm, 30nm and 25nm at pH3.8, 7.4 and 9.0, respectively.

The broadening of the methylene signal ($\Delta\delta_{1/2}$: 0.12 ppm) was observed in the ^{1}H-NMR of 1 dispersion in D_2O at 60°C in comparison to that in $CDCl_3$ at 20°C ($\Delta\delta_{1/2}$:0.03 ppm). ^{31}P-NMR measurements at 60°C showed the sharp double peaks at 0.0 and -0.2 ppm ($\Delta\delta_{1/2}$<0.5 ppm) indicating the ordered molecular structure and the spherical geometry of phosphoryl groups (10).

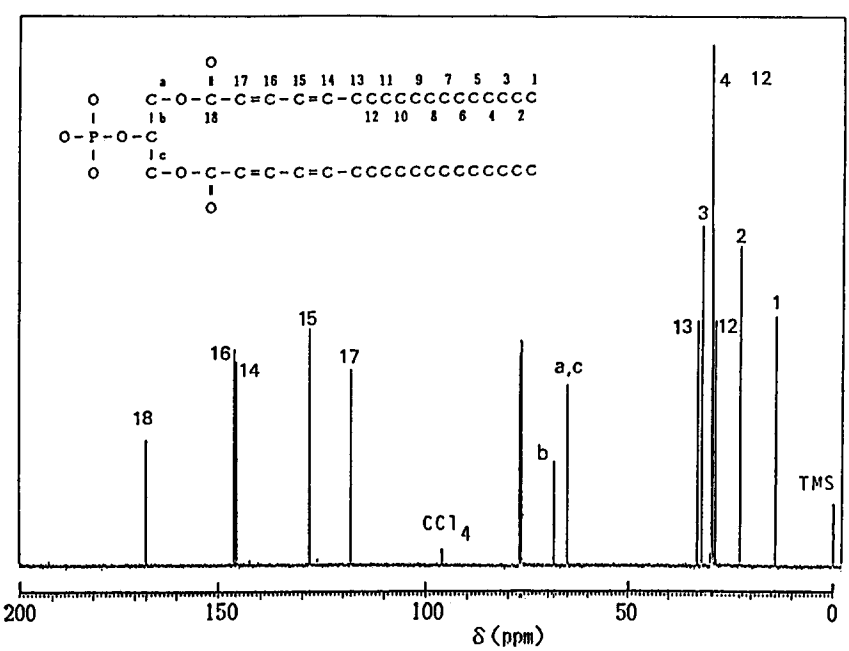

Figure 1. Proton-decoupled ^{13}C-NMR spectrum of 1 ($CDCl_3$, TMS)

The phase transitions of the lipid dispersions of 1 were studied in the Tris buffer of pH 3.8, 7.4 or 9.0 by three different methods and compared with those of the neutral phospholipid (1,2- and 1,3-phosphatidylcholines). The pH values of the medium were determined from the general pKa_1 ($\approx$3.5) and pka_2 (8.0-9.0) values (1).

Firstly, they were determined by fluorescence measurements using N-phenyl-1-naphthylamine (NPN) as a fluorescence probe (11). NPN is a polarity-dependent and uncharged probe and well known to be entrapped in the fatty region of lipid bilayers. Figure 2 shows the histograms of the temperature dependence of the relative fluorescence intensity of various lipid dispersions. Figure 2 shows that the 1 dispersion has a distinct phase transition temperature and that the transition is strongly dependent on pH of the medium, while the neutral lipid (1,3-bis(octadeca-2,4-dienoyl)-rac-glycero-2-phosphocholine, 2) is independent. Nextly, the phase behavior was measured by diffrential scannning calorimetric measurements (DSC). Figure 3 shows the DSC curves. With increasing pH, the phase transition temperature became lower as in the case of common phosphatidic acids (1). Thirdly, the transition was studied by using the ultraviolet chromophores (diene groups) of the acyl chain of 1 as a probe. For example, Figure 4 shows the spectral change of the 1 dispersion (pH3.8) in the ultraviolet region with temperature. With raising temperature, the absorption at 225 nm decreased, while the new absorption band at 254 nm appeared. The ratio of absorbance at 250 nm to that at 230 nm changed with temperature (Figure 5). Figure 5 also indicates distinct phase transitions.

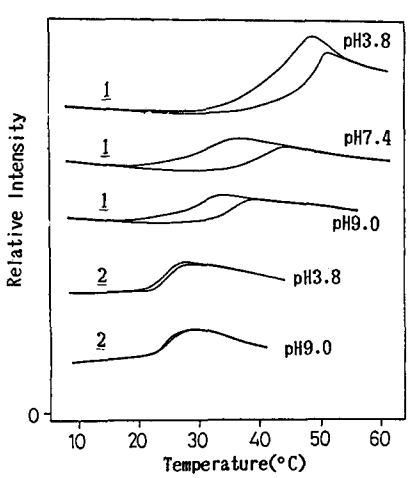

Figure 2. Fluorescence indication of phase transition for 1 and 2 at different pH vakues.

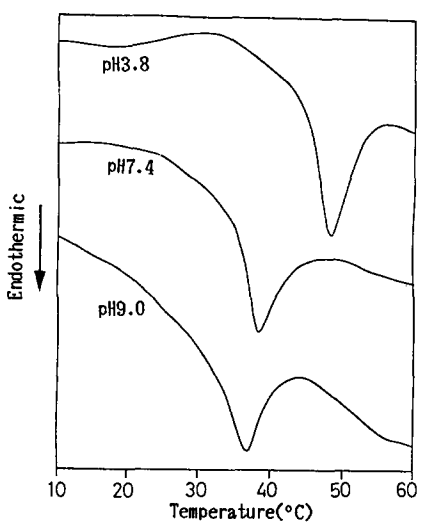

Figure 3. DSC curves for 1 dispersion at different pH values.

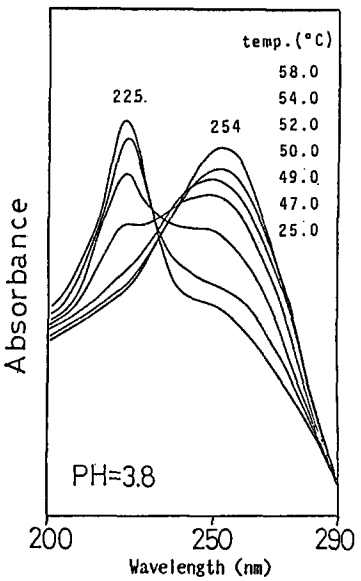

Figure 4. Change of UV Absorption Spectra of 1 dispersion with temperature.

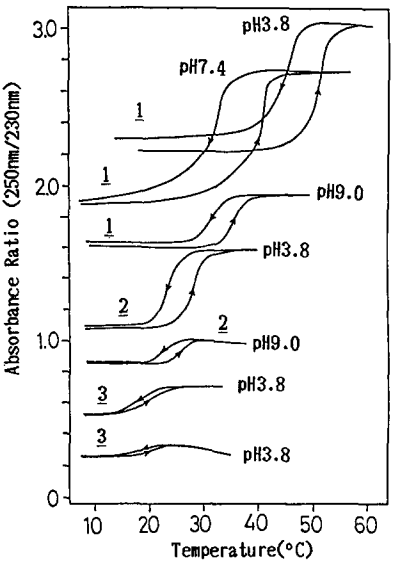

Figure 5. Change of Absorbance Ratio with Temperature.

The phase transition temperatures determined by three different methods are summarized in Table 1 with the UV absorption maxima (λ_{max}) due to the diene chromophores of the lipids. Tc values coincide with each others. Tc values for the charged lipid 1 are strongly dependent on pH, but not for the neutral lipids, 2 and 3. The λ_{max} value above phase transition temperature (TC) is 253-256 nm, which is independent on lipid structure. The λ_{max} value below Tc for the neutral lipids, 2 and 3, is about 242-245 nm, which corresponds to the value for 1 at pH 9.0, where the phosphoric acid residue dissociates considerably. The absorption maxima of 1 at pH 7.4 (half-dissociated) and at pH 3.8 (less dissociated) are 231 nm and 225 nm.

Table 1. Phase Transition Temperatures (Tc) and UV Absorption Maxima of Aqueous Dispersions of Diene-group Containing Phospholipids

Lipids	pH of medium	Tc(°C) DSC	Tc(°C) Fluorescence	Tc(°C) UV	λ_{max}(nm) below Tc	λ_{max}(nm) above Tc
1	3.8	44.8	47.0	43.0	225	254
	7.4	35.4	37.0	33.0	231	255
	9.0	32.5	33.0	30.0	241	253
2	3.8	-	25.0	23.0	242	255
	7.4	28.0	26.0	24.0	244	256
	9.0	-	24.5	24.0	243	252

at below Tc, respectively. The absorption bands at 242-245 nm and at 253-256 nm could be assigned to the $L_{\beta'}$ state and the L_{α} state of bilayer assemblies, respectively (4-7,12). The new bands at 231 nm and at 225 nm reflecting the diene groups exsisting in a more hydrophilic envi-ronment are characteristic of the charged lipid 1. The aggregation states of 1 with one negative net charge and with no charge correspond to the another $L_{\beta'}$ state and the L_{β} state, respectively (1,12). The 231 nm and 225 nm bands then correspond to the former and the latter, respectively.

Photopolymerization Behavior

The pH-dependent change of bilayer structure of 1 is expected to affect the photopolymerization reaction. As shown in Figure 6, the rate was dependent on the state of bilayers and increased with the degree of dissociation of the phosphoric acid group of 1, while the neutral and zwitterion-type lipid, 2, was not influenced upon pH.

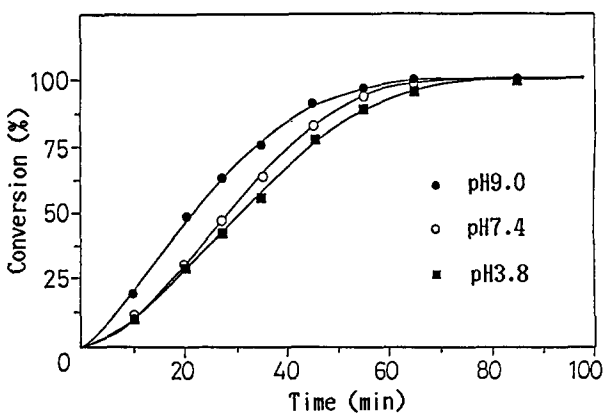

Figure 6. pH-dependence of photo-polymerization of 1 suspension at 20°C. [1]=0.32 mM.

Experimental

Reagents 1,2-Bis(octadeca-trans-2,trans-4-dienoyl)-sn-glycero-3-phospho-choline 3 (3) and 1,3-bis(octadeca-trans-2,trans-4-dienoyl)-rac-glycero-2-phosphocholine 2 (9) were synthesized according to literatures. N-Phenyl-1-naphthylamine was purchased from Kodak. 25mM Tris buffers (pH 3.8, 7.4 and 9.0) having 5mM EDTA were used as solvent.
Apparatus and Methods DSC, FABms, IR and particle size measurements were carried out as described (8). Fluorescence spectra were recorded on a JASCO FP-770 spectrofluorometer. N-Phenyl-1-naphthylamine (NPN) was used as a fluorescence probe (Excitation: 350 nm, Emission: 420 nm). The molar ratio of lipids to NPN was 100 ([NPN]=1.2 uM). ^{13}C- and ^{31}P-NMR spectra were

measured with a JEOL GSX-400 NMR spectrometer. Photopolymerization was carried out by a low-vacuum mercury lamp as deacribed (9).

1,3-Bis(octadeca-trans-2,trans-4-dienoyl)-rac-glycero-2-phosphoric acid 1

To the ice-cooled and stirred phosphorous oxytrichloride (0.24 ml, 2.35 mmol) in dry dichloromethane (10 ml) were added 1,3-bis(octadeca-trans-2,trans-4-dienoyl)-rac-glycerol (1.0 g, 1.6 mmol) in dichloromethane (10 ml) and triethylamine (0.36 ml, 1.65 mmol) in dichloromethane (10 ml) simulateneously, slowly and dropwise. It was stirred for 12 hr at room temperature. Benzene was added to the solution and the precipitates were removed by filtration. After concentration, the residue was hydrolyzed with 0.5M $NaHCO_3$ (50 ml)/0.25M EDTA (pH10.8) (25 ml)/tetrahydrofuran (70 ml) for 12 hr. To the mixture was added brine (50 ml) and the organic phase was collected and evaporated under reduced pressure. The residue was taken in chloroform/methanol/water (50ml/50ml/50ml) and stirrered. The organic layer was collected and the solvents were removed. The residue was taken in chloroform and filtered. The filtrate was concentrated and dried. Reprecipitation from a chloroform-acetone mixture gave 1 (0.50 g, 41%). IR(KBr): 1720($\nu_{C=O}$), 1650, 1610($\nu_{C=C}$), 3400, 1260,1160, 1100 cm^{-1} (phosphate); ^{1}H-NMR($CDCl_3$, TMS):δ(ppm) 4.25(4H,-CH_2-), 2.60(1H,-CH), 5.80, 7.30,6,16(2H,2H,4H,-CH=CH-CH=CH-); FABms: 763 $[M+Na]^+$,741 $[M+H]^+$; UV($CHCl_3$) λ_{max} 261 nm, ϵ_{max} 52500 (ℓ/mol·cm); TLC (Silicagel, $CHCl_3$/methanol/water (65/25/4)) Rf: 0.3; Anal.($C_{39}H_{67}O_8P_1Na_2 \cdot H_2O$): C 61.7(61.72), H 9.0(9.17).

References

1. J.M.Boggs, Biochim. Biophys. Acta, 906, 353 (1987).
2. M.Shimomura, R.Ando, T.Kunitake, Ber. Bunsenges. Phys. Chem., 87, 1134 (1983).
3. H.Ringsdorf, B.Schlarb, J.Venzmer, Angew. Chem. Int. Ed. Eng., 27, 113 (1988).
4. P.N.Tyminski, I.S.Ponticello, D.F.O'Brien, J. Amer. Chem. Soc., 109, 6541 (1987); B.Hupfer, H.Ringsdorf, H.Schupp, Chem. Phys. Lipids, 33, 355 (1983).
5. H.Ohno, S.Takeoka, E.Tsuchida, Macromolecules, 20, 929 (1987).
6. S.Takeoka, E.Hasegawa, H.Ohno, E.Tsuchida, Bull. Chem. Soc. Jpn., 61, 2007 (1988).
7. H.Ohno, S.Takeoka, H.Iwai, E.Tsuchida, Macromolecules, 21, 319 (1988).
8. A.Singh, J.M.Schnur, J. Amer. Chem. Soc., 108, 2785 (1986).
9. E.Hasegawa, K.Eshima, Y.Matsushita, H.Ohno, E.Tsuchida, Polymer Bull., 18, 65 (1987).
10. P.R.Cullis, A.C.McLaughlin, Trends Biochem. Sci., 2, 196 (1977); J. Magn. Res., 20, 146 (1975).
11. E.Traeube, H.Eibl, Proc. Nat. Acad. Sci., USA, 71, 214 (1974).
12. A.Tardiev, V.Luzzati, J. Mol. Biol., 75, 711 (1973).

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