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Application of liposome membrane as the reaction field: A case study using the Horner–Wadsworth–Emmons reaction

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The properties of the liposome membrane as a reaction field were investigated by focusing on the Horner–Wadsworth–Emmons reaction as a case study. Use of the liposomes existing in the gel phase resulted in the enhanced activity of the substrates and furnished the products with same *E*/*Z* stereoselectivity as in the liposome-free system. The membrane environment in the gel phase most likely assisted the formation of adducts that induced selective generation of the *E*-isomer. The possible role of liposomes is to assist the proton removal from the reactant, rather than providing the basic interfacial environment.

[**Key words:** Liposome; Phospholipid; Reaction field; Horner–Wadsworth–Emmons reaction; Stereochemistry]

Phospholipids can form molecular self-assemblies such as micelles and vesicles. The phospholipid vesicle (liposome) is a closed lipid bilayer that has the ability to encapsulate the soluble or hydrophobic reagents (1,2). Liposomes bearing biocompatible interfaces are being actively applied in the drug delivery systems (2). Moreover, they can exhibit catalytic properties due to their assistance in proton transfer (3) and the ordered ligand structure that can act as the reaction center (4). In addition, liposome membranes provide the reaction field that controls the enzymatic polymerization reaction, resulting in the template effect (5). Thus, in light of the above applications of vesicles, liposomes have been widely studied.

The membrane interior of liposomes has been reported to control the oxidation reaction (3,6,7). The proton transfer between the substrates and the catalysts, which is a key process in the above reaction, is mediated by liposome membranes. Besides, these membranes can also concentrate the hydrophobic compounds in a water system due to their bilayered structure. The Horner–Wadsworth–Emmons (HWE) reaction (8–12), as well as aldol and Wittig reactions (13–15) result in the production of α,β-unsaturated esters (8). These compounds are useful for drug design in chemical and cosmetic industries. The HWE reaction is usually performed in an organic solvent; hence, an improved method which bypasses the use of an organic solvent is desired considering the environmental concerns. Recently, the HWE reaction under the solvent-free conditions has been reported using potassium carbonate and catalytic amount of 1,8-diazabicyclo[5,4,0] undec-7-ene (DBU) (9). DBU has relatively high basicity and displays the proton sponge effect when a proton is adsorbed onto it (9). If the liposome that mediates the proton transfer can behave similar to DBU, it would not only provide the organic solvent-like reaction environment in the water system but also mediate the deprotonation of reactant. This could be possible because the liposome membranes possess hydrophobic acyl chains and water molecules that are bound to lipids.

In this study, we focused on the HWE reaction as the model one to study the effect of liposomes. Ethyl cinnamate, the product of the HWE reaction, was monitored to estimate the kinetic parameters. Thereafter, the effect of physicochemical properties of liposome membranes on the HWE reaction is discussed using these kinetic parameters. Finally, we discuss the role of liposome membranes in the HWE reaction.

MATERIALS AND METHODS

Materials Lipids used herein were 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC; $T_m = -20°C$), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC; $T_m = -3°C$), 1,2-dimyristoyl-*sn*-glycero-phosphocholine (DMPC; $T_m = 23.4°C$), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC; $T_m = 41.4°C$), Sphingomyelin (SPH), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE; $T_m = 25°C$) (these lipids were purchased from Wako Pure Chemical Co. Ltd., Osaka, Japan). Chemical structure of lipids is shown in Fig. 1. Potassium carbonate, triethyl phoshonoacetate, benzaldehyde and 1,8-diazabicyclo[5,4,0] undec-7-ene (DBU) were also purchased from the above company.

Liposome preparation For the liposome preparation, hydration method was used as in the previous report (16,17). Lipids were dissolved in chloroform and then dried overnight onto the walls of a round-bottomed flask under vacuum to ensure the complete removal of the solvent. The dried thin lipid film was hydrated with water to form multilamellar vesicles (MLVs). Large unilamellar vesicles (LUVs) were formed from MLVs after five cycles of freeze-thaw treatment. The obtained vesicle suspension was extruded 15 times into a polycarbonate filter of 100 nm pore size to adjust the liposome diameter. The mean liposome diameter was found to be 110 ± 15 nm as measured by a dynamic light scattering method.

HWE reaction The HWE reaction was performed according to the previous report (9). In short, triethyl phoshonoacetate (5 mmol) and benzaldehyde (5 mmol) were mixed at 25°C with aqueous potassium carbonate (K_2CO_3) solution (10 mL) and

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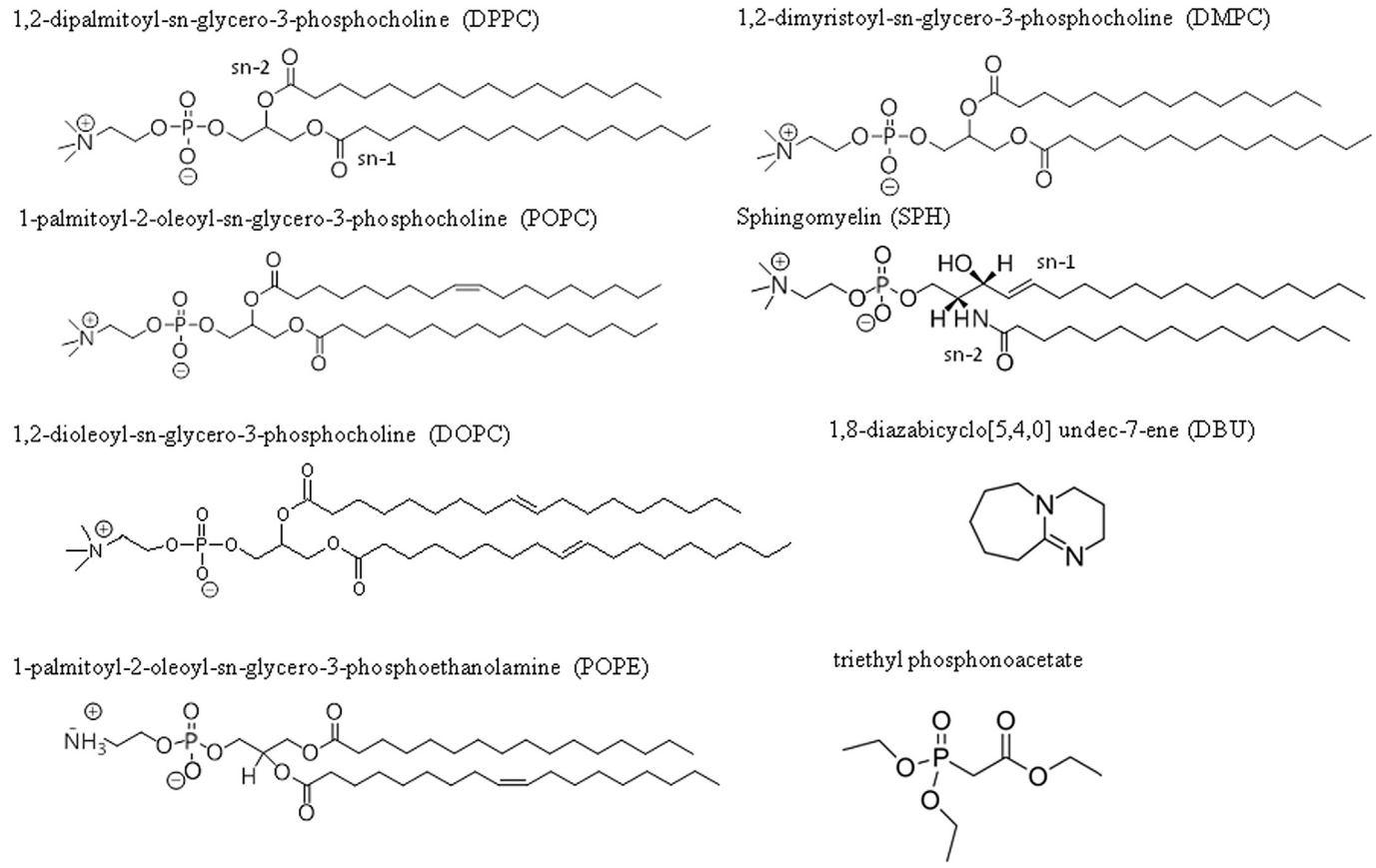

FIG. 1. Chemical structure of phospholipids, reactants, and DBU.

DBU to start the reaction, both in the absence and presence of liposome suspension. The final concentrations of K_2CO_3, DBU and liposome were 10 mM, 0.15 mM, and 0.1 mM, respectively. The suspension was stirred for 10 h at 25°C and the product in the suspension was extracted with ethyl acetate and thereafter evaporated to remove ethyl acetate. The residue was dissolved in tetramethylsilane/chloroform-d and analyzed by ^{1}H NMR spectroscopy. The NMR signals of *E*- and *Z*-isomers of ethyl cinnamate were separately detected at around 6.5 and 6.0 ppm, respectively (9). Therefore, a further separation of *E*- and *Z*-isomers in the solution was not required after the reaction.

The amount of product obtained, $B(t)$, at various times, t was monitored. The maximal productivity of ethyl cinnamate (B_{max}) [%] and the rate constant (k) [h^{-1}] of the reaction were then analyzed using the equation: $B(t) = B_{max}(1\text{-}\exp(-kt))$.

RESULTS

Kinetic analysis of HWE reaction In the first series of experiments, we attempted the reaction in water in the presence and absence of DBU, K_2CO_3, and liposomes. The representative reaction conditions and their results are summarized in Fig. 2. In the absence of both DBU and K_2CO_3, no reactivity was observed (entry 1). A comparison of entries 2 and 3 revealed that K_2CO_3 was indispensable in this reaction, which is consistent with the previous report (9). So, the effect of other additives on reaction

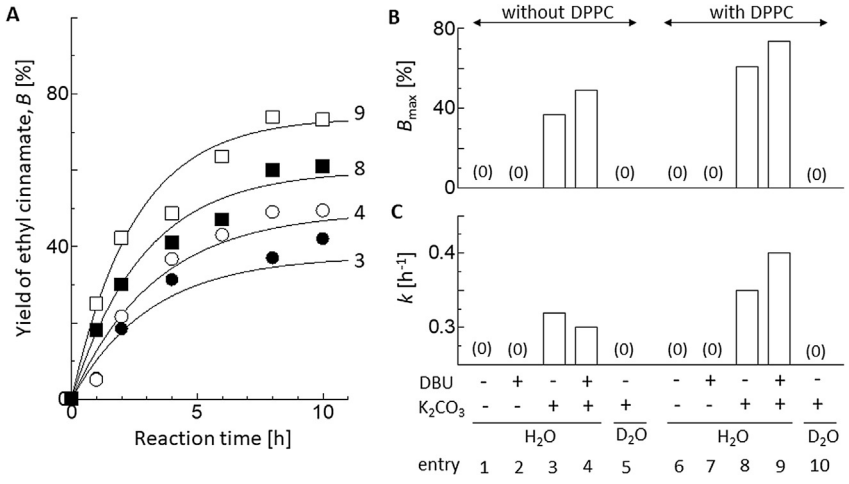

FIG. 2. (A) Time-course of ethyl cinnamate. The reaction was carried out at 25°C in the presence of DBU and/or DPPC liposomes. Reaction conditions are shown at the bottom of panel C. (B) Maximal yield (B_{max}) and (C) reaction rate constant (k) under conditions with and without DPPC liposomes. Reaction was carried out at 25°C for 8 h. Symbols: +, addition; -, no addition. Solid curves are the best fit with experimental data (entry 3, $r^2 = 0.938$; entry 4, $r^2 = 0.978$; entry 8, $r^2 = 0.981$; entry 9, $r^2 = 0.987$).

outcome was examined in the presence of K_2CO_3. The time course of the yield of ethyl cinnamate ($B(t)$) for four conditions (entries 3, 4, 8, 9) was monitored as shown in Fig. 2A. The *E*-isomer was confirmed to be the major product in this study, both in the absence and presence of liposomes. The kinetic parameters B_{max} and k were then extrapolated by a fitting. In the presence of DBU (entry 4), B_{max} = 49.2% and k = 0.30 h^{-1} (r^2 = 0.978) were obtained whereas B_{max} = 37.0% and k = 0.32 h^{-1} (r^2 = 0.938) were obtained in its absence (entry 3). Furthermore, the addition of DPPC liposome (entries 8 and 9) enhanced the reactivity resulting in B_{max} = 59.2%/k = 0.35 h^{-1} (r^2 = 0.981) and 73.7%/k = 0.40 h^{-1} (r^2 = 0.987), respectively. These results suggested a positive contribution of the liposome to the kinetics of the HWE reaction. Fig. 2B and C shows the B_{max} and k values for each condition. Even in the presence of DPPC liposomes, no reactivity was observed without K_2CO_3. Addition of DBU along with K_2CO_3 elevated the B_{max} value, both in the presence and absence of DPPC liposomes (ΔB_{max} ~13%). Alternatively, the enhanced effect by DPPC liposomes (ΔB_{max} ~25%) was observed, both in the presence and absence of DBU. Thus, it was likely that DPPC liposomes were independent of DBU in assisting the HWE reaction and that the contribution of DPPC liposomes was higher than DBU in elevating the B_{max} value.

In the HWE reaction, the proton removal from the substrate by a general base (K_2CO_3) occurs in the first step. A reaction in the D_2O system can give the key information to judge if the proton transfer from the substrate is the rate-determining step of the HWE reaction. D_2O has weaker polarization than H_2O and reduces the reaction rate relative to H_2O, resulting in D_2O solvent isotope effect. As shown in Fig. 2B and C, no reactivity in D_2O was observed even in the presence of K_2CO_3 (entry 5). This influence by the D_2O solvent isotope effect indicated that K_2CO_3 played a role in the proton transfer from the substrate in the rate-determining step, which is consistent with the previous report (9). Similarly, no reactivity in D_2O was observed even in the presence of DPPC liposomes (entry 10). The enhanced ΔB_{max} of ~25% and significant increase in k value can be considered a result of the enhancement of the proton transfer by liposomes, as well as by K_2CO_3 (compare entries 4 and 9).

Effect of lipid composition The effect of lipid composition was investigated to reveal the contribution of the physicochemical properties of liposome membranes as shown in Table 1. In case of entries 1–10 (Fig. 2A), the time course of $B(t)$ indicated the maximum value at 8 h. Therefore, the yield at 8 h was measured as B_{max} value in each lipid composition. The HWE reaction in the presence of liposomes predominantly yielded the *E*-isomer. DOPC, POPC, and DMPC liposomes enhanced the reactivity when compared to the control reaction (entry 4 in Fig. 2B), although they resulted in lower yields in the range of 49–55.3% (entries V1-V3) relative to DPPC liposome (entry V4: 73.7%). Judging from the phase transition temperatures of phospholipids, the liposomes prepared by DOPC (T_m = −20°C, entry V1) and POPC (T_m = −3°C, entry V2) exist in the liquid-crystalline phase while DMPC (entry V3) exists in a mixed phase at 25°C (16). However, DPPC liposome (T_m = 41.4°C) exists in a gel phase (entry V4) (16) which was the likely reason to enhance the HWE reaction.

Another contribution of liposomes in terms of their basicity was also investigated. SPH bears the amide group in sn-2. The occurrence of strong H-bond between the secondary amine group (NH group) of the sn-2 and OH of the sn-1 results in the stiffness of lipid membrane (solid phase) (16). Also, the NH group is responsible for the basicity in the membrane environment. The SPH liposome resulted in 49.7% yield of the *E*-isomer (entry V5), which suggested that the amino group in the interior of membrane contributed minimally to the HWE reaction. Phosphoethanolamine (POPE), bearing the primary amino group in the headgroup, exhibits relatively higher basicity than other lipids bearing the phosphocholine group in the headgroup (entries V1-V4). However, POPE alone cannot form the liposome (16); hence 30 mol% of it was mixed with the phospholipids and tested in the HWE reaction. This resulted in no enhanced reactivity as compared to the original systems (entries V4 and V6; V5 and V7). Thus, the amino group in lipids was unlikely to contribute as an alternative to K_2CO_3 and also did not alter the *E*/*Z* selectivity (stereoselectivity).

TABLE 1. Effect of liposome on HWE reaction (at 25°C for 8 h).

Entry	Lipid system	T_m (°C)	Yield (B_{max}) (%)		Phase	Note
			E-isomer	*Z*-isomer		
V0	None		49.2	Trace		Entry 4 in Fig. 2B
V1	DOPC	−20	50	Trace	L	
V2	POPC	−3	55.3	Trace	L	
V3	DMPC	23.4	49	Trace	L + G	
V4	DPPC	41.4	73.7	Trace	G	
V5	SPH	37	49.7	Trace	G	Secondary amine in SPH
V6	DPPC/POPE (7/3)	>50	73.7	Trace	$l_d + l_o$	Primary amine in POPE
V7	SPH/POPE (7/3)	n.d.	43	Trace	$s_o + l_d$	Primary amine in POPE

L, liquid-crystalline phase; G, gel phase; l_d, liquid-disordered phase; l_o, liquid-ordered phase; s_o, solid-ordered phase. n.d., not determined.

DISCUSSION

Generally, the stereoselectivity of the HWE reaction is a result of both kinetic and thermodynamic controls upon the reversible formation of the *erythro* and *threo* adducts and their decomposition into products (8–10), as shown in Fig. 3A. The predominant formation of the *E*-isomer (*trans*-olefin) in the usual HWE reactions can be explained as a result of the predominant formation of thermodynamically more stable *threo* adduct (18). In our conditions, the ethyl diethylphosphonoacetate substrate in presence of liposomes predominantly yielded the *E*-isomer (Table 1) as expected in the HWE reaction in the absence of liposomes. Hence, it was evident that the liposome membrane did not interfere with the formation of *threo* adduct from the *E*-selective substrate.

The process of the HWE reaction in liposome membrane is discussed in this section. A possible reaction mechanism based on previous studies (9,10,18) is shown in Fig. 3B. Benzaldehyde molecule shows the diffusion behavior similar to benzene. The measurements of lateral diffusion for benzaldehyde and benzene yielded the values 4.81 $\mu m^2/s$ and 4.06 $\mu m^2/s$, respectively (19). Study by Bassolino-Klimas et al. (20,21) revealed that a large migration of benzene could be explained by the diffusion mechanism being a possible kind of jump inside a bilayer, i.e., a mechanism in which the molecule diffuses over its own size from one void to another within a short time scale. This void in a bilayer, available for diffusion, is usually generated by a motion of acyl chain of phospholipid (22,23) and the rotational Brownian motion of its headgroup (24). The void can be estimated by a free volume profile. MD simulations (22,23) have shown that a free volume profile is smallest in the headgroup region, limiting the formation of void near headgroup. Thus, benzaldehyde (log P = 1.48), as indicated by its large diffusivity, appeared to use a void in acyl chain region. It should be noted that P is the partition coefficient of the substrate in the biphasic system of 1-octanol and water. The log P value is often used as the hydrophobicity index.

Alternatively, the reactant, triethyl phosphonoacetate (log P = 0.5), readily uses the void in the headgroup region rather than in

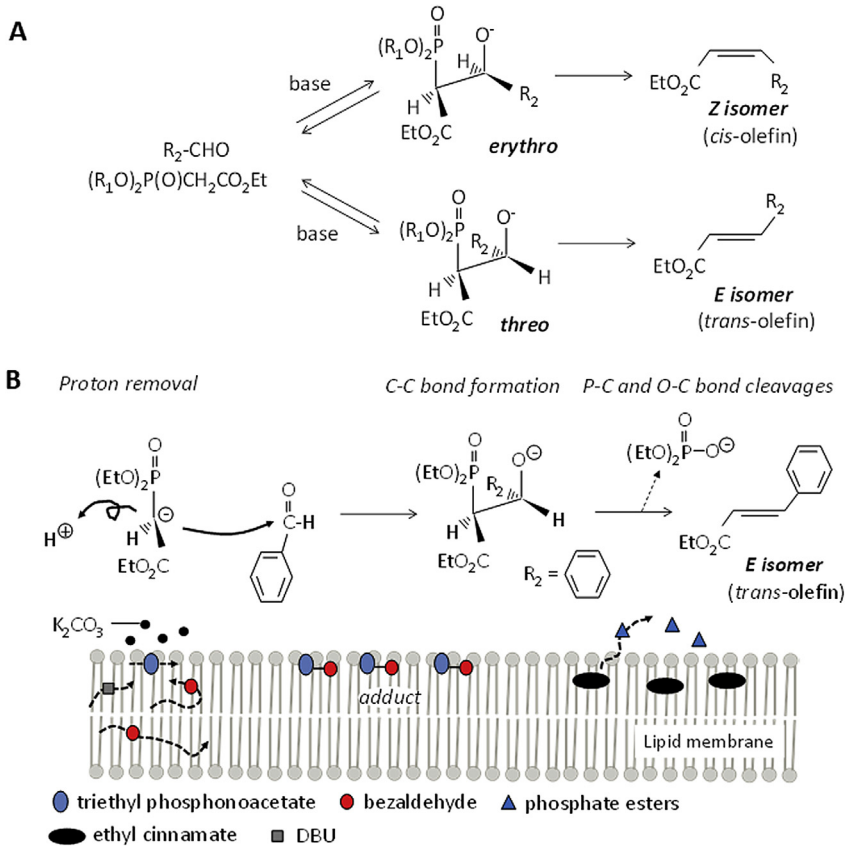

FIG. 3. (A) Reaction mechanism of HWE reaction. (B) Possible mechanism of HWE reaction in the presence of liposomes. These mechanisms are based on the study by Ando (18).

acyl region, because of its polarity (Fig. 3B). It is therefore plausible that the HWE reaction occurs by the diffusion of both benzaldehyde and K_2CO_3 (with DBU bearing log P = 1.4) into triethyl phosphonoacetate present at the membrane interface, for the formation of adducts and subsequent release of water-soluble $P(OEt)_2O_2^-$ (log P = −0.3) into the outer aqueous phase. As a consequence, the hydrophobic product, ethyl cinnamate (log P = 3.0), remains within the membrane. K_2CO_3, as usual, generates the anion of triethyl phosphonoacetate. Moreover, liposomes alone did not induce the HWE reaction (entry 6 in Fig. 2B,C), whereas K_2CO_3 alone could (entry 3 in Fig. 2B,C). It was therefore unlikely that liposomes induced the deprotonation of triethyl phosphonoacetate as effectively as K_2CO_3.

Considering no difference in *E/Z* selectivity both in presence (entry V1-V7) and absence of liposomes (entry V0), a void in the headgroup region is not preferred for *erythro* adducts. Ando (18) has revealed that the formation of the *Z*-isomer from *erythro* adducts requires the formation of oxaphosphetane and pseudorotation. The headgroup moiety might attribute steric hindrance to prevent the above processes. Besides, the voids are enriched with hydrated water or protons derived from K_2CO_3. Thus, the liposome supplies the environment required for the enhanced reactivity in the HWE reaction even in the water system, by well adopting the void within the liposome membrane.

In the calculations by Kupiainen et al. (23), SPH liposomes bear the low-content void, in particular, in the acyl chain region, due to the binding of the NH group in the sn-2 with OH of the sn-1, reducing the diffusion of benzaldehyde. Thus, SPH bearing the same headgroup as other phospholipids has a similar void environment (entry V5) which enhances the production of the *E*-isomer, as seen with other lipid compositions (entries V1-V4).

Previously, a high yield of 96% was demonstrated under the solvent-free HWE reaction conditions using K_2CO_3 and a catalytic amount of DBU (19). It was also demonstrated that DBU alone (1.5 equivalent to substrate) could also promote the reaction under the solvent-free conditions (25). DBU is a relatively stronger base which preferably adsorbs a proton (proton sponge effect). Lack of reactivity when DBU alone was used (compare entries 2 and 3 in Fig. 2B,C) suggested that the water molecules most likely diminished the proton sponge effect of DBU. Besides, DBU molecule (log P = 1.4), that preferably distributes within the membrane, would easily indicate the proton sponge effect in the hydrophobic region of lipid membranes (Fig. 3B). It is considered that liposome membrane supplied the environment where DBU could exert the proton sponge effect, thus resulting in the first possible mechanism of the liposome-enhanced HWE reaction. Alternatively, a second possible mechanism could involve the contribution of the basicity of liposomes, similar to DBU, that strongly adsorbs protons (proton sponge effect). Water molecule binds to the ester PO_2 group and ester C=O group of the sn-2 gets polarized (26). This water bound to lipids at the membrane interface acts as the base to remove the proton from the reactant. Thus, the liposome membranes directly or indirectly abstract the proton from the reactant. Lack of reactivity in the D_2O system (entries 5 and 10 in Fig. 2B,C) also supports the above-mentioned contribution of water bound to lipids in the HWE reaction. Notably, the liposome alone displayed no reactivity (entry 6) in contrast to the case where DBU alone induced the solvent-free HWE reaction (25). Thus, liposome was likely less basic compared to DBU in the HWE reaction.

In conclusion, the liposome considerably enhanced the HWE reaction of the ethyl diethylphosphonoacetate substrate even in the water system. The enhanced effect of reactivity by the liposome was observed in terms of both the yield and the reaction rate, while

no alteration of *E*/*Z* selectivity was observed. The observed effects of liposomes were likely to result from the use of their voids for the stable formation of intermediate adducts and their activity similar to the basicity of DBU. The former effect depended on the phase state of liposome membranes while the latter was derived from the hydrated water bound to the membrane interface. Thus, this finding describes the use of molecular self-assemblies, such as vesicles, as the reaction fields.

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