How Does the Mobility of Phospholipid Molecules at a Water/Oil Interface Reflect the Viscosity of the Surrounding Oil?

Makiko Negishi, Hideki Seto,† Masahiko Hase, and Kenichi Yoshikawa*

Department of Physics, Graduate School of Science, Kyoto University, Kyoto 606-8502, Japan

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The mobility of phospholipid molecules at a water/oil interface on cell-sized phospholipid-coated microdroplets was investigated through the measurement of diffusion constants by fluorescence recovery after photobleaching. It is found that the diffusion constant of phospholipids showed the relation $D \sim (\eta_{water} + \eta_{oil})^{-0.85}$, where D is the diffusion constant, η_{water} is the viscosity of water, and η_{oil} is the viscosity of oil. This observation indicates that the viscosity of the surrounding oil is the primary factor that determines the diffusibility of phospholipids at a water/oil interface.

1. Introduction

Biological membranes are a self-assembled two-dimensional system with phospholipid molecules embedded in three-dimensional bulk liquids.[1] The Brownian motion of particles in biological membranes is expected to play a crucial role in many life processes such as signal transduction, transport, receptor recognition, and so forth.[2,3] The Brownian motion of phospholipid molecules in biological membranes has been investigated by fluorescence recovery after photobleaching (FRAP), which is a useful method for measuring the Brownian motion of particles in a lateral membrane[4−7] by the direct observation of fluorophores which are incorporated into the membranes.[4] The diffusion constant of phospholipid molecules is 8×10^{-9} cm^2/s in myoblast membranes,[4] 1×10^{-9} cm^2/s in human intact red blood cell membranes,[8] 9×10^{-10} cm^2/s in human skin fibroblast membranes,[8] and 5×10^{-11} cm^2/s in thylakoid membranes.[6]

Whereas, the diffusion constants of lipid molecules in artificial model membranes are much larger than those in cell membranes: 1.5×10^{-7} cm^2/s for a 1,2-dilauroryl-*sn*-glycero-3-phosphocholine (DLPC) monolayer at an air/water interface or at a heptane/water interface[9] and 1.25×10^{-7} cm^2/s for a 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) bilayer at a water/water interface.[10]

The significantly lower diffusion constants in biological membranes compared to those in artificial model membranes have been attributed to "complexity" in biological membranes; for example, cytoskeleton matrixes are anchored to the lipid molecules in membranes through contact with membrane-associated proteins[11] and multilamellar stacked structures such as thylakoid membranes.[6] These structures peripheral to the membrane could produce extra frictional resistance against the membrane. In contrast, three-dimensional liquids peripheral to artificial membranes, such as water or air, show low viscosity, which could produce the low frictional resistance. Currently, there were some studies to measure the lateral mobility by using polymer-tethered or polymer-cushioned lipids under the different condition of "friction" with the adjacent solutions.[12,13] However, the effects of bulk viscosity on the membrane fluidity remain unclear, because the various undefined physicochemical factors are included in these systems. In the present study, we adapted a monolayer at a water/oil interface to shed light on this unsolved problem.

As a model membrane system, we have chosen cell-sized phospholipid-coated microdroplets (CPMDs). CPMDs have frequently been used as cell models, as well as microreactors.[12−16] These pioneering works have shown that, comparing to liposomes with phospholipid bilayer membranes, CPMDs are more useful because they have several advantages such as high resistance to osmotic and physical stress, ease of manipulation, ease of encapsulation of biological molecules, and the ability to kinetically control chemical reactions through the fusion of CPMDs.[14−18] The diffusion of lipid molecules can be measured by using the FRAP method. In this letter, we report the results of FRAP measurements to evaluate the diffusion constants of lipid molecules at a water/oil interface with changes in the viscosity of the oil phase from 10^{-7} to 10^{-10} cm^2/s.

2. Materials and Methods

2.1. Materials. 1,2-Dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE), and 1-oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-glycero-3-phosphoethanolamine (NBD-PE) were purchased from Avanti Polar Lipids. Mineral oil, linseed oil, rapeseed oil, and castor oil (analytical grade) were purchased from Nacalai

* To whom correspondence should be addressed. E-mail: yoshikaw@scphys.kyoto-u.ac.jp. Telephone: +81 75 753 2812. Fax: +81 75 753 3779.

† Present address: Institute of Materials Structure Science, High Energy Accelerator Research Organization, Tsukuba 305-0801, Japan.

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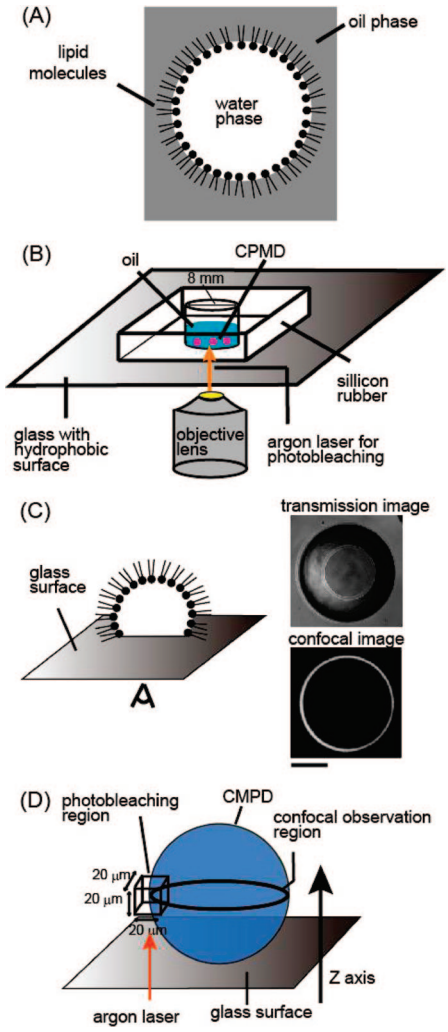

Figure 1. FRAP experiment on a cell-sized phospholipid-coated microdroplet (CMPD). (A) Schematic representation of a CPMD. (B) Experimental setup of the microscopic observation. (C) Schematic representation of a CPMD located on a glass slide (left) and an actual microscopic image of a CPMD with a DOPE membrane before photobleaching (top right, transmission image; bottom right, confocal fluorescence image). The inner ring on the transmission microscopic image is the area in contact with the glass surface. Scale bar is 100 μm. (D) Schematic image of the photobleaching region.

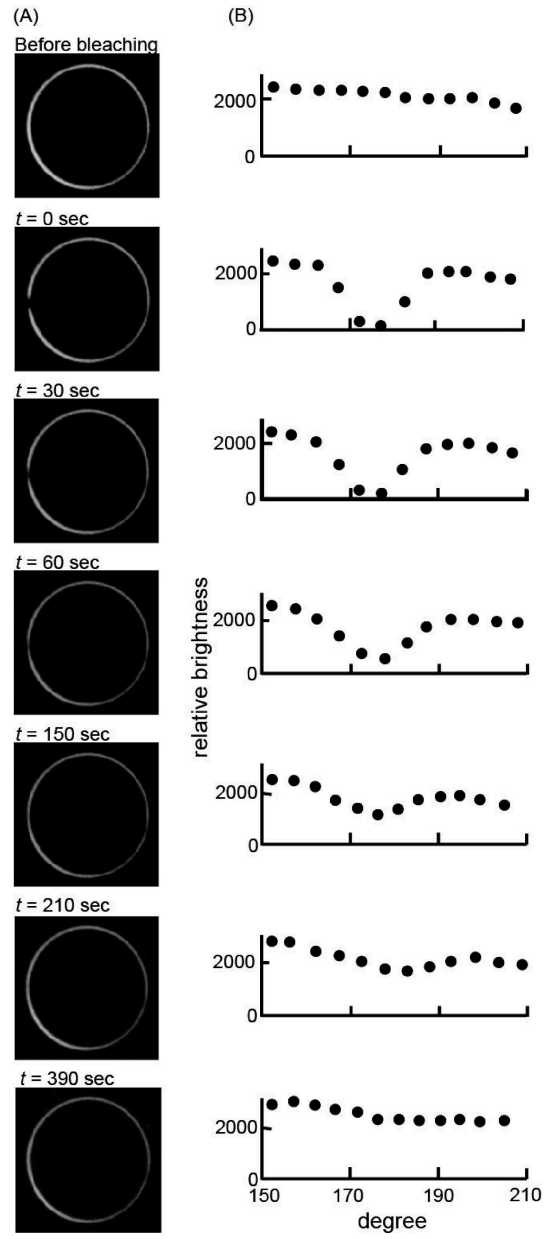

Figure 2. Example of a FRAP experiment on a CMPD. (A) Time-sequence confocal images of CMPD with a DOPE membrane in rapeseed oil. Scale bar is 50 μm. (B) Bleaching profile.

Tesque. All of the chemical compounds were used without further purification. Glass with a hydrophobic surface was purchased from Matsunami Glass Ind.

2.2. Preparation of CPMDs. CPMDs were prepared as follows. First, DOPE or DPPE including 0.5 mol % NBD-PE was dissolved in an oil under ultrasonication for 60 min at 50 °C. The concentrations of total phospholipids were 0.25 mM in all the cases. NBD-PE is a fluorescent lipid analogue to observe the diffusion of lipid molecules directly. After the temperature of oil completely cooled down from 50 to 20 °C, 2 μL of PBS buffer solution (10 mM sodium phosphate including 0.9% NaCl, pH 7.4) was added to 100 μL of oil containing phospholipids in a test tube and mechanically agitated by repeated pipetting for ~100 s, which resulted in the formation of CPMDs dispersed in the oil phase. Figure 1A shows a schematic picture of a CPMD.

2.3. FRAP Measurements. The oil phase with CPMDs prepared as described above was transferred to an observation chamber. This circular chamber (diameter, 8 mm; height, 5 mm) was surrounded by silicon rubber, being situated on a glass with a hydrophobic surface (Figure 1B). This hydrophobic surface made it possible to prevent CPMDs from sticking and spreading on the surface. CPMDs were left still for 1 h at 20 °C before FRAP measurements for the steady state observations. Under these conditions, we have obtained reproducible results in a statistical manner. The lipid membranes of CPMDs stained by the fluorophore NBD-PE was observed by using a confocal laser-scanning microscope (LSM510, Carl Zeiss) with an argon laser of $\lambda = 458$ nm, equipped with a 20× objective lens. The laser was focused on the surface of CPMDs by passing through a 20 μm pinhole. The fluorescence emitted from the sample was monitored through a 505 nm long-pass filter. The transmission image and the confocal image of CPMD located on the glass surface are exemplified in Figure 1C. For the bleaching, the diameter of the laser spot was ~20 μm (Figure 1D). The bleaching time is 34.7 s except for the sample with mineral oil (311.4 s for the sample with mineral oil to perform enough bleaching of NBD-PE spices; the complete bleaching is necessary for the reliable analysis on the diffusion, by adapting the methodology as explained in the following paragraph. As the diffusion of phospholipid molecules is very fast with mineral oil, we performed long-time bleaching in order to attempt the complete bleaching.).

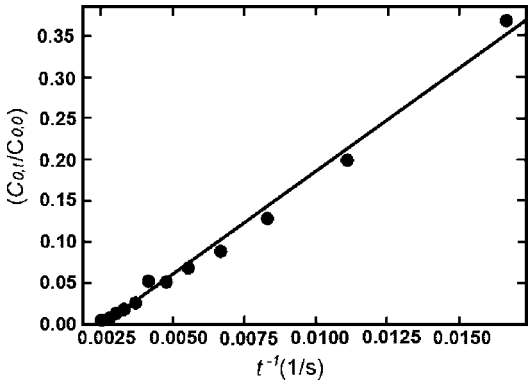

Figure 3. Maximum bleach depth versus inverse of time after photobleaching for a CPMD with DOPE in rapeseed oil. From the slope, the diffusion constant can be determined to be $D = 4.7 \times 10^{-9}$ cm^2/s.

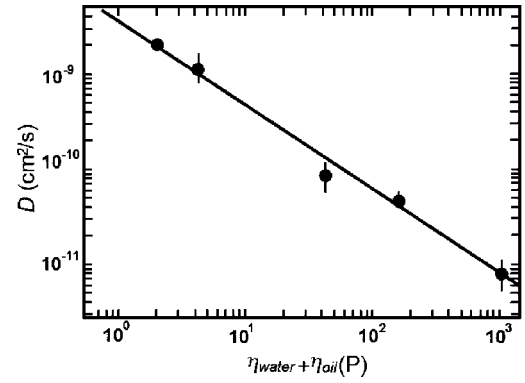

Figure 4. Diffusion constant D of DOPE molecules on the surface of a CPMD versus the viscosity of both bulk phases $\eta_{water} + \eta_{oil}$, which is expressed in units of 1 P = 1 dyn·s/cm^2 = 0.1 P·s. The solid shows the relationship of ln $D = -0.85$ ln($\eta_{water} + \eta_{oil}$) − 6.5.

Table 1. Diffusion Constants D of Lipid Molecules on the Surface of CPMD in Various Kinds of Oil

phospholipid	oil phase	D (cm^2/s, 20 °C)
DOPE	mineral oil	1.2×10^{-7}
	linseed oil	8.3×10^{-9}
	rapeseed oil	4.4×10^{-9}
	castor oil	7.7×10^{-10}
DPPE	rapeseed oil	1.2×10^{-9}

All FRAP experiments were performed at 20 °C. CPMDs with diameters above 200 μm were selected for FRAP measurements. FRAP data were analyzed with a one-dimensional diffusion equation

$$\frac{1}{D}\frac{\partial C}{\partial t} = \frac{\partial^2 C}{\partial r^2} \quad (1)$$

where D is the diffusion constant of lipid molecules, C is the concentration of diffusing species at position r and time t. When the initial bleaching profile is assumed to be described by a Gaussian profile, eq 1 can be solved as

$$C(r,t) = \frac{1}{4\pi Dt}\exp\left(\frac{-r^2}{4Dt}\right) \quad (2)$$

at time t, where the width of the profile is given by $(4Dt)^{1/2}$ and the maximum bleach depth by $1/4\pi Dt$. When the time dependence of the half-width was plotted to calculate the diffusion constants, the regression coefficient by linear least-squares fitting was insufficiently low. In contrast, we confirmed that the regression coefficient is above 90% when the maximum bleach depth was plotted against time. Therefore, we selected the maximum bleach depth to calculate diffusion constants.

3. Results

Figure 2A shows the typical time evolution of confocal fluorescence images of CPMDs coated with DOPE in rapeseed oil. We have chosen DOPE because of the following reason. As PE lipids are inverted-cone shaped phospholipids, we expected that membranes with negative intrinsic curvature will easily form. The white rings show the fluorescence from NBD-PE at the water/oil interface of CPMD, which is uniform before the bleaching. Just after photoirradiation for up to 34.7 s, the bleached spot of ~20 μm appeared. As shown in time-sequence images from "30 sec" to "390 sec", the bleached region gradually recovered. And finally (at 390 s), the bleached region recovered almost completely. This time evolution is attributed to the diffusion of unbleached NBD-PE species into the bleached spot. Figure 2B shows fluorescence intensity profiles. Similar behavior on the recovery shown in Figure 2 was also observed in the other samples.

Figure 3 shows the time dependence of the maximum bleach depth for the sample shown in Figure 2. The maximum bleach depth at each time $C_{0,t}$ is described in relative values based on the depth before bleaching $C_{0,0}$. The data were analyzed by a linear least-squares fitting equation. The diffusion constants of DOPE molecules on the surface of CPMDs in various kinds of oil are summarized in Table 1.With mineral oil, the diffusion constants are on the order of 10^{-7} cm^2/s, which is comparable to those for lipid molecules at an air/water interface, a heptane/water interface,[9] and a water/water interface.[10] Meanwhile, with castor oil, these values are on the order of 10^{-10} cm^2/s, which corresponds to the value seen in biological membranes, 9×10^{-10} cm^2/s.[8]

Figure 4 shows the log−log plot of the diffusion constants D of lipid molecules at a water/oil interface and the viscosity of both bulk phases $\eta_{water} + \eta_{oil}$. In Figure 4, the diffusion constants of DLPC molecules at a heptane/water interface (diffusion constant = 1.5×10^{-7} cm^2/s)[9] were used together with the values we obtained for DOPE molecules. The viscosities of the oil solutions are as follows: 0.6 cP for heptane, 1.9 cP for mineral oil, 43 cP for linseed oil, 164 cP for rapeseed oil, and 1059 cP for castor oil.[19] The viscosity of water is 1.0 cP.[19] When the viscosity of the bulk oil phase is small, the diffusion constant of lipid molecules is large, and the diffusion constants of lipid molecules decrease with an increase in the viscosity of the bulk oil phase. This indicates that the diffusion of phospholipids is limited according to the viscosity of the bulk oil phase. Figure 4 indicates that the relation between the diffusion constants D of lipid molecules and the viscosities of both bulk phases $\eta_{water} + \eta_{oil}$ follows the power law $D \sim (\eta_{water} + \eta_{oil})^{-0.85}$.

To investigate the difference between the diffusion constants of lipids in the gel phase and those in the liquid crystalline phase, the behaviors of DOPE and DPPE in rapeseed oil were compared. The main transition temperature from the gel phase to the liquid crystalline phase of DOPE is −16 °C, and that of DPPE is 61.5 °C.[20] Under the experimental conditions (T = 20 °C), DOPE is in the liquid crystalline phase and DPPE is in the gel phase. As shown in Table 1, the diffusion constant of DPPE in the gel phase is about one-quarter of that of DOPE molecules in the

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liquid crystalline phase, which corresponds to the results of a previous study at an air/water interface.[21]

4. Discussion

It has been well-established that diffusion constants are related to the motility of particles by the Einstein relationship,

$$D = k_B T/f \tag{3}$$

where k_B is the Boltzmann constant, T is the absolute temperature, and f is the friction coefficient in the manner of Stokes. However, when we consider a viscous, two-dimensional membrane, it is impossible to satisfy all of the boundary conditions, that is, Stokes' paradox.[22] Saffman and Delbrück reported a theoretical study to avoid Stokes' paradox. In their approach, a viscous two-dimensional membrane, with a small thickness h and a surface shear viscosity η_s, is embedded in a pair of liquid mediums with a much lower, but finite, viscosity η_1 and η_2, which makes it possible to derive an expression for the friction coefficients:[23]

$$f = 4\pi\eta_s h\left(\ln\frac{\eta_s h}{\eta_1 R} - \gamma\right)^{-1} \tag{4}$$

where $\eta_1 = \eta_2$, γ is Euler's constant (0.5772), and R is the apparent radius of a diffusive molecule.[23]

However, in actual living cellular systems, it is well-known that the membrane is modified by various kinds of networks, such as the cytoskeleton and sugar protein/sugar lipids, inside and outside the cytoplasmic membrane, namely the viscosity of the inside membrane is different from that of the outside membrane. An extended model under the condition $\eta_1 \neq \eta_2$ was proposed by Hughes et al.

$$f = 4\pi\eta_s h\left(\ln\frac{2}{\varepsilon} - \gamma + \frac{4}{\varepsilon} - \frac{\varepsilon^2}{2}\ln\frac{2}{\varepsilon}\right)^{-1} \tag{5}$$

where $\varepsilon = R(\eta_1 + \eta_2)/h\eta_s$.[24] From the experimental result as interpreted above, the diffusion constant is given by $D \sim (\eta_{water} + \eta_{oil})^{-0.85}$. Since the friction coefficient is inversely proportional to D under the Einstein relationship, we obtain

$$(R(\eta_{water} + \eta_{oil}))^{-1} \sim (\eta_{water} + \eta_{oil})^{-0.85} \tag{6}$$

In both models proposed by Saffman and Delbrück[23] and Hughes et al.,[24] the theoretical framework is limited to the condition where the bulk viscosity is much smaller than the membrane viscosity. It is obvious that these theoretical models cannot be applicable for the general living cells and in our CPMDs. In such situations, the most important dissipative effect is considered to be interfacial drag, that is, the transfer of momentum through friction due to the local velocity of the fluid membrane with respect to the bulk phase. In other words, the fluctuation−dissipation relationship is dominated by viscous friction at the surface of the fluidic membrane. Under the approximation that a two-dimensional membrane is an incompressible fluid, we obtain the following empirical relationship:

$$R \sim (\eta_{water} + \eta_{oil})^{0.15} \tag{7}$$

This indicates that the effective size of a lipid molecule increases slightly with an increase in the viscosity of the bulk phase. This is attributable to the two-dimensional specificity of diffusion in contact with a highly viscous medium, which suggests an effect by the collective motion of neighboring phospholipid molecules together with surrounding oil molecules when the viscosity of oil is high.

5. Conclusion

It has become clear that an increase in the viscosity of an environmental solution effectively decreases the diffusibility of phospholipid molecules on a monolayer film at a water/oil interface, $D \sim (\eta_{water} + \eta_{oil})^{-0.85}$. The fractal exponent in this relationship is most probably due to the collective motion of phospholipid molecules together with the bathing oil molecules. Such a significant effect of the environmental solution on the mobility of lipid molecules may be associated with the large decrease in the diffusion constant of a cytoplasmic membrane compared to that of a model phospholipid bilayer membrane, since in living cells the bilayer membrane is supported by a cytoskeleton with very low mobility. Further studies on the effect of the outer environment on membranes may clarify the long-standing problem of the low diffusibility of the cytoplasmic membrane.

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