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Dynamic hydration of phospholipid films in aqueous environments

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Abstract

A dynamic study of the hydration of phospholipid films attached to solid substrates when exposed to liquid water at room temperature is presented. The phospholipids used were 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine (DPPE), and a modification of DPPE with a fluorescent molecular probe: *n*-(5-fluoresceinthiocarbamoyl)-1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylethanolamine, triethylammonium salt (FITC-DPPE). Three different experimental techniques were used: rates of hydration were measured by depositing the phospholipid film on a quartz crystal microbalance (QCM), total internal reflection fluorescence (TIRF) studies were carried out with FITC-DPPE films deposited on the internal surface of a cylindrical quartz tube, and X-ray diffraction analysis was used to determine possible changes of the film's crystalline structure during hydration. The DPPE films exhibited different successive hydration stages: within the first 2 h, the films uniformly hydrate towards a limiting water uptake (short-time behavior); however, at one point, hydration rates suddenly increase and the hydration process continues for longer periods of time, of the order of 24 h. No evidence of change in the film crystalline structure was found when dry and hydrated DPPE films were analyzed by wide-angle X-ray diffraction. The FITC-DPPE films showed a completely different hydration pattern: water uptake reached a maximum value at short times and then decreased continuously until an asymptotic value was reached. The TIRF results on FITC-DPPE films show that the evolution of fluorescence with time closely resembles the hydration results obtained in the QCM. This is attributed to the self quenching occurring in the phospholipid films.

Keywords: Phospholipid hydration; Quartz crystal microbalance; Total internal reflection fluorescence

1. Introduction

Phospholipid membranes provide barriers that regulate the exchange of materials and information between a cells internal compartments and the external environment. The study of the molecular structure of phospholipid aggregates is there-

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fore of great practical interest. In the presence of a polar solvent, such as water, phospholipids have the tendency to form bilayers with the polar head groups exposed to the solvent. This leads to the formation of vesicles, which are spherical aggregates where the lipid bilayer surrounds an aqueous core. Phospholipid vesicles have been used as model membranes. There is also interest in the study of supported membranes, obtained by depositing layers of phospholipids on a solid substrate. A specific number of well-ordered molecular layers can be obtained by using the Langmuir–Blodgett technique to generate the phospholipid film. Alternatively, supported structures can be generated by evaporation of a solution of phospholipid in an appropriate solvent.

Phospholipid headgroups hydrate when exposed to aqueous environments. This phenomenon has been a subject of interest because it is believed to stabilize bilayer structures [1], and may be an essential property in the fusion and endocytosis of biological membranes [2]. The swelling of such membranes has also been studied to determine their ability to absorb and release drugs in medical applications [3,4], and to characterize passive transport across them [5]. It is well known that phospholipids exhibit lyotropic mesomorphism when exposed to water [6].

Hydration and solvent partitioning into phospholipid films have been studied by a variety of experimental techniques [7]. Structure changes during hydration have been monitored by differential scanning colorimetry (DSC) [1,8–11] and X-ray diffraction [7–11]. The dependence of partition coefficients on surface density has been measured by ^{2}H-NMR [5]. Phase transitions have also been identified by NMR techniques [5,12,13]. Surface properties of hydrated films have been ascertained by ellipsometry [14] and SEM [15]. The total amount of water absorption during hydration can be measured directly by gravimetric techniques [15]. Recently, Ariga and Okahata [16], and Wakamatsu et al. [17] measured total water absorption and hydration rates using a quartz crystal microbalance (QCM). In a recent study [18], we have presented results on hydration of phospholipid films in aqueous solutions of surfactants using a QCM to determine film hydration and removal rates due to micellar solubilization.

Most of the studies on phospholipid hydration have been carried out at temperatures greater than the main phase transition temperature (T_c). It is well known that phospholipids exhibit their largest hydration capabilities at temperatures close to T_c [8,16]. However, hydration can also occur below T_c.

In this work, we explore the hydration of supported phospholipid films below T_c. The phospholipids used for this purpose were 1,2-dipalmitoyl - *sn* - glycero - 3 - phosphatidiethanolamine (DPPE), for which $T_c = 63$°C [19] (transition from gel to liquid crystalline phase in the presence of excess water), and DPPE with a fluorescent group covalently bound to the molecule: fluorescein isothiocyanate (FITC-DPPE). The addition of the FITC probe to the DPPE molecule is expected to affect substantially the hydration capabilities of the phospholipid due to the hydrophilic nature of the FITC. This would mean that the use of FITC-DPPE as a fluorescent label when mixed with DPPE would have serious limitations whenever hydration processes take place.

The purpose of this work is to lay the foundation for a study of the removal of phospholipid films from tubes using aqueous media. As a preliminary step, this work will not only analyze water uptake during hydration, but will also report on measurement of hydration rates and structural changes that may occur in the phospholipid film as hydration proceeds. Water uptake as a function of time was measured by means of a QCM, and film structure was analyzed by wide-angle X-ray diffraction. In addition, we performed total internal reflection fluorescence (TIRF) studies during the hydration of films prepared with the fluorescently-labeled phospholipid.

2. Experimental

2.1. Materials

The DPPE used in the experiments was obtained in powder form from Sigma, and was stored in a dessicator at temperatures below −20°C. Stock solutions were prepared by dissolving

10 mg DPPE in 5 ml of a 9:1 v/v chloroform/methanol solution. The DPPE concentration in the stock solution was 2 mg cm^{-3}.

The molecular probe used to make the DPPE molecules fluorescent was fluorescein isothiocyanate (FITC). This probe was selected because its excitation spectrum peak is located at 491 nm, which is very close to the wavelength of an Argon laser (488 nm). Its emission spectrum peak is located at 520 nm. The emission and absorption spectrum peaks are separated enough so that they can be easily distinguished using a narrow band filter. The fluorescent phospholipid is *N*-(5-fluoresceinthiocarbamoyl)-1,2-dipalmitoyl-*sn*-glycero-3-phosphatidyethanolamine, triethylammonium salt (FITC-DPPE). It was also obtained in powder form with a purity above 99.9%. The solutions were prepared by a similar procedure as the DPPE solutions. Both phospholipids (DPPE and FITC-DPPE) are insoluble in water, but the fluorescent probe makes the FITC-DPPE molecule more hydrophilic than the DPPE molecule.

2.2. Quartz crystal microbalance experiments

A quartz crystal microbalance (QCM) was used to determine the dynamic hydration of DPPE and FITC-DPPE films at room temperature (25°C). The balance consisted of a polished At-cut quartz crystal slide. The weighing area had two gold electrodes attached to opposite sides of the quartz slide. An oscillating circuit changes the polarity of the electrodes at the crystals resonant frequency. Any mass that is rigidly bound to the electrode causes a frequency decrease. Changes in mass (Δm) are directly proportional to frequency changes, according to the following relation [20]

$$\Delta m = -\frac{A\sqrt{\rho_q \mu_q}}{2F_0^2}\Delta F \quad (1)$$

where A is the electrode weighing area (consisting of a disk 0.5 cm in diameter), ρ_q is the density of the quartz (2.65 g cm^{-3}), μ_q is its shear modulus (2.95×10^{10} Pa), and F_0 is the crystals resonant frequency ($F_0 = 10$ MHz).

The quartz slide was attached to the side of a reservoir using silicone glue. The frequency shift was measured by means of an Elchema FC-299 frequency meter/generator. The signal was passed through an Elchema DAQ-616 analog/digital converter and data acquisition board mounted in a personal computer.

For these experiments, phospholipid films were obtained by depositing on the electrode 2 μl of a 1 mg cm^{-3} phospholipid solution. The solution was spread over the whole electrode area and allowed to evaporate at room temperature. The amount of material and concentration were selected on the basis of previous studies with a similar system [17]. To record the mass of dry phospholipid on the balance, the frequency was measured before and after film deposition. The balance was then immersed in deionized water and frequency changes were measured as a function of time for a typical period of 20 h. Increases in the measured mass correspond directly to water uptake by the film.

To verify that no portions of the film were removed from the balance during the hydration process, the film was dried after the experiment by removing it from water and placing drops of acetone on the film and allowing the acetone/water to evaporate. The mass of dry film was in all cases within 10% of that of the original film.

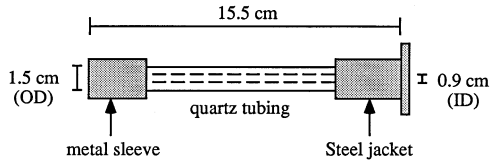
2.3. Total internal reflection fluorescence experiments

Total internal reflection fluorescence (TIRF) was used with the purpose of determining possible changes in structure and thickness of the FITC-DPPE films when they were exposed to static or moving aqueous environments.

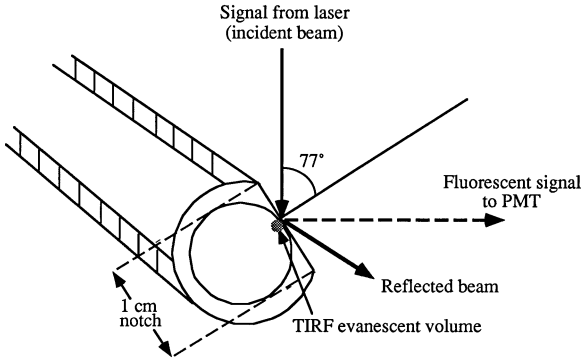
The TIRF experiments were conducted in a fused quartz (General Electric type 214) cylindrical cell with internal and external diameters of 0.9 and 1.5 cm, respectively, and a length of 15.5 cm (Fig. 1(a)). A stainless steel jacket and a stainless steel sleeve were epoxied to the ends of the cell as connections to a solvent flow system. Total internal reflection is obtained when an incident laser beam passes through the relatively high refractive index quartz material and strikes the interface

between the quartz and the water at an angle larger than the critical angle ($\theta_c = \arcsin(n_2/n_1)$, where n_1 and n_2 are the refractive indices of the solid and the liquid, respectively). In order to have the incident laser beam hit a flat surface to simplify the optics, one side of the cylindrical cell was modified by cutting a 1 cm wide notch along the outer surface (Fig. 1(b)). The notch was mechanically polished to minimize light scattering associated with defects on the surface.

The incident light beam was focused to a point on the surface of the test cell and an evanescent wave was generated at the quartz–water interface on the inner surface of the cell (Fig. 1(b)). An incident angle of 77° was selected since it optimized the fluorescent signal measured in this experimental system [21]. The penetration depth of the evanescent wave was determined to be 128 nm, which is always thicker than the phospholipid films used in this study. The evanescent wave



(a) Test cell



(b) Schematic of evanescent volume and beam directions

Fig. 1. Schematic of test cell and generation of evanescent wave.

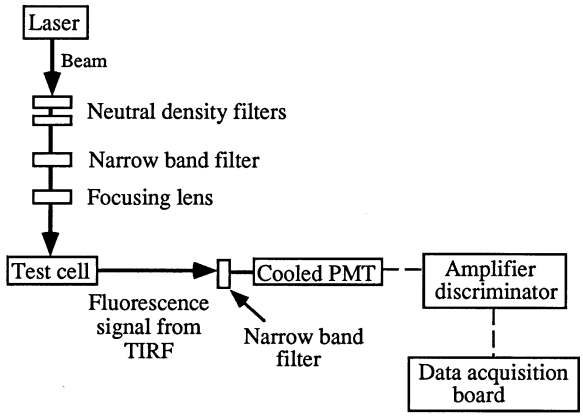


Fig. 2. Schematic diagram of the optical system.

excites the fluorescent material deposited on the internal wall of the cell and the fluorescent signal generated is directed to a photomultiplier tube (PMT).

A schematic of the optical train used is presented in Fig. 2. The optical system was mounted, along with the experimental equipment, on a Newport vibration isolation table; the entire assembly was in a dark enclosure. The incident light beam was generated by a 5 mW Spectraphysics 161-C Ar laser, with a wavelength of 488 nm. The beam was passed through two neutral density filters to lower its intensity, and a narrow band filter to control its wavelength. The beam then passed through a focusing lens before it reached the cell surface. The fluorescent emissions were passed through a narrow band filter and then detected by a photomultiplier tube (Thorn Emi 9924A). The signal from the PMT was amplified by a C604 amplifier/discriminator which sends the analog signal to a C660 counter/timer board installed in a PC.

Supported phospholipid films can be generated by the well-known Langmuir–Blodgett technique. However, Seul and Sammon [22] have reported that uniform films of phospholipids are very difficult to obtain with this technique, and perfectly organized membranes with minimal defects are not achieved. In the TIRF experiments, the phospholipid film is deposited on the inside wall of a cylinder, which would be difficult to achieve using the Langmuir–Blodgett technique. For

these reasons we have selected the evaporative method to prepare the films used in this work. This technique has been widely employed to generate phospholipid films [17,22]. In this method the phospholipid solution is spread on the substrate and the solvent is allowed to evaporate. Seul and Sammon [22] have shown that, although there seems to be more long-term spatial order in membranes created by the Langmuir–Blodgett technique, there is short-term order in membranes generated by evaporation.

The films were prepared by spreading 1 ml of a 25 mM phospholipid solution in chloroform/methanol (obtained by dilution of the corresponding stock solution) on the internal surface of the cylindrical cell while the cell was mounted on a rotating roller. The cell was inclined 25° with respect to the horizontal plane to allow excess solution to run off. This generated films with thicknesses ranging between 80 and 120 nm, as verified by measuring the total mass of phospholipid deposited in the cell. The coating was prepared at room temperature.

The quartz cell was part of a flow system capable of generating a maximum water flow rate of 132 cm^3 min^{-1} (Fig. 3). This flow system was used to fill the test cell with water and to study the effect of laminar flow on the TIRF measurements. It consisted of a peristaltic pump and viton tubing. The cell was mounted in a rotation stage coupled to a precision positioning motor to adjust the incident angle of the laser beam on the cell surface.

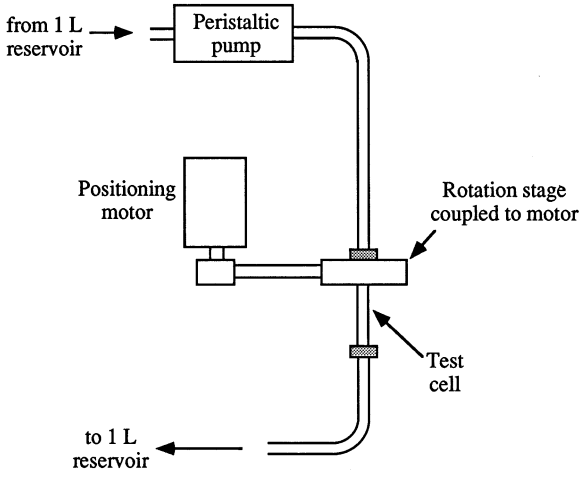


Fig. 3. Diagram of the flow system.

In a typical TIRF experiment, the cell with the FITC-DPPE coating is slowly filled with deionized water so that the phospholipid film is not perturbed by the flow. The film is then allowed to hydrate for a period of approximately 24 h while recording simultaneously the fluorescence emitted as a function of time. In order to study the effects of water flow on the film fluorescence, in some hydration experiments, the pump was turned on and the water allowed to flow over the film for a given period of time. The water flow rate was 60 cm^3 min^{-1} in all cases. The TIRF experiments were performed at room temperature (25°C).

2.4. X-ray diffraction experiments

Wide-angle X-ray diffraction was conducted on dry and hydrated DPPE and FITC-DPPE films to determine their crystalline structural properties and structural changes due to the hydration process.

A Diano 8500 wide-angle diffractometer was used in these experiments. All measurements were performed at room temperature (25°C). For these experiments, the films were made thicker than those used in the TIRF and QCM experiments, to increase the number of structural layers diffracting X rays. These films were prepared by repeated deposition and solvent evaporation of 0.2 cm^3 of a 25 mM solution on a quartz slide 3 cm in diameter. The final mass of phospholipid on the slide ranged between 1.5 and 6 mg. The quartz slide with the deposited phospholipid film was kept in a dessicator prior to the X-ray diffraction measurement of the dry film. X-ray diffraction analyses were done with unresolved $Cu–K_2$ radiation, and all pertinent instrumental parameters, including tube voltage and scanning rate, were kept constant for all experiments.

For the measurement of hydrated films, the quartz slide was immersed in deionized water for a specific period of time, and then it was removed from the water and immediately subjected to X-ray diffraction analysis. Hydration times were of the same order as the periods used in the TIRF and QCM experiments.

3. Results and discussion

3.1. Hydration of DPPE

Previous studies of DPPE hydration in liquid water at a temperature of 20°C using a QCM [17] have shown that films of DPPE and similar phospholipids swell very rapidly and the mass uptake of water stabilizes in ~ 30 min. At this temperature, Wakamatsu et al. [17] report that the final mass of water in a hydrated DPPE film was ~ 5–10% of the original phospholipid mass. Water uptake increases dramatically when the temperature approaches T_c. Our QCM results of DPPE film hydration are shown in Fig. 4(a), where mass of water in the film (m_w) is plotted as a function of time for various values of dry phospholipid mass in the film (m_p, value adjacent to each curve), which is approximately the mass of the film at the beginning of the experiment. In all cases we observed a rapid increase in the water mass followed by a stabilization after ≈ 30 min. This behavior is consistent with the results presented by Ariga and Okahata [16], and Wakamatsu et al. [17]. However, our results indicate that, if the hydration test is continued beyond the first 30 min, there are further increases in water uptake with time. Note that, after the signal has stabilized, there is an abrupt change in the slope of the curves, followed by a new period of stabilization. In fact, for two of the cases depicted in Fig. 4(a), there seems to be three consecutive hydration cycles (curves corresponding to $m_p =$ 1.5 and 1.9 μg).

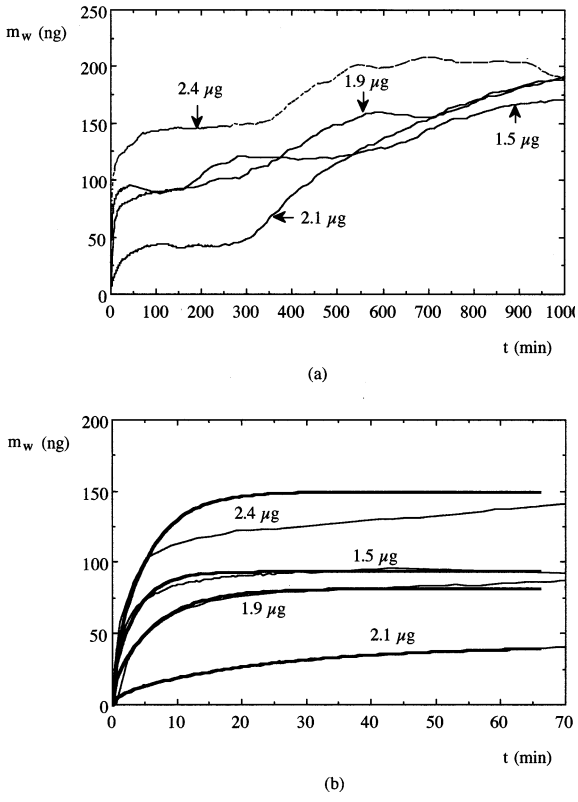


Fig. 4. Hydration of DPPE films in deionized water. Curve labels indicate the mass of the dry film (m_p). (a) Long term hydration. (b) Short term hydration, thick solid lines correspond to the model.

The water mass uptake during the first hydration cycle is between 5 and 10% of the dry phospholipid mass, which is consistent with results reported previously [17]. Table 1 shows the water uptake after the first cycle (m_1) and the ultimate water uptake observed during the time period of these experiments (m_∞). These numbers indicate that after the first cycle there are typically two molecules of water per phospholipid molecule in the hydrated film. This parameter is almost doubled in the final film conformation.

Since the total water uptake during the first cycle is relatively low, we used a diffusion model to characterize the hydration process in which we neglected changes in film volume throughout the process (Fig. 5). Under these conditions, the transient water concentration profiles inside the film, $c(t,z)$, are the solution to the following boundary value problem,

$$\frac{\partial c}{\partial t} = D_w \frac{\partial^2 c}{\partial z^2} \tag{2}$$

$$c(0,z) = 0 \tag{3}$$

$$c(t,\delta) = c_s \tag{4}$$

$$\frac{\partial c}{\partial z}(t,0) = 0 \tag{5}$$

where D_w is the diffusion coefficient of water in the phospholipid film, and c_s is the saturation concentration of water in the film, which is assumed to be achieved instantaneously at the film–

Table 1
Hydration mass of DPPE films as determined by QCM experiments

	Phospholipid mass m_p (μg)	Water uptake in first stage m_1 (ng)	Mols of water per mol of phospholipid	Long term water uptake m_∞ (ng)	Mols of water per mol of phospholipid
Current work	1.5	110	2.8	170	4.4
	1.9	95	1.9	193	3.9
	2.1	40	0.7	195	3.6
	2.4	140	2.2	205	3.3
Wakamatsu et al. [17]	1.3	82[a]	2.7	–	–
	2.1	126[a]	2.1	–	–

[a] T = 20°C.

water interface ($z = \delta$). We will assume that the asymptotic water concentration at the end of the first hydration cycle is also c_s.

The solution of the above problem is [23]

$$\frac{c}{c_s} = 1 - \frac{4}{\pi}\sum_{n=0}^{\infty}\frac{(-1)^n}{2n+1}\,e^{-[(2n+1)\pi/2]^2 D_w t/\delta^2}\cos\left[\frac{(2n+1)\pi z}{2\delta}\right] \quad (6)$$

The total mass of water in the film (m_w) as a function of time can be obtained by a volume integration of this solution. If we assume that the area of film is not a function of time, we have,

$$\frac{m_w(t)}{m_1} = \frac{1}{\delta c_s}\int_0^{\delta} c\,dz \quad (7)$$

(note that the mass of water at the end of the hydration cycle, m_1, corresponds to a film completely saturated). Upon substitution of Eq. (6) and integration we obtain

$$\frac{m_w}{m_1} = 1 - \frac{8}{\pi^2}\sum_{n=0}^{\infty}\frac{1}{(2n+1)^2}\,e^{-[(2n+1)\pi/2]^2 D_w t/\delta^2} \quad (8)$$

This equation has been used to fit the experimental data of the first hydration cycle of DPPE corresponding to the results presented in Fig. 4(a) by using D_w/δ^2 as an adjustable parameter. The results are presented in Fig. 4(b). The model represents adequately the behavior, except for the thickest phospholipid film. By estimating the film thickness using the initial phospholipid mass, we find a diffusion coefficient $D_w = 6.4 \times 10^{-13} \pm 2.5 \times 10^{-13}$ m^2 s^{-1}. This diffusion coefficient is of the order of magnitude of the diffusivity of a small molecule in a crystalline solid, which confirms that the mechanism of water transport into the phospholipid film is by diffusion through the films crystalline structure.

One possible explanation for the existence of two or three consecutive hydration cycles in deposited DPPE films is the occurrence of a change in the film structure as hydration proceeds. The results of X-ray diffraction measurements on the DPPE films, before and after hydration, are presented in Fig. 6. The diffraction traces shown for the dry film show lamellar periodicity up to the 19th order. An average over all the peaks ob-

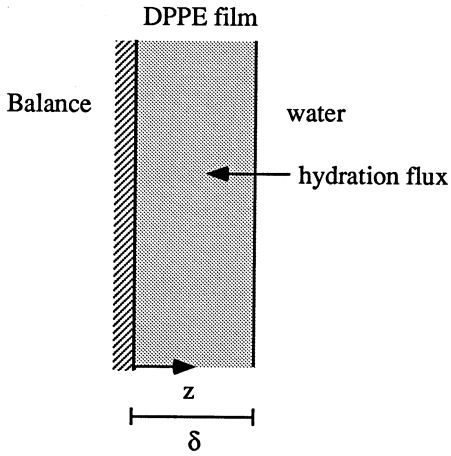


Fig. 5. Coordinate system for the diffusion model of water uptake in DPPE film.

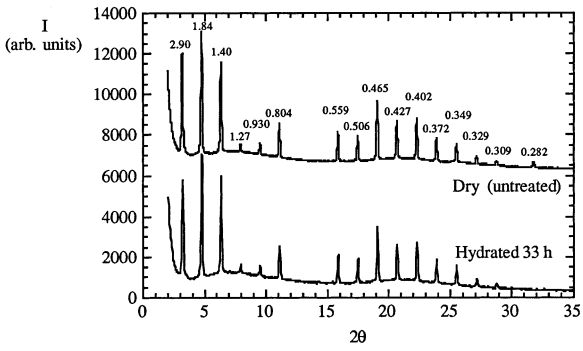


Fig. 6. X-ray diffraction patterns of DPPE films. Hydrated sample was exposed to deionized water for 33 h. Peak values are d-spacings in nm.

tained yields a first order d-spacing of 5.53 nm (the primary peak would be at lower angles than those shown in Fig. 6). This value coincides with those reported by Wakamatsu et al. [17], and Williams and Chapman [6] for DPPE (5.52 nm). This suggest that the films exhibit a multilayer lamellar structure.

The X-ray diffraction pattern obtained after exposure to deionized water for 33 h (Fig. 6) shows that there was essentially no change in the crystalline structure of DPPE upon hydration, except for the disappearance of the 0.282 nm peak. This result indicates that there were no major structural changes in the basic lamellar structure of the film upon hydration, which was expected since the hydration temperature was substantially below T_c. Hydration beyond the first cycle (as evidenced by the QCM results) might be a consequence of the formation of water pockets within the film, i.e., there might be phase separation between water and lamellar phospholipid structures, leading to an increase in the water uptake.

3.2. Hydration of FITC-DPPE

Fig. 7 shows the results of hydration of FITC-DPPE films by QCM analysis. Both long (Fig. 7(a)) and short (Fig. 7(b)) time behaviors are presented. Qualitatively, the pattern of water uptake is different than that observed in the DPPE films (Fig. 4). The film reaches a maximum hydration limit (characterized by a water uptake that we will term m_m) after which the water mass decreases until it reaches an asymptotic value. This behavior is obtained in all films except the thicker film (which corresponds to an initial mass of 3.2 μg of FITC-DPPE). It is interesting to notice that the higher the initial mass, the less sharp the peak in water uptake (Fig. 7). It is important to emphasize that drying the film after the experiments showed no significant loss in phospholipid mass, so that the decrease in water content observed after the maximum is not due to film flaking.

The presence of a maximum in the hydration curves suggests first, that as water is absorbed, the film structure changes to a new conformation whose equilibrium hydration capacity is lower than that of the original structure, and second, that these structural changes occur over times that

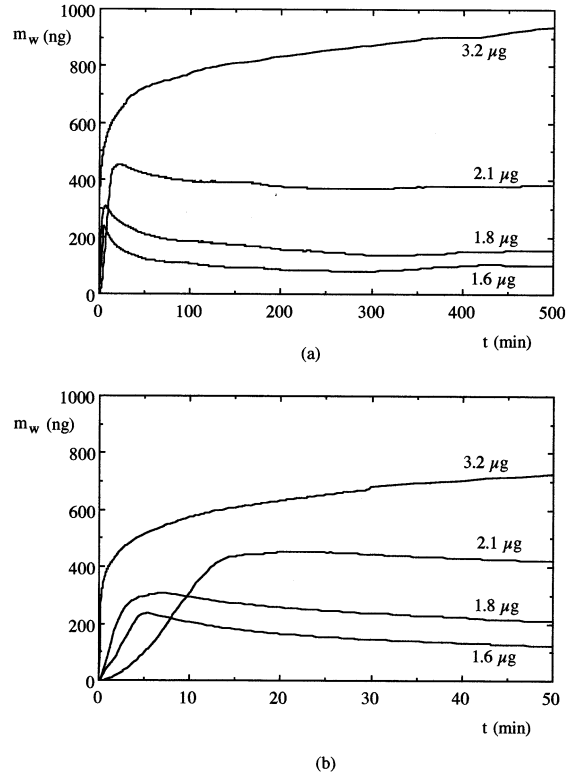


Fig. 7. Hydration of FITC-DPPE films in deionized water. Values adjacent to measured curves indicate the mass of the dry film. (a) Long term hydration. (b) Short term hydration.

Table 2
Hydration mass of FITC-DPPE films as determined by QCM experiments

Phospholipid mass m_p (μg)	Maximum water uptake m_m (ng)	Mols of water per mol of phospholipid	Long term water uptake m_∞ (ng)	Mols of water per mol of phospholipid
1.6	240	4.9	70	1.4
1.8	315	6.5	177	3.6
2.1	450	9.2	410	8.4
3.2	–	–	1000	20.6

are of the same order of magnitude as those of the diffusion process. This leads to a competing effect between the hydration of the parts of the film with high water uptake capability and the rejection of water from the parts of the film adopting the new structure. A reduction in hydration capability of phospholipid films caused by spontaneous phase changes has been observed before for 1,2-dipalmitoyl-L-phosphatidylcholine (DPPC) films [8,24]. In this case, the structure of the phospholipid changed from a gel structure to an orthorhombic crystalline form.

Table 2 shows the water uptake at the point of maximum hydration (m_m), along with the ultimate amount of water present at long times (m_∞) for the films whose hydration curves are presented in Fig. 7. These results show that FITC-DPPE films tend to absorb more water than DPPE films in terms of their maximum hydration (compare with Table 1). This is not surprising if one considers that the fluorescent FITC probe in the phospholipid molecule is hydrophilic. From a practical point of view, this behavior shows the limitations of using FITC-DPPE as a fluorescence substitute for DPPE in processes that include hydration. An interesting observation is that, for FITC-DPPE, the number of water molecules per phospholipid molecule in the film exhibits a substantial increase as the initial film mass increases. Recall (Table 1) that this parameter did not change substantially with initial mass in DPPE films. The increase in hydration capability with film thickness might be an indication that thicker films do not have a uniform structure as hydration proceeds. If the region of the film close to the wall exhibits a structural pattern that is affected by the attachment of the phospholipid molecules to the wall, it is possible that far from the wall the structure resembles more what would be obtained in an unsupported film. Different molecular arrangements between supported films and unsupported swollen gels have been observed in polymeric materials [25].

Total internal reflection fluorescence was performed on FITC-DPPE films deposited in the quartz cell. The results of fluorescence as a function of time under static conditions (i.e. no net water flow) are presented in Fig. 8. The initial point ($t = 0$) in these results corresponds to the moment at which the phospholipid film is brought in contact with water. The three curves for FITC-DPPE represent three different experiments at the same conditions. In addition, we show the response of the system with a coating of DPPE (non-fluorescent) to give an idea of the amount of light scattering detected by the PMT in the experiments. The three curves obtained for the FITC-

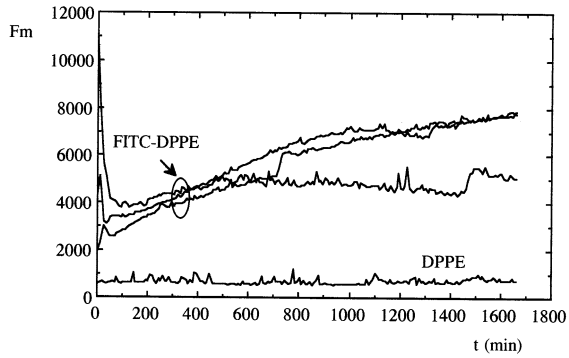


Fig. 8. Fluorescence signal (Fm, in arbitrary units) for phospholipid films exposed to deionized water. The curves for FITC-DPPE correspond to three different experiments at the same conditions. The non-zero DPPE results are due to scattered light.

DPPE film follow approximately the same trend, although substantial quantitative differences exist among them. The trend consists of a sudden increase in fluorescence until a maximum is reached. This is followed by a rapid decrease in fluorescent levels with a subsequent slower continuous increase. The difference observed for the three experiments might be due to differences in the local thicknesses of the three films. The measured fluorescence is sensitive to film thickness, as discussed below. Since the phospholipid layers used in these experiments are obtained by evaporating a flowing film over a curved surface, it is not difficult to imagine that the films are not completely uniform and reproducible. Furthermore, the fact that fluorescence is measured at a point (where the laser light is focused) implies that any variations in film thickness will lead to different fluorescence levels.

We postulate that the observed changes in film fluorescence with time are a result of the effects of hydration and dehydration on the self quenching of FITC-DPPE. An increase in film volume due to swelling after water is absorbed induces a decrease in the local concentration of the fluorophore in the film, which leads to an increase in fluorescence emissions due to inhibition of self quenching. In what follows we will elaborate on quantitative arguments that describe this behavior.

The fluorescence intensity measured by TIRF (F) can be quantified as the convolution of the fluorophore emission profile, $E(z,t)$, and the intensity of the evanescent wave that excites the fluorophore, $I(z)$ [26],

$$F(t) = A_1 \int_0^{\delta} E(z,t) I(z)\, \mathrm{d}z \tag{9}$$

where A_1 is a constant for a given experiment, whose value depends on system geometry and characteristics of the optical detection system, and δ is the film thickness, which is smaller than the penetration depth of the evanescent wave. In Eq. (9) we are assuming that the contribution to the measured fluorescence from possible dissolved fluorescent material present beyond the film is negligible.

The intensity of the evanescent wave decays exponentially with distance from the wall,

$$I = I_0\, \mathrm{e}^{-z/\Lambda} \tag{10}$$

where I_0 is the intensity at $z = 0$ (wall), and Λ is the penetration depth, given by

$$\Lambda = \frac{\lambda_0}{4\pi\sqrt{n_1^2 \sin^2\theta - n_2^2}} \tag{11}$$

where λ_0 is the wavelength of the light in vacuum, n_1 and n_2 are the refractive indices of quartz and water, respectively, and θ is the angle of incidence of the light at the quartz–water interface. In our experiments, $\Lambda \approx 128$ nm and $\theta = 77°$ [21].

If the fluorophore is present in small concentrations and we assume that the film is isotropic, E would be directly proportional to fluorophore concentration, so that a reduction in concentration, caused by swelling of the film due to hydration, would lead to a reduction in measured fluorescence. However, at high fluorophore concentrations, fluorescence self-quenching occurs. In this case, the relation between the fluorescence emission and concentration is nonlinear and it can be quite complex for fluorescently labeled phospholipids [27]. Furthermore, an anisotropic structure of the hydrated film consisting, for example, of alternating layers of possibly hydrated phospholipid and liquid water, would also make the relation between the emission profile and global fluorophore concentration in the film more difficult to obtain theoretically.

As an illustration, and only for qualitative purposes, we will neglect effects of film anisotropy with respect to the hydration process, and assume that Stern–Volmers equation is applicable,

$$E = \frac{K c_{\mathrm{p}}}{1 + k_{\mathrm{s}} c_{\mathrm{p}}} \tag{12}$$

where $c_{\mathrm{p}}(z,t)$ is the global concentration of FITC-DPPE in the film, and K and k_{s} are constants. The parameter k_{s} quantifies the self-quenching mechanism.

Substituting Eqs. (10) and (12) into Eq. (9) leads to

$$F(t) = A_2 \int_0^{\delta} \frac{c_{\mathrm{p}} e^{-z/\Lambda}}{1 + k_{\mathrm{s}} c_{\mathrm{p}}} \tag{13}$$

where $A_2 = A_1 I_0 K$.

The substantial swelling that the FITC-DPPE film experiences, coupled with its internal change of structure with time makes the application of Eq. (13) for quantification purposes difficult. However, in order to have a qualitative idea of the effect of self-quenching on fluorescence, let us consider a uniform phospholipid concentration in the film, so that

$$c_p = \frac{m_p}{A\delta} \tag{14}$$

where m_p is the total mass of phospholipid (which is a constant) and A is the film surface area. In this case, Eq. (13) can be integrated to obtain

$$F(t) = \frac{A_2 m_p \Lambda}{A} \frac{1}{\delta + k_s m_p / A}(1 - e^{-\delta/\Lambda}) \tag{15}$$

where the only parameter that depends on time is the film thickness, δ.

Eq. (15) shows that, for large enough values of the quenching constant k_s, F will be a monotonically increasing function of the film thickness. Since the film thickness increases with water uptake, we can conclude that F will be proportional to the mass of water absorbed by the film, i.e., there is a direct relation between fluorescence intensity and hydration. This is confirmed by the results shown in Fig. 9. In this plot we have superimposed one of the hydration curves obtained in the QCM for FITC-DPPE with one of the TIRF curves. The similarity between the two curves indicates that fluorescence intensity can be considered an indirect measure of film hydration in this case.

X-ray diffraction experiments were conducted with FITC-DPPE films to study the structure of the dry films and to analyze possible structural changes upon hydration. However, within the range of accuracy of the instrument used, the film did not exhibit an ordered structure.

A dehydration–rehydration experiment was performed with the hydrated FITC-DPPE films in order to study the reversibility of the structural changes that may occur during the hydration process. For this purpose, a film was hydrated in the QCM for a period of 24 h and then it was dehydrated by exposing it for 12 h to an atmosphere at equilibrium with a concentrated sulfuric acid solution. Independent mass measurements showed that practically all the water absorbed during the hydration process was removed from the film with this procedure: the initial film mass was 3.2 μg, and its mass after consecutive hydration/dehydration cycles was 3.4, 3.4 and 3.3 μg. Fig. 10 shows the results of an experiment involving three consecutive hydration/dehydration cycles. These results show that after the first dehydration, the new dry film does not absorb appreciable amounts of water. This indicates that the new structure achieved in the film due to hydration/dehydration has a much lower capability to absorb water, and it suggests that there are structural changes occurring in the phospholipid film during the hydration process.

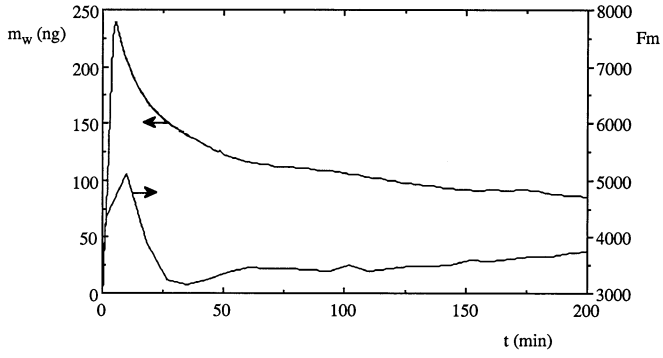


Fig. 9. Combined QCM and TIRF results for FITC-DPPE films. The QCM curve corresponds to 1.6 μg of initial phospholipid mass.

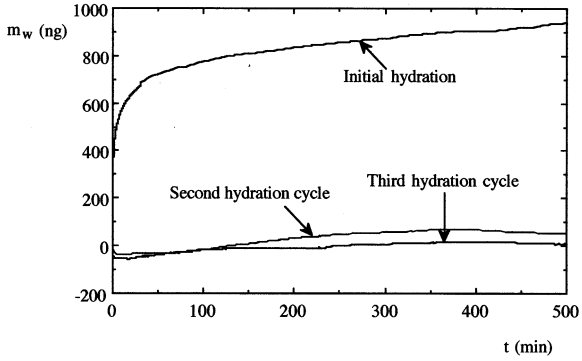


Fig. 10. Successive exposures to water of a 3.2 μg FITC-DPPE film. After initial hydration, the film was dehydrated by exposure to an atmosphere in contact with concentrated sulfuric vapor during 12 h and then subjected to a second hydration cycle in deionized water. The procedure was repeated for a third hydration cycle.

3.3. Behavior of FITC-DPPE films during flow

The results presented in the previous section suggest that TIRF can be used as a technique for measuring dynamic FITC-DPPE film hydration due to the dequenching that occurs during exposure of the film to water. In order to further explore the use of this technique, we recorded fluorescence changes observed when the film is exposed to flowing water. A long-term objective of this type of study is to use TIRF to measure film swelling and removal under applied shear.

Fig. 11 shows the fluorescence intensity recorded when a FITC-DPPE film is exposed to

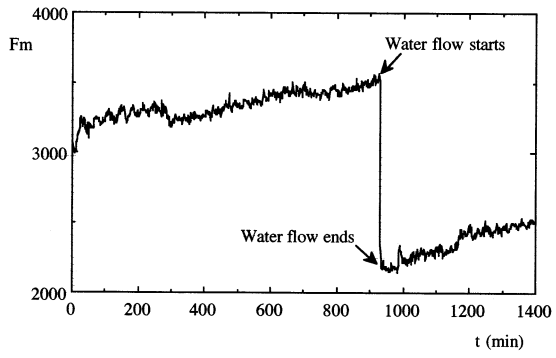


Fig. 11. Typical fluorescence response of FITC-DPPE film when water flow is started and maintained for 5 min at a flow rate of 60 cm^3 min^{-1}.

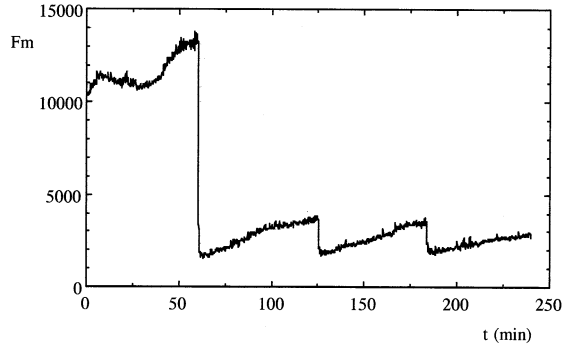


Fig. 12. Fluorescence response of FITC-DPPE film subjected to intermittent water flow every 55 min, maintaining the flow for 5 min at a flow rate of 60 cm^3 min^{-1}.

laminar flow. Initially, without flow, the fluorescent signal increases with time as hydration proceeds. When the flow is started, the intensity abruptly decreases. After 5 min of continuous flow, the flow is stopped and the intensity starts to gradually increase again in the absence of flow. We believe that the reduction in fluorescence intensity is caused by a sudden compression of the film that occurs when flow is started. In fact, if the flow is activated and deactivated repeatedly in succession, we observe a cyclical pattern in the fluorescence signal, as shown in Fig. 12. In these experiments there is no film removal, since the original fluorescence level can be recovered by waiting enough time after the flow is stopped. To confirm that the reduction in fluorescence levels is due to film compression, we performed an experiment in which the exit of the cell was closed and the pressure inside it was increased by turning the pump on for a few seconds. In this experiment, a sudden decrease in fluorescence similar to those shown in Figs. 11 and 12 was observed. After opening the exit so that the pressure relaxed, the original signal was recovered.

4. Concluding remarks

The hydration behavior of supported DPPE and FITC-DPPE films differs substantially, even though the only difference in the structure is due to the addition of a fluorescent group to the

DPPE molecule. The fluorescent probe is hydrophilic, which explains larger water uptake by FITC-DPPE films observed in QCM experiments. X-ray diffraction data showed that the structures of the dry films are substantially different: the DPPE film exhibits a regular lamellar crystalline structure, whereas the FITC-DPPE film appears to be amorphous. Upon hydration, the FITC-DPPE film evolves to a structure with a lower water uptake capability than the original film, so that hydration curves go through a maximum. The complex hydration pattern of the FITC-DPPE films was also reflected in total internal reflection fluorescence measurements due to the self-quenching capability of the FITC-DPPE.

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References

[1] T.J. McIntosh, S.A. Simon, Biochemistry 25 (1986) 4058.
[2] R.P. Rand, Annu. Rev. Biophys. Bioeng. 10 (1981) 277.
[3] A. De la Maza, J.L. Parra, M.T. García, I. Ribosa, J. Sánchez-Leal, J. Am. Oil Chem. Soc. 68 (1991) 315.
[4] I.A. Maxwell, J. Kurja, Langmuir 11 (1995) 1987.
[5] L.R. De Young, K.A. Dill, Biochemistry 27 (1988) 5281.
[6] R.H. Williams, D. Chapman, Prog. Chem. Fats Other Lipids 11 (1970) 3.
[7] G. Cevc, in: E. Westhof (Ed.), Hydration of Biological Macromolecules, Macmillan Press, New York, 1993, p. 338.
[8] M.J. Ruocco, G.G. Shipley, Biochim. Biophys. Acta 691 (1982) 309.
[9] J.M. Seddon, K. Harlos, D. Marsh, J. Biol. Chem. 258 (1983) 3850.
[10] J.M. Seddon, G. Cevc, R.D. Kaye, Biochemistry 23 (1984) 2634.
[11] S.A. Simon, C.A. Fink, A.K. Kenworthy, T.J. McIntosh, Biophys. J. 59 (1991) 538.
[12] F. Borle, J. Seelig, Biochim. Biophys. Acta 735 (1983) 131.
[13] P.R. Cullis, B. de Kruyff, Biochim. Biophys. Acta 436 (1976) 523.
[14] B.U. Rucha, J. Rabe, G. Schneider, W. Knoll, J. Colloid Interface Sci. 114 (1986) 1.
[15] G.L. Jendrasiak, J.H. Hasty, Biochim. Biophys. Acta 337 (1974) 79.
[16] K. Ariga, Y. Okahata, Langmuir 10 (1994) 2272.
[17] K. Wakamatsu, K. Hosoda, H. Mitomo, M. Ohya, Y. Okahata, K. Yasunaga, Anal. Chem. 67 (1995) 3336.
[18] M.T. Colberg, K. Carnes, A.E. Sáez, C.S. Grant, K. Hutchinson, D. Hesterberg, Thin Solid Films, 327–329 (1998) 247.
[19] J.M. Seddon, G. Cevc, D. Marsh, Biochemistry 22 (1983) 1280.
[20] G.Z. Sauerbrey, Physics 155 (1959) 206.
[21] R.M. Addy, A System for the Non-Invasive, Continuous Study of the Removal of a Contaminant Film from the Inner Surfaces of Tubes, M.Sc. Thesis, North Carolina State University, 1995.
[22] M. Seul, M.J. Sammon, Thin Solid Films 185 (1990) 287.
[23] J. Crank, The Mathematics of Diffusion, Clarendon Press, Oxford, 1971.
[24] H.H. Füldner, Biochemistry 20 (1981) 5707.
[25] M.M. Santore, M.J. Kaufman, J. Polym. Sci. B Polym. Phys. 34 (1996) 1555.
[26] F. Rondelez, D. Ausserre, H. Hervet, Annu. Rev. Phys. Chem. 38 (1987) 317.
[27] J.D.A. Shrive, J.D. Brennan, R.S. Brown, U.J. Krull, Appl. Spectroscopy 49 (1995) 304.