Temperatured dependent transitions in several phospholipids measured by ellipsometry

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TEMPERATURE DEPENDENT TRANSITIONS IN SEVERAL PHOSPHOLIPIDS MEASURED BY ELLIPSOMETRY

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The technique of producing regular multilayers of phospholipids according to the Langmuir–Blodgett method is developed. The temperature dependency of thickness and refractive index of multilayers of 1,2-dimyristoyl-sn-glycero-3-phosphoserine (14 : O/14 : O PS) as a function of ionic strength is described and compared with the behavior of randomly oriented layers of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (16 : O/16 : O PC). Multilayers of 14 : O/14 : O PS show several transitions that are dependent on ionic strength. Some of these transitions are not detectable by the usual technique of differential scanning calorimetry because the enthalpy consumed in the transitions is too low.

1. Introduction

The interaction between proteins and phospholipids plays a crucial role in the process of bloodclotting, yet our understanding on a molecular level is still fragmentary.

Although there is conclusive evidence that a negative surface charge of the phospholipids is required for coagulant activity, neither the magnitude of the charge nor the charge density alone are sufficient to account for the ability of the lipids to form a procoagulant surface.

For instance, a direct relationship with the lipid phase transition was recently established by Tans et al. [1] who compared the clot promoting behavior of synthetic mixtures of phosphatidyl serine and phosphatidyl choline with their phase transitions as observed in calorimetry. The results demonstrate that membrane fluidity is as important as a small negative surface charge for full procoagulant activity of the phospholipid/water interface. To study protein–phospholipid interactions occurring at the surface of phospholipid vesicles by means of ellipsometry, we have to construct a surface simulating the outer vesicle membrane which is

known to consist of ordered phospholipid multilayers.

To this end we first obtained a preparation consisting of regular phospholipid multilayers deposited upon a chromium slide. This preparation allows detailed study of temperature-dependent transitions which have been the object of much calorimetric work.

2. Materials and methods

2.1. Materials

The following phospholipids were used:
1,2-dipalmitoyl-sn-glycero-3-phosphocholine (16 : O/16 : O-phosphatidylcholine);
1,2-dimyristoyl-sn-glycero-3-phosphoserine (14 : O/14 : O-phosphatidylserine).

Phosphatidylcholine was prepared by reacylating the cadmium chloride adduct of sn-glycero-3-phosphocholine with palmitic-acyl chloride according to the method of Bear and Buchnea [2]. Phosphatidyl serine was prepared from phosphatidylcholine by enzymatic synthesis as described by Comfurius and Zwaal [3]. All chemicals used were Merck P.A. Chromium-coated glass slides were manufactured by Stabilix, The Hague, The Netherlands.

2.2. Methods

2.2.1. Production of multilayers

Phosphatidylserine (14 : O/14 : O PS) can be spread as a monomolecular layer on water and then transferred to a solid substrate, layer by layer, by repeatedly dipping and withdrawing the substrate vertically through the air/water interface. With this so called Langmuir–Blodgett technique [4] any number of layers can be deposited on such a substrate. The solid substrate was a chromium coated glass slide.

2.2.2. Production of randomly oriented films

Chromium-coated glass slides were cleaned with chloroform, and a small drop (5 μl) of phospholipid dissolved in chloroform (7 mg/ml) was put near the end of one slide, the holder, which is kept horizontal. A second slide, the spreader, is held at an angle of (say) 30° to the horizontal, its long axis parallel to that of the holder. The lower edge of the spreader is placed on the surface of the holder so that the drop is beneath it. The spreader slide is then moved slowly toward the drop until contact is made, whereupon the solution flows evenly along the area of contact. The spreader is then pulled back rapidly and smoothly, but still on the surface of the holder. The thickness of the resulting film depends on the rate of movement of the spreader slide and the angle at which the spreader is held; a larger angle makes a thicker film.

The principle of this technique is used in hematology to study blood films.

2.2.3. Ellipsometer (instrument)

We modified a Rudolf ellipsometer, type 43303-200E. The instrument is automatized by computer-steered stepping motors on the analyzer and polarizer. The minimalization procedure is an automatized manual procedure. The procedure consists of finding the analyzer position that gives minimal light transmission at a fixed polarizer position, then fixing the analyzer, changing the polarizer, and so on. This procedure continuously follows changes of the minimum up to a velocity of about 1.5 deg/s, but changes observed in actual experiments did not exceed 0.2 deg/s.

By this method of automatization we can measure one position every two or three seconds. The accuracy and reproducibility is within 0.01°. Complete description of the apparatus and method we use are given in refs. [5,8].

2.2.4. Calculation technique

The refractive index n and thickness d are calculated directly from observed Δ and ψ values by an iterative procedure [7]. Substitution of the explicit expression for the total reflection coefficients R^p and R^s into the equation

$$R^p/R^s = \tan \psi \exp(j\Delta) ,$$

yields a quadratic form from which a complex value of d can be calculated for given n. The correct value of d is real, so n is adjusted such that the imaginary part of d is minimized.

3. Results

3.1. Stacked multilayers of 14 : O/14 : O PS

Any number of 14 : O/14 : O PS layers can be deposited on a chromium slide in water containing less than 1 μmol Ca^{2+}. The conditions to be fulfilled are a subphase (water) pH between 5 and 5.4, a surface pressure of 25–40 dyn/cm and a low diping speed (4–5 mm/min).

The optical properties of these multilayers show some variation when measured in air by ellipsometry. Thickness and refraction index of the first several layers show fluctuations (see fig. 1 and table 1). While the refractive index and thickness per layer of a 30-layer system range between n = 1.49–1.51 and d = 24.5–28.0 Å. Similar variations are found for multilayers of Ba-stearate [6–8]. When measured in water these variations were much smaller and no significant deviation of the first few layers were observed. The results of 12 experiments in this study were d = 25.9 ± 0.4 Å and n = 1.503 ± 0.002 (mean ± SEM, n = 12).

Preparating multilayers of 16 : O/16 : O PC was not feasible. The first layer is transferred to the chromium slide under the same conditions as 14 : O/14 : O PS. This layer remains on the slide but no other layers adhere to the first. The thick-

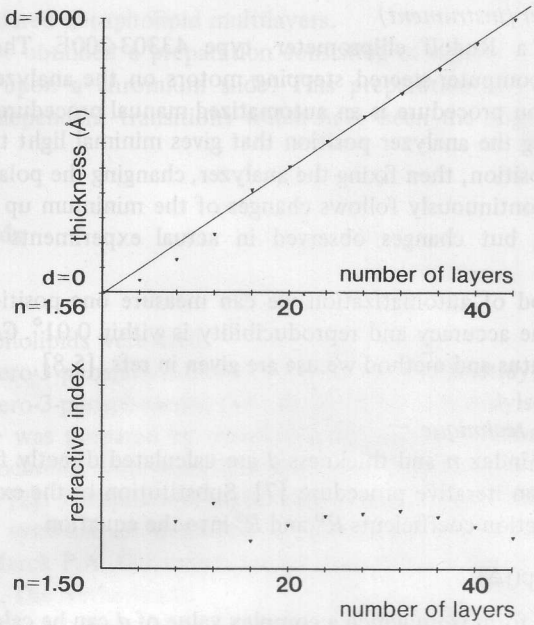

Fig. 1. Stacked layers of 14 : O/14 : O phosphatidylserine on a chromium coated glass slide. Measured in air.

Table 1
Stacked layers of di-$C_{14\text{-}O}$PS

Number of layers	Refractive index	Thickness
4	1.560	67.9
8	1.510	177.1
12	1.514	277.5
16	1.506	454.3
20	1.509	547.1
24	1.511	647.8
28	1.512	746.0
32	1.512	837.9
36	1.511	933.8
40	1.509	1035.4
44	1.507	1131.3

ness and refractive index of the first monolayer is hard to calculate because the accuracy is not high enough to discriminate between different refractive indexes. These thin layers of 16 : O/16 : O PC could not be used to study temperature behavior, so randomly oriented phospholipid films were used.

3.2. Randomly oriented 16 : O/16 : O PC

As a rough impression of the films we used, some numerical values of thickness and refractive index are:

$d = 741$ Å , $n = 1.393$; $d = 1156$ Å , $n = 1.351$; $d = 1058$ Å , $n = 1.367$.

Starting with these layers we studied their temperature behavior.

3.3. Temperature effects

3.3.1. Temperature effect on stacked monolayers of 14 : O/14 : O PS

The temperature was varied over a range of 20–50°C. If the medium in the cuvette contains only Hepes buffer 0.001 M pH 7.0, two transitions at temperatures

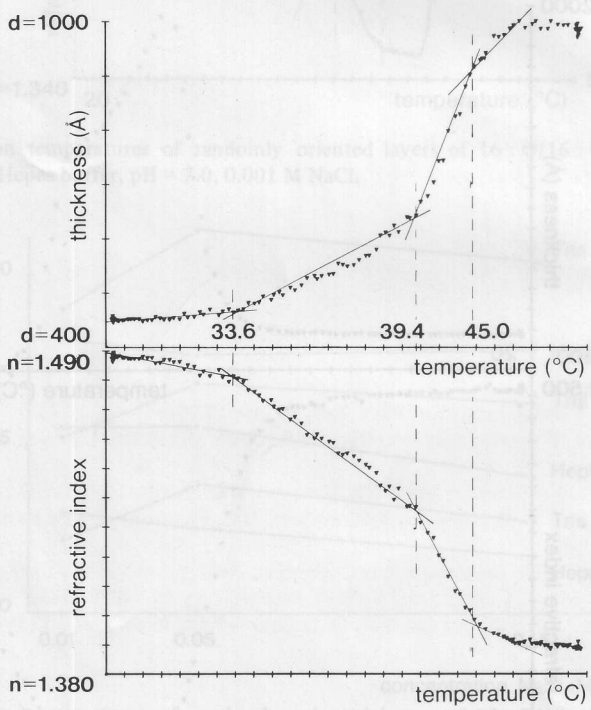

Fig. 2. Transition temperatures of stacked monolayers of 14 : O/14 : O phosphatidylserine; 0.01 M Hepes buffer, pH = 7.0, 0.02 M NaCl.

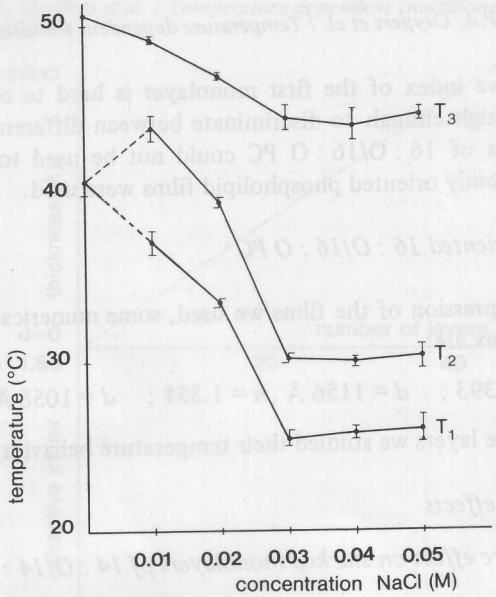

Fig. 3. Transition temperatures of stacked monolayers of 14 : O/14 : O phosphatidylserine as a function of the sodium chloride concentration; 0.001 M Hepes buffer pH = 7.0.

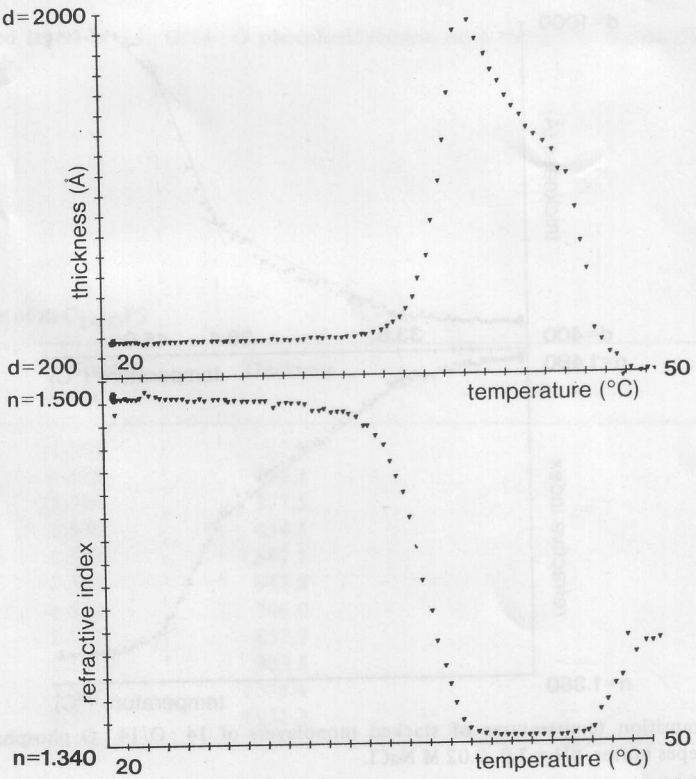

Fig. 4. Transition temperatures of stacked monolayers of 14 : O/14 : O phosphatidylserine in the presence of 10 μM Ca^{2+}; 0.001 M Hepes buffer, pH = 7.0, 0.05 M NaCl.

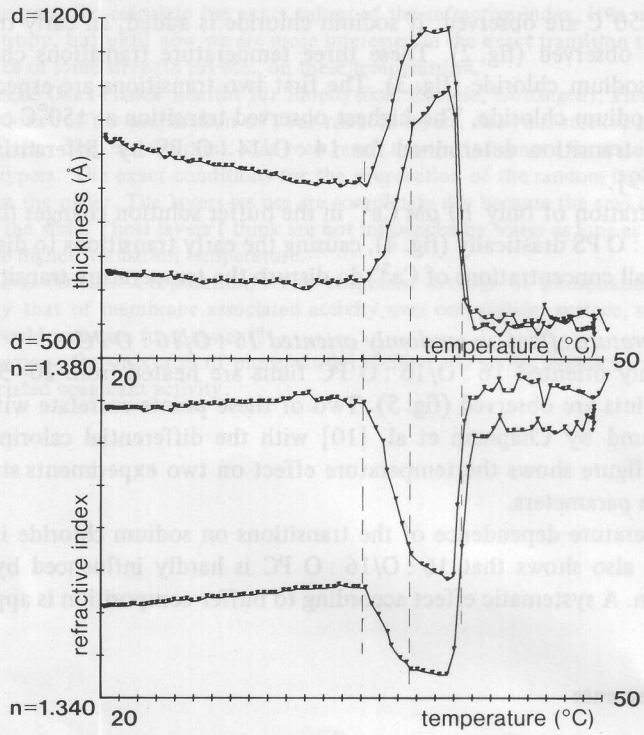

Fig. 5. Transition temperatures of randomly oriented layers of 16 : O/16 : O phosphatidylcholine; 0.01 M Hepes buffer, pH = 7.0, 0.001 M NaCl.

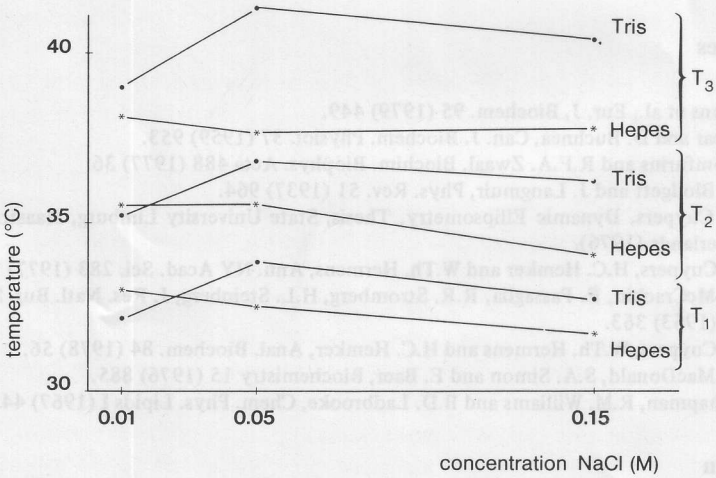

Fig. 6. Transition temperatures of randomly oriented layers of 16 : O/16 : O phosphatidylcholine as a function of the sodium chloride concentration; 0.01 M Hepes buffer, pH = 7.0.

at ±40 and ±50°C are observed. If sodium chloride is added, an early transition at ±30° is also observed (fig. 2). These three temperature transitions change upon addition of sodium chloride (fig. 3). The first two transitions are especially more sensitive to sodium chloride. The highest observed transition at ±50°C corresponds to the phase transition determined for 14 : O/14 : O PS by differential scanning calorimetry [9].

A concentration of only 10 μM Ca^{2+} in the buffer solution changes the behavior of 14 : O/14 : O PS drastically (fig. 4), causing the early transitions to disappear. So even very small concentrations of Ca^{2+} do disturb the temperature transitions.

3.3.2. Temperature effect on randomly oriented 16 : O/16 : O PC

If randomly oriented 16 : O/16 : O PC films are heated from 20–50°C, three transition points are observed (fig. 5). Two of these points correlate with the temperatures found by Chapman et al. [10] with the differential calorimetry techniques. This figure shows the temperature effect on two experiments starting with different film parameters.

The temperature dependence of the transitions on sodium chloride is shown in fig. 6, which also shows that 16 : O/16 : O PC is hardly influenced by the NaCl concentration. A systematic effect according to buffer composition is apparent.

Acknowledgements

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Discussion

G.A. Bootsma (Twente University of Technology): Should you not treat the layers of adsorbed elongated molecules as anisotropic instead of isotropic phases?

P.A. Cuypers: To calculate the exact values of the refractive index, it is necessary to consider anisotropy, but until now we are more interested in the exact transition temperatures and the influence of ionic strength pH etc., on these temperatures.

E. Kruecke (Max-Planck Institut für Biophysikal. Chemie, Göttingen): Please let me know the instructions for the preparation of your random layers. Are your stacked layers dry or wet and, in the latter case, do you think that your result may be influenced by water?

P.A. Cuypers: The exact conditions for the preparation of the random layers is completely described in the paper. The layers we use are completely dry because the acyl chains are on the outside of the slide. These layers I think are not influenced by water as long as the temperature is below the highest transition temperature.

R.B. Davis (Dionex Corporation): The coagulant activity of phosphatidyl serine is not matched by that of membrane associated activity over one platelet surface, so called platelet factor 3. Would you care to comment?

P.A. Cuypers: Our mixtures of phosphatidyl choline and serine appear to reproduce membrane associated coagulant activity.