Production of thrombin as a probe for mixing of phospholipids in membranes on solid supports

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Abstract

Phospholipid-covered solid supports have been used successfully as model membranes in studies on blood coagulation and other research fields. In order to produce such membranes, simple exposure of the support to suspensions of phospholipid vesicles was recently introduced, but questions have remained about the process of vesicle adherence to the surface and the physico-chemical properties of the resulting membranes. Using a new technique, mixing of phospholipids in such membranes was demonstrated. A rotating, hydrophilic, silicon disc was exposed in a two-step procedure to vesicles prepared from mixtures of dioleoylphosphatidylserine (DOPS) and dioleoylphosphatidylcholine (DOPC). Factor Xa, factor Va and prothrombin were added and the transport-limited production rate of thrombin was measured. For low surface coverage with 40% DOPS/60% DOPC, a much higher conversion rate was found if, prior to addition of coagulation factors, excess DOPC vesicles were added to fill up vacant surface area. It is concluded that DOPS is spread over the entire surface and that confluent bilayers are formed. The presented technique may also be used to measure lateral diffusion constants.

Keywords: Thrombin; Phospholipid membrane; Coagulation factor

1. Introduction

Phospholipid membranes on solid supports are becoming increasingly important model systems in various research fields such as protein–lipid interactions in blood coagulation [1,2], antibody–membrane interactions [3,4], production of blood-compatible surfaces [5,6], and charge transport by integral membrane proteins [7,8]. In most of these studies, planar phospholipid mono- and double-layers were deposited on slides, using a film balance and the classical Langmuir-Blodgett dipping technique [9]. A more simple technique, exposure of the support to a suspension of phospholipid vesicles, was first introduced in membrane receptor studies [10] and has recently been used more frequently in other areas [11–16]. Although this technique is simple, few details on the mechanism of membrane formation and physico-chemical properties of the resulting membrane are known. It was demonstrated recently that, in the resulting membrane, only proteins on the outside of vesicles are exposed [17], but although proteins apparently do not undergo significant transmembrane flip-flop, this does not prove that the lipids from outer and inner bilayer leaflets are not scrambled during membrane formation. For hydrophilic glass surfaces, it has been suggested that vesicle adsorption results in bilayers separated from the surface by a water layer of about 2 nm thickness [18]. This would explain normal values for the lateral diffusion constants of lipids [14]. On the other hand it was reported that proteins incorporated in such layers are immobilized, indicating direct interaction between membrane and surface [16]. In this study, normal lateral mobility was only found when the surface was first covered with a separate monolayer of lipids.

In the presence of calcium and proper (procoagulant) phospholipids, the complex of activated coagulation factors Xa and Va (prothrombinase complex) will adsorb on the phospholipid membrane, and will convert prothrombin to thrombin. This conversion is so efficient that, even for Xa-Va surface concentrations as low as a few fmol per cm^2, it becomes transport-limited, that is, limited by the rate of transport of prothrombin from the buffer solution to the membrane surface [15]. Because such transport is dependent on surface area, a few small 'hot spots' of procoagulant lipid will produce thrombin much slower,

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than when this procoagulant lipid is allowed to diffuse into a larger surface area of non-procoagulant phospholipid. In the present study, this principle is used to demonstrate lateral mixing of phospholipids in vesicle-deposited membranes on hydrophilic silicon surfaces.

2. Materials and methods

2.1. Preparation of SUV

Phospholipids were purchased from Avanti Polar Lipids. A mixture of dioleoylphosphatidylserine (DOPS) and dioleoylphosphatidylcholine (DOPC) in chloroform was dried under nitrogen, and 1 ml of buffer (50 mM Tris-HCl, pH 7.5, with 0.1 M NaCl) was added to a final phospholipid concentration of 1 mM. The turbid mixture was sonicated for 10 min with a MSE Soniprep at 150 W and 7.5 μm amplitude. The clear vesicle suspensions thus obtained were characterized by thin film cryo-electron microscopy demonstrating small unilamellar vesicles (SUV) with a size distribution of 10–40 nm (median 19 nm) diameter [19].

2.2. Ellipsometry

Adsorption of phospholipids on silicon surfaces was measured by ellipsometry. This is an optical technique for the measurement of changes in light polarization due to reflection. Such changes are strongly influenced by the presence of the lipid layer on the reflecting silicon surface and allow measurement of phospholipid surface mass Γ with a precision of about 5 ng/cm^2. Measurements were made in a cuvette containing 4–6 ml of buffer and a glass holder in which a reflecting silicon disc, described below, was rotated at stable velocity of 745 rpm. The instrument and analysis of data have been described [1,20].

2.3. Coverage of silicon discs with phospholipids

Silicon discs of 1.6 cm diameter and 0.4 mm thickness were cut from polished silicon wafers (Wacker-Chemitronic, n-type, phosphorus-doped) and, with a drop of melted paraffin, attached to PETP (polyethylene terephthalate or Arnite, AKZO Plastics) cylinders of 1.4 cm diameter and 0.3 cm thickness. These cylinders each contained two tiny magnets. The ensemble was thoroughly cleaned with detergent (Sparkleen, Calgon) and water. The polished silicon(oxide) surface was kept overnight in 30% chromic sulfuric acid (Merck), applied to the surface of horizontally placed discs, flushed with excess deionized water (Millipore Milli-Q3 system), and stored in deionized water. Prior to use they were again rinsed with detergent and deionized water, resulting in a highly hydrophilic surface, and placed in a cylindrically shaped polyacrylate vessel. This vessel was filled with 2 ml of buffer, containing 3 mM of calcium (CaCl$_2$), and contained at its bottom a cylindrical cavity enclosing the PETP cylinder and supporting the slightly protruding edge of the silicon disc. This allowed rotation of the ensemble by means of a rotating magnet below the vessel. Due to a larger distance between the rotating magnet and the PETP cylinder, a lower rotation rate of 286 rpm was used. SUV were added to a final concentration of 20 mM and allowed to adsorb for 5 min. Thereafter the vessel was thoroughly flushed with 50 ml of buffer. A glass slide (2.5 × 8 cm) with a magnet glued on one side was dipped into the vessel and the silicon disc was attracted towards its other side. Because of the buffer layer entrapped between silicon and glass surfaces, the silicon disc could thus be transported to the ellipsometer cuvette without exposure of phospholipids to air.

In experiments in which the silicon disc was only partially covered with phospholipids, such coverage was not performed in the polyacrylate vessel but in the ellipsometer cuvette, while the degree of coverage was directly followed by ellipsometry. When the required surface mass was reached, adsorption was terminated by rapidly flushing the cuvette with 50 ml of buffer in 5 s. In order to get rid of phospholipids adhering to the cuvette wall, which may significantly contribute to prothrombin conversion, see below, the disc was removed with the glass slide from the cuvette, the cuvette was rinsed with detergent and excess water and the disc was reintroduced into the clean cuvette filled with fresh buffer.

2.4. Measurement of transport-limited conversion of prothrombin

Bovine prothrombin and activated coagulation factors Xa and Va were prepared as described [15]. The buffer used in these experiments contained 0.5 g/l of bovine serum albumin (Sigma, No. A7030, fatty-acid free), in order to minimize aspecific adsorption of coagulation factors to the cuvette walls. Factor Va (0.5 nM), factor Xa (0.5 nM) and CaCl$_2$ (3 mM) were added to the buffer and the assembly of the prothrombinase complex on the phospholipid membrane was allowed to proceed for 10 min [21]. Thereafter, 20 nM prothrombin was added and the rate of thrombin production was measured by determination of thrombin concentrations in 0.1 ml samples taken from the cuvette 1, 2, 3, 4, 6 and 8 min after addition of prothrombin. Thrombin was measured by spectrophotometry, using the chromogenic substrate S-2238 (Kabi Diagnostics) as described [15]. Under these conditions, conversion of prothrombin is strictly dependent on the presence of calcium.

Under these circumstances, the prothrombin-converting capacity formed on the membrane is so high that the conversion rate is limited by the maximal transport rate of prothrombin from the buffer solution towards the surface (transport-limited conversion). The rotating disc has the

special feature of uniform accessibility, that is, the flow component towards the surface is identical all over the surface and the rate of transport of prothrombin by diffusion and convection will therefore not be site-dependent. This implies that, in order to obtain the rate of conversion per cm^2, the overall rate of thrombin production can be simply divided by the total surface area of the disc. Also, the maximal transport-limited conversion rate equals ΔC_{bulk} with C_{bulk} the buffer concentration of prothrombin and Δ the mass-transfer coefficient equal to $\Delta = 0.62\,D^{2/3}\nu^{-1/6}\omega^{1/2}$, with D the diffusion constant of prothrombin ($D = 6.2 \cdot 10^{-7}\ cm^2\ s^{-1}$), ν the kinematic viscosity of the solution ($\nu = 0.01\ cm^2\ s^{-1}$) and ω the rotation rate of the disc ($\omega = 78\ rad\ s^{-1}$) [20]. Inserting these values, the maximal conversion rate obtained for $C_{bulk} = 20$ nM and a total disc surface of $\pi(0.8)^2 = 2.0$ cm^2 becomes 34 fmol s^{-1}.

3. Results

Fig. 1 presents examples of 40% DOPS/60% DOPC-vesicles adsorbing on rotating (78 rad s^{-1}) silicon discs, as directly measured by ellipsometry. The upper curve shows adsorption up to a maximal surface coverage of 0.43 ± 0.02 $\mu g\ cm^{-2}$ (mean ± S.D.) after addition of 10 μM of phospholipid. Using lower phospholipid concentrations, such as 3 μM in the lower curve, a required degree of coverage of the surface was obtained by allowing the adsorption to proceed for sufficient time and then flushing the cuvette as indicated. After such flushing, no desorption of phospholipids was observed. In the concentration range of 1 to 10 μM of phospholipid, the mass transfer coeffi-
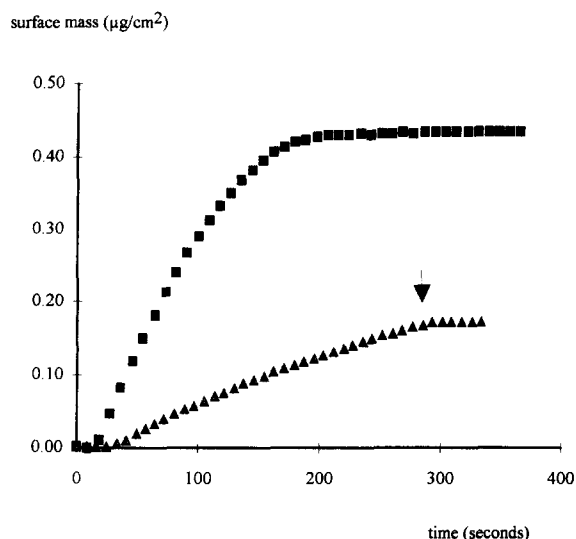
surface mass ($\mu g/cm^2$)

Fig. 1. Adsorption of 10 μM (squares) and 3 μM (triangles) vesicles (40% DOPS/60% DOPC) on a hydrophilic rotating silicon disc, measured by ellipsometry. In the latter experiment, adsorption was terminated by flushing the cuvette with fresh buffer at the time indicated by the arrow.
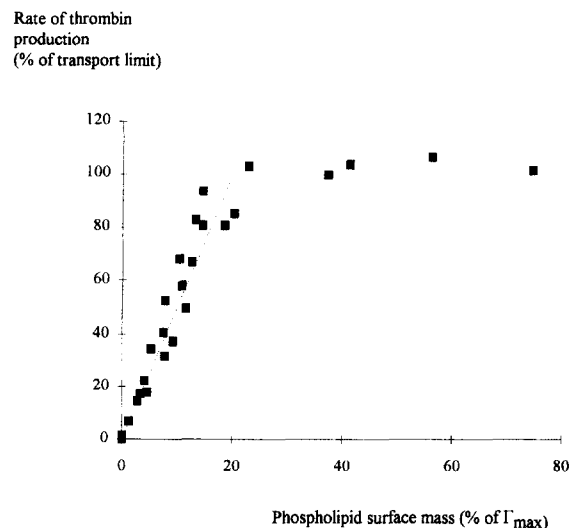
Rate of thrombin production (% of transport limit)

Phospholipid surface mass (% of Γ_{max})

Fig. 2. Rate of thrombin formation, expressed as percentage of the transport limit of 34 fmol s^{-1}, as a function of surface coverage with 40% DOPS/60% DOPC, expressed as percentage of the maximal surface mass of 0.43 $\mu g/cm^2$. The best fit straight line through data points up to 5% surface coverage is indicated.

cient Δ, calculated as the rate of phospholipid adsorption ($\mu g\ cm^{-2}\ s^{-1}$) divided by the bulk concentration of phospholipid ($\mu g\ cm^{-3}$), was constant and equal to (5.3 ± 0.3)·$10^{-4}\ cm\ s^{-1}$.

Fig. 2 presents rates of prothrombin conversion as a function of the degree of surface coverage with 40% DOPS/60% DOPC-vesicles. Conversion rates were expressed as a percentage of the theoretical maximal value of 34 fmol s^{-1}, obtained for transport-limited conversion (cf. Materials and methods). It follows from this figure that the maximal value is already obtained at about 25% surface coverage and this situation is schematically depicted in Fig. 3. Apparently, at low surface coverage the phospholipid layer consists of small separate patches, acting as local sinks that attract substrate from all directions. Each patch is thus able to convert the amount of prothrombin transported to a considerably greater surface area than the patch area itself. If, in contrast, the phospholipid layer would consist of a few large fused areas, boundary effects would be negligible and the conversion rate would simply correspond to the percentage of total surface covered.

If for very low surface coverage with phospholipids, the lipid patches would consist of isolated vesicles and would be so few that the concentration of prothrombin at the surface remained close to the buffer concentration C_{bulk}, the maximal conversion rate would be equal to the collisional-limited rate $v_c = 4\pi n(D_v + D_p)(R_v + R_p)C_{bulk}$ [22], with n the number of adsorbed vesicles and D and R the diffusion constant and radius of vesicles (v) and prothrombin (p), respectively. A factor 1/2 should be added to this expression because prothrombin molecules can only approach the surface from one side. Also the value of D_v for the vesicles attached to the surface can be neglected
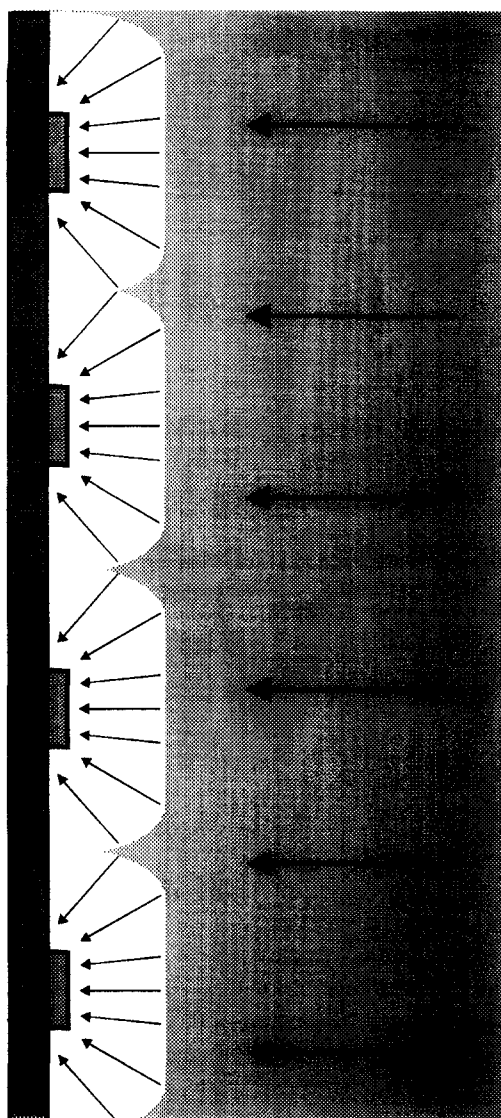
Fig. 3. Schematic illustration of prothrombin transport towards isolated 'hot spots' of procoagulant phospholipid (see text).

$S = 4\%$ of 2 cm^2 disc area $= 0.08$ cm^2, one obtains $v_c = 640$ fmol s^{-1}. This value could of course not be observed, because it much exceeds the transport limit of 34 fmol s^{-1}, but the best fit straight line through the initial data points in Fig. 2 shows a conversion rate of only 18% of the transport limit, or 6.1 fmol s^{-1}, for 4% surface coverage with lipid. (In that case, the prothrombin concentration in the buffer at the surface will indeed be more than 80% of C_{bulk}, as was assumed.) Apparently only about 1% of the collisions between prothrombin molecules and adsorbed vesicles will result in conversion, and this collisional efficiency is in the same range as the value of 5% measured in vesicle suspensions [15].

Table 1 shows the effect of enlarging available phospholipid area on the conversion rate of prothrombin. The upper half shows experiments from Fig. 2 with approx. 4% surface coverage, while the lower half shows similar experiments in which, after removal of the 40% DOPS/60% DOPC-vesicles by flushing, 20 μM of 100% DOPC vesicles was added to the cuvette and allowed to adsorb for 5 min. This procedure results in additional adsorption of phospholipid to a maximal value of 0.43 μg cm^{-2}, similar to the value found for maximal surface coverage with 40% DOPS/60% DOPC. It was not attempted to determine prothrombin conversion rates before and after suppletion with DOPC in the same experiment, because irreversible adsorption of albumin on the empty part of the silicon surface inhibits subsequent adsorption of DOPC vesicles.

It follows from Table 1 that suppletion with DOPC increased the rate of prothrombin conversion with a factor of 5 to the transport limit. Control experiments with 2% DOPS/98% DOPC, approximately equal to the final composition of the membrane if complete mixing of the lipids would occur, also resulted in transport-limited conversion of prothrombin. Together, these results strongly indicate lateral mixing of DOPS with suppleted DOPC. If no such mixing would have occurred, the rate of prothrombin conversion would have remained unaltered or even would have decreased because of steric hindrance of lateral transport. Control experiments with complete coverage of the

compared to D_p. The lowest surface coverages studied were about 4% (see Fig. 2). Taking $D_p = 6.2 \cdot 10^{-7}$ cm^2 s^{-1}, $R_v = 10$ nm, $R_p = 3$ nm and $n = S/4\pi R_v^2$, with

Table 1
Effect of suppletion with DOPC on prothrombin-converting activity

Initial surface mass Γ of 40% DOPS/60% DOPC expressed as percentage of Γ_{max} (0.43 μg/cm^2)	Suppletion with 100% DOPC vesicles	Rate of thrombin production expressed as percentage of transport limit (34 fmol s^{-1})
3.6	no	16
4.3	no	21
4.8	no	17
4.4	yes	104
5.0	yes	106
4.2	yes	88
Thrombin production in three control experiments after maximal surface coverage (0.42 ± 0.03 μg/cm^2) with 2% DOPS/98% DOPC vesicles		97
		96
		106

surface with 100% DOPC did not produce any measurable conversion of prothrombin.

4. Discussion

4.1. Lateral and transmembrane (flip-flop) mixing of phospholipids

The present demonstration of lipid mixing in vesicle-deposited membranes does not contradict the finding that, after membrane formation, only outwardly directed transmembrane proteins were exposed to the surrounding medium [17]. In fact, only lateral mixing of lipids is required to explain the enhancement of thrombin conversion by diffusion of procoagulant lipid into the non-procoagulant area, and such lateral mobility was also demonstrated for fluorescent phosphatidylethanolamine by means of photobleaching recovery [14]. Also, the absence of transmembrane flip-flop of proteins does not imply a similar restriction for phospholipids.

The data on prothrombin conversion in the present study indicate that differences between the DOPS content of the inner and the outer membrane leaflet must be limited. For low percentages of DOPS, such as 2% DOPS/98% DOPC, the prothrombin converting capacity of the bilayer is a sensitive function of DOPS content. The membranes formed by initial adsorption of 40% DOPS vesicles, up to 5% of total bilayer mass, and suppletion of 100% DOPC vesicles, should contain 2% DOPS in the outer leaflet after complete mixing of lipids. Indeed, the prothrombin converting capacity of these membranes equalled the capacity of the bilayers formed directly from 2% DOPS vesicles (Table 1). It is concluded that either the DOPS/DOPC ratio in both leaflets of the original 40% DOPS vesicles is not different [19], or such a difference vanishes during the formation of the bilayer.

4.2. Evidence for transport-limited adsorption of phospholipid vesicles on the silicon disc

Assuming transport-limited adsorption, the mass transfer coefficient for vesicle adsorption, $\Delta = 5.3 \cdot 10^{-4}$ cm s^{-1}, corresponds to a diffusion coefficient $\Delta = 3 \cdot 10^{-7}$ cm^2 s^{-1}. This is somewhat higher than the value of $(1.8–2.2) \cdot 10^{-7}$ cm^2 s^{-1}, reported in most studies on sonicated vesicles [23–25]. Also, inserting D into the Stokes-Einstein relation $D = kT/6\pi\eta R$, one obtains a value of $R = 7.3$ nm for vesicle radius which is in the small range of the distribution of vesicle size as determined by electron microscopy [19]. If the adsorption would not be transport-limited, an even higher value of the diffusion constant would be required to explain the observed rate of vesicle adsorption. It is concluded that the data are compatible with a high affinity of the hydrophilic silicon surface for sonicated phospholipid vesicles, result-ing in transport-limited adsorption. Apparently, this affinity is not dependent on net membrane charge because 100% DOPC vesicles adsorbed equally fast.

4.3. Evidence for the formation of confluent bilayers from adsorbed vesicles

Formation of a confluent phospholipid layer from adsorbed vesicles is suggested by the observation that, for low surface concentrations of Xa-Va (0.3 fmol cm^{-2}), the conversion rate of prothrombin is such that a single Xa-Va complex converts the amount of prothrombin adsorbing to about 0.3 μm^2 of surface area, that is about 1000-times as large as the surface area of the Xa-Va complex itself [15]. Apparently, prothrombin adsorbing at some distance of the Xa-Va complex is laterally transported towards this complex and the simplest mechanism for such transport would be lateral diffusion across a confluent membrane. However, this argument is not compelling because such transport could also be effected by short-distance diffusion through the solution or 'hopping' of protein molecules, which are of the same order of magnitude as the vesicles, across vesicle clusters adhering to the surface (see Fig. 3). Maximal, transport-limited, production of thrombin was also observed for only 25% of maximal surface coverage with lipid (Fig. 2), and a confluent bilayer can obviously not exist in this situation.

Assuming homogeneous coverage with circular 40% DOPS islands, the finding that only 25% surface coverage is required for transport-limited conversion of prothrombin (Fig. 2) could in principle be used for estimation of island dimensions. Such estimation, however, asks for prior accurate determination of the intrinsic K_m and k_{cat} values for prothrombin conversion on 40% DOPS membranes, and would also require a major computational effort, falling outside the scope of the present study.

The standard Blodgett-technique for deposition of confluent bilayers on solid supports uses dipping of the support into a film balance covered with a monolayer of phospholipid. Using this technique, ellipsometric measurements [26] have produced values of 0.40–0.44 μg/cm^2 for the surface mass of DOPS/DOPC bilayers deposited on silicon surfaces similar to the ones used in the present study. These values correspond to surface area's of 0.65–0.70 nm^2 per phospholipid molecule. The similarity between these values and the value for vesicle-deposited membranes in the present study suggests that the adsorbed vesicles also form a confluent bilayer, but again this evidence is not compelling. It follows from simple geometry that a surface covered with close-packed vesicles would contain about 2.3-times more phospholipid than if covered with a bilayer. This estimate takes into account that, due to the packing constraints in highly curved surfaces, a vesicle contains only about 65% of the amount of phospholipid present in a flat bilayer of (outer) vesicle surface area [27,28]. But such a close-packed configuration could not

occur if the vesicles would remain fixed at their adsorption sites. In that case, part of the surface area would be excluded from adsorption due to steric hindrance. Using a Monte-Carlo simulation with hard-core discs, it was found that a final surface coverage of about 50% of the theoretical close-packed maximum could then be expected [29]. So it is concluded that random coverage with intact vesicles could indeed produce a lipid mass per surface area close to the value for a continuous bilayer.

However, together with the demonstration of actual mixing of phospholipids from different vesicles in the present study, these data strongly indicate that a confluent bilayer is formed. It would indeed be difficult to imagine another configuration for a layer allowing free lateral mixing of lipids and exactly the same surface mass as a bilayer. After adsorption of a vesicle to the surface, the process of deformation, rupture and spreading of lipid over the surface must be rapid compared to the overall rate of vesicle adsorption, otherwise the lipid mass would tend to exceed a bilayer.

4.4. Limitations and possibilities of the technique

After flushing of DOPC vesicles from the cuvette and 10 min incubation with factors Xa and Va, the fivefold increase in the rate of prothrombin conversion was found immediately after addition of prothrombin. So the rate of increase in the conversion rate, and thereby the value of the diffusion constant of DOPS, could not be measured. This can also be understood theoretically. It follows from diffusion theory that for an initial flat DOPS source with radius r, surrounded by DOPC, the time course of disappearance due to diffusion is determined by $r^2/(4Dt)$ [30]. Considering the incubation delay, the first measurement of prothrombin conversion can be made after about 1000 s. The value of D is approximately equal to 10^{-8} cm^2 s^{-1} [14]. For a measurable effect in 1000 s we thus must have $r^2 \approx 4 \cdot 10^{-8} \cdot 1000$ or $r \approx 60$ μm. So the DOPS vesicles with $r \approx 10$ nm are much too small and will loose their excess DOPS within milliseconds. On the other hand, this limitation is not fundamental. If spots of about 60 μm radius could be covered with DOPS, this technique would also allow measurement of diffusion constants.

Acknowledgements

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