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Formation of tethered supported bilayers via membrane-inserting reactive lipids

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Abstract

Fluid phospholipid bilayers partially bound to a supporting polymer cushion (tethered supported membranes) have been widely discussed as model systems for studying biomembrane structure and function. We have synthesized a new isothiocyanate-functionalized lipid from dimyristoylphosphatidylethanolamine, which closely resembles naturally occurring membrane lipids. Monolayers containing the reactive lipid at the air–water interface covalently bind to the amino functionalities of branched polyethylenimine (PEI) added to the water subphase which could be shown by infrared spectroscopy. At neutral pH, PEI is cationically charged which guarantees the transfer of a polymer-supported lipid monolayer onto a mica substrate during Langmuir–Blodgett deposition. A second layer of pure dimyristoylphosphatidylcholine can be deposited by vesicle adsorption. This system should allow the application of techniques such as the surface forces apparatus (SFA) technique to investigate interbilayer forces, membrane–polymer interactions, and other dynamic membrane properties under near in-vivo conditions.

Keywords: Reactive lipid synthesis; Monolayer reaction; Polymer support; Tethered supported membrane

1. Introduction

Phospholipid bilayers supported by a polymer cushion which can swell and act as a deformable and mobile substrate, thus resembling the cytoskeletal support in living cells [1], are a field of growing research interest. These gel-supported membranes are used as model systems for the basic study of biomembrane structure and function, and their technical and medical interest arises from their potential use in biosensor systems [2,3]. To improve the long-term stability of such systems, the partial covalent fixation of the lower (proximal) lipid layer of the membrane onto the polymer has been discussed in the literature [4–6].

The surface forces apparatus allows us to investigate adhesion and fusion processes between model membranes in real time [7–9]. Apart from measuring static and equilibrium forces between supported bilayers, it is also interesting to study membrane deformations and lipid rearrangements. In this context, important questions arise from the coupling of the proximal lipid layer to the supporting surface [10,11].

Most recently, reactive membrane-inserting amphiphiles have been used for the formation of tethered lipid bilayers on glass substrates [6]. Adopting this approach for our purposes, we have synthesized a new isothiocyanate-functionalized lipid (Fig. 1) from dimyristoylphosphatidylethanolamine (DMPE) and carbon disulfide. Compared to most of the synthetic amphiphiles used so far, this reactive lipid resembles natural occurring lipids and thus favors a homogeneous mixing of the substrate-bound units into the bilayer membrane. Its reactive isothiocyanate head group can selectively react with amino groups in the presence of water which has been used, e.g. to bind proteins to functionalized surfaces [12,13]. This allows the partial attachment of an interfacial reactive lipid monolayer with the amino functions of branched polyethylenimine (PEI) dissolved in the water subphase. At neutral and acidic pH, the positively charged polyelectrolyte in turn guarantees the strong electrostatic binding of a tethered monolayer system to the mica substrates used in SFA studies (Fig. 1). In this communication, we describe the details of the lipid synthesis, as well as the monolayer characterization. Tethered supported bilayers can be formed, e.g. by adsorption of dimyristoylphosphatidylcholine (DMPC) vesicles onto transferred, polymer-supported monolayers containing this newly synthesized reactive lipid.

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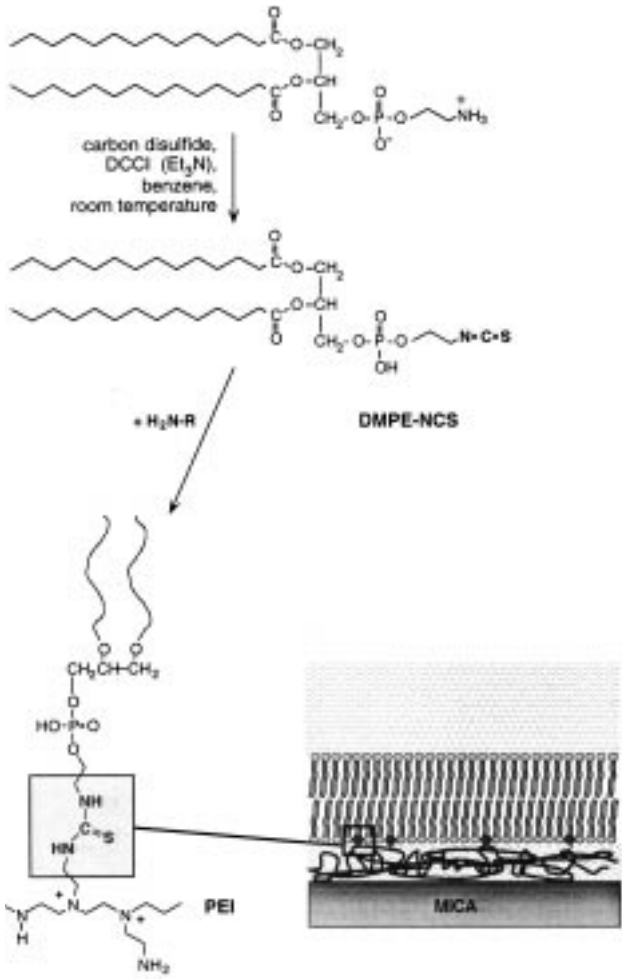

Fig. 1. Formation of tethered DMPC monolayers and bilayers partially attached to a supporting polyethylenimine gel. Bilayers are formed by adsorption of small unilamellar vesicles on the monolayers.

2. Experimental

2.1. Materials

All materials, if not otherwise noted, were purchased from Fisher. Branched polyethylenimine (1800 g mol^{-1}) was obtained from Polysciences.

2.2. Synthesis of 1,2-dimyristoylphosphatidyl-ethanolisothiocyanate (DMPE-NCS)

A suspension of 126 mg (0.2 mmol) dimyristoylphosphatidyl-ethanolamine (DMPE, Fluka), 100 μl (126.3 mg, 1.66 mmol) carbon disulfide (Aldrich), three drops of triethylamine, 10 ml dichloromethane and 2 ml benzene was stirred at 0°C for 30 min. 250 μl of a 1 M solution of *N,N′*-dicyclohexylcarbodiimide (0.25 mmol) in dichloromethane were added dropwise, resulting in a clear reaction mixture which was then stirred at room temperature overnight. The following day, complete conversion of DMPE was indicated by thin-layer chromatography (TLC) from chloroform, chloroform/methanol (1:1, v/v), and chloroform/methanol/water (64:25:4, v/v/v). The solvents were evaporated and the resulting white residue was treated with as much ethanol as necessary (ca. 2–3 ml) to achieve complete dissolution at room temperature. While keeping the solution at 4°C overnight, a white precipitate was formed (*N,N′*-dicyclohexylthiourea) which was filtered off. The filtered ethanol solution was distilled in vacuo, and the resulting white material was purified by column chromatography over silica gel. First, remaining side products were separated with chloroform, and after increasing the solvent polarity (addition of methanol), 80 mg of the desired product were obtained which could be further purified by preparative TLC (solvent: chloroform/methanol/water = 65:25:4). Yield: 37 mg (28%); ^{1}H NMR (200 MHz, $CDCl_3$/MeOD, 9:1) δ [ppm] 0.87 (t, 6H, CH_3), 1.25 (br, 40H, $CH_3(CH_2)_{10}CH_2CH_2COO$), 1.6 (br, 4H, CH_2CH_2COO), 2.30 (m, 4H, CH_2CH_2COO), 3.79 (t, 2H, CH_2NCS), 4.0 (br, 4H, $CH_2OPO_2OCH_2CH_2NCS$), 4.40 and 4.17 (dd, 2H, $RCOOCH_2CH$), 5.25 (br, 1H, $RCOOCH_2$-$CH(OOCR)CH_2OP$); FTIR (KBr), ν [cm^{-1}] 2924, 2853 (CH_2), 2215, 2110 (NCS, strong, fermi dublet), 1740 (s, COO), 1457, 1248, 1113, 1074; MS (FAB, M = 676.9 g mol^{-1}), 700.2 (Na-salt), 495.3 ($C_{13}H_{27}COOCH_2$–$CH(OOCC_{13}H_{27})=CH_2$), 285.2, 253.1 and 254.2 (OCH_2–OCH–$CH_2OPO_2OCH_2CH_2NCS$), 211.2 ($C_{13}H_{27}CO$), 183.9 and 205.9 ($OP(OH)_2OCH_2CH_2NCS$ and Na-salt).

2.3. Monolayer characterization and deposition

All lipids or lipid mixtures were spread from chloroform solutions at a concentration of 0.5–1 mg ml^{-1} (approximately 10^{-3} mol l^{-1}) on double-distilled water ultimately purified through a Milli-Q filter system (Millipore). Pressure–area (π–A) isotherms were measured on a temperature-controlled Wilhelmy trough (Joyce–Loebl) which was placed in a laminar flow box (Labconco) with a compression rate of typically 0.03–0.05 nm^2 (rep.unit $min)^{-1}$. The same film balance was used for the Langmuir–Blodgett (LB) deposition of the monolayers from the air–water interface onto freshly cleaved mica supports as well as onto Germanium substrate for infrared (IR) investigations (see below). Prior to deposition, the monolayers were compressed to the desired transfer pressure, expanded, compressed again, and finally equilibrated for at least 30 min. The dipping speed during transfer was 5 mm min^{-1}; in all cases, reproducible transfer of the monolayers onto the substrates was observed (calculated transfer ratios between 0.95 and 1.1).

2.4. Attenuated-total-reflection Fourier transform infrared spectroscopy (ATR-FTIR)

The substrates used in the IR study of transferred monolayers were rectangular (50 × 10 × 0.71 mm) germanium (Ge) wafers with 45° bevels at each of the short sides (i.e. trapezoidal ATR crystals). Infrared radiation generated by

an infrared spectrometer (Nicolet Magna 550) was focused on one of the beveled edges of the ATR crystal with a lens (KBr). It traversed through the Ge substrate, and exited from the opposite beveled edge undergoing total internal reflections. The transmitted IR beam was collected by another KBr lens and was sent to a HgCdTe detector by an off-axis parabolic mirror.

3. Results

3.1. Monolayer preparation and characterization

3.1.1. π–A curves

Fig. 2a shows the temperature-dependent *π–A* curves for monolayers of the newly synthesized lipid DMPE-NCS on the surface of pure Milli-Q water. At room temperature and above, only a liquid phase was observed, similar to DMPC, which should favor a homogeneous mixing of the two lipids in mixed monolayers. The films collapsed at areas below 52 $Å^2$ mol^{-1}. At 11°C, an additional monolayer phase was formed as indicated by the steep increase of the surface pressure at 42 $Å^2$ mol^{-1}. So far, the stability of this 'solid' phase has not been investigated further.

Besides its reactivity towards amino functions, the head group of DMPE-NCS is negatively charged at pH values above the pK_s of the phosphatidic acid group (pK_s~3–4), which resulted in a slight pH dependency of the *π–A* isotherms. However, a noticeable effect was only observed on an alkaline subphase (Fig. 2b).

The addition of branched polyethylenimine (100 ppm) to the subphase gave rise to more pronounced changes in the isotherms (Fig. 2c). As compared to the curves of DMPE-NCS on pure water, the onset of the surface pressure was shifted to higher areas per lipid molecule. At the same time, the stability of the films was enhanced as shown by their increased collapse pressures. Since pH changes had almost no effect on the monolayer behavior in the acidic to neutral region, these differences obviously did not result from the alkaline pH of the polyethylenimine subphase alone. In addition, extending the time that the reactive lipid was kept on the polymer subphase before compression resulted in an expansion of the liquid-expanded region to larger surface areas (at constant compression rate). We assume that

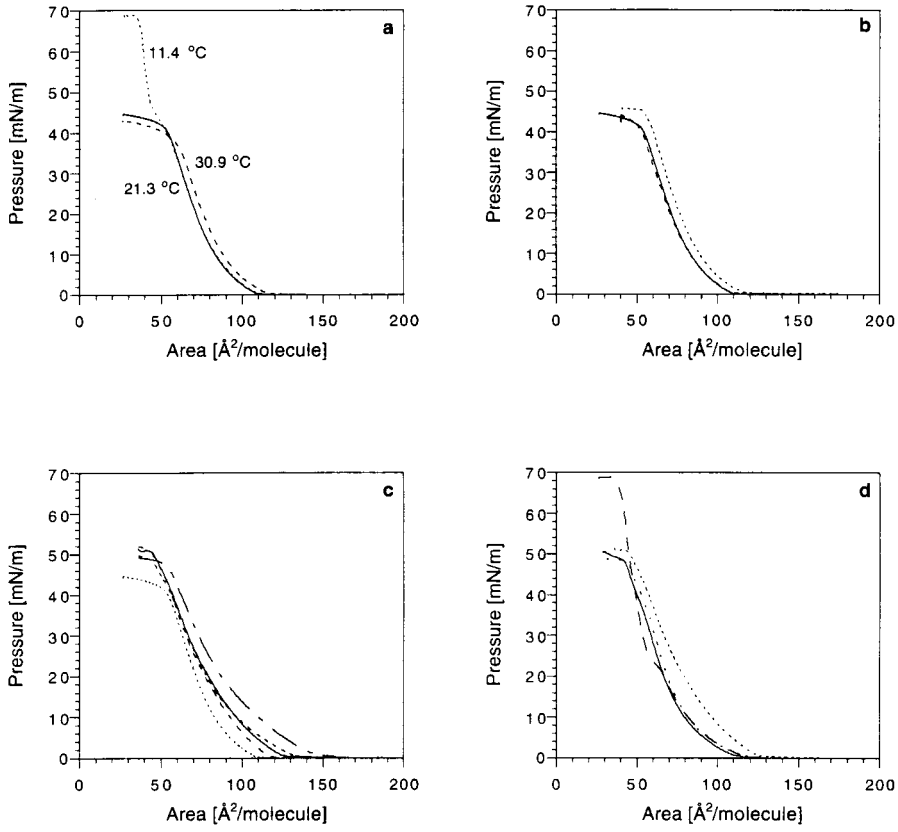

Fig. 2. *π–A* isotherms of DMPE-NCS at the air–water interface. (a) Temperature dependency on pure water subphase. (b) pH dependency at room temperature, pure water (full line), 0.5 mM HNO_3 (pH 1.3, dashed line), 0.5 mM KOH (pH 12.7, dotted line). (c) On polyethylenimine solution (100 ppm), kept for 30 min before compression (dashed line, second curve from the left), kept for 90 min before compression (full line), kept on PEI subphase containing 0.5 mM KOH for 30 min followed by addition of stoichiometrical amounts of HNO_3 (dashed line, second curve to the right), kept on PEI subphase containing 0.5 mM KOH for 30 min (broken curve at the right), DMPE-NCS on pure water (dotted line to the left). (d) DMPC/DMPE-NCS (9.6:1) on polyethylenimine solution (100 ppm) at room temperature (full line) and at 11°C (dashed line), compared to pure DMPC and pure DMPE-NCS on PEI subphases at room temperature (dotted curves to the left and right, respectively).

this observation reflects the proceeding covalent attachment of DMPE-NCS to the amino functions of polyethylenimine, thus giving an increased polymeric character to the monolayer. Again, as observed on aqueous subphases without polymer, an increase in the mean molecular area was found after addition of potassium hydroxide (approx. 0.5 mM). This pH effect could be reversed by adding equivalent amounts of nitric acid. However, note that the same broadening of the liquid-expanded region was achieved after 30 min on a KOH/PEI subphase followed by neutralization, as during 90 min reaction time on PEI in pure water. Thus, the monolayer reaction seems to be accelerated on alkaline PEI subphases.

Fig. 2d shows the π–A isotherms of a lipid mixture DMPC/DMPE-NCS (9.6:1) on a water subphase containing polyethylenimine (100 ppm) in comparison with the curves obtained for the two pure lipids. The lipid mixture (full line) showed no noticeable difference from pure DMPC (dotted line), indicating that the reactive lipid was distributed homogeneously within the DMPC layer. At lower temperature (11°C), at 64 $Å^2$ mol^{-1} and 22 mN m^{-1}, a transition to a solid phase was observed. It should be mentioned that, at both temperatures, the π–A curves for the lipid mixture on a polyethylenimine subphase showed no significant difference from those measured on pure water.

3.1.2. LB-transfer onto mica supports

DMPE-NCS as well as the lipid mixture were transferred from a polyethylenimine subphase onto freshly cleaved mica supports. As discussed above, the films were kept in the gas analogous phase for 90 min before compression to achieve a high degree of covalent binding to the polymer. The applied deposition pressure was 35 mN m^{-1} in both cases which corresponds to a mean molecular area at the air–water interface of approximately 60 $Å^2$ mol^{-1} (compare Fig. 2); close to the known area per lipid of DMPC within the fluid bilayer phase.

3.1.3. Contact angle measurements

Contact angle measurements on the transferred monolayers showed the influence of the underlying polyelectrolyte, as well as of the covalent attachment of the monolayer to the polymer. The contact angles of water droplets on mixed monolayers of DMPC containing approximately 10 mol.% DMPE-NCS on polyethylenimine were rather low. The advancing angle was $\theta_A = 43°$ and the receding angle was $\theta_R < 15°$. Moreover, the static contact angle of a water drop left on the substrate decreased with time, starting at a value of approximately 20°, until within a few minutes the water spread on the surface and wetted the support such that the contact angle was no longer measurable ($\Theta \approx 0$). Obviously, the mixed monolayer consisting mainly of DMPC is not stable against water and was ‘washed off’ the substrate or rearranged, presumably into bilayer patches. In contrast, a monolayer of pure reactive lipid on PEI/mica was much more hydrophobic and stable: the measured dynamic values were $\theta_A = 89°$ and $\theta_R = 18°$. Despite this rather high hysteresis, the static contact angle of initially 76° dropped to only 64° after 1 h. These observations point to an increased stability of the PEI-supported monolayers at higher covalent binding densities of the lipid headgroups.

3.1.4. ATR characterization of transferred monolayers

In order to obtain a direct structural proof of the chemical binding of the reactive lipid head groups to polyethylenimine, we investigated the ATR-IR spectra of monolayers deposited onto a germanium crystal. As references, next to an empty Ge sample, the crystals were covered with polyethylenimine adsorbed from solution, or with a monolayer of pure DMPE-NCS transferred from the air–water interface at room temperature and 30 mN m^{-1}. Fig. 3 shows the spectra obtained.

The IR spectrum of polyethylenimine (upper curve) contains two broad adsorptions at 2930 and 2820 cm^{-1} resulting from the CH stretching vibrations of the methylene groups of the polymer (note that there is also a broad, weak peak with a maximum around 3300 cm^{-1}, not shown in Fig. 3, which can be attributed to the NH stretching band). In addition, a broad range of absorption bands between 1600 and 1200 cm^{-1} resulting from NH and CH deformation modes was observed. In the spectrum of a DMPE-NCS monolayer (Fig. 3, second curve from the top), the CH stretching vibrations arising from the methylene groups of the lipid tails give rise to two strong, sharp peaks at 2920 and 2860 cm^{-1} (with an additional weaker signal attributed to the methyl end groups at 2970 cm^{-1}). Next to that, two characteristic bands at 1740 cm^{-1} (C=O stretching mode in carboxylic esters) and 2210 cm^{-1} (N=C=S antisymmetric stretch) were found. The latter adsorption, indicated with an arrow in Fig. 3, proves the presence of the reactive isothiocyanate head groups in the transferred monolayer and their stability against hydrolytic cleavage on a pure water subphase.

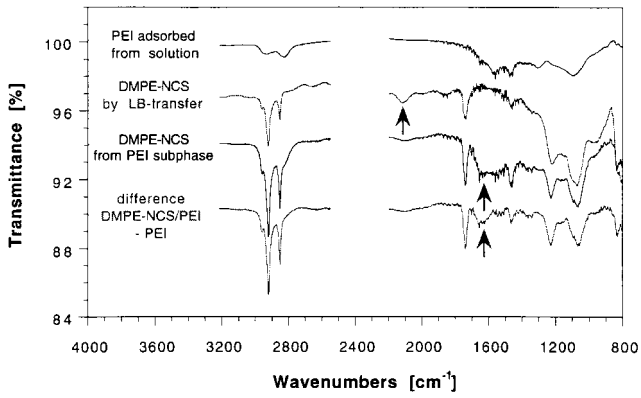

Fig. 3. ATR-IR spectra of thin films on Ge single crystals measured at 40°C. Upper curve: polyethylenimine film adsorbed from solution at room temperature; 2nd and 3rd curves from top: DMPE-NCS monolayer transferred from pure water as well as from PEI subphase; bottom curve: Subtraction of the polymer signal (normalized) from DMPE-NCS/PEI spectra. Significant bands indicating the monolayer reaction from the isothiocyanate (2210 cm^{-1}) to the thiourea group (1630 cm^{-1}) are highlighted by arrows.

The spectrum of a monolayer of DMPE-NCS transferred from a polyethylenimine subphase (second curve from the bottom in Fig. 3) shows the presence of DMPE-NCS as well as polyethylenimine on the Ge support, especially in the range of the CH and NH stretching modes from 3000 to 2700 cm^{-1} which appears as the sum of both polymer and lipid contributions. However, there are two distinct differences: first, the isothiocyanate band has almost disappeared in the spectrum of the polymer-supported lipid film. Moreover, an additional shoulder in the range of the polymer absorption bands was found at 1630 cm^{-1} (indicated by an arrow in the two bottom curves of Fig. 3, more clearly visible in the difference spectrum, bottom curve). This additional band can be attributed to the *N,N′*-disubstituted thiourea structure (compare Fig. 1) formed during the reaction of the reactive lipid with the amino functions of polyethylenimine, which is known to give a characteristic peak in the region around 1600 cm^{-1} [14]. It can be viewed as providing direct evidence for the conversion of the isothiocyanate head groups in contact with a polyethylenimine subphase.

3.2. Formation of tethered supported bilayers

Deposition of a second, upper (distal) lipid monolayer onto the lower (proximal) monolayer, thereby forming a bilayer, was achieved by adsorbing bilayer vesicles onto the monolayer-covered substrates in solution [5,15–17]. The resulting bilayers were analyzed using fluorescence microscopy. These results will be published elsewhere.

4. Conclusions

We have synthesized a reactive lipid deriving from DMPE which resembles naturally occurring membrane lipids. The isothiocyanate function introduced into the head group reacts selectively with amino groups in the presence of water. It was thus possible to covalently attach a reactive lipid monolayer onto polyethylenimine dissolved in the water subphase as shown by isotherm, contact angle, and IR measurements. With this approach we have constructed polymer-supported monolayers and bilayers on molecularly smooth mica substrate surfaces. The tethered bilayer system should allow investigation of model membranes using the surface forces apparatus and other techniques under controllable and near in-vivo conditions.

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