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Reconstitution of Rhodopsin into Polymerizable Planar Supported Lipid Bilayers: Influence of Dienoyl Monomer Structure on Photoactivation

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Abstract

G-protein-coupled receptors (GPCRs) play key roles in cellular signal transduction and many are pharmacologically important targets for drug discovery. GPCRs can be reconstituted in planar supported lipid bilayers (PSLBs) with retention of activity, which has led to development of GPCR-based biosensors and biochips. However, PSLBs composed of natural lipids lack the high stability desired for many technological applications. One strategy is to use synthetic lipid monomers that can be polymerized to form robust bilayers. A key question is how lipid polymerization affects GPCR structure and activity. Here we have investigated the photochemical activity of bovine rhodopsin (Rho), a model GPCR, reconstituted into PSLBs composed of lipids having one or two polymerizable dienoyl moieties located in different regions of the acyl chains. Plasmon waveguide resonance spectroscopy was used to compare the degree of Rho photoactivation in fluid and poly(lipid) PSLBs. The position of the dienoyl moiety was found to have a significant effect: polymerization near the glycerol backbone significantly attenuates Rho activity whereas polymerization near the acyl chain termini does not. Differences in cross-link density near the acyl chain termini also do not affect Rho activity. In unpolymerized PSLBs, an equimolar mixture of phosphatidylethanolamine and phosphatidylcholine (PC) lipids enhances activity relative to pure PC; however after polymerization, the enhancement is eliminated which is attributed to stabilization of the membrane lamellar phase. These results should provide guidance for the design of robust lipid bilayers functionalized with transmembrane proteins for use in membrane-based biochips and biosensors.

Introduction

Planar supported lipid bilayers (PSLBs) composed of phospholipids have been explored as biosensor coatings by numerous research groups,¹⁻³ in part because PSLBs are biocompatible hosts for tethering and reconstitution of water soluble and membrane associated receptors.⁴⁻¹¹ Additionally the highly hydrated phosphatidylcholine (PC) lipid headgroup is particularly effective in minimizing the undesirable nonspecific adsorption of nontarget proteins^{12,13} which is important for the design of biocompatible molecular devices. Advances in patterning lipid

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Supporting Information Available: Example sets of PWR curves for formation, Rho incorporation, and YL activation of unpolymerized PSLBs composed of mono-SorbPC, bis-DenPC, and bis-SorbPC/monoSorbPC, and polymerized PSLBs composed of mono-SorbPC and bis-DenPC. Plots of shifts in the *p*-polarized PWR minimum measured as a function of the molar ratio of Triton X-100 to lipid for PSLBs composed of bis-SorbPC/monoPC, bis-DenPC, and bis-SorbPC:mono-SorbPE. This material is available free of charge via the Internet at <http://pubs.acs.org>.

bilayer surfaces have demonstrated the potential utility of PSLB arrays for high-throughput biosensing and drug screening applications.¹⁴⁻¹⁹

A potential problem associated with implementing lipid structures in molecular devices is the inherent lack of stability that arises from the exclusively noncovalent forces that are responsible for formation of the lipid lamellar phase. As a result, partial or complete loss of the lamellar structure occurs upon exposure to surfactants or organics, or upon removal from the aqueous environment.^{20,21} In addition, PSLB instability has been observed upon extended storage in and exchange of the contacting buffer, in the presence of soluble proteins, and with variations in buffer pH and temperature.^{22,23} This lack of stability is a significant impediment to technological implementation of PSLBs in devices such as biochips and biosensors, especially when regeneration and reuse of the recognition surface is desired.^{14,24-26}

We have investigated cross-linking polymerization of synthetic lipid monomers as a strategy to create PSLBs that are stable to conditions that would destroy a fluid membrane, while retaining some of the biocompatible properties of a native lipid bilayer.^{13,21,27,28} Results from these studies show that PSLB systems based on the dienoyl polymerizable moiety are stable to conditions that would destroy a fluid membrane (e.g., repeated drying/rehydration, exposure to surfactants and organic solvents), despite the absence of a covalent tether between the membrane and the underlying substrate. Insolubility is attributed to a high degree of cross-linking polymerization coupled with multivalent interactions between polymer segments and the substrate surface. These cross-linked PSLBs were also found to resist nonspecific adsorption of proteins, similar to fluid PSLBs.^{13,16} Thus these poly(PSLBs) possess both the stability and inertness required for implementation in molecular devices (e.g., biosensors and biochips). Nonetheless it is unclear if extensive lipid cross-linking can be achieved without adversely affecting the activity of reconstituted transmembrane proteins, particularly because polymerization significantly reduces the lipid lateral mobility.²⁹ The conventional view is that a membrane must be highly fluid to maintain protein activity.^{30,31} Elastic membrane deformation (compression and/or bending) appears to be required to accommodate conformational changes in a transmembrane protein^{32,33} but membrane compression and/or bending does not necessarily require lateral lipid mobility.

A few previous studies have explored the effects of lipid cross-linking on the activity of transmembrane proteins by reconstituting bovine rhodopsin (Rho) or bacteriorhodopsin into vesicles composed of bis-diacetylenic lipids, followed by UV photopolymerization.^{34,35} This procedure was found to inactivate both Rho and bacteriorhodopsin; however, it was unclear if the lack of activity was caused by UV exposure and/or receptor immobilization in a cross-linked membrane. Subsequent studies showed that receptor activity could be maintained by reconstitution into vesicles composed of prepolymerized and fluid domains,³⁶⁻³⁸ presumably because the receptor inserted into the fluid domains. However, this approach undermines the strategy of using lipid cross-linking to achieve bilayer stability, because although the poly (lipid) domains should withstand exposure to destabilizing conditions, the fluid lipid domains will not.

In a recent communication,⁷ we investigated the effect of lipid polymerization on the photoactivity of Rho reconstituted into a PSLB composed only of the polymerizable lipid bis-SorbPC (structure in Figure 1). UV irradiation was used to initiate lipid polymerization, yielding a cross-linked bilayer that is stabilized to conditions that would destroy a fluid lipid bilayer.²¹ The photoactivity of Rho in poly(bis-SorbPC) was found to be comparable to that of Rho reconstituted into PSLBs composed of unpolymerized bis-SorbPC and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC). Thus it appears that cross-linked bis-SorbPC retains sufficient flexibility to accommodate the conformational change(s) that accompany photoactivation of Rho.

Here we have expanded our initial investigation to include other types of polymerizable lipids. The photochemical activity of Rho in PSLBs composed of lipids with polymerizable moieties located at different regions in the acyl chains, having different head groups, and differing degrees of cross-linking is examined. Significant differences among the different lipid compositions are observed. The results provide guidance for the design of robust lipid bilayers functionalized with transmembrane proteins for use in membrane-based biochips and biosensors.

Experimental Section

Materials

The polymerizable lipids bis-sorbyl phosphatidylcholine (bis-SorbPC), bis-dienoyl phosphatidylcholine (bis-DenPC), monosorbyl phosphatidylcholine (mono-SorbPC) and monosorbyl phosphatidylethanolamine (mono-SorbPE) (structures in Figure 1) were synthesized following procedures by Lamparski et al.,^{39,40} Dorn et al.,⁴¹ Bondurant,⁴² and Liu.⁴³ DOPC was obtained from Avanti Polar Lipids (Alabaster, AL). Frozen bovine retinas were purchased from W. L. Lawson (Lincoln, NE). Triton X-100 and octylglucoside were purchased from Sigma-Aldrich and Anatrache (Maumee, OH), respectively. Buffers were prepared using 18.2 M Ω -cm water from a Barnstead Nanopure system.

Purification of Retinal Rod Membranes and Solubilization of Rho

Native rod outer segment (ROS) membranes were isolated from frozen bovine retinas by the method of Papermaster and Dreyer⁴⁴ in a discontinuous sucrose density gradient. Purified ROS membranes appear as a dense red band at the 1.11/1.13 g·mL⁻¹ interface in the sucrose step gradient after 1h of centrifugation at 25,000 rpm. The preparations typically had a A_{280}/A_{500} absorbance ratio in the range of 2.4–2.8. Rho was extracted by homogenizing ROS membranes in 10 mM phosphate buffer (pH 7.0) containing 200 mM octylglucoside and centrifuged at 6400 rpm for 1h at 4 °C. All rhodopsin samples were handled in dim red light (Kodak safelight filter No. 1, 15-W bulb). The purified Rho was stored at -70 °C under argon, and thawed immediately before use.

Plasmon Waveguide Resonance Spectroscopy

Plasmon waveguide resonance (PWR) spectroscopy was used to characterize conformational changes accompanying Rho photoactivity in PSLBs.⁴⁵ PWR theory and instrumentation are described in previous papers.⁴⁶⁻⁵¹ Briefly, from an instrumental perspective, PWR is similar to surface plasmon resonance spectroscopy. Using a Kretschmann configuration, *s*- and *p*-polarized modes are excited using a HeNe laser (632.8 nm) in a thin planar waveguiding layer⁵² (SiO₂, ca. 400 nm) that is coated over a metal film (Ag, ca. 40 nm). These resonant modes are highly sensitive to changes in the optical properties (complex refractive index, thickness) of thin films deposited on the waveguide surface. A change in film properties is detected as a shift in the angle of incidence at which a resonant mode is excited, which corresponds to the minimum reflectivity measured as a function of incidence angle. The angular width and depth of the resonance are also affected. PWR has been used to detect mass density and conformational changes accompanying protein–lipid and protein–ligand interactions occurring at or within PSLBs, including Rho photoactivation.^{47-51,53}

Formation of Lipid Bilayers on the Prism Surface

Supported lipid bilayers were formed on the SiO₂ waveguide surface by the self-assembly method described by Alves and co-workers.⁴⁷ Vacuum dried lipids were dissolved in *n*-butanol:methanol:squalene (0.95:0.05:0.005, v/v) to a final concentration of 7 mg/mL. A small volume of this lipid solution (~2 μ L) was spread over the 2 mm diameter orifice in the Teflon

spacer that separates the waveguide surface from the main body of the PWR. Filling the cell with buffer (10 mM phosphate buffer, pH 5.5) initiates the next steps of bilayer formation, which are thought to involve a thinning process and formation of a plateau-Gibbs border that anchors the lipid membrane spanning the orifice to the Teflon spacer.⁴⁵ These steps were monitored by acquiring PWR curves (scans of reflectivity vs incidence angle) as a function of time until a steady-state response was observed.

Incorporation and Photoactivation of Rho in PSLBs

Incorporation of Rho into a preformed PSLB was carried out by introducing 25 μ L aliquots of the protein (1 mg/mL Rho solubilized in 30 mM octylglucoside (OG) containing 10 mM phosphate, pH 7.2) into the PWR cell, which was filled with 0.5 mL of buffer. Upon injection of the solubilized Rho, the OG was diluted below its critical micelle concentration (25 mM), resulting in spontaneous transfer of the protein from OG micelles into the PSLB.⁴⁷ Four aliquots were injected into the cell sequentially, and Rho incorporation into the PSLB was monitored by acquiring PWR curves continuously until a steady-state response was obtained. Photoactivation of Rho incorporated into a PSLB was accomplished by illuminating the PWR cell with yellow light (wavelength >500 nm) from a 150 W tungsten-halogen light source equipped with a fiber optic cable (Ace Light Source, Schott) and yellow long pass filter (Schott A08072). A PWR curve was acquired after each exposure (5 s) until no further angular shifts were observed, which usually occurred after the second exposure.

Polymerization of PSLBs and Evaluation of Stability to Surfactant Dissolution

PSLBs of bis-DenPC, bis-SorbPC/mono-SorbPC (1:1 mol/mol), bis-SorbPC/mono-SorbPE (1:1 mol/mol) were UV polymerized by directing light from a mercury lamp^{7,21} through a port in the PWR cell for 45 min. A band-pass filter (Hoya U-330 $\pm$ 80 nm) was used to remove visible light (>450 nm) that photoactivates Rho. Under these conditions, $\geq$ 95% lipid polymerization was achieved, based on measuring the attenuation of the monomer UV absorbance of vesicles composed of these lipids, as described previously.⁷ In most cases, Rho was incorporated into PSLBs before polymerization. However, in some cases incorporation was performed after PSLB polymerization, as follows: PSLBs were formed and polymerized as described above. After polymerization, aliquots of OG-solubilized Rho were injected into the aqueous volume of the PWR cell, above the PSLB as described above. Rho incorporation was monitored by acquiring PWR curves and was deemed complete when a steady-state response was observed. PSLBs were exposed to dissolved surfactant to assess if UV irradiation caused lipid polymerization and enhanced bilayer stability. PSLBs were photopolymerized as described above, then aliquots of Triton X-100 solution (1% v/v) were added to the cell and PWR curves were acquired after each addition. For comparison purposes, this procedure was also performed on PSLBs that were not exposed to UV radiation.

Results and Discussion

Rho is a model for G-protein coupled receptors (GPCRs) and is one of only two for which high resolution crystal structures are available.⁵⁴⁻⁶⁰ The activated state of Rho is formed when the covalently bound 11-*cis*-retinal absorbs a photon and isomerizes to the trans form.^{61,62} Several thermal decay steps take place within the ensuing few milliseconds, leading to establishment of a metastable equilibrium between two conformational states of the receptor, metarhodopsin I (MI) and metarhodopsin II (MII).⁶³⁻⁶⁶ Formation of MII is accompanied by a conformational change that triggers G-protein (transducin) binding and activation.^{67,68} The interaction of Rho with the surrounding lipids modulates the extent of this conformational change,^{33,69-72} thus making Rho an appropriate model protein for studying the effect of lipid polymerization on GPCR activation.

PSLB formation, Rho incorporation, and UV polymerization

PWR angular scans were recorded before and after PSLB formation, after incorporation of Rho into the PSLB, and after photopolymerization of lipids. The PSLB structure at each step of a typical experiment is illustrated schematically in Figure 2. Examples of typical *p*- and *s*-polarized curves obtained for poly(PSLBs) composed of equimolar mixtures of bis-SorbPC/mono-SorbPC and bis-SorbPC:mono-SorbPE are shown in Figures 3 and 4, respectively. Example PWR curves for poly(mono-SorbPC) and poly(bis-DenPC) PSLBs are presented in Supporting Information (SI), as are sets of curves for unpolymerized PSLBs composed of mono-SorbPC, bis-DenPC, and bis-SorbPC/mono-SorbPC. For unpolymerized bis-SorbPC/mono-SorbPE, an example set of curves is shown in Figure 5. The respective shifts in the resonance minima obtained from at least three trials with each of these lipids and lipid mixtures, along with those observed for bis-SorbPC and DOPC PSLBs, are listed in Table 1.

For all lipid compositions studied, upon PSLB formation the resonance minimum shifted to higher angles of incidence (~ 100 mdeg for *p*- and ~ 80 mdeg for *s*-polarized light) relative to the respective resonance minimum in buffer (e.g., shifts between curves 1 and 2 in Figure 3; all data listed in Table 1). The increase in angle is due to displacement of water by lipid at the waveguide surface,⁴⁷⁻⁵¹ and the magnitude of the shifts is consistent with formation of a single lipid bilayer based on previous experiments.^{47,50} The anisotropy in the *p*- and *s*-polarized shifts is expected based on the known birefringence of lipid bilayers.^{51,73}

Injection of OG-solubilized Rho into the PWR cell caused further positive shifts in the resonance minimum (e.g., shifts between curves 2 and 3 in Figure 3). For all lipids studied, the shifts were ca. ~ 70 and ~ 50 mdeg for *p*- and *s*-polarized light, respectively (Table 1). These changes are consistent with insertion of Rho into the PSLB, which displaces water and forces redistribution of lipids, resulting in an overall increase in membrane optical thickness. It is reasonable to assume that the magnitude of the observed shift is correlated to the amount of Rho inserted into the PSLB. Given that the shifts were approximately equal for all lipids and lipid mixtures, it appears that lipid structure has little effect on the amount of Rho inserted.

The anisotropy in the *p*- and *s*- shifts is consistent with oriented insertion of Rho into PSLBs with its long axis perpendicular to the membrane plane. This is consistent with recent studies using angle-resolved X-ray photoelectron spectroscopy (XPS) on dried poly(bis-Sorb PC) PSLBs containing Rho.⁷⁴ The N 1s signal was found to be independent of the XPS takeoff angle which shows that the protein is inserted into the bilayer (i.e., uniformly distributed throughout its thickness) rather than adsorbed on its surface.⁷⁴ However, the degree of vectorial orientation of Rho cannot be ascertained from the XPS (or the PWR) data.

UV light was used to polymerize PSLBs containing Rho. Irradiation induced small shifts of ~ 2 – 4 mdeg in the resonance minimum for both *p*- and *s*-polarizations (e.g., shifts between curves 3 and 4 in Figure 3) for all polymerizable lipids that were tested (Table 1). Thus lipid polymerization minimally altered the optical thickness of the proteo-lipid membranes. In summary, the PWR angular shifts caused by PSLB formation and rhodopsin insertion for bis-SorbPC:mono-SorbPC, bis-SorbPC:mono-SorbPE, mono-SorbPC, bis-DenPC, bis-SorbPC, and DOPC were all very similar. The same is true for shifts induced by photopolymerization (excepting DOPC, which was not polymerized). These results show that differences in the position and number of polymerizable groups in the lipid chains, as well as differences in the headgroup structure (PC vs PE), do not measurably influence the formation and polymerization of these proteo-lipid membranes, as detected by PWR.

Stability of PSLBs in the Presence of Triton X-100

The mechanism of lysis of a lipid bilayer by a single-chain surfactant is thought to involve incorporation of the surfactant into the bilayer, followed by eventual detachment of mixed micelles composed of surfactant and lipid.⁷⁵⁻⁷⁸ A number of studies on poly(lipid) vesicles and supported membranes have shown that polymerization increases their resistance to solubilization by surfactants.^{13,29,43} In a previous study (see Supporting Information in ref 7), this property was used to assess if lipid polymerization in a PSLB was achieved. PWR scans were recorded as a function of increasing amounts of Triton X-100 injected into the aqueous volume above the bilayer. Minimal shifts in the resonance minimum were observed for UV polymerized bis-SorbPC, whereas for unpolymerized bis-SorbPC and DOPC, significant shifts attributable to PSLB dissolution were observed. Results from another previous study²¹ in which ellipsometry and atomic force microscopy was performed on air-dried poly(PSLBs) also confirmed that UV-initiated polymerization was achieved.

A similar strategy was employed here to assess if UV irradiation resulted in polymerization of other types of lipids and lipid mixtures. Plots of shifts in the *s*-polarized PWR minimum measured as a function of the molar ratio of Triton X-100 to lipid are presented in Figure 6 for PSLBs composed of bis-SorbPC/mono-SorbPC, bis-DenPC, and bis-SorbPC/mono-SorbPE. The corresponding *p*-polarized plots are shown in the Supporting Information. For bilayers that were not exposed to UV light, in all cases the resonance minimum shifted to lower incidence angles at Triton X-100/lipid molar ratios greater than five. The change corresponds to a decrease in mass density at the waveguide surface, which is consistent with bilayer dissolution. For bis-SorbPC/mono-SorbPC and bis-DenPC, small positive shifts in the resonance minimum were observed at Triton X-100/lipid molar ratios ≤ 5 . This increase in mass density is likely due to insertion of surfactant into the bilayer, consistent with the early stages of the lysis mechanism described above. In the case of bis-SorbPC:mono-SorbPE, a sharp decrease in the resonance minimum occurred upon addition of only ca. two molar equivalents of Triton X-100. This lower apparent stability is likely caused by the presence of the PE headgroup, which is smaller and less hydrated than the PC headgroup.⁷⁹ In mixtures of DOPC and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), the inverted hexagonal (H_{II}) and other nonlamellar phases are thermodynamically favored as the DOPE content increases.⁸⁰ Assuming that the phase behavior of mono-SorbPE is similar to that of DOPE, its presence at high concentration in a PSLB may enhance the susceptibility of the bilayer to surfactant dissolution.

The response of UV irradiated PSLBs to Triton X-100 was much different than that of nonirradiated bilayers. In the case of bis-SorbPC/mono-SorbPC (Figure 6a), a initial increase in the resonance minimum was observed at low Triton X-100/lipid ratios but it was smaller than that measured for unpolymerized bis-SorbPC/mono-SorbPC, which implies a lower degree of surfactant insertion. At Triton X-100/lipid ratios greater than five, gradual negative shifts in the resonance minimum were observed, however these shifts were much less than those observed for unpolymerized bis-SorbPC/mono-SorbPC. These data clearly show that UV polymerization increases the resistance of bisSorbPC/mono-SorbPC PSLBs to surfactant dissolution. Similar observations have been made for UV polymerized vesicles composed of these lipids.^{29,81}

Similar trends were observed for polymerized bis-DenPC PSLBs (Figure 6b). At low Triton X-100/lipid ratios, small increases in the PWR minimum were measured, whereas at ratios greater than five, gradual negative shifts occurred but again these shifts were smaller than those observed for unpolymerized bis-DenPC. However the difference in the magnitude of the negative shifts of unpolymerized and poly(bis-DenPC) was significantly less than those of bis-SorbPC:mono-SorbPC PSLBs (Figure 6a). These comparisons indicate that although polymerization of bis-DenPC enhances its resistance to Triton X-100 solubilization, the

enhancement is less than that achieved upon polymerization of bis-SorbPC⁷ or bis-SorbPC/mono-SorbPC. This finding is consistent with previous studies in which the air stability of these poly(lipids) was examined.²¹ The difference can be attributed to the location of the polymerizable group—in sorbyl PSLBs, interleaflet cross-linking can occur due to interdigitation of the acyl chains, whereas in bis-DenPC PSLBs, cross-linking can occur only within each leaflet.

Enhanced resistance to Triton X-100 solubilization was also observed for poly(bis-SorbPC/mono-SorbPE) PSLBs (Figure 6c). However, as was the case for poly(bis-DenPC), the enhancement was less than that observed for poly(bis-SorbPC)⁷ or bis-SorbPC/mono-SorbPC (Figure 6a). As discussed above, this finding is attributed to the tendency of PE lipids to favor the H_{II} phase. A caveat is that for all lipid compositions studied, the degree of polymerization (X_n) is not known. Therefore the greater stability of pure poly(sorbyl) bilayers could be due to a larger X_n which would make them more resistant to surfactant dissolution. In summary, the data in Figure 6 show that although Triton X-100 can insert into and structurally alter poly(PSLBs), the degree of disruption is significantly less than that of the corresponding unpolymerized lipid bilayers. Moreover these data also demonstrate that the UV irradiation conditions used in this study are sufficient to achieve PSLB polymerization.

Characterization of Yellow Light Activation of Rho in PSLBs

Illuminating Rho with yellow light (YL) leads to formation of a metastable equilibrium between the MI and MII intermediates. This equilibrium decays irreversibly to generate the MIII state and/or the apoprotein opsin and free retinal.^{54,82} The conformational change accompanying the MI to MII transition is thought to involve elongation of the protein along the axis normal to the membrane plane along with a significant increase in partial molar volume.⁸³⁻⁸⁵ This elongation causes local deformation of the surrounding bilayer and infusion of lipid molecules from the Gibbs border, as depicted schematically in Figure 2. These changes increase the optical thickness of the proteo-lipid membrane, which can be measured as a shift in the PWR minimum to a larger incidence angle.^{7,47} As shown previously in comparative studies of YL activation of Rho reconstituted in lipid bilayers as a function of pH, the magnitude of the angular shift measured using PWR parallels the extent of MII formation measured directly by flash photolysis.^{47,69} This correlation therefore establishes that PWR provides a quantitative measure of Rho photoactivity in lipid bilayers.

Here DOPC was used as a benchmark for comparison of Rho photoactivity in PSLBs. Typical shifts in PWR minima measured after exposing Rho in a DOPC PSLB to YL were reported in a previous communication⁷ and are also listed in Table 1. Photoactivation of Rho caused a positive shift of 13 mdeg for *p*- and 8 mdeg for *s*-polarized light, similar to results reported by Alves et al.⁴⁷

Table 1 presents a comparison of Rho photoactivity in PSLBs composed of sorbyl lipids before and after polymerization. The magnitude of the PWR shifts measured for poly(bis-SorbPC), ca. 12 mdeg in both polarizations, was equivalent to those measured for YL activation of Rho in unpolymerized bis-SorbPC, and similar to those of Rho in a fluid DOPC bilayer. Thus the extent of the conformational change induced by Rho photoactivation was unaffected by bis-SorbPC polymerization, specifically the formation of a cross-linked network in the center of the bilayer. With respect to unpolymerized bis-SorbPC, it is also apparent that dienoyl moieties in the center of the bilayer do not adversely affect Rho activity.

The effect of lipid cross-linking density on Rho photoactivity was assessed by performing comparative experiments on PSLBs containing mono-SorbPC. Studies of mixed vesicles composed of bis-SorbPC and mono-SorbPC have shown that when the mole fraction of bis-SorbPC is ≥ 0.3 , the lipid bilayer is cross-linked.^{29,43} Thus PSLBs composed of equimolar bis-

SorbPC:mono-SorbPC should also be cross-linked, but less so than that of a PSLB composed of pure bis-SorbPC. In pure mono-SorbPC bilayers, only linear polymers are formed. PWR shifts measured upon photoactivation of Rho incorporated into equimolar bis-SorbPC/mono-SorbPC and pure mono-SorbPC PSLBs are listed in Table 1, and example curves are shown in Figure 3 for poly(bis-SorbPC/mono-SorbPC) and in SI for poly(mono-SorbPC) and unpolymerized lipids. In both cases, the shifts of ca. 10 mdeg were equivalent before and after polymerization, and statistically indistinguishable from the shifts observed for pure bis-SorbPC. These data demonstrate that (i) polymerization of sorbyl lipid bilayers has no significant effect on the photoactivity of incorporated Rho and (ii) variations in the density of cross-links also have no effect.

A growing body of evidence indicates that the material properties of lipid bilayers, such as thickness, elastic stretching and bending moduli, and intrinsic lipid curvature, modulate the conformational changes of transmembrane receptors via hydrophobic coupling between the bilayer-spanning portion of the protein and the surrounding lipids.^{32,33} Clearly, lipid polymerization should alter the elastic moduli of a PSLB, e.g., the curvature (bending) elastic modulus. Although it is known that polymerization of dienoyl lipids creates an elastomeric polymer,⁸⁶ the mechanical properties of these membranes have not been measured. Nevertheless the data in Table 1 clearly show that the material properties of poly(sorbyl) bilayers are not altered to the extent that they can no longer accommodate the membrane deformation that accompanies the conformational change associated with the MI to MII transition of rhodopsin, as illustrated schematically in Figure 2.

In Figure 5 is shown a set of PWR curves for Rho incorporated into an unpolymerized equimolar bis-SorbPC/mono-SorbPE bilayer. The PWR shifts caused by YL activation, 23 mdeg for *p*- and 15 mdeg for *s*-polarized light, were larger than those observed for all other bilayer compositions examined (Table 1). Similarly large shifts were observed by Alves and co-workers upon photoactivation of Rho in mixed DOPC/DOPE bilayers relative to pure DOPC.⁴⁷ These larger shifts are indicative of a greater reorganization of the proteolipid membrane. Due to their negative spontaneous curvature, lipids with PE head groups favor the H_{II} phase. The curvature at the protein–lipid boundary in the elongated MII conformation of Rho is predicted to be negative; thus PE lipids shift the MI-MII equilibrium to favor MII formation.^{32,33,47,82,87}

In contrast, UV polymerized bis-SorbPC/mono-SorbPE bi-layers behaved much differently, as illustrated by a comparison of Figures 4 and 5 and the data listed in Table 1. The shifts in the PWR minima upon YL exposure were 10 mdeg for *p*- and 9 mdeg for *s*-polarized light, approximately half of those measured for unpolymerized bis-SorbPC:mono-SorbPE PSLBs, and comparable to those observed for Rho in poly(sorbyl) PSLBs. Previous work has suggested that both lipids and protein contribute to the PWR shifts measured upon photoactivation of Rho in mixed DOPC/DOPE membranes, whereas in pure DOPC membranes having zero spontaneous curvature, only the protein conformational change contributes; thus the measured shifts are smaller.⁴⁷ Extending this hypothesis to the present case, polymerizing monoSorbPE apparently restricts its ability to match the negative curvature of the protein–lipid boundary of the MII conformation. Thus covalently linking the tails of mono-SorbPE and bis-SorbPC stabilizes the lamellar phase of the membrane.

Bilayers composed of bis-DenPC were also examined as a matrix for reconstitution of Rho. YL activation of unpolymerized bis-DenPC PSLBs containing Rho produced PWR shifts of 8 mdeg for *p*-polarized and 5 mdeg for *s*-polarized light (data listed in Table 1; example curves are presented in SI), which are smaller than those observed for DOPC and unpolymerized sorbyl PSLBs. In contrast to the sorbyl-functionalized lipids, in bis-DenPC the polymerizable groups are adjacent to the glycerol backbone (Figure 1) and the center of the acyl chain region

in a bis-DenPC bilayer is saturated. Several previous studies have examined the effect of acyl chain saturation on the activity of Rho reconstituted in artificial lipid membranes;^{33,69,88,89} In general, the degree of unsaturation is positively correlated with Rho photochemical activity. The lack of unsaturation in the center of a bis-DenPC PSLB is a likely cause for the reduced Rho activity observed here.

YL activation of poly(bis-DenPC) PSLBs containing Rho gave rise to even smaller shifts of ~2 mdeg in both polarizations (Table 1 and Supporting Information). The cause of the lack of Rho activity indicated by these minimal shifts is not clear. However it is evident that a poly(bis-DenPC) bilayer that contains two polymeric networks, one in each leaflet adjacent to the glycerol backbone, does not accommodate the conformational change that accompanies the MI-MII transition. In contrast, sorbyl lipid polymerization, which creates a polymeric network in the center of the bilayer, does permit this conformational change. Thus a possible explanation for the minimal activity in poly(bis-DenPC) is that polymerization near the headgroup region in each leaflet generates a relatively inflexible bilayer that cannot conform to the hydrophobic mismatch^{32,33} which is a consequence of elongation of the protein to form the MII intermediate.

Here we expand on this hypothesis: Cross-linking the lipids near the glycerol backbone/polar headgroup stiffens the bilayer and increases the curvature elastic modulus (bending rigidity) close to the neutral surface (pivotal plane) where bending occurs.^{32,82} As a consequence, the MI-MII equilibrium is back-shifted toward the inactive MI state. In contrast, cross-linking the lipids near the center of the bilayer, well removed from the neutral surface (pivotal plane), has a smaller effect on the curvature elastic modulus. Thus Rho photoactivity is better maintained by cross-linking the lipids deeper in the bilayer.

Experiments were also conducted to determine if photoactive Rho could be incorporated into a bilayer *after* the lipids were polymerized. PWR angular shifts corresponding to each step in experiments with polymerized bis-SorbPC, bis-SorbPC/mono-SorbPC, bis-DenPC and bis-SorbPC/mono-SorbPE are listed in Table 2. Shifts accompanying PSLB formation and UV polymerization were consistent with previous experiments (Table 1). In all cases, incubation of OG-solubilized Rho with polymerized PSLBs produced substantial shifts in the resonance minimum, showing that Rho does associate with poly(lipid) bilayers. The nature of this association is unknown but a combination of adsorption and partial insertion is likely based on the anisotropy in the observed shifts ($p > s$). However, both the magnitude and anisotropy of the shifts differed considerably among the several lipid compositions, e.g., the *s*-polarized shifts were 22 and 61 mdeg for poly(bis-SorbPC) and poly(bis-SorbPC/mono-SorbPE), respectively. In contrast, incorporation of Rho into unpolymerized PSLBs produced shifts that were independent of lipid composition (see Table 1). A comparison of the incorporation data in Tables 1 and 2 indicates that the nature of Rho-poly(lipid) association is clearly different from that of unpolymerized lipids, and also differs among the various poly(lipids) examined, but the structural basis of these differences is unknown.

YL activation of Rho associated with prepolymerized bis-SorbPC and bis-SorbPC:mono-SorbPC PSLBs produced smaller PWR shifts (Table 2) than PSLBs to which Rho was introduced before polymerization (Table 1). Analogous experiments performed with (poly)bis-DenPC and poly(bis-SorbPC/monoSorbPE) produced small positive and small negative PWR shifts, respectively. The smaller shifts in the resonance minima correspond to less or negligible Rho activity, which is a probable result if the protein is not associated with the bilayer in a manner that promotes the MI-MII transition. We speculate that Rho is partially inserted or merely adsorbed to the surface of these poly(PSLBs).

Summary and Conclusions

In this study, planar supported bilayers composed of lipids with one or two dienoyl moieties located at different regions in the acyl chains and having different head groups were investigated. Direct UV irradiation was used to polymerize these PSLBs. After irradiation, the resistance of PSLBs to Triton-X 100 solubilization was enhanced, showing that photopolymerization was achieved.

PSLBs with varying degrees of cross-linking were prepared from bis-SorbPC, mono-SorbPC, and mixtures thereof. Rho was incorporated into these PSLBs prior to lipid polymerization and its photochemical activity was characterized using PWR, which is sensitive to the changes in the optical thickness of the proteolipid membrane that accompany the MI to MII transition. The PWR shifts upon YL activation were equivalent in polymerized and unpolymerized PSLBs, demonstrating that sorbyl lipid polymerization and cross-linking density do not significantly affect the photoactivity of incorporated Rho.

It is well established that the MI-MII equilibrium is shifted toward MII in mixed PC/PE lipid bilayers relative to pure PC bilayer,^{33,47,87} and similar behavior was observed here for Rho incorporated into unpolymerized bis-SorbPC/mono-SorbPE PSLBs. However, the photoactivity of Rho was attenuated significantly after lipid polymerization, to a level comparable to that observed for sorbyl PSLBs. Among the lipids examined, bis-DenPC was the least favorable for maintenance of Rho activity. The protein was essentially inactive in poly(bisDenPC). We hypothesize that formation of two cross-linked networks, one in each leaflet adjacent to the glycerol backbone in poly(bisDenPC), produces a less flexible polymer that cannot accommodate the membrane deformation^{32,33} resulting from elongation of the protein to form the MII intermediate. In contrast, polymerization of sorbyl lipids generates a polymeric network in the more disordered center of the bilayer, and these polymers appear to retain sufficient elasticity to accommodate the membrane deformation that accompanies MII formation, as depicted in Figure 2. However copolymerizing mono-SorbPE with SorbylPC lipids restricts the capability of the membrane to match the negative curvature at the MII-lipid boundary. Although morphological observations of vesicles indicate that polymerization of dienoyl lipids creates an elastomeric polymer,⁸⁶ the mechanical properties of these membranes have not been measured. Thus our hypotheses relating membrane mechanical properties to Rho photochemical activity remain to be tested, and will be the subject of future investigations.

Supported lipid membranes and membrane arrays functionalized with proteins have been fabricated by numerous research groups, primarily for use as cell-surface models or in biosensing and drug screening applications (see for example refs 1, 2, and 8–11 and references therein). In most cases, these membranes have been composed of fluid lipids. In situations where fluidity is not a requirement for the sensing device to function, lipid polymerization could be a useful strategy to enhance device lifetime. The results reported here provide guidance for designing robust poly(lipid) bilayers functionalized with transmembrane proteins for membrane-based biosensing and other applications.

Finally it is clear from both this and prior studies that a conformational change in a transmembrane protein reconstituted into a PSLB can be detected using PWR, assuming that the change generates a detectable difference in optical thickness. However, with respect to Rho activation, PWR is an indirect method because it does not directly monitor formation of MII, the key intermediate in visual transduction. Rather PWR spectroscopy provides only a relative measure of Rho activity in PSLBs, e.g., our measurements show that Rho activity is comparable in DOPC and poly(bis-SorbPC). Furthermore the total surface coverage of Rho in these membranes and the fraction that is photoactive is unknown. Rho surface coverage as well as MI and MII formation and decay can be directly measured using absorbance spectroscopy,

^{38,69,87} assuming that the measurements could be performed with adequate sensitivity. In this regard, ATR spectroscopy using a thin planar waveguide as the internal reflection element should provide the required sensitivity.^{90,91}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Structures of polymerizable lipids.

Figure 2.

Illustration of the major steps in formation and photoactivation of a poly(PSLB) with incorporated Rho. The numbers **2–5** correspond to the points in a PWR experiment at which angular scans are acquired (e.g., see corresponding curves in Figure 3b): A PSLB is formed on the SiO₂ waveguide surface (**2**); Rho is incorporated into the PSLB (**3**); the lipids are photopolymerized with UV light (**4**); and Rho is photoactivated with yellow light (**5**). The latter process leads to MII formation and a conformational change involving elongation of the protein along the axis normal to the membrane plane. Membrane deformation occurs to accommodate the hydrophobic mismatch resulting from the conformational change.

Figure 3.

PWR curves (A, *p*-polarized; B, *s*-polarized) acquired at each stage of an experiment with a poly(bis-SorbPC/mono-SorbPC) bilayer (1:1 (mol/mol)). Curve obtained with only buffer in the PWR cell (1), after PSLB formation (2), after Rho incorporation (3), after UV polymerization (4), and after yellow light activation of Rho at pH 5.5 and 25 ± 1 °C (5).

Figure 4.

PWR curves (A, *p*-polarized; B, *s*-polarized) acquired at each stage of an experiment with a poly(bis-SorbPC/mono-SorbPE) bilayer (1:1 (mol/mol)). Curve obtained with only buffer in the PWR cell (1), after PSLB formation (2), after Rho incorporation (3), after UV polymerization (4), and after yellow light activation of Rho at pH 5.5 and 25 ± 1 °C (5).

Figure 5.

PWR curves (A, *p*-polarized; B, *s*-polarized) acquired at each stage of an experiment with an unpolymerized bilayer composed of bis-SorbPC/mono-SorbPE (1:1 (mol/mol)). Curve obtained with only buffer in the PWR cell (1), after PSLB formation (2), after Rho incorporation (3), and after yellow light activation of Rho at pH 5.5 and 25 ± 1 °C (4).

Figure 6.

Shifts in *s*-polarized PWR resonance minima for unpolymerized (triangles) and polymerized (squares) PSLBs measured as a function of added molar equivalents of Triton X-100: (A) bis-SorbPC/mono-SorbPC (1:1 (mol/mol)); (B) bis-DenPC; (C) bis-SorbPC/mono-SorbPE (1:1 (mol/mol)).

Table 1

Angular Shifts in PWR Resonance Minima (*p*- and *s*-Polarized; in mdeg) Due to PSLB Formation, Rho Incorporation, PSLB Polymerization, and Yellow Light Activation for Different Types of Lipids

lipid composition	PSLB formation (<i>p</i> -, <i>s</i> -)	Rho incorporation (<i>p</i> -, <i>s</i> -)	UV irradiation (<i>p</i> -, <i>s</i> -)	yellow light activation (<i>p</i> -, <i>s</i> -)
DOPC ^a	113 (±4)	71 (±2)		13 (±2)
	78 (±3)	51 (±2)		8 (±2)
bis-SorbPC ^a	116 (±3)	70 (±2)		11 (±1)
	80 (±3)	52 (±2)		11 (±2)
poly(bis-SorbPC) ^{a,b}	113 (±4)	70 (±3)	4 (±1)	13 (±1)
	77 (±3)	51 (±2)	3 (±1)	12 (±2)
bis-SorbPC/mono-SorbPC (1:1)	110 (±2)	69 (±2)		10 (±1)
	80 (±3)	48 (±2)		10 (±1)
poly(bis-SorbPC/mono-SorbPC) (1:1) ^b	105 (±4)	73 (±2)	4 (±1)	11 (±1)
	81 (±2)	48 (±3)	4 (±1)	10 (±2)
mono-SorbPC	110 (±4)	74 (±3)		10 (±2)
	82 (±4)	52 (±2)		10 (±1)
poly(mono-SorbPC) ^b	113 (±4)	71 (±3)	2 (±1)	10 (±1)
	85 (±3)	53 (±3)	2 (±1)	11 (±2)
bis-SorbPC/mono-SorbPE (1:1)	104 (±2)	70 (±2)		23 (±2)
	78 (±2)	51 (±3)		15 (±2)
poly(bis-SorbPC/mono-SorbPE) (1:1) ^b	107 (±3)	72 (±3)	3 (±1)	10 (±2)
	79 (±2)	51 (±3)	4 (±1)	9 (±1)
bis-DenPC	112 (±4)	71 (±2)		8 (±1)
	83 (±3)	50 (±3)		5 (±1)
poly(bis-DenPC) ^b	106 (±4)	69 (±1)	4 (±1)	2 (±1)
	79 (±2)	46 (±2)	4 (±1)	2 (±1)

^aData from ref 7.

^bPSLBs were polymerized by UV irradiation after incorporation of Rho.

Table 2

Angular Shifts in the PWR Resonance Minimum (*p*- and *s*-Polarized; in mdeg) Due to PSLB Formation, PSLB Polymerization, Rho Incorporation, and Yellow Light Activation for Different Types of Lipids

lipid composition ^a	PSLB formation (<i>p</i> -, <i>s</i> -)	UV irradiation (<i>p</i> -, <i>s</i> -)	Rho incorporation (<i>p</i> -, <i>s</i> -)	yellow light activation (<i>p</i> -, <i>s</i> -)
poly(bis-SorbPC) ^b	117 (±4)	4 (±2)	64 (±4)	7 (±2)
	82 (±3)	3 (±1)	22 (±4)	6 (±2)
poly(bis-SorbPC/mono-SorbPC) (1:1)	110 (±2)	4 (±1)	71 (±2)	8 (±2)
	80 (±3)	3 (±1)	26 (±2)	4 (±1)
poly(bis-SorbPC/mono-SorbPE) (1:1)	107 (±3)	3 (±1)	90 (±5)	-3 (±1)
	79 (±4)	4 (±1)	61 (±3)	-4 (±2)
poly(bis-DenPC)	107 (±4)	4 (±1)	64 (±3)	2 (±1)
	81 (±2)	4 (±1)	46 (±3)	2 (±2)

^a All PSLB compositions were polymerized before incorporation of Rho.

^b Data from ref 7.