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Photopolymerization of dienoyl lipids creates planar supported poly(lipid) membranes with retained fluidity

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Abstract

Polymerization of substrate-supported bilayers composed of dienoyl phosphatidylcholine (PC) lipids is known to greatly enhance their chemical and mechanical stability, however the effects of polymerization on membrane fluidity have not been investigated. Here planar supported lipid bilayers (PSLBs) composed of dienoyl PCs on glass substrates were examined to assess the degree to which UV-initiated polymerization affects lateral lipid mobility. Fluorescence recovery after photobleaching (FRAP) was used to measure the diffusion coefficients (D) and mobile fractions of rhodamine-DOPE in unpolymerized and polymerized PSLBs composed of bis-sorbyl phosphatidylcholine (bis-SorbPC), mono-sorbyl phosphatidylcholine (mono-SorbPC), bis-dienoyl phosphatidylcholine (bis-DenPC) and mono-dienoyl phosphatidylcholine (mono-DenPC). Polymerization was performed in both the L_{α} and L_{β} phase for each lipid. In all cases, polymerization reduced membrane fluidity, however measurable lateral diffusion was retained which is attributed to a low degree of polymerization. The D values for sorbyl lipids were less than those of the denoyl lipids; this may be a consequence of the distal location of polymerizable group in the sorbyl lipids which may facilitate inter-leaflet bonding. The D values measured after polymerization were 0.1 to 0.8 of those measured before polymerization, a range that corresponds to fluidity intermediate between that of a L_{α} phase and a L_{β} phase. This D range is comparable to ratios of D values reported for liquid-disordered (L_d) and liquid-ordered (L_o) lipid phases, and indicates that the effect of UV polymerization on lateral diffusion in a dienoyl PSLB is similar to the transition from a L_d phase to a L_o phase. The partial retention of fluidity in UV polymerized PSLBs, their enhanced stability, and the activity of incorporated transmembrane proteins and peptides is discussed.

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SUPPORTING INFORMATION AVAILABLE

Chemical structures of polymerizable lipids, determination of UV polymerization time for PSLBs, structures of linearly polymerized lipids, and a comparison of fitting mono- and biexponential models to a recovery curve. This material is available free of charge via the Internet at <http://pubs.acs.org>.

INTRODUCTION

Artificial lipid membranes have been extensively studied, primarily due to their importance as models for natural cell membranes and their use as membrane-mimetic constructs in a variety of technological applications, including biocompatible coatings, therapeutic and diagnostic vehicles, and chemical and biological sensors.^{1–6} Creation of artificial membranes that provide a suitable environment for reconstitution of integral membrane proteins and peptides with retention of structure and bioactivity has been a very active area of research.^{7–13} Much of this work has focused on solid supported and suspended membranes because these constructs are compatible with many surface analytical/physical characterization techniques and biosensor transduction strategies. Both the chemically-specific properties of the lipid constituents (e.g., head group structure) as well as the lipid bilayer continuum properties (e.g., elasticity, curvature, thickness, fluidity) are thought to be important in determining if a reconstituted transmembrane protein is functional in an artificial lipid membrane.^{14–17}

An additional consideration that is relevant for use of artificial membranes in technological applications is bilayer stability.¹⁸ Lipids self-organize into bilayers by non-covalent intermolecular forces that may be compromised by exposure to chemical conditions, thermal instability, and mechanical disruptions encountered during device use and storage.^{1,19–21} Numerous methods to stabilize lipid membranes have been investigated,^{11,18,22,23} including linear and cross-linking polymerization of lipid monomers.^{3–5,24–26}

Synthetic lipids functionalized with reactive dienoyl groups have been used to create several types of poly(lipid) supramolecular assemblies, including vesicles, suspended planar membranes, and planar supported lipid bilayers (PSLBs).^{3,19,26–39} Numerous parameters, such as the chemical structure of the lipid, the polymerization method, and the type of supramolecular assembly, influence the degree to which polymerization alters bilayer properties. Remarkably enhanced membrane stability, altered permeability to ions and small molecules, changes in thermotropic behavior, and other important properties have been observed. Planar bilayers composed of polymerized dienoyl lipids also have been used as hosts for reconstitution of membrane proteins and peptides.^{31,32,36–38} A key question is how the polymerization conditions and the altered properties of the bilayer affect the incorporated protein or peptide. Subramaniam et al.^{37,38} reconstituted bovine rhodopsin (bRho) into dienoyl PSLBs that were subsequently polymerized using UV irradiation. bRho photoactivity was retained when the polymeric network was formed in the center of the bilayer but not when it was adjacent to the glycerol backbone, which was ascribed to differences in the effect of polymerization on bilayer bending rigidity.^{37,38} Alamethicin, an ion channel-forming peptide, loses activity in a UV polymerized dienoyl lipid bilayer; this finding was attributed to the lower fluidity of the poly(lipid) which inhibits peptide oligomerization.³¹ In a mixed bilayer composed of a poly(lipid) and a nonpolymerizable lipid, however, alamethicin channel activity increased after polymerization, presumably because the peptides were localized in the nonpolymerized, fluid lipid domains.

The effects of lipid polymerization on membrane fluidity, which is most frequently assessed by measuring the lateral diffusion coefficient (D) of fluorescent lipid probes using

fluorescence recovery after photobleaching (FRAP),⁴⁰ have been the subject of only a handful of publications. Gaub et al.³⁵ reported that UV polymerization of vesicles of dioctadecadienoyl ammonium bromide, a cross-linkable lipid, caused a four-fold reduction in D which was attributed to a low number-average degree of polymerization (X_n). Diacetylene lipids, both pure and mixed with nonpolymerizable, fluid-phase lipids, have been the subject of several studies.^{41–43} Upon exposure to a high dose of UV irradiation, pure bis-diacetylene lipid bilayers are highly cross-linked which results in $D \approx$ zero on the timescale of a FRAP measurement.^{42,43} Lower UV doses, however, produce partially polymerized bilayers in which restricted lateral diffusion of co-incorporated, nonpolymerizable lipids is observed.^{42,43} Fahmy et al.⁴⁴ reported that thermal polymerization of bilayers composed of a zwitterionic lipid with a methacryloyl group in one tail caused a 500-fold increase in the time constant for fluorescence bleaching recovery. Kölchens et al.²⁷ examined diffusion in membranes composed of mono- and bis-acryloylphosphatidylcholines that were polymerized using thermal initiation. Their work demonstrated an inverse correlation between D and X_n for linear poly(lipids). When the mole fraction of the bis lipid was greater than 0.3 in mixed mono/bis bilayers, the decrease in D was much greater. In all of these studies, lipid polymerization caused a reduction in D compared to the unpolymerized membranes, and in some cases, a decrease in the mobile fraction was also reported. However degree of reduction varied considerably which is not unexpected based on differences in lipid structure and polymerization method and conditions.

Studies of diffusion behavior in polymerized PSLBs of dienoyl-functionalized phosphatidylcholine lipids have not been published, and are of particular interest in light of reports of greatly enhanced chemical/mechanical stability and retained protein activity in these membranes.^{30–32,36–39} Here lateral diffusion in unpolymerized and UV polymerized PSLBs formed from dienoyl lipids was characterized using FRAP. The variables examined included the location and number of polymerizable groups in the lipid tails and the effect of polymerization above and below the main phase transition temperature (T_m). In all cases, UV polymerization reduced membrane fluidity; the D values measured after polymerization were 0.1 to 0.8 of those measured before polymerization. This range is comparable to ratios of D values reported for liquid-disordered (L_d) and liquid-ordered (L_o) lipid phases, and suggests that the effect of UV polymerization on lateral diffusion in a dienoyl PSLB is similar to the transition from a L_d phase to a L_o phase. These results provide guidance for creating highly stable poly(lipid) membranes in which the activity of reconstituted transmembrane proteins and peptides is maintained.

EXPERIMENTAL

Materials

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (rho-PE) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). The structures of bis-sorbyl phosphatidylcholine (bis-SorbPC), mono-sorbyl phosphatidylcholine (mono-SorbPC), bis-dienoyl phosphatidylcholine (bis-DenPC) and mono-dienoyl phosphatidylcholine (mono-

DenPC) are shown in Figure S1. bis-SorbPC and mono-SorbPC were prepared via a modified version of that described by Lamparski et al.^{45,46} Preparatory scale purification of bis-SorbPC was performed by reverse phase HPLC.³⁹ Synthesis of bis-DenPC and mono-DenPC was performed using the methods reported by Jones et al.⁴⁷ and Liu et al.⁴⁸ respectively. Lipids were kept at -80°C for long-term storage and -20°C for short-term storage. Polymerizable lipids were always handled under yellow light or in darkness during preparation. All water, referred to as DI water, was obtained from a Barnstead Nanopure system (Thermolyne Corporation, Dubuque, IA) with a measured resistivity of greater than $17.5\text{ M}\Omega\text{-cm}$.

PSLB formation and UV polymerization

All substrates were $25 \times 75\text{ mm}$ microscope slides (Gold Seal, Portsmouth, NH). Slides were cleaned by briefly scrubbing with 1% Liquinox (Alconox, Jersey City, NJ) and a cotton pad, followed by rinsing with DI water and drying under nitrogen. They were then soaked for 5 minutes in 70:30 concentrated sulfuric acid /30% hydrogen peroxide (EMD, Gibbstown, NJ; *Caution: this solution is highly corrosive*), then rinsed with copious amounts of DI water and blown dry under nitrogen. After cleaning, slides were immediately mounted into a custom sample holder.

Vesicles were prepared from the lipid of interest and $\sim 0.6\text{ mol \%}$ rho-PE. A gentle stream of Ar (g) was used to remove chloroform from the lipid solution, followed by drying under vacuum for 4 hours. Lipid mixtures were used within 2 days of drying. If not immediately used, the dried lipid mixture was stored at -20°C . Immediately before use, the lipid mixture was reconstituted to 0.5 mg/ml in 10 mM phosphate buffer, pH 7.0, then sonicated (at $5\text{--}10^{\circ}\text{C}$ above the T_m for polymerizable lipids) with an ultrasonicator fitted with a cuphorn (W-380, Heat Systems Ultrasonics, Inc, Farmingdale, NY) until the solution became clear and no visible traces of suspended material remained. Several drops of the solution were then quickly deposited onto the freshly cleaned slide. For polymerizable lipids, the slide was pre-heated to $5\text{--}10^{\circ}\text{C}$ above the T_m of the respective lipid, and vesicle fusion^{20,49} was allowed to occur for 30 minutes at $5\text{--}10^{\circ}\text{C}$ above the T_m of the respective lipid. The sample chamber was then rinsed with at least 20 ml of phosphate buffer at room temperature without exposing the PSLB to air. The PSLB was then reheated to $5\text{--}10^{\circ}\text{C}$ above the T_m of the respective lipid and examined by epifluorescence microscopy to locate uniform areas on which FRAP was performed. PSLBs composed of DOPC were prepared similarly, except that sonication was performed at $25\text{--}35^{\circ}\text{C}$ and subsequent manipulations were performed at room temperature.

UV polymerization was carried out at either $5\text{--}10^{\circ}\text{C}$ above or $5\text{--}10^{\circ}\text{C}$ below the T_m of the respective polymerizable lipid. The PSLB was illuminated for 30 minutes by a low pressure Hg pen lamp (rated $4500\text{ }\mu\text{W/cm}^2$ at 254 nm) mounted 7.6 cm above the slide. These conditions were sufficient to photoreact nearly 100% of the dienoyl groups in the PSLB (see Supporting Information (SI) for further details) which means that the extent of monomer-polymer conversion was nearly 100%. Minor decreases in rho-PE fluorescence intensity in PSLBs were measured after 30 minutes of UV irradiation.

FRAP

FRAP was performed using a Nikon TE2000-U inverted microscope (Nikon Instruments, Inc., Melville, NJ) with a 20× phase contrast objective. Samples were photobleached in an epi-illumination geometry for < 1 s using the 488 nm line of an Innova 70 Ar ion laser (Coherent, Santa Clara, CA) at a power of ~ 200 mW measured after a neutral density filter placed at the laser exit aperture. Images were captured with a Princeton Instruments CCD camera controlled by an ST-133 controller using WinSpec/32 software (Trenton, NJ). The laser intensity profile was a Gaussian with a radius at $1/e^2$, ω , between 5 and 17 μm in the first image captured after photobleaching. Regions of interest (ROIs) inside (I_{in}) and outside (I_{out}) the bleached spot were monitored before and after bleaching to determine the diffusion coefficients and percent recoveries. To normalize recovery curves, the intensity ratio ($F = I_{in}/I_{out}$) immediately before bleaching was set to unity. A biexponential model (equation 1) composed of two diffusing rho-PE populations, denoted fast and slow, was used to fit recovery curves,

$$F(t) = 1 - (A + B_1 e^{-k_1 t} + B_2 e^{-k_2 t}) \quad (1)$$

where $F(t)$ is the intensity ratio at a given time, t , after bleaching at $t = 0$, k_x are the rate parameters characteristic of the fast ($x = 1$) and slow ($x = 2$) populations, A is the unrecovered fraction, and B_x are the fractions of the fast and slow population. Goodness of fit was evaluated based on the adjusted coefficient of determination (R_{adj}^2) and the residuals plot. Residuals from fitting a monoexponential model to recovery curves displayed systematic deviations and lower R_{adj}^2 values for all PSLBs studied, while the residuals from biexponential fits lacked these deviations and R_{adj}^2 values were greater (see SI for a comparison of residuals and R_{adj}^2 values).

Fast and slow diffusion coefficients (D_1 and D_2 , respectively) were computed for each PSLB using⁴⁰

$$D_x = \gamma_D \omega^2 (4\tau_x)^{-1} \quad (2)$$

where γ_D is a correction factor related to bleach depth (1.1 in all cases) and τ_x is the half-time for recovery ($\tau_x = (\ln 2)k_x^{-1}$). Percent recoveries for the fast and slow components were calculated as

$$\%_1 = 100 B_1 (A + B_1 + B_2)^{-1} \text{ for fast} \quad (3)$$

$$\%_2 = 100 B_2 (A + B_1 + B_2)^{-1} \text{ for slow} \quad (4)$$

The total percent recovery for the PSLB, $\%_{tot}$, was the sum of $\%_1$ and $\%_2$. The percent of the bleached fluorescence that did not recover (the percent immobile) is $100 - \%_{tot}$. A weighted average diffusion coefficient for the mobile fractions, D_{avg} , was also calculated

$[D_{avg} = \%_1 (D_1) + \%_2 (D_2)]$ for each PSLB to permit simpler comparisons of the diffusion data.

RESULTS AND DISCUSSION

Unpolymerized PSLBs

To assess the effects of polymerization on diffusion, FRAP measurements were first performed on PSLBs composed of the unpolymerized dienoyl lipids. Measurements were performed at 5–10 °C above the T_m of the respective lipid which ensured that all PSLBs were in the liquid crystalline-like (L_α) phase. DOPC PSLBs served as a reference sample. Figure 1 shows examples of recovery curves for unpolymerized lipids and fits of equation 1 to these data. Table 1 lists the T_m , the measurement temperature (T_{fr}), fast and slow diffusion coefficients (D_1 and D_2 , respectively), percent recoveries for the fast and slow components ($\%_1$ and $\%_2$, respectively), the total percent recovery ($\%_{tot}$), and D_{avg} for all five PSLB types.

All unpolymerized PSLBs as well as DOPC PSLBs were found to recover entirely ($\%_{tot} \approx 100$), with the fast component accounting for 68–75% of the total recovery. Fast and slow diffusing populations with unequal amplitudes were also reported by Scomparin et al.⁵⁰ for PSLBs of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) formed on glass via vesicle fusion. A possible source of this behavior is an unequal distribution of rho-PE in the upper and lower leaflets of the bilayer. To probe this distribution, the emission intensity of a DOPC PSLB was measured by epifluorescence microscopy before and after the addition of 500 mM CoCl_2 to the aqueous phase above the PSLB. Co^{2+} quenches the emission of fluorophores in upper leaflet which allows the percentage of fluorophores in each leaflet to be determined by difference.⁵⁴ After correcting for background intensity and photobleaching, the percent of emission intensity quenched by Co^{2+} was 68% (± 1.2), which agrees well with $\%_1 = 69$ (± 2.3) for DOPC listed in Table 1. Ajo-Franklin et al.⁵⁴ reported similar results; they found that 70% of Texas Red DHPE, a fluorescent lipid structurally similar to rho-PE, is in the upper leaflet of egg phosphatidylcholine PSLBs formed by vesicle fusion on glass. The asymmetric distribution may be due to repulsive interactions between the negative charges on the glass surface and the lipid headgroups⁵⁴ (both rho-PE and Texas Red DHPE have a -1 charge). These results suggest that the biexponential recoveries listed in Table 1 reflect an asymmetric distribution of fast and slow diffusing fluorophores in the upper and lower leaflets, respectively; however, a more extensive study involving other lipids and probes would be required to verify these assignments.

The D_{avg} value for DOPC listed in Table 1 is comparable to results found in the literature.^{e.g. 55,56} The D_{avg} values of mono-substituted lipids were similar to DOPC, while those of bis-substituted lipids were 2–5 fold lower. However it is difficult to directly compare the diffusion coefficients of these lipids because the FRAP measurements were performed at different temperatures. To perform a temperature-normalized comparison, D_{avg} values were estimated for each lipid at 25 °C, assuming that each lipid is in the L_α phase at this temperature. In a recent paper, diffusion coefficients were measured as a function of temperature for rho-PE in DOPC PSLBs formed by vesicle fusion on glass.⁵⁶ Using those data, $\ln(D)$ vs temperature was plotted over the range of 298–313 K, from which a slope of

$0.0215 \ln(D) \cdot (K^{-1})$ was obtained for DOPC in the L_α phase. The D_{avg} values in Table 1 were then converted to normalized values at 25 °C using

$$\ln D_{25} = (\ln D_{avg}) - 0.0215(T_{fr} - T_{ref}) \quad (5)$$

where T_{ref} is 25 °C and D_{25} is the estimate of D_{avg} at 25 °C in the PSLB composed of the respective dienoyl lipid.

The D_{25} values of the polymerizable lipids, listed in Table 1, range from 15% to 100% of D_{25} for DOPC, with the denoyl lipids showing more rapid recovery than the corresponding sorbyl lipids. This difference is likely due to differences in intermolecular forces between the lipids in the center of the bilayer. The sorbyl tail groups can interact via dipole-induced-dipole and dipole-dipole mechanisms, whereas the denoyl tails interact only via dispersion forces in that region of the bilayer. Comparing the sorbyl lipids, diffusion in bis-SorbPC is significantly slower than diffusion in mono-SorbPC, which may be attributable to: *a*) two sorbyl tail groups are capable of participating in dipole-induced-dipole and dipole-dipole interactions in bis-SorbPC vs only one in mono-SorbPC; and *b*) the 1-palmitoyl tail in mono-SorbPC is one bond shorter than the corresponding 10-(2',4'-hexadienoyloxy)decanoyl tail in bis-SorbPC. Comparing the denoyl lipids, the 1-palmitoyl tail in mono-DenPC is two carbons shorter than the corresponding octadeca-2,4-dienoyl tail in bis-DenPC; this difference suggests that diffusion in mono-DenPC should be more rapid which matches the experimental results.

UV polymerization above T_m

Diffusion data for PSLBs polymerized at 5–10 °C above the T_m (i.e., in the L_α phase) are summarized in the upper half of Table 2. To compare changes in the diffusion coefficient resulting from polymerization, FRAP measurements were performed at the same T_{fr} values and D_{25} values were computed as described above. Table 2 also includes the D_{ratio} , which is the D_{avg} of a polymerized PSLB divided by the D_{avg} of an unpolymerized PSLB. The range of D_{ratio} values, from 0.19 to 0.79, shows that UV polymerization above the T_m attenuates lateral diffusion to a variable degree; however in all cases, measureable diffusion is retained. In a control experiment, a DOPC PSLB was irradiated for 30 minutes. The D_{avg} values before and after irradiation were $4.2 \mu m^2/s$ and $4.1 \mu m^2/s$, respectively, showing that UV exposure had a minimal effect on lateral diffusion. To further verify that UV polymerization was responsible for the attenuation of diffusion in dienoyl PSLBs, partial polymerization of a bis-SorbPC PSLB was performed by irradiating it for 45 s. The resulting D_{ratio} was 0.42, whereas D_{ratio} for fully polymerized bis-SorbPC was 0.27.

Both mono-DenPC and mono-SorbPC PSLBs maintained 100% recovery after polymerization. There was a minor change in D_{avg} for poly(mono-DenPC), while a moderate decrease was observed for poly(mono-SorbPC), and this occurred entirely in D_I . The respective D_{ratio} values of 0.79 and 0.54 suggest that oligomers are formed upon UV polymerization of these lipids. Results reported by several groups provide support for this interpretation.^{27–29,35} Lamparski and O'Brien²⁸ showed that UV polymerization of mono-SorbPC vesicles produced oligomers with $X_n = 3–10$, although this result was not correlated with diffusion measurements. Kölchens et al.²⁷ reported both diffusion coefficients and X_n

values for mono-acryloyl lipid bilayers polymerized using thermal initiation. Before polymerization, $D = 3.8 \mu\text{m}^2/\text{s}$, whereas in polymerized bilayers having X_n values of 233 and 695, D was $1.4 \mu\text{m}^2/\text{s}$ and $0.28 \mu\text{m}^2/\text{s}$, respectively. Thus the respective D_{ratio} values were 0.37 and 0.07, significantly less than the D_{ratio} values measured here for poly(mono-SorbPC) and poly(mono-DenPC). Taken together, these findings indicate that the minor to moderate decrease in probe mobility observed here upon polymerization of the mono-substituted dienoyl PSLBs is attributable to a low X_n . (Note: X_n is problematic to determine for a PSLB with an area of a few cm^2 because it contains a small number of molecules and their ionization efficiency is very low.³⁹)

The location of the dienoyl groups in these lipids provides a possible explanation for the difference in the D_{ratio} values of poly(mono-DenPC) and poly(mono-SorbPC) bilayers. In the former, polymeric networks are formed adjacent to the glycerol backbone in each leaflet, whereas in the latter, polymerization occurs at the distal ends of the lipid tails (see example structures in SI). Polymerization across the two leaflets also may be possible in sorbyl bilayers, as suggested by Ross et al.¹⁹ Inter-leaflet polymerization should present a greater barrier to diffusion than polymerization confined to a single leaflet, in which the number of nearest neighbor molecules should be less compared to inter-leaflet polymerization. This difference is a probable cause for the finding that the D_{ratio} for mono-SorbPC is less than that of mono-DenPC.

Cross-linking polymerization of PSLBs composed of bis-DenPC and bis-SorbPC above their respective T_m values produced changes in diffusion behavior greater than those observed for the mono-substituted lipids (Table 2). Example recovery curves for bis-SorbPC before and after polymerization are shown in Figure 2. The $\%_{tot}$ was less than 100 for both poly(bis-DenPC) (7% immobile) and poly(bis-SorbPC) (14% immobile). Incomplete recovery is likely caused by rho-PE trapped in cross-linked lipid structures that are immobile on the time scale of the FRAP measurement. Relative to the corresponding unpolymerized PSLBs (Table 1), D_1 , D_2 and $\%_1$ decreased, while $\%_2$ slightly increased.

The D_{25} values for the mobile fraction in poly(bis-DenPC) and poly(bis-SorbPC) were approximately 11% of the corresponding D_{25} values for the mono-substituted, polymerized PSLBs. The D_{ratio} values for the cross-linked PSLBs were also significantly less than those observed for the linearly polymerized PSLBs. These trends are consistent with the expectation that cross-linking will generate larger lipo-polymers, resulting in a lower rate of lateral diffusion arising from a more restricted diffusion path.^{27,29,35} For example, Kölschens et al.²⁷ reported that D in polymerized bilayers composed of an equimolar mixture of mono- and bis-acryloylphosphatidylcholine lipids was more than 10-fold lower than D in polymerized bilayers composed of only the mono-substituted lipid. Gaub et al.³⁵ studied UV polymerized vesicles composed of dioctadecadienoyl ammonium bromide, a lipid with a tail structure similar to that of bis-DenPC. They reported a D_{ratio} of 0.25, quite similar to the values of 0.19 and 0.27 observed here for poly(bis-DenPC) and poly(bis-SorbPC). The authors attributed this “rather small reduction” in diffusion coefficient to a low X_n which was estimated to be <100 .³⁵ Likewise, a low X_n is the most probable explanation for the moderate decrease in lateral diffusion that occurs upon polymerization of bis-DenPC and bis-SorbPC.

In summary, the data in the upper half of Table 2 clearly show that after nearly 100% conversion of monomer to polymer (see SI), measurable lateral diffusion is still present in linearly polymerized mono-DenPC and mono-SorbPC and cross-linked bis-DenPC and bis-SorbPC. This result is a marked contrast to bis-diacetylene lipids which, upon exposure to a high dose of UV irradiation that results in near-complete polymerization, form a cross-linked bilayer in which lateral probe diffusion is essentially eliminated on the timescale of a FRAP measurement.⁴³

UV polymerization below T_m

The greater molecular order in the solid-like (L_β) phase of a lipid bilayer, relative to the L_α phase, may affect lipid polymerization:⁵² In the L_β phase, the more ordered lipid tails may be in conformations more favorable for propagating the polymerization reaction. However, slower diffusion in the L_β phase will reduce the collision frequency, possibly reducing X_n .

Some properties of dienoyl lipids polymerized at temperatures above and below the T_m have been compared in a few studies. Lei et al.⁵² investigated redox-initiated radical polymerization (redox) of mono-SorbPC vesicles; they found that the rate of polymerization was moderately higher when the reaction was performed above the T_m relative to below the T_m , whereas the difference in X_n was minor (51 in the L_α phase vs 43 in the L_β phase). In contrast, Lamparski and O'Brien reported that the rate of UV polymerization of sorbyl vesicles is relatively insensitive to the phase of the bilayer, which they ascribed to the low X_n characteristic of this polymerization method.²⁸

Tsuchida et al.³³ studied polymerization of mono-DenPC vesicles using redox and visible sensitization methods, and found that both methods generated a larger X_n when the lipids were in the L_α phase. Lipid diffusion was not measured in any of these studies.

Here FRAP of dienoyl lipid PSLBs that were UV polymerized in the L_β phase was performed to assess if polymerization temperature influences lipid mobility. Polymerization was performed at 5–10 °C below the respective T_m of each lipid and FRAP measurements were made at the same T_{fr} used in the previous set of experiments. The results, summarized in the lower half of Table 2, show that UV polymerization below the T_m attenuates lateral diffusion to a variable degree; however in all cases, measureable diffusion is retained.

Both mono-DenPC and mono-SorbPC PSLBs maintained 100% recovery after polymerization. The D_{avg} for poly(mono-DenPC) was equivalent to the D_{avg} for this lipid polymerized in the L_α phase. In contrast, the D_{avg} and D_{ratio} values for mono-SorbPC polymerized in the L_β phase were two-fold less than the corresponding values in the L_α phase. Decreases in both D_1 and D_2 were observed. This indicates that L_β phase polymerization of mono-SorbPC produces a more viscous membrane, suggesting a larger X_n .

The locations of the polymerizable groups in mono-DenPC and mono-SorbPC and changes in molecular order in lipid tail region in the L_α and L_β phases provide a probable explanation for the different results obtained with these lipids. Both theoretical and experimental studies show that in a lamellar lipid bilayer, the lipid tail order parameter, S_{CD} , decreases along the

acyl chain from the glycerol backbone to the distal end, showing that the center of the bilayer is more disordered.⁵⁷ Molecular dynamics simulations as a function of temperature indicate that when a transition from the L_α phase to the L_β phase occurs, the increase in S_{CD} is greater at the distal ends.⁵⁸ This suggests that the change in order accompanying the L_α to L_β phase transition should be greater in the vicinity of the dienoyl group in a mono-SorbPC bilayer relative to a mono-DenPC bilayer. If this increased order promotes polymerization,⁵² perhaps aided by stronger intermolecular forces between the sorbyl groups, then an increase in X_n and consequent decrease in lateral diffusion may be expected from polymerization of mono-SorbPC in the L_β phase.

Polymerization of bis-SorbPC PSLBs in the L_β phase increased the immobile fraction to 25%, indicative of greater rho-PE entrapment in cross-linked domains; this increase was reflected in a corresponding decrease in $\%I$. Relative to polymerization in the L_α phase, D_I and D_2 decreased by factors of 2 and 2.7, respectively. These results are consistent with the above discussion regarding the change in S_{CD} at the distal ends of the lipid tails accompanying the L_α to L_β phase transition. The increase in S_{CD} likely promotes polymerization of sorbyl groups, resulting in a greater X_n and more restricted lateral diffusion. In contrast, when bis-DenPC was polymerized in the L_β phase, the $\%_{tot}$ was equivalent and the D_{avg} was moderately greater in comparison to the results obtained when polymerization was performed in the L_α phase. This result is consistent with the findings of Tsuchida et al.³³ discussed above. The difference in the D_{avg} was due primarily to a greater D_I in the L_β phase. The reasons for this difference are not clear and require further study.

Comparison with diffusion in nonpolymerized lipid phases

The data listed in Tables 1 and 2 show that UV polymerization of PSLBs composed of dienoyl lipids attenuates the lateral mobility of rho-PE to varying degrees. Depending on the lipid and polymerization conditions, up to 25% immobile populations and a 10-fold decrease in D_{avg} was observed; however in all cases, measurable lateral diffusion was retained. As demonstrated in past work, UV polymerization also greatly enhances bilayer stability; for example, the lifetime of a suspended planar membrane composed of bis-DenPC is extended by cross-linking from a few hours to a few weeks.³⁶

It is instructive to compare the D_{ratio} data listed in Table 2 to relative rates of lateral diffusion in the L_α and L_β phases of a PC lipid bilayer which typically differ by two orders of magnitude. For example, D measured in the L_β phase of a DMPC PSLB formed by vesicle fusion on glass is about 50-fold less than D measured in the L_α phase.⁵⁰ The resulting D_{ratio} , 0.02, is less than all D_{ratio} values listed in Table 2, which indicates that UV polymerization of dienoyl PSLBs produces bilayers with lateral mobility intermediate between the L_α and L_β phases of a model lipid such as DMPC. Only for bis-SorbPC polymerized in the L_β phase ($D_{25} = 0.08 \mu\text{m}^2/\text{s}$) is diffusion attenuated to a degree comparable to that in the L_β phase.

A comparison to lipid mixtures that form liquid ordered (L_o) and liquid disordered (L_d) is also instructive. Kayha et al.⁵⁹ measured diffusion coefficients in giant unilamellar vesicles (GUVs) composed of DOPC as a function of added cholesterol, which causes a gradual transition from the L_d phase, at high DOPC content, to the L_o phase at high cholesterol

content. The D value declined from $6.3 \mu\text{m}^2/\text{s}$ for pure DOPC (L_α phase) to $2.4 \mu\text{m}^2/\text{s}$ for 2:1 (mol/mol) cholesterol:DOPC (L_o phase), yielding a D_{ratio} of 0.38. The same group also studied ternary GUVs composed of DOPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), and cholesterol, a mixture that is a model for “lipid rafts.”⁶⁰ GUVs composed of 0.4 DOPC/0.4 DPPC/0.2 cholesterol (mol/mol) form a L_d phase enriched in DOPC and a L_o phase enriched in DPPC/cholesterol. The respective D values were $5.2 \mu\text{m}^2/\text{s}$ and $0.44 \mu\text{m}^2/\text{s}$ which equate to a D_{ratio} of 0.08. These D_{ratio} values, 0.38 and 0.08, are in the same order of magnitude of the D_{ratio} values listed in Table 2. This comparison suggests that the effect of UV polymerization on lateral diffusion in dienoyl PSLBs can be likened to a transition from a L_d phase to a L_o phase, and is attributed to the low X_n characteristic of this polymerization method. It is important to emphasize, however, that this comparison of rho-PE diffusivity does not imply that there is any structural similarity between a poly(dienoyl) lipid bilayer and a L_o -phase lipid bilayer; for example, lipid packing and order in these two types of bilayers are certainly dissimilar.

These findings provide additional perspective to published studies reporting that in some cases, proteins incorporated into UV polymerized dienoyl lipid bilayers retain activity: *a*) bRho, the canonical G-protein-coupled receptor (GPCR), retains photoactivity in poly(mono-SorbPC) and poly(bis-SorbPC),^{37,38} which shows that the lower fluidity in these membranes does not inhibit the conformational change that accompanies bRho activation. This suggests that membrane properties that are thought to modulate GPCR activation, such as intrinsic lipid curvature and elastic moduli,^{14–16} are not significantly altered upon polymerization of sorbyl lipid bilayers. In contrast, bis-DenPC polymerization eliminates bRho activity; the formation of cross-linked networks near the glycerol backbone may stiffen the bilayer such that it cannot accommodate the conformational change associated with bRho activation.³⁸ *b*) When protein or peptide subunits must reversibly associate in a membrane to form a functional transmembrane ion channel (IC), the lower fluidity of a poly(lipid) may attenuate or eliminate IC activity. For example, alamethicin oligomerizes to form ICs in a unpolymerized bis-DenPC bilayer but the IC activity is nearly eliminated after UV polymerization.³¹ The lower fluidity of poly(bis-DenPC) appears to exceed a threshold for reversible peptide oligomerization. However mixtures of poly(bis-DenPC) and a nonpolymerizable lipid such as diphytanoyl phosphatidylcholine are sufficiently fluid to support alamethicin activity.³¹

SUMMARY AND CONCLUSIONS

UV polymerization of dienoyl lipid PSLBs was performed in both the L_α and L_β phases and, in all cases, caused a reduction in membrane fluidity. However measurable lateral diffusion was present in all four types of poly(lipid) bilayers, which is attributable to a low X_n that appears to be characteristic of UV-initiated polymerization of these lipids. D_{25} values were lower for the poly(sorbyl) lipids vs the poly(dienoyl) lipids which may be due to inter-leaflet polymerization. Polymerization in the L_β phase caused a marked reduction in D_{25} , relative to polymerization in the L_α phase, for sorbyl lipid bilayers. This result is attributed to the increase in S_{CD} at the distal ends of the lipid tails in the L_β phase which is hypothesized to produce dienoyl group conformations more favorable for polymerization, resulting in a greater X_n .

The D_{ratio} range of 0.11 to 0.81 indicates that UV polymerization of dienoyl PSLBs produces bilayers with lateral mobility intermediate between the L_{α} and L_{β} phases of a conventional lipid such as DMPC. This range of D_{ratio} values is comparable to ratios of D values reported for L_o vs L_d lipid phases. This comparison suggests that the effect of UV polymerization on lateral diffusion in a dienoyl PSLB is similar to a transition from a L_d phase to a L_o phase. It also suggests why some transmembrane proteins retain function in poly(dienoyl lipid) membranes. The activity of a protein reconstituted in a UV polymerized dienoyl lipid bilayer will depend on a number of factors, some of which are specific to the protein. Thus a generalization in terms of protein characteristics cannot be stated; however, the comparison to the L_o phase may be appropriate with respect to membrane mechanical properties and their effect on protein function. In other words, if a protein is active when reconstituted in a L_o phase, then we hypothesize that it might be active in a UV polymerized dienoyl lipid bilayer. Future studies will be designed to address this hypothesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Representative unpolymerized PSLB recovery curves for a mono-DenPC PSLB (black diamonds) and a bis-SorbPC PSLB (red triangles). Solid lines are fits of equation 1 to the data.

Figure 2.
Representative recovery curves for an unpolymerized bis-SorbPC PSLB (black diamonds) and a bis-SorbPC PSLB polymerized above the T_m (red triangles). Solid lines are fits of equation 1 to the data.

Table 1

Phase transition temperature (T_m), the temperature at which the FRAP measurement was performed (T_{fr}), diffusion coefficients and percent recoveries for the fast and slow components (D_1 , %1, D_2 , %2), total percent recovery (% $_{tot}$), weighted average diffusion coefficient (D_{avg}), and weighted average diffusion coefficient normalized to 25 °C (D_{25}) of unpolymersized PSLBs.

Lipid	T_m (°C)*	T_{fr} (°C)	%1	%2	D_1 (μm ² /s)	D_2 (μm ² /s)	% $_{tot}$	D_{avg} (μm ² /s)	D_{25} (μm ² /s)	$n^{\dagger}$
DOPC	-17	25	69 ± 2.3	31 ± 2.7	6.2 ± 0.80	0.62 ± 0.096	99	4.4	4.4	5
mono-DenPC	26	35	74 ± 7.9	26 ± 7.5	7 ± 2.3	0.5 ± 0.12	100	5.5	4.4	6
mono-SorbPC	32-36	43	74 ± 2.1	26 ± 2.6	6 ± 1.5	0.31 ± 0.020	100	4.4	3.0	5
bis-DenPC	20	30	75 ± 2.3	28 ± 3.2	2.7 ± 0.30	0.41 ± 0.062	103	2.2	1.9	5
bis-SorbPC	29	37	68 ± 2.5	31 ± 2.9	1.2 ± 0.21	0.11 ± 0.020	100	0.89	0.69	4

* T_m data for lipids obtained from published sources^{48,51-53} and avantilipids.com.

[†] Number of trials.

Table 2
 T_f , D_1 , D_2 , $\%_1$, $\%_2$, $\%_{tot}$, D_{avg} , D_{25} , and D_{ratio} for PSLBs polymerized above and below T_m .

Lipid	T_f (°C)	$\%_1$	$\%_2$	D_1 (μm ² /s)	D_2 (μm ² /s)	$\%_{tot}$	D_{avg} (μm ² /s)	D_{25} (μm ² /s) [^]	D_{ratio} [*]	n [†]
polymerization $T > T_m$										
mono-DenPC	35	71 ± 2.1	28 ± 4.2	6 ± 1.0	0.65 ± 0.086	99	4.3	3.5	0.79	4
mono-SorbPC	43	74 ± 4.6	25 ± 1.4	3.1 ± 0.22	0.37 ± 0.051	99	2.4	1.6	0.54	3
bis-DenPC	30	55 ± 2.6	38 ± 3.6	0.7 ± 0.18	0.10 ± 0.034	93	0.41	0.37	0.19	4
bis-SorbPC	37	52 ± 4.5	34 ± 2.5	0.4 ± 0.11	0.06 ± 0.016	86	0.24	0.19	0.27	3
polymerization $T < T_m$										
mono-DenPC	35	73 ± 11	23 ± 8.7	6 ± 1.0	0.6 ± 0.29	96	4.5	3.6	0.81	4
mono-SorbPC	42	70 ± 3.6	28 ± 2.0	1.6 ± 0.21	0.20 ± 0.038	98	1.2	0.84	0.28	5
bis-DenPC	30	59 ± 4.9	36 ± 4.9	1.2 ± 0.27	0.120 ± 0.0091	95	0.73	0.65	0.34	3
bis-SorbPC	37	43 ± 11	32 ± 5.4	0.2 ± 0.11	0.022 ± 0.0090	75	0.10	0.08	0.11	6

[^] D_{avg} value normalized to 25 °C.
[†] Number of trials.
^{*} $D_{ratio} = (D_{avg} \text{ for UV polymerized}) / (D_{avg} \text{ for unpolymolymerized})^{-1}$.