Plasmon-waveguide resonance (PWR) spectroscopy has been used to examine solid-supported lipid bilayers consisting of dioleoylphosphatidylcholine (DOPC), palmitoyloleylophosphatidylcholine (POPC), sphingomyelin (SM), and phosphatidylcholine/SM binary mixtures. Spectral simulation of the resonance curves demonstrated an increase in bilayer thickness, long-range order, and molecular packing density in going from DOPC to POPC to SM single component bilayers, as expected based on the decreasing level of unsaturation in the fatty acyl chains. DOPC/SM and POPC/SM binary mixtures yielded PWR spectra that can be ascribed to a superposition of two resonances corresponding to microdomains (rafts) consisting of phosphatidylcholine- and SM-rich phases coexisting within a single bilayer. These were formed spontaneously over time as a consequence of lateral phase separation. Each microdomain contained a small proportion (∼20%) of the other lipid component, which increased their kinetic and thermodynamic stability. Incorporation of a glycosylphosphatidylinositol-linked protein (placental alkaline phosphatase) occurred within each of the single component bilayers, although the insertion was less efficient into the DOPC bilayer. Incorporation of placental alkaline phosphatase into a DOPC/SM binary bilayer occurred with preferential insertion into the SM-rich phase, although the protein incorporated into both phases at higher concentrations. These results demonstrate the utility of PWR spectroscopy to provide insights into raft formation and protein sorting in model lipid membranes.

The classical textbook model of biomembrane structure, usually referred to as the fluid-mosaic model, envisons a two-dimensional solution of integral membrane proteins in a homogeneous lipid solvent, albeit one composed of many molecular lipid species and possessing inside-outside asymmetry with respect to both protein and lipid components. However, in recent years, there has been a growing recognition that lateral segregation of lipids and proteins occurs within regions of biomembranes called rafts (cf. Refs. 1 and 2). Along with caveolae, which are invaginations of raft regions stabilized by interactions with oligomers of the protein caveolin, these microdomains have been suggested to play important roles in cell polarity, protein sorting, signal transduction, and membrane trafficking (cf. Refs. 3 and 4).

One of the key properties of rafts is their high content of sphingomyelin (SM) and cholesterol, which leads to their being organized into what are referred to as liquid-ordered domains. These are characterized by being more highly ordered and somewhat thicker than the surrounding liquid-disordered regions of the membrane. This is a consequence of the ordering influence of cholesterol and the presence in SM of a larger proportion of saturated fatty acyl chains. However, it should be noted that studies of model membranes have shown that microdomain formation also occurs in phosphatidylcholine (PC)/SM mixtures in the absence of cholesterol (2). These lipid phases can coexist within a single bilayer, giving rise to a heterogeneous pattern of islands of differing composition. Protein enrichment in raft/caveola microdomains (cf. Refs. 4 and 5) is thought to be a consequence of selective interactions between the liquid-ordered microdomains and various types of membrane anchors, such as glycosylphosphatidylinositol (GPI) moieties bound to the C terminus or fatty acyl chains attached to serine residues, as well as interactions occurring between proteins and caveolin oligomers or via hydrophobic matching to the slightly thicker rafts in the case of transmembrane proteins. Such enrichment can have two consequences, i.e., co-localization of various signaling components (e.g. receptors and G-proteins) and microenvironmental effects on protein functional properties, both of which have important pharmacological implications (cf. Refs. 3 and 4). Examples of the former include the translocation of transducin to lipid rafts upon activation by rhodopsin (6) and the movement of the β2-adrenergic receptor out of rafts upon agonist binding (7, 8); examples of the latter include an increased affinity of agonist for the human oxytocin receptor (9, 10) and for the Drosophila metabotropic glutamate receptor (11) by raft localization.

Model membrane studies have shown that lipid-lipid interactions are sufficient to induce the formation of raft-like domains (12, 13). It is well established that phase separation can occur in binary lipid mixtures consisting of lipids that have different phase transition temperatures. Typically, a gel phase, which is characterized by tightly packed lipids that have limited mobility, coexists with a fluid or liquid-disordered phase in which the lipids are loosely packed and have a high degree of lateral mobility. Addition of cholesterol has been reported to modify the gel phase component of such systems, resulting in the formation of a liquid-ordered phase in which the lipids are still tightly packed, but acquire a relatively high degree of

*This work was supported by National Institutes of Health Grants GM59630, DA06284, and DA13449. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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To investigate lipid raft characteristics in model membranes, SM is commonly combined with phospholipids having unsaturated and therefore kinked fatty acyl chains, such as dioleoylphosphatidylcholine (DOPC), with or without cholesterol. As noted above, SM lipids typically have long saturated acyl chains that facilitate close packing, an important feature of lipid raft organization (18). For this reason, SM-enriched domains in a bilayer are thicker than areas enriched in more fluid, unsaturated lipids, which have kinked chains that effectively shorten the molecular length. These structural differences between diverse types of membranes create significant modifications in their mechanical properties that in turn can lead to segregation of transmembrane proteins (19, 20).

A number of biochemical and biophysical methodologies have been used to investigate microdomain systems in both whole cells and model membranes, such as low temperature extraction with nonionic detergents; effects of cholesterol depletion; and various fluorescent microscopies. All of these methods have potential problems (5); and thus, despite a great deal of effort in recent years, there is no consensus yet on the size, lifetime, composition, or cell biological effects (5); and thus, despite a great deal of effort in recent years, there is no consensus yet on the size, lifetime, composition, or cell biological effects. Enzymatic methods on the optical properties of the bilayer membrane, which can be evaluated by thin film electromagnetic theory based on Maxwell’s equations (27, 28). Inasmuch as both excitation wavelengths (632.8 and 543.5 nm) in this work are far removed from the absorption bands of the lipids and proteins used, a $k$ value different from zero reflects a decrease in reflected light intensity due only to scattering resulting from imperfections in the membrane film. It is important to recognize that for anisotropic thin films, such as the lipid bilayer membranes in this work, scattering will be different for different exciting light polarizations.

The refractive index is a macroscopic quantity and is related to the properties of individual molecules through the molecular polarizability tensor as well as to the environment in which these molecules are located (e.g. packing density and internal organization) (29). Environmental properties are especially important when molecules are located in a matrix (such as a biomembrane) that has a nonrandom organization and thus possesses long-range spatial molecular order. Such molecular ordering creates an optically anisotropic system with a uniaxial optical axis having two (different) principal refractive indices, $n_1$ and $n_2$ (also referred to as $n_1$ or $n_2$) (29). The first of these indices is associated with a linearly polarized light wave in which the electric vector is polarized parallel to the optical axis. The second one is observed for light in which the electric vector is perpendicular to the optical axis. This is the fundamental basis upon which measurement of refractive indices with polarized light can lead to the evaluation of the structural parameters of anisotropic systems such as a lipid bilayer membrane. In the simplified case in which the molecular shape is rod-like (e.g. phospholipid molecules) and the molecules are ordered such that their long axes are parallel, one has an optically anisotropic system whose optical axis is perpendicular to the plane of the bilayer (30). The values of the refractive indices measured with two polarizations of light (i.e. parallel ($n_p$) and perpendicular ($n_s$) to the optical axis) will describe this optical anisotropy ($A_p$) as follows (Equation 1),

$$A_p = (n_p^2 - n_s^2)/(n_p^2 + 2)$$

where $n_p$ is the average value of the refractive index. A uniaxial system in which the optical axis is parallel to the membrane normal is described by Equation 2.

$$n_p = 1/3(n_p^2 + 1/n_s^2)$$

In summary, $A_p$ reflects the spatial mass distribution created by both the anisotropy in the molecular polarizability and the degree of long-range order of molecules within the system (30).

Furthermore, as can be seen from the Lorentz-Lorenz relation, $n_\text{av}$ is also directly related to the mass surface density (for details, see Refs. 31 and 32) (Equation 3),

$$m = 0.1M/A(t(n_p^2)^2 - 1)(n_\text{av})^2 + 2$$

where $M$ is molecular weight, $A$ is molar refractivity, and $t$ is the thickness of the membrane. For the lipid molecules used in this work, a reasonable approximation of $M/A$ is 3.6 (32). Thus, from the thickness of the membrane ($t$) and $n_\text{av}$, one can calculate the surface mass density (or molecular packing density), i.e. mass per unit surface area (or number of molecules/unit surface area (30)), which reflects the surface area occupied by a single molecule.

In these experiments, the plasmon-generating device was calibrated by measuring the PWR spectra of the resonator using two laser light wavelengths, green ($\lambda = 543.5$ nm) and red ($\lambda = 632.8$ nm), with both p- and s-polarized light and then simulating these spectra with theoretical curves. Both of these wavelengths produced essentially equivalent results in our experiments. The goal of such a calibration is to obtain the optical parameters of the sensor. This provides an input set of data that is used in analyzing the resonance spectra obtained with the lipid membrane system deposited on the resonator surface.

**Spectral Simulation**—The purpose of simulating experimental spectra by theoretical resonance curves is to evaluate the optical parameters of the bilayer membrane (i.e. $n_p$, $n_s$, $t$, and $k$) and then to use these values (see Equations 1–3) to calculate the surface mass density or packing density) and the refractive index anisotropy. This provides a detailed description of the membrane structure. Simulation is based on two...
facts. First, the PWR spectrum can be described by the classical electromagnetic theory of thin films (27), and the equations describing such resonance curves can be used in the simulation to obtain the component curves. Second, the number of contributed components (i.e., positions, widths, and depth of the curves) equals the number of unknown optical parameters. Hence, one can uniquely determine the latter from the simulated spectra. We used this approach in our previous study (33) by nonlinear least-squares fitting of a theoretical resonance curve to the experimental spectra. This is relatively easy to do when the spectrum corresponds to a single resonance. The current application of such an approach is made more complicated by the fact that the experimental spectrum is a complex one, i.e. it consists of more than one (usually two) single resonance curves. Therefore, the simulation has to be done in two steps. First, one must calculate single resonance curves for the components; and second, one must sum such single resonance curves using appropriate ratios to fit the complex spectrum.

It is important to note that the first step results in an evaluation of the optical parameters that describe the physical properties of those parts of the lipid membrane that contribute to this particular single resonance curve. As discussed in our previous studies (26, 30, 33), the three optical parameters characterizing an immobilized lipid bilayer (n, k, and t) can be obtained with a high degree of accuracy because they are well separated in their effects on the plasmon resonance. Several different analytical methods (26) make it possible to use each of the parameters (26). The values of such errors have been included in the analysis of the experimental results presented below.

The second step provides information about the ratio of the bilayer surfaces covered by the two different kinds of membrane that are exposed to the excitation laser beam. This is based on the fact that the area of a single resonance curve is proportional to the ratio of the number of lipid molecules to the optical properties of the component membranes. Therefore, a single resonance will result in a narrow and deep resonance curve, whereas two resonances occurring simultaneously will broaden the spectrum and make it shallower. There is another important consequence of the simulation process in the case of PWR measurements with a single lipid membrane. Although repetition of the measurements in separate experiments with the same type of membrane will result in similar values of the parameters, the ratio of the areas covered by the different membrane components that are exposed to the excitation laser beam may vary from membrane to membrane; and therefore, the final complex spectrum may change its visual characteristics (see below). This implies that one can average the parameter values obtained from different measurements and use them to calculate physical quantities describing the properties of such types of bilayers.

In the present case, because we were able to measure and simulate the spectra of membranes formed from a single component lipid (DOPC, POPC, and SM), we could use such curves as a starting point in simulation for spectra of lipid membranes consisting of mixtures of these components. Iteration of such simulation was performed by variation of the parameters of the optical properties of the lipid component spectrum until appropriate agreement with the experimental spectra was obtained. As we will demonstrate below, the final deconvoluted component spectra obtained from the mixture that describe the separate phases were always different from those of the single component curves, indicating that each of the separate phases contained small amounts of the other lipid. This will be discussed further.

Formation of Lipid Membranes and Incorporation of Protein—In this study, self-assembled solid-supported lipid membranes were used. The details of sample compartment design and the protocols for membrane formation and protein incorporation have been described previously (for example, see Refs. 25, 26, and 33). Here, we present a short summary of these descriptions. The method for membrane preparation involves spreading a small amount of lipid solution across an orifice in a Teflon block separating the silica surface of the PWR resonator from the aqueous phase. The hydrated silica surface attracts the polar groups of the lipid molecules to form a monolayer with the hydrocarbon chains oriented toward the excess lipid solution. Spontaneous bilayer formation is initiated when the sample compartment of the resonator is filled with an aqueous solution, resulting in a thinning process to form the lipid membrane. The hydrocarbon chains of the lipid molecules to form a monolayer with the hydrocarbon chains oriented toward the excess lipid solution. Spontaneous bilayer formation is initiated when the sample compartment of the resonator is filled with an aqueous solution, resulting in a thinning process to form the lipid membrane. The hydrocarbon chains of the lipid molecules associate to form a monolayer on the silica surface of the PWR resonator.

When the appropriate lipid compositions were used, the bilayers produced PWR spectra that displayed two resonances. As we will demonstrate below, these can be ascribed to the spontaneous formation of microdomains within the bilayer due to lateral segregation of lipid molecules. As has been shown by atomic force microscopy (13, 17), SM-enriched microdomains are randomly distributed within the lipid bilayer. In the PWR device, the size of the microdomain is about 100 nm in diameter (i.e., the width, thickness, and depth of the resonance curve) is much larger than the individual microdomain sizes. Thus, in a typical PWR experiment, we are averaging across many such microdomains. Since the microdomain distribution, as well as the sampling of the population by the laser probe, can vary from one experiment to another, some variability is expected in the observed ratio between resonances associated with the liquid-ordered and liquid-disordered domains. As will be described below, this was indeed observed. However, the analysis of the resonance spectra in terms of the optical properties of the components will not be affected by this variability. It is important to point out that diffusion of these microdomains within the bilayer is relatively slow (on the order of minutes; see below for examples), so an individual PWR spectrum, which is obtained in a few seconds, is not influenced by such movement.

Further, we estimated that it would be observable by PWR at the wavelength lengths we used, microdomain sizes must be larger than ~100 nm.

After lipid membrane equilibration, GPI-PLAP molecules were incorporated into the bilayer by addition of microliter aliquots of the protein, solubilized in buffer containing 30 mM octyl glucoside, to the aqueous compartment of the PWR cell (1-ml volume). This resulted in dilution of the detergent to well below the critical micelle concentration (CMC). As we will describe below, each detergent micelle dissolved in the lipid membrane (28). The corresponding p- and s-polarized spectra were measured after each addition of GPI-PLAP solution into the sample cell. As we will demonstrate below, incorporation proceeded initially into the SM-rich microdomain, although when larger amounts of protein were added, incorporation into both microdomains occurred. All PWR spectral measurements reported here were obtained with a 543.5- or 632.8-nm laser light source using a Beta PWR instrument from Proterion Corp. (Piscataway, NJ).

Materials—Solid-supported lipid bilayers (DOPC, POPC, SM, and their mixtures; Avanti Polar Lipids, Birmingham, AL) were made using solutions of either the single components or mixtures of different ratios of lipids (10 mg/ml lipid concentration) in butanol/squalene (10:0.1, v/v). The buffer solution in contact with the bilayer in the sample cell for all the experiments was 10 mM Tris, 10 mM KCl, and 0.5 mM EDTA (pH 7.3).

The procedure employed for the purification and isolation of GPI-PLAP was similar to a previously published method (34). Fifty mg of PLAP (Sigma) was dissolved in 2% (v/v) Triton X-114 in phosphate-buffered saline and subjected to phase separation. This procedure enables membrane-associated proteins to partition into the detergent-rich phase, allowing their separation from hydrophilic proteins (35). Ten ml of 35% (w/v) CHAPS in phosphate-buffered saline was added to the detergent phase and incubated overnight at 4 °C. The protein was loaded onto a Sephacryl S200 column (Amersham Biosciences) pre-equilibrated with 2% CHAPS in phosphate-buffered saline. Fractions were removed and tested for their absorbance at 280 nm (Triton X-114 and CHAPS absorb at 280 nm). The apparent molecular mass of GPI-PLAP was similar to a previously published method (34). Fifty mg of protein was recovered as GPI-PLAP in good agreement with values reported by Udenfriend and co-workers (37).

RESULTS AND DISCUSSION

Lipid Bilayer Membranes Composed of a Single Lipid—To characterize lipid bilayer membranes containing microdomains, it was necessary to first examine bilayers formed from single lipid components. Fig. 1 shows both experimental and simulated PWR spectra obtained with DOPC, POPC, and SM using green p-polarized (panel A) and s-polarized (panel B) exciting light. It is important to note that similar results were obtained with red light excitation. A key observation is that experimental spectra obtained with different single lipid components were distinctively different in both their position and shape. These differences clearly indicate significant variations...
in membrane properties. To characterize the properties of each of these membranes, the experimental spectra were analyzed by theoretical simulation. These simulations are also included in Fig. 1. It is evident that the positions, widths, and depths of the spectra were well reproduced by the simulations. However, significant deviations are seen in the wing regions of the spectra; these can be ascribed to minor amounts of lipid phases having slightly different long-range order than the majority component. These will be ignored in our analysis.

Optical parameters obtained from the simulation were averaged for both exciting light wavelengths. In addition, the thickness of the membrane was averaged for both polarizations. These values were then used to calculate the packing density (or surface area occupied by one molecule) and the refractive index anisotropy. The results of these calculations are shown in Table I and clearly show that these three bilayers differ in these parameters. The thickest and most densely packed is the SM membrane, whereas the thinnest and least densely packed is the DOPC membrane, with the POPC membrane lying in between.

The structural parameters of the lipid membranes (i.e. thickness, average surface area per lipid membrane, and degree of long-range molecular order) presented in Table I are in good agreement with our previous work (30). To compare these results with those obtained using conventional structural methodologies such as x-ray diffraction and NMR (for review, see Ref. 38), it is important to recognize some important factors that cause the comparison to be rather difficult. First, there is still a large degree of uncertainty in the structural characterization of lipid membranes. Although recent applications of new theoretical approaches (see Ref. 39) have allowed medium resolution structural descriptions of both the gel and liquid phases of membrane phospholipid bilayers, the uncertainty in these parameters for the benchmark lipid dipalmitoylphos-
phatidylcholine is still at a very high level. This not only makes it difficult to compare bilayers of different lipids, but also presents even more problems in comparing different kinds of bilayers. Thus, whereas x-ray and NMR structural measurements have been made with lipid suspensions in water, the results presented in this study were obtained with a single solid-supported lipid bilayer created in the same manner as so-called black (or freely suspended) bilayers. The latter types of membranes are characterized by both a larger degree of ordering of hydrocarbon chains, producing a higher packing density (i.e., a decrease in surface area per lipid molecule), and a larger thickness as compared with lipid suspensions in water.

It is also important to point out that the PWR measurement results in a thickness that comprises both the hydrophobic chain length and the length of hydrated polar groups, including bound water molecules. Therefore, the average lipid membrane thickness obtained with this technique is larger than that acquired with classic structural methodologies, which usually refer to the hydrophobic chain length. PWR values will be more comparable with the so-called steric thickness of the membrane, as defined in the x-ray diffraction measurements, which is $4.7 \text{ nm}$ for the fully hydrated dipalmitoylphosphatidylcholine liquid phase. This will be larger than the hydrophobic chain length, but still less than the length of the fully hydrated

### Table I

Optical parameters obtained with lipid membranes consisting of single lipids

|          | $n_p$ (±0.005) | $n_s$ (±0.005) | $t_{av}$ (average of $p$- and $s$-polarizations) | Surface area/molecule | $A_n$ (±0.003) |
|----------|----------------|----------------|-----------------------------------------------|----------------------|----------------|
| DOPC     | 1.450          | 1.435          | 5.0                                           | 0.01                 |
| POPC     | 1.470          | 1.445          | 5.4                                           | 0.02                 |
| SM       | 1.525          | 1.465          | 6.1                                           | 0.04                 |

### FIG. 2

*PWR spectra obtained for a supported lipid bilayer containing a 1:1 mixture of DOPC and SM.* All spectra were measured with 632.8-nm (red) exciting light using either $p$-polarized (A and B) or $s$-polarized (C and D) light. All other conditions were as described in the legend of Fig. 1. A and C, the open circle curve shows the experimental spectra; short dashed and long dashed curves represent the deconvoluted single lipid component spectra for DOPC and SM, respectively, obtained from the simulated fit to the open circle curve, which is shown by the solid line. Note that the solid line is the sum of the short and long dashed curves, appropriately weighted to fit the experimental spectrum. B and D, shown are PWR spectra obtained from a summation of the experimental spectra obtained with single lipid component bilayer membranes, as shown in Fig. 1 for DOPC (short dashed curve) and for SM (long dashed curve). The solid line represents an appropriately weighted sum (1:1) of the short and long dashed curves. deg, degrees.
lipid molecules. The range of values for surface areas per lipid molecule obtained with phospholipid membranes using conventional methodologies extends from $0.48$ to $0.72$ nm$^2$ for dipalmitoylphosphatidylcholine (38).

Despite the above-mentioned uncertainties, the differences in the structural parameters obtained with different lipids and presented in Table I are in very good agreement with previous observations that phospholipids with unsaturated (and there-
fore kinked) fatty acyl chains will produce thinner and less densely packed bilayers as compared with ones composed of more saturated fatty acyl chains. Thus, it has been demonstrated that the thickness of a bilayer composed of C18:1 PC is 10 Å thinner than that of C18:0 SM (40). As the results in Table I show, the difference between DOPC and SM membrane thickness obtained by our analysis is 11 Å, in excellent agreement with the previous result.

The differences in thickness are consistent with alterations in packing density and refractive index anisotropy. Thus, significantly smaller values of $A_n$ and larger values of surface area occupied per molecule obtained with the DOPC bilayer as compared with the SM bilayer indicate that both the average lipid molecular length and the degree of long-range order in the molecular organization of the bilayer are much decreased, creating a more fluid and disordered phase. Note that the values for the POPC bilayer are intermediate between those of the DOPC and SM bilayers, as expected. This demonstrates that the simulation procedure provides an accurate picture of the bilayer structure for these systems.

**Binary mixture**

| Binary mixture     | $t_{av}$ (nm) | Surface area/molecule | $A_n$ ($\pm 0.003$) |
|--------------------|---------------|-----------------------|---------------------|
| DOPC/SM (1:1)      |               |                       |                     |
| DOPC-rich lipid domain | 5.2          | 0.51                  | 0.015               |
| SM-rich lipid domain | 5.9          | 0.40                  | 0.025               |
| DOPC/SM (1.5:1; Fig. 3A) | 5.1          | 0.52                  | 0.015               |
| DOPC-rich lipid domain | 6.0          | 0.39                  | 0.025               |
| DOPC/SM (1.5:1; Fig. 5C) | 5.0          | 0.52                  | 0.015               |
| DOPC-rich lipid domain | 5.9          | 0.40                  | 0.025               |
| POPC/SM (1:1)      |               |                       |                     |
| POPC-rich lipid domain | 5.4          | 0.46                  | 0.025               |
| SM-rich lipid domain | 6.1          | 0.39                  | 0.025               |

**TABLE II**

**Optical parameters of lipid membranes consisting of mixtures of lipids**

*PWR Studies of Microdomains in Lipid Bilayers*

shown in panels $B$ and $D$ of Figs. 2–4 clearly differ from the experimental spectra presented in panels $A$ and $C$ (open circle curves). This indicates that the experimental results are not well approximated by a superposition of pure single component spectra. To properly simulate the experimental spectra, one has to significantly modify the single component spectra. This is demonstrated in panels $A$ and $C$ with the short dashed and long dashed curves. Thus, as can be clearly seen from comparison of the single component and modified spectra, the DOPC and POPC spectra (short dashed curves in panels $A$ and $C$) have to be narrower and have a larger depth, whereas the SM spectrum (long dashed curves in panels $A$ and $C$) has to be broader and shallower as compared with the component spectra obtained from simulation of the single lipid spectra (short dashed and long dashed curves in panels $B$ and $D$).

Table II presents the parameters resulting from the simulations of the PWR spectra in Figs. 2–4 of lipid domains existing within bilayers consisting of DOPC and SM and POPC and SM. The average thicknesses, calculated packing densities (surface area per molecule), and refractive index anisotropies can be compared with those shown in Table I obtained with membranes consisting of the single lipid components. Although the absolute differences in the thickness and packing density values are small, the pattern is clear that both the DOPC and POPC components have increased values for both of these quantities, whereas the SM values are decreased for the microdomains as compared with the pure lipids. Also important to note are the opposite and larger changes in the refractive index anisotropies, which are consistent with the packing density and thickness alterations. These results lead to the conclusion that the experimental spectra for the mixtures can be interpreted as a sum of two resonance curves that are produced by small modifications of the single component parameters, suggesting that the lipid compositions in the microdomains are modified by the inclusion of very small amounts of the other component. It must be pointed out, however, that these small amounts are significant enough to considerably alter the resonance spectra of the pure single components and to allow formation of two stable membrane phases. This would suggest that microdomains composed of the pure lipids are not thermodynamically or kinetically stable. It is possible that mixing in small amounts of the other component can act to stabilize the system by filling in the gaps in the bilayer structure, somewhat like the mixture of amino acid side chains in the interior of a globular protein molecule allows a more compact structure to be formed.

These results confirm earlier observations obtained by fluorescence and atomic force microscopies indicating that rafts can be formed in supported lipid monolayers as well as in both supported and unsupported bilayers (41–43). Both of those techniques have provided information as to the thickness and overall dimensions of the membrane domains. The PWR tech-
nique provides another important capability, viz. it allows one to quantify important structural parameters such as packing density and internal organization, which permit a fuller description of the properties of such membrane domains.

In addition to the characterizations described above, PWR used in a kinetic mode allows us to quantify the formation process of lateral segregation. Fig. 5 shows the dynamics of domain formation using mixtures of DOPC/SM at two molar ratios, 4:1 (panel A), and 1.5:1 (panel B). There are several important conclusions that can be drawn from the comparison of the results in these two panels. First, the process of phase segregation occurred for both mixtures, i.e. even with a large excess of DOPC as in panel A, there was still enough SM to form, although transiently, a microdomain composed mainly of this lipid component. It is also important to note that the final spectrum obtained after 35 min was much different from either the DOPC or SM microdomain spectra presented in Figs. 2 and 3. This indicates that these microdomains contained much less than 20% of the second component. Second, as shown in panel A, the kinetics of the initial formation of laterally segregated phases was significantly faster in the case of an excess of DOPC than when a larger amount of SM was present (panel B), perhaps as a consequence of a lower viscosity in the former than in the latter. However, the thermodynamic equilibrium of the DOPC-rich system was shifted away from that of segregated microdomains, probably as a result of coalescence, i.e.
after transient lateral separation, the system equilibrated into what appeared to be a single phase consisting of a mixture of these two lipids. In contrast, the system with a larger amount of SM was able to form stable segregated microdomains.

A comparison of the experimental PWR spectrum shown in Fig. 5B with that in Fig. 3A reveals that, although both were obtained with DOPC/SM ratios of 1.5:1, the relative amplitudes of the resonances corresponding to the two microdomains are different. This is an illustration of the point noted under “Experimental Procedures” regarding differences in the sampling of the microdomains by the laser probe in separate experiments with similar bilayers. To verify that simulations of these two spectra result in comparable individual microdomain properties, a deconvolution of the spectrum of Fig. 5B is presented in Fig. 5C, and the calculated values obtained from this analysis are given in Table II. As is evident, the parameters resulting from the simulation of Fig. 5C and those from Fig. 3A are essentially identical, thereby providing direct support for the contention that the spectral differences were due to sampling effects and not to an inherent difference in the microdomain compositions.

**GPI-PLAP Insertion into Bilayers**—Successive aliquots of GPI-PLAP solutions were added to the sample compartment of a PWR cell containing preformed bilayers composed of each of the three lipids used in these experiments. In all cases, PWR spectral changes were observed, indicating protein interaction with the bilayer. These spectra are shown for \( p \)-polarization in Fig. 6. For both POPC and SM bilayers, shifts occurred to larger resonance angles, whereas for DOPC bilayers, the shifts were to smaller angles. Shifts to larger angles are expected when net mass increases occur, as would be the case upon protein incorporation into a bilayer with a relatively small displacement of lipid. This is reasonable for a protein such as GPI-PLAP, if only the fatty acyl chains of the phosphatidylinositol portion of the GPI linker were inserted into the lipid bilayer. In contrast, the negative shifts that occurred in both POPC and SM could only be due to a decrease in mass density within the membrane caused by PLAP insertion. A possible rationalization of this is that, as we have noted above, the DOPC bilayer, by virtue of the disordering effect of the unsaturated fatty acid chains, is much more loosely packed than either the POPC or SM bilayer (see Table I). This may allow the PLAP molecules to penetrate more deeply into the bilayer, thereby displacing more lipid and resulting in a net negative change in mass density.

Plots of the resonance angle changes as a function of added PLAP for the three bilayer systems are also shown in Fig. 6. These can be fit with a single hyperbolic function, indicating that saturable binding occurred. However, accurate equilibrium binding constant values cannot be obtained from these
plots. This is due to uncertainties regarding the equilibration of the PLAP with the bilayer resulting from nonspecific binding of the protein to non-observable surfaces within the sample compartment and possible precipitation of the protein after detergent dilution. However, higher protein concentrations were required for incorporation into the DOPC bilayer, indicating a less effective insertion process.

Incorporation of PLAP was also performed with bilayers composed of lipid mixtures. This is shown for p-polarization in a bilayer containing equimolar amounts of DOPC and SM in Fig. 7. When only small amounts of the protein were added, spectral effects occurred predominantly in the SM-enriched portion of the spectrum, indicating preferential incorporation into this microdomain. However, upon addition of larger quantities, spectral shifts to larger angles were observed to occur in both portions of the spectrum (not shown), indicating protein incorporation into both microdomains.

A plot of the spectral shifts as a function of the concentration of added PLAP is also presented in Fig. 7. This again indicates saturable binding. Note also that the spectral shifts were to larger angles at all protein concentrations, consistent with an ordering of the DOPC-rich microdomain by inclusion of a small amount of SM.

Conclusion—The results presented above have shown that spontaneous lateral segregation of lipids into microdomains (rafts) occurs in solid-supported bilayers containing binary mixtures of PC (DOPC or POPC) and SM, producing regions within the bilayer whose different optical properties allow observation of separate resonances in a PWR experiment. Lateral movement of the lipid molecules into separated regions occurs on a time scale of minutes under the conditions of these experiments. Simulation of the PWR spectra of lipid bilayers consisting of individual lipids and binary mixtures has allowed the structure and composition of these systems from which information regarding the molecular composition of the segregated microdomains has been obtained. Thus, we have shown that the resonance occurring at smaller angles is due to a microdomain containing mainly PC molecules, with a small admixture of SM, whereas the higher angle resonance consists predominantly of SM, with a small amount of PC incorporated. The thermodynamic and kinetic stability of these microdomains appears to be greatly improved by this mixing. The ability to resolve resonances corresponding to these bilayer regions is due to their differing microstructure, with the PC areas less well ordered, less tightly packed, and thinner than the SM areas. We attribute this to the varying proportions of unsaturated fatty acyl chains in these lipids. Furthermore, the intrinsically larger disorder in a pure DOPC bilayer is decreased by the inclusion of a small amount of SM in the DOPC-rich microdomain. Correspondingly, including a small amount of DOPC decreases the higher degree of order of an SM bilayer.

We have also shown that the GPI-linked protein PLAP can insert into bilayers composed of either the single lipids or binary mixtures of the lipids. Incorporation occurred relatively efficiently and was observed to be more effective into the POPC and SM bilayers than into the DOPC bilayers. Evidence was also obtained that the insertion process was different in the latter case. This was suggested to be a consequence of the looser packing of lipids in the DOPC bilayer, which allowed greater penetration of the bilayer by the GPI linker region. Insertion of PLAP into a bilayer composed of a binary mixture of DOPC and SM occurred predominantly in the SM-rich domain at low protein concentrations. This provides evidence supporting the observations made in other laboratories (17, 44) of selective interaction of GPI-linked proteins with SM-enriched raft microdomains and demonstrates that PWR spectroscopy is able to obtain information on protein sorting within bilayers.

In summary, we have shown that PWR spectroscopy has distinct advantages over other methods of visualizing lipid microdomains in that it allows information to be obtained in real-time regarding the domain structure and composition, including the ability to distinguish between domains formed from various PC species. Future studies along these lines will be directed toward examination of the functional properties of proteins that have been incorporated into raft regions of a lipid bilayer. The ability to obtain such information is yet another advantage of the PWR methodology.

Acknowledgments—We are grateful to Ian Stevenson and Denton Vacuum Co. for the preparation of thin film coatings.

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