Confocal fluorescence microscopy and fluorescence correlation spectroscopy (FCS) have been employed to investigate the lipid spatial and dynamic organization in giant unilamellar vesicles (GUVs) prepared from ternary mixtures of dioleoyl-phosphatidylcholine/sphingomyelin/cholesterol. For a certain range of cholesterol concentration, formation of domains with raft-like properties was observed. Strikingly, the lipophilic probe 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI-C18) was excluded from sphingomyelin-enriched regions, where the raft marker ganglioside GM1 was localized. Cholesterol was shown to promote lipid segregation in dioleoyl-phosphatidylcholine-enriched, liquid-disordered, and sphingomyelin-enriched, liquid-ordered phases. Most importantly, the lipid mobility in sphingomyelin-enriched regions significantly increased by increasing the cholesterol concentration. These results pinpoint the key role, played by cholesterol in tuning lipid dynamics in membranes. At cholesterol concentrations >50 mol%, domains vanished and the lipid diffusion slowed down upon further addition of cholesterol. By taking the molecular diffusion coefficients as a fingerprint of membrane phase compositions, FCS is proven to evaluate domain lipid compositions. Moreover, FCS data from ternary and binary mixtures have been used to build a ternary phase diagram, which shows areas of phase coexistence, transition points, and, importantly, how lipid dynamics varies between and within phase regions.

More than 10 years ago, the hypothesis was formulated that cellular membranes are organized in discrete dynamic entities, called lipid rafts (1, 2). Studies on epithelial cell polarity revealed that lipids, in particular sphingolipids and cholesterol, were laterally organized in the exoplasmic leaflet of the apical plasma membrane according to a variable short and long range order. Furthermore, distinct proteins were shown to be selectively partitioned into lipid rafts, indicating that rafts could serve as specific sites for molecular sorting and polarized transport. They also function as platforms for intra- and intercellular signaling (3, 4), e.g. in T-cells and basophils (5–10), and play an important role in sorting, occurring in the trans-Golgi network of polarized epithelial cells (1, 11, 12) and neurons (13), as well as in pathways originating from the cell surface, i.e. involving caveolae (14, 15) and endocytic pathways (3, 12, 16). In addition, rafts may be important in cell surface proteolysis (17) and virus infection (18).

Commonly, lipid rafts are enriched in sphingolipids and cholesterol (1–4). The presence of long and saturated acyl chains in sphingolipids allows cholesterol to become tightly intercalated with such lipids, resulting in the organization of liquid-ordered (lo) phases. By contrast, unsaturated phospholipids are loosely packed and form a disordered state (usually indicated as liquid crystalline lα or liquid-disordered lβ) (19, 20). The difference in packing ability leads to phase separation (21, 22).

Model membrane studies carried out on ternary mixtures of cholesterol with phospholipids and sphingolipids showed that lβ phases, enriched in sphingolipids, separate from lα phases, enriched in phospholipids (19, 23). Several observations indicate that these “artificial rafts” are a reasonable, though crude, model of raft-containing cell membranes (24).

More recently, along with a number of techniques employed to address questions on rafts (11, 21, 25–27), important contributions have also come from optical microscopy (28, 29). Direct visualization of raft-like domains in model bilayer membranes has provided a tangible proof for the coexistence of liquid-ordered and liquid-disordered phases (30–33). However, rafts are by no means static structures. If it is true that their main function consists of forming platforms to concentrate certain proteins, then a detailed characterization of lipid and protein dynamics in the different phases is essential to understand mobility-dependent protein organization (34). Single particle tracking (SPT) has been applied to follow raft-associated proteins in vivo (29) and lipid mobility in cell membranes and in vitro (31, 35). Additional contributions have come from fluorescence recovery after photobleaching (FRAP) (32) and fluorescence resonance energy transfer (FRET) (28). However, a detailed characterization of cholesterol-containing membranes from a dynamic point of view is still lacking.

Fluorescence correlation spectroscopy (FCS) is based on the time-correlation of temporal fluorescence fluctuations detected in the focal volume, which are governed by dynamic parameters of the system at equilibrium (36, 37). The power of FCS relies on the single molecule sensitivity and the capability of exploring a wide range of dynamic events with high temporal resolution and good statistical accuracy (38). In the past, this technique has been proven to be a powerful tool to follow lipid
dynamics in domain-forming giant unilamellar vesicles (GUVs) (39), which serve as excellent model membranes for single molecule optical microscopy (40).

In this study, we present a detailed characterization of lipid dynamics in raft-forming GUVs prepared from a ternary mixture of cholesterol, dioleoyl-phosphatidylcholine, and sphingomyelin. By combining confocal optical microscopy and FCS, insight is gained in the static and dynamic organization of lipids, partitioning in different phases. It is evident that cholesterol plays a key role in promoting raft formation and, most importantly, in tuning membrane lipid mobility. Finally, we show that FCS provides information on lipid raft composition, allowing for a mapping of the lipid phase diagram, entirely based on dynamic parameters.

MATERIALS AND METHODS

Chemicals—1,2-Dioleoyl-sn-glycero-3-phosphocholine (dioleoyl-phosphatidylcholine; DOPC), N-stearoyl-o-erythrosphingosylphospho-rhylcholine (stearoyl sphingomyelin, SM), cholesterol, porcine brain ganglioside GM1 (GM1) were purchased from Avanti Polar Lipids. 1,1′-Diocadecyl-3,3′,3″-tetramethylindocarbocyanine perchlorate (DiIC18) and the Alexa-Fluor 488 conjugate of cholera toxin B subunit (AF-CTB) were from Molecular Probes. The cholesterol-sequestering agent methyl-β-cyclodextrin (MβCD) was from Sigma. All other chemi-cals were of reagent grade.

Preparation of GUVs—GUVs were prepared by electroformation (40, 41). With this approach, truly unilamellar vesicles are produced with sizes ranging from 10 to 100 μm (42, 43). The flow chamber (closed-bath perfusion chamber, RC-21, Warner Instruments Co.) used for vesicle preparation was equipped with two microscope slides, each coated with optically transparent and electrically conductive indium tin oxide (ITO). Lipids in chloroform/methanol 9:1 (5 mM, prepared freshly under nitrogen atmosphere as much as possible. Lipids were checked for oxidation by UV/VIS spectroscopy and thin layer chromatography. Unless otherwise stated, lipids were always prepared from fresh lipid mixtures and kept under a nitrogen atmosphere as much as possible. Lipids were checked for oxidation by UV/VIS spectroscopy and thin layer chromatography. Unless otherwise stated, lipids were always prepared from fresh lipid mixtures and kept under a nitrogen atmosphere as much as possible. 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of the vesicles allowed us to look for phase interlayer coupling and it was found that, in all of the GUVs, the phase domains comprised both apposing membrane leaflets. Phase separation was also visualized at higher amounts of cholesterol (SM/DOPC = 0.5/0.5), as shown in Fig. 1D for 20 mol% and in Fig. 1E for 33 mol% of cholesterol. The domain morphology was the same as described for 10 mol% cholesterol, except that the total surface area of the SM-enriched phase increased with the amount of cholesterol. At 50 mol% cholesterol, rafts were no longer observed within the optical resolution (Fig. 1F).

Similarly, uniform fluorescence from DiI-C₁₈ and GM₁-bound AF-CTB was detected in GUVs with 65 mol% cholesterol (not shown).

Membrane Lipid Mobility Is Controlled by Cholesterol—We assessed the membrane lipid mobility of GUVs made from ternary mixtures of DOPC/SM/cholesterol by measuring the diffusion coefficient of DiI-C₁₈ by FCS. In Fig. 2A, correlation curves are shown for the liquid-disordered, DOPC-enriched domain, where DiI-C₁₈ preferentially partitioned, and in Fig. 2B those for the liquid-ordered, SM-enriched domain, from which DiI-C₁₈ was largely excluded. Note that the sensitivity of FCS allows one to measure lipid diffusion with the fluorescent marker at very low concentrations in both phases. As soon as phase separation occurred, in the presence of 10 mol% of cholesterol (Fig. 2A, dash (d)), the lipid mobility in liquid-disordered domains ($D = 4.9 ± 0.3 \times 10^{-8}$ cm²/s) almost matched the one of pure DOPC membranes ($D = 6.3 ± 0.2 \times 10^{-8}$ cm²/s, Fig. 2A, dot (a)). This mobility was significantly higher than that measured in DOPC/SM (0.5/0.5) GUVs in the absence of cholesterol ($D = 2.6 ± 0.2 \times 10^{-8}$ cm²/s, Fig. 2A, solid (e)). An increase in the cholesterol concentration hardly affected the mobility value of DiI-C₁₈ relative to values measured for pure DOPC. On the other hand, cholesterol greatly varied the lipid mobility in the SM-enriched phase (Fig. 2B), where lipid diffusion was significantly slower than in the fluid-disordered phase and in the SM/DOPC (0.5/0.5) mixture without cholesterol (Fig. 2B, solid (a)). However, by increasing the amount of cholesterol, the membrane lipid mobility in SM-enriched domains greatly increased, from $D = 0.105 ± 0.031 \times 10^{-8}$ cm²/s (10 mol% cholesterol, Fig. 2B, dash (f)) up to $D = 0.795 ± 0.108 \times 10^{-8}$ cm²/s (33 mol% cholesterol, Fig. 2B, dash dot (d)) approaching that of SM/DOPC (0.5/0.5) mixtures. By further increasing the amount of cholesterol, the domains disappeared but the lipid mobility remained higher than that of the SM-rich domains (50 mol% cholesterol, Fig. 2B, short dash (b)), though lower than in SM/DOPC = 0.5/0.5 GUVs. Any further increase in cholesterol concentration made the whole membrane stiffer (e.g. 65 mol% cholesterol in Fig. 2B, short dash dot (c)). Taking different SM/PC molar ratios $\geq 1$ (e.g. 0.5/0.13), the domain morphology and the lipid diffusion were unchanged (see Table I). On the other hand, in the case of SM/PC molar ratios $< 1$, no domains were visualized by confocal microscopy and the lipid dynamics measured was rather high and very close to that in pure DOPC (e.g. SM/DOPC 0.13/0.53, see Table I). For all of the FCS curves, excellent fits were produced with a one-component normal Brownian diffusion model (37). The diffusion coefficients, calculated from the fitting of FCS curves shown in Fig. 2, are reported as a function of mol% of cholesterol in Fig. 3 (see also Table I).

Lipid Mobility in Binary Mixtures—In order to investigate in more detail the lipid spatial organization in raft-exhibiting membranes, lipid mobility in GUVs prepared from ternary mixtures of SM/DOPC/cholesterol was compared with that in GUVs from binary mixtures of DOPC/cholesterol, SM/cholesterol and DOPC/SM. For all of these binary compositions, GUVs showed no phase separation by confocal microscopy and the lipid dynamics measured was rather high and very close to that in pure DOPC (e.g. SM/DOPC 0.13/0.53, see Table I). For all of the FCS curves, excellent fits were produced with a one-component normal Brownian diffusion model (37). The diffusion coefficients, calculated from the fitting of FCS curves shown in Fig. 4, are reported as a function of mol% of cholesterol in Fig. 3 (see also Table I).
were recorded for the fluid-ordered, SM-enriched phase at increasing cholesterol concentration (dash (d) indicates 10 mol%, dash dot (e) indicates 20 mol%, and dash dot dot (f) indicates 33 mol%). The correlation decays almost matched the one from GUVs of pure DOPC (dash dot dot (f) indicates 33 mol%). Values for the DOPC-enriched phase at increasing cholesterol concentration (dash (f) indicates 10 mol%, dash dot (e) indicates 20 mol%, and dash dot dot (d) indicates 33 mol%). Short dash (b) indicates 50 mol% cholesterol, short dash dot (c) 65 mol% cholesterol, and solid (a) SM/DOPC = 0.5/0.5.

Table I

Translational diffusion coefficients for the ternary SM/DOPC/cholesterol system

Values of diffusion coefficient of DiI-C18, as obtained from the fitting of FCS curves, in GUVs prepared from DOPC/SM/cholesterol mixtures (see “Materials and Methods”).

<table>
<thead>
<tr>
<th>Composition, molar fraction</th>
<th>No phase separation</th>
<th>Phase separation</th>
</tr>
</thead>
<tbody>
<tr>
<td>SM</td>
<td>DOPC</td>
<td>Chol</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>0.45</td>
<td>0.45</td>
<td>0.1</td>
</tr>
<tr>
<td>0.4</td>
<td>0.4</td>
<td>0.2</td>
</tr>
<tr>
<td>0.33</td>
<td>0.33</td>
<td>0.33</td>
</tr>
<tr>
<td>0.25</td>
<td>0.25</td>
<td>0.5</td>
</tr>
<tr>
<td>0.175</td>
<td>0.175</td>
<td>0.65</td>
</tr>
<tr>
<td>0.1</td>
<td>0.8</td>
<td>0.1</td>
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<tr>
<td>0.13</td>
<td>0.53</td>
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<tr>
<td>0.53</td>
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DiI-C18 are reported for GUVs composed of SM/DOPC at different ratios. For the data at 80 mol% SM, only FCS curves in the less bright fluid-disordered regions could be recorded, as the FCS measurements in the SM gel-phase were strongly affected by photobleaching. These latter results confirm that, in SM/DOPC membranes with ≳ 80 mol% of SM, an equilibrium is established at room temperature between a SM-enriched gel-phase and a SM/DOPC-containing, liquid-disordered phase characterized by high lipid mobility.

Phase Diagram—The FCS measurements were used to build the phase diagram shown in Fig. 5. Starting from the left axis (DOPC/cholesterol), the membrane lipid mobility continuously decreases upon increase of cholesterol concentration. Consistent with previous findings for phospholipid/cholesterol mixtures (23, 47), a transition from liquid-disordered to liquid-ordered phase can be identified around ~40 mol% of cholesterol. As the lipid diffusion coefficients in DOPC-enriched domains of DOPC/SM/cholesterol GUVs almost match that of pure DOPC, we can conclude that the DOPC-enriched phase is largely devoid of cholesterol and that the SM-enriched phase takes up most, if not all, of the cholesterol present in the membrane. The slight mismatch could be simply due to the presence of small amounts (~5–10%) of SM/cholesterol clusters in the DOPC-rich phase. In contrast to DOPC membranes, lipid dynamics in SM membranes (right axis in Fig. 5) increases upon addition of cholesterol and undergoes a transition from gel-phase to a liquid-ordered phase around 40 mol% of cholesterol.

Fig. 2. Ternary lipid mixtures of SM/DOPC/cholesterol exhibit phase separation. A, FCS curves were recorded for the fluid-disordered, DOPC-enriched phase at increasing cholesterol concentration (dash (d) indicates 10 mol%, dash dot (e) indicates 20 mol%, and dash dot dot (f) indicates 33 mol%). The correlation decays almost matched the one from GUVs of pure DOPC (dash dot dot (f) indicates 33 mol%). B, FCS curves were recorded for the fluid-ordered, SM-enriched phase at increasing cholesterol concentration (dash (f) indicates 10 mol%, dash dot (e) indicates 20 mol%, and dash dot dot (d) indicates 33 mol%). Short dash (b) indicates 50 mol% cholesterol, short dash dot (c) 65 mol% cholesterol, and solid (a) SM/DOPC = 0.5/0.5.

Fig. 3. Average diffusion coefficients, as determined from fitting the autocorrelation curves in Fig. 2, A and B, as a function of cholesterol concentration. Values for the DOPC-enriched phase are indicated by open circles, those for the SM-enriched phase and for mixtures that do not give rise to phase separation (within the optical resolution) are indicated by filled squares. The dashed line, which corresponds to the value of lipid diffusion coefficient in GUVs of DOPC, is shown as a reference.
The trend of diffusion coefficients is comparable to that of the SM-rich phase in DOPC/SM/cholesterol GUVs and, remarkably, much steeper than what estimated in previous reports (31, 32). However, the values of diffusion coefficient are larger in the SM-rich areas of ternary mixtures than in the binary SM/cholesterol. Therefore, we can conclude that the liquid-ordered phase in SM/DOPC/cholesterol membranes is mainly composed of SM/cholesterol but, most likely, also contains some DOPC, which further increases the lipid mobility. Finally, the lipid dynamics in DOPC/SM GUVs is regulated by the amount of SM soluble in the fluid DOPC membrane. The trend of lipid diffusion coefficients as a function of mol% of SM suggests the presence of two transition points, the first being around 10 mol% SM and the second around 45 mol%. The difference in lipid mobility between these ranges may be due to different molecular packing and spatial distributions of gel-phase and liquid-disordered lipid clusters.

**DISCUSSION**

We have characterized the morphology of raft-like microdomains in GUVs, prepared from ternary mixtures of dioleoylphosphatidylcholine (DOPC), sphingomyelin (SM), and choles-
terol, by confocal fluorescence microscopy and the lipid dynamics by FCS. Cholesterol promotes phase separation of DOPC-enriched and SM-enriched domains by engaging a stable and strong interaction with SM, as demonstrated by the partitioning of the lipophilic probe DiI-C18. Most importantly, cholesterol plays a pivotal role in tuning the lipid mobility, in particular in the SM-enriched domains.

Previously, domains with raft-like properties were visualized by one- and two-photon fluorescence microscopy in model membranes (31, 32). Depletion and repletion of cholesterol in membranes composed of SM/DOPC/cholesterol (1/1/1) resulted in disappearance and reappearance of lipid rafts in supported monolayers (32). However, a systematic investigation of the morphology of raft-like domains as a function of cholesterol concentration has never been attempted. From the confocal images shown here, it is evident that cholesterol is the determining factor in causing phase separation of sphingolipids and unsaturated phospholipids. Confocal images of GUVs made of SM/DOPC/cholesterol (0.5/0.5) and different amounts of cholesterol show that, at room temperature, extended phase separation starts to occur at 10 mol% cholesterol. Consistent with previous studies reporting phase separation in model membranes with similar lipid mixtures (31, 32), the round shape of the domains suggests the coexistence of a liquid-ordered and a liquid-disordered phase, as the circular borders of the domains minimize the line energy. GUVs with less than 10% or more than 50% cholesterol did not exhibit phase separation, at least within the optical resolution. As previously observed in artificial membranes (31, 38), ordered phase domains in apposing leaflets were always perfectly coincident. Therefore, at least in the case of SM/DOPC/cholesterol mixtures, where the long fatty acid chains of SM in opposite leaflets can superimpose by interdigitation, the lipid component alone is able to create strong coupling between inner and outer leaflet.

It has been proposed that cholesterol-rich membranes exhibit formation of a sphingolipid-rich, liquid-ordered phase, which separates from a phospholipid-rich, liquid-disordered phase (1–4). Lipid segregation is driven by the tendency of sphingolipids to engage special molecular interactions with cholesterol and to organize in a more ordered manner than unsaturated phospholipids. By adding a certain amount of cholesterol to the SM/DOPC mixture, the lipophilic probe DiI-C18 is squeezed out of the SM-enriched regions and greatly favors the unsaturated phospholipid-enriched domain. In contrast, in GUVs prepared from SM/DOPC mixtures, with ≥80 mol% of SM, DiI-C18 presents a slight preference for the SM-rich gel-phase.

We have used FCS to systematically analyze lipid mobility and identify the effect of cholesterol in rafts. FCS has been successfully applied to study diffusion of lipids and proteins in membranes (39, 48). Quantitative information on the average number of the particles in focus and their dynamic properties, e.g. diffusion coefficients, can be obtained with excellent statistical accuracy (38). FCS has been shown to be sensitive to deviations from single-phase behavior, e.g. caused by heterogeneities in the sample (39). As lipid rafts are thought to be dynamic assemblies in membranes, the assessment of lipid dynamic properties is an important step toward the understanding of how lipids modulate membrane lipid mobility and, thereby, possibly control the timing of cellular events, such as sorting or signaling. This technique is less time-consuming than SPT and, in contrast to FRAP, FCS works at single molecule regimes. This is a great advantage in experiments on domain formation in membranes, because of the following.

(i) Lipid analogs do not need to be introduced at high amounts, which have been shown to affect, in some cases, the lipid organization (48, 49), and (ii) at a single molecule level, clustering of the dye may be readily spotted. FCS illustrates, here, the important role of cholesterol in tuning the membrane lipid mobility in raft-containing membranes. Consistent with previous studies (32), lipid diffusion in liquid-disordered phase is ~2 times faster than in cholesterol devoid GUVs (SM/DOPC 0.5/0.5). However, the most remarkable effect is found for the lipid diffusion in SM-enriched phases, where the mobility increases by a factor of ~8 as the cholesterol concentration is increased from 10% up to 33%. This implies that cholesterol acts as raft-promoting component and, most importantly, is able to control the lipid dynamics in domains. On the other hand, the SM level (for SM/DOPC molar ratios ≥ 1) does not affect very much the lipid dynamics in domains. This result might have some physiological implications, as it implies that cells can alter the SM levels without altering the dynamic properties of the domains.

The lipid diffusion coefficient characterizes a certain lipid phase composition, given the data reproducibility, the good statistical accuracy of the results and the excellent properties of GUVs as model membranes. Vesicle unilamellarity ensures that the diffusion components in the autocorrelation curve belong only to molecules diffusing within a single bilayer. On the basis of our data, we constructed a phase diagram for the DOPC/SM/cholesterol mixture. The classic method for studying equilibrium between phases in membranes is Differential Scanning Calorimetry (DSC), often combined with infrared and fluorescence spectroscopy (47). Additional information can be extracted by Atomic Force Microscopy (in the sub-micrometer scale) (46), or one- and two-photon fluorescence microscopy (in the μm scale) (30–32). These techniques describe the static lipid organization, whereas, here, we exploit the time dimension and use the dynamic parameters obtained by FCS as a
Lipid Dynamics in Domain-forming Membranes

We have identified regions of lipid compositions that give rise to phase separation and obtained information on the phase transition points. A large amount of literature has been previously reported on phase diagrams of similar ternary systems (Refs. 47 and 51, see Ref. 52 for an excellent review). Our data on lipid dynamics add new information as we show how membrane lipid mobility changes, not only between different phase regions but also within a particular region.

In conclusion, FCS has been proven to be a valuable tool to assess the molecular basis of lipid mobility in raft-like domains, which is crucial for our understanding of the dynamics of many biological processes. Here, we focused on the role of cholesterol in promoting phase separation and increasing the lipid mobility in SM-enriched phases. In addition, by using the dynamic parameters obtained by FCS, we built a phase diagram, which reports on the lipid dynamic properties within different lipid phases.

Acknowledgment—We thank Dick Hoekstra for useful discussions.

REFERENCES