J Fluoresc (2006) 16:671–678
DOI 10.1007/s10895-006-0108-6

ORIGINAL PAPER

How Phospholipid-Cholesterol Interactions Modulate Lipid Lateral Diffusion, as Revealed by Fluorescence Correlation Spectroscopy

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Received: 1 November 2005 / Accepted: 12 July 2006 / Published online: 1 August 2006

Abstract Cholesterol is a key player in regulating physicochemical properties of cellular membranes and, thereby, ensuring cell viability. In particular, lipid-cholesterol interactions may provide important information on the spatio-temporal organization of membrane components. Here, we apply confocal imaging and Fluorescence Correlation Spectroscopy (FCS) to Giant Unilamellar Vesicles (GUVs) composed of binary mixtures of lipids and cholesterol.

The effect of cholesterol on lipid dynamics and molecular packing order of unsaturated, monounsaturated, fully saturated (with both low and high phase transition temperatures, T_m) glycero-phospholipids and sphingomyelin has been investigated. We show that, for unsaturated glycerophospholipids, the decrease of the lipid diffusion coefficient as a result of the interaction with cholesterol does not depend on the fatty acid chain length. However, the values of the diffusion coefficient change as a function of chain length. The monounsaturated phospholipid palmitoyl-oleoyl-phosphatidylcholine (POPC) exhibits a dynamic behavior very similar to the unsaturated dioleoyl-phosphatidylcholine (DOPC). By contrast, for saturated (low T_m) glycero-phospholipids, cholesterol causes a decrease of lipid mobility in a chain length-dependent manner.

FCS can be employed as a valuable tool to study lipid-sterol interactions and their effect on lipid dynamics, molecular packing and degree of conformational order.

Keywords FCS · Confocal fluorescence microscopy · Giant unilamellar vesicles · Lipid-sterol interaction · Cholesterol · Glycerophospholipid

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Introduction

The plasma and other membranes of eukaryotic cells contain a significant amount of cholesterol, which has been shown to play a key role in regulating the "bulk" properties, such as bilayer thickness, membrane fluidity and permeability, as well as more specific lipid-lipid and lipid-protein interactions [1–3]. A range of mechanisms is available in the cell for modulating the cholesterol content and, thereby, controlling the physical properties of the bilayer. The level of cholesterol synthesis can be modulated by the cell to balance between the synthesis of saturated and unsaturated fatty acids and phospholipids, to ensure membrane fluidity in any environmental condition the cell may have to face. In the past decade, much attention has been focused on the idea that a significant fraction of lipids in the plasma and other membranes of eukaryotic cells is organized as liquid domains, called lipid rafts [4–6], which are highly enriched in cholesterol.

Optical microscopy on giant vesicles has contributed to the understanding of the lipid spatio-temporal organization

in various binary and ternary mixtures [7]. In particular, Giant Unilamellar Vesicles (GUVs) provide an excellent model bilayer for (single-molecule) optical microscopy [8, 9]. They comprise of single closed bilayers, which freely stand in the medium and span the focal spot, as they have a diameter of 10 up to 100 μm. We and other groups have visualized lipid microdomains in GUVs composed of binary and ternary mixtures [10–12]. In particular, we used a combination of confocal imaging and Fluorescence Correlation Spectroscopy (FCS) [13, 14] to systematically characterize domain formation and lipid dynamics in coexisting lipid phases. FCS also allows for detection of domains (within the optical resolution), whenever the fluorescent probes equally partition in the lipid phases and, hence, do not provide any contrast in the image [15]. FCS provides an unambiguous phase assignment, thereby identifying solid phases, liquid-disordered and liquid-ordered phases [12]. In addition, FCS gives information on the phase lipid composition and on the degree of molecular packing and conformational order. It can therefore be used as an experimental tool to measure lipid diffusion coefficients and relate them to lipid-lipid interaction energies.

In this paper, we report on a systematic study of interactions that cholesterol engages with various phospholipids, i.e. fully unsaturated, fully saturated, monounsaturated glycerophospholipids and sphingomyelin. The effect of cholesterol on the lipid dynamics of different membrane matrices has been investigated and characterized in detail.

Experimental procedures

Materials

1,2-Dioleoyl-sn-glycro-3-phosphocholine (dioleoyl-phosphatidylcholine; 18:1 PC (cis), DOPC), 1,2-Dilaureoyl-sn-glycero-3-phosphocholine (dilaureoyl-phosphatidylcholine; 12:0 PC, DLPC), 1,2-Dimiristeoyl-sn-glycero-3-phosphocholine (dimiristeoyl-phosphatidylcholine; 14:0 PC, DMPC), 1,2-Dimyristoleoyl-*sn*-Glycero-3-Phosphocholine (dimiristoleoyl-phosphatidylcholine, C14:1 (cis), DMirPC), 1,2-Dierucoyl-*sn*-Glycero-3-Phosphocholine (dierucoyl-phosphocholine, C22:1 (cis), DEPC), and cholesterol were purchased from Avanti Polar Lipids. 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI-C_{18}), 2-(4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-pentanoyl)-1-hexadecanoyl-*sn*-glycero-3-phosphocholine (BODIPY® 581/591 C_5-HPC, Bodipy-PC), and 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate ('DiD' oil; DiD-C_{18}) were from Molecular Probes. All other chemicals were of reagent grade.

Preparation of giant unilamellar vesicles (GUVs)

GUVs were prepared by electroformation [8] with diameter varying from 10 up to 100 μm. As previously described [12], the flow chamber (closed-bath perfusion chamber, RC-21, Warner Instruments Co.) for GUV preparation was equipped with two microscope slides, each coated with indium tin oxide (ITO), which is electrically conductive and optically transparent in the visible range. For lipid mixtures with high phase transition temperature (T_m), GUVs were formed in the sample chamber at high temperature (60°C) under a AC field. After lipid swelling, the chamber was put directly at room temperature. The experiments were performed in the presence of a reducing agent, dithiotreitol (DTT; 2mM, final concentration), to prevent lipid oxidation. Lipids were checked for oxidation by UV/Vis spectroscopy and thin layer chromatography before and after electroformation. DiI-C_{18} was added in the amount of 0.1 mol% for confocal imaging and 0.001 mol% for FCS. As a control, other fluorescent lipid analogs (Bodipy-PC, DiD-C_{18} and Rhodamine-PE) were employed to check for data reproducibility. The cholesterol content of the prepared GUVs was assayed by using a fluorometric method (Amplex Red Cholesterol Assay Kit, #A12216, Molecular Probes, Eugene, OR).

Confocal fluorescence microscopy and fluorescence correlation spectroscopy

Confocal fluorescence microscopy and FCS were performed on a commercial ConfoCor2 (Zeiss, Jena, Germany), as previously described [12]. Confocal images were taken with the excitation light of a He-Ne laser at $\lambda = 543$ nm, which was reflected by a dichroic mirror (HTF 543) and focused through a Zeiss C-Apochromat 40x, NA = 1.2 water immersion objective onto the sample. The fluorescence emission was recollected by the same objective and, after passing a 560 nm longpass filter, focused into a photomultiplier (PMT). The confocal geometry was ensured by pinholes (60 μm) in front of the PMT.

FCS measurements were performed by epi-illuminating the sample with the 543 nm HeNe laser ($I_{ex} \approx 1.2$ kW/cm^2). The excitation light was reflected by a dichroic mirror (HTF 543) and focused onto the sample by the same objective as for the LSM. The fluorescence emission was recollected back and sent to an avalanche photodiode via a 560–615 nm bandpass filter. Out-of-plane fluorescence was reduced by a pinhole (90 μm) in front of the detector (APD). The laser focus was positioned on the topside/bottomside of GUVs, by performing an axial (z-)scan through the membrane prior to the FCS recording. In order to acquire reliable FCS data for lipid diffusion, the positioning of the laser focus on the

membrane was crucial, as the fitting model relies on a two-dimensional diffusion in a bilayer, which is planar at the focus site. A giant vesicle on the top/bottom side can be approximated to a plane perpendicular to the optical axis. Furthermore, along with the z-scans through the membrane to identify the z-position that gave the maximum fluorescence intensity (corresponding to the top/bottom of GUVs), we also performed routine checks for each sample [16] to increase the accuracy in the laser beam positioning. Membrane undulations sometimes appeared in the FCS curve as an apparent "diffusive" component with much longer decay times than the lipid diffusive times. This "artefactual" component was readily recognized because of the great variability within one sample due to the different vesicle size and drifting movements. All the data retained in our study referred to measurements of diffusion with no or negligible (maximum fraction of 2%) component due to membrane undulations.

The fluorescence temporal signal was recorded and the autocorrelation function $G(\tau)$ was calculated, according to Magde et al. [17]. The apparatus was calibrated by measuring the known diffusion coefficient of rhodamine 6G in solution. The detection area on the focal plane was approximated by a Gaussian profile and had a radius of $\approx 0.18\ \mu$m at $1/e^2$ relative intensity. Data fitting was performed with the Levenberg-Marquardt nonlinear least-squares fit algorithm (ORIGIN, OriginLab, Northampton, MA). The fitting equation made use of a two-dimensional Brownian diffusion model, assuming a Gaussian beam profile [17]:

$$G(\tau) = \frac{\left(\sum_i \langle C_i \rangle \left(\frac{1}{1+\tau/\tau_{d,i}}\right)\right)}{A_{\text{eff}}\left(\sum_i \langle C_i \rangle\right)^2},$$

where $<C_i>$ is the two-dimensional time average concentration of the species i in the detection area A_{eff} ($\sim 0.1\ \mu\text{m}^2$)and $\tau_{d,i}$ is the average residence time of the species i. The diffusion coefficient D_i for the species i is proportional to $\tau_{d,i}$.

Three independent GUVs preparations for every lipid composition were analyzed by FCS and, for each of them, data from ~ 30 different GUVs were recorded with 100 s acquisition time per FCS measurement. All the measurements were carried out at room temperature (23 °C).

Results

Lipid dynamics in binary mixtures of fully unsaturated glycerophospholipids and cholesterol

Giant Unilamellar Vesicles (GUVs) represent the ideal model membrane for (single-molecule) optical microscopy, as they provide a single spherical closed bilayer visible under the light microscope (diameter of 10–100 μm). As they span the laser focus in the lateral direction, GUVs are ideally suited for FCS studies.

Binary mixtures of fully unsaturated glycero-phospholipids (DOPC, 18:1; DMirPC, 14:1; DEPC, 22:1) and cholesterol yield homogeneous fluorescence signal in the GUVs (Fig. 1). The lipid probe used was DiI-C_{18}, but other fluorescence lipid analogs (Bodipy-PC, and DiD-C_{18}) gave consistent results (not shown). Fluorescence fluctuations were recorded from GUVs composed of the above-mentioned fully unsaturated glycero-phospholipids and various amounts of cholesterol. In Fig. 2A, the FCS curves are reported for the DMirPC/cholesterol mixture. The FCS curves were all fitted to a one-component two-dimensional Brownian diffusion model. As for the DOPC/cholesterol [12], also for the DMirPC/cholesterol and the DEPC/cholesterol (FCS curves are not shown) mixtures, the lipid diffusion coefficient gradually decreases as a function of cholesterol concentration (Fig. 2B), in a linear fashion. Remarkably, the slope of the linear decrease is similar for all of these lipids. On the other hand, the absolute values of the lipid diffusion coefficient are different, and depend linearly on the length of the fatty acid chain (Fig. 2B, inset). The diffusion coefficient for the pure

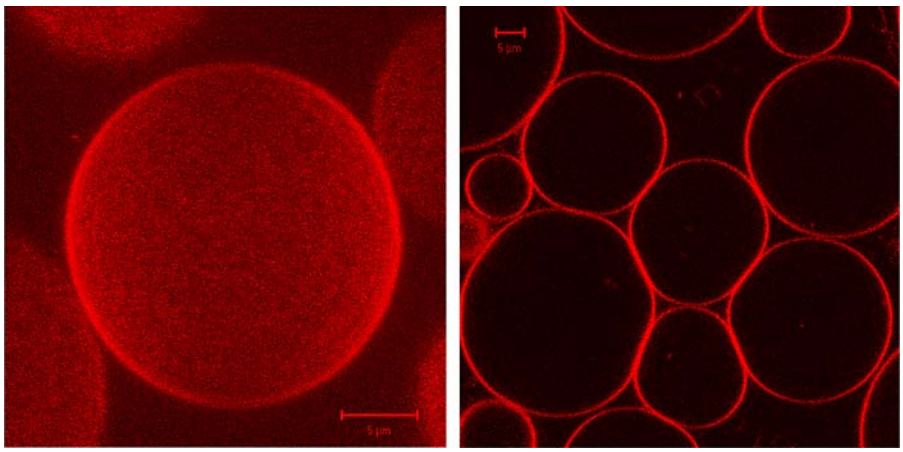

Fig. 1 GUVs from binary lipid mixtures do not exhibit microdomains under the light microscope. (A) Confocal image of GUVs composed of DmirPC/cholesterol 1:1 and 0.1 mol% of DiI-C_{18} (left panel) and 3D projection of a GUV reconstructed from a stack of confocal images at different z-positions (0.4 μm thick)

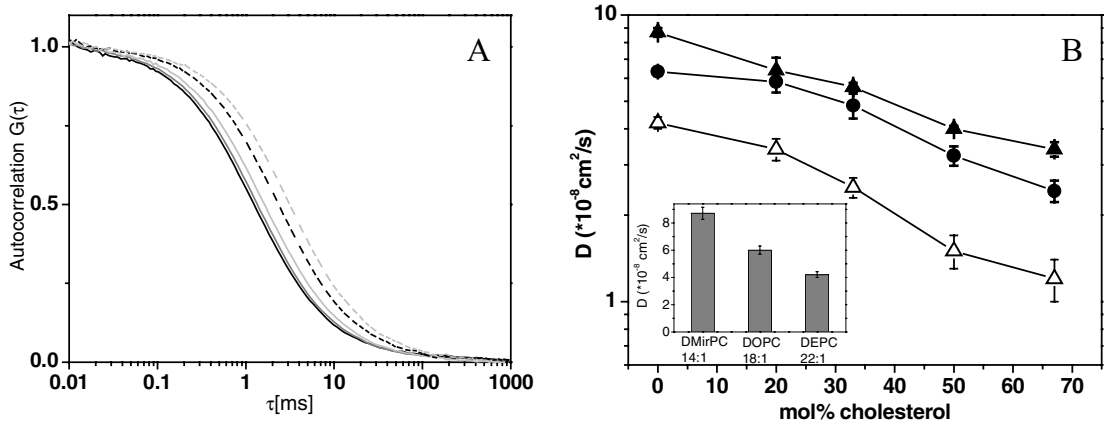

Fig. 2 Lipid mobility as a function of cholesterol concentration for binary mixtures of fully unsaturated glycerophospholipids and cholesterol. (A) FCS autocorrelation curves are shown for DiI-C_{18} mobility in DOPC/cholesterol GUVs, black solid line (a) for 0 mol%, grey solid (b) for 20 mol%, light grey solid (c) for 33 mol%, black dashed line (d) for 50 mol% and grey dashed (e) for 67 mol% of cholesterol. (B) Average diffusion coefficients, as determined from the fitting of the autocorrelation curves, are reported as a function of cholesterol concentration for DMirPC (14:1) (closed triangles), DOPC (18:1) (circles) and DEPC (22:1) (open triangles). Bars represent the standard deviation from the average values (see *Materials and Methods* for details). Inset: Absolute values of lipid diffusion coefficient measured in GUVs composed of pure DMirPC (14:1), DOPC (18:1) and DEPC (22:1)

DMirPC (14:1) is higher than that for DOPC (18:1), which is in turn higher than that for DEPC (22:1).

Lipid dynamics in binary mixtures of fully saturated (low T_m) glycero-phospholipids and cholesterol

We wanted to compare lipid diffusion for mixtures of cholesterol and unsaturated glycero-phospholipids and mixtures of cholesterol and saturated, low T_m glycero-phospholipids. Confocal imaging of GUVs from these mixtures exhibited homogeneous fluorescence signal, hence no domains (at least within the optical resolution). In Fig. 3A, FCS curves for DLPC/cholesterol mixtures are reported. As for DMPC/cholesterol mixtures (not shown), the curves were well fitted to a one-component two-dimensional Brownian diffusion model. The corresponding fitting values of diffusion coefficient are reported as a function of cholesterol concentration in Fig. 3B (DLPC, closed inverted triangles; DMPC, open inverted triangles) and compared to those from DOPC/cholesterol mixtures (closed circles). Note that for DLPC/cholesterol, the diffusion coefficient continuously decreases as increasing of cholesterol concentration, whereas the trend for DMPC/cholesterol mixtures may follow a steep decrease at low cholesterol amount (<33mol% of cholesterol) and decreases more slowly at high cholesterol density. Compared to DOPC/cholesterol mixtures, lipid dynamics

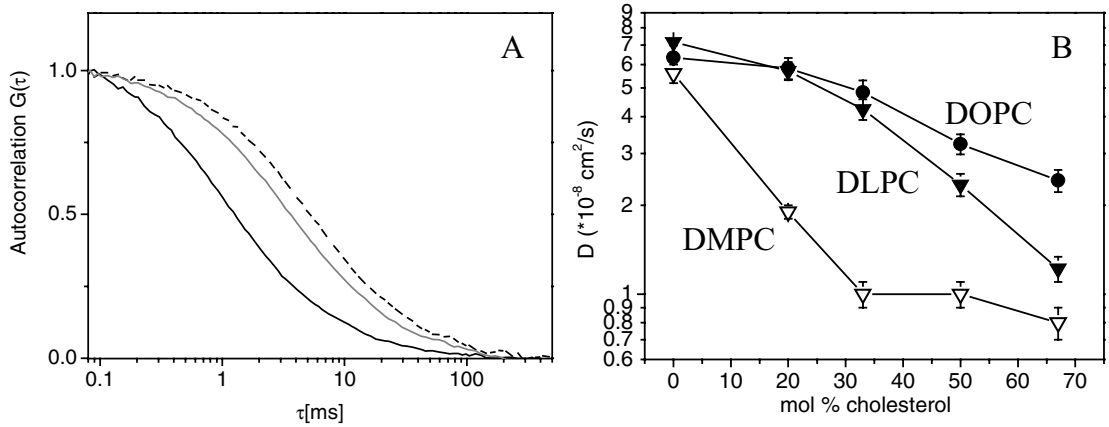

Fig. 3 Lipid mobility as a function of cholesterol concentration is compared between fully saturated (low T_m) and fully unsaturated glycerophospholipids. (A) FCS autocorrelation curves are shown for DiI-C_{18} mobility in DLPC/cholesterol GUVs, black solid line (a) for 0 mol%, grey solid (b) for 20 mol%, light grey solid (c) for 33 mol%, black dashed line (d) for 50 mol% and grey dashed (e) for 67 mol% of cholesterol. (B) Average diffusion coefficients, as determined from the fitting of the autocorrelation curves, are reported as a function of cholesterol concentration for DLPC (12:0) (closed inverted triangles), DOPC (18:1) (circles) and DMPC (14:0) (open inverted triangles). Bars represent the standard deviation from the average values (see *Materials and Methods* for details)

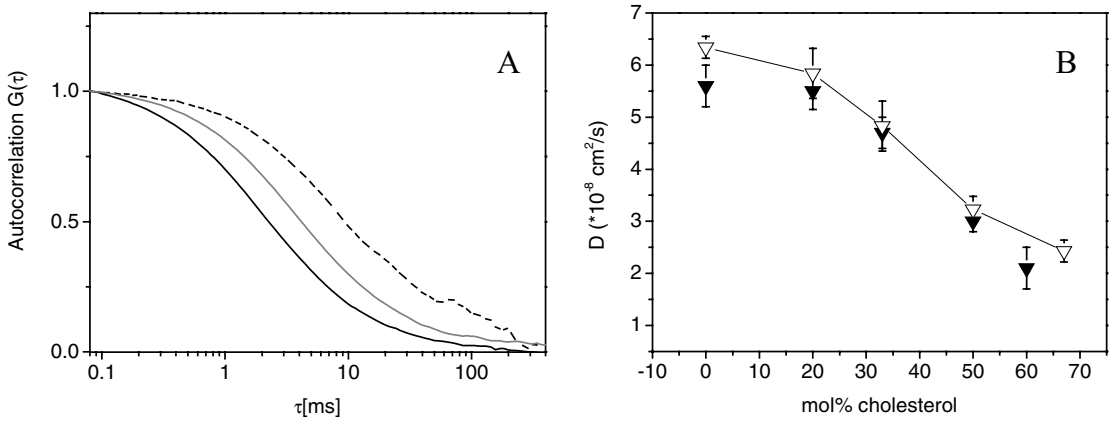

Fig. 4 Lipid mobility as a function of cholesterol concentration is compared between fully unsaturated (DOPC, 18:1) and monounsaturated glycerophopsholipid (POPC, 16:0, 18:1). (A) FCS autocorrelation curves are shown for DiI-C_{18} mobility in POPC/cholesterol GUVs, black solid line (a) for 0 mol%, black solid (b) for 50 mol%, grey solid (c) for 33 mol%, black dashed line. (B) Average diffusion coefficients, as determined from the fitting of the autocorrelation curves, are reported as a function of cholesterol concentration for POPC (closed triangles), and DOPC (open triangles). Bars represent the standard deviation from the average values (see *Materials and Methods* for details)

slows down more rapidly for saturated PC (for DMPC more than for DLPC).

Lipid dynamics in binary mixtures of POPC (16:0; 18:1) and cholesterol

In the case of mixtures of cholesterol and POPC, a glycerophospholipid with one saturated fatty chain (16:0) and one unsaturated (18:1), FCS measurements were recorded (Fig. 4A) and yielded excellent fits to a one-component two-dimensional Brownian diffusion. The corresponding lipid diffusion coefficients are reported as a function of cholesterol concentration in Fig. 4B (closed inverted triangles) and compared to those for DOPC/cholesterol mixtures (open inverted triangles). Except for the values in the absence of cholesterol, the trends are very close.

Discussion

The goal of this work was to provide a systematic characterization of lipid mobility in giant unilamellar vesicles by Fluorescence Correlation Spectroscopy (FCS). The effect of cholesterol on lipid dynamics of various lipids, including several glycero-phospholipids and sphingomyelin, was examined. Depending on the specific chemical structure of the lipid and on the interaction engaged with cholesterol, different lipid diffusion coefficients were observed. These changes reflect different molecular packing and degree of conformational changes due to different lipid-cholesterol and lipid-lipid interaction energies.

It has been previously reported that vesicles from binary mixtures of various lipids and cholesterol do not exhibit micrometer-sized domains [7, 12], although we cannot exclude the presence of small domains, with a size beyond the diffraction-limited optical resolution. A small length- and time-scale domain structure was reported for binary lipid mixtures in ESR [18], FRET [19, 20], and NMR studies [21]. The minimal amount of components needed to visualize domains with the light microscope is three, as shown for ternary mixtures of an unsaturated phospholipid, a fully saturated phospholipid and cholesterol [10–12, 22, 23]. Consistent with this data, we did not observe micrometer-sized domains on GUVs composed of various amounts of cholesterol together with unsaturated, saturated and monounsaturated glycero-phospholipids, and sphingomyelin.

The application for FCS to probe the lipid environment in artificial membranes has been shown previously [12, 15, 23, 24]. Correlation traces recorded in GUVs composed of lipid binary mixtures always yielded one-component fitting, although we cannot rule out the presence of small domains, beyond the optical resolution. FCS performed with one color (auto-correlation) gives a measure of the lipid translational diffusion mobility in the plane of the bilayer on the large (micrometer) length-scale, and it gives a measure of the lipid-cholesterol interactions involved in the temporal lipid organization. The values we found for the diffusion coefficients are consistent with previous measurements with other techniques, such as Single Particle Tracking (SPT) [25], Fluorescence Recovery After Photobleaching (FRAP) [10] and diffusion NMR [21, 26]. Information on lipid organization at higher spatial resolution can only be obtained with dual-color correlation spectroscopy (cross-correlation), in the case the domains are stable over the time which is needed to cross the focal area, and/or other techniques, such as FRET, polarization spectroscopy, and SPT.

Note that an absolute estimate of the diffusion coefficient would require precise measurements of the focal spot radius

w_0, but that is not needed in this case, as we are only interested in the relative changes of the diffusion coefficient as a function of cholesterol content in the membrane. Diffusion coefficients are thus computed by using a reasonable estimate of w_0 (see Experimental Procedures), which is therefore considered a simple scaling factor. In addition, all of the FCS curves exhibited one Brownian diffusion component, at least at very low excitation powers, thereby suppressing potential complications due to photobleaching and triplet kinetics.

We found that the values of lipid diffusion coefficient do not depend on the lipid probe used. Given the complexity of the membrane organization, which is governed by a large variety of physical forces, minimal changes in the chemical molecular structure might induce significant changes in intra- and intermolecular interactions. It is therefore reasonable to think that different lipid probes may diffuse at different rates in the same bilayer. We tested various lipid probes and measured their diffusion coefficients in GUVs composed of single lipid components and of binary mixtures. These lipid probes diffused at approximately the same rate, implying that our results are independent of the fluorescent probe used.

Cholesterol is known to have a "condensing" effect on the fully unsaturated glycero-phospholipid DOPC [12]. Here, we probed this effect for unsaturated glycero-phospholipids of different chain length and we observed a continuous, monotonic decrease of lipid diffusion coefficient with increasing of cholesterol content, without detecting phase transitions. The molecular picture behind this change in lipid dynamics is not fully understood. Cholesterol may help lipid molecules pack tighter and/or suppress conformations of the fatty acid chains, as shown for fully saturated phospholipids [27]. The decrease of the diffusion coefficient as a function of cholesterol concentration is approximately linear and the slope does not depend on the fatty acid chain length, at least for the lipids examined in this paper. This implies that the contribution from lipid-cholesterol interactions does not change with the chain length. On the other hand, lipid lateral dynamics does change upon changing the chain length, as the absolute values of the lipid diffusion coefficients are significantly different. This implies that the lipid mobility is governed by lipid-lipid interactions rather than by lipid-cholesterol interactions. Moreover, the longer the fatty chain, the slower the lipid mobility. From a molecular point of view, this translates into an increase in the ordered packing of the phospholipids with increasing of the chain length, as a result of an increase of the efficiency of van der Waals forces among the hydrocarbon chains. Increasing the chain length (and therefore the bilayer thickness) improves the lipid packing and reduces the partial free volume in the bilayer, thereby reducing the lipid lateral speed.

From the analysis of the FCS data, the role played by cholesterol in modulating lipid packing and dynamics is stronger for fully saturated, low T_m glycero-phospholipids than for unsaturated glycero-phospholipids. Qualitatively the effect is similar, as in both cases, cholesterol fills cavities and defects left from the poor packing of these lipids, thereby exerting a condensing effect on the lipid bilayer. However, in the case of fully saturated short-chain glycero-phospholipids, the decrease of lipid diffusion coefficient as a function of cholesterol content does depend on the fatty acid chain length. The slope observed for DLPC is smaller than that for DMPC, indicating that cholesterol is more effective in ordering DMPC molecules rather than DLPC lipids. The DLPC-cholesterol interaction is then weaker than DMPC-interaction. This is consistent with the idea that cholesterol interacts poorly with short chain lipids, as the effective length of cholesterol has been estimated to correspond to an all-*trans* hydrocarbon chain of 17°C atoms [28]. Moreover, cholesterol is shown to be more effective in reducing the partial free volume in bilayers of fully saturated (low T_m) phospholipids than fully unsaturated ones, as demonstrated by the shallower decrease of the molecular speed for the latter lipids.

This might indicate that lipid-cholesterol interactions are stronger when saturated lipids are involved, as discussed previously [29].

We tested the effect of cholesterol on diffusion in membranes composed of POPC, a phospholipid with one saturated 16:0 and one unsaturated 18:1 fatty acid chain (T_m of POPC: -5°C [30]). A smooth decrease of the lipid diffusion coefficient with increasing of cholesterol density was observed, as for the fully unsaturated and low T_m saturated glycero-phospholipids. No phase transition was apparent from the lipid mobility data. This is consistent with some recent optical microscopy studies [31], which found only one uniform phase in POPC/cholesterol mixtures. However, other techniques, i.e. FRET [32, 33], reveal the presence of a broad phase coexistence region at intermediate cholesterol density. The controversy on this point may be due to the ability of different techniques to probe lipid clustering at different length-scales. FCS and optical imaging, on one hand, detect micrometer-size domains and long-range order, whereas FRET can detect nanometer-sized domains and short-range order. These controversial results reflect the lack of a full understanding of small versus large-scale lipid ordering in simple lipid mixtures and the difficulty of determining small domains as true lipid phases. From our data, the slowing down of lipid dynamics in POPC/cholesterol membranes matches well that in DOPC/cholesterol bilayers. The differences between POPC and DOPC may be more apparent only when considering ternary lipid mixtures, e.g. with sphingomyelin (or DPPC) and cholesterol, as shown recently [31].

Finally, how does the effect of cholesterol on unsaturated, monounsaturated or low T_m saturated glycero-phospholipids compare with that on high T_m phospholipids? For these latter lipids, cholesterol is known to "fluidize" the bilayer, at

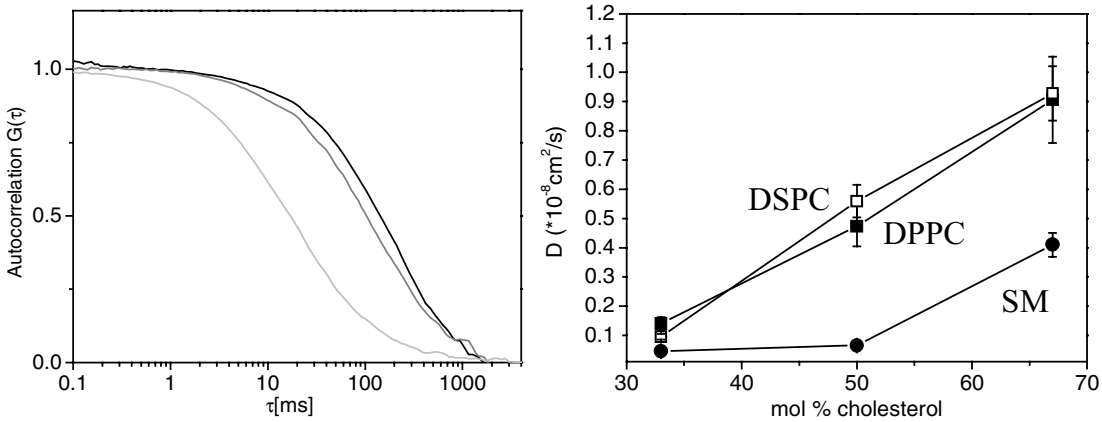

Fig. 5 Lipid mobility as a function of cholesterol concentration is compared between fully saturated (high T_m) glycerophospholipids and sphingomyelin [12, 15]. (A) FCS autocorrelation curves are shown for DiI-C_{18} mobility in SM/cholesterol GUVs, black line for 33 mol%, grey line for 50 mol%, light grey line for 65 mol% of cholesterol. (B) Average diffusion coefficients, as determined from the fitting of the autocorrelation curves, are reported as a function of cholesterol concentration for DSPC (open squares), and DPPC (closed squares) and SM (circles). Bars represent the standard deviation from the average values (see *Materials and Methods* for details)

temperature below the T_m of the mixture [34]. Consistent with previous work, we found an increase of lipid diffusion coefficients for DSPC, DPPC and SM (18:0) with increasing of cholesterol density (see Fig. 5A & B) [12, 15]. Here, fatty acid chains whose length differs by of 2 C atoms have virtually identical dynamic behavior when mixed with cholesterol, implying similar lipid-lipid and lipid-sterol interactions, within the accuracy of the measurements. Consistent with previous work [35], a striking difference was observed between glycero-phospholipids and SM of comparable chain length [15], suggesting a stronger network of SM molecules engaged in tight H-bond interactions compared to the interactions among glycero-phospholipids.

In conclusion, FCS provides valuable information on lipid diffusion in lipid/cholesterol mixtures and gives a measure of long-range lipid dynamics, which reflects the degree of molecular packing and conformational ordering. Given the high sensitivity of the technique, statistically accurate data on lipid dynamics can be extracted from membranes with very low dye concentrations, thereby overcoming possible changes in lipid organization in the presence of a foreign fluorescent probe. Further investigations will focus on employing this data to yield lipid-sterol interaction energies for a better understanding of the lipid molecular packing in the presence of cholesterol.

Acknowledgments We thank Dick Hoekstra, Lucie Kalvodova, and Dennis Merkle for stimulating discussions.

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