# Use of Cyclodextrin for AFM Monitoring of Model Raft Formation

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ABSTRACT The lipid rafts membrane microdomains, enriched in sphingolipids and cholesterol, are implicated in numerous functions of biological membranes. Using atomic force microscopy, we have examined the effects of cholesterol-loaded methyl-$\beta$-cyclodextrin (M$\beta$CD-Chl) addition to liquid disordered ($l_d$)-gel phase separated dioleoylphosphatidylcholine (DOPC)/sphingomyelin (SM) and 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC)/SM supported bilayers. We observed that incubation with M$\beta$CD-Chl led to the disappearance of domains with the formation of a homogeneously flat bilayer, most likely in the liquid-ordered ($l_o$) state. However, intermediate stages differed with the passage through the coexistence of $l_o$-$l_d$ phases for DOPC/SM samples and of $l_o$-gel phases for POPC/SM bilayers. Thus, gel phase SM domains surrounded by a $l_o$ matrix rich in cholesterol and POPC could be observed just before reaching the uniform $l_o$ state. This suggests that raft formation in biological membranes could occur not only via liquid-liquid but also via gel-liquid immiscibility. The data also demonstrate that M$\beta$CD-Chl as well as the unloaded cyclodextrin M$\beta$CD make holes and preferentially extract SM in supported bilayers. This strongly suggests that interpretation of M$\beta$CD and M$\beta$CD-Chl effects on cell membranes only in terms of cholesterol movements have to be treated with caution.

## INTRODUCTION

There is now strong evidence that the in-plane organization of plasma membrane is heterogeneous and contains domains associated with specific functions (Brown and London, 2000; Brown and Rose, 1992; Jacobson and Dietrich, 1999; Jacobson et al., 1995; Kusumi and Sako, 1996; Simons and Ikonen, 1997; van Meer, 2002). Among these, rafts are microdomains rich in cholesterol (Chl) and sphingolipids (Spl), which have a distinct protein composition, being enriched in lipid-anchored proteins with posttranslational modifications like glycosylphosphatidylinositol (GPI) or acyl groups while relatively depleted in most transmembrane proteins. Because rafts play a key role in the expression and regulation of numerous cellular processes including signal transduction and membrane traffic (Dykstra et al., 2001; Ikonen, 2001; Simons and Ikonen, 1997), numerous studies have focused on the understanding of the principles that underlie raft formation.

Biophysical studies of the detergent-resistant fraction of both cell membranes (DRM) that most likely are made of aggregated rafts (Mayor et al., 1994) and model membranes have shown that resistance to the detergent was linked to the membrane physical state. Lipids in the gel or in the liquid-ordered ($l_o$) phase were insoluble, whereas lipids in the liquid-crystalline disordered phase ($l_d$) were solubilized (Ahmed et al., 1997). The $l_o$ phase is formed by the interaction of phospholipids with Chl (Ipsen et al., 1987; McMullen and McElhaney, 1995; Sankaram and Thompson, 1991; Vist and Davis, 1990). It is characterized by a high degree of acyl chains order associated with lateral diffusion properties close to those determined for lipids in the liquid-crystalline or fluid phase ($L_\alpha$ or $l_d$) where the acyl chains are kinked and loosely packed. The DRM physical properties correspond to those of the $l_o$ phase. The actual hypothesis proposes that rafts formation is essentially driven by a $l_o$-$l_d$ phase separation process in which Spl/Chl-enriched $l_o$ lipid domains are surrounded by a $l_d$ matrix enriched in more unsaturated glycerophospholipid species (Ahmed et al., 1997; Brown and London, 1998; Silvius et al., 1996; Simons and Ikonen, 1997).

The characteristics of the $l_o$-$l_d$ phase separation process have been studied with fluorescent probes in model membranes made of various ternary lipid mixtures containing (Ahmed et al., 1997; Dietrich et al., 2001b; Xu and London, 2000) or not (Korlach et al., 1999; Parasassi et al., 1997; Silvius et al., 1996) sphingomyelin (SM), the most abundant natural sphingolipid. The topology of the formed domains at a mesoscopic resolution, however, escapes the possibilities of fluorescence microscopy. Atomic force microscopy (AFM) gives access to details of reconstituted membrane structures at a lateral resolution better than 1 nm and a vertical resolution of ~0.1 nm, under ''physiological'' conditions (Engel et al., 1997; Hoh and Hansma, 1992; Shao and Zhang, 1996). This technique allows to image domains in binary or ternary mixtures of lipids in monolayers and bilayers (Dufrêne et al., 1997; Janshoff and Steinem, 2001; Milhiet et al., 2003; Rinia and de Kruijff, 2001). Although local viscoelastic properties are accessible through oscillating modes (Cleveland et al., 1998), AFM detection of membrane lipid domains generally relies on the thickness difference between lipids under different phases. This property seems particularly appropriate for the detection of ''rafts,'' which are predicted to protrude from their $l_d$ environment (van Meer, 2002). Size, shape, and growth law of individual microdomains upon gel-fluid phases separation process, as well as the predicted passage from the discon-

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nected gel to the disconnected fluid in bilayers made of a phospholipid binary mixture, can be determined in real time (Giocondi et al., 2001a,b). First AFM studies on the behavior of dioleoylphosphatidylcholine (DOPC)/SM/Chl bilayers, a mixture commonly used to model rafts, have been published recently, which show the coexistence of ordered and disordered lipid domains for a wide range of Chl concentrations (Dietrich et al., 2001a; Milhiet et al., 2002a; Rinia et al., 2001; Saslowsky et al., 2002). Diunsaturated phosphatidylcholine (PC) species are a very minor constituent of biological membranes. Rather, long saturated and long unsaturated fatty acyl chains occupy the sn-1,2 position, respectively, of natural PC. Presence of a sn-1 long saturated acyl chain facilitates strong van der Waals attractive interaction with Chl planar steroid ring resulting in a much more marked Chl condensing effect than for DOPC (Smaby et al., 1994).

In this AFM study we compared the evolution, from the gel-$l_d$ phase separation to uniform $l_o$ state, of bilayers made of either a DOPC/SM (1:1) or a 1-palmitoyl-2-oleoylphosphatidylcholine (POPC)/SM (1:1) mixture when providing Chl via incubation with methyl-$\beta$-cyclodextrin preloaded with Chl (M$\beta$CD-Chl). The use of M$\beta$CD-Chl provides a convenient method to deliver Chl to cultured cells (Christian et al., 1997), isolated biological membranes (Niu et al., 2002), and model membranes (Dietrich et al., 2001b; Lawrence et al., 2003; Leventis and Silvius, 2001; Niu and Litman, 2002). Reversibility of the process was investigated by adding unloaded M$\beta$CD, which is commonly used to deplete Chl from cell membranes (Kilsdonk et al., 1995; Yancey et al., 1996) and model membranes (Lawrence et al., 2003; Ohvo and Slotte, 1996; Ohvo-Rekila et al., 2000; Radhakrishnan et al., 2000).

## MATERIALS AND METHODS

Stock solutions of 10 mM Chl, POPC, DOPC, and bovine brain SM (Sigma-Aldrich, Saint Quentin, France and Avanti Polar Lipids Inc., Alabaster, AL) were prepared in chloroform/methanol 2:1 (v/v). M$\beta$CD was from Sigma-Aldrich. M$\beta$CD-Chl was either prepared from Chl water soluble (Sigma-Aldrich) or from Chl dissolved in a M$\beta$CD solution according to a previously published protocol (Christian et al., 1997).

### Supported bilayers

Supported bilayers were prepared as previously described (Giocondi et al., 2001a,b). Briefly, SM and DOPC or POPC (1:1) in solvent were mixed by vortexing. The solvent was evaporated under nitrogen at 30°C. Multilamellar vesicles (MLV) were prepared by adding phosphate buffered saline (PBS, pH 7.4), at 70°C, to a final phospholipid concentration of 0.5 mM, while vortexing in the presence of glass beads. Unilamellar vesicles were obtained, always at the same temperature, by extrusion of the MLV through a 0.1 $\mu$m polycarbonate membrane (Avanti Polar Lipids). Vesicles were deposited on a freshly cleaved mica disk enclosed in a swinney holder, sheltered from air, and allowed to fusion in a water bath for 2 h at 70°C (Giocondi et al., 2001a).

### Atomic force microscopy

Samples laying under PBS with 0.5 mM $Ca^{2+}$ and $Mg^{2+}$ were imaged with a Bioscope (Digital Instruments, Santa Barbara, CA) in contact mode, at room temperature (Giocondi et al., 2001b; Milhiet et al., 2002b). Divalent cations were added to provide to the bilayer an environment closer to cell extracellular medium. Silicon nitride cantilevers with a nominal spring constant of 0.01 or 0.03 N/m (Park Scientific Instruments, Sunnyvale, CA) were used. The scanning force was adjusted at ~200 pN and scan rates ranged from 1 to 2 Hz. Images were obtained from at least three different samples prepared on different days with at least five macroscopically separated areas on each sample.

## RESULTS

### Model rafts formation upon cholesterol addition to DOPC/SM bilayers

DOPC/SM/Chl mixtures have been previously used to model the behavior of rafts (Dietrich et al., 2001a; Milhiet et al., 2002a; Rinia et al., 2001; Saslowsky et al., 2002; Yuan et al., 2002). To follow the in situ formation of model rafts, M$\beta$CD-Chl was added, under the AFM, to the solution bathing supported bilayers made of DOPC/SM equimolar mixtures. As recently described (Lawrence et al., 2003; Milhiet et al., 2002a; Rinia et al., 2001) and in accordance with differential scanning calorimetry data (Demel et al., 1977), the topology of DOPC/SM (1:1) samples observed before M$\beta$CD-Chl addition was characteristic of a bilayer under phase separation (Fig. 1 *a*) with lighter (thicker) gel phase domains protruding from the darker fluid phase by a single step of 1.0 ± 0.1 nm apparent height ($\delta h$). Corrugated gel phase domains made of closely packed globular particles were also frequently imaged. The size of gel domains, which accounted for 29 ± 3% of the total surface, varied between a few hundred nanometers and a few microns. Defects in the bilayers allowed to estimate the distance between the mica substrate and the top of the bilayers. This distance varied between ~5.2 and 8.5 nm, according to the experiments. Few vesicles, which did not fuse with the bilayer, appeared as brighter dots. Addition of M$\beta$CD-Chl (1–2 mM) induced a marked reorganization of the bilayer, with the frequent formation of holes and a decrease in the size of gel domains (Fig. 1 *b*), indicating the net uptake of bilayer material by M$\beta$CD-Chl. The holes were then rapidly filled in by the lateral extension of the bilayer, which accompanied the formation and growth of protruding domains, likely regrouping gel + $l_o$ phases (Fig. 1 *c*). After 45-min incubation there were no more holes, and the protruding domains appeared more elongated and, for most of them, branched (Fig. 1 *d*). They occupied 32 ± 2% of the total surface and $\delta h$ was significantly reduced (0.6 ± 0.2 nm). Prolonging the incubation time ($t = 60$ min), the branched domains fragmented into smaller (200–500 nm) domains that occupied the majority of the bilayer surface (Fig. 1 *e*). The difference in height with their surrounding was further reduced ($\delta h = 0.3 \pm 0.1$ nm), which precluded

an accurate determination of their relative area. Because of the time-dependent decrease in $\delta h$, the vertical grayscale in Fig. 1 had to be reduced from 15 nm (Fig. 1, *a* and *b*) to 10 nm (Fig. 1 *c*) and to 7.5 nm (Fig. 1, *d–f*) to allow domains visualization. Finally, after 80 min of incubation (Fig. 1 *f*), the surface of the bilayer became homogeneous with no detectable domains, which strongly suggests that the whole bilayer was now in the $l_o$ phase. Reversibility of the phenomenon was checked by adding 1 mM M$\beta$CD after rinsing of the bilayer (Fig. 2). Under these conditions, lateral heterogeneity of the bilayer was rapidly (10 min) restored, with domains protruding from the matrix by 0.4 ± 0.1 nm. Addition of unloaded M$\beta$CD was also frequently associated with the formation of holes in the bilayer.

## Effects of M$\beta$CD-Chl on POPC/SM bilayers

Similar M$\beta$CD-Chl-mediated cholesterol loading experiments were performed starting with POPC/SM (1:1) supported bilayers. Like for the DOPC/SM mixture, gel domains enriched in SM protruding from the POPC enriched matrix were observed. Both flat and corrugated SM domains were again visualized. Fig. 3 *a* is representative of such corrugated SM domains that protruded from the matrix by a $\delta h$ of 1.4 ± 0.4 nm. Some globular and elongated structures that constituted the corrugations could reach up to 2.5 nm above the matrix. The first step after M$\beta$CD-Chl addition was a decrease in the size of SM domains accompanied by a change in their topography (Fig. 3 *b*). Formation of holes upon M$\beta$CD-Chl addition was observed in several occasions (data not shown). In contrast with the diunsaturated-PC containing mixture, in POPC/SM bilayers the size of protruding domains decreased continuously as a function of the incubation time (Fig. 3, *c* and *d*) before they disappeared (Fig. 3 *e*). Thus, no formation of new emerging domains, no branching, and no fragmentation into multiple small-size domains as observed for DOPC/SM samples occurred. This could suggest that M$\beta$CD-Chl had taken up SM from the bilayer, leading to a bilayer in a homogeneous $l_d$ phase. Alternatively, Chl delivered by the cyclodextrin could have interacted with POPC as well as with some SM species giving rise to a $l_o$ (POPC/SM/Chl) matrix surrounding domains of SM species still in the gel state. To distinguish between these two possibilities, after washing M$\beta$CD-Chl and rinsing, M$\beta$CD was added to the topographically homogeneous sample. Even if it took, as

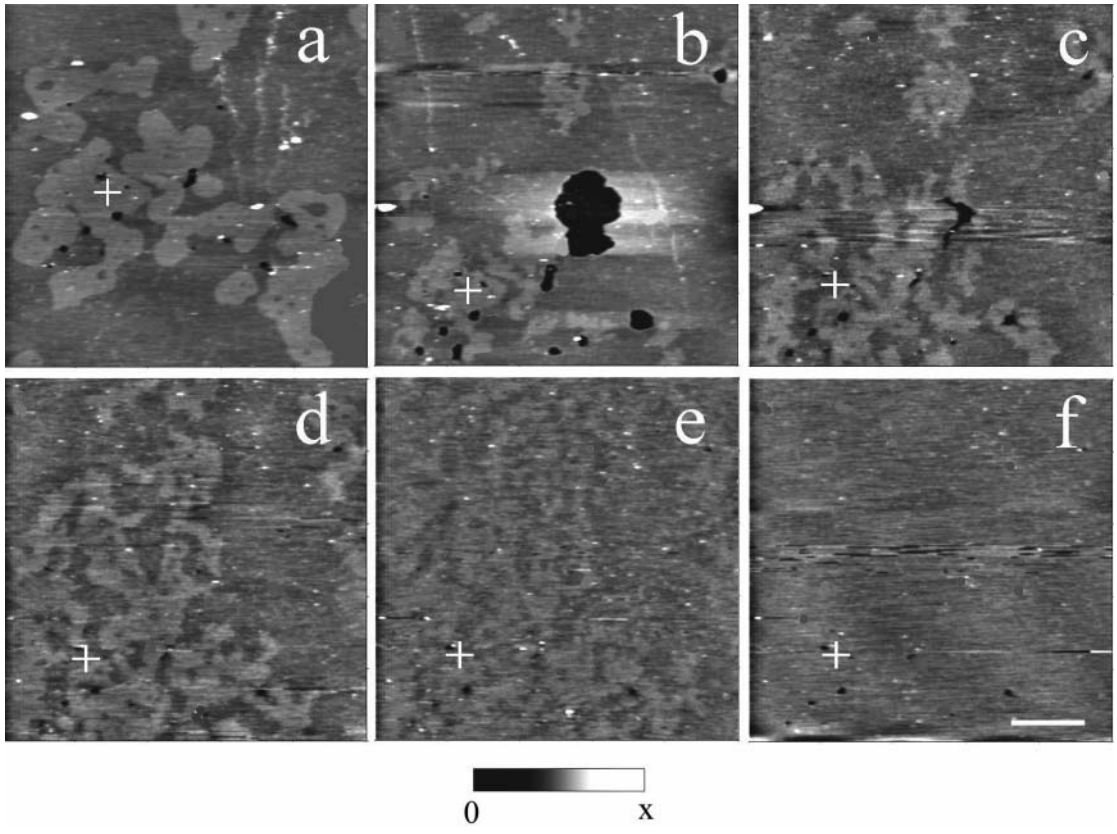


FIGURE 1 Effect of M$\beta$CD-Chl treatment on DOPC/SM supported bilayers. A DOPC/SM (1:1) bilayer in buffer was imaged before (*a*) treatment with 1 mM M$\beta$CD-Chl. The same zone was imaged 6 min after M$\beta$CD-Chl addition (*b*). Drift between these two scans is indicated by the position of the cross-hair. Images *c–f* were obtained 12, 45, 60, and 80 min after the cyclodextrin addition. *Z*-grayscale: 15 nm (*a* and *b*), 10 nm (*c*), and 7.5 nm (*d–f*). Height image, bar: 2.5 $\mu$m.

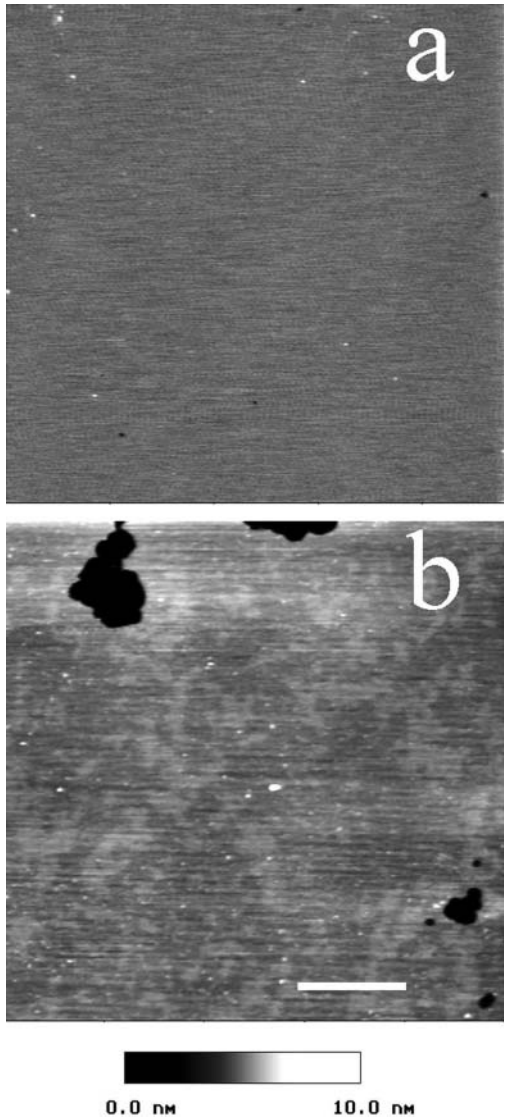


FIGURE 2 Reversibility of M$\beta$CD-Chl treatment. DOPC/SM sample incubated with M$\beta$CD-Chl, until a homogeneous single phase was obtained, was rinsed under the microscope (*a*) and 1 mM M$\beta$CD was added. The sample was examined 10 min later (*b*). Height image, bar: 2.5 $\mu$m.

compared to DOPC, higher M$\beta$CD concentration (5 mM) and longer incubation time, up to 3 h, reappearance of protruding domains in samples containing POPC (Fig. 4) clearly identified the $l_o$ phase as the starting homogeneous phase. This reversibility of $l_o$ formation, at least partial, was accompanied by the destruction of large membrane zones (Fig. 4, *c* and *d*).

### Effects of M$\beta$CD on PC/SM bilayers

Formation of holes after treatment with either M$\beta$CD or M$\beta$CD-Chl raised questions about the specificity of the cyclodextrin toward Chl. To answer this question, a large zone (50 $\times$ 50 $\mu$m) of a DOPC/SM sample was scanned before M$\beta$CD (1.25 mM) addition (Fig. 5 *a*). When using such large scan sizes it is difficult, even after rinsing, to avoid the presence of liposomes, which did not fuse and are just adsorbed on the bilayer. Keeping the scan rate at a reasonable value (1 Hz) reduces the feedback efficiency. Liposomes are then moved by the AFM tip during scanning and appear as bright elongated spots and scratches on the figure. Cyclodextrin was added during the next downward scan. As shown by Fig. 5 *b*, the top of the image, before M$\beta$CD addition, was practically identical to that of the first scan. Within 2 min after the treatment with cyclodextrin, the majority of SM domains disappeared from the bilayer surface and a hole was formed (*white arrow*). Gel domains had disappeared from the top of the image and two additional holes have been formed in the next scan (Fig. 5 *c*). Similarly, holes appeared and the number and size of SM gel domains were drastically reduced by M$\beta$CD addition to POPC/SM samples (data not shown).

## DISCUSSION

Cholesterol and sphingolipids play key roles in the formation of rafts in the plasma membrane. To better understand the principles that underlie this formation, we studied in real time the topology of microdomains starting from either a DOPC/SM or a POPC/SM fluid-gel phase-separated bilayer to which Chl was delivered via M$\beta$CD-Chl. For both categories of samples, incubation with M$\beta$CD-Chl ultimately led to the formation of a homogeneous bilayer, devoid of domains, in the liquid-ordered phase. Intermediate stages, which could be affected by an uptake of SM from bilayers by both M$\beta$CD and M$\beta$CD-Chl, however, differed with the passage through the coexistence of liquid disordered-liquid ordered phases for DOPC/SM samples and of liquid ordered-gel phases for POPC/SM bilayers. These data suggest that raft formation in biological membranes could occur not only via liquid-liquid but also gel-liquid immiscibility. They also strongly suggest that interpretation of M$\beta$CD and M$\beta$CD-Chl effects on cell membranes only in terms of cholesterol movements have to be treated cautiously.

### Structure of DOPC/SM and POPC/SM bilayers

In the absence of cholesterol, AFM data showed the presence of protruding domains in both DOPC/SM and POPC/SM bilayers. Existence of SM enriched gel phase domains emerging from the DOPC enriched fluid phase in similar equimolar DOPC/SM mixture was recently reported (Lawrence et al., 2003; Milhiet et al., 2002a; Rinia et al., 2001). In accordance with these data, we observed not only flat domains of a size varying from a few tens of nanometers to a few micrometers protruding from the fluid phase by $\sim$1 nm, but also corrugated SM domains. That SM in the gel phase

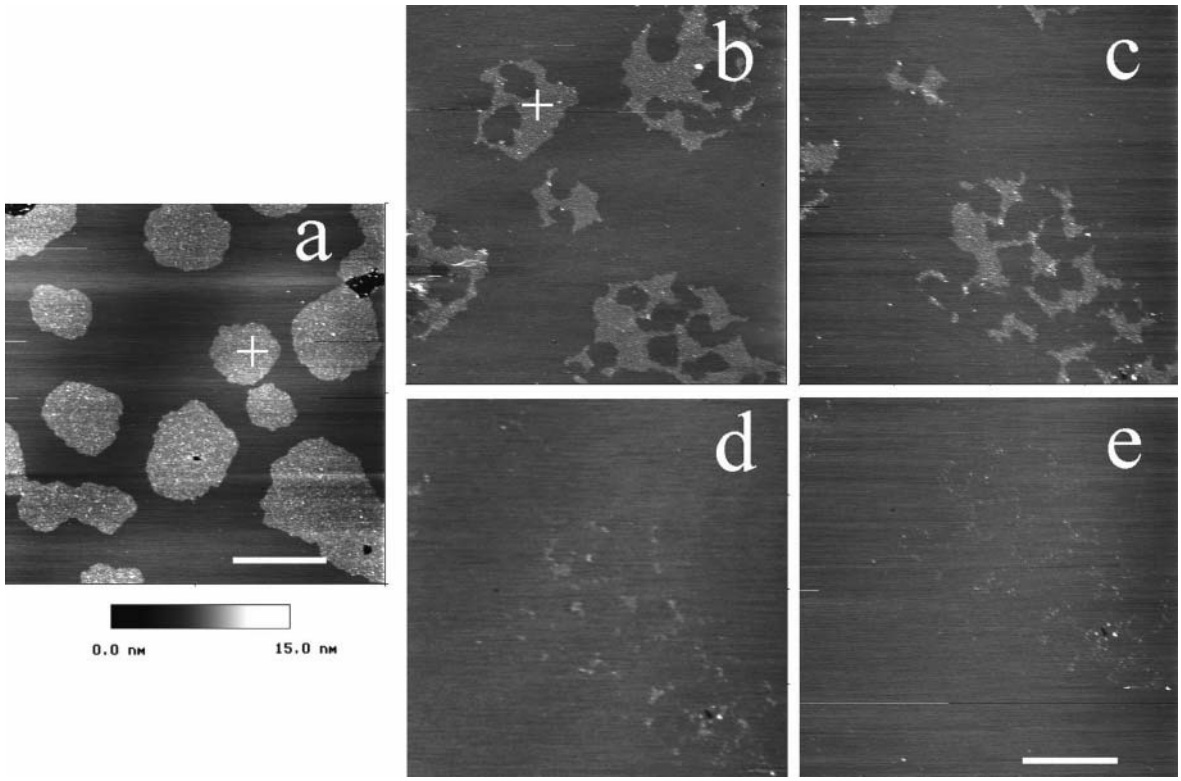


FIGURE 3 Effect of MβCD-Chl treatment on POPC/SM supported bilayers. A POPC/SM (1:1) bilayer in buffer was imaged before (*a*) and after 20 min (*b*), 60 min (*c*), 100 min (*d*), and 150 min (*e*) 1.25 mM MβCD-Chl addition. The scan size has been reduced by twice between the control and the MβCD-Chl treated sample. Height image, bar: 5 μm for *a* and 2.5 μm for *b–e*.

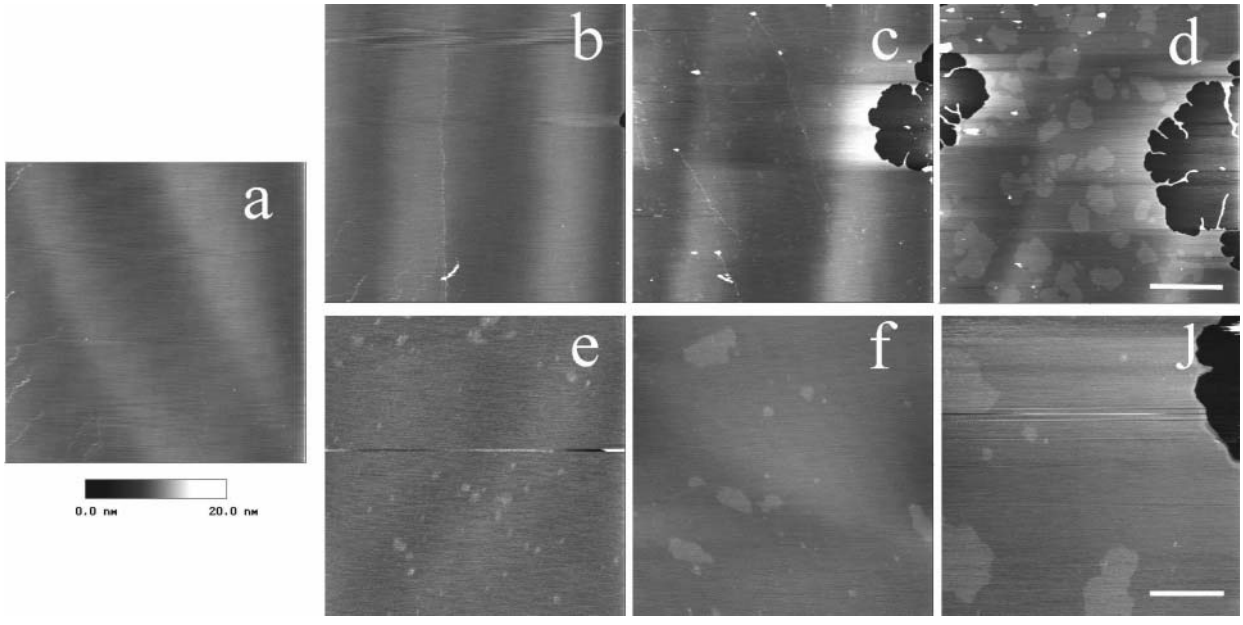


FIGURE 4 Slow reversibility of MβCD-Chl treatment in POPC/SM bilayers. POPC/SM sample incubated with MβCD-Chl, until a homogeneous single phase was obtained, was rinsed under the microscope (*a*). Lipid demixing induced by treatment with 5 mM MβCD was followed as a function of time on the same zone. (*b–d*) correspond to scans acquired 5, 165, and 290 min after the cyclodextrin addition. Height image, bar for *a–d*: 5 μm. Images *e*, *f*, *j* are 5-μm scans of the same zone at 155, 180, and 235 min; bar: 1 μm.

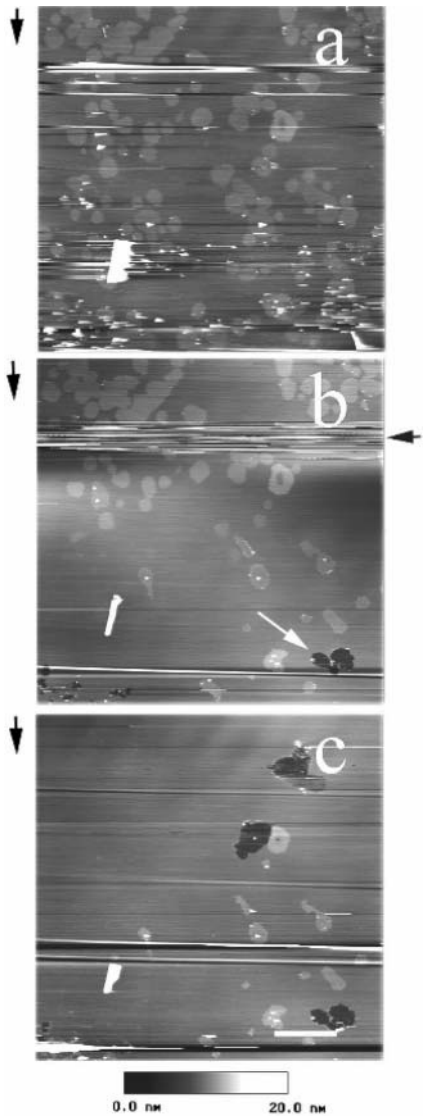


FIGURE 5 M$\beta$CD induced hole formation in DOPC/SM bilayers. Scanning of a large zone (50 $\times$ 50 $\mu$m) of a DOPC/SM (1:1) bilayer confirms the presence of numerous SM enriched gel phase domains emerging from the DOPC matrix. Note also the presence of a contaminant in the lower left part of the image (*a*). Arrows on the left of the image indicate the slow axis scan direction. In the next scan (*b*), 1.25 mM M$\beta$CD was added at the position marked by the horizontal arrow, on the right side of the image. Image *c* was acquired 8 min after starting the second scan. Height image, bar: 10 $\mu$m.

can adopt different structures, from regular ripples to ''egg carton'' and ''sponge-like,'' was reported earlier using freeze-fracture electron microscopy (Hui et al., 1980; Meyer et al., 1999). Thus, the globular or elongated structures forming the corrugated gel domains, observed in both DOPC/SM and POPC/SM bilayers, likely result from two-dimensional relaxation processes within a highly frustrated bilayer in the gel state. Our recent data show that in sphingomyelin/unsaturated phosphatidylcholine two-phase mixtures, SM enriched microdomains can adopt a variety of morphologies, from flat homogeneous surfaces to branched filaments decorated domains (Giocondi et al., 2004). Determined from defects or holes in the gel phase domains, the surface of the supported bilayer was ~5.2–8.5 nm above the substrate. Such a variation was likely due to differences, from one experiment to the other, in the thickness of the buffer layer between the support and the bilayer, a parameter poorly controlled when forming the supported bilayer (Milhiet et al., 2003). More generally, visualization by AFM of a gel-fluid phase separation in DOPC/SM (1:1) mixture, at room temperature, was expected from the early data obtained by DSC (Demel et al., 1977). Existence of a gel-fluid phase separation in POPC/SM (1:1) bilayers under similar condition was also expected from x-ray diffraction results on egg yolk PC (which contains POPC as the principal component)/brain SM multilamellar system (Untracht and Shipley, 1977), from the spontaneous transfer of SM between bilayers (Frank et al., 1983), and from DSC and AFM data on kidney brush border membrane models (Milhiet et al., 2002b).

## Effect of M$\beta$CD-Chl on DOPC/SM supported bilayers

Independently of flat or corrugated gel domain aspect, addition of M$\beta$CD-Chl to DOPC/SM bilayers resulted in a triphasic response with a marked decrease in the size of the SM domains accompanied by the formation of holes in the bilayer (i), followed by the reformation of protruding domains that slowly increased in size and then connected (ii). Further incubation resulted in the disconnection of most domains, which adopt a mesoscopic size, followed by their disappearance giving the bilayer a uniform aspect (iii). The most likely interpretation of this observation is the following. In the first stage, material, and preferentially SM, is taken up from the bilayer by M$\beta$CD-Chl. Cholesterol is then delivered to the bilayer where it interacts preferentially with the remaining SM to form domains that in the beginning can contain both gel and $l_o$ phases. Addition of more Chl results in the formation of enriched SM/Chl domains likely in the $l_o$ phase, surrounded by a Chl poor DOPC matrix in the fluid $l_d$ phase. Further Chl addition increases its concentration in the DOPC matrix, resulting in the fragmentation of branched SM/Chl structures into domains of mesoscopic size to finally form a topographically uniform bilayer in the $l_o$ phase. Thus, starting with DOPC/SM mixture, formation of $l_o$ phase would follow this way: fluid (DOPC) + gel (SM) phases $\rightarrow$ fluid (DOPC) + $l_o$ (SM + cholesterol) phase $\rightarrow$ $l_o$ phase.

Our data confirm the recent finding that, upon addition of M$\beta$CD-Chl, DOPC/SM (1:1) ultimately forms a single raft-like lipid phase (Lawrence et al., 2003). However, they differ by the existence of a net uptake of phospholipids, preferentially SM, from DOPC/SM bilayers by both M$\beta$CD and

MβCD-Chl. In what concerns the net uptake of bilayer phospholipids (i), our use of rather large scans certainly facilitated the detection of holes formation, which was observed with the different batches of cyclodextrin we tested. For instance, the probability to image the hole present in Fig. 5 *b* would have been very low using scans of a few micrometers. This spatial distribution of holes might explain why we did not report such a phenomenon from our previous experimental series where MβCD-Chl was added to DOPC/SM bilayers preincubated with AP, the GPI-anchored alkaline phosphatase (Milhiet et al., 2002a). The presence of AP, concentrated at the fluid/gel interface, and/or the use of a lower concentration of MβCD-Chl may also account for the absence of holes and the slower kinetics for forming a uniform $l_o$ phase. Accompanying the hole formation, treatment with cyclodextrin resulted in a marked reduction in the area occupied by SM enriched gel domains. Starting from DOPC/SM binary mixture this can hardly occur otherwise than by a preferential uptake of SM by the cyclodextrin. In contrast to the early effect of MβCD-Chl on DOPC/SM, large branched gel-$l_o$ domains are formed when including Chl during the preparation of supported bilayers (DOPC/SM/Chl 1:1:0.35) (Milhiet et al., 2002b). Accordingly, preferential uptake of SM is again the most likely explanation for the initial decrease in the size and area of SM domains upon MβCD-Chl addition. Uptake of lipids from the bilayer by MβCD is not unexpected because cyclodextrins have been shown to encapsulate various hydrophobic molecules (Pitha et al., 1988), which is in accordance with the data obtained both in liposomes (Niu and Litman, 2002) and biological membrane (Niu et al., 2002). Furthermore, Leventis and Silvius (2001) have demonstrated that MβCD accelerates the transfer of dipalmitoyl-PC between large unilamellar lipid vesicles (LUV) by a much greater factor than it does the transfer of cholesterol under the same conditions. Indeed, in the liposomes experiments, the presence of both donor and acceptor vesicles resulted in an exchange of material between the two populations, which prevented holes formation. On the other hand, in our experiments the use of a single supported bilayer resulted in a much larger cyclodextrin to lipid molar ratio, which explains the formation of large holes, even at millimolar cyclodextrin concentration.

The second phase (ii), i.e., the increase in the size of the remaining-SM enriched domains followed by their connection, can be explained by MβCD-Chl mediated delivery of cholesterol to the bilayer. Experiments on LUV have shown that addition of cholesterol to bilayers made of binary mixtures combining long-chain PC or SM and short-chain PC, like dilauroyl PC (DLPC), its bromo-substituted derivative (12BrPC), or diunsaturated DOPC, greatly promotes lateral segregation (Ahmed et al., 1997; Silvius et al., 1996). Cholesterol-induced interconnection of domains was previously reported for supported bilayers made by fusing DOPC/SM/cholesterol vesicles on mica (Milhiet et al., 2002a; Rinia et al., 2001; Saslowsky et al., 2002). Like in these experiments, the progressive incorporation of cholesterol in the bilayer as a function of time was associated with a decrease in the apparent difference in thickness between the lipid domains. Taking into account the marked preference of Chl for interacting with SM rather than with DOPC (Demel et al., 1977; Milhiet et al., 2002a), connected domains were likely to be made of SM/Chl mostly in a $l_o$ phase.

Further Chl addition via MβCD-Chl (iii) provoked the disconnection of $l_o$ domains that fragmented into mesoscopic scale microdomains whose size fall within the range described for rafts. When raising the bilayer cholesterol concentration, fragmentation of branched domains into smaller domains results in an increase of the interfacial area between domains and the bulk, as predicted from theoretical analysis (Cruzeiro-Hansson et al., 1989). Finally, prolonging the incubation with MβCD-Chl resulted in a complete disappearance of domains, which strongly suggests that the totality of the bilayer was in $l_o$ phase, a view supported by the reversibility of the phenomenon upon treatment with MβCD. This could indicate an increased affinity toward gel-phase rigid phospholipid species. The fact that even MβCD-Chl was capable of extracting SM from DOPC/SM bilayers is likely related to the presence of unloaded cyclodextrin molecules in such complexes: upon maximum cholesterol loading, the cyclodextrin to cholesterol molar ratio is ~3:1 (Christian et al., 1997). Despite these effects, when applied to cholesterol enriched bilayers in the $l_o$ phase, MβCD restored the DOPC/SM phase separation, a situation that agrees with numerous literature data showing a preferential uptake of cholesterol from such mixtures.

## Coexistence of gel-liquid ordered phases in DOPC/SM/Chl bilayers

Starting with POPC/SM rather than with DOPC/SM, we observed a different evolution of the bilayer upon MβCD-Chl addition. Formation of holes also frequently occurred at early time but, instead of a subsequent growth of domains, it was followed by a continuous decrease in the size of SM-enriched domains, until they disappeared with the formation of a homogeneous bilayer. Accordingly, the protruding domains whose size decreased as a function of MβCD-Chl incubation time most likely corresponded to gel phase SM-enriched domains surrounded by a $l_o$ POPC-enriched matrix. Existence of a $l_o$ phase in POPC/cholesterol mixtures was established by NMR (Thewalt and Bloom, 1992). Identification of the sample physical state as a $l_o$ phase came from the reversibility of the domains formation when adding MβCD to the topographically homogeneous bilayers obtained after MβCD-Chl treatment. It is worth noting that higher concentrations of cyclodextrin and longer incubation time were required to extract the cholesterol from the POPC than from the DOPC containing bilayers, an observation in accordance with the lower affinity of cholesterol for the

diunsaturated PC species (Ohvo-Rekila et al., 2002; Silvius, 2003; Smaby et al., 1994). This slower process allowed the observation of the appearance of small gel domains emerging from the matrix and then growing slowly before extensive phase separation occurred. Such a behavior could be explained by the following sequence of events, $l_o$ phase → gel (SM) + $l_o$ (POPC+SM+Chl) phase → gel (SM) + fluid (POPC) phase, in the reverse order of that leading to the $l_o$ phase formation. This strongly suggests that, in a mixture were SM is in the gel and PC in the fluid phase, cholesterol interacts with POPC as well as with the SM species, in contrast with the DOPC containing samples. This is in agreement with the recent finding that cholesterol is in fast exchange between two or more membrane regions in such mixtures (Aussenac et al., 2003).

## Implications for plasma membranes

The AFM data show that gel phase domains enriched in sphingolipids can coexist with a liquid ordered matrix rich in cholesterol and POPC. They suggest that in a biological membrane variation in the cholesterol can locally promote formation of lipid microdomains by either liquid-liquid or gel-liquid phase separation phenomena. They also call for comments about the use of cyclodextrins in the identification of rafts- or nonrafts-associated events in cell biology. Observation that, not only M$\beta$CD, but also M$\beta$CD-Chl make holes in supported bilayers and preferentially extract SM from a fluid PC/SM supported bilayer, indicates that one has to be cautious when attributing exclusively to cholesterol concentration changes the biochemical and functional modifications associated with a cyclodextrin treatment of biological membranes.

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