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# Cholesterol modulation of membrane resistance to Triton X-100 explored by atomic force microscopy

Karim El Kirat [a], Sandrine Morandat [b,*]

[a] Laboratoire de Biomécanique et Génie Biomédical, UMR-CNRS 6600, Université de Technologie de Compiègne, BP 20529, 60205 Compiègne Cedex, France
[b] Laboratoire de Génie Enzymatique et Cellulaire, UMR-CNRS 6022, Université de Technologie de Compiègne, BP 20529, 60205 Compiègne Cedex, France

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**Abstract**

Biomembranes are not homogeneous, they present a lateral segregation of lipids and proteins which leads to the formation of detergent-resistant domains, also called "rafts". These rafts are particularly enriched in sphingolipids and cholesterol. Despite the huge body of literature on raft insolubility in non-ionic detergents, the mechanisms governing their resistance at the nanometer scale still remain poorly documented. Herein, we report a real-time atomic force microscopy (AFM) study of model lipid bilayers exposed to Triton X-100 (TX-100) at different concentrations. Different kinds of supported bilayers were prepared with dioleoylphosphatidylcholine (DOPC), sphingomyelin (SM) and cholesterol (Chol). The DOPC/SM 1:1 (mol/mol) membrane served as the non-resistant control, and DOPC/SM/Chol 2:1:1 (mol/mol/mol) corresponded to the raft-mimicking composition. For all the lipid compositions tested, AFM imaging revealed that TX-100 immediately solubilized the DOPC fluid phase leaving resistant patches of membrane. For the DOPC/SM bilayers, the remaining SM-enriched patches were slowly perforated leaving crumbled features reminiscent of the initial domains. For the raft model mixture, no holes appeared in the remaining SM/Chol patches and some erosion occurred. This work provides new, nanoscale information on the biomembranes' resistance to the TX-100-mediated solubilization, and especially about the influence of Chol.


*Keywords:* Raft; DRM; Real-time AFM; Lipid bilayer; Solubilization; Non-ionic detergent

## 1. Introduction

The plasma membrane of mammalian cells is mainly composed of glycerophospholipids (GPLs), sphingolipids (SphLs) and cholesterol (Chol) that are not homogeneously distributed within the bilayer as proposed by the fluid mosaic model [1] but they are rather organized into microdomains also called "rafts" [2,3]. These membrane microdomains are especially enriched in SphLs and Chol and they play a pivotal role in cellular processes such as signal transduction and membrane trafficking [4–8].

GPLs are especially enriched in unsaturated acyl chains that tend to adopt a kinked structure. Consequently, GPLs present a loosely packed conformation yielding a liquid-disordered or fluid phase (Ld or Lα) [9,10]. On the contrary to GPLs, SphLs bear long and saturated acyl chains. This property is responsible for their tight packing into gel phases in which only very little lateral diffusion can occur [11,12]. Furthermore, Chol can fill the voids between the acyl chains of SphLs leading to their association into a phase that is significantly more fluid than the gel phase: the liquid-ordered phase (Lo) [13–15].

Generally, rafts can be purified as detergent resistant membranes (DRMs) by cold extraction (4 °C) of eukaryotic cell plasma membranes. Usually, this protocol is based on the use of non-ionic detergents, such as Triton X-100 (TX-100) [3,5,16–18]. TX-100 has a low critical micelle concentration (CMC) of 0.24 mM and can also be useful for the purification and the reconstitution of integral or lipid modified proteins in biomembranes [19,20]. Furthermore, a growing body of literature describes model membranes mimicking DRMs such as liposomes, Langmuir monolayers or supported bilayers [21–26].

Atomic force microscopy (AFM) is a powerful technique that allows the high resolution imaging of biological specimens under physiologically compatible conditions (buffered solutions, temperature, …) [27]. Therefore, AFM is widely employed

* Corresponding author. Tel.: +33 3 44 23 44 18; fax: +33 3 44 20 39 10.
*E-mail address:* sandrine.morandat@utc.fr (S. Morandat).

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to explore phase separations in membranes [28] and the interaction of supported lipid bilayers with peptides [29–31], proteins [32–34], drugs [35], solvents [36] and buffers [37]. So far, only few articles have described detergent interaction with membranes observed by AFM [26,38–40]. In a previous study, TX-100 solubilization of dioleoylphosphatidylcholine/dipalmitoylphosphatidylcholine (DOPC/DPPC 1:1 mol/mol) bilayers was followed by AFM in real-time [39]. This lipid composition is known to produce fluid/gel phase separation at room temperature and can thus be considered as a simple model to investigate membrane resistance to detergents. Time-lapse AFM imaging revealed that when TX-100 was added at a concentration above the CMC, it was always able to dramatically alter DOPC/DPPC bilayers by instantly removing the DOPC fluid phase. The remaining DPPC gel phases then appeared more or less swollen depending on TX-100 concentration. The swelling of the gel domains was directly correlated with detergent intercalation within DPPC molecules. When DPPC gel phases were swollen, then holes appeared within the patches. This desorption of bilayer parts left the silhouette of the initial gel phase still recognizable even at the end of the incubation [39].

In this study, TX-100 solubilization of bilayers mimicking lipid rafts was followed in real-time by AFM. To this end, we have prepared three different bilayers: DOPC/sphingomyelin (SM) 1:1 (mol/mol), and DOPC/SM/Chol 2:1:1 (mol/mol/mol) or 4:3:1 (mol/mol/mol). Different TX-100 concentrations were tested to better understand its interaction with membranes.

## 2. Materials and methods

### 2.1. Materials

L-α-dioleoylphosphatidylcholine (DOPC), egg sphingomyelin (SM), cholesterol (Chol) and Triton X-100 were purchased from Sigma (St. Louis, MO) and used without any further purification. Other chemicals were purchased from Merck (Darmstadt, Germany). For all experiments, the distilled water was purified with a Millipore filtering system (Bedford, MA), yielding an ultrapure water with a resistivity of 18.2 MΩ × cm.

### 2.2. Preparation of supported lipid bilayers

Supported DOPC/SM 1:1 (mol/mol), DOPC/SM/Chol 4:3:1 (mol/mol/mol) and DOPC/SM/Chol 2:1:1 (mol/mol/mol) bilayers were prepared using the vesicle fusion method [26,41–43]. To this end, lipids were dissolved in chloroform at 1 mM final concentration. The mixture of these lipids was then evaporated under nitrogen and dried in a dessicator under vacuum for 2 h. Multilamellar vesicles (MLV) were obtained by resuspending the lipidic dried film at room temperature in a buffer containing calcium (10 mM Tris, 150 mM NaCl, 3 mM $CaCl_2$, pH 7.4; Tris/calcium buffer) at 1 mM final lipid concentration. To obtain small unilamellar vesicles (SUV), the suspension was sonicated to clarity (3 cycles of 2 min 30 s) using a 500 W titanium probe sonicator (Fisher Bioblock Scientific, France; 35% of the maximal power; 13 mm probe diameter) while being kept in an ice bath. The liposomal suspension was then filtered on a 0.2 μm Acrodisc® (Pall Life Sciences, USA) to eliminate titanium particles. Freshly cleaved mica squares (16 $mm^2$) were glued onto steel sample discs (Agar Scientific, England) using Epotek 377 (Polytec, France). A 150 μL portion of the SUV suspension was then deposited onto the mica samples, and the SUVs were allowed to adsorb and fuse on the solid surface for 1 h at 60 °C. Subsequently, samples were rinsed with 3 mL of buffer (10 mM Tris, 150 mM NaCl pH 7.4; Tris buffer) and slowly cooled to room temperature.

### 2.3. Atomic force microscopy

Supported bilayers were investigated using a commercial AFM (NanoScope III MultiMode AFM, Veeco Metrology LLC, Santa Barbara, CA) equipped with a 125 μm × 125 μm × 5 μm scanner (J-scanner). A quartz fluid cell was used without the O-ring. Topographic images were recorded in contact mode using oxide-sharpened microfabricated $Si_3N_4$ cantilevers (Microlevers, Veeco Metrology LLC, Santa Barbara, CA) with a spring constant of 0.01 N/m (manufacturer specified), with a minimal applied force (<200 pN) and at a scan rate of 5–6 Hz. The curvature radius of silicon nitride tips was ~20 nm. Images were obtained at room temperature (21–24 °C) either in a Tris buffer or in a Tris buffer containing TX-100. All images (256 × 256 pixel) shown in this paper are flattened raw data.

## 3. Results

DOPC/SM biomembranes were prepared with varying amounts of Chol. Preliminary experiments permitted to determine that DOPC/SM/Chol 1:1:1 (mol/mol/mol) produced interconnected small domains (data not shown) as previously described [26]. To allow the comparison among the different bilayers, we first managed to obtain well-delimited domains with a sufficient size (diameter between 2 and 15 μm). In the end, to better understand the mechanism of resistance to TX-100 related to the Chol content of membranes, we have selected three different lipid compositions. The DOPC/SM 1:1 (mol/mol) corresponded to the membrane easily solubilized by TX-100. The DOPC/SM/Chol 2:1:1 (mol/mol/mol) bilayer was considered as the raft-mimicking membrane. Indeed, as previously shown, the ternary mixture made of phospholipids with unsaturated acyl chains, sphingomyelin and cholesterol could crudely imitate the phase separation of cell membranes [12,15,21,32,34]. An intermediate composition was also tested: DOPC/SM/Chol 4:3:1 (mol/mol/mol). It is noteworthy that previous descriptions of these lipid compositions were shown to produce: gel phases of SM for the DOPC/SM membranes [26,44], and Lo phases of SM/Chol for the ternary mixtures [26,32]. These three lipid membranes were imaged by real-time AFM in buffer solution and in the presence of TX-100 at different concentrations.

### 3.1. Time-lapse AFM of DOPC/SM bilayers incubated with TX-100

Fig. 1A presents a typical AFM topographic image of a DOPC/SM bilayer. It reveals the coexistence of two phases: the brighter areas correspond to the domains enriched in SM forming a gel phase while the surrounding darker matrix can be attributed to DOPC in a fluid phase. SM domains protruded from the fluid phase by 1.0 ± 0.1 nm which is in accordance with previous descriptions of this lipid system [26,32,44].

Supported DOPC/SM bilayers were incubated with TX-100 at a concentration two times greater than the CMC (2CMC, 0.48 mM, Fig. 1A to D) and successive AFM images of the same area were then recorded: after 5, 30 and 120 min (Fig. 1B to D). Immediately after the TX-100 addition (Fig. 1B), the DOPC fluid phase was totally solubilized as attested by their thickness 5.5 ± 0.2 nm corresponding to apparently unmodified SM domains. At 30 min incubation time (Fig. 1C), the SM patches remained unaltered while some material, presumably