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Real-time imaging of drug–membrane interactions by atomic force microscopy

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Abstract

Understanding drug–biomembrane interactions at high resolution is a key issue in current biophysical and pharmaceutical research. Here we used real-time atomic force microscopy (AFM) imaging to visualize the interaction of the antibiotic azithromycin with lipid domains in model biomembranes. Various supported lipid bilayers were prepared by fusion of unilamellar vesicles on mica and imaged in buffer solution. Phase-separation was observed in the form of domains made of dipalmitoylphosphatidylcholine (DPPC), sphingomyelin (SM), or SM/cholesterol (SM/Chl) surrounded by a fluid matrix of dioleoylphosphatidylcholine (DOPC). Time-lapse images collected following addition of 1 mM azithromycin revealed progressive erosion and disappearance of DPPC gel domains within 60 min. We attribute this effect to the disruption of the tight molecular packing of the DPPC molecules by the drug, in agreement with earlier biophysical experiments. By contrast, SM and SM-Chl domains were not modified by azithromycin. We suggest that the higher membrane stability of SM-containing domains results from stronger intermolecular interactions between SM molecules. This work provides direct evidence that the perturbation of lipid domains by azithromycin strongly depends on the lipid nature and opens the door for developing new applications in membrane biophysics and pharmacology.

Keywords: AFM; Drug; Lipid bilayer; Phase-separation; Real-time imaging

1. Introduction

Due to their unique properties, supported lipid bilayers are attracting more and more attention in basic and applied research. These systems are widely used as models to investigate the properties of biological membranes and associated processes such as molecular recognition, enzymatic catalysis, cell adhesion and membrane fusion [1–3]. Supported lipid bilayers are also important for a number of applications, including the design of biosensors, the biofunctionalization of solid surfaces, the crystallization of proteins and the immobilization of DNA [3–5]. While liposomes have been extensively used in pharmaceutical research as drug-delivery systems and assays for studying drug–membrane interactions [6], the potential of supported lipid bilayers in this area has been largely underestimated so far.

In pharmacology, the biological activity of a number of drugs is known to directly depend on their interaction with biological membranes [6]. Experimental work on artificial membranes using a variety of methods has demonstrated that the membrane properties may strongly be affected by the presence of membrane associated molecules. Examples of parameters that can be affected by drug–membrane interactions include the conformation of acyl groups, the membrane surface and thickness, the phase transition temperature, the membrane potential and hydration of head groups and the membrane fusion properties [7]. Evidence is also increasing that the presence of a heterogeneous lateral organization may have a number of important consequences on this interaction, including enhancement of penetration and insertion of the molecules at the domain boundary [8]. Monte Carlo calculations on model membranes have also revealed that application of a drug may itself have an influence on the lateral organization of the lipid membrane [8]. Accordingly, understanding the interaction of drugs with lipid membranes has long been a key issue in membrane biophysics and pharmaceutical research.

A number of techniques are available to characterize membrane domain topology, such as fluorescence micros-

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copy, Brewster angle microscopy, X-ray diffraction, and freeze-etch electron microscopy. Despite this variety of methods, little was known until recently about the structure and properties of lipid films at the nanometer level. In this context, atomic force microscopy (AFM) has recently become an essential tool in lipid film research [9]. The instrument has been used to characterize the molecular structure of lipid films in aqueous environment [10,11], the organization of phase-separated films [12–16], the surface of crystalline protein arrays anchored to lipid bilayers [13,17] or reconstituted in the presence of lipids [18], and the interaction with external agents such as alcohols [19], peptides [20] and drugs [21,22]. In addition, the unique real-time imaging capability of AFM has been used to monitor time-dependent processes such as the insertion of proteins into model membranes [23–25] and the growth of single lipid domains in lipid bilayers [15].

In this report, we present the first real-time observation of the interaction of antibiotics with lipid domains in supported lipid bilayers using AFM. Mixed dioleoylphosphatidylcholine/dipalmitoylphosphatidylcholine (DOPC/DPPC) bilayers were used because choline phospholipids are commonly found in eukaryotic cell membranes and this specific mixture is known to separate at room temperature into fluid and gel phases [15]. DOPC/DPPC bilayers were compared with bilayers containing sphingomyelin (SM) because these are known to form domains which can be considered as simple models of lipid microdomains (rafts) found in natural biomembranes [26–28]. Supported bilayers were imaged in real time by AFM, in the absence or presence of the dicationic antibiotic azithromycin (Fig. 1). We chose azithromycin because this amphiphilic antibiotic is known to interact with lipid bilayers. Conformational analysis showed that the drug is capable of interacting both with the hydrophilic and hydrophobic regions of a lipid monolayer [29]. More recently, equilibrium dialysis and nuclear magnetic resonance (NMR) spectroscopy experiments revealed that this antibiotic is able to bind to phospholipid bilayers and especially to interact with the phosphate heads [30]. Despite the accumulation of experimental data on azithromycin–membrane interactions, direct information at high resolution is still lacking.

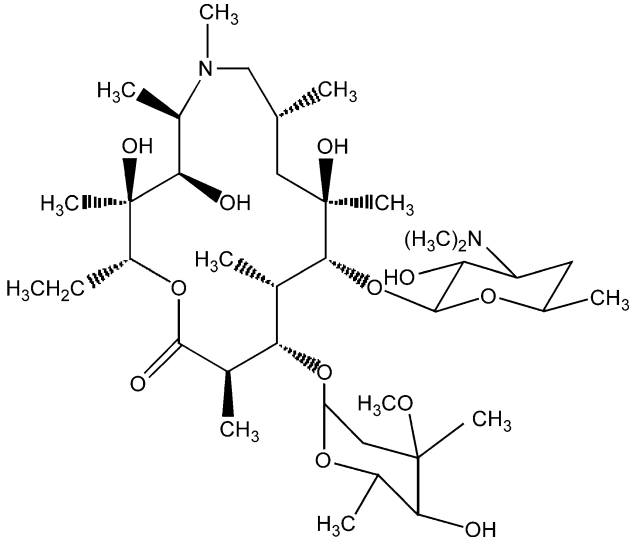

Fig. 1. Structural formula of azithromycin.

2. Materials and methods

2.1. Materials

DOPC, DPPC, SM and Chl were purchased from Sigma (St. Louis, MO). Azithromycin (dihydrate free base for microbiological standard; 94% purity) was generously supplied by Pfizer s.a. (Brussels, Belgium) on behalf of Pfizer Inc. (Groton, CT, USA). Other chemicals were purchased from Merck (Darmstadt, Germany). Azithromycin was dissolved in 0.1 N HCl and thereafter diluted to the desired final concentrations. Three different solutions were used for preparing and imaging the lipid bilayers, i.e. Tris/NaCl/$CaCl_2$ (10 mM Tris, 100 mM NaCl, 3 mM $CaCl_2$, pH 7.4), Tris/NaCl (10 mM Tris, 100 mM NaCl, pH 7.4) and Tris/NaCl/azithromycin (10 mM Tris, 100 mM NaCl, 1 mM azithromycin, pH 7.4).

2.2. Preparation of lipid bilayers

Supported bilayers were prepared as follows. Lipid mixtures were dissolved in $CHCl_3$/MeOH 2:1 (v/v) in glass tubes, evaporated with nitrogen and dried in a desiccator under vacuum for 2 h. The lipids were then resuspended from the walls of the glass tube by vigorous vortexing in Tris/NaCl/$CaCl_2$ buffer (~ 5 ml). To obtain small unilamellar vesicles (SUVs), the suspension was sonicated to clarity (five cycles of 2 min) using a 500-W probe sonicator (Fisher Bioblock Scientific, France; 35% of the maximal power; 13 mm probe diameter) while keeping the suspension in an ice bath and the suspension was finally filtered on 0.2-μm nylon filters (Whatman Inc., USA) to eliminate titanium particles. The SUV solution was then put into contact with freshly cleaved mica substrates for 45 min at 60 °C. After slowly cooling down the system to room temperature, the samples were carefully rinsed to remove the SUV excess using either a Tris/NaCl solution or a Tris/NaCl/azithromycin solution. Samples were then attached to a steel sample puck (Digital Instruments, Santa Barbara, CA) using a small piece of adhesive tape and transferred into the AFM liquid cell while avoiding dewetting.

2.3. AFM imaging

AFM measurements were made at room temperature (~ 23 °C), using a commercial microscope (Nanoscope III, Digital Instruments). Contact mode topographic images

were taken in the constant-deflection mode using oxide-sharpened microfabricated Si_3N_4 cantilevers (ThermoMicroscopes, Sunnyvale, CA) with typical radius of curvature of 20 nm and spring constants of 0.01 and 0.03 N/m (manufacturer-specified). The applied force was kept as low as possible (< 1 nN) and the scan rate was 3–5 Hz. All images were flattened (flattening order of 1 or 2).

3. Results and discussion

With the aim to gain insight into azithromycin–membrane interactions, supported lipid bilayers made of DPPC/DOPC, SM/DOPC and SM/Chl/DOPC mixtures were prepared by fusion of unilamellar vesicles on mica. Time-lapse AFM images were recorded in buffer solution, either in the absence or presence of azithromycin.

3.1. DPPC/DOPC bilayers

Fig. 2A presents a typical topographic image of a mixed DPPC/DOPC (1:1, mol/mol) bilayer supported on mica recorded in buffer solution. The bilayer clearly showed phase-separation in the form of well-defined elevated domains surrounded by a continuous matrix. In the light of the known phase diagrams of the lipids and of previous work [15], the elevated domains may unambiguously be attributed to DPPC in the gel phase and the matrix to DOPC in the fluid phase. Consistent with earlier AFM studies [15,25], we note that the shape of the DPPC domains was irregular, with a size ranging from 100 to 1500 nm. The step height measured between the DPPC and DOPC phases was 1.1 ± 0.05 nm, which is larger than the value expected from the DPPC and DOPC film thicknesses. This behavior is likely to result from a difference in the film mechanical properties [12]. It is also worth noting that only two discrete topographic levels were observed in all images, suggesting that for DPPC/DOPC bilayers prepared by vesicle fusion the gel domains in the two monolayers are coupled [15].

To assess the stability of DPPC/DOPC bilayers during consecutive scanning in buffer, successive images of the same bilayer location were recorded. As can be seen in Fig. 2B, scanning the bilayer surface for 60 min did not cause any significant change of the surface morphology, indicating that the bilayer was stable in these conditions.

3.2. DPPC/DOPC bilayers incubated with azithromycin

Supported DPPC/DOPC bilayers were incubated with a 1 mM azithromycin solution and successive AFM images were then recorded at the same location. A series of topographic images recorded after 2, 12, 18, 26, 31, 36, 44, 58 and 63 min is shown in Fig. 3. After 2 min of incubation, the bilayer morphology, i.e. domain geometry and surface coverage, was similar to that observed in the absence of azithromycin (Fig. 2A). After 12 min, however, the smallest DPPC gel domains disappeared, yielding a topographic level which was similar to that of the DOPC matrix. Increasing the incubation time led to a progressive decrease of the size of the DPPC domains and to their disappearance. After 63 min, gel domains were no longer visible, the bilayer surface being totally homogeneous. We note that for all incubation times the bilayer surface was devoid of defects, i.e. holes in the upper monolayer or in the bilayer were never observed.

One may argue that the observed morphological changes are due to consecutive scanning by the AFM tip rather than to the action of the drug. To test this hypothesis, two control experiments were carried out. First, at the end of the time-dependent experiment (Fig. 3), the tip was moved ~ 1 mm aside and a topographic image of a new zone was recorded. The image revealed a totally homogeneous surface morphology that was similar to that observed at the first location at the end of the time-dependent experiment (63 min).

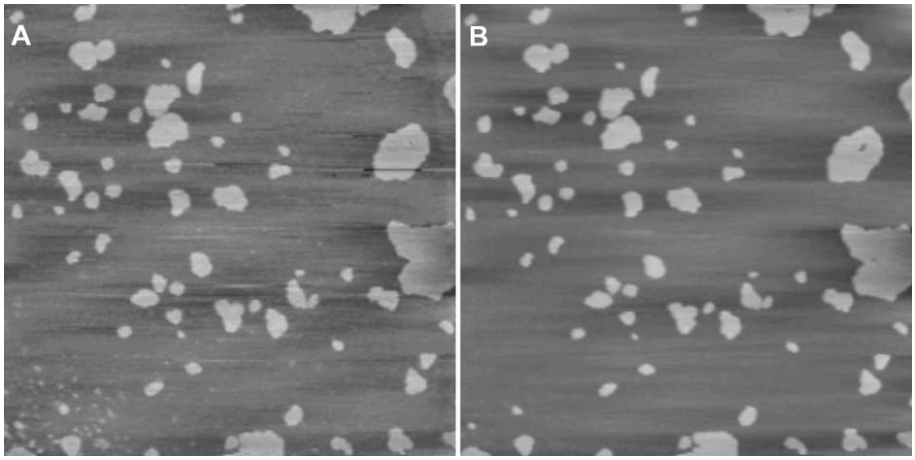

Fig. 2. AFM height images (7.5 × 7.5 μm; *z*-scale : 10 nm) of a mixed DPPC/DOPC (1:1, mol/mol) bilayer in Tris/NaCl buffer. (A) Image recorded almost immediately after sample mounting. (B) Image obtained after scanning the surface for 60 min.

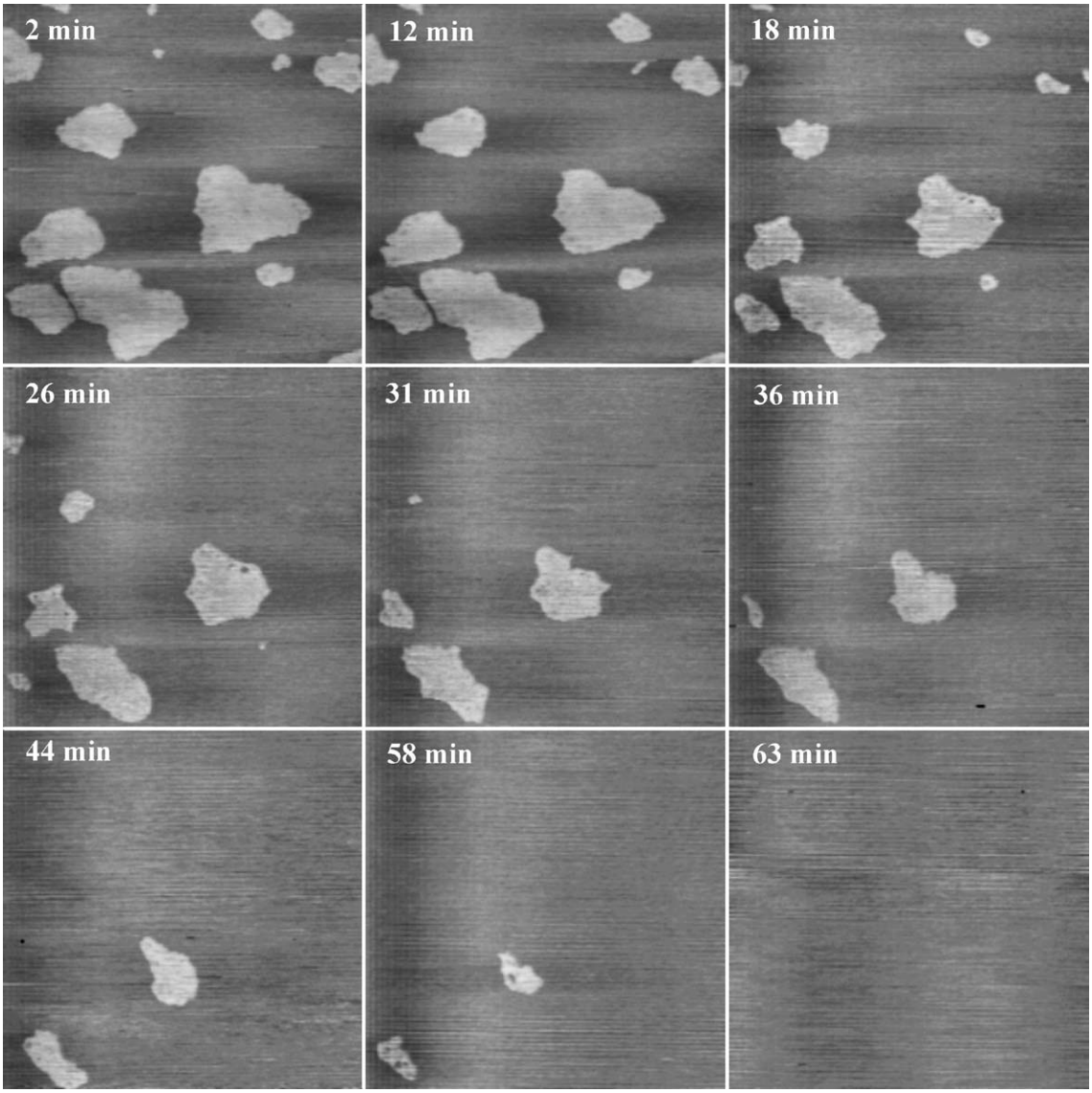

Fig. 3. AFM height images (7.5 × 7.5 μm; *z*-scale : 10 nm) of a mixed DPPC/DOPC (1:1, mol/mol) bilayer recorded in a Tris/NaCl/azithromycin solution at increasing incubation times.

Second, a DPPC/DOPC bilayer was immersed for 60 min in a well containing a 1 mM azithromycin solution and transferred into the AFM fluid cell for subsequent imaging. Images recorded in different locations did all present a homogeneous surface morphology. Note that in these two experiments the presence of a lipid bilayer on the mica substrate was confirmed using force–distance curves and scratching experiments (data not shown). Taken together, these data indicate that the time-dependent erosion of the DPPC gel domains is due to the action of azithromycin rather than to a simple scanning artifact.

Several pieces of evidence suggest that the progressive erosion and disappearance of the DPPC gel domains reflect a modification of the molecular packing of individual DPPC molecules resulting from the interaction with the drug. First, several differential scanning calorimetry (DSC) studies have shown that cationic amphiphilic drugs are able to lower the phase-transition temperature of liposomes made of phospholipids, an effect which was related to the penetration and intercalation of the drug into the lipid bilayer. For instance, the transition temperature of dipalmitoylphosphatidic acid (DPPA) liposomes was shown to decrease from 64 to 11 °C in the presence of cationic amphiphilic drugs [31]. For DPPC liposomes, Kursch et al. [32] demonstrated that the transition temperature is depressed by 16 different cationic compounds; for example, addition of chlorphentermine at a 5:1 ratio (mol drug/mol DPPC) decreased the transition temperature from 41 to 25 °C. Second, equilibrium dialysis and NMR experiments showed that azithromycin is able to bind to phospholipid bilayers at neutral pH by interacting with the phosphate groups [30]. Third, molecular modeling studies of azithromycin in interaction with phospholipids

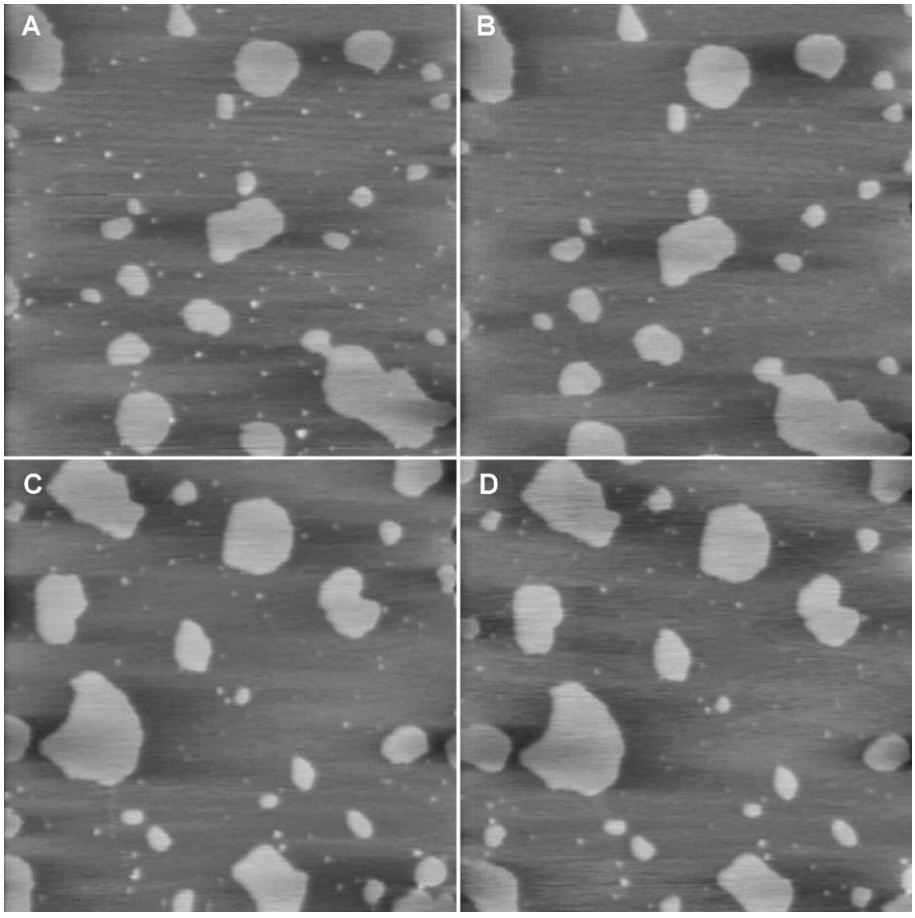

Fig. 4. AFM height images (5 × 5 μm; *z*-scale : 10 nm) of a mixed SM/DOPC (1:1, mol/mol) bilayer recorded in Tris/NaCl (A, B) and Tris/NaCl/azithromycin (C, D) solutions. (A, C) Images recorded almost immediately after sample mounting. (B, D) Images obtained after scanning the surface for 60 min.

revealed that the drug molecules establish contact both with the hydrophilic and hydrophobic domains of a phospholipid monolayer [29]. Taken together, the above observations lead us to believe that the time-dependent erosion of the gel-phase domains observed here is due to the interaction between individual azithromycin and DPPC molecules. Presumably, disruption of the interactions between the lipid polar headgroups, and possibly between the hydrocarbon chains, due to the intercalation of the drug, is responsible for the progressive disappearance of the domains. In future work, it would be interesting to investigate the influence of azithromycin concentration on the erosion process.

Interestingly, we also note in the set of images of Fig. 3 that the inner region of most gel domains remained essentially homogeneous during the course of the experiment, indicating that the erosion process proceeds essentially at the boundary of the gel and fluid phases. The preference of the drug for the interface is consistent with the expectation that accessibility of the drug should be enhanced due to the molecular packing disorder associated with this region [8]. This finding is also in agreement with previous real-time AFM studies of the interaction of proteins with lipid membranes. Milhiet et al. [24,25] reported that when phase-separated SM/DOPC and DPPC/DOPC bilayer systems are incubated with alkaline phosphatase, the proteins insert preferentially at the gel-fluid phase boundary, a behavior that was related to a decrease in the interfacial tension at the phase boundary.

3.3. SM/DOPC and SM/Chl/DOPC bilayers in the absence and presence of azithromycin

Evidence is growing that natural biomembranes contain lipid microdomains (rafts) composed of SM, Chl and glycosphingolipids [26,27]. These structures are thought to play important roles in processes such as cellular signaling and membrane trafficking. The raft hypothesis suggests that the lipids aggregate in the plane of the membrane driven only by distinct intermolecular forces including van der Waals interactions and hydrogen bonding. These interactions would explain the detergent resistance of lipid rafts. SM and Chl are known to preferentially interact and to form domains in the liquid-ordered phase [24]. In this phase, the lipid molecules are tightly packed as in the gel phase but the lateral diffusion is almost as high as in the fluid phase.

This prompted us to compare the behavior of DPPC domains with that of SM and SM/Chl domains. A topographic image of a mixed SM/DOPC (1:1, mol/mol) bilayer

recorded in buffer is shown in Fig. 4A. Two phases were observed, the elevated domains being attributed to SM in the gel phase and the surrounded matrix to DOPC in the fluid phase. The domain geometry (shape, size) was found to be similar to that of the DPPC/DOPC system but the height difference was somewhat smaller, i.e. 0.85 ± 0.03 nm. Comparable observations were recently reported by Milhiet et al. [24]. As shown in Fig. 4B, scanning the surface repeatedly for 60 min did not change significantly the domain morphology. Strikingly, Fig. 4C and D reveals that the morphology of the SM/DOPC bilayer incubated with azithromycin was not perturbed, even after 60 min. This indicates that, as opposed to the DPPC behavior, the organization of SM molecules in the gel phase is not significantly affected by the drug.

As can be seen in Fig. 5A and B, the morphology of mixed SM/Chl/DOPC (1:1:1, mol/mol/mol) bilayers was not too different from that observed in the absence of cholesterol except that the domain size ranged from 3 to 5 μm. We attribute these large domains to SM/Chl in the liquid-ordered phase [24,33]. Scanning the surface for 60 min did not alter the domain morphology, a finding which is in contrast with previous AFM studies [24] and with the notion that cholesterol increases the lipid mobility in liquid-ordered phases. Further work is needed to assess whether the nature and composition of the lipid bilayers and buffer solution used here may account for this discrepancy. Another interesting question is whether the SM/Chl domains, owing to their enhanced lateral diffusion properties, would be more sensitive to azithromycin compared to SM domains. Fig. 5C and D reveals that the domain organization was not altered even after 60 min, indicating that SM/Chl liquid-ordered domains are resistant to the drug.

At first, the higher membrane stability of SM-containing domains compared to DPPC domains may be surprising since both lipids have the same polar headgroups. However, they are known to differ significantly in the other regions [28,34]. In the interfacial region of SM, the amide and hydroxyl groups give the molecule the unique ability to form intra- and intermolecular hydrogen bonding, a property that has been speculated to provide a driving force for lipid raft formation. In comparison, PC through its carbonyl esters can only accept hydrogens. Furthermore, the trans double bond between the C-4 and C-5 atoms of the

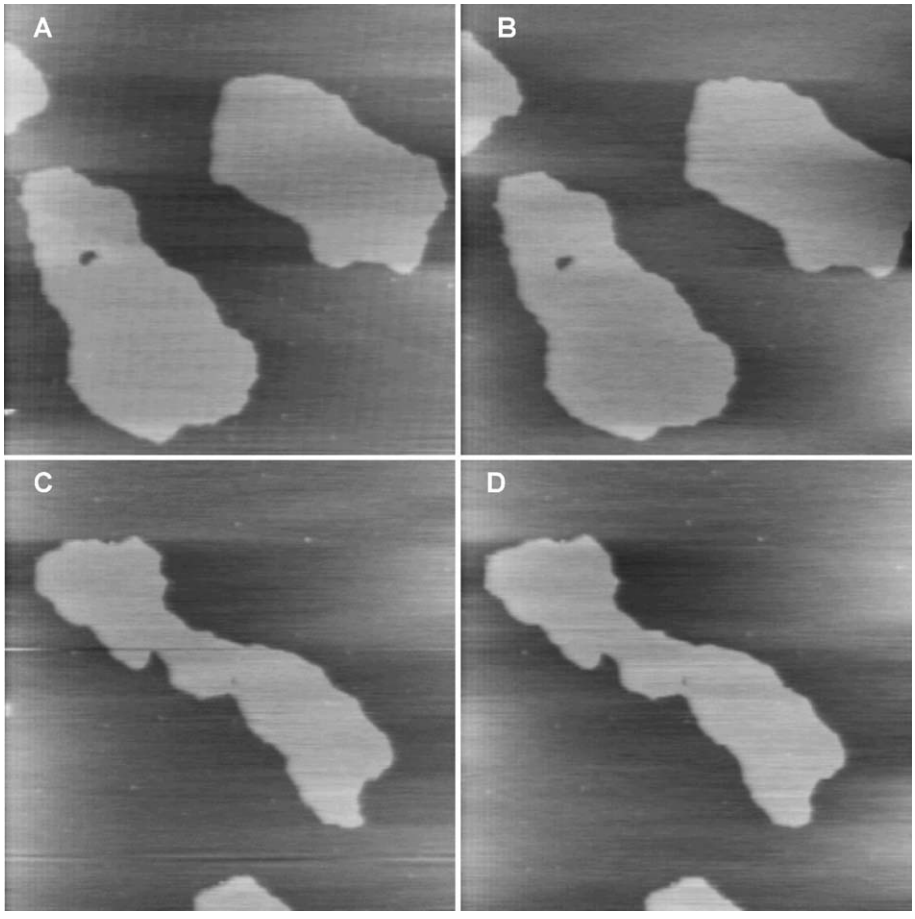

Fig. 5. AFM height images (5×5 μm; *z*-scale : 10 nm) of a mixed SM/Chl/DOPC (1:1:1, mol/mol/mol) bilayer recorded in Tris/NaCl (A, B) and Tris/NaCl/azithromycin (C, D) solutions. (A, C) Images recorded almost immediately after sample mounting. (B, D) Images obtained after scanning the surface for 60 min.

sphingosine moiety has the ability to induce dipoles in the interfacial region. Several important consequences may result from this, including better chain stacking, closer lipid packing, membrane stabilization and lower membrane permeability [34]. We therefore suggest that the higher stability of SM-containing domains reflects tighter lipid packing owing to stronger intermolecular interactions between the lipid molecules.

4. Conclusion

We have shown that real-time AFM imaging is a valuable approach to probe the dynamic interaction of drugs, such as antibiotics, with lipid domains in model membranes. Incubation of DPPC/DOPC bilayers with azithromycin causes a time-dependent erosion and disappearance of the DPPC domains attributed to the disruption of the molecular packing by the drug. By contrast, SM and SM-Chl domains are not modified by azithromycin. In agreement with the literature data, we propose that the higher membrane stability observed for SM-containing domains results from stronger intermolecular interactions between SM molecules. Our results point to the power of real-time AFM imaging for monitoring the interaction of biologically active molecules such as drugs and peptides with lipid membranes.

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