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AFM characterization of solid-supported lipid multilayers prepared by spin-coating

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Abstract

Lipids are the principal components of biologically relevant structures as cellular membranes. They have been the subject of many studies due to their biological relevance and their potential applications. Different techniques, such as Langmuir–Blodgett and vesicle-fusion deposition, are available to deposit ordered lipid films on etched surfaces. Recently, a new technique of lipid film deposition has been proposed in which stacks of a small and well-controlled number of bilayers are prepared on a suitable substrate using a spin-coater.

We studied the morphological properties of multi-layers made of cationic and neutral lipids (DOTAP and DOPC) and mixtures of them using dynamic mode atomic force microscopy (AFM). After adapting and optimizing, the spin-coating technique to deposit lipids on a chemically etched Silicon (1,0,0) substrate, a morphological nanometer-scale characterization of the aforementioned samples has been provided. The AFM study showed that an initial layer of ordered vesicles is formed and, afterward, depending on details of the spin-coating preparation protocol and to the dimension of the silicon substrate, vesicle fusion and structural rearrangements of the lipid layers may occur.

The present data disclose the possibility to control the lipid's structures by acting on spin-coating parameters with promising perspectives for novel applications of lipid films.

Keywords: AFM; Tapping mode; Lipid film; Spin-coating

1. Introduction

Solid supported lipid layers are of practical and scientific interest for many reasons. They are used as model membranes to study protein binding to lipid ligands [1], cell–cell recognition [2], insertion of proteins into membranes [3] and other studies of bio-modeling. Frequently, they are used as substrate to immobilize biomolecules such as DNA or proteins [4,5]. They also provide a method to build many optical and electrical-based biosensors [6] and are expected to be important in the construction of novel biomolecular materials. Many investigations have been focalized on the assembly of these systems with DNA (DNA–lipids complexes or CL–DNA), because of the potential use of these complexes as gene vectors [7,8].

In order to obtain a surface with suitable features, great importance must be devoted to the preparation and characterization of samples. To date, the methods used to prepare these kind of samples are essentially three: Langmuir–Blodgett [9,10], vesicle-fusion [11,12] and direct spreading [13]. The first two allow the deposition of one or two bilayers assuring an ordered and defect-free surface, but the samples are easily damaged and strong interactions with the substrate are present. On the other hand, the direct deposition on the substrate allows the deposition of stacks of hundreds of bilayers, but in this case, only averaged information can be obtained and there is no precise control of the total number of layers deposited.

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Recently, a new deposition technique based on the use of a spin-coater has been proposed [14–16] in order to obtain a well-controlled number of layers on the substrate. With this technique, lipids are dissolved in an organic solvent, spread on the surface and immediately accelerated to rotation. This procedure allows the formation of large-scale ordered samples with a number of bilayers that can variate between 2 and ≈30.

Different techniques are used to characterize the structure, composition and properties of lipid films: fluorescence microscopy allows the study of molecular organization and domain morphology of layers [17]; a good description of the film's fine structure is possible by X-ray reflectometry [18], diffraction and neutron reflectivity [19,20]; Raman and infrared spectroscopy allow to monitor the formation of lipid film and the binding of biomolecules [21,22].

Obviously, a morphological approach is particularly effective in describing the lipid arrangement in term of, for instance, film homogeneity and presence and occurrence of peculiar structure, defects, etc. Thus, a high-resolution surface characterizing technique, such as Atomic Force Microscopy (AFM) [23] can be particularly useful. Among the main advantages of such a non-invasive technique are: the possibility to perform quantitative morphological measurements, the ability to study in real-time and with high spatial resolutions the surface nanostructure of lipids, to directly measure physical properties at nanometer level, to perform measurements in, ideally, any kind of environment and to perturb biological surfaces in a controlled way. In particular, the introduction of the more "gentle" Tapping Mode AFM [24], has allowed the study of samples catalogued as "ultra-soft" and, thus, the demand of applications in the field of lipid films [25] is continuously growing. In spite of this, to date, there have been only two AFM approaches to lipid samples deposited with spin-coating technique on solid supports [15,16]. These works show the possibility, by using spin-coating, to obtain large and defects-free lipid films. Furthermore, they gave a complete description of hydration and dehydration picture of lipid films on mica substrate and optimized the choice of a critical parameter for film deposition, such as the lipid concentration. In this landscape, our AFM morphological study takes advantage of some optimized parameters, such as lipid concentration and amount of deposited solution, and extend the analysis toward other parameters that are presently proved important for the resulting lipid arrangement.

Aims of the present paper are, consequently, two-fold: (i) optimizing the spin-coating technique to obtain, for the first time, ordered lipid films on silicon substrates and (ii) extending the study to the effect, on the produced lipid films, of parameters such as spinning velocity and substrate dimensions.

2. Materials and methods

2.1. Sample preparation

Dioleoylphosphatidylcholine (DOPC or $C_{44}H_{84}NO_8P$, MW=786.1) and 1,2-Dioleoyl-3-Trimethylammonium-Propane (Chloride Salt) (DOTAP or $C_{42}H_{80}NO_4Cl$, MW=698.55) were solved in a 5 mg/ml solution with chloroform and used without further purification.

Silicon (1,0,0) substrates were prepared by chemical etching according to a chemical method [26] based on the alternate action of nitric (HNO_3) and fluoridric (HF) acids. This procedure provides a clean H-terminated silicon surface suitable for interaction with the head-groups of lipids. After this treatment, the substrates are rinsed in water to eliminate traces of acids and placed under vacuum for 15 min to obtain completely dry surfaces. Then, a constant amount (150 μl) of lipid solution are deposited on the etched surface with a Convac spinner model 1001 (Convac Technologies, Sichuan China). The studied samples have been prepared following different spin-coating cycles summarized in Table 1.

2.2. Atomic force microscopy

The AFM measurements were performed using a home built microscope described in detail elsewhere [27,28] and modified to allow operating in Tapping-mode (vertical resolution 2 Å). All images have been obtained in air with Nanodevices (Santa Barbara, CA, USA) Tap150 cantilevers (k=5 N/m, L=125μm, f_{res}=150 kHz) at constant scanning frequency. Experiments have been carried out in controlled environment conditions (e.g., temperature of 22–24°C and relative humidity of 30–35%).

3. Results and discussions

The experiments, as seen in Table 1, can be summarized in three series. At first, we measured DOPC and DOPC–DOTAP mixtures deposited with the same spin-coating

Table 1
Summary of sample preparation: spin-coating cycles and samples label

Lipid species	Step 1 (1 s)	Step 2 (30 s)	Substrate size (mm^2)	Sample order
I series				
DOPC	No	3000 rpm	10×10	1
DOPC–DOTAP	No	3000 rpm	10×10	2
II series				
DOPC	No	3000 rpm	5×5	3
DOPC–DOTAP	No	3000 rpm	5×5	4
III series				
DOPC	500 rpm	3000 rpm	5×5	5

All spin velocity are measured in rpm.

cycles on a square surface of 10×10 mm^2 (samples labeled as 1 and 2 in Table 1). Then, in order to evaluate the influence of the substrate dimension, we deposited the same lipid mixtures on a smaller substrate (5×5 mm^2) leaving unchanged the spin-coating parameters (samples 3 and 4). Finally, the influence on lipid films of the introduction of a boosting step, which is probably the most important parameter of the spin-coating procedure, has been tested by depositing DOPC on the small substrate and introducing a short initial step (1 s–500 rpm). Such a procedure change the balancement of the chemical and physical interaction during the film deposition and the physical effect of the introduction of a boosting step, will be deduced by comparing the samples n. 3 and n. 5.

Fig. 1 shows typical images taken on DOPC sample deposited on a 10×10 mm^2 square surface: there is no evidence of a planar or multi-planar array of lipids which, indeed, are almost completely arranged in vesicle-like structures. Referring to results proposed by Leonenko et al. [29], we performed a statistical analysis of the structure's dimensions to estimate the vesicle's mean radius. The distribution of vesicle's height has been fitted using a Gaussian function: the resulting mean radius of 6 ± 1 nm is consistent with the hypothesis of unilamellar vesicles. Histogram and fitting curve are shown in Fig. 1(c).

The second sample of this series, DOPC–DOTAP (sample 2 in Table 1), shows a similar predominance of vesicle-like structures even though some differences, compared to sample 1, can be observed. For instance Fig. 2, which is representative of the sample, shows a partial planar disposition of lipids that covers a large portion of the measured surface.

These data indicate that when DOTAP is present, the formation of layer is easier than in the case of pure DOPC. Comparing the vesicles formed using DOPC and DOPC–DOTAP, we conclude that in this latter case, they are unilamellar as well.

In the second series of experiments, we changed the substrate area but left the spin-coater parameters unchanged. We considered, at first, DOPC sample (sample 3 in Table 1) that exhibits an apparently astonishing behavior. As shown by the image in Fig. 3, we found a multi-planar disposition of lipids but with a peculiar internal arrangement. In particular (see Fig. 3(a)), the sample surface seems to exhibit a multi-planar array of ordered superposed bilayers (as observed in previous works [15,16]), but a higher resolution image (Fig. 3(b)) clearly demonstrate that planes are not completely ordered. A quantitative analysis of the dimension of the sample's feature allow to propose that the planes are made by semi-fused vesicles because the measure of the surface structures gives a results (40 ± 2Å) that is smaller than the value expected for unilamellar vesicles. By direct comparison with the results obtained with DOPC sample of the first series of experiment, we deduced that lipid disposition, in this latter case, represents an intermediate configuration between completely ordered superposed bilayers and a surface uniformly covered by non-fused unilamellar vesicles.

To obtain completely ordered lipid multilayers, we completed this series of experiments studying a DOPC–DOTAP mixture sample spread on a square surface of 5×5 mm^2 with the same spin-coating treatment. To motivate this choice, we assumed that the role of DOTAP is critical for

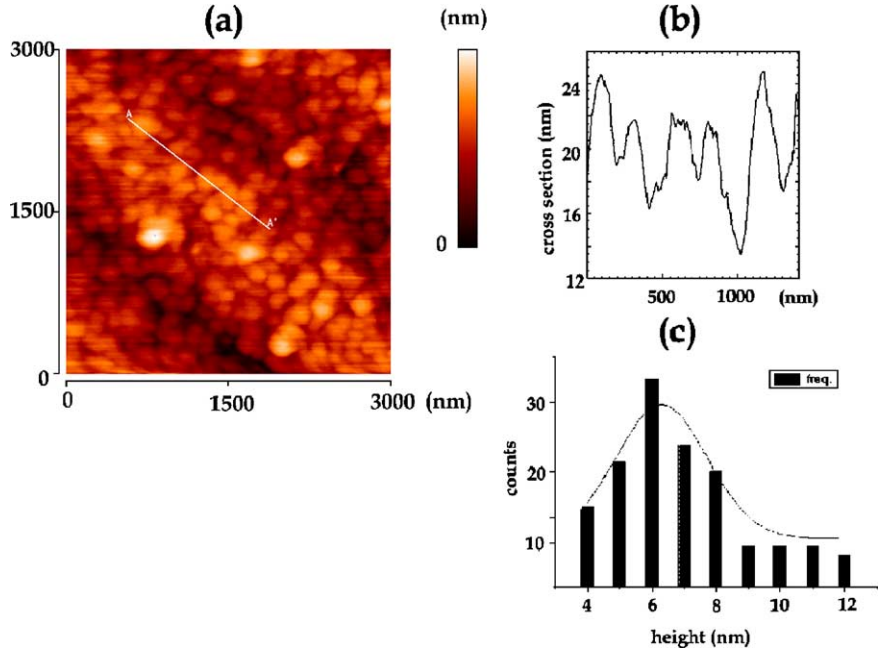

Fig. 1. Images of DOPC deposited on a surface of 10×10 mm^2; (a) topographic image of 3×3 μm^2, 200 points per line; (b) cross-section line between AA′ points; (c) statistical estimate of vesicle dimensions.

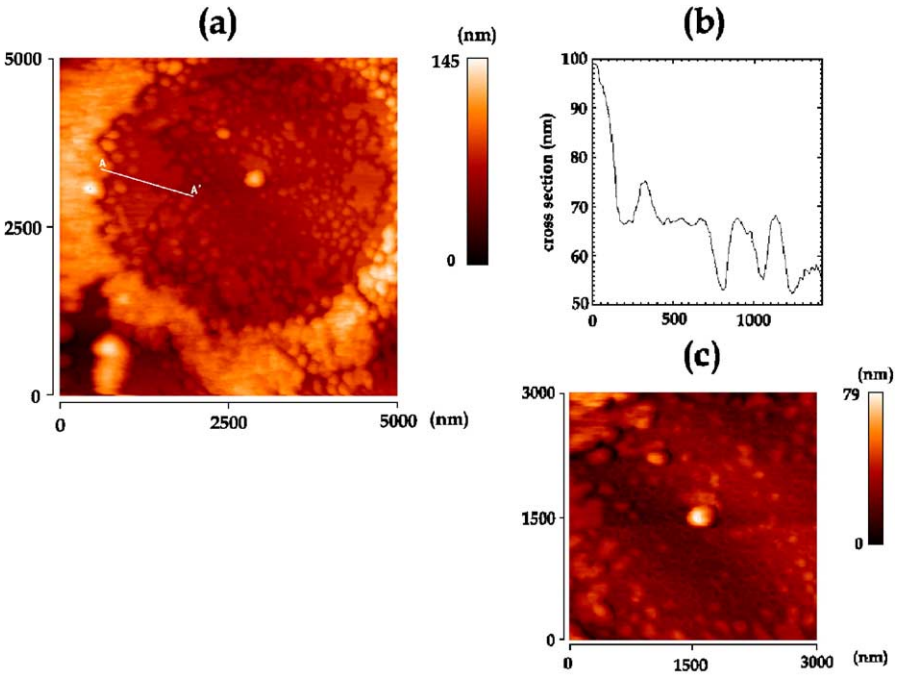

Fig. 2. Images of DOPC–DOTAP with same preparation deposited on a surface of 10×10 mm^2; (a) 5×5 μm^2 image, 300 points per line; (b) AA′ cross-section; (c) higher resolution image, 3×3 μm^2, 300 points per line.

the internal bilayer order because of the presence, in this molecule, of an electrical charged head. We can suppose that electrostatic head–head interactions, already analyzed in case of liposomes in water solution [9], in our system are balanced by other packing factors such as the lipids stack static pressure and the spin-coater action but play an important ordering role. Consequently, during the film formation, DOTAP molecules are forced to assume a planar array under the spin-coating action and to minimize the electrostatic repulsion increasing the head–head distance. Thus, this double action enhances the fusion of lipid vesicles and, differently to the DOPC case in which only

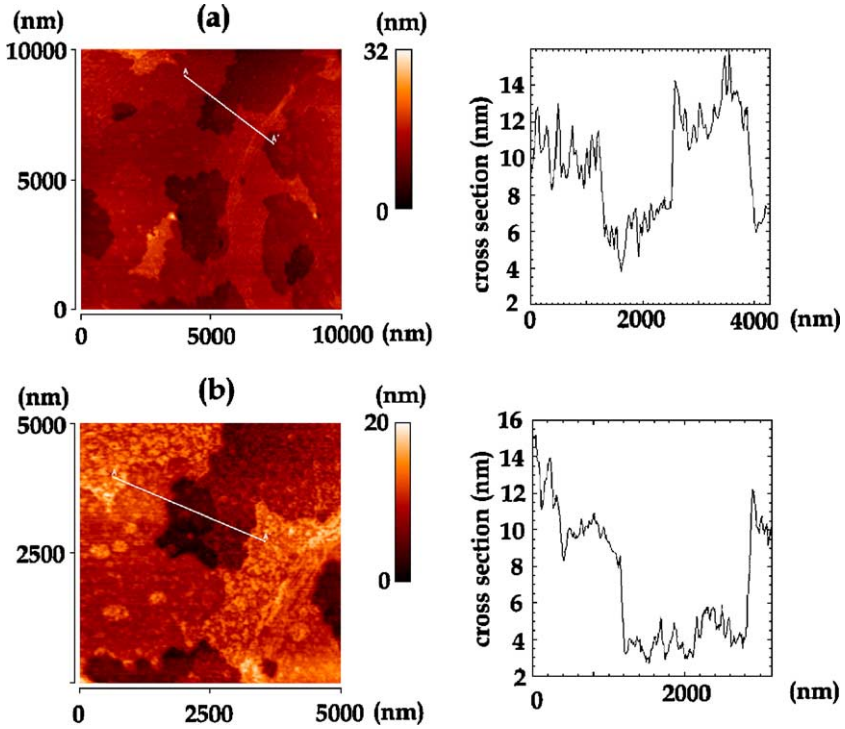

Fig. 3. DOPC sample deposited on a surface of 5×5 mm^2; (a) large scale image, 10×10 μm^2 and 300 points per line; (b) 5×5 μm^2 and 300 points per line. In this case, planes are made by semi-fused vesicles, as stressed in the cross-section.

spin-coating action is present, allows the formation of completely ordered bilayers.

In Fig. 4, typical structures of the investigated sample are shown: micron-sized terraces are overlapped to make a multi-planar ordered surface. A measurement of the terrace heights is provided by the cross-section (see Fig. 4(a)) and is shown to be 100 Å. These structures have already been observed in the past and have been interpreted in terms of the standard thin film dehydration picture [16,30] in which the terraces are formed by solvent evaporation.

As demonstrated in Fig. 4(b), samples obtained in this way show an ordered surface on a large scale (20 μm in this case) without significant defects, in good agreement with precedent results [15]. Nanometer-size defeats with a depth of (100–300 nm) are also present: these "holes" have already been observed and interpreted as effects due to irregular hydration.

Fig. 5 shows a higher resolution image of the same surface to provide a more detailed measure of the terraces' height. As stressed by cross-section and three dimensional view we estimate the plane's height in (100±2)Å. Since DOPC–DOTAP bilayer has been measured to be [7] 39 Å, we can conclude that the present planes are not composed by a single lipid bilayer but rather by a double bilayer.

A comparison between the present sample and DOPC–DOTAP studied in the first series (sample n. 2) allows to associate the strong influence of the substrate area with the order of the lipid's surface. Only reducing the substrate's surface, in fact, we found a dramatic increase in the order of the lipids disposition: we change a surface almost uniformly covered by unilamellar vesicles (see Fig. 2(a)) into a large-ordered multi-planar structures (see Fig. 4).

Moreover, a comparison of the present sample with the characterization of sample 3 (pure DOPC), which has been deposited on the same surface with the same spin-coating cycles, reveals the effects due to different lipids composition. In particular, we found a completely ordered multi-planar array in DOTAP–DOPC, while planes composed by semi-fused vesicles was find in the case of simple DOPC. Such a result confirms the previously cited hypothesis of strong ordering rules due to the presence of DOTAP.

In the last series of experiments, we covered the substrate's surface (5×5 mm^2 area) with DOPC solution while introducing a boosting step in the spin-coating cycles. Results obtained are shown in Fig. 5. The effect of the boosting step on the resulting film features is clear if we analyze the process of lipid deposition. In the other commonly used methods of deposition (i.e., Langmuir–Blodgett and vesicles deposition), the formation of layers is driven by two dominant processes: the hydrophobic effect and the interaction with the substrate. The hydrophobic effects, i.e., the property of amphiphilic molecules to expose the hydrophilic face to the solvent while avoiding direct contact between the hydrophobic one and water, is dominant in aqueous environment and drives to the formation of vesicles. Ordered lipid layers can be obtained depositing a correct amount of vesicles on a reactive substrate, which blocks vesicles and allows their rupture and layers formation.

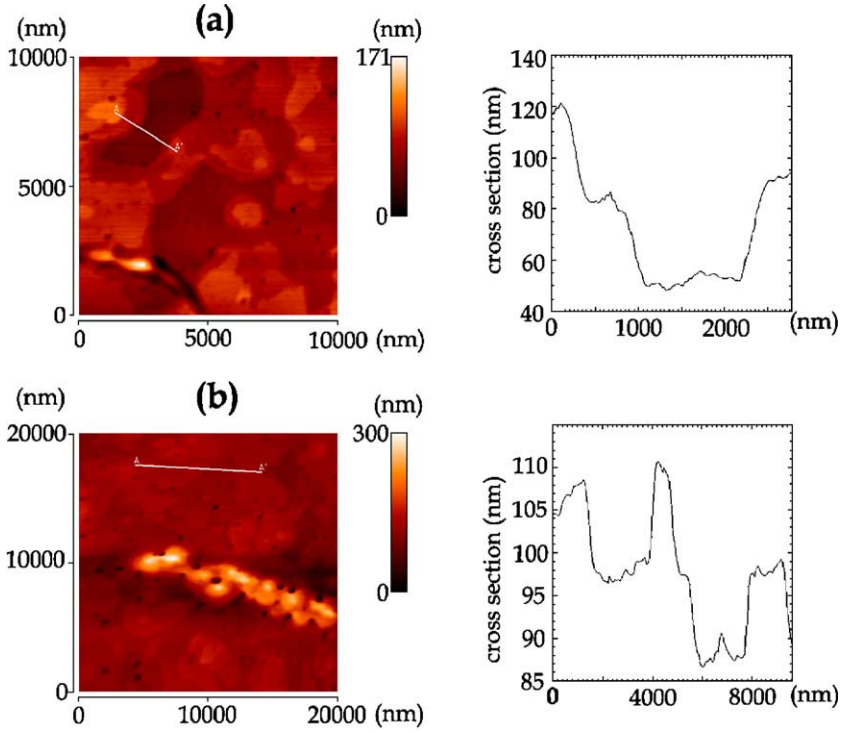

Fig. 4. Images of DOPC–DOTAP sample deposited on a surface of 5×5 mm^2; (a) 10×10 μm^2 and 300 points per line, cross-section showing a terrace profile; (b) large scale image (20×20 μm^2, 300 points per line) showing the global order of surface.

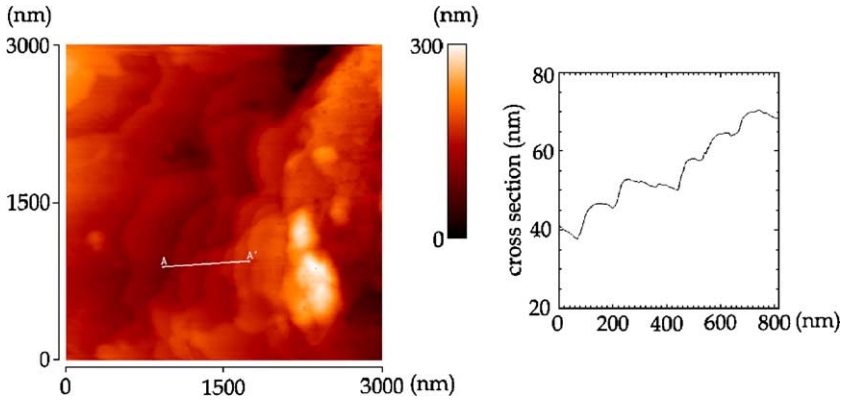

Fig. 5. High resolution image (300 points per line) of DOTAP–DOPC sample: the stack of planes can be measured and AA′ cross line indicate that planes are 100 Å height.

On the other hand, using the spin-coating method there are some differences: an aqueous environment exist only in the first stages of the deposition (because spin-coating action separates solvent and solute) and the interactions with the substrates are relevant only for the lipids close to its surface. Lipids far from substrate surface, in a non-aqueous environment, are not forced to assume a planar ordered disposition, thus, we propose that spin-coating plays, in this case, a critical ordering role and that by varying spin-coating parameters, we can modify the lipid disposition.

This is demonstrated in Fig. 6(a), where a 5×5 μm^2 image of DOPC deposited with a two step spin-coating procedure (referring to Table 1) is shown. The AFM images reveal a semi-ordered disposition of lipids in superposed planes made by unilamellar vesicles. A comparison between the measured vesicle size observed for sample 1 and for the present sample 4 shows that the current vesicles are unilamellar.

In Fig. 6(b), a higher resolution image clarifying the fine structure of lipid film is shown: on this scale the planes show no evidence of substructures other then the vesicles. The planes themselves result to be effectively composed by unilamellar vesicles.

As previously noted, a comparison between samples 3 and 5 allows to deduce the effect of the first spin-coating step introduced in latter case. In principle, the boosting step add a “separating force” which act decreasing the lipid–

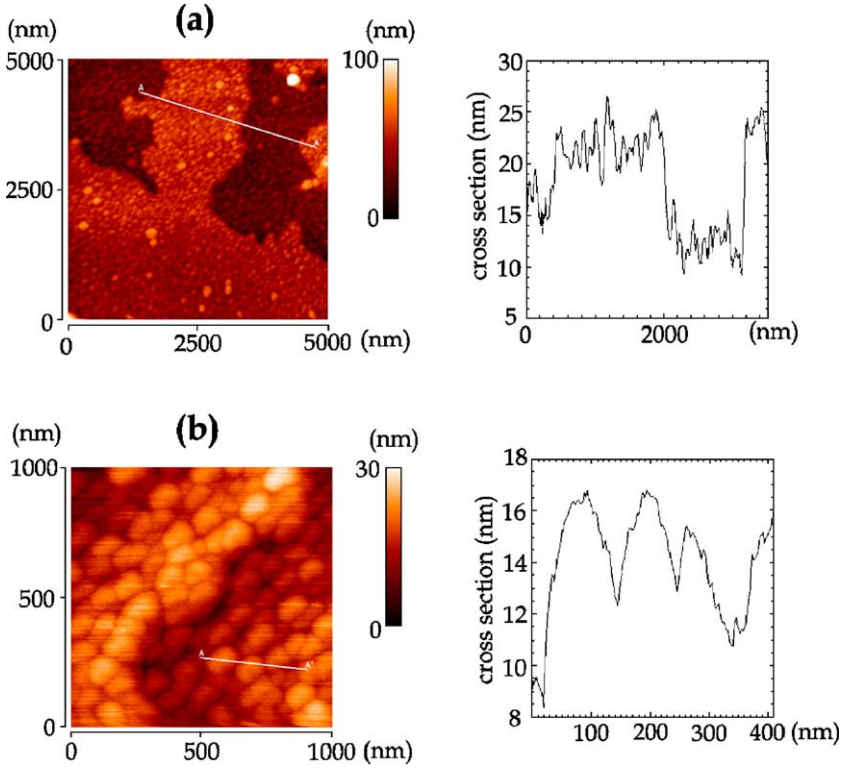

Fig. 6. DOPC sample deposited with a two-step spin-coating preparation. (a) The surface is covered by planes made by unilamellar vesicles superposed (5×5 μm^2, 300 points per line); (b) higher resolution image (1×1 μm^2, 300 points per line).

lipid or lipid–solvent interaction in the first, and probably most critical, instants of the deposition. Thanks to that, when we accelerate the rotation to the second faster step, lipid vesicles are intact and the action of the second step is enough to establish a vertical order between vesicles that compose stacks but, differently from sample 3, is unable to act as a force driving vesicle to fusion.

As a whole, the reported data show the effectiveness of combining a high resolution characterization with the investigation of the structures obtained by using a novel lipid deposition technique, namely spin-coating. Beyond the specific information here reported, the approach based on a punctual characterization of the dependence of the lipid arrangement on the details of preparative method seems very appropriate. Indeed, the present results show the possibility to manipulate the films not only in term of number of bilayers but also in term of the much more important control of the morphological characteristics of the layers (e.g., vesicles, fused vesicle, simple layers, etc.). A circumstance, which demand a more detailed chemical and physical characterization of the observed structure for putative future application like, for instance, the use of planes of vesicle as selective membranes, as traps for specific biomolecules, etc.

4. Conclusions

In the present work, we adapted and optimized a technique to deposit lipids on chemically etched silicon substrates using a spin-coater. The AFM study provided a nanometer scale morphological characterization of the DOTAP and DOTAP–DOPC lipids confirming the general known features and describing the dependence of the observed structures by the details of the chosen preparative method. Varying substrate dimension, we obtained a dramatic change in lipid film features: there is an increase of order in lipid deposition only decreasing substrate surface. Depending on the spin-coater parameters, we can obtain a stack of ordered bilayers superposed, a stack of unilamellar vesicles superposed or semi-ordered planes composed by semi-fused vesicles. Lipid mixture composition have been already considered: we confirm that in presence of DOTAP, the order of lipid bilayers is enhanced thanks to interlayer electrostatic repulsions.

In more general terms, the present results show the possibility to take advantage of the strong sensitivity of the samples from the spin-coating protocol to manipulate, in morphological term, the shape and arrangement of the resulting lipid films.

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