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Study of the surface morphology of a cholesteryl tethering system for lipidic bilayers

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Abstract

The immobilization of functional molecules embedded in lipidic membranes onto inorganic substrates is of great interest for numerous applications in the fields of biosensors and biomaterials. We report on the preparation and the morphological characterization of a tethering system for lipidic bilayers, which is based on cholesteryl derivatives deposited on hydrophilic surfaces by self-assembling and microcontact printing techniques. The investigation of the structural properties of the realized films by atomic, lateral, and surface potential microscopy allowed us to assess the high quality of the realized cholesteryl layers.

Keywords: Cholesteryl derivative; Lipid bilayer; Scanning probe microscopy; Soft lithography

1. Introduction

The functional coupling of lipid bilayers with solid substrates has become a very popular issue in recent years for a wide number of fundamental (from structural biology to biophysics) [1–4] as well as applied (surface modification, biosensor technology) research fields [5–9]. Indeed, solid-supported lipid bilayers, formed by the spontaneous fusion of vesicles floating on an ultrathin (10–20 Å) aqueous film covering hydrophilic surfaces [10], resulted suited for a broad range of biophysical investigations, as they retained many of the properties of free membranes [11,12]. This is mainly due to the lubricating (and somewhat de-coupling) effect of the interface water layer, which not only allows the surface-deposited bilayers to show thermodynamic properties similar to those of a free bilayer [13], but also leads to a significant long-range lateral mobility of the membrane components. This is particularly important for embedding active biomolecules, which require that the membrane is fluid enough to allow the movement into correct orientation of multiple binding sites [10,14,15].

Notwithstanding these advantages, the close proximity of the solid substrate can create several limitations and difficulties. In particular, a significant frictional coupling between the bilayer and the underlying substrate may occur, thus leading to a slowing of the lateral diffusion and to a remarkable defect density, which is a major disadvantage for biosensor applications [16]. In fact, the lipid–substrate interactions and their specific dependence on the separation distance may directly affect the folding and functionality of some proteins, which are suitable for sensor applications. For instance, the immobilization of dopamine neuroreceptors on a solid support can represent an appealing strategy for the realization of sensing elements for dopamine and dopaminergic drugs related to many important pathologies, including schizophrenia, Parkinson's disease, and Huntington's chorea. The preservation of a correct biological activity is therefore dramatically important. Since these biomacromolecules have

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intracellular loops protruding out from the bilayer surface into the adjacent water phase, parts of these functional units could interact strongly with the hydrophilic substrate, thus inhibiting the lipid lateral mobility [17] or leading to a partial loss of functionality, or even to the complete denaturation of the biomolecule [18].

A possible solution to overcome these drawbacks is the introduction of a tethering layer, which guarantees a mechanically and chemically robust attachment of the lipid film to the support, preventing the detachment or the replacement by surface-active elements which can decrease the membrane lifetime, and also acts as a spacer, determining a spatial and functional decoupling between the membrane and the substrate [19]. In particular, the morphology of such tethering layers, essential for the correct attachment of the supported membrane, certainly deserves to be investigated in depth.

Among the possible tethering systems for the lipidic bilayers, cholesteryl derivatives, presenting two cholesterol units attached to a polysulfide terminated diethylene glycol chain [20], are very suitable for providing a certain hydrophylicity in the proximity of the inorganic surface.

In this study, we investigated the morphological and structural properties of this tethering system by scanning probe microscopy (SPM) techniques, in order to test its quality and integrity, employing in particular lateral force microscopy (LFM) as sensitive method for detecting different chemical components or packing densities. In particular, the cholesteryl derivatives were deposited on gold substrates and patterned by microcontact printing (μCP) in order to create textured layers to support lipidic membranes, for the functional reconstitution of dopamine receptors [21,22].

2. Experimental

2.1. Deposition procedure

Au(111)-on-mica substrates (Molecular Imaging, AZ) were annealed by ethanol flame and cleaned by O_2 plasma treatment, in order to remove organic residues on the surfaces. Then they were immersed into a freshly prepared solution (1 mg/mL) of cholesteryl derivatives in methylene chloride for 24 h, rinsed abundantly with the same solvent and dried in a N_2 stream.

2.2. Imprinting procedure

The microcontact printing was carried out according to the scheme of Fig. 1. After producing master patterns by electron beam lithography, elastomeric replicas were realized by composite stamps of hard PDMS (*h*-PDMS) and ordinary PDMS (Sylgard 184, Dow Corning, MI), aiming to increase the pattern stability at μm-scale

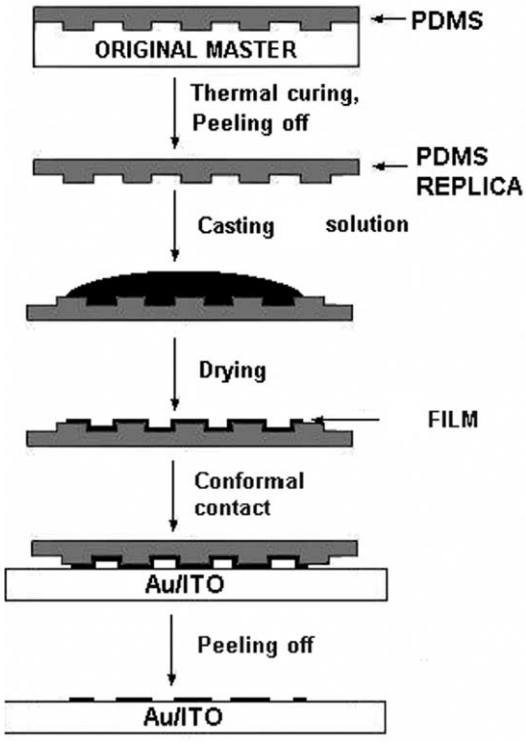

Fig. 1. Schematic diagram of the performed μCP process (features not in scale).

resolution. The *h*-PDMS solution was prepared by mixing trimethylsiloxy-terminated vinylmethylsiloxane-dimethylsiloxane (VDT-731, ABCR, Karlsruhe, Germany), a Pt catalyst (platinum divinyltetramethyldisiloxane, SIP6831.1, ABCR, Karlsruhe, Germany) and a modulator (2,4,6,8 tetramethyltetravinylcyclotetrasiloxane, Sigma Aldrich, St. Louis, MO). We then spin-coated a thin *h*-PDMS layer onto the master surface, and cured it at 60 °C for 30 min. A few drops of a freshly prepared solution of cholesteryl derivatives in absolute ethanol (1 mg/mL) filtered through a 0.2-mm PTFE syringe, were applied to the surface of the PDMS stamps, which were then dried under nitrogen stream until the complete solvent evaporation. Then, the polysulfide-coated elastomeric elements were placed under their own weight onto gold substrates. After peeling-off the replicas, the patterned surfaces were vigorously rinsed several times by ethanol in order to remove the unattached molecules.

2.3. Preparation of biomimetic membrane

Cholesteryl-derivatized substrates were incubated in a freshly prepared solution of liposomes in 50 mM Tris–HCl, pH = 7.4 for 30′ at room temperature and then rinsed several times with the same buffer.

2.4. SPM measurements

Air and fluid tapping-mode AFM (TM-AFM) images were obtained using a Bioscope microscope (Digital Instru-

ments) with a Nanoscope IIIA controller. TESP-NCH silicon probes (Veeco) with a resonant frequency of about 320 kHz and square-pyramidal Si_3N_4 sharpened microlevers (Veeco) were employed.

LFM measurements were performed in air and at room temperature, in contact mode by a Digital Nanoscope microscope (Digital Instruments) equipped with a Nanoscope IIIA controller, integrated with a scanner having a maximum scan size of 12 μm (E scanner) and of 90 μm (J scanner), employing square-pyramidal Si_3N_4 sharpened microlevers (Veeco) with a tip deflection setpoint comprised between −2 and 2 V. During LFM scanning the sample was first scanned at an angle of about 90° with respect to the long axis of the cantilever. The friction signals for both scan directions (trace and retrace) were registered, as actual difference in friction force can only be identified by a contrast reversal for trace (for instance data collected while scanning from left to right) and retrace (right to left). The influence of the scan direction with respect to the printed features on the detected

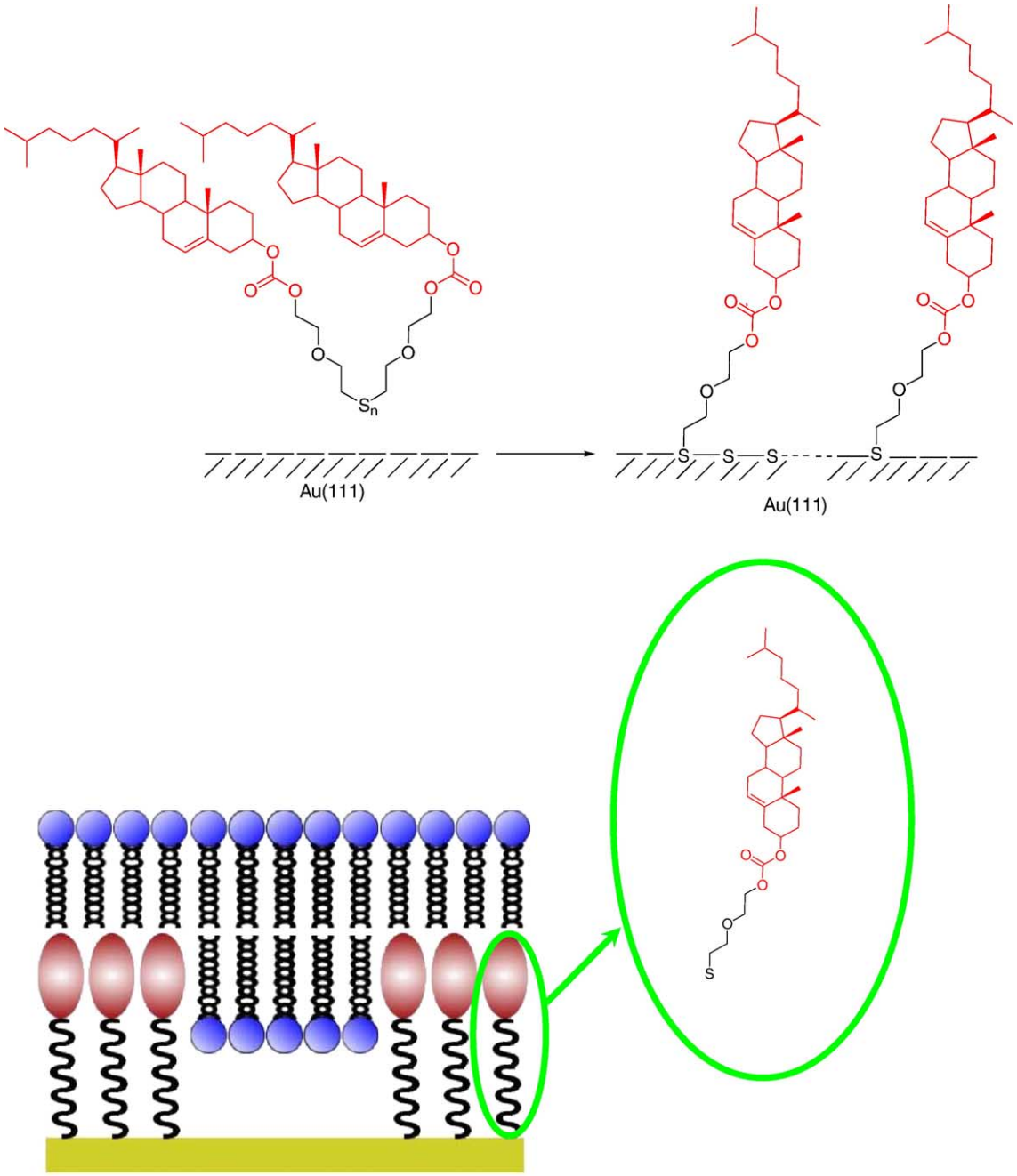

Fig. 2. Molecular structure of the employed tethers, and schematic model of lipid bilayer on μ-patterned tethering molecules (features not in scale).

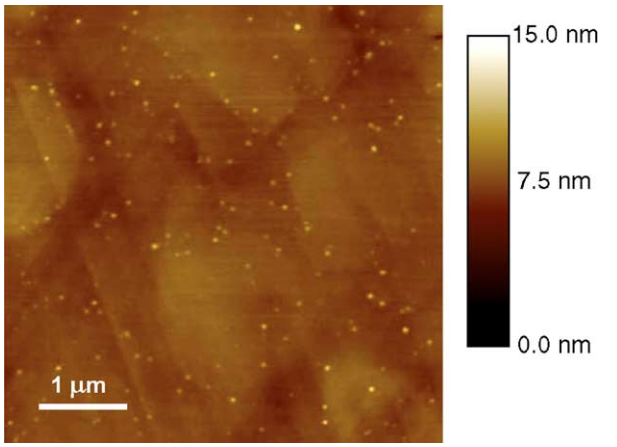

Fig. 3. Air TM-AFM image of Au (111) surface with cholesteryl derivatives. Scan size = 5 × 5 μm^2, scanning frequency = 0.5 Hz.

friction was also investigated, by varying the scan angle up to 180°. EFM and Phase Contrast images were collected in air using a Thermomicroscope Autoprobe CP research and probes with metallic coating (PtIr5) on both sides of the cantilever for enhancing the electrical conductivity of the tip and a spring constant of 3.4 N/m (Nanosensors). The phase contrast image is that defined by tapping mode AFM. Samples were imaged at a bias voltage of 0.5 V, by applying a sinusoidal voltage to the tips. For all the realized experiments, the samples were prepared in many duplicates (≥5), and the reproducibility of the achieved topologies was carefully checked. The reported values of the round-mean-square (rms) roughness and heights were obtained as the averages of at least three independent measurements or images in different regions of the sample.

3. Results and discussion

In aqueous solutions, natural lipid bilayers are arranged in such a way that hydrophilic groups of the lipid molecules

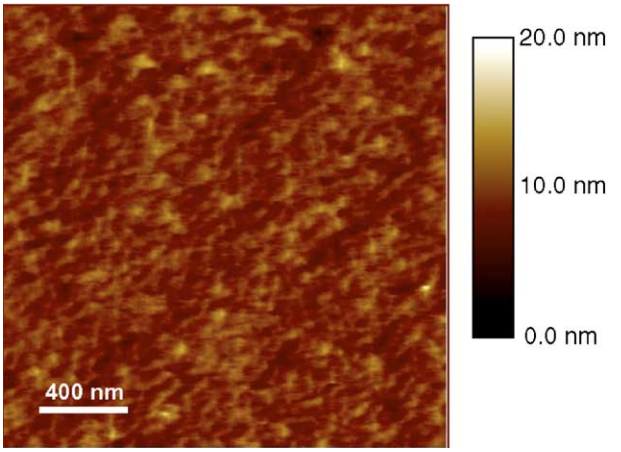

Fig. 4. Fluid TM-AFM image of Au (111) surface with cholesteryl derivatives (in water). Scan size = 2 × 2 μm^2, scanning frequency = 0.4 Hz.

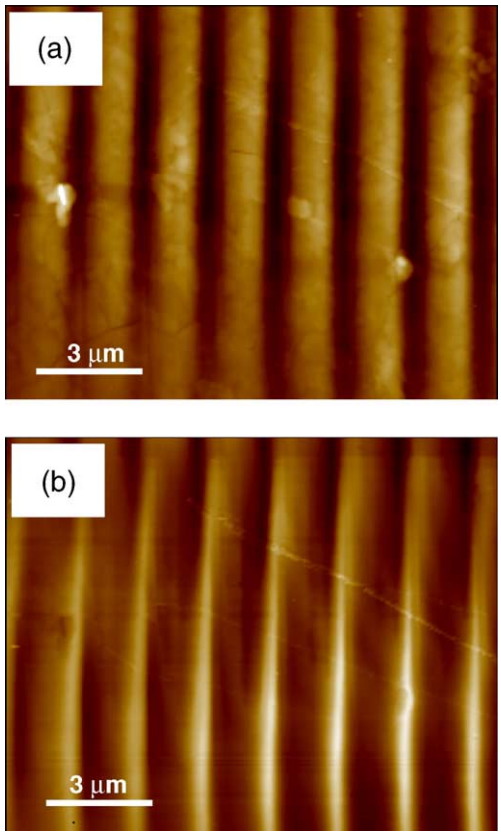

Fig. 5. (a) AFM image of Au (111) surface with patterned cholesteryl derivatives. Scan size = 14 × 14 μm^2 (vertical scale = 50 nm); scanning frequency = 1 Hz. (b) LFM image of the same patterned surface (vertical scale = 1 V).

are oriented toward the outside of the membrane while the more hydrophobic chains form the middle of a lamellar structure. For this reason, the construction of an artificial membrane anchored to a substrate and designed for incorporation of biomolecules requires a stable tether with the function of anchoring it to the solid surface which should be stable in water.

In particular, cholesteryl derivatives, composed by two cholesterol units attached to a polysulfide through a

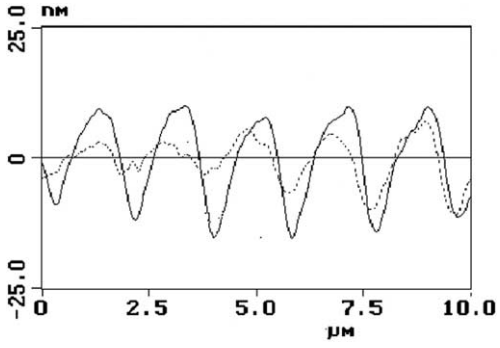

Fig. 6. AFM cross-sectional profiles showing the height of the patterned sample in the two different regions: “A” (continuous line) and “B” (dotted line), respectively.

diethylene glycol chain, exhibit suitable terminations for anchoring phospholipids on gold substrate, thus allowing the formation of biomimetic immobilized bilayers and retaining a hydrophilic environment on solid support (Fig. 2) [23,24].

A typical air TM-AFM image of layers of the cholesteryl derivative self-assembled onto gold surfaces is shown in Fig. 3. Some bumps (with a characteristic density of 5×10^6 $1 \times 10^7/cm^2$) are present in the deposited layers, exhibiting an average height of about 6 nm. This is attributable to the formation of molecular aggregates, likely induced by the interactions between the alkyl chains ending of cholesteryl moiety [25]. The resulting surface morphology, obtained by imaging the sample in bidistilled water (namely in the hydrated state), is quite different (Fig. 4). In this case, the shape and the relative height of the observed features suggest that the tethering molecules significantly swell because of the aqueous environment, as found also on Langmuir–Blodgett phospholipid monolayers [26]. In particular, water molecules are strongly attracted by the grafted polyethyleneglycol-like terminations, which can provide a suitable liquid region inserted between the solid surface and the standing membrane. Indeed, since dopamine receptors contain seven transmembrane domains, which form a narrow hydrophobic cleft surrounded by three extracellular and three intracellular loops [27], this interposing liquid layer is very desirable, as it allows the hydrophilic loops to be easily inserted and settled within the membrane.

To further favor the correct insertion and folding of transmembrane receptor, a patterning of the cholesteryl molecules layer could be required and microcontact printing (μCP) techniques was used for this purpose. The control of the surface geometry in terms of size, shape, and distribution of bilayer areas allows the confinement of the incorporated transmembrane proteins within the bilayer regions in which they are adsorbed (i.e., to have a controlled restriction of their lateral motion) [28]. In Fig. 5a, we report the AFM image of the patterned disulfides onto gold surface, showing periodic stripes of thiolipids having a width of 2 μm (faithfully reproducing the master features) and a height of about 20 nm, indicating the presence of several packed lipid molecules in a multilayer structure. The same surface morphology as within the printed features, with a transferred multilayer of rms roughness of about 1.5 nm, was observed in unpatterned thiolipid regions, obtained by microcontact printing with flat elastomeric stamps.

In order to assess in depth the degree of uniformity and the quality of the printed patterns, we performed a

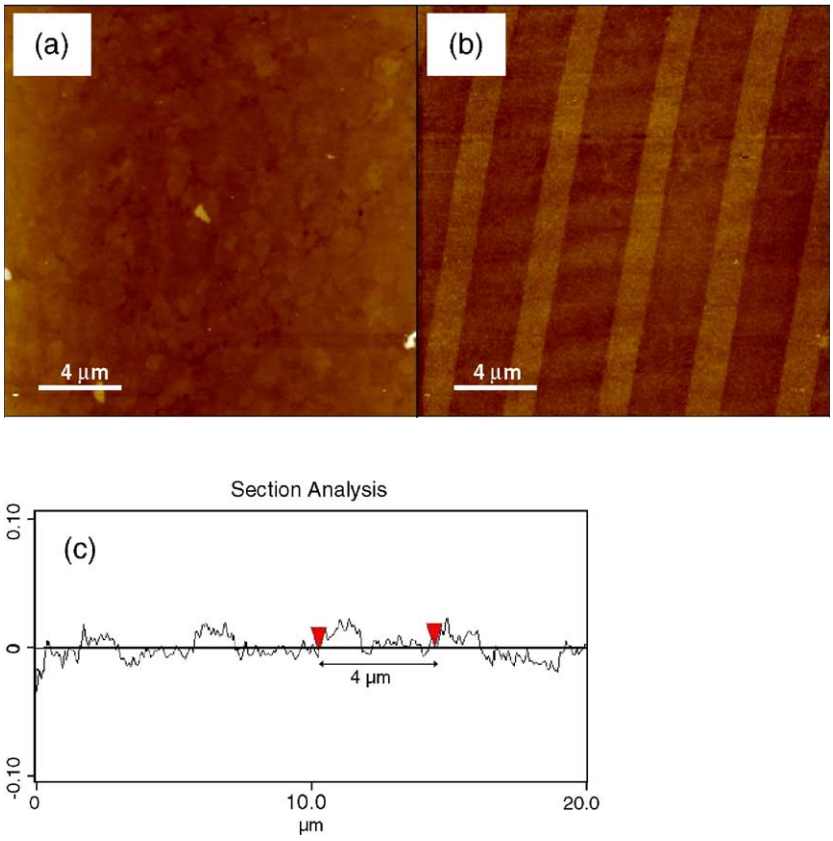

Fig. 7. (a) AFM image of Au (111) surface with patterned cholesteryl derivatives (optimized procedure). Scan size = 20 × 20 μm^2 (vertical scale = 35 nm); scanning frequency = 1 Hz. (b) LFM image of the same patterned surface (vertical scale = 0.2 V). (c) LFM cross-sectional profiles showing the periodicity of the patterned sample.

LFM investigation, aiming at identifying and mapping the different sample regions in terms of frictional forces between the probe tip and the surface (brighter LFM regions correspond to higher frictional interactions). The LFM allows one to reach an unequalled sensitivity to the water layer adsorbed on surfaces under ambient conditions onto hydrophilic surfaces [29]. In our study, we employed untreated silicon nitride tips, which present a well-established hydrophilicity (water contact angle <30°) [30]. Consequently, hydrophilic areas should present a higher interaction (friction) with these hydrophilic tips, with respect to the hydrophobic [20] printed cholesteryl stripes. This effect should result in bright LFM regions on the uncovered, hydrophilic gold areas [31], and dark LFM regions on the printed hydrophobic features. However, our study highlighted that in some regions the friction forces felt over the thiolipid stripes are quite higher than those measured over the surrounding gold areas (see for instance the top part of Fig. 5b). In particular, comparing the top ("A") and the bottom ("B") regions of Fig. 5b, a change in the image contrast is evident, leading to a clear distinction between the supramolecular organization of the printed molecules in "A" and "B". In the first area, the probe tip undergoes higher friction on the cholesteryl stripes, whereas in second region the larger force is exerted by the bare gold (as expected).

This observation indicates that the frictional properties of surfaces do not depend only on the chemical nature of the molecular compounds (although these do determine the surface energy), but also on the chemical order, suggesting that the amorphous structural organization of the deposited layer can determine strong friction, and therefore it has to be taken into account [32,33]. In particular, we note that the region "A" corresponds to a higher cross-sectional profile in the topographical image with respect to region "B" (Fig. 6), suggesting that a different degree of disorder and/or different packing densities are exhibited by different regions of chemically identical molecules [31,33–35].

Many different mechanisms can contribute to friction force, including packing energy, packing density, elasticity and local disorder [36]. In fact, the observed difference in frictional forces dependence can be explained by a higher degree of disorder in the region "A". In the formation of the layers during printing, the molecule–substrate and molecule–molecule interactions both play an important role, determining the quality of the resulting films. A more

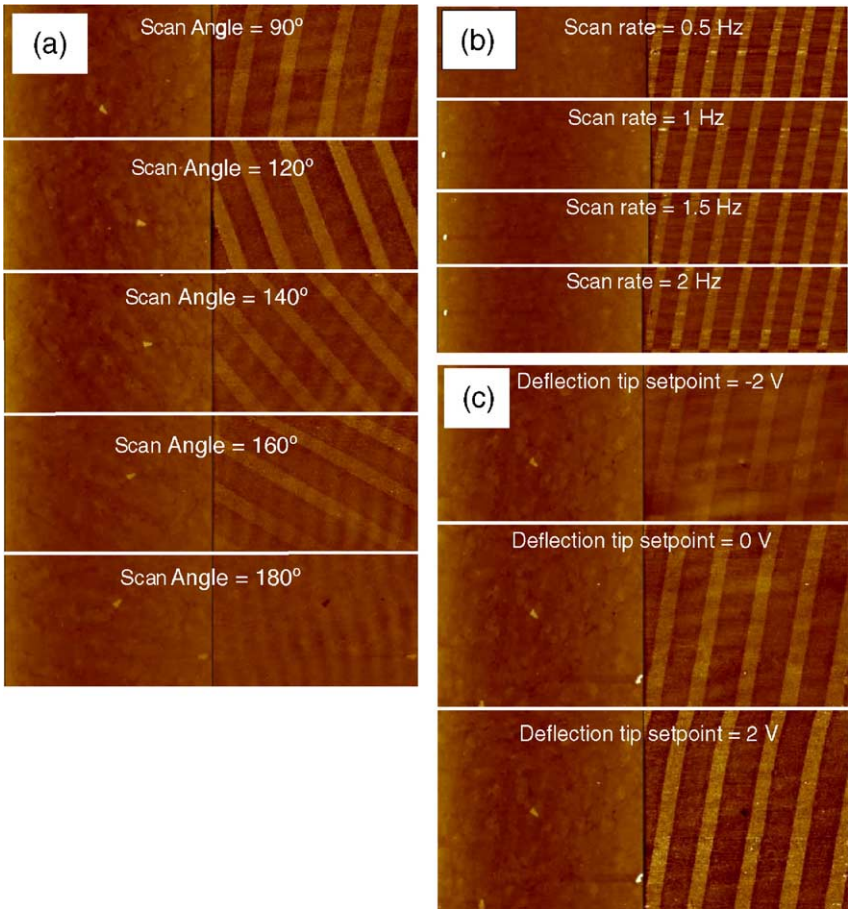

Fig. 8. AFM and LFM images of Au (111) surface with patterned cholesteryl derivatives (optimized procedure), for various scanning angles (series a), scan rates (b), and tip deflection setpoints (c). The printed features have a period of 4 μm, vertical scale = 35 nm for AFM images and 0.2 V for LFM images.

amorphous organization may favor the occurrence of numerous defects, providing many excitation modes to efficiently absorb energy and thus giving rise to a high value of friction [32,33].

In addition, different packing energies could mainly arise from the hydrophobic attraction between the alkyl chains ending of cholesteryl moiety [24]. This behavior, commonly observed in supported lipidic layers, is enhanced in more ordered molecular layers, in which the alkyl chains undergo a higher degree of alignment. Due to such interaction, a higher packing density can result in the region "B", hence a higher penetration barrier to the probe, resulting in a lower measured friction [35].

This study enlightens that very careful optimization is needed for achieving uniform printed biomolecular layers

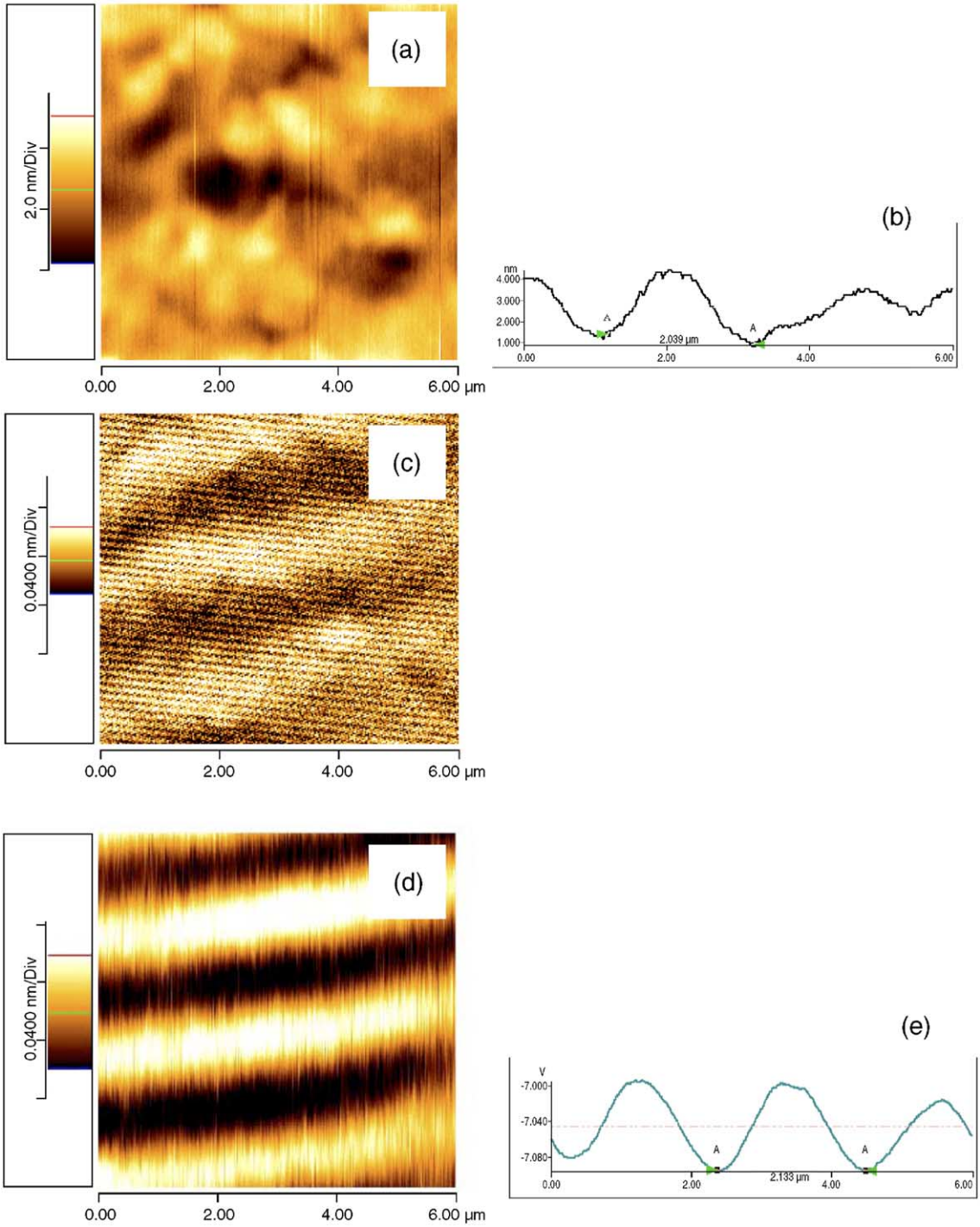

Fig. 9. (a) AFM image of Au (111) surface with patterned cholesteryl derivatives (solution concentration 0.1 mg/mL). Scan size = 6 × 6 μm^2, scanning frequency = 1 Hz. (b) AFM cross-sectional profile. (c) Corresponding phase-contrast and EFM (d) image. (e): EFM cross-sectional profile.

by μCP, especially by strongly interacting compounds. For our cholesteryl derivatives, more diluted solutions (0.1 mg/mL in filtered absolute ethanol) were employed to avoid the presence of conglomerates within the printed stripes, and to achieve more ordered and less thick molecular layers.

Fig. 7a and b show the AFM topography and friction images of an optimized patterned sample. The well-defined LFM features clearly reproduce the printed cholesteryl derivative stripes, with very sharp boundaries and a periodicity of 4 μm (Fig. 7c). For the optimized monolayer the untreated silicon nitride tip experienced higher friction in the gold regions than in areas with cholesteryl derivatives groups, thus indicating a good degree of order in the printed hydrophobic layers. In addition, we checked the dependence of the friction force on the scan angle, the scan rate, and the tip deflection setpoint, finding that higher frictions are detected by normal scans, slow scan rates, and positive deflection setpoints (Fig. 8).

Simultaneously acquired topographical, phase-contrast and surface potential microscopy images of an optimized patterned surface are displayed in Fig. 9a, c, and d, respectively. The vertical scale in the AFM image is comparable with the expected dimension of cholesteryl derivatives monolayers, which gives rise to a poor morphological contrast with respect to the underlying substrate. Other patterned regions of the same sample exhibit similar topological profiles. However, we point out that such poorly defined surface contrast is not due to uncomplete or unfaithful pattern transfer, as clearly demonstrated by Figs. 7 and 8.

Hence, the patterned features can be clearly identified only by a comparison with Fig. 9c and d. The phase image, being sensitive to the chemical composition of the surface groups by measuring the phase (simultaneously with the amplitude) of the cantilever oscillation relative to the driving signal during scan [37], provides the initial evidence of the formation of the cholesteryl derivatives patterned monolayers on the gold surface. In addition, the achievable brightness contrast achievable by EFM is directly related to the surface chemical composition and terminal moieties [38–40], therefore this approach reveals very useful for assessing the chemical contrast achieved by printing-like soft lithographies. The observable contrast in our case is indeed enhanced by surface potential microscopy (Fig. 9d). The pattern is quite regular with darker region corresponding to a stronger tip-sample interaction. The surface potential profile clearly shows stripes having a lateral dimension of 2 μm, and a signal height of 90 mV (Fig. 9e).

Finally, in order to directly assess the suitability of our tethering systems for supporting lipid bilayers, we investigated the surface morphology of self-assembled membranes both on top of, and in the absence, of our tethers. The attaching mechanism of the bilayers (generally formed in aqueous media by unilamellar vesicles, see [19–26] and references therein) to the tethering system can be justified by considering that the cholesteryl residues insert into the bilayer by substituting a lipid molecule (Fig. 2). The lipid bilayer is generally formed in aqueous media by unilamellar vesicles. The height of the tethering molecules, being larger than that of other systems already employed for supporting biomimetic membranes [31], certainly provides the space needed for the membrane protein dynamics.

A typical topology of the membrane upon fusion on the cholesteryl derivatives optimized patterns is displayed in Fig. 10b, showing a uniform and smooth surface with a rms roughness as low as (1.4 ± 0.1) nm (on the top of the thiolipid features), whereas on bare gold substrates (Fig. 10a) we found a value of (1.8 ± 0.2) nm. In addition, we found a maximum height contrast of 1.7 nm between the membrane regions on the printed stripes (i.e., with the bilayer on top of the cholesteryl molecule features), and the unprinted features (i.e., with the standing bilayer). These data, together with the vertical size of the tethering monolayer (≅3.5–4 nm), and the thickness of the assembled lipid bilayer (≅5 nm), confirms the very good degree of conformability of the achieved membrane to the

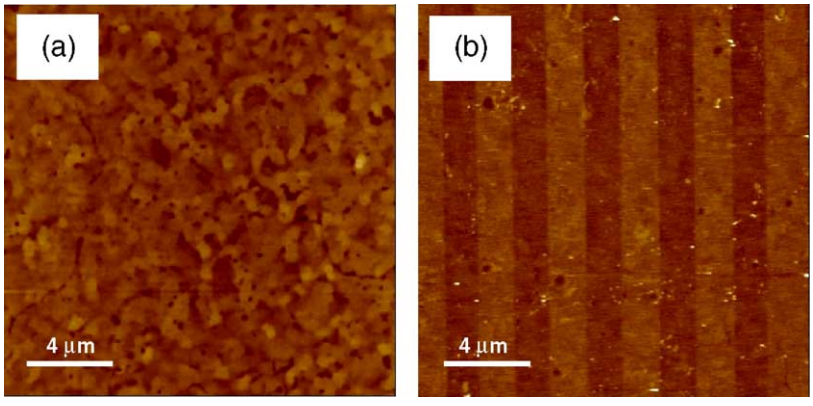

Fig. 10. Fluid TM-AFM image of Au (111) surface with lipid bilayer formed in absence (a) and on the top of patterned cholesteryl derivatives (b) (in water). Scan size = 20 × 20 μm^2, vertical scale = 35 nm, scanning frequency = 1.19 Hz for (a) and 1.5 Hz for (b).

underlying profile. In addition, one can easily obtain the bottom room required to confine transmembrane proteins within the lithographically printed tethering domains. Different geometries are currently being tested, in order to anchor the membrane by a more confining geometry, namely wells (≅μm^2) surrounded by larger printed cholesteryl regions, able to support the lipid bilayer and decreasing its conformability to the underlying height profile [31].

4. Conclusion

Lipid bilayers are very important, since they are the natural environment of membrane proteins and relevant supports in biosensor technology. In this work, we investigated deposited and patterned cholesteryl derivatives by SPM, aiming at achieving high-quality tethering layers. In particular, the AFM morphological analysis is not sufficient to characterize in depth the supramolecular organization of the printed molecules. LFM allowed instead to directly studying the different properties of the realized layers, in which can give rise to multilayer structures exhibiting different packing densities and disorder degrees, thus leading to significant surface defects. The careful optimization of the process of formation of cholesteryl molecules monolayers, allowed us to unambiguously visualize the fine structure of the tethering monolayers, by phase-contrast and surface potential microscopy. This work confirms that chemical-sensitive SPM are well suited for the investigation of high-quality tethering monolayers.

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