Phase Behavior and Structure Properties of Supported Lipid Monolayers and Bilayers in Interaction with Silica Nanoparticles

Ndeye Rokhaya Faye, Ibtissem Gammoudi, Fabien Moroté, Christine Grauby-Heywang, and Touria Cohen-Bouhacina

Abstract—In this study we investigate silica nanoparticle (SiO$_2$-NP) effects on the structure and phase properties of supported lipid monolayers and bilayers, coupling surface pressure measurements, fluorescence microscopy and atomic force microscopy. SiO$_2$-NPs typically in size range of 10nm to 100 nm in diameter are tested. Our results suggest first that lipid molecules organization depends to their nature. Secondly, lipid molecules in the vicinity of big aggregates nanoparticles organize in liquid condensed phase whereas small aggregates are localized in both fluid liquid-expanded (LE) and liquid-condensed (LC). We demonstrated also by atomic force microscopy that by measuring friction forces it is possible to get information as if nanoparticle aggregates are recovered or not by lipid monolayers and bilayers.

Keywords—Atomic force microscopy, fluorescence microscopy, Langmuir films, silica nanoparticles, supported membrane models.

I. INTRODUCTION

Nanoparticles are widespread used in our environment, since a lot of products containing them are available in various technological fields, (paints, cosmetics, clothing,...). These last years, an increasing interest has been motivated by their physico-chemical properties differing from those of the same materials in bulk. However, an important question concerns the potential toxic effects of these NPs in cells, since it has been shown that they are able to cross the cellular membrane and to interact with intracellular biological compounds [1]. A possible mechanism of their toxicity would lie in the interaction of NPs with membranes, since recent papers demonstrate clearly that NPs are able to induce nanoscale holes in membranes and their thinning or disruption [2], [3]. Some studies suggest that various parameters such as the size, shape, surface chemistry, composition, and aggregation state of NPs would be determining in NP-membrane interaction [2], [4], [5], [6], [7].

To date, majority of nanotoxicity studies were focused on cell cultures or in vivo systems [8], [9], [10]. However, because of the structural complexity and the large wide of cellular processes which take place in cell membranes, in vivo studies of these interactions are often difficult to assess. In particular, it is well-accepted now that membrane lipids are not randomly distributed in membranes: some of them are rather concentrated in domains called rafts, which are probably involved in different cellular processes, whereas other lipids are present in the fluid phase of membranes [11]. It explains that in recent years, new approaches have been developed to study the NP-membrane interaction by using models of biological membranes, mimicking their bilayer structure, but limited to only a few lipids in composition. Among these models, two-dimensional ones are particularly interesting, such as lipid monolayers and bilayers deposited on planar supports, usually built by Langmuir-Blodgett (LB) and Langmuir-Schaeffer (LS) transfer or vesicle spreading [12], [13], [14], [15]. Indeed they are simple to prepare and it is possible to access the lateral packing of lipid molecules (and thus the lipid phase) in the case of a LB-LS transfer by controlling the surface pressure in the monolayer during its transfer. At last, these models are particularly well-adapted to surface-sensitive physical techniques, such as atomic force microscopy (AFM) and fluorescence microscopy (FM) [16]. These two methods enable to characterize the morphology of lipid monolayers and bilayers at different lateral resolutions, in the micrometer range for FM and in the nanometer range for AFM. Moreover, AFM does not require the presence of a fluorescent probe, and offers the opportunity to study the topography of the samples by determining height profile with a sub-nanometer range. At last, this technique offers the opportunity to probe locally the mechanical properties of soft matter with less damage[17], [18], [19], [20], [21], [22].

In our case, we applied these methods to the study of lipid planar monolayers and bilayers deposited on nude mica
surfaces and mica previously nanostructured by silica NPs (SiO$_2$-NPs), in order to mimic the NPs-membrane interaction. Different lipids characterized by different transition temperatures ($T_m$) were tested. The $T_m$ value, depending on different parameters such as the length of lipid chains and their degree of unsaturation, defines the lipid phase at the experimental temperature $T$: roughly lipids are in a disordered liquid expanded (LE) phase if $T>T_m$ whereas they are in an ordered condensed (LC) phase in the opposite case. In this work, we describe results obtained with three different lipids: POPC, an unsaturated lipid in the liquid expanded phase at ambient temperature ($T_m$=2°C); DMPC, a saturated lipid with an ambient transition temperature ($T_m$=23°C); SM, a lipid characterized by a high $T_m$ ($T_m$=41°C) and preferentially concentrated in raft. These lipids organized in monolayer or bilayer were deposited on mica surfaces previously nanostructured with SiO$_2$-NPs of various diameters from 10 to 100 nm, thus giving different local curvatures. Understanding the effects of surface defects and patterns on supported lipid monolayers or bilayers is a valuable step to assess information about NP-membrane interaction.

II. MATERIALS AND METHODS

Lipids (Fig. 1) were purchased from Avanti Polar Lipids (USA): N-palmitoyl-D-erythro-sphingosylphosphorylcholine (SM), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphatidylcholine (POPC). They were >99% pure and used without further purification. The fluorescent probe (2-(12-(7-nitrobenz-2-oxa-1,3-diazol-4-yl-amino)dodecanoyl-1-hexadecanoyl-sn-glycero-phosphocholine) (NBD-PC) was purchased from Molecular Probes (USA). Ethanol and chloroform used to solubilize lipids and the probe were both HPLC grade and purchased from Fluka and Sigma, respectively. Tris buffer and salts were purchased from Sigma. Millipore water (pH 5.5, resistivity $>18.2$ MΩ·cm) was used as subphase for surface pressure measurements and buffer preparation.

Freshly cleaved ruby muscovite mica (Electron Microscopy Sciences) was used as solid support for Langmuir-Blodgett transfers. SiO$_2$-NPs (10 nm, 50 nm and 100 nm in diameter) were purchased from Biovalley (Marne-la-Vallée, France). The other SiO$_2$-NPs at 4 nm and 20 nm in diameter were purchased from Alfa Aesar (Schiltigheim, France). QCM-D sensor crystal (AT-cut, 5 MHz) with gold electrodes and thin upper layer of silicon oxide (30nm) were purchased from Lot-Oriel (Massy-France).

NP suspensions were first cleaned by three cycles of centrifugation, NPs being dispersed in ultra pure water after each centrifugation to ensure to remove surfactant impurity from the final colloidal suspensions. After their dilution at a final concentration of about 0.1mg/mL, 5µL were deposited on freshly cleaved mica following two methods: in the first one, samples were simply dried, whereas in the second one, the spreading of NPs on the surface was favored by spin-coating (300 rpm, 1 minute). In all cases, these nanostructured surfaces were dried under a desiccator overnight at ambient temperature, before being used for lipid monolayers and bilayers deposition. Consequently, the main part of NPs was washed from the surface, but a sufficient amount remained on the surface, as shown by AFM experiments performed at different steps of the procedure (data not shown).

B. Surface Pressure Measurements and LB Transfer

Surface pressure experiments were carried out in air, at a temperature of 20±1°C, using a Langmuir trough (KSV NIMA, Finland) equipped with a Wilhelmy balance. Π-A isotherms were obtained by measuring the surface pressure Π ($Π = γ_w - γ$, where $γ_w$ and $γ$ are the surface tensions of water in the absence and in the presence of lipids, respectively) as a function of the mean molecular area A occupied by each lipid molecule at each step of the monolayer compression (Fig. 2). Briefly, lipids were dissolved in a 1/1 (v/v) mixture of ethanol and chloroform at a concentration of 1mM. Nude or nanostructured mica was immersed in water, perpendicular to the interface. Lipid solution was spread at the air-water interface using a Hamilton syringe. After evaporation of solvents (15 minutes), lipids were compressed continuously at a rate of 5 cm$^2$.min$^{-1}$, until a surface pressure of 30 mN.m$^{-1}$, this value being in the estimated range of membrane surface pressures. The surface pressure was then kept constant thanks to a control system adjusting continuously the surface occupied by the lipid monolayer. After the stabilization of the monolayer (a few minutes), the mica surface was removed.
from the subphase at a rate of 2 mm.min\(^{-1}\) by using an automatic dipper from NIMA (Fig. 2).

![Diagram](image)

**Fig. 2** Langmuir-Blodgett principle. (B) Theoretical isotherm (II-A) obtained by compressing an insoluble lipid monolayer formed at an air / water (or buffer) interface

The LB film was then kept protected from dust under a desiccator until its imaging by AFM and FM. It is also possible to transfer a second layer, in order to have a bilayer. In this case, the mica surface with the first monolayer is placed parallel to the air-water interface and dipped in order to put it in contact with the monolayer spread at the interface. The second layer is transferred on the first one, forming a bilayer in contact with water. At least three LB films or bilayers were prepared under the same conditions to ensure reproducibility.

### C. Unilamellar DMPC Vesicle Preparation

Unilamellar DMPC vesicles were prepared using the film re-hydration and extrusion methods. Briefly, an appropriate mass of lipids was dissolved in chloroform in a round bottom flask. A thin film of lipids was obtained after evaporating the solvent by rotating the flask for at least 3 hours. The lipid film was then hydrated in Tris-Buffer (10mM Tris, 150mM NaCl, 2mM CaCl\(_2\), pH 7.6) at a temperature of 40\(^\circ\)C, thus above the T\(_m\) of DMPC, with a final lipid concentration of 5 mg/mL. At this step, multilamellar vesicles were obtained. To obtain unilamellar vesicles, the solution was vortexed, then extruded 15 times at 40\(^\circ\)C through a polycarbonate membrane (pore diameter of 200 nm) (Avanti Polar Lipids Inc). Unilamellar vesicle solution was immediately stored at 4\(^\circ\)C and used within 2 weeks. Vesicle size was characterized using dynamic light scattering (ALV-7000, Germany). The measured average hydrodynamic diameter of DMPC unilamellar vesicles was 208 ± 6 nm.

### D. QCM-D Monitoring

The QCM-D principle is based on real time measurement of the frequency of piezoelectric quartz crystal oscillator sensor on which a thin layer of (soft) material is deposited. The variation of the frequency is directly proportional to the change in mass (due to molecules adsorbed on the surface) and to the density and viscosity of the liquid in contact with the quartz [14]. We applied this method to the characterization of DMPC bilayers obtained by vesicle spreading. QCM-D sensor crystal (AT-cut quartz) was rinsed in water and ethanol successively and exposed to UV-ozone for 10 min. This process was repeated twice, and to make sure to remove all traces of water a last cleaning step was done by rinsing the crystal in iso-propanol, then exposing it to UV-ozone for 30 min. All QCM-D measurements were made at the first overtone (\(n = 1\), 5MHz) using SiO\(_2\)-coating quartz crystal (Lot Quantum Design, France), a commercial liquid cell (QCM 500W-KSV) and a homemade experimental setup. First TRIS-buffer was injected into the measurement cell thanks to the flow pump (REGLO Digital MS-2/6, ISMATEC) and changes in the frequency (\(\Delta f\)) and dissipation (\(\Delta D\)) as function of the time were measured. After frequency and dissipation stabilization, vesicles suspension was injected and \(\Delta f\) and \(\Delta D\) were recorded. All measurements were performed at the working temperature of 32 \(^\circ\)C, thus higher to the T\(_m\) value to promote vesicles spreading on the quartz crystal surface.

### E. Fluorescence Microscopy (FM) Experiments

NBD-PC probe was dissolved in chloroform/ethanol 1/1 (v/v) and was added at 0.5 mol% in the lipid solution before spreading. This probe is currently used to characterize phase separation in lipid systems, since it concentrates in fluid phases and is expelled from condensed ones [23], [24], [25]. After its transfer, the LB film, enclosed in a black box, was placed on the stage of a fixed-stage upright microscope (BX51WI from Olympus) equipped with a 100 W mercury lamp (U-LH100HG), a BX-RFA illuminator, an U-MWBV2 filter cube (adapted to the absorption and emission spectra of NBD-PC centred at 470 nm and 540 nm, respectively) and LMPF1 x20 and x100 objectives. Images were recorded using a ORCA-Flash 2.8 CCD camera from Hamamatsu (Japan) and the HCImage Live V3.0 software. Taking into account the probe photobleaching, acquisition times were limited within 50 to 100 ms range, and several images were taken at different points of the samples to ensure homogeneity. The lateral resolution of our setup is in the 0.4-0.6 \(\mu\)m range.

### F. Atomic Force Microscopy (AFM) Experiments

Atomic force microscopy is a high resolution scanning probe microscopy to characterize surface at the nanometer scale. Its principle consists in a sharp cantilever tip interacting with the sample surface and sensing the local forces with the sample surface (Fig. 3). Typically, these tip-sample forces are measured directly by the deflection of the cantilever on which a laser spot is reflected into an array of photodiodes. AFM can be performed through two modes: the contact and the dynamic mode. The first is used to obtain information on the topography (height signal) and rheological properties (friction signal) of different surface samples (hard, soft, conductor, non conductor,…). The second mode is in turn well suited for soft matter imaging and provides information such as the topography (height signal), mechanical and rheological properties such as heterogeneity and viscoelasticity through the phase signal. The error signal can also contain interesting
informations. Samples were investigated using two commercial AFM setups: (1) the BioscopeII AFM one (Veeco-Brucker, Santa Barbara, CA) equipped with a G scanner (maximum XYZ scan range of 150 µm × 150 µm × 12 µm), (2) the Nanoscope III one (Multimode Veeco-Brucker) equipped with a J (maximum XYZ scan range of 125µm × 125µm × 5µm) and E (maximum XYZ scan range of 10 µm x 10 µm × 2.5µm) scanners. In this work, sample surfaces were scanned in tapping mode using PPP-NCL silicon probes (NANOSENSORS™) with a spring constant and a corresponding measured resonance frequency \( f_o \) ranging between 21 mN/m and 98 mN/m, and 146 KHz and 236 KHz respectively. In contact mode, triangular SNL silicon nitride probes (Brucker, France) with a nominal spring constant of 0.12 N/m were used. AFM measurements were performed in air for monolayers, and in liquid (buffer) for the bilayer study, at room temperature (20 ± 1°C) with scan rates between 0.3 Hz and 1 Hz (according to the scan size and the scanning mode). Monolayer samples are scanned just after deposition in both tapping and contact modes whereas bilayers are scanned only in contact mode. AFM data were processed using the Nanoscope (Nanoscope 7.30, Veeco) and the Gwiddion softwares. Images at different places were recorded as in the case of FM experiments. Each time, six images were recorded at the same time: trace and retrace height images (topography), trace and retrace amplitude images (error signal) and trace and retrace phase images (mechanical properties) in tapping mode; trace and retrace height images (topography), trace and retrace deflection images (error signal) and trace and retrace friction images (mechanical properties) in contact mode. We point out that according the expected information we want to highlight we will show one or few of these images and for clarity only flattened images (512 x 512 pixels) are shown in this paper.

III. RESULTS AND DISCUSSION

A. Nanostructured Surfaces Characterization by AFM

Fig. 4 shows examples of AFM images obtained with 100 nm-SiO\(_2\) NPs deposited on mica with the spin coating deposition method.

They show small aggregates (few hundred nanoparticles in size) where NPs are regularly organized in a one-layer structure, and isolated NPs (Fig. 4 A and Fig. 4 C). In the case of the direct deposition method, NPs are assembled in very dense and multilamellar networks (data not shown). These results are confirmed by images obtained by scanning electron microscopy (Fig. 4 B).

B. Surface Pressure Measurements

Fig. 5 shows \( \Pi \) isotherms for POPC, DMPC and SM. Both isotherms show a rather regular increase of surface pressure with compression, but the DMPC and SM isotherms are shifted to lower mean molecular areas. For instance at the surface pressure of transfer (30 mN/m), mean molecular areas are 105 Å\(^2\), 80 Å\(^2\) and 72 Å\(^2\) for POPC, SM and DMPC, respectively. Under our experimental conditions, POPC is clearly in a fluid liquid-expanded (LE) phase during all the compression.
In the case of DMPC and SM, the isotherms suggest the existence of a phase transition because of a subtle change in the slopes of the isotherm. At low surface pressure, molecules would be in a LE phase. A further compression would be responsible for a transition in a more organized liquid condensed (LC) phase. Under these conditions, FM and AFM images are very useful to precise this point.

C. Supported Lipid Monolayers Study

Fig. 6 shows a very homogenous FM image of a POPC monolayer transferred at 30 mN/m, confirming that POPC is in a LE phase. On the contrary, images of DMPC and SM monolayers show clearly the co-existence of two phases, one concentrating the probe and corresponding to lipid molecules in the LE phase, and the other one expelling the probe and assigned to the LC phase. SM molecules in LC phase organize in flower-like domains with an average size of about 17.3 µm, whereas DMPC form rather circular domains of heterogeneous size and having an average diameter of about 6.4 µm. Previous studies of SM monolayers at the air-water interface or on planar support report the same heterogeneous organization and domain morphology [25]. As shown in Fig. 7, AFM images of POPC, DMPC and SM monolayers confirm fluorescence results. Indeed AFM images reveal homogeneous and flat surface for POPC monolayers with an averaged roughness of about 1 Å, and the co-existence of LE and LC phases in the case of SM and DMPC with the specific patterns previously described.

These results are in agreement with literature [26], [27], [28]. The different behavior of POPC and SM arises from differences in their structure. First, the sn2 chain of POPC molecule bears a cis-double bond, preventing a regular molecular organization to install during the compression and explaining its low \( T_m \). In the case of DMPC, the formation of domains in LC phase is favored by the saturation of its chains. SM chains are also saturated and elongated, favoring Van der Waals interactions between chains. Moreover, SM molecules are able to form intermolecular hydrogen bonds, whereas POPC and DMPC molecules are not. These bonds strengthen the molecular cohesion. For all the following results on the study of supported monolayers, we will focus POPC and SM.

After the characterization of nanostructured substrates and lipid monolayers, we studied the same monolayers transferred on nanostructured mica surfaces. In all cases, FM and AFM show no change in the organization of lipids in the presence of NPs, whatever their dimensions are, as shown for instance in Fig. 8 in the case of POPC and SM monolayers interacting with NPs of 100 nm in diameter. In particular, our imaging methods reveal the same shape of LC domains and quite the same distribution in the case of SM monolayers and DMPC (data not shown).

FM and AFM enable also to reveal that the size of the NP aggregate influence locally the lipid phase. Thus, SiO\(_2\)-NPs small aggregates (less than 10 nanoparticles in size) creating locally a high curvature are localized in both LE and LC phases as well as in LC domain boundary regions. On the contrary, bigger aggregates are surrounded by lipid molecules in LC phase in the case of SM.

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D. Supported Lipid Bilayers Study

After this first step devoted to the optimization of experimental protocols (sample preparation, characterization conditions...), it is necessary to turn to supported lipid bilayers, which are more realistic membrane models, in order to extrapolate to mechanisms taking place in the interaction between NPs and membranes at the cell scale. Supported lipid bilayers consist in two lipid leaflets deposited on a planar substrate. Two main methods are possible, a combination of LB-LS methods or the vesicles spreading. In the first method, the first monolayer is transferred by pulling vertically the solid support, whereas the second one is transferred by dipping horizontally the solid surface covered with the first leaflet. The vesicle spreading method is the most widely used method and consists basically in the adsorption and rupture of unilamellar lipid vesicles on a solid surface. We first concentrated our study on DMPC bilayers, which are widely described in literature [4], [29], [30].

In a first step, we studied by AFM the quality of DMPC planar bilayers prepared by the combination of LB-LS methods or the vesicle spreading method. AFM images (Fig 9) show that bilayers built with the first method cover bigger areas on mica surface. However, height profiles show that in some areas of the sample, the first monolayer is not covered by the second one (data not shown). Such an observation is recurrent in the case of lipid planar bilayers built by the LB-LS method. In the case of the vesicle spreading, AFM images show the presence of bilayer discontinuous patches. Their size depends on different parameters, such as the diameter of vesicles and the incubation time. These observations clearly show that the preparation method used to build the planar bilayers are particularly important, and led us to use the spreading method, which is widely applied, after optimization of different parameters such as the size of our lipid vesicles.

Supported lipid bilayer formation was optimized by QCM-D measurements, useful to determine the pertinent parameters for the bilayer building, by following the spontaneous adsorption and rupture of unilamellar vesicles thanks to changes of the resonance frequency and the dissipation energy, $\Delta f$ and $\Delta D$, respectively. Fig. 10 shows typical curves obtained when a SiO$_2$-coated quartz crystal is put in contact with a solution of DMPC vesicles at 32°C. Just after the vesicle injection, a decrease of $\Delta f$ and an increase of $\Delta D$ are systematically observed, because of the vesicle adsorption on the surface, inducing an increase of the mass in contact with the crystal surface. After a brief stabilization of both parameters, $\Delta f$ increases and $\Delta D$ decreases, without reaching their initial values. These variations are due to the deformation and to the rupture of vesicles, once a critical concentration is reached on the quartz crystal surface, and more particularly to the water release during the rupture (mass of the trapped water in and between vesicles) [14], [31]. $\Delta f$ and $\Delta D$ remain finally stable, once the bilayer is formed and stable. We are currently optimized supported lipid bilayer study by coupling AFM and QCM-D experiments.

The second step consists in the characterization of DMPC bilayers in interaction with nanostructured surfaces by AFM. In this case, the contrast observed in friction images can be really useful, since it can help to discriminate between NPs recovered or not by the bilayer because of different viscoelastic behaviors. Indeed, in the last case, the lipid bilayer forms a pore around the NP, this one being exposed to water. This information is thus complementary of morphological information shown previously. Some results, shown in Fig. 11, concern the interaction of DMPC bilayers with NPs of 10 nm in diameter: the contrast observed in different part of the images reveal different viscoelastic properties, suggesting two modes of interaction of these NPs with the bilayer. As shown in Fig 11A we observe both small and big aggregates interacting with DMPC bilayer.
Behavior of these systems, and thus about the possible membrane models made of various lipids. At last, all the techniques described here can be applied to NPs, different in size, chemical composition.

IV. CONCLUSION

In conclusion, we demonstrate in this work the potential of combining various surface-sensitive techniques to characterize qualitatively and quantitatively cell membrane models and their interaction with NPs. In particular AFM and FM enable to observe the effect of NPs on the lipid organization. AFM gives also very interesting information about the viscoelastic behavior of these systems, and thus about the possible recovering of NPs by the membrane model. Our first results encourage us to explore more deeply the effects of SiO$_2$-NPs on membrane models made of various lipids. At last, all the techniques described here can be applied to NPs, different in size, chemical composition.

ACKNOWLEDGMENT

This work was funded by the Région Aquitaine-Limousin and the CNRS through the PhD grant of N.R. Faye. Authors thank Gérard Couturier and NSI platform (CPER COLA2) for atomic force microscopy equipment and technical help.

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 Schaeffer methods or vesicle spreading, and made of lipids supposed to be present in rafts (glycolipids, sphingolipids, sterols). She studies also molecular planar systems made of organic molecules such as functionalized hemicyanines with particular optical properties, which can be applied for the specific detection of cations or light to electrical energy conversion. She turned two years ago to the interaction of nanoparticles with membrane models, studied by fluorescence microscopy and atomic force microscopy.

Grauby-Heywang (Dr) is a member of the French Biophysical Society.

Touria Cohen-Bouhacina Touria Cohen-Bouhacina is born in Algeria in September 1961. After making all her schooling (until Master 1) in Tlemcen - Algeria- she went to Toulouse-France- for continuing her graduate studies (Master 2 and PhD). Her thesis, defended in October 1989 at the Paul-Sabatier University, was focused on the relaxation in the insulating glass and semiconductors studied by electron paramagnetic resonance. She taught as master auxiliary in colleges and high schools, held a temporary assistant-professor position at the Paul-Sabatier University. In September 1991, she became assistant-professor at the University of Bordeaux 1 -France- and in September 2005 she was promoted to Professor at the same university.

Her research has always been multidisciplinary, at the interface between physics, chemistry and biology using atomic force microscopy - AFM for characterization. For example, she became interested in the physico-chemical treatment of surfaces (organosilane and / or polymers grafting, adsorption of molecules on surfaces), polymer conductors, adsorption of water on silica layers, biomaterials and cell adhesion, ATP-synthase, structure and sliding of fluid layers near a solid surface, supported models of cellular membranes. Since 2009, Cohen-Bouhacina created her research group "Biophysics & Nanosystems" and she conducts studies related to toxicity problems: nanoparticle-membrane or bacteria interaction, bacteria-based biosensors as detectors of heavy metals, etc ... All of these studies combined different techniques of characterization such as atomic force microscopy, fluorescence microscopy, quartz microbalance dissipation, surface energy, contact angle, etc ... Pr Touria Cohen-Bouhacina is a member of several scientific communities; she is, among other things, member of the Scientific Council of the "forum of local probes" and the "French Society of Microscopy".