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Characterization of Silica Nanoparticles in Interaction with *Escherichia coli* Bacteria

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

***Abstract*—**The objective of the present investigation was to evaluate the morphology of *Escherchia coli* bacteria in interaction with SiO_2 nanoparticles.

This study was made by atomic force microscopy and quartz crystal microbalance using SiO_2 nanoparticles with 10nm, 50nm and 100nm diameter and bacteria immobilized on polyelectrolyte multilayer films obtained by spin coating or by "layer by layer" (LbL) method.

***Keywords*—**Atomic Force Microscopy, *Escherichia coli*, Quartz Crystal Microbalance, polyelectrolyte, silica nanoparticle.

I. Introduction

THE development of nanosciences is a technological revolution, and many industrial and medical applications are already available, using for instance nanoparticles (NPs). Today, although NPs are permitted in numerous technologies in the world, their long-term toxicity on the environment and on humans is still poorly understood. It is clear from studies to date that NPs may affect the biological behavior at the cellular, subcellular and protein levels. Some NPs easily penetrate in the body, cross cell membranes, lodge in mitochondria and can trigger adverse reactions [1].

A major emphasis regarding the impact of nanoparticles focuses on their toxicity to microorganisms such as bacteria, and many studies aim to examine SiO_2 toxicity mechanisms on microorganisms [2], [3].

Whilst there are many powerful techniques for studying biofilm formation, including confocal optical microscopy [4], scanning electron microscopy, transmission electron microscopy (TEM) [5], etc., the ability to examine in details their interfacial properties especially under in situ conditions is still limited. In comparison with optical microscopy, atomic force microscopy (AFM) can probe structural details with a high spatial resolution (atomic and molecular scales). The AFM not only gives us access to the topography (height image) but can also go back to the mechanical properties (elasticity, viscosity) through tribological (friction in contact mode) or phase images (surface charge, elasticity in tapping mode). Spectroscopy mode can also be used leading to the force curves (in contact) and approach-withdrawal curves (by tapping). This technique has been used in liquid medium and under ambient conditions for imaging different bacterial systems [6], [7].

In our study, we want to follow by AFM the interaction of the *Escherichia coli* (*E. coli*) bacteria with SiO_2-NPs with various diameters from 10 to 100nm and more particularly their potential stress, giving information about the NPs toxicity. Indeed, some studies suggest that various parameters such as the size, shape, surface chemistry, composition, and aggregation state of NPs would be determining in NPs-bacteria membrane interaction [8], [9].

E. coli bacteria were immobilized on polyelectrolyte multilayer (PEM) films [10], [11], realized using the layer by layer technique (LbL), each layer being alternatively positively or negatively charged. AFM method allows first to characterize the PEM deposition process, and secondly the morphology of bacteria in the absence or in the presence of SiO_2-NPs, in the nanometer range. The monitoring of PEM deposition was also followed by the quartz-crystal microbalance method (QCM-D) in real time.

II. Materials and Methods

A. Material

SiO_2-NPs (10nm, 50nm and 100nm in diameter) were purchased from Biovalley (Marne-la-Vallée, France).

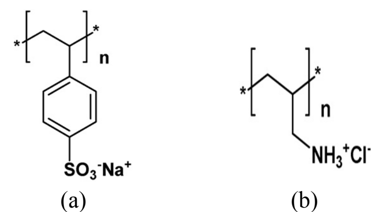

Fig. 1 Chemical structures of (A) PSS and (B) PAH

Commercial polyelectrolytes used for bacteria immobilization were cationic *poly allylamine hydrochloride* (PAH, MW =56kDa), and anionic *polystyrene sulfonate* (PSS,

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MW =70 kDa) (Fig. 1). They were purchased from Sigma Aldrich.

E. coli bacteria were obtained from the *Centre d'Etudes du Bouchet* (DGA, France). Millipore water (pH 5.5, resistivity > 18.2MΩ.cm) was used for the preparation of all solutions. Mica was used as support for AFM measurements, and was purchased from Electron Microscopy Sciences©. QCM-D sensor crystal (AT-cut) with a fundamental resonance frequency of 5MHz and gold electrode (area: 0.1cm^2) and thin upper layer of silicon oxide (50nm) were purchased from LOT-Quantum Design.

B. Methods

1. Bacteria Biofilm Preparation on a PEM Surface

Polyelectrolytes (PE) were dissolved in Millipore water at a concentration of 0.5g/l. Each PE layer was deposited through the LbL method resulting in self-assembled molecular multilayers PAH-(PSS-PAH)$_n$. Typically, PAH and PSS solutions were alternatively deposited, adsorption and rinsing steps being repeated to obtain the desired number of PE layers (Fig. 2). In this work the last layer was made of positively charged PAH layer to immobilize negatively charged *E. coli* bacteria.

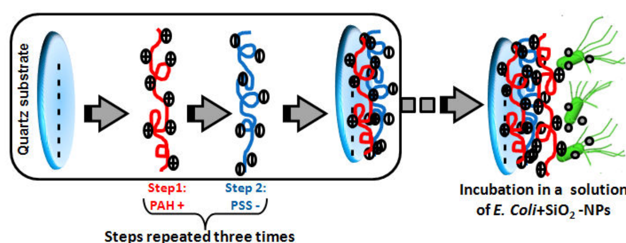

Fig. 2 LbL method for *E.coli* bacteria immobilization on a surface

For all measurements, the conditions were maintained at pH=5.5 and T=28°C. The injection was made in macro-flow with an optimal through put set at 65µl/min for both PE alternatively, followed by signal stabilization in a few minutes after each PE layer deposition indicating the end of the adsorption step. A baseline was systematically made using an injection of Millipore water.

E. coli were grown overnight in Petri dishes containing Luria-Bertani (LB) solid agar nutritive medium at 37°C. They were suspended in ultrapure water to obtain an optical density of 0.6 (at 600nm) corresponding to a cell concentration of 10^8 cells/ ml.

2. Quartz Crystal Microbalance (QCM-D)

The QCM technique (thoroughly described by Rodahl et al. [12]) is known as a very sensitive mass sensor. Its principle is based on the piezoelectric effect of a quartz crystal oscillating at a characteristic resonant frequency, f_0. An increase of the mass in contact with the crystal surface shifts this frequency [13]. It is thus possible to measure in real time sub-nanogram changes at the liquid-crystal interface thanks to the Sauerbrey equation [14]:

$$\Delta m = -\frac{\rho_q t_q \Delta f_n}{n f_0}$$

where Δm is the apparent mass, ρ_q is the specific density of the quartz crystal (2.648g·cm^{-3}), f_0 is the fundamental frequency of the crystal in air (4.95 MHz), t_q is the thickness of the quartz crystal (30nm) and Δf_n is the change of resonance frequency at the first overtone (n=1).

It is also possible to follow in real time the change in energy dissipation, ΔD, the dissipation energy being defined as:

$$D = E_{dis} / (E_{st} \times 2\pi)$$

where E_{dis} is the energy dissipated during one period of oscillation and E_{st} is the energy stored in the oscillating system.

3. Optimization of Concentration of the NPs Solution

We tried first to optimize the concentration of SiO_2-NPs used in order to obtain a homogeneous distribution devoid of NPs aggregates.

NPs 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. Then different dilutions were achieved. In order to determine the presence or not of NPs aggregates, the obtained solutions were deposited on substrates by spin-coating (300rpm, 1 minute) leading to nanostructured surfaces which were dried under a desiccator overnight at ambient temperature, before AFM observation.

In addition, bacteria were also put in contact with NPs before the formation of the biofilm. In this case, the bacteria suspension previously described was diluted to a concentration of 10^{6}cells/ml, and 500µL of this diluted suspension was added to 4.5mL of Millipore water. Bacteria were then incubated in the presence of an equal volume of SiO_2-NPs suspension during 1 to 24 hours. Finally they were deposited on a surface functionalized by PEM and kept protected from dust under a desiccator overnight.

4. Atomic Force Microscopy (AFM)

Briefly, AFM technique [15] (described in Fig. 3) is based on the detection of weak forces existing between a tip and an object (the surface sample) which is scanned along the tip with a piezo scanner (maximum XYZ scan range of 150µm*150 µm*12µm). The tip is attached on a cantilever, and the tip-sample forces are measured directly by the deflection of the cantilever on which is reflected a laser spot into an array of photodiodes. This technique can generate information such as topography and mechanical properties (adhesion, elasticity, viscoelasticity or chemical composition) at the nanometer level, thanks to friction and/or phase images.

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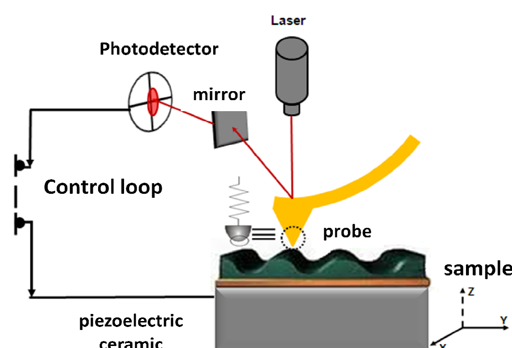

Fig. 3 Atomic Force Microscopy (AFM) principle

Two AFM setups were used in this work, a MultiMode NanoScope II apparatus (AFM imaging), and a Bioscope II, mounted on an Olympus inverted optical microscope and operating with the NanoScope V controller (Veeco-Brucker, Santa Barbara, CA). The AFM characterization of samples was performed in tapping mode under ambient conditions in air, depending on the nature of the samples.

All data presented in this paper were generated with the same cantilever (with a spring constant and a corresponding measured resonance frequency (f_o) ranging between 21mN/m and 98mN/m, and 146 KHz and 236 KHz respectively). For each experiment, four images were recorded at the same time: trace and retrace height images (topography), trace deflection images (error signal) and phase images (mechanical proprieties).

III. Results and Discussion

A. Optimization of the NPs Organization and PEM Film Formation

Several nanoparticles size have been used the corresponding NPs solution have been optimized such as Φ= 4 nm, Φ=10nm, Φ=50nm and Φ=100nm. In this paper we present only the results for NPs with Φ= 100nm

To optimize the concentration of NPs while avoiding the formation of aggregates, different solutions dilutions were tested and the corresponding solution were deposited on mica leading to nanostructured surfaces which were observed by AFM.

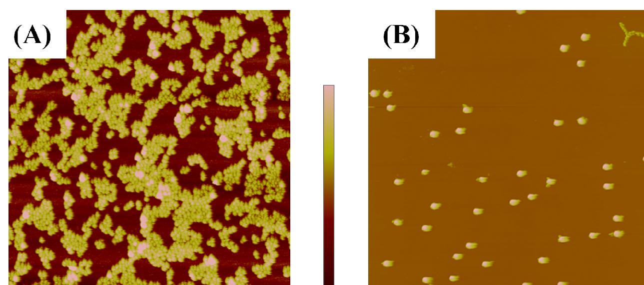

Fig. 4 AFM results on nanostructured surface with SiO_2-NPs (Φ=100nm). Amplitude images 10µm*10µm obtained with the tapping mode (A: dilution 500×, B: 1000× dilution)

Let us note that the main part of NPs was washed from the surface, only NPs adsorbed on the mica (in a sufficient amount) remaining on the surface. As shown in Fig. 4, the suitable NPs solution, avoiding aggregates, was diluted 1000 times from a stock solution of concentration 5.7 % (w/w). Bacteria were incubated in the optimized NPs solution. Also the mixtures Bacteria-NPs is deposited on a surface functionalized by a polyelectrolytes film which has been optimized its construction by QCM-D.

The preparation of the PE multilayers was followed by the QCM-D technique and the obtained multilayer film was observed with AFM.

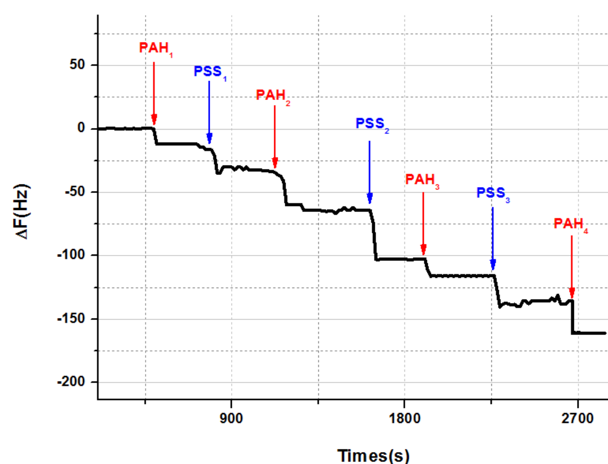

Fig. 5 Frequency shifts (Hz) as a function of time, due to the PEM deposition on the crystal surface

Typical in situ frequency shifts obtained during the alternative deposition of PAH and PSS layers are shown in Fig. 5. After each injection, the frequency shift decreases rapidly, due to the adsorption of the polymer on the surface, and then stabilizes in a few minutes, showing the saturation of the surface. Each adsorption step lasts between 5 and 10 minutes.

Then, AFM experiments enabled us to characterize the morphology of these samples and to confirm QCM-D results.

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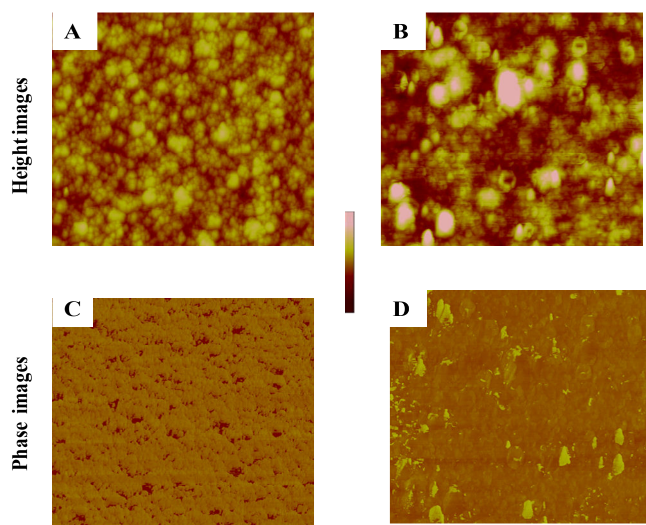

Fig. 6 Topographic images (5µm * 5µm) obtained in AFM tapping mode on bare surface (A and C, height and phase respectively), and on surface recovered with PEM (B and D, height and phase respectively). Scale bar for height and phase image are 50nm and 360°, respectively

AFM results on the substrate before and after the PEM deposition show an evolution of the surface topography (height images) after the deposition of the film. The increasing of the grain sizes and of the average roughness suggests that the polyelectrolyte multilayers ((PAH-PSS)$_3$-PAH) have been effectively deposited on the surface. The low variations in the contrast of phase images indicate that the sample surface is relatively homogeneous and hence confirms that the PEM covers the entire surface.

B. Morphology of E. coli Bacteria Observed by AFM

Let us recall that the biofilm of bacteria was formed using two methods: first on bare surface by spin coating, and secondly by immobilization through the PEM process (Fig. 2). The first method is limited, since the surface density of immobilized bacteria is weak and a lot of them are removed during the rinsing phase (results not shown) leading to poorly reproducible results.

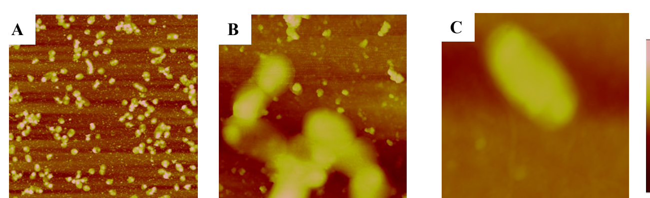

Fig. 7 AFM results obtained in tapping mode in air. Topography of bacteria immobilized on a surface functionalized by PAH-(PSS-PAH)$_3$. Bacteria population (A: 30µm *30µm, B: 5µm * 5µm); an isolated bacterium (C: 3µm * 3µm)

The second method, as shown in Fig. 7A and 7B, gives more reproducible results, thanks to the formation of a stable biofilm, even after three rinsing steps. Fig. 7C enabled us to determine the following bacterium dimensions: length, width (half bacterium height) and height of 1.8µm, 1.2µm and 150 nm, respectively. These dimensions are in agreement with those obtained in the case of healthy bacteria [16].

C. NPs-Bacteria Interaction

The first experiments to test the bacteria-NPs interaction and to implement the protocol were performed on high size nanoparticule (Φ=100nm). The choice, at this first step, of such a large size of NPs is done in order to have images with sufficient contrast, where it is possible to observe both bacteria and NPs.

In Fig. 8, height images (8A and 8B) including topographical information, shows that the bacteria morphology (shape and size) does not change after their incubation in the presence of NPs. This suggests that bacteria are always healthy. We note also the presence of aggregated NPs on the surface of the bacteria membrane. Moreover, we guess that NPs are aggregated at the interface between the bacterium contours and the substrate interface. The corresponding amplitude images (8C and 8D) confirm these observations by providing higher contrast. This result was expected since the size of NPs is large enough to prevent the penetration of NPs into bacteria.

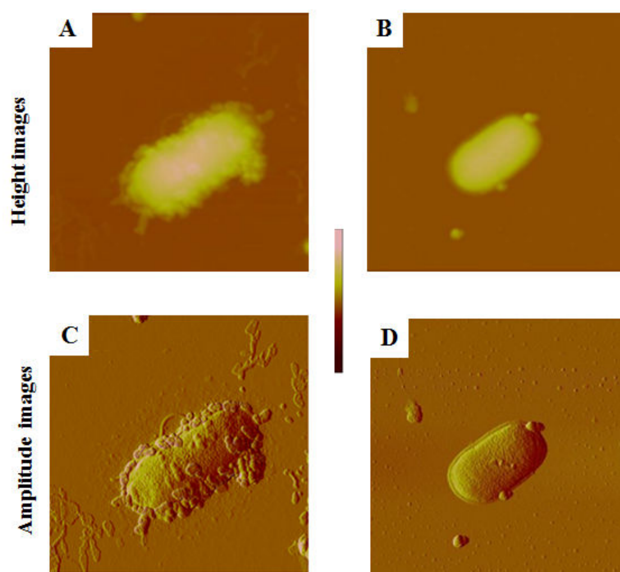

Fig. 8 AFM topography images (5µm * 5µm) obtained in tapping mode. Bacteria were incubated in a SiO_2-NPs solution (Φ=100nm) during 1 hour on mica substrate for two separate bacteria (A, C) and (B, D). Scale bar for height and amplitude images are 500 nm and 100mV, respectively

Other information may be extracted from the AFM experiments. For example, phase images provide information such as the heterogeneity of the surface sample (in terms of charge, hardness, viscoelasticity, etc.). In this study, one can expect that phase images tell us more about the evolution of the mechanical properties of the bacteria according to the presence or absence of nanoparticles.

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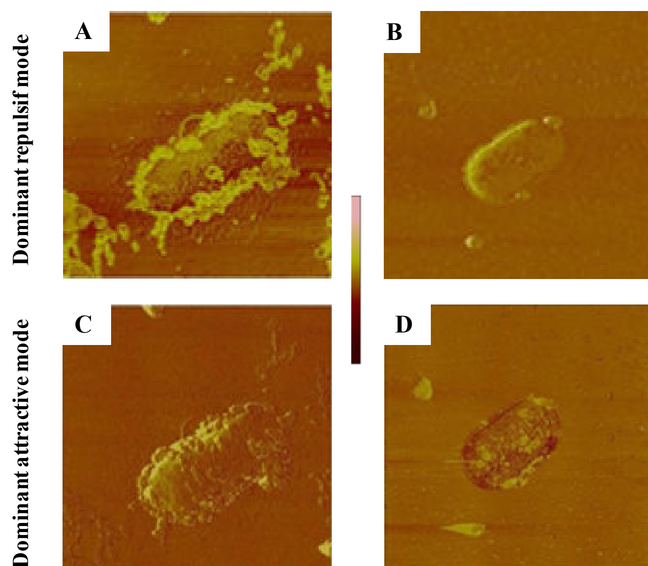

Fig. 9 AFM Phase images (5µm * 5µm) of two distinct *E. coli* bacteria (A and B) interacting with SiO_2-NPs (Φ=100nm) Dominant repulsive AFM mode (A and B) and dominant attractive AFM mode (C and D) Scale bar for phase 90°

Indeed, a variation of the oscillating AFM probe induces a variation of its interaction with the bacterium surface. So, small oscillating amplitudes correspond to dominant attractive mode (mechanical contrast) while high amplitudes correspond to repulsive dominant mode (topographic contrast). Fig. 9 shows such typical results for two different bacteria interacting with a high amount of NPs (9A, 9C) or a low one (9B, 9D). In the first case, the bacterium shows no change in the phase image (both in repulsive and attractive modes) suggesting that this bacterium is rigid and its surface is smooth. The presence of aggregates of NPs (hard matter) on its surface enhanced the contrast of the phase image between bacterium and NPs (repulsive interaction). In the case of the second bacterium (Fig. 9B and 9D) the evolution of the corresponding phase contrast between the repulsive and attractive mode is a sign of its low stiffness which leads to greater dissipation between the AFM probe and the bacterium surface.

The contact of NPs and the bacterial membrane and their aggregation is probably due simply to electrostatic interactions. However, the hypothesis related to the toxicity of Nano cannot be eliminated definitively. Indeed, although the passage of NPs through an intact bacterial membrane seems unlikely, because the great size of the latter (Φ=100nm), their accumulation in the cytoplasm, probably after the rupture of membranes, is often observed. So, the difference observed between all these cases (9A & 9B, 9C & 9D) indicates a relationship between rigidity of the bacteria and the attachment (or not) of NPs on the membrane.

Now that the full protocol for the study of NPs-bacteria interaction is established, we have attempted to test the effect of NPs sizes. We used the diameters: Φ=4nm, 100nm and 50 nm. As an example Fig. 10 shows the results obtained recently on NPs with a diameter of 10nm. Different image sizes are shown in this figure. The amplitude (A2, B2, C2) and phase ones (A3, B3, C3) complete information obtained with the height ones (A1, B1, C1) with providing better contrast.

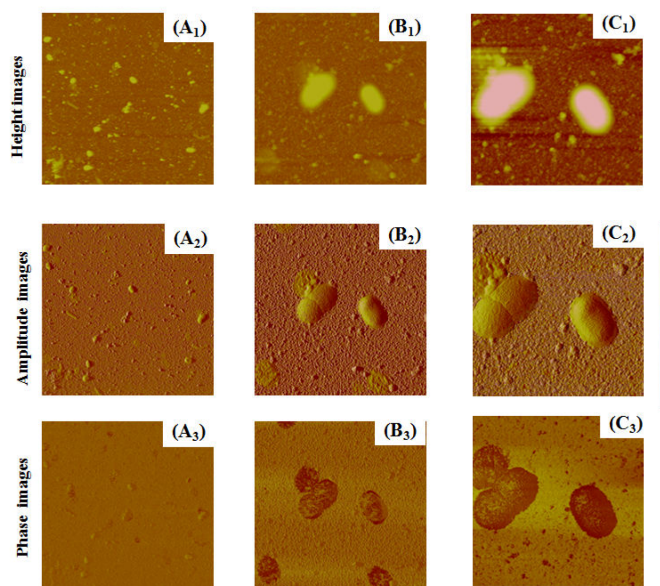

Fig. 10 AFM images in tapping mode for *E. coli* bacteria after incubation in SiO_2-NPs solution (Φ=10nm) during 24 hours on mica substrate for three different scale (A: 20µm x 20 µm; B: 8µm x8 µm; C: 5µm x5µm). Scale bar for height, amplitude and phase image are 500nm, 100mV and 92° respectively

A first observation can be made when analyzing all of these results: the coverage of the surface by bacteria is much less important than in other cases such as bacteria in the absence or in the presence of NPs of 100nm in diameter. Furthermore, the surface is covered with an inhomogeneous bacterial population: some bacteria show the typical cylindrical morphology of a healthy state previously described, whereas as other ones are rather spherical. Other bacteria have less contrast and seem to have much lower heights as if they were emptied of their content. The toxic effect of these small NPs (Φ=10nm) is thus clearly visible. These preliminary results are encouraging and need other complementary experiments such as fluorescence microscopy and will also be deepened with a study of other sizes of NPs (Φ=4nm and 20nm).

IV. Conclusions

In this work we have first optimized our protocol namely the optimization of the NPs concentration and the immobilization of the bacteria on our surface. In a second step, the study by AFM shows that NPs are able to interact with bacteria, the mode of interaction, and more particularly their ability to penetrate into the membrane, depending probably on the size of the NPs. This study may provide insightful information toward our understanding of the NPs-biosystems interactions and other interfacial phenomena at nanoscale.

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Ibtissem Gammoudi is born in Tunisia in Febrary 1980. After making Master in Physical Chemistry of Condensed Matter (2007-2008) at the Monastir University she obtained her thesis, defended in June 2012 at the Bordeaux 1 University, entitled: "Development of bacteria based micro-biosensor for environmental monitoring", when she made her research at the IMS laboratory (laboratoire de l'Intégration du matériaux au système) in collaboration with the Laboratoire Ondes et Matières d'Aquitaine (LOMA). Actually she held an ATER position in University of Bordeaux 1 –France.
Her research domain has been multidisciplinary, at the interface between physics, chemistry and biology. The aim of her project is the design of a micro-biosensor based on bacteria that is sensitive and useful as a disposable sensor for environmental control and for industrial applications, using several methods for this study such as the Love wave, QCM-D, AFM and electrochemistry. To date, her results have been the subject of several oral presentations and posters in different national and international congresses.. For example can be cited two articles where it was a first author: (1) Love wave bacterial biosensors for the detection of heavy metal toxicity in liquid medium. Biosensors and Bioelectronics (2010) 26 (4), pp. 1723-1726. (2). Love wave bacterial biosensors and microfluidic network for detection of heavy metal toxicity. Sensor Letters9 (2010) (2), pp. 816-819. "Enhanced bio-inspired microsensor based on microfluidic / bacteria / love wave hybrid structure for continuous control of heavy metals toxicity in liquid medium". *Biosensors and bioelectronics* (Accepted 2013).

Ndeye Rokhaya Faye is born in Senegal in Poût. She obtained a Master 2 degree in Cutaneous Physiology, Pharmacology and Bioavailability at the University of Lyon 1 (France) in 2010. Before her master, Ndeye Rokhaya Faye got a BSc degree in biochemistry and a "Maîtrise" in structural biochemistry at the interface of biology, physics and chemistry at the University of Bordeaux 2 (France) in 2007 and 2009, respectively.
During her education, she applied her knowledge in Biochemistry and Biophysics to characterize the different conformations adopted by an antibacterial peptide during its insertion in lipid films at the air-water, during an internship in the European Institute of Chemistry and Biology of Bordeaux. During her Master, she also worked for almost 8 months at the Therapeutic Research Center of the School of Medicine-University of Queensland in Brisbane (Australia). During this internship, she studied in vivo and ex vivo redox state of keratinocytes (human skin) before and after penetration of silver nanoparticles, using a multiphoton microscope. Currently, she is in her third year of Ph.D. in the Laboratoire Ondes et Matières d'Aquitaine (LOMA, University of Bordeaux 1), funded by the Centre National de Recherche Scientifique (France) and the Aquitaine-Limousin region. Her thesis subject, at the interface between biology, physics and chemistry, focuses on the interaction of nanoparticles with simplified membrane models studied mainly by Atomic Force Microscopy (AFM) and fluorescence microcopy. To date, her results have been the subject of several oral presentations and posters in different national and international congresses. They have also been the theme of 2 publications: (1) "Oxidation of Langmuir-Blodgett films of monounsaturated lipids studied by Atomic Force Microscopy" Int. J. Nanotechnology. 2013; 10 (5):390 - 403, (2) "Nanoparticles and microparticles for skin drud delivery, advanced Drug Delivery" Adv Drug Deliv Rev. 2011 May 30; 63 (6):470-91. Two more publications are expected in the following weeks.

Fabien Moroté is born in France in 1987. He obtained his technical degree in physics at the University of Bordeaux 1 (France) in 2007. After this, Fabien Moroté got a professional BSc degree in physical measurements at the University of Albi in 2008.

He benefitted from two internships during his education in the Lyonnaise des eaux company, first in the metrology field, and secondly in the field of recovery and energy savings in a water treatment station. He was recruited in 2008 at the University of Bordeaux 1, in the technical staff of the Laboratoire Ondes et Matière d'Aquitaine (LOMA). He is in charge of the Nano-Spectro-Imaging technical platform, and particularly involved in the maintenance of AFM setups and all the experimentations made on them. His collaboration with different teams led to several publications such as (1) "Oxidation of Langmuir-Blodgett films of monounsaturated lipids studied by atomic force microscopy" *int. J. Nanotechnology.* 2013; 10 (5):390 - 403, (2) " Enhanced bio-inspired microsensor based on microfluidic / bacteria / love wave hybrid structure for continuous control of heavy metals toxicity in liquid medium". *Biosensors and bioelectronics* (Accepted).

Daniel Moynet Daniel Moynet received his PhD from Université Paul Sabatier (Toulouse) in 1984. He is an assistant professor of microbiology in Université de Bordeaux since 1981. His research activity is focused on human inflammatory pathologies (autoimmunes or viro induced), comprehension of the inflammatory response and drug discovery.

Christine Grauby-Heywang is born in France in 1971. She has a degree in biochemistry and molecular biophysics from the University of Paris 6 (France) and received her Ph.D. in molecular biophysics in this university in 1998.

After a temporary assistant-professor position at the University of Paris-Nord (France) and a post-doctoral position of one year in the group of E. Sackmann at the Technische Universität München (Germany), she became assistant-professor at the University of Bordeaux 1 (France) in 1999. Her research fields concern mainly Langmuir monolayers and supported models of cellular membranes obtained by Langmuir-Blodgett and Langmuir-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.
Dr Grauby-Heywang 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

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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 ...

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".

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