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Directed Assembly of Living *Pseudomonas aeruginosa* Bacteria on PEI Patterns Generated by Nanoxerography for Statistical AFM Bioexperiments

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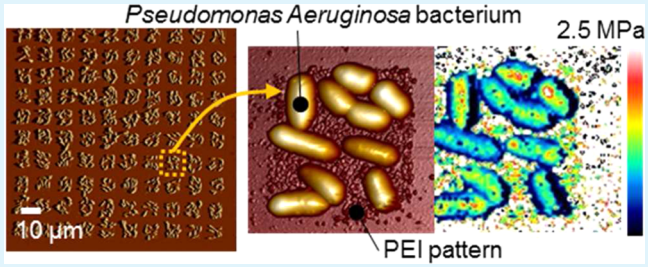
ABSTRACT: Immobilization of living micro-organisms on predefined areas of substrates is a prerequisite for their characterizations by atomic force microscopy (AFM) in culture media. It remains challenging since micro-organisms should not be denatured but attached strongly enough to be scanned with an AFM tip, in a liquid phase. In this work, a novel approach is proposed to electrostatically assemble biological objects of interest on 2 nm thick polyethylenimine (PEI) patterns fabricated by nanoxerography. This nanoxerography process involves electrostatic trapping of PEI chains on negatively charged patterns written on electret thin films by AFM or electrical microcontact printing. The capability of this approach is demonstrated using a common biological system, *Pseudomonas aeruginosa* bacteria. These negatively charged bacteria are selectively assembled on large scale arrays of PEI patterns. In contrast to other PEI continuous films commonly used for cell anchoring, these ultrathin PEI patterns strongly attached on the surface do not cause any denaturation of the assembled *Pseudomonas aeruginosa* bacteria. AFM characterizations of large populations of individual living bacteria in culture media can thus be easily performed through this approach, providing the opportunity to perform representative statistical data analysis. Interestingly, this process may be extended to any negatively charged micro-organism in solution.



KEYWORDS: *directed assembly, Pseudomonas aeruginosa bacteria, PEI, atomic force microscopy, nanoxerography*

1. INTRODUCTION

Atomic force microscopy (AFM) is a powerful tool in microbiology, allowing nanoscale topographical, mechanical, and chemical characterizations of biological objects of interest within their culture media. Immobilization of living micro-organisms on a substrate is one of the main prerequisites for AFM measurements in liquid media. This is very challenging because the strength of the micro-organism immobilization needs to be optimal: it has to be strong enough to maintain the position of the biological objects which can potentially be altered or moved by the AFM tip interaction during the scanning of the surface and yet not too strong to cause their denaturation. Ideally, a technique that would allow the immobilization of living biological objects of interest, on predefined periodic arrays over large areas of a substrate would largely facilitate the localization of the zones of observation by AFM and the collection of statistical data on heterogeneous populations in their culture media.

Several approaches for assembling biological objects on substrates have been developed in the literature[1] and can be divided into two main groups: (i) mechanical trapping in gel matrix,[2,3] topographically patterned substrates obtained by lithography,[4−6] or porous membranes.[7−9] (ii) Immobilization using localized functionalization of a substrate (based on electrostatic interactions,[10−12] covalent bonds,[13−15] antibody−antigen linking,[16] or hydrophobic−hydrophilic contrasts[17]). Among this range of available assembly techniques, electrostatic immobilization is direct and convenient for micro-organisms. Indeed, most of them are negatively charged and therefore polycation films can be used to create positively charged substrates. Poly L Lysine and Polyethylenimine (PEI) films are probably the most largely employed since they provide an efficient immobilization and are very simple to implement. However, such technique suffers from two main disadvantages: (i) it generates random deposition of studied micro-organisms, (ii) polycations dissolved in buffer media can be cytotoxic.[18,19]

In the present study, we propose a novel approach to electrostatically assemble biological objects of interest on PEI patterns prefabricated by nanoxerography. The nanoxerography process, usually employed for directed assembly of colloids on predefined areas of substrates,[20−23] is used in this work to assemble PEI chains on negatively charged patterns written on

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electret thin films by AFM or electrical microcontact printing (e-μCP). The potential of this approach is evaluated by applying it to a chosen biological system, the medical relevant *Pseudomonas aeruginosa* bacterium. This bacterium is indeed responsible for hospital acquired infections, and is able to gain resistance to all the known antibiotics. We have investigated if AFM characterizations in culture media can be carried out on bacteria arrays obtained in this way, checked the viability of assembled bacteria and studied how to control the number of bacteria immobilized on each PEI pattern. Such developments are of first interest to generate statistically relevant AFM data in a reasonable time scale.

2. EXPERIMENTAL SECTION

Fabrication of PEI Patterns by Nanoxerography. Figure 1 illustrates the two-step protocol of nanoxerography used in this work

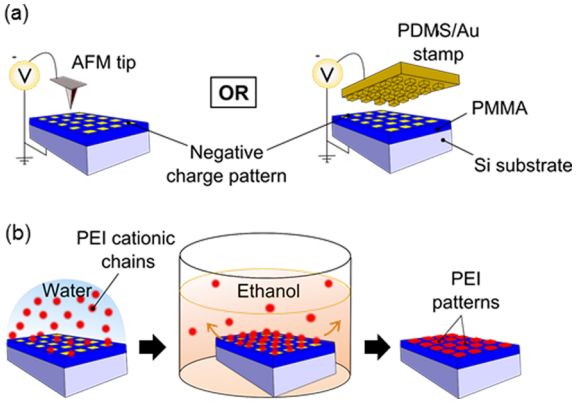

Figure 1. Schematics of the fabrication of PEI patterns by nanoxerography: (a) charge writing by AFM (left) or e-μCP (right), (b) development by incubation in an aqueous PEI solution and immersion in ethanol.

to fabricate PEI patterns. First, negatively charged patterns of desired geometry were written written on 100 nm thick poly(methyl methacrylate) (PMMA) films spin-coated on 1 × 10^{16} cm^{-3} p-doped silicon substrates, by applying 1 ms voltage pulses at a frequency of 50 Hz, either to a highly n-doped silicon AFM tip or a 1 cm^2 microstructured gold−metallized polydimethylsiloxane (PDMS) stamp (so-called electrical microcontact printing (e-μCP) process) (Figure 1a). Voltage pulses were varied from −10 to −80 V. These specific writing conditions are reliable and reproducible, causing no tip, stamp and/or sample damage at the high voltages used, as demonstrated previously.[24] After the charge writing step, the surface potential of the charge patterns was systematically measured in air by the electrical derived mode of amplitude modulation Kelvin Force Microscopy (KFM). AFM and e-μCP charge writings are complementary: AFM charge writing is versatile since AFM allows both to draw charge patterns of complex geometry in a few minutes and to quantify directly their surface potential by KFM. Charge writing by e-μCP is very useful to fabricate in parallel in a few minutes, hundreds of thousands of charge patterns covering large areas of substrates or to perform repetitive charge writings with the same stamp on various substrates, a requirement for further scaling-up and automation of the protocol. In the second step (Figure 1b), a 10 μL droplet of 0.2% solution of aqueous PEI (Fluka P3143 from Sigma-Aldrich, molecular weight (M_w = 750 000)) was incubated on the electrostatically patterned substrates for 90 s. The substrates were subsequently immersed in absolute ethanol for 30 s, rinsed with pure water and dried under nitrogen. As previously demonstrated,[20] the immersion in ethanol, which has a lower dielectric constant compared to water, helps in increasing the amplitude and the range of the electric field generated by charge patterns. This second step led to the selective electrostatic trapping of cationic PEI polymer chains on the negative charge patterns, thanks to electrophoretic forces.

Assembly of *Pseudomonas aeruginosa* Bacteria on PEI Patterns. Cultures of *Pseudomonas aeruginosa* ATCC 27853 bacteria were conducted at 35 °C in Mueller Hinton media (difco, 275730−500 g) during 24 h under static conditions. Bacteria at their stationary phase of growth were used. One mL of media at a 10^8 cells/mL concentration was collected and centrifuged at 5000 rpm for 10 min. Supernatant was then removed and replaced by 1 mL of deionized water. This washing step was repeated three times. The directed assembly of negatively charged *Pseudomonas aeruginosa* bacteria on positively charged PEI patterns was performed by incubating a 10 μL droplet of the aqueous bacteria suspension for 10 min on arrays of PEI patterns, followed by rinsing with deionized water. The samples were then transferred into 10 mM phosphate buffered saline (PBS) to avoid any denaturation of the bacteria immobilized on the PEI patterns. It is worth noting that it would not be possible to directly fix the negatively charged bacteria on positively charged patterns written on PMMA because immersion of bacteria in ethanol would denature them.

AFM Characterizations. AFM images and force spectroscopy on the bacteria assembled on PEI patterns were all carried out in buffer medium, in contact mode using an ICON from Bruker AXS Instruments, equipped with a Nanoscope V controller. Si_3N_4 AFM probes (MLCT manufactured by Bruker AXS Instruments) were used for all the experiments. Their cantilever spring constants were systematically measured using the thermal tune method. They ranged from 10 to 30 pN/nm. The maximum force applied to the cells was limited to 1 nN in order to probe the cell wall elasticity (Young's Modulus) and not the cell turgor pressure that is made at higher loading forces.[25,26] The topography and surface potential of PEI patterns were determined by AFM imaging in tapping mode and KFM mapping under ambient conditions.

Fluorescence Optical Microscopy Characterizations Using a LIVE/DEAD Cell Viability Assay. In order to test and compare the viability of *Pseudomonas aeruginosa* bacteria immobilized on PEI patterns fabricated by nanoxerography, PEI continuous film and bare silicon substrate, the bacteria were tagged using a LIVE/DEAD cell viability assay, purchased from Life technologies. This kit is composed of two fluorophores: (i) the Cyto9 fluorophore (green), which enters the cytoplasmic membrane of any bacteria; and (ii) the propidium iodide fluorophore (red), which penetrates the cytoplasmic membrane only if bacteria cannot longer control osmotic flow. Observations in buffer medium by fluorescence optical microscopy of bacteria immobilized on these three substrates using the same experimental conditions, allowed us to quantify the percentage of damaged (red colored) and live, intact (green colored) bacteria.

3. RESULTS AND DISCUSSION

PEI Patterns Fabricated by Nanoxerography. Figure 2 shows examples of PEI patterns fabricated by AFM and e-μCP nanoxerography. The first one, consisting of a 17 μm large Occitane cross, illustrates the flexibility in the pattern design offered by AFM nanoxerography (Figure 2a). The KFM image on the left panel reveals that this charge pattern, written using −30 V voltage pulses, presents a surface potential of −1.5 V. After development in the PEI solution, the AFM topographical image (middle panel) shows that a 2 nm thick PEI layer is selectively assembled on the charge pattern, reproducing it very accurately. This PEI pattern presents a positive surface potential of +80 mV (right panel). Figure 2b shows similar results for a different pattern geometry obtained by e-μCP nanoxerography. In this case, hundreds of 5 μm wide PEI square patterns were fabricated on a 1 cm^2 zone of the PMMA film. Each PEI square exhibits a 2 nm mean thickness and a surface potential of about +80 mV. It is important to note that the 2 nm thick PEI patterns fabricated by nanoxerography present a surface potential similar to that observed on regular,

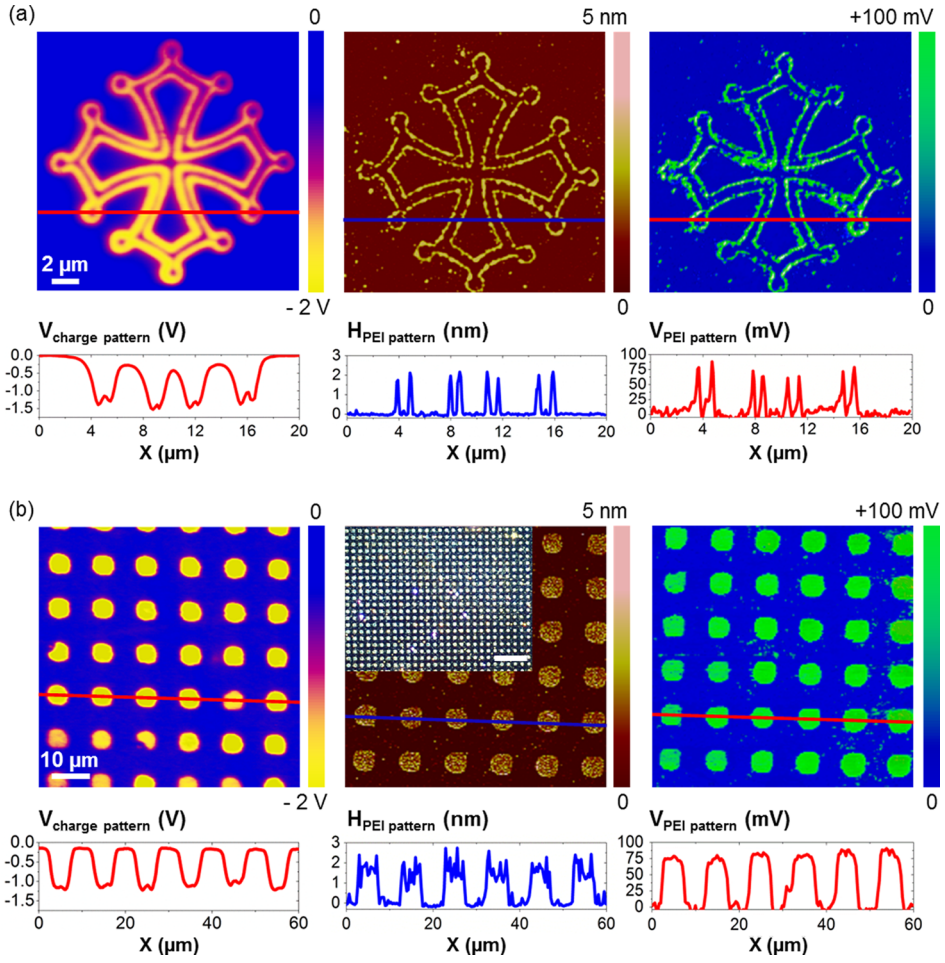

Figure 2. (a) Occitane cross-shaped PEI pattern fabricated by AFM nanoxerography and (b) array of 5 μm square PEI patterns fabricated by e-μCP nanoxerography. In each case, KFM surface potential images of the associated charge patterns written on PMMA thin films are presented on the left panel, AFM height images and KFM images of the PEI patterns obtained after development of the charge patterns in an aqueous PEI solution are shown on the middle and the right panels, respectively. Representative sections are systematically shown under each image. Optical microscopy image presenting a larger area of the array of PEI squares is shown as the inset in the middle panel of b (scale bar = 50 μm).

100−200 nm thick PEI films obtained by incubation of a PEI solution. This indicates that such PEI patterns, composed of a very limited number of PEI chains have the same electrostatic trapping capability as that of thicker PEI films.

Assemblies of *Pseudomonas aeruginosa* Bacteria on PEI Patterns. Figure 3a presents typical AFM images in buffer medium, of *Pseudomonas aeruginosa* bacteria assembled on an array of 5 μm square PEI patterns fabricated by e-μCP nanoxerography. Ten bacteria are immobilized on each PEI square, demonstrating the repeatability of the process. Firmly fixed on the patterns, the bacteria can be imaged in contact mode in buffer medium over several hours, indicating that they neither suffer any damage nor get displaced. Figure 3b shows a zoom-in AFM deflection image of bacteria assembled on the PEI pattern (marked by a dashed square in Figure 3a). It shows ten *Pseudomonas aeruginosa* bacteria immobilized on one 5 μm PEI pattern. The mean height of 500 nm measured on the cross sections of the AFM height images is very typical of living *Pseudomonas aeruginosa* bacteria.[27,28] Force volume mapping experiments performed in buffer medium reveal heterogeneities of Young's modulus within the bacteria (Figure 3c).[29] Force volume mappings on 200 nm scan in the middle of the five bacteria labeled in Figure 3b allowed us to extract, the distribution of Young's modulus for each bacterium, using the Sneddon's model.[30] The elasticity values vary from 740 to 967 kPa, in agreement with the values usually reported in the literature.[27,28] These heterogeneities of elasticity from one bacterium to another, pointed out by these distributions, indicate the absolute necessity to perform statistical analysis on a large population of micro-organisms to obtain accurate and representative mechanical characteristics.

To verify if the cell cytoplasmonic membrane of the immobilized bacteria was not damaged by PEI patterns fabricated by nanoxerography, we employed a LIVE/DEAD cell viability assay (find details in the Experimental Section). *Pseudomonas aeruginosa* bacteria tagged with this assay were immobilized by incubation for 30 s on three different silicon substrates: (i) one covered by 5 μm square PEI patterns fabricated by e-μCP nanoxerography, (ii) one covered by a 150 nm thick PEI film obtained by incubation of the same PEI solution used for fabricating PEI patterns by nanoxerography, and (iii) one bare substrate. Images a and b in Figure 4 present typical fluorescence optical microscopy images, taken in buffer medium, of assemblies of bacteria on the PEI patterns and the PEI film, respectively. These images recorded after 20 min in buffer medium show that a large proportion of the bacteria assembled randomly on the PEI film are colored in red, meaning that their cell walls are permeable to propidium iodide

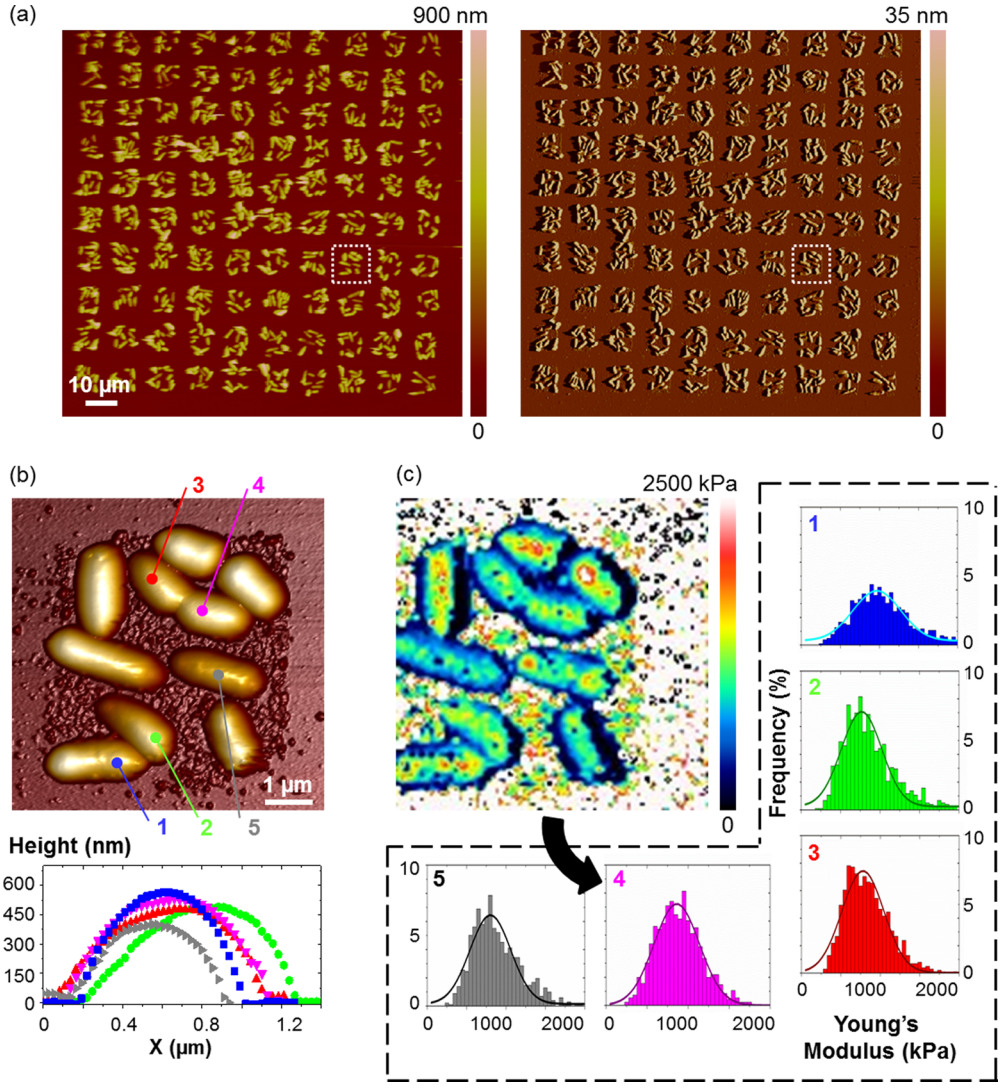

Figure 3. (a) AFM height image (left) and deflection image (right) in buffer medium of *Pseudomonas aeruginosa* bacteria assembled on an array of 5 μm square PEI patterns similar to the one presented in Figure 2b, (b) deflection image of ten *Pseudomonas aeruginosa* bacteria assembled on the square PEI pattern marked by a dashed square on a, with height profiles of the five bacteria labeled on the deflection image, (c) Young's modulus force volume mapping in buffer medium, of the area observed in b with Young's modulus histograms of the five bacteria labeled in b.

and thus damaged. On the contrary, all the bacteria selectively assembled on the PEI patterns fabricated by nanoxerography are colored in green, confirming that their cell walls are not damaged. Figure 4c quantifies the percentage of damaged bacteria as a function of time in the case of the three tested substrates. The rate of damaged bacteria is about 25% after only 25 min and strongly increases with time in the case of bacteria immobilized on the PEI film. After 1 h of immobilization on the PEI film, 40% of the bacteria are damaged. As reported in the literature, this high cytotoxicity is probably due to the dissolution of PEI chains in the buffer medium.[18,19] Mobile PEI chains are indeed responsible for the disruption of the bacteria membrane leading to necrotic cell death.[31] The amount of damaged bacteria assembled on the bare silicon substrate, around 10%, is weak and stable with time. It probably corresponds to the naturally dead bacteria in the starting culture media. Similar results are observed in the case of bacteria assembled on PEI patterns, indicating that PEI patterns are not toxic for bacteria. This observation points out a major advantage of PEI patterns fabricated by nanoxerography: PEI patterns, composed of a limited amount of PEI chains selectively assembled on charge patterns (no PEI chains are deposited outside charge patterns), are strongly attached to the substrate through electrostatic interactions. The antibacterial activity of PEI thus vanishes since PEI molecules, losing their mobility, are unable to penetrate into the bacteria.

To control the number of bacteria immobilized on each PEI pattern, two main experimental parameters of AFM nanoxerography were varied: the surface potential of charge patterns and their lateral size. Figure 5a presents a first series of ten PEI patterns fabricated from 5 μm square charge patterns written with decreasing (in absolute value) voltage pulses (−80, −75, −70, −65, −60, −50, −40, −30, −20, and −10 V, from left to right). The surface potential of such charge patterns varies respectively from −3 V down to −500 mV. No charge pattern was visible when −10 and −20 V voltage pulses were used, indicating that there is a pulse voltage threshold between −20 and −30 V, in agreement with previous results.[24] The development by incubation of a PEI solution conducts to 2 nm thick PEI patterns, independently of the surface potential of charge patterns (between −3 V and −1.15 V). All these PEI patterns present a surface potential of +80 mV. No PEI

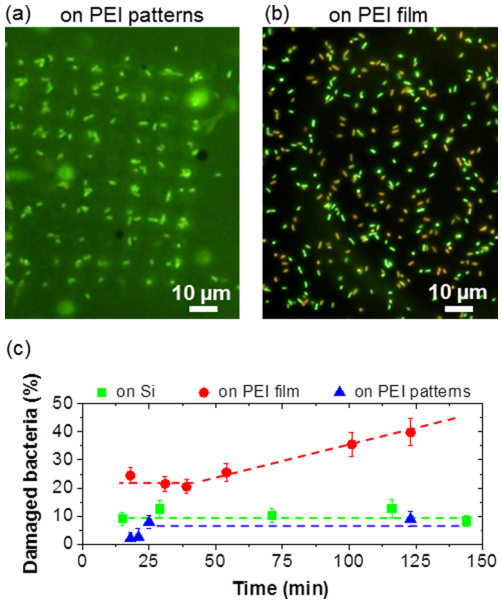

Figure 4. Fluorescence microscopy images in buffer medium of *Pseudomonas aeruginosa* bacteria tagged with the LIVE/DEAD assay, 20 min after their assembly on (a) an array of 5 μm square PEI patterns similar to the one presented in Figure 2b and (b) a continuous 150 nm thick PEI film, (c) percentage of damaged bacteria as a function of time when assembled on a bare silicon substrate (green square symbols), a 150 nm PEI film (red disk symbols), and PEI patterns (blue triangle symbols). Dashed lines are plotted as visual guides.

assembling was observed on the −770 mV charge pattern, revealing that the electric field generated by this charge pattern was not strong enough to trap PEI chains. This indicates the existence of a threshold of surface potential of charge patterns (around −1 V) for effective PEI adsorption. A more precise analysis of topographical AFM images of PEI patterns reveals a significant decrease of the roughness of PEI patterns with increase in absolute value of the surface potential of charge patterns (data not shown). This indicates that increasing surface potential in absolute value, i.e., electric field generated by charge patterns, increase the density of assembled PEI chains per pattern. Similar results were reported on directed assembly of charged colloidal nanoparticles by AFM nanoxerography,[32,33] where an increase of the surface potential of charge patterns led to an increase of nanoparticle density assembled on charge patterns. Figure 5b illustrates a second series of ten PEI patterns fabricated from square charge patterns of various sizes ranging from 5 μm down to 500 nm (from left to right) by 500 nm steps. As expected, whatever the initial dimension of the charge pattern, the thickness of the obtained PEI patterns is constant at about 2 nm. Their surface potentials are also constant at around +80 mV; the observed smaller values for the 1 μm and 500 nm patterns come from the average artifact effect inherent to KFM measurements.[34−36]

Pseudomonas aeruginosa bacteria were then assembled on ten series of PEI patterns similar to the ones depicted in Figure 5. Figure 6 reports the mean number of bacteria immobilized per PEI pattern while independently varying the surface potential of the charge patterns (red triangle symbols) and their lateral size (blue disk symbols). The mean number of bacteria fixed per pattern increases with the surface potential in absolute value of the charge patterns. As previously mentioned, an increasing density of PEI chains assembled on charge patterns creates more anchoring points for bacteria. On the other hand, we observed that the mean number of bacteria assembled per pattern increases with the lateral size of the PEI patterns. The reason for this fine control of the bacteria number per pattern is purely geometrical: once attached, a single micron-sized bacterium does not leave sufficient room for others to be attached on the same pattern. The small interbacteria distance observed in Figure 3b indicates that electrostatic repulsion between negatively charged bacteria is not a major factor

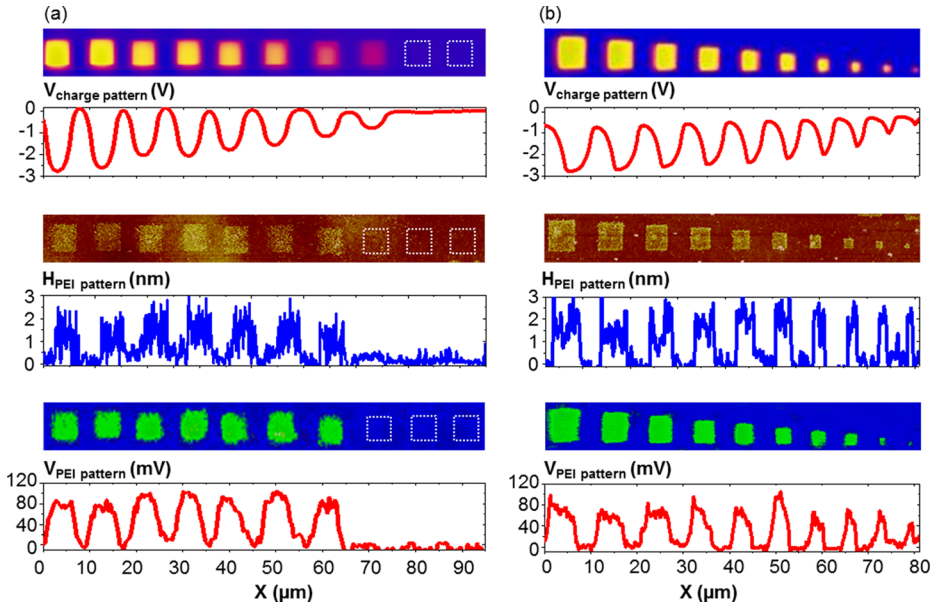

Figure 5. PEI patterns fabricated by AFM nanoxerography using charge patterns of (a) decreasing surface potentials in absolute value and (b) decreasing lateral sizes (from the left to the right). Data reported are KFM surface potential images and associated sections of the charge patterns (top), AFM height images and associated sections of the PEI patterns after development (middle) and KFM images and associated sections of the PEI patterns (bottom).

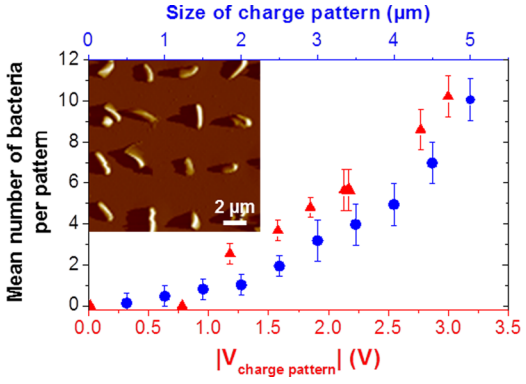

Figure 6. Evolution of the mean number of bacteria assembled per PEI pattern with the lateral size (blue disk symbols) or the absolute value of the surface potential of the charge patterns (red triangle symbols). A typical AFM deflection image in buffer medium of an array of single *Pseudomonas aeruginosa* bacteria immobilized on 1 μm PEI patterns is shown in the inset.

limiting the number of bacteria per pattern. The fine-tuning of any of these two parameters (surface potential and lateral size of charge pattern) allows adjusting the number of trapped bacteria immobilized per PEI pattern down to a single bacterium, as illustrated in the inset of Figure 6.

4. CONCLUSION

We present a novel approach to tackle a vital prerequisite of AFM characterizations of living micro-organisms in their culture media: their immobilization on predefined areas of a substrate. This approach consists in electrostatic immobilization of biological objects of interest on PEI patterns generated by electrostatic trapping of PEI chains on charge patterns written on electret thin films by AFM or e-μCP (so-called nanoxerography process). As a proof of concept, we validated it using opportunistic pathogens, *Pseudomonas aeruginosa* bacteria. The flexibility of nanoxerography allowed us to design PEI patterns with various geometries, sizes and densities of PEI chains. Obtained PEI patterns, 2 nm thick, present a constant positive surface potential similar to that of 150 nm thick PEI films. But contrary to such continuous films, the strong and selective attachment of a very limited amount of PEI chains prevents micro-organisms immobilized on these PEI patterns from being damaged. We demonstrated that the number of bacteria immobilized on each PEI pattern can be controlled down to unity by either tuning the lateral size or the surface potential of charge patterns. This approach allows user-friendly AFM characterizations in buffer medium, on large populations of individual bacteria without denaturing them. More importantly, this technique may constitute a generic route for electrostatically assembling any live biological object of interest for subsequent statistical characterizations by AFM, within its culture medium.

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Notes

The authors declare no competing financial interest.

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