Electric polarization properties of single bacteria measured with electrostatic force microscopy

Theoretical and practical studies of Dielectric constant of single bacteria and smaller elements

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DOCTORAL THESIS

5 Experimental methodology for single bacteria dielectric characterization

5.1 Materials

The main instrument used for the experimental part of the thesis was an Easyscan 2 FlexAFM by Nanosurf™. It consists of a sample stage (with passive vibration isolatoion), a sample holder (with connection to ground) that is mounted magnetically to the sample stage. The AFM head has a tip holder were the tip is mounted by the help of small notches that automatically align the laser (if the same tips are mounted) called easyalign. To maintain an electrical contact a conductive clip is used to be able to bias the tip with an electric potential. The controller is in charge to electrical conversions, amplifications, piezo control, etc. and it is connected to a breakout box from where we have access to different signals, X, Y, Z piezo input/output, tip bias input/output, deflection, 2 auxiliar imputs/outputs, etc. Finally there is an environmental control chamber, with a N_2 input and an outlet (to manually lower the humidity) and a humidity sensor (Rotronic AG™) to monitor the desired relative humidity value. A picture of the system can be seen in Figure 5.1.

Besides the AFM system we used a desktop active anti vibration table (Table Stable Ltd.). The table successfully passed the quality test (data not shown). Finally, an external lock-in amplifier (Anfatec™) is connected to the breakout box.

5.2 Sample mounting

The samples were prepared over a conductive magnetic circular sample holder of about 1 cm in diameter. The sample was glued from the bottom with silver paint to the holder. A small cable was then glued from one side with the same paint. Once dry the other side of the cable was screwed to the conductive sample stage. This stage is connected to the AFM head ground through a cable with a soldered resistor of several giga Ohm's to avoid large current flux through the small tip apex that could damage it when in contact to a conductive substrate.

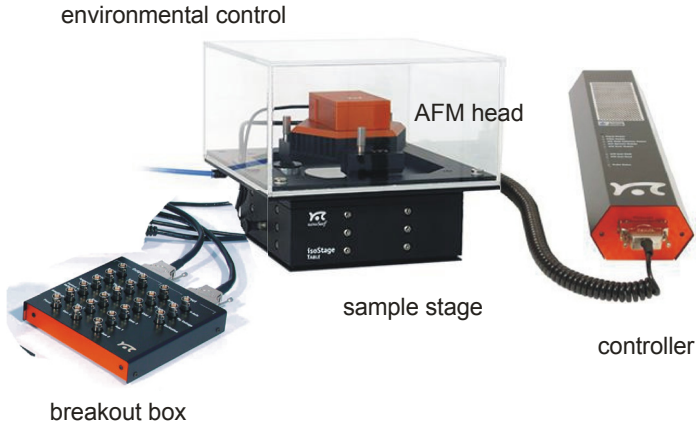

Figure 5.1 *Photography of the Nanosurf AFM system with its sample stage, breakout box, controller and environmental chamber. (image from nanosurf webpage).*

5.3 Tip calibration

One of the crucial aspects for quantitative measurements is to have a good calibration of the tip geometry, so that effects associated to it can be taken into account. The tip geometry calibration procedure has been developed by the research group first based on DC electric current measurements (capacitance) [33]. It consist in measuring an approach curve on a metallic substrate and use it to fit a theoretical model with the tip geometry (apex radius and cone angle mainly) as fitting parameters.

In our case the AFM tips used are doped diamond tips CDT-FMR from Nanosensors™. The CDT-FMR nominal values are, H = 15 μm, R = 100-200 nm (smaller locally), θ = 30°, K = 3-5 N/m. A scanning electrical microscope (SEM) image of one tip can be seen in *Figure 5.2*. The values are within the nominal.

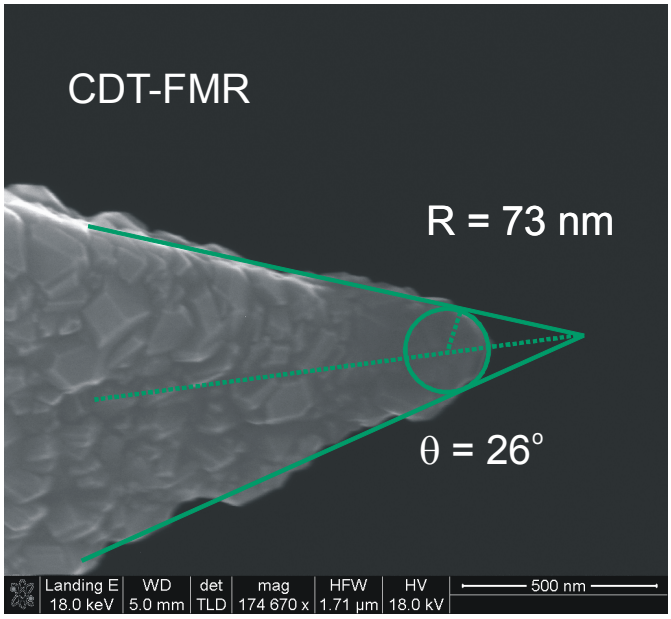

Figure 5.2 *SEM image of a CDT-FMR (nanosensors) giving a (local) radius of 73 nm and an aperture angle (θ) = 26°.*

The specific procedure implemented in the experimental set up used in the present work of thesis is described on what follows. We started in dynamic topographic mode (to avoid high loads to the biological samples) with a set point of about 70% of the free vibration amplitude. Once in the intermittent contact regime the feedback is activated and a topographic image is taken. In the obtained image a bare part of the metallic substrate is identified and the tip is positioned in this region. The imaging mode is changed to contact mode with a certain deflection as set point. Then an AC potential is applied through the external lock-in at a certain frequency lower than the resonance (~2 kHz). The gain at this frequency is calculated with a recorded frequency

spectrum which is used by our Matlab™ routines. All the other gains (AFM, Lock-in, etc.) have to be very carefully recorded so a correct unit conversion can be done in the analysis with the Matlab™ scripts.

From here the spectroscopy mode in the Easy Scan 2 software is used to do several curves (N=10-15) retracting and approaching a quite long range 1 μm since later the value of z + h (sample height) will be used as reference and will be subtracted. The approach stops when a certain deflection (set point is reached) and it is repeated until the number of curves is reached. In this case both, the capacitance gradient and the deflection-piezo extension are recorded. This is important since later our custom scripts will obtain the real zero deflection (to know the actual z from the sample) linearizing both, the contact and the non-contact regions. The intersection point is the zero deflection that is used to calibrate each of the curves. In Figure 5.3 we see were the zero deflection point is taken.

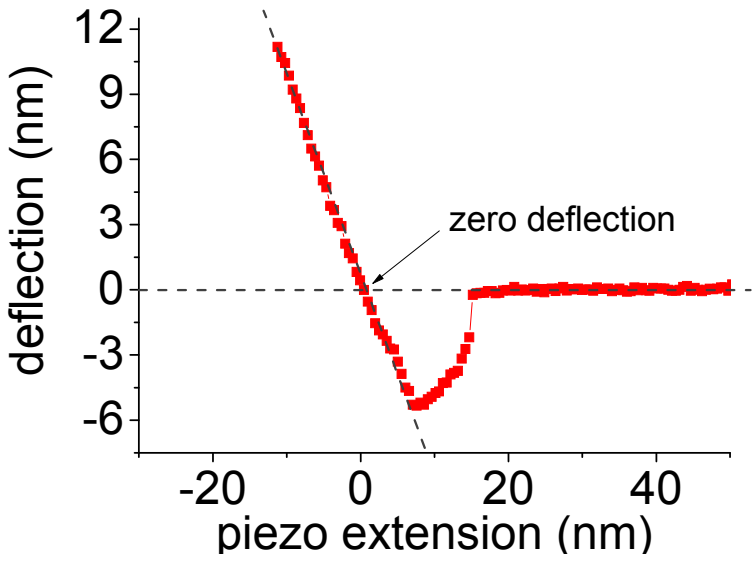

Figure 5.3 *A deflection-piezo extension curve (red symbols) where the non-contact region (horizontal dashed line) and the contact region (diagonal dashed line) are linearized. The intersection point is taken as the z = 0 (zero deflection).*

Using the conversion factors and normalizing the capacitance gradient using the obtained zero the curves can be fitted with least squares error routines programed in Matlab. These routines take the data from many simulations performed with R as a parameter. Simulations are done for R = 50 nm to 300 nm, in steps of 1 nm and with $\theta = 30^{o}$ fixed to the nominal value. The range of distances taken is from 5 nm up to 1 μm, to resemble all the parameters used in the experiments. The fitting routines, though, are zeroed at 100 to 150 nm since this is enough to obtain a good approximation of the tip radius. There are analytical approximations for the calibration [34] although we decided not to use them since they underestimate the radius by about an 18% [29]. All the points of the N (10-15) curves are joined together and then fitted using a least square root error. See Figure 5.4 for an example of tip calibration.

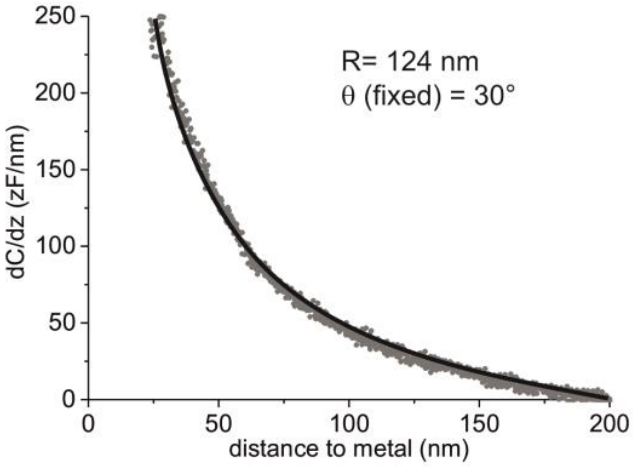

Figure 5.4 *Example of the fitted data of 10 approach curves to metal. The grey symbols correspond to 10 curves with 200 points each. The solid black line is the fitting function with R = 124 nm and $\theta = 30^{o}$.*

The main problem when using biological samples could be a tip modification after or in the middle of an experiment since proteins,

lipids, liquid medium, high loads, etc. could mess or change the aspect of the apex. That is why we calibrate it in the metal region before and after the dielectric extraction on the insulator region. Whenever the tip radius changes more than 20% all the data in the experiment was discarded and a freshly new cantilever was mounted. An example of the stability of the tip radius can be seen in Figure 5.5 where several approach curves to the conductive substrate previously, during and after the experiment. First 5 curves were fitted (θ fixed to 30^{o}) over clean freshly cleaved HOPG. During the experiment the sample substrate (doped silicon) was used for the tip calibration. And finally after the experiment the sample was changed again with a clean HOPG substrate. All the curves were giving very similar values (170-190 nm) showing that neither the substrate nor the change of the sample or the experiments performed were giving a substantial tip modification.

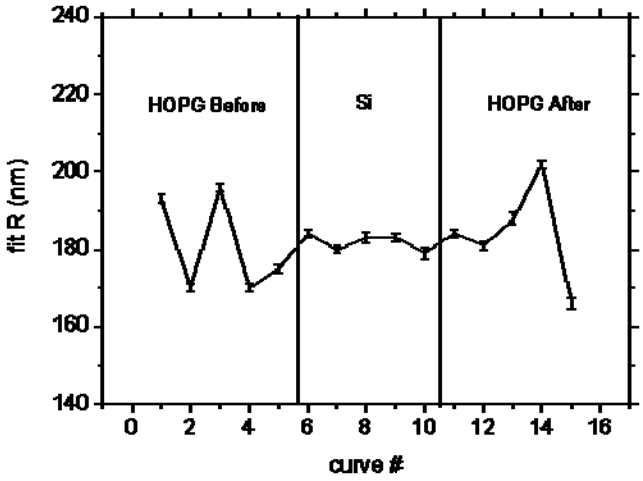

Figure 5.5 *Calibration tip curves before (clean HOPG), during (doped silicon) and after (again clean HOPG) the experiment. The obtained value was 182 +/- 2.*

5.4 Force distance methodology

The chosen methodology to extract the permittivity of the bacteria is based on the force distance method. This method was originally proposed by members of our research team in the context of capacitance measurements on dielectric thin films [33]. This procedure is similar to the tip calibration, but in this case the curves are taken over the bacteria and the ones closer to the middle are taken since the simulation is axial symmetric and there could be effects if we go to the laterals.

In this case what we fit is the obtained capacitance gradient on the bacteria minus the capacitance gradient on the metal at z + h (height of the bacteria) which is similar to what we do with the constant height mode also used in our group [27].

The fitting routine in this case is a two-dimensional function (z, and ε_r) and the permittivity is extracted curve by curve (N=10-15) and later averaged obtaining a mean value and its standard deviation.

To see if the contact region could affect the results (since bacteria are expected to be softer than metal), several deflection-piezo extension curves were performed in both bacteria and HOPG substrate and the gradient in the contact region was compared. In Figure 5.6 we see that the difference is not very significant and the force-distance curves were almost similar. This could be an effect of the strong cell walls of bacteria.

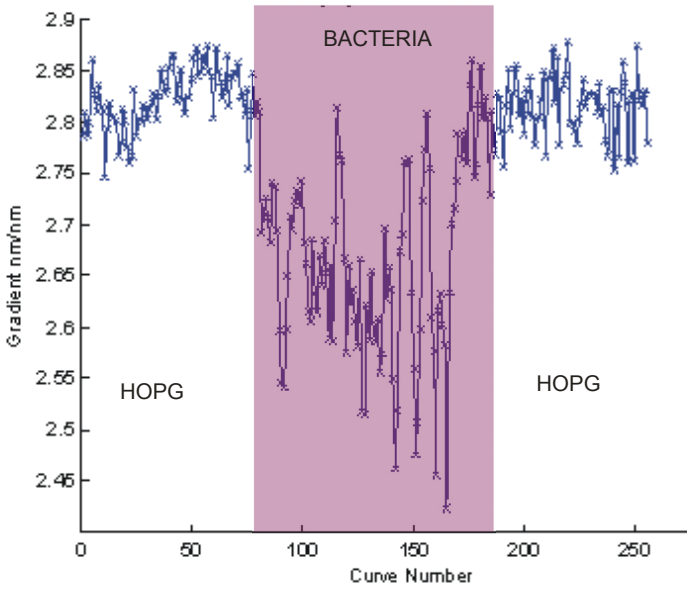

Figure 5.6 *Gradient of the contact region in HOPG and bacteria. The slope is quite similar and both are quite lineal (data not shown).*

We chose this methodology since when using the constant height function implemented in the AFM software does not give us access to all the force curves at each line. Also a quite different contrast line by line was observed which could be meaning that each line is taken at slightly different height (because of drift, set point not properly recovered, and speed). In image a constant height measurement is done at different heights over a silicon oxide (SiO_2) and different contrasts can be seen line by line, most evident when closer to the silicon (top middle of the image).

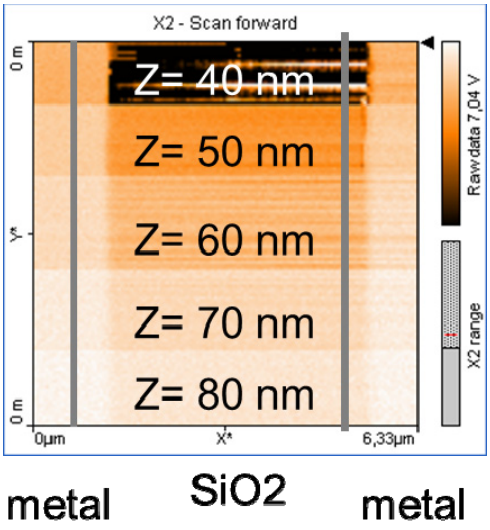

Figure 5.7 *Constant height contrast done on a silicon oxide film over a metal at different heights z= 40, 50, 60, 70, 80 nm. We can see that the contrast change within the same height, probably due to slight uncontrolled differences from line to line.*