UNIVERSITÀ DEGLI STUDI DI MILANO
DIPARTIMENTO DI FISICA

Investigation of bio-mechanical interactions in cellular system by atomic force microscopy

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The role of forces is fundamental in a wide variety of biological processes, such as cell adhesion with the micro-enviroment, migration, cell proliferation and differentiation[1]. The bio-mechanical processes through which the cell perceives, interacts and reacts to physical and morphological stimuli, induced by the surrounding environment, like the extracelluar matrix (ECM), is called mechano-transduction[2]. The cell's ability to perceive the nano-topographical features, of the surrounding enviroment, is mediated by specific proteins (integrins) clustered together in the so called **Focal Adhesions (FAs)**. A quantitative analysis of cellular interaction with the extracellular matrix is necessary to understand how cells regulate adhesion in order to develop and mantain multicellular tissues. Due to its complex configuration, unraveling the role of the extracellular matrix is particularly challenging. Previous works have demonstrated the fundamental role of morphological disorder on nanometer scale in order to mimic properties of the ECM[3,4]. Here we present a novel approach based on force spectroscopy to quantify the cell reaction induced by a nanostructured micro-environment. We developed a new kind of colloidal nanostructured probes for atomic force microscopy able to simulate topographical properties of ECM and measure the cell-probe interaction forces. Results are in good agreement with previous works and demonstrate the high dynamical behavior of cells focal adhesion spots, and how morphology-induced frustrated interaction seems to be responsible for mechano-trasductive processes.

Mechano-sensitive signaling and induced differentiation via mechanical stimuli

Figure 1. The nostructured Zirconium Oxide (ns-ZrOx) is a thin film grown be the Supersonic Cluster Beam Deposition (SCBD) technique with specific morpholigy and disorder on the nanoscale dimension, controlled via the time deposition, able to simulate the topographical features of the extracellular matrix.

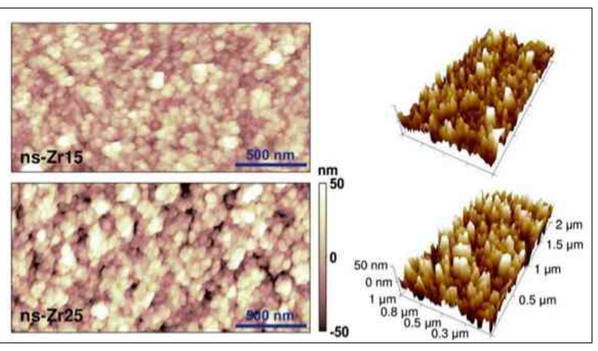
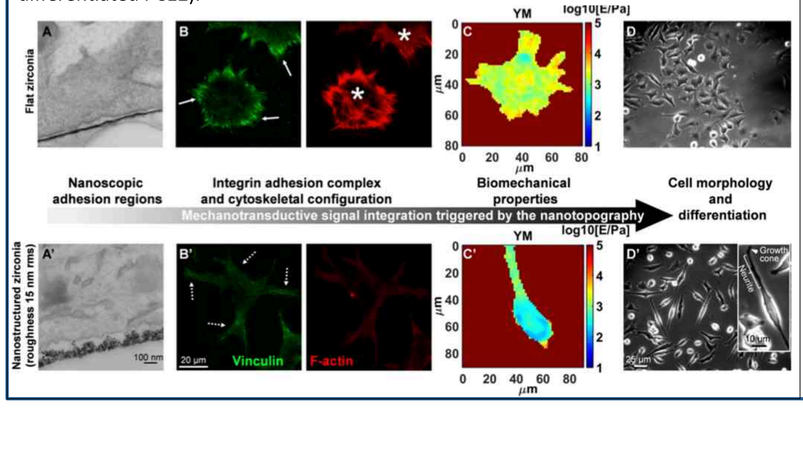


Figure 2[4]. (A, A') TEM images shows the interface between PC12 cells and (A) flat zirconia (produced by E-beam evaporation) or (A') nanostructured zirconia surface fabricated by SCBD. Cells interact **only with the upper part of the surface asperities, leading to spatial confinement in the growth of FAs** . (B, B') The staining of vinculin (a crucial FAs component) recorded by TIRF microscopy shows (B) FA formation (arrows) on flat zirconia, compared to (B') the smaller structures with smaller dimensions (dashed arrows) on the nanostructured surface. On the right, the corresponding fluorescence images of filamentous actin cytoskeleton are shown. The asterisks indicate zones of stress fiber formation on flat zirconia, not present on the nanostructured zirconia. (C, C') Young's modulus maps **the cellular elasticity** on (C) flat or (C') nanostructured zirconia and **demonstrate a lower cellular rigidity in the latter condition.** (D, D') The phase contrast images shows the effect of the mechanotransductive signaling on PC12 differentiation, (D') like morphological changes (neurite outgrowth) on the nanostructured surface (inset, typical morphology of differentiated PC12).



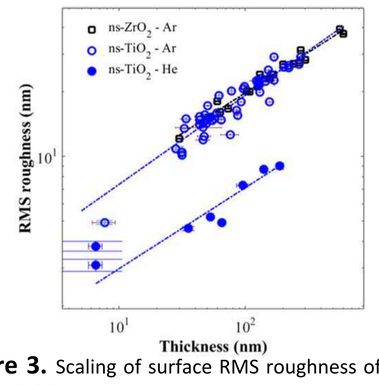
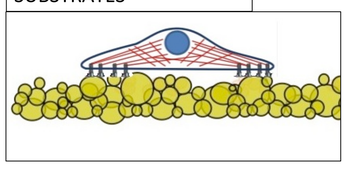
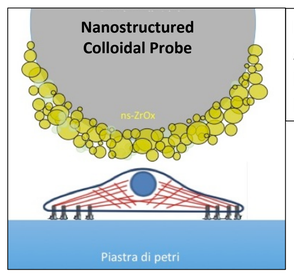


Figure 3. Scaling of surface RMS roughness of ns-TiO2 and ns-ZrO2 films deposited using different carrier gases. A linear fit in log–log scale highlights the power law character of the roughness evolution with film thickness (deposition time)

Inverted geometry ➔Quantification of interaction force exerted/felt by cells

Standard geometry experiment: CELL ON NANOSTRUCTURED SUBSTRATES





Novel approach with AFM: CELL UNDER NANOSTRUCTURED PROBE

In the standard cells - ns-ZrOx interaction experiment, cells are deposited on the nanostructured thin film and examined via optical imaging (fluerent microscopy/phase contast) or via standard indentation method (AFM). The new approach we present, is based on the idea that the **interaction region,** where the most interesting molecular precesses happens, is hidden under the cell itself. With this new techinque we build a totally new kind of AFM's probe in order to move the interaction region on the top of the cell; **opening a new window on the understanding of mechano-trasductive events.** Our purpose are reported below:

- Quantify the adhesion force between **cell and nanostructured ZrOx;**
- Observe **IN SITU** the growth of FAs and measure their strenght;
- Get into relation the adhesion force with specific cellular reaction;

Production of Nanostructured colloidal probes (ns-CP)

We developed a protocol for production and characterization for a new kind of polymeric colloidal nanostructured probes. The steps through witch this new kind of probes are built are reported and below.

1. Polymer spheres, with radius 15.1 µm, are first extracted and cleaned from their buffer. The cleaning procedure consist in a three sequential centrifugation in a 1:1 water and ethanol solutions, replacing the old with new solution after every centrifuge. The cleaned spheres are then mono-dispersed in a toluene solution and later deposited on a glass slide.
2. Microspheres are attached to cantilever through epoxy adhesive glue (RS, 9845, epoxy hardener).
3. A nanostructured thin film of zirconium oxide is then grown on the probes via a cluster assembling technic called supersonic cluster beam deposition (SCBD). The morphological properties of the film is analysed and quantified using standard AFM tapping technic, the roughness-thickness relation of the film growth is evaluated in order to obtain probes with desired topographical features.

Experiment

Experiments are performed putting the ns-CP gently in contact with the top of the cell and waiting until Fas starts to grow (in this context contact time was 60, 120, 240, 600 sec) and then pulled away from the cell in order to break bonds and quantify their strenght. This procedure is repeated in **three different configuration**: with ns-ZrOx Probe, with Flat probe, to compare differences in cellular reactions when stimulated with different morpholigies; in the end a controll experiment was performed, the *4b4* antibody was given to cells to inhibit the Fas formation.

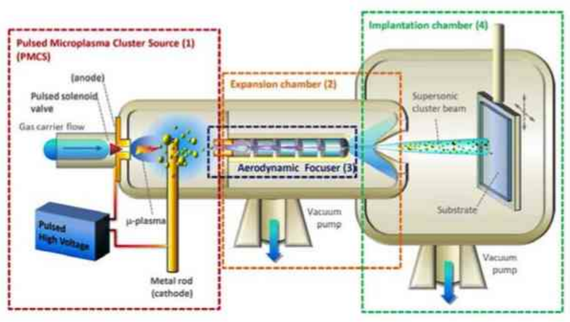


Figure 5. Schematic representation of the **SCBD.** (1) The pulsed microplasma cluster source. (2,3) The expansion chamber with aerodynamic focuser lenses. (4) The deposition chamber.

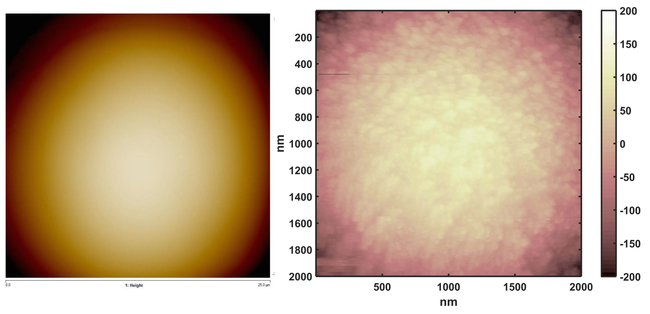


Figure 6. (A) AFM image of a microsphere cleaned before the supersonic deposition. (B) AFM image after the deposition of nanostructured ZrOx thin films.

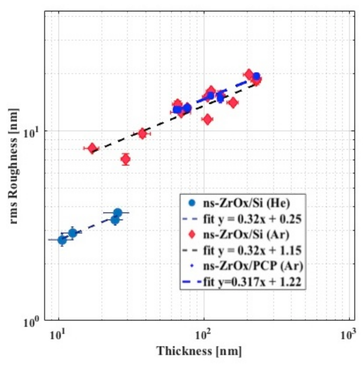
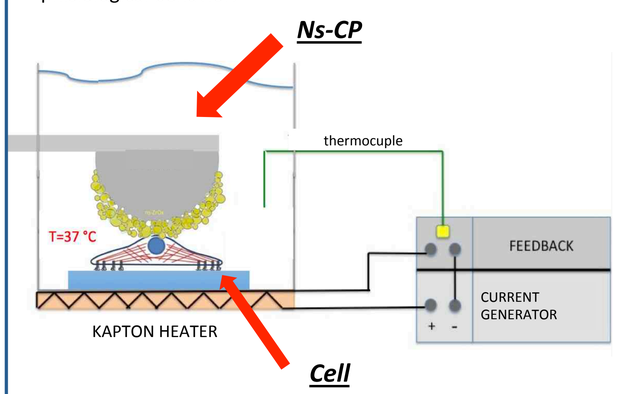


Figure 7. Scaling law of ns-ZrOx with different carrier gas on plane substrate (red and light blue dots) and on the colloidal probe (blue dots). Despite the difference in geometry of the substrates (Plane Vs Spherical), all films obey the same scaling law, compatible with the ballistic deposition regime.With the rms Roughness/Thickness relation is possible to obtain probes with specific and extremely controlled morphological properties.

Figure 8. Schematic representation of the experiment procedure**.** Feedback and current generator are needed to keep cells in the correct temperature (37 °C) to ensure phisiological behavior.



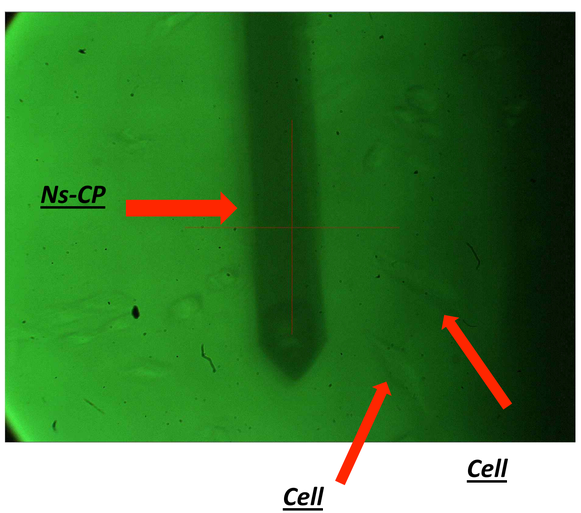
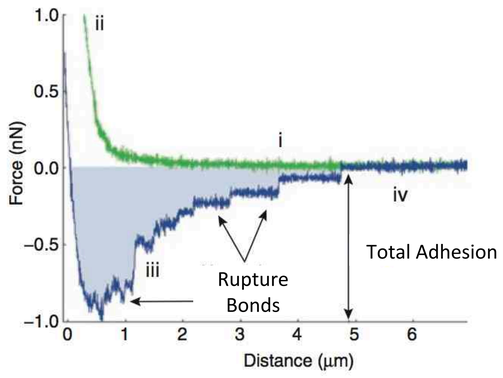
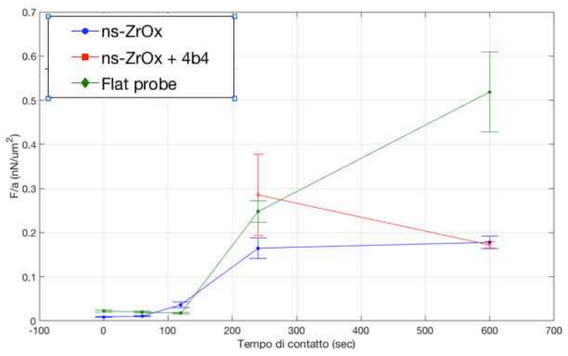


Figure 9. Image made by transmission optical microscopy of the nanostructured colloidal probe over PC12 cells during the experiment.

Results

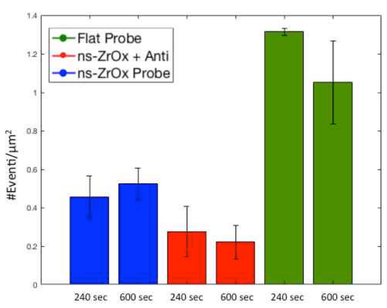
We measured the total adhesion of cell in function of time on different morpholgies and then we analyzed the density and the strenght of the single bond rupture events.



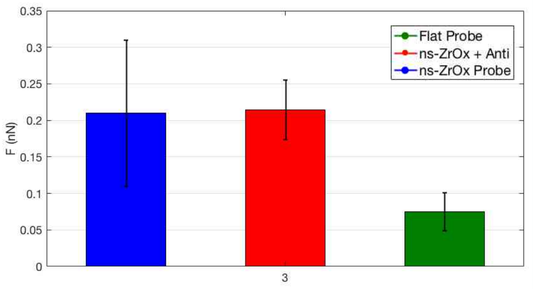


Total Adhesion: In order to compare the values of the three experiments (ns-CP, flat-CP, ns-CP+4b4) we renormalized al the adhesion values in function of the effective cell-probe contact area, evaluated by the Hertz model. The adhesion with the flat probe is higher than the nanostructured one caused by a bigger spreading of FAs with no spatial confinement. The **first** formation of FAs are represented by the jumps in the adhesion around 150 s.

Rupture events per contact area: In cells establishing probes contact, integrins are frequently clustered into small focal complexes that may later maturate into larger focal adhesions structures. Purpose of this analysis is to understand and quantify the difference in cells FAs creation stimulated by particular morphologies. Figure shows the number of unbinding events per contact area.



Addiction of the β1 integrin inhibitors antibody 4b4 lead to a consistent reduction of detaching events compared to the physiological conditions. The number of events for the flat probes is much higher than in the case of nanostructured one, again this could be explained considering that in the flat interaction there is no spatial confinement of contact regions, enabling cell to grow bigger integrin-clusters.



Single bond strenght: The intensity of single bond with nanostructured surface is the same with or without antibody 4b4, meaning that, where they survived the bonds are of the same nature. On the contrary bonds with flat surface are more weak and the values reflects the single integrin binding.

Conclusions

- We built a totally new kind of nanostructured colloidal probes able to simulate ECM's topographical features.
- We developed a setup to measure IN SITU the growth of FAs in living cells.
- Measurment successfully demonstrated a behavior referable to an integrins-mediated adhesion.

Results are in good agreement with previous work and show the early steps of Focal Adhesion formation. In partucular we saw how frustration, in FAs formation, due to the spatial confinement applied by asperities, leads to a **stronger single integrin adhesion.** At the same time **bigger spatial FAs are made by weaker single integrin adhesion**.

References

[1] Eyckmans, J et al. A Hitchhiker's Guide to Mechanobiology. *Dev Cell* **21,** 35–47 (2011).
[2] Dalby, M. et al.Harnessing nanotopography and integrin-matrix interactions to influence stem cell fate. *Nat Mater* **13,** 558–569 (2014).
[3] Schulte, C. *et al*. Conversion of nanoscale topographical information of cluster-assembled zirconia surfaces into mechanotransductive events promotes neuronal differentiation. *Journal of Nanobiotechnology* **14,** 18 (2016).
[4] Schulte, C. et al. Quantitative Control of Protein and Cell Interaction with Nanostructured Surfaces by Cluster Assembling. *Acc. Chem. Res.* **50,** 231–239 (2017)