AFM BioMed Conference
Barcelona 2007

International Meeting on AFM in Life Sciences and Medicine

19, 20, 21 April 2007
Barcelona, Spain

Abstracts

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Book of abstracts

International Meeting on AFM in Life Sciences and Medicine

BARCELONA
Thursday 19 - Saturday 21 April 2007
Programme

Day 0 – Wednesday 18, April
16.00-20.00  Registration (NH Constanza hotel)

Day 1 – Thursday 19, April
08.00-12.30  Registration (NH Constanza hotel)
10.00-12.30  Posters set up (CosmoCaixa, Conference site, Science Museum)
10.00        Transfer 1 from hotel to conference site for all Posters and Speakers of Day 1
10.30        Transfer 2 from hotel to conference site
11.00        Transfer 3 from hotel to conference site
11.30-12.30  Coffee/Snack/lunch available at conference site
12.30        General introduction of the conference, Organizing committee
12.40        Opening keynote address
             What is the biological relevance of the specific bond properties revealed by single molecule studies?
             Pierre Bongrand, INSERM, Marseille, France
13.40        Break
Day 1 – Thursday 19, April (cont.)

**SESSION I | CELLS, CELLULAR INTERACTIONS**

13.55 *Yves Dufrêne* introduces the session and Invited speakers

14.00 Adapting AFM for studying living cells  
*J.K. Heinrich Hörber* University of Bristol, Bristol, United-Kingdom

14.30 AFM measurement of leukocyte adhesion to endothelial cells  
*Vincent Moy* University of Miami, Miami FL, USA

15.00 Measurement of cell mechanical properties by atomic force microscopy  
*Daniel Navajas* Universitat de Barcelona, Barcelona, Spain

15.30 Coffee Break / Posters

16.00 Mechanical dynamics during cell death  
*Andrew Pelling* University College London, United-Kingdom

16.15 Elastic membrane heterogeneity of living cells revealed by stiff nanoscale membrane domains  
*Frank Lafont* Pasteur Institute, Lille, France

16.30 Probing microbial interfacial properties by force spectroscopy and microelectrophoresis  
*Fabien Gaboriaud* Nancy-University, CNRS, Villers-lès-Nancy, France

16.45 Monitoring of biomechanical cellular activity induced by vascular active agonists with AFM  
*Charles M. Cuerrier* Université de Sherbrooke, Canada

17.00 Cell topometry analysis can replace direct measurement of fluid permeability  
*Christoph Riethmuller* University of Munster, Germany

17.15 Exploring the surface of living microbial cells using AFM  
*Yves Dufrêne* Université catholique de Louvain, Louvain-la-Neuve, Belgium
17.45  Coffee break / Poster session # 1 (Sessions I and IV)
18.45  Departure for the NH Constanza hotel
19.30  Arrival at the NH Constanza hotel
20.00  Departure for the concert
20.30  Concert in the church (Monastir de Pedralbes)
21.30  Tapas in « refectori » of the Monastery
23.00  Departure for the NH Constanza hotel
Day 2 - Friday 20, April

**SESSION II | SINGLE MOLECULAR RECOGNITION, AFFINITY, UNFOLDING FORCES**

08.55  **Peter Hinterdorfer** introduces the session and Invited speakers

09.00  Nanobiotechnological drug screening: imaging, sensing and locating ligands that drive cellular machines
       **Daniel J. Müller** Center of Biotechnology, Dresden, Germany

09.30  Molecular devices: sensors, grabbers and actuators
       **Hermann Gaub** Ludwig-Maximilians-Universität, München, Germany

10.00  A rotaxane based method of determining hairpin location and kinetics in nucleic acids with an AFM
       **Brian Ashcroft** University Tempe, Arizona, USA

10.15  Single molecule force spectroscopy mapping
       **Arturo M Baró** Instituto de Ciencia de Materiales de Madrid (CSIC), Spain

10.30  Coffee Break / Posters

11.00  Molecular mechanisms contribute to the fracture resistance of bone: repeatable energy dissipation by sacrificial bonds and hidden length in molecular networks
       **Georg E. Fantner** University of California Santa Barbara, CA, USA

11.15  Mechanical properties of glucans/Dectin-1 interactions: Implications for pathogen recognition
       **Liz Adams** University of Delaware, USA

11.30  Myomesin: a molecular spring with adaptable elasticity
       **Patricia Bertoncini** CNRS Institut des Matériaux Jean Rouxel, Nantes, France

11.45  Atomic Force Microscopy study of interactions between supercoil-dependent gene regulatory proteins and DNA
       **Sergey Chasovskikh** Georgetown University Washington, USA
12.00 Single molecule recognition force microscopy
Peter Hinterdorfer Johannes Kepler Universität Linz, Linz, Austria

12.30 Lunch

SESSION III | HIGH RESOLUTION IMAGING

13.55 Simon Scheuring introduces the session and Invited speakers

14.00 G-Protein coupled receptors are active as dimers and higher oligomers: a lesson from rhodopsin
Andreas Engel University of Basel, Basel, Switzerland

14.30 Dynamic behaviors of proteins at work captured by high-speed AFM
Toshio Ando Kanazawa University, Kanazawa, Ishikawa, Japan

15.00 Imaging of individual protein molecules with femto newton force sensitivity
Ricardo Garcia Instituto de Microelectronica de Madrid, Madrid, Spain

15.30 Coffee Break / Posters

16.00 The supramolecular architecture of junctional microdomains in native lens membranes
Nikolay Buzhynskyy Institut Curie, UMR-CNRS, Paris, France

16.15 High resolution AFM imaging of native single-standed DNA binding (SSB) protein – DNA complexes
Olivier Piétrement Institut Gustave-Roussy, Villejuif, France

16.30 Specific patterning of LH2 and LH1 protein complexes
M. Escalante-Marun University of Twente, The Netherlands
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| 16.45 | Characterization of transcription regulatory complexes of bacteriophage Phi29 by AFM  
Paloma Gutiérrez del Arroyo, Universidad Autónoma de Madrid, Spain |
| 17.00 | Structure and assembly of membrane proteins in native membranes by AFM  
Simon Scheuring, Institut Curie, Paris, France |
| 17.30 | Coffee break / Poster session #2 (Sessions II and III)               |
| 18.30 | Departure for the NH Constanza hotel                                |
| 19.00 | Arrival at the NH Constanza hotel                                   |
| 19.50 | Departure for Hotel Casa Fuster                                     |
| 20.15 | Cocktail and dinner in Hotel Casa Fuster                             |
|       | Keynote address                                                      |
|       | High speed AFM and quantitative indentation testing                  |
|       | Paul Hansma, UC Santa Barbara, California, USA                       |
| 23.15 | Departure for the hotel                                             |
Day 3 – Saturday 21, April

SESSION IV | MODEL MEMBRANES AND PROTEIN–MEMBRANE INTERACTIONS

08.55 Christian Le Grimellec introduces the session and Invited speakers

09.00 Fingerprinting membranes with force spectroscopy
Fausto Sanz Universitat de Barcelona, Barcelona, Spain

09.30 Quantitative analysis of coarsening and spatial domain distribution in a ternary membrane
Adam Cohen Simonsen University of Southern Denmark, Denmark

09.45 How can Atomic Force Microscopy help to understand sepsis?
Thomas Gutsmann Research Center Borstel, Germany

10.00 Dynamic strength of the interaction between lung surfactant protein D (SP-D) and saccharide ligands
Esben Thormann University of Southern Denmark, Odense, Denmark

10.15 Atomic force microscopy characterization of supported planar bilayers that mimic the mitochondrial inner membrane
Jordi Hernandez-Borrell Universitat de Barcelona, Spain

10.30 Coffee Break / Posters

11.00 Concept of dynamic DNA network dedicated to DNA-Protein interactions studies
Céline Elie-Caille FEMTO-ST Institute, CNRS, Besançon, France

11.15 Using Atomic Force Microscopy to Quantify Amyloid Formation at the Nanoscale
Martijn van Raaij University of Twente Enschede, The Nederlands
Day 3 – Saturday 21, April (cont.)

11.30  Alkaline phosphatase interactions with domains in supported bilayers
       Christian Le Grimellec  INSERM, Montpellier, France

12.00  Closing keynote address
       A little can go a long way!
       Mike Horton, UCL. Dept. of Medicine, United-Kingdom

13.00  End of the conference
       Transfers to the NH Constanza hotel and to the airport will be available
Organizing committee

Pierre Parot  
CEA/DSV, Marcoule, France

Jean-Luc Pellequer  
CEA/DSV, Marcoule, France

Daniel Navajas  
Universitat de Barcelona, Barcelona, Spain

Yves Dufrêne  
Université catholique de Louvain, Louvain-la-Neuve, Belgium

Peter Hinterdorfer  
Johannes Kepler Universität Linz, Linz, Austria

Simon Scheuring  
Institut Curie, Paris, France

Christian Le Grimellec  
Inserm, Montpellier, France

CEA is a French government-funded technological research organization. A prominent player in the European Research Area, it is involved in setting up collaborative projects with many partners around the world.

The University of Barcelona (UB), founded in 1450, can lay claim to being the leading university in Catalonia. It is the university with the most students and offers the widest and most complete range of courses. The UB is the leading centre for university research in Spain and is one of the largest in Europe in terms of the number of research programs and the excellence of its results.

The Bioengineering Institute of Catalonia (IBEC) was established in 2005 by the Government of Catalonia, the University of Barcelona and the Technical University of Catalonia. IBEC seeks to further the development of multidisciplinary research of excellence, from basic studies to medical applications, in the field of biomedical engineering.

*CEA: French Atomic Energy Commission - IBEC: Bioengineering Institute of Catalonia
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Keynote presentations

What is the biological relevance of the specific bond properties revealed by single molecule studies?

**Pierre Bongrand**

INSERM U600 - CNRS FRE2059  Laboratoire d'Immunologie  Hôpital de Sainte-Marguerite  Marseille, FRANCE

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High Speed AFM and Quantitative Indentation Testing

**Paul Hansma**

Department of Physics, University of California,  Santa Barbara, California, United States

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A little can go a long way!

**Mike Horton**

London Centre for Nanotechnology and Department of Medicine  University College London, United Kingdom
Journal of Molecular Recognition

The Journal of Molecular Recognition will broaden the scientific scope of the journal and in future will include AFM applications in the biological sciences. This will be initiated with a Special Issue presenting peer-reviewed papers selected from presentations made at the AFM BioMed 2007 Barcelona conference.

- Deadline submission: April 21 2007
- Submission of final version: July 2007
- Special Issue Editors: Yves Dufrêne, Pierre Parot, Jean-Luc Pellequer

Wiley - Journal of Molecular Recognition
Invited talks

Sessions

Session I | Cells, Cellular interactions
Session II | Single molecular recognition, affinity, unfolding forces
Session III | High resolution imaging
Session IV | Model membranes and protein-membrane interactions
SESSION I

Cells, cellular interactions

Session chair
Yves Dufrêne
Université catholique de Louvain
Louvain-la-Neuve, Belgium

Topics
Cell imaging, cell mechanics, cell adhesion
Session I: Invited talk

Exploring the surface of living microbial cells using AFM

Yves F. Dufrêne

Unité de Chimie des Interfaces – Université catholique de Louvain – Croix du Sud 2/18 – 1348 Louvain-la-Neuve – Belgium

There is a need in current microbiological and biophysical research to develop new, high resolution tools for probing the structure, properties and interactions of microbial cell surfaces. The advent of atomic force microscopy (AFM) has recently opened a wide range of novel possibilities for probing the surface of microbes (bacteria, yeast, fungi) in their native environment (1-3). Using AFM imaging in aqueous solution, microscopists can visualize cell surface nanostructures (surface layers, appendages), follow physiological changes (germination, growth) and monitor the effect of external agents (antibiotics, metals) in real-time. Further, using force spectroscopy researchers can learn about local biomolecular interactions and physical properties. For instance, spatially-resolved force mapping offers a means to determine variations of elasticity and chemical properties at the subcellular level, thereby providing complementary information to classical characterization methods. Functionalizing the AFM tip with chemical groups or biomolecules enables quantitative measurements of surface charge, surface energy and receptor-ligand interactions. Finally, force spectroscopy can be applied to single cell surface molecules to gain insight into their mechanical properties. Clearly, these novel AFM-based experiments contribute to improve our understanding of the structure-function relationships of microbial cell surfaces and open the door to new applications in biotechnology and medicine.

Session I: Invited talk

Adapting Atomic Force Microscopy (AFM) for studying living cells

J. K. H. Hörber

HH Wills Physics Laboratory, Tyndall Avenue, Bristol BS8 1TL, UK

In the last 20 years, AFM has emerged as a powerful technique for biological research giving ultra high resolution structural information and allowing measurements of molecular forces at the single molecule level. An important aspect for AFM studies on living cells from the very beginning was the integration of the instrument into an optical microscope. Furthermore, a combination of more techniques in one instrumental set-up is desirable to reduce the problems caused by variations in sample preparations. A patch-clamp pipette as a sample holder for individual cells along this line is a first step to combine AFM with electrophysiological measurements on ion channels in the membrane of whole cells. A logical step further is a set-up that can be used to investigate also excised membrane patches allowing studies on single ion-channels in the membrane, especially on those activated by mechanical stimulation with the cantilever tip. However, living cells are 3-D structures and AFM is a surface technique with its performance directly coupled to the flatness of the surface investigated. Imaging inside cells is impossible due to the mechanical connection of the instrument with the imaging tip. Therefore, a scanning probe microscope without a mechanical connection to the tip would be an ideal complementary technique. The Photonic Force Microscope (PFM) is such an instrument, where the mechanical cantilever is replaced by the 3-D trapping potential of a laser focus.
Session I: Invited talk

ATOMIC FORCE MICROSCOPY MEASUREMENT OF LEUKOCYTE ADHESION TO ENDOTHELIAL CELLS

Vincent T. Moy
Department of Physiology and Biophysics, University of Miami School of Medicine

Leukocyte adhesion to vascular endothelium is a key initiating step in the pathogenesis of many inflammatory diseases such as atherosclerosis. This process requires the activation of leukocyte integrins and the upregulation of the integrin ligands on the endothelial cells. Here we present real-time force measurements of the interaction between monocytic HL-60 cells and a monolayer of human umbilical vein endothelial cells (HUVECs) acquired by atomic force microscopy (AFM). The detachment of HL-60/HUVEC conjugates involved a series of rupture events with force transitions of 30-100 pN. These rupture forces are consistent with values measured for the unbinding of individual ligand-receptor pairs. Integrated force of individual rupture events provided a quantitative measure of the adhesion strength on a whole cell level. The AFM measurements revealed that HL-60 cells adhered more tightly to the borders formed by adjacent HUVECs than to the cell body of a HUVEC. The average force and mechanical work required to detach a single HL-60 cell from the cell borders of a TNF-α activated HUVEC layer were twice as high as those of the HUVEC bodies. HL-60 adhesion to the monolayer was significantly reduced by a monoclonal antibody against integrin β1, and partially inhibited by function blocking antibodies against P-selectin, E-selectin, ICAM-1 and VCAM-1, but was not affected by a monoclonal antibody against αVβ3.
Measurement of cell mechanical properties by atomic force microscopy

Daniel Navajas
Unitat de Biofísica i Bioenginyeria. Facultat de Medicina
Universitat de Barcelona, Spain

Mechanical properties of the cell play an important role in critical cell functions including migration, contraction, mechanotransduction and gene expression. Atomic Force Microscopy (AFM) probes single cell mechanics by indenting the surface of the cell with the cantilever tip and measuring the force-displacement relationship (F-z). Assuming a pure elastic behavior, an estimate of the Young modulus of the cell (E) can be obtained by fitting F-z with an appropriate cell-tip contact model that takes into account the rise in contact area as indentation increases. On the other hand, constant contact area indentation can be performed with a flat-ended cylindrical tip obtained by modifying the pyramidal tip of commercial AFM cantilevers using focused ion beam technology. The value of E computed from F-z provides a rough estimate of cell stiffness. Cells, however, exhibit viscoelastic behavior, which is more suitably probed by applying small amplitude vertical tip oscillations around the operating indentation. The oscillatory force recordings require correction for the viscous friction of the cantilever with the liquid. This hydrodynamic artifact can be readily estimated by oscillating the cantilever in the liquid at different cell-tip distances and extrapolating the value of the viscous drag to the cell surface. The viscoelastic modulus is computed in the frequency domain from the ratio of force and indentation oscillations after correction for the hydrodynamic artifact. The cell exhibits scale free dynamics with a viscoelastic modulus increasing with frequency as a weak power law.
SESSION II

Single molecular recognition, affinity, unfolding forces

Session chair
Peter Hinterdorfer
Johannes Kepler Universitat Linz
Linz, Austria

Topics
DFS, Folding-Unfolding, protein-ligand, DNA, single molecules, molecular recognition
Session II: Invited talk

Single Molecule Recognition Force Microscopy

Peter Hinterdorfer

Institute for Biophysics, Johannes Kepler University of Linz, Altenbergerstr. 69, A-4040 Linz, Austria

In molecular recognition force microscopy (MRFM), ligands are covalently attached to atomic force microscopy tips for the molecular recognition of their cognitive receptors on probe surfaces. Using an appropriate tip surface chemistry protocol, the ligand density on the AFM tip is sufficiently dilute for the allowance of single molecule studies. Interaction forces between single receptor-ligand pairs are measured in force-distance cycles. A ligand-containing tip is approached towards the receptors on the probe surface, which possibly leads to formation of a receptor-ligand bond. The tip is subsequently retracted until the bond breaks at a certain force (unbinding force). In force spectroscopy (FS), the dynamics of the experiment is varied, which reveals a logarithmic dependence of the unbinding force from the force velocity. These studies give insight into the molecular dynamics of the receptor-ligand recognition process and yield information about the binding pocket, binding energy barriers, and kinetic reaction rates. Applications on isolated proteins, native membranes, viruses, and cells will be presented.

We have also developed a method for the localization specific binding sites and epitopes with nm positional accuracy by combining dynamic force microscopy with single molecule recognition force spectroscopy. A magnetically driven AFM tip containing a ligand covalently bound via a tether molecule was oscillated at 5 nm amplitude while scanning along the surface. Since the tether had a length of 8 nm, the ligand on the tip was always kept in close proximity to the surface and showed a high probability of binding when a receptor site was passed. The recognition signals were well separated from the topographic signals arising from the surface, both in space (z ~ 5 nm) and time (half oscillation period ~ 0.1 ms). Topography and recognition images were obtained simultaneously using a specially designed electronic circuit. Maxima (U_{up}) and minima (U_{down}) of each sinusoidal cantilever deflection period were depicted, with U_{down} driving the feedback loop to record a height (topography) image and U_{up} providing the data for the recognition image. In this way, topography and recognition image were gained simultaneously and independently with nm lateral resolution.
Session II: Invited talk

Nanobiotechnological drug screening: Imaging, sensing and locating ligands that drive cellular machines

Daniel J. Müller
Center of Biotechnology, University of Technology, Dresden, Germany

Using the example of vertebrate Gap Junctions and the sodium/proton antiporter from Escherichia coli NhaA we review the capabilities of high-resolution atomic force microscopy (AFM) and single-molecule force spectroscopy (SMFS) to observe structural and functional insights of membrane proteins, which are not attainable by other traditional methods. While we use AFM to observe the ligand induced gating of native and single membrane proteins, we apply SMFS to detect molecular interactions that switch the functional state of the protein. The sensitivity of both methods makes it possible to detect and locate interactions that stabilize secondary structures such as transmembrane alpha-helices, polypeptide loops and segments within them. Controlled refolding experiments using single-molecule force spectroscopy observed individual secondary structure segments folding into the functional protein. Various folding pathways of NhaA were detected each one exhibiting a certain probability to be taken. Time-lapse refolding experiments enabled determining the folding kinetics and hierarchy of individual secondary structural elements. Recent examples detected and located the ligand binding of an antiporter. Similarly, inhibitor binding and location can be detected which in future guides towards comparative studies of agonist and antagonist determining the functional state of a protein. We sketch current and future potentials of these approaches to characterize the action of pharmacological molecules on the membrane protein function. Further membrane proteins investigated and discussed are G-protein coupled receptors (GPCRs), communication channels, aquaporins, and others.

Figure 1. Architecture of Na⁺-binding sites of sodium transporters. SMFS experiments detects molecular interactions established upon ligand binding to the functional domain of NhaA (on the left) that involves α-helix V, and two partly unwound α-helices IV and XI. Core of leucine/sodium transporter LeuTAa (on the right) contains two Na⁺-binding sites embedded by partly unwound α-helices I and VI, and neighboring α-helix VIII. Positions of a bound Na⁺ (yellow) and a leucine molecule are shown. Functionally important residues of both proteins were highlighted.

Key words: GPCR, ion channels, gap junctions, aquaporin, single-molecule approach, stable segments, ion exchange, protein folding, two-stage folding model
Session II: Invited talk

Molecular Devices: Sensors, Grabbers and Actuators

Hermann E. Gaub

Chair for Applied Physics - Biophysics and Molecular Materials Ludwig-Maximilians-Universität München

Molecular devices are promising building blocks for functional nanosystems. Sensors, grabbers and actuators may be designed based on DNA and proteins or other biopolymeric systems. Azobenzen units in polypeptides may be employed as bi-stable photo-switchable actuators. Lipases may be programmed to act as force sensors. Nucleic acid oligomers may be used to implement assembler concepts based on hierarchical forces. This talk will summarize our current activities in this field.
SESSION III

High resolution imaging

Session chair
Simon Scheuring
Institut Curie
Paris, France

Topics
High resolution imaging, high speed imaging, coupling with other methods
Session III: Invited talk

Structure and assembly of membrane proteins in native membranes by atomic force microscopy (AFM)

Simon Scheuring, Nikolay Buzhynsky, Rui Pedro Goncalves, Szymon Jaroslawski

Institut Curie, UMR-CNRS 168, 26 rue d’Ulm, 75248 Paris, France.

The atomic force microscope (AFM) has become a powerful tool in structural biology allowing the investigation of biological samples under native-like conditions: experiments are performed in physiological buffer at room temperature and under normal pressure. Topographies of membrane proteins can be acquired at a lateral resolution of ~10Å and a vertical resolution of ~1Å. Importantly, the AFM features an extraordinary signal-to-noise ratio allowing imaging of individual membrane proteins in prokaryotic ¹ and eukaryotic ² native membranes that participate in supramolecular assemblies. These images can be docked with high precision by high-resolution structures resulting in atomic models of multiple proteins working together. The development of a novel 2-chamber AFM setup, in which membranes are deposited on nano-patterned surfaces, allows probing non-supported functional membrane proteins ³.

G-Protein Coupled Receptors Are Active as Dimers and Higher Oligomers: A Lesson From Rhodopsin

Andreas Engel ¹, B. Jastrzebska ², A. Philippsen ¹, D.J. Müller ³, K. Palczewski ², and D. Fotiadis ¹

¹) M.E. Müller Institute for Microscopy, Biozentrum, University of Basel, CH-4056 Basel, Switzerland.
²) Department of Pharmacology, Case School of Medicine, Case Western Reserve University, Cleveland, Ohio 44106-4965, USA.
³) Center for Biotechnology, University of Technology, 01307 Dresden, Germany.

G protein-coupled receptors (GPCRs) participate in many physiological processes and represent the largest and most structurally conserved family of signaling molecules. Growing evidence indicates that many, if not all, GPCRs are active as dimers and/or higher-order oligomers. High-resolution crystal structures are available only for the detergent-solubilized light receptor rhodopsin, the archetypal class A GPCR. Rhodopsin is the only GPCR for which the higher-order oligomeric state has been demonstrated by imaging native disk membranes using atomic force microscopy (AFM). Based on these data and the X-ray structure, an atomic model of rhodopsin dimers has been proposed, and the AFM has also been used to measure forces required to unfold single rhodopsin molecules, demonstrating which residues dictate rhodopsin’s stability. Functional analyses of fractions from solubilized disk membranes revealed that higher-order Rho oligomers are the most active species. These recent results have enhanced our understanding of GPCRs structure and function.
Session III: Invited talk

Dynamic behaviors of proteins at work captured by high-speed atomic force microscopy

**Toshio Ando**\(^{1,2}\), Takayuki Uchihashi\(^{1,2}\), Noriyuki Kodera\(^1\), Daisuke Yamamoto\(^2\), Hayato Yamashita\(^1\)

\(^1\)Department of Physics, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan
\(^2\)Core Research for Evolutional Science and Technology (CREST) of the Japan Science and Technology Agency (JST), Kawaguchi, Saitama 332-0012, Japan

Various experimental techniques have been invented to study the mechanisms of protein functions. Because experimental data on protein functions are often indirect, theoretical interpretations and inference have been necessary to speculate the mechanism of protein functions from indirect experimental data. However, ideas derived from such analyses do not necessarily converge onto one conclusion. Therefore, people have longed for a means to view directly the nanometer-scale dynamic behaviors of single protein molecules at work. High spatial resolution imaging of the nanometer-scale world in aqueous solutions is possible only with atomic force microscopy (AFM). However, its temporal resolution was too low to trace the dynamics. Therefore, in the last few years, various efforts\(^{1-5}\) have been carried out to enhance the scan speed. Owing to these efforts and other efforts to reduce the tip-sample interaction force\(^6\), it has recently become possible to image at (near) video rate, without disturbing protein’s physiological functions. For example, hand-over-hand movement of myosin V along actin filaments is clearly imaged. The negatively cooperative binding events between Gro-ES and the two rings of GroEL are successfully captured. These demonstrate that high-speed AFM is truly useful for studying protein’s dynamic action and will surely open a new way of elucidating the mechanisms of protein functions.

Atomic force microscopes have deeply transformed the study of materials. However, high resolution imaging of biological systems has proved more difficult than obtaining atomic resolution images of crystalline surfaces. The reasons that explain those shortcomings are nonetheless established. The forces exerted by the tip on the molecules (1-10 nN) either displace them laterally or break the noncovalent bonds that held the biomolecules together. Here, we present a force microscope concept based on the simultaneous excitation of the first two flexural modes of the cantilever. The coupling of the modes generated by the tip-molecule forces enables imaging under the application of forces (~35 pN) which are smaller than those needed to break non-covalent bonds. With this instrument we have resolved the intramolecular structure of antibodies in monomer and pentameric forms. Furthermore, the instrument has a force sensitivity of 0.2 pN which enables the identification of compositional changes along the protein fragments.
SESSION IV

Model membranes and protein-membrane interactions

Session chair
Christian Le Grimellec
INSERM
Montpellier, France

Topics
Membrane imaging, protein-membrane interactions, possible applications
Session IV: Invited talk

Alkaline Phosphatase Interactions with domains in supported bilayers

Marie-Cécile Giocondi 1, Françoise Besson 2, Patrice Dosset 1, Pierre-Emmanuel Milhiet 1, and Christian Le Grimellec 1.

1 INSERM U554, Nanostructures et Complexes Membranaires, Centre de Biochimie Structurale, F34090 Montpellier, France; CNRS UMR 5048, F34090 Montpellier, France; Universités Montpellier 1 et 2, F34090 Montpellier, France.
2 Laboratoire Organisation et Dynamique des Membranes Biologiques, CNRS UMR 5013, Université Claude Bernard Lyon I, 43 boulevard du 11 novembre 1918, F-69622 Villeurbanne Cedex, France.

GPI-anchored proteins preferentially localize in the most ordered regions of the cell plasma membrane. Acyl and alkyl chain composition of GPI-anchors determine the association with the ordered domains. This suggests that changes in the fluid and in the ordered domains lipid composition affect the interaction of GPI-anchored proteins with membrane microdomains. Atomic force microscopy (AFM) shows that the spontaneous insertion of the GPI-anchored intestinal alkaline phosphatase (BIAP) into the gel phase domains of dioleoylphosphatidyl-choline / dipalmitoylphosphatidyl-choline (DOPC/DPPC) and DOPC/sphingomyelin (DOPC/SM) also occurred in palmitoyloleoylphosphatidylcholine /SM (POPC/SM) gel-fluid phase separated membranes. However changes in the lipid composition of membranes had a marked effect on the bilayer topography: BIAP insertion was associated with a net transfer of phospholipids from the fluid to the gel (DOPC/DPPC) or from the gel to the fluid (POPC/SM) phases. For DOPC/SM bilayers, transfer of lipids was dependent on the homogeneity of the gel SM phase. In POPC/SM binary mixtures with the coexistence of fluid, gel and liquid ordered phases induced by cholesterol (POPC:SM:Chl, 1:1:0.35), BIAP preferentially localized in the more ordered phase, at room temperature. However, this distribution of BIAP between fluid and ordered phases was a function of temperature. How the AFM imaging of BIAP in model systems could contribute to the understanding of the behaviour of GPI-anchored proteins in biological membranes and what are the limitations of AFM in such studies will be discussed.
Understanding the effect of mechanical stress on biological membranes is of fundamental importance since cells are known to naturally perform their function under the effect of a complex combination of forces. Indeed, the chemical composition of such membranes is the ultimate responsible for determining their architecture, while guaranteeing the cell mechanical stability. Micro-scale assays have revealed a wealth of information regarding the overall membrane mechanical resistance. Nonetheless, the diversity in the chemical composition of such membranes makes it difficult to test the mechanical contribution of every particular membrane component. Thanks to the capability of accessing contact areas in the nanometer scale, force spectroscopy can precisely probe the mechanical resistance of substrates under the application of force [1]. In particular, this technique has allowed us to study the effect of solution ionic strength and temperature on the nanomechanics of supported lipid bilayers [2]. A small increase in ionic strength (up to 200 mM) gives rise to a 7-fold increase in bilayer (nano)mechanical resistance due to an ion-binding effect. Mechanical resistance exhibits a discontinuity at temperatures where phase transitions occur. We have extended our work towards individually testing the mechanical role of different phospholipid headgroups and measured the resistance that a change in the hydrophobic tail adds to the overall bilayer stability. The effect of different sterols on a liquid (DLPC) and a solid (DPPC) membrane has been also addressed. This work paves the way for the mechanical characterization of membrane components, suggesting that mechanical stability can be regarded as the ‘sum of its parts’.

Selected talks

Sessions

Session I  |  Cells, Cellular interactions
Session II  |  Single molecular recognition, affinity, unfolding forces
Session III |  High resolution imaging
Session IV  |  Model membranes and protein-membrane interactions
Session I: Cells, cellular interactions

Selected talk

Mechanical dynamics during cell death

Andrew Pelling¹, Farlan Veraitch², Carol Chu², Chis Mason², Michael Horton¹

1) London Centre for Nanotechnology and Department of Medicine, University College London
2) Department of Biochemical Engineering, University College London

Measurement of the dynamic mechanical characteristics of living cell membranes can often reveal surprising insights into cell biology in addition to quantifying material properties. In this study, we induced early apoptosis in human skin fibroblasts with Staurosporine (STS) and studied how the local cell membrane stiffness changed over a two hour period with atomic force microscopy (AFM). It was found that the cell membrane underwent a single oscillation in local stiffness with a period of ~30 minutes as opposed to controls. Utilizing combined fluorescence-AFM and confocal microscopy, we observed that the oscillation in mechanical stiffness was a consequence of early apoptosis and was marked by significant morphological and structural changes. The underlying chemo-mechanical basis for the oscillation is shown to be dependent cytoskeletal remodeling in concert with nuclear collapse. The initial decay in stiffness is likely caused by Rho-kinase inhibition by STS, followed by an apparent increase in stiffness due to microtubule condensation into a meshwork dome surrounding the nucleus. Inhibiting caspase activity delayed the mechanical dynamic but early apoptosis was not prevented. Using confocal microscopy we observed that the final decay in stiffness was caused by nuclear condensation and translocation. Caspase inhibition revealed that the nuclear collapse was part of a cell death program but the initial cytoskeletal remodeling was a response to kinase inhibition. Therefore, the dynamic mechanical signature is a caused by a complex interplay of kinase inhibition by STS and a caspase mediated cell death program. Therefore, caspase dependent and independent signaling pathways are activated in parallel during early apoptosis and their relative effects can be distinguished in dynamic mechanical signatures.
Session I: Cells, cellular interactions

Selected talk

Elastic membrane heterogeneity of living cells revealed by stiff nanoscale membrane domains

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For several years, multiple approaches have been developed to characterize membrane heterogeneity in living cells. We present here a study of the elastic properties of plasma membrane domains in living cells using atomic force microscopy. This work demonstrates the existence of nanometric scale heterogeneous domains with specific biophysical properties. In particular, we focused on glycosylphosphatidylinositol (GPI)-anchored proteins, which play important roles in membrane trafficking, cell signalling and diseases and which were shown, using a wealth of methods, to preferentially partition in cholesterol rich microdomains. We found that GPI-anchored proteins resided in domains stiffer than the surrounding membrane, whereas membrane domains containing the transferring receptor, which do not partition in cholesterol rich domains showed no such features. The observed increase in stiffness with GPI-domains is consistent with previously documented specific lipid condensation and slow diffusion rate of proteins/lipids within these domains. These new data quantitatively document elastic membrane heterogeneity unveiling a possible link between membrane stiffness, molecular diffusion and signalling activation.
Session I: Cells, cellular interactions

Selected talk

Probing microbial interfacial properties by force spectroscopy and microelectrophoresis

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The surface properties of microbes in aqueous media play an important role in controlling interfacial phenomena such as bacterial adhesion, biomineralization and biofilm formation. There is a paucity of information on the relationship between the physico-chemical properties of the bacterial surface and the development of microbial communities and allied community phenomena both at the nano-scale (as relates to the cell-substrate interaction) and the micro-scale (as relates to the environmental conditions of the system), warranting further multidisciplinary and multiscale research. In recent years, remarkable advances have been made in applying force spectroscopy to quantify the interaction forces and physical properties of microbial surfaces. In this talk, we focus on the quantification of surface forces, hydrodynamic features and mechanical properties for different type of microbial cells (bacteria and yeast). In particular, the complementarity between microelectrophoresis data and force spectroscopy by means of advanced soft particle theory allowed to infer the interaction forces between the hard AFM probe and the soft cells. Such theoretical model taking into account the heterogeneous and soft characters allowed, for the first time, a means to differentiate between the contribution of surface forces and surface mechanics to the net interaction measured between a hard AFM tip and a compliant microbial surface. The results reveal new insights into the relationships between microbial adhesion processes and physico-chemical composition of the electrolyte solution, specifically pH and ionic strength.
Session I: Cells, cellular interactions

Selected talk

Monitoring of biomechanical cellular activity induced by vascular active agonists with AFM

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Angiotensin II (AngII), thrombin (TB) and bradykinin (BK) are agonists implicated in vascular processes such as the regulation of vascular permeability and blood pressure. These peptides activate cellular signaling pathways, involving the mobilization of intracellular calcium store as well as cytoskeleton reorganization leading to extensive cellular morphology remodeling. We have use the AFM to monitor the cellular response induced by these agonists on individual cells. AFM time course measurement of cellular reorganization and the simultaneous measurement of intracellular Ca$^{2+}$ release using a calcium sensitive fluorescent indicator show that these phenomenons are intimately related. Cellular activation by AngII (1µM) produces an elevation of the apical cell surface of the cell measured by the AFM corresponding to a maximum in the Ca$^{2+}$ intracellular concentration. This initial response is then followed by a significant mechanical oscillation of the cell surface associated with extensive cell cytoskeleton remodeling. Furthermore, time course analysis of TB (10nM) and BK (1µM) actions show an increase in the cell stiffness (8.5 and 6.8 kPa, respectively) and the membrane tether elongation forces (48 and 51pN, respectively) after 30 to 45 minutes in comparison to the control (5.6 kPa and 42 pN), also suggesting a reorganization of the cytoskeleton and an increase of its interaction with the membrane which clearly indicates an important biomechanical remodeling of the cells. These results show that AFM can be used to perform time monitoring of intracellular signalization induced by vascular active agonists through their effects on the mechanical integrity of the cell.
Session I: Cells, cellular interactions

Selected talk

Cell topometry analysis can replace direct measurement of fluid permeability

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Endothelial cells control water and solute flux between blood and tissue. Short term changes of paracellular permeability are regulated by actomyosin interaction. A higher intracellular tension leads to formation of paracellular gaps and hence to a breakdown of barrier function. Fluid permeability is usually quantitated by passage of macromolecules across cell layers - a time consuming procedure, applicable only to cells grown on permeable supports. Assuming that intracellular tension would affect the three-dimensional shape of cells, we tried to assess paracellular permeability indirectly by a topometric approach. Therefore we applied AFM to primary endothelial cells isolated from human umbilical veins (HUVEC). As well, the electrical resistance across the layer was measured using electrical impedance spectroscopy (ECIS). The Ca-ionophore ionomycin was used for inducing cellular contraction. In samples treated with ionomycin, AFM was able to detect paracellular gaps. Analogously, the electrical resistance decreased to virtually zero. Moreover, the topometric parameters “maximal height”, “surface roughness” and “surface area difference” were taken in order to enumerate the topographic information of the scanned region. Values of all three parameters augmented with increasing ionomycin concentrations, indicating a widening of cellular clefts and a rounding of the cells due to elevated intracellular tension. We conclude that AFM topometric data indicate the paracellular permeability properties of a cell layer. This approach might also apply to substrates which are not suitable for classical assay procedures.
Session II: Single molecular recognition, affinity, unfolding forces

Selected talk

A Rotaxane Based Method of Determining Hairpin Location and Kinetics in Nucleic Acids with an AFM

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Hairpins on DNA and RNA have also been implicated in a number of genetic diseases by the hairpin mediated polymerase slippage model. The causes and dynamics of the slippage are poorly understood. We have used cyclodextrin to build a rotaxane system to directly measure the strength and dynamics of the interaction of a ring molecule and a nucleic acid hairpin. The dynamics of a DNA molecule passing through the ring are measured, as well as several hairpin structures. This method is beneficial that we can measure both the location and strength of the hairpins on complicated structures. The force required to open the hairpins corresponds to predictions made by mFold.
Session II: Single molecular recognition, affinity, unfolding forces

Selected talk

**Single Molecule Force Spectroscopy Mapping**

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In this work we apply the AFM jumping operation mode, JM, [1] to the study of molecular recognition. JM works by performing a force versus distance curve at each point of the surface, recording simultaneously surface topography and tip-sample adhesion force. In this way when imaging the sample, the adhesion force on top of a receptor molecule corresponds to the unbinding force of the ligand-receptor complex. The main difficulty is to deal with the appearance of multiple adhesion peaks of diverse origin. To solve this problem we were working in the repulsive electrical double layer regime and make use of the spacer technology [2] so that the ligand can reach the receptor without establishing mechanical contact between tip and sample. Although rupture events in force spectroscopy (FS) are done in well-defined molecules, so that the experiments are named as single molecule, these are of stochastic nature, so that a statistic analysis is needed. In FS this is done by averaging single unbinding events over multiple ligand-receptor pairs, i.e. several molecules. In JM, thanks to its capability to obtain topographical information and to vary the loading rate within a wide range without loosing image stability we are able to do the statistics on a true single molecule. We have successfully applied this method to the avidin-biotin complex.

Session II: Single molecular recognition, affinity, unfolding forces

Selected talk

**Molecular mechanisms contribute to the fracture resistance of bone: repeatable energy dissipation by sacrificial bonds and hidden length in molecular networks**

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Properties of the organic matrix of bone as well as its function in the microstructure could be the key to the remarkable mechanical properties of bone. It was found that, calcium-mediated sacrificial bonds enhanced the binding strength of bone constituent molecules. By investigating the nanoscale arrangement of the bone constituents and their interactions by AFM molecular force spectroscopy, AFM and SEM we found evidence how this sacrificial bond-hidden length mechanism contributes to the mechanical properties of the bone composite. We find that bone consists of mineralized collagen fibrils and a non fibrillar organic matrix which acts as “glue” that holds the mineralized fibrils together. Energy dissipation in this glue comes, in large part, from work against the entropic elasticity of molecules with sacrificial bonds and hidden lengths. The proteins involved, however, unlike titin and fibronectin, lack the folded modular domains commonly associated with sacrificial-bonds and hidden-lengths. We show that human osteopontin molecules, as found in bone and arterial plaque buildup, can dissipate large amounts of energy, without the need for folded domains within the protein. Osteopontin monomers can join together to form networks that can be pulled for several microns using AFM force spectroscopy. The strength of these networks can be increased by recruiting divalent ions from the solution surrounding them, just as for the adhesive molecules previously detected in bone. Other phosphorylated proteins show similar behavior in our experiments. This suggests that strongly anionic flexible proteins, combined with Ca^{2+} ions, enable the formation of ion-mediated, self healing networks.
Selected talk

Mechanical properties of glucans/Dectin-1 interactions: Implications for pathogen recognition

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The recognition of pathogens by the innate immune system is fundamental in controlling an appropriate immune response. Glucans are structurally diverse biopolymers that are major components of fungal cell walls. Glucans stimulate innate immunity and are thought to be fungal PAMPs. Glucans are bound and internalized by the leukocyte β-glucan specific receptor, Dectin-1. The glucan/Dectin ligand receptor pair plays a pivotal role in the innate immune response to fungal pathogens. The purpose of this study was to determine the influence of glucan structure on recognition and binding affinity by recombinant murine Dectin-1 and Dectin expressing cells using AFM. A library of natural product (1→3)-β and/or (1→6)-β-glucan ligands were employed. Mannan, an α-linked mannose polymer, was employed as carbohydrate polymer controls. The primary structure and MW of each carbohydrate was confirmed by NMR and GPC/MALLS. Binding interactions were established using AFM. The binding of glucan to Dectin expressing cells was following using AFM and confocal. Binding affinities for natural product glucan ligands were observed over a very broad range (20-100 pN). In addition, rDectin-1 showed differential recognition of glucan ligands. These data indicate that Dectin is highly specific for glucan ligands that have a (1→3)-β-D-linked backbone structure. Furthermore, Dectin-1 can clearly differentiate between (1→3)-β-glucan ligands based on structural determinants. These data suggest that Dectin-1 immunosurveillance of fungal pathogens may be dependent upon the presence of (1→3)-β-D-glucopyranosyl backbone structures in the fungal cell wall glucans.
Myomesin is the most prominent structural component of the sarcomeric M-Band that is expressed in mammalian heart and skeletal muscles. Like titin, this protein is an intracellular member of the Ig-fibronectin superfamily, which has a flexible filamentous structure and which is largely composed of two types of domain that are similar to immunoglobulin (Ig)-like and fibronectin type III (FNIII) domains. Several myomesin isoforms have been identified, and their expression patterns are highly regulated both spatially and temporally. Particularly, alternative splicing in the central part of the molecule gives rise to an isoform, EH (embryonic heart)-myomesin, containing a serine and proline-rich insertion with no well-defined secondary structure, the EH segment. EH-myomesin represents the major myomesin isoform at embryonic stages of mammalian heart and is rapidly down-regulated around birth, but it is re-expressed in the heart of patients suffering from dilated cardio-myopathy. Here, we explore the mechanical stability and force-driven structural changes of human myomesin’s sub-molecular segments using single-molecule force spectroscopy and protein engineering. We find that human myomesin molecules are composed of modules (Ig and FNIII), that are designed to withstand force and we demonstrate that the human cardiac EH segment functions like an additional elastic stretch in the middle part of the EH-myomesin and behaves like a random coil. So, we provide the evidence that not only titin but also other sarcomeric proteins have complicated visco-elastic properties depending on the contractile parameters in different muscle types.
Session II: Single molecular recognition, affinity, unfolding forces

Selected talk

Atomic Force Microscopy study of interactions between supercoil-dependent gene regulatory proteins and DNA

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Modulating the extent of DNA supercoiling has been proposed as a mechanism for regulation of the gene expression. Secondary structures of DNA, such as cruciforms, can effect in transcription by creating new protein binding sites. Several proteins have been shown to bind to supercoiled DNA, including high mobility group protein B1 (HMGB1) and DEK protein, by recognizing specific DNA structures (cruciform and bent). Poly(ADP-ribose) polymerase (PARP-1) has an affinity for binding to supercoiled DNA. We have used supercoiled topoisomers of pUC8F14 plasmid, containing one cruciform structure, for investigation of proteins interactions with DNA. Using volume measurement analysis of molecules of HMGB1, DEK and PARP-1 proteins, we determined the numbers of protein molecules interacting with supercoiled plasmid. We found that HMGB1, predominantly binds as large multiprotein complexes (1-5 dimers) to the nodes in supercoiled DNA. We determined that HMGB1 can bend DNA after binding to the top of a DNA loop of a supercoiled plasmid. DEK protein can binds to nodes or top of the DNA loop as 1-2 dimers. In 2% of complexes, we observed the interaction of HMGB1 protein with junction regions of cruciform the structures. In contrast, PARP-1 protein binds to the ends of the hairpin arms of the cruciform structures and does not interact with junction regions. We found that PARP-1 protein can binds to cruciform structures maximum as two dimmers.
Session III: High resolution imaging

Selected talk

The supramolecular architecture of junctional microdomains in native lens membranes

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Gap junctions formed by connexons and thin junctions formed by lens-specific aquaporin 0 (AQP0) mediate the tight packing of fibre cells necessary for lens transparency. Gap junctions conduct water, ions and metabolites between cells, whereas junctional AQP0 seems to be involved in cell adhesion. High-resolution atomic force microscopy (AFM) showed the supramolecular organization of these proteins in native lens core membranes, in which AQP0 forms two-dimensional arrays that are surrounded by densely packed gap junction channels. These junctional microdomains simultaneously provide adhesion and communication between fibre cells. The AFM topographs also showed that the extracellular loops of AQP0 in junctional microdomains adopt a conformation that closely resembles the structure of junctional AQP0, in which the water pore is thought to be closed. Finally, time-lapse AFM imaging provided insights into AQP0 array formation. This first high-resolution view of a multicomponent eukaryotic membrane shows how membrane proteins self-assemble into functional microdomains.
Session III: High resolution imaging

Selected talk

HIGH RESOLUTION AFM IMAGING OF NATIVE SINGLE-STRANDED DNA BINDING (SSB) PROTEIN – DNA COMPLEXES

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Single-stranded DNA binding proteins (SSB) play a central role in cellular processes involving the generation and protection of single-stranded DNA (ssDNA). To characterize binding mode transition, cooperativity or DNA wrapping around oligomers, imaging nucleoprotein filaments formation at high resolution in native conditions is of particular interest. However, only few atomic force microscopy (AFM) investigations of ssDNA nucleoprotein filaments have been conducted up to now, due to the difficulty of spreading them on mica properly, which leads to a poor resolution. In this study, we present AFM images of native ssDNA / SSB complexes (E. coli SSB, Bacteriophage T4 gene 32 protein) on mica obtained by using spermidine at a low concentration (<0.3mM). Trivalent cations like spermidine induce a stronger adsorption of the complex than divalent ones, which are ineffective in the ssDNA / SSB complex adsorption on mica. The additional benefit is its ability to adsorb partially or fully saturated nucleoprotein filaments within a large range of monovalent salt concentrations. This new method opens perspectives for the AFM characterization of SSB proteins behavior on ssDNA, in association with conventional biochemical techniques.
Specific patterning of LH2 and LH1 protein complexes

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The biological roles of proteins are extraordinarily diverse, and include catalysis, mechanical support, signaling, and sensing. Proteins are also of great interest because of their potential to be integrated in hybrid structures to create devices of unprecedented small scale and high efficiency. However, both fundamental research and applications of protein-nanodevices are continuously challenged by the inherent difficulty of positioning these small objects. We are investigating strategies to assemble biological molecules on a surface to create (nano)patterns. Here we present optically active patterns of single photosynthetic membrane proteins (light harvesting antennas LH1, LH2) from Rhodobacter Sphaeroides purple bacteria. Protein complexes were immobilized via electrostatic interactions onto chemically patterned glass substrates created by Nanoimprint Lithography (NIL). The micro- and nanometer scale structures created are investigated with a custom-built combined atomic force and confocal fluorescence microscope (AFFM) which provides simultaneous topographical, optical and spectral information. Spectral images of the patterned protein complexes indicated that the LH1 and LH2 complexes remain functional after the patterning and immobilization procedure. This work points towards the possibility to create biophotonic devices in a controlled manner with nanometer spatial resolution.
Session III: High resolution imaging

Selected talk

Characterization of transcription regulatory complexes of bacteriophage Phi29 by Atomic Force Microscopy

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Atomic Force Microscopy (AFM) is used in this project to study DNA-protein complexes that appear during the transcription of the genome of Bacillus subtilis bacteriophage Phi29. Phage early promoters A2c and A2b and late promoter A3 are coordinately regulated by a multimeric complex of viral proteins p4 and p6, which elicits the switch from the early to the late transcription, repressing promoters A2c and A2b, and simultaneously activating promoter A3. Here we present the results obtained on the structural characterization of the nucleoprotein complexes formed with a 1189bps fragment of the phage DNA containing all three promoters and the two transcriptional factors, proteins p4 and p6. We have found a local bend induced in the DNA by protein p4 (specifically bound), as well as a conformational rearrangement of the p4-DNA complex into a hairpin-like structure that brings in closer proximity promoters A2c and A3 and occludes the A2b), due to the consecutive binding of the nucleoid protein p6. Further modifications on the ternary complex related to the additional interaction with the RNA polymerase were also observed. A plausible model for the functional conformation of the nucleoprotein complex required for the regulation of transcription is proposed.
Session IV: Model membranes and protein-membrane interactions

Selected talk

Quantitative analysis of coarsening and spatial domain distribution in a ternary membrane

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A number of morphological and statistical aspects of domain formation in a single and double supported ternary membrane have been investigated. Such ternary membranes are typically composed of an unsaturated phospholipid, a saturated phospho- or sphingolipid and a sterol. They display macroscopic phase separation in two fluid phases and are widely used as raft models. We find that membrane interactions with the support surface can have a critical influence on the domain shapes if measures are not taken to screen these interactions. Combined AFM and fluorescence microscopy demonstrates small (500 nm) irregular domains and incomplete formation of much larger round domains. The interaction-artefacts can be effectively removed by employing double supported membranes under physiological salt concentrations. These fully decoupled supported membranes display macroscopic circular domains closely mimicking the domains found in free-standing membranes. The planar membrane geometry allows a quantitative characterization of domain coarsening upon rapid cooling into the coexistence region. We determine the domain growth exponent $\alpha=0.31$ which is in close agreement with the theoretical value of 1/3. Analysis of the spatial domain pattern in terms of Voronoi polygons demonstrates a close similarity to equilibrated cellular structures with a maximized configurational entropy.
Session IV: Model membranes and protein-membrane interactions

Selected talk

How can Atomic Force Microscopy Help to Understand Sepsis?

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There is a battle between the immune system and invading pathogens, e.g. microorganisms such as bacteria, or pathogenic factors, i.e. structures released from the microorganisms. We focused on two important aspects of the Gram-negative sepsis, which causes about 50% of all septic diseases. Gram-negative bacteria are surrounded by a cell envelope, consisting of the cytoplasmic or inner membrane, a thin peptidoglycan layer, and an additional barrier, the outer membrane (OM). The lipid composition of the OM is asymmetric: the inner leaflet is composed of a mixture of phospholipids and the outer leaflet of glycolipids, in most cases lipopolysaccharides (LPS). The OM and in particular the LPS plays a dual role: (i) All antimicrobial agents attacking Gram-negative bacteria have to permeate through the OM or to destroy it; (ii) When LPS is released from the OM during cell division or induced by antibacterial agents, it interacts with immune cells and simulates them to release mediators, e.g. proinflammatory cytokines. The activation of the immune cells can finally lead to sepsis. To get insight into the molecular interaction mechanisms between lipid membranes and peptides or proteins, the complex biological system of human cells or bacteria has often to be reduced. Therefore, membranes are reconstituted from purified lipid preparations to perform biophysical experiments on simplified models. We used the atomic force microscope to investigate the structure and function of these lipid membranes and their interaction with antimicrobial peptides as well as with proteins being involved in the LPS-induced signal transduction in human immune cells.
Dynamic strength of the interaction between lung surfactant protein D (SP-D) and saccharide ligands

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In order to investigate the dynamic strength of the interaction between lung surfactant protein D (SP-D) and different sugars, maltose, mannose, glucose and galactose, we have used an atomic force microscope to monitor the interaction on a single molecule scale. The experiment is performed by measuring the rupture force when the SP-D--sugar bond is subjected to a continuously increasing force. Under these dynamic conditions, SP-D binds strongest to D-mannose and weakest to maltose and D-galactose. These results differs from equilibrium measurements wherein SP-D exhibits preference for maltose. The binding site is optimized for monosaccharide binding and we propose that binding of the disaccharide maltose changes the interactions potential in a way which lowers the dynamic stability but not the equilibrium stability. We emphasize that determining the strength of a protein-ligand bond under a dynamic stress using an atomic force microscope is possible more relevant for mimicking the actual non-equilibrium physiological situation in the lungs.
Session IV: Model membranes and protein-membrane interactions

Selected talk

Atomic force microscopy characterization of supported planar bilayers that mimic the mitochondrial inner membrane

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In this study we examined the properties of supported planar bilayers (SPBs) formed with phospholipid components comprising the mitochondrial inner membrane. We have used 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) and cardiolipin (CL). Liposomes of binary POPE:POPC (0.6:0.4, mol:mol) and ternary (POPE:POPC:CL (0.5:0.3:0.2, mol:mol:mol) composition were used for the formation of SPBs onto mica. Characterization of the SPBs was carried out below (4°C) and above (24°C and 37°C) the phase transition temperature (Tm) of the mixtures in solution. We have observed: (i) that bilayers’ thicknesses, calculated from cross-sectional analysis decreased as the visualization temperature increased; (ii) the existence of laterally segregated domains, that respond to the temperature, in SPBs of POPE:POPC:CL; (iii) increases in height and roughnesses (Ra) of SPBs after cyt c injection at room temperature. The get further insight on the nature of the interaction between cyt c and the bilayers the competition between 8-anilino-1-naphthalene sulfonate (ANS) and the protein for the same binding sites in liposomes was monitored by fluorescence. The changes in the surface potential of liposomes containing CL (1.5 mV) revealed the existence of an electrostatic interaction which is dependent of the amount of CL. Taking together these results and previous works published by the group we will discuss the preferential adsorption of cyt c in CL domains. This would provide means for the relevance of this phospholipids as a proton trap in the oxidative phosphorylation in the energy transducing membranes.
Session IV: Model membranes and protein-membrane interactions

Selected talk

Concept of dynamic DNA network dedicated to DNA-Protein interactions studies.

Celine ELIE-CAILLE\textsuperscript{1}, Eric LESNIEWSKA\textsuperscript{2}, Alexandre BERTHIER\textsuperscript{3}, Regis DELAGE-MOURROUX\textsuperscript{3}, Wilfrid BOIREAU\textsuperscript{1}

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The approach lies on the development of an original DNA biosensor. This self-assembled DNA biochip is constituted of a tethered lipid membrane presenting « protein/DNA » complexes. This unique assembly, using a highly flexible bifunctional molecular spacer and a fluidic matrix, allows the control of oligonucleotides surface density and their mobility thus favouring hybridization of the DNA probes in liquid environment and under weak constraints. Recent Surface Plasmon Resonance experiments lead to building of protein/DNA dimers presenting two special DNA sequences recognized by regulation proteins. We demonstrated the capacity and relevance of this biosensor in the case of an estrogen receptor/DNA specific recognition, opening the possibility of specific drugs screening. We present here preliminary characterizations of these thin biomolecular films through Atomic Force Microscopy in liquid. The homogeneity of the lipidic film was controlled, the surface coverage in protein was evaluated and specificity of binding is under examination at the present time. Indeed, the visualization of the self-assembled « edifice » at a molecular scale and in a bio-mimetic environment is a precious and complementary way to improve the understanding of the protein/DNA interaction mechanisms.
Session IV: Model membranes and protein-membrane interactions

Selected talk

Using Atomic Force Microscopy to Quantify Amyloid Formation at the Nanoscale

Martijn van Raaij, Ine Segers-Nolten, Vinod Subramaniam

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The aggregation of proteins inside or outside neural cells is the pathological hallmark of many human neurodegenerative disorders. We use both commercial and custom-built atomic force microscopes to investigate the morphology of aggregates formed by alpha-synuclein, a protein implicated in Parkinson’s disease. We developed a quantitative morphological analysis procedure (1) to precisely measure differences between fibrillar aggregates formed by disease-related mutants and wildtype forms of alpha-synuclein. Current work extends these procedures to measure fibril length distributions as a function of initial protein concentration. These data will be used to test a statistical-mechanical model of fibril formation to assess the energies of various interactions between alpha-synuclein monomers as they aggregate. An inherent limitation of AFM is that it probes only the exterior surface of any structure on a support. To overcome this restriction, a combined AFM and single molecule confocal fluorescence microscope was developed in our group. This instrument allows us access to the ‘inside’ of the fibrils by fluorescent labeling of alpha-synuclein and its mutants. In this way we expect to shed light on cofibrillation of fluorescently labelled protein variants. (1) van Raaij, M. E., G. M. J. Segers-Nolten, V. Subramaniam (2006). "Quantitative Morphological Analysis Reveals Ultrastructural Diversity of Amyloid Fibrils from alpha-Synuclein Mutants." Biophys. J. 91(11): L96-98.
Selected abstracts for poster presentation

Sessions

Session I | Cells, Cellular interactions
Session II | Single molecular recognition, affinity, unfolding forces
Session III | High resolution imaging
Session IV | Model membranes and protein-membrane interactions
Session I: Cells, cellular interactions

Study of bioactive composites based on polylactide/pseudowollastonite for bone tissue engineering

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Novel bioactive and biodegradable composites based on semi-crystalline poly(L,L-lactide) (PLA) and pseudowollastonite (psW) particles were fabricated by melt blending. The bioactivity of these PLA/psW composites was evaluated by soaking in a simulated body fluid (SBF) at 37 °C and pH: 7.25. The hydrolytic degradation of the polylactide matrix, the evolution of the pH and the ion concentrations in the SBF solutions were evaluated by techniques such as gel permeation chromatography (GPC) and atomic emission spectroscopy inductively coupled plasma (AES-ICP). The results show that the immersion of the composites does not affect the local pH values of the solutions, which remain within the physiological range. The materials release Ca2+ and (SiO4)4- ions in a controlled way over time. In this work, we also characterized the degradation of the polylactide matrix, the dissolution of psW crystals and the formation of hydroxyapatite (HA) at the surface of the composites, by means of atomic force microscopy (AFM) measurements. The results show that the PLA/psW composites exhibit a significant bioactivity because the formation of HA is induced after immersion in SBF. These morphological studies, coupled with the characterization of the molecular parameters, and the analysis of the evolution of the pH and ion concentrations allow to establish a comprehensive model of the bioactivity of the PLA/psW composites in the framework of their use in bone-guided regeneration.

Mechanical Properties of Human Lamina Cribrosa Cells of the Optic Nerve Head

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Purpose: To investigate the nanomechanical properties and mechanoresponsiveness of Lamina Cribrosa (LC) cells. Methods: LC cells harvested from normal human optic nerve heads were imaged and indented using Atomic Force Microscopy (AFM). Firstly, we scanned individual LC cells to acquire a detailed topographical image of our LC cells. We then indented cells at up to 20 points, 2-5µm apart, in a line across a number of cells to calculate the variation in cell elasticity throughout the cell body. We applied repeated, point-specific, 5-10 nN forces to a number of cells to investigate the mechanoresponsiveness over time. For each indentation a force curve was formed and
using the Hertz model we calculated the elasticity of the cell. To account for cell variability, both within the cell and between cells, large populations of cells were examined. We used a force mapping model to quantify the variation in elasticity throughout the individual cells. We also transfected our LC cells with a fluorescent tag for F-actin and using these fluorescent cells focussed our AFM nanoprobe to indent actin rich regions of our cells. Results: We found a large variation in elasticity across the cell surface. Repeated force-specific indentations to areas of high elasticity resulted in an almost linear increase in elastic modulus. This response to repeated AFM indentation was rapid with a four-fold increase in elasticity in responding cells over a ~20 second period. Cells indented in areas of a lower elasticity did not respond to repeated AFM indentation. Conclusion: The variation in elasticity across the cell surface corresponds to the distribution of the cytoskeletal filaments intracellularly. We believe that responding cells are indented at actin filament rich areas. These results highlight the importance of the actin cytoskeletal filaments as mechanosensing and mechanoresponding units of the LC cell.

A TEST BED FOR BIO CELL MANIPULATION

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Bio, micro and nano technologies are among the most important of the recently emerging domains in science and engineering. The focus of this paper is on biological cell manipulation and the creation of a simulation framework to assist in such activities. The field of biology presents interesting challenges especially in the context of using micro / nano technological tools and approaches. One of the recent areas of interest is the exploration of methods to manipulate (pull, push, move, etc) individual cells. This paper outlines the design of a Bio Manipulation Research Test Bed for cell manipulation contexts; the development of interactive force models to enable the understanding of the mechanics underlying cell manipulation is also discussed. The adhesive forces can be minimized through design of innovative gripping approaches and gripper/probe shapes. The design of an integrated Virtual-Physical interface involving a Virtual Reality environment and a physical AFM based manipulation environment is also discussed. The creation of a simulation framework holds the potential of enabling the virtual comparison of alternate cell manipulation approaches prior to conducting physical activities.
The Development of Scanning Ion Conductance Microscope Module for Imaging Live Cells

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A new scanning ion conductance microscope (SICM) module has been developed from a conventional Atomic Force Microscope (AFM) platform, which uses a glass micropipette as a sensitive probe instead of silicon-based stylus. Since it utilizes a conventional AFM platform, SICM module is freely exchangeable with the AFM Module. Therefore, we introduce the first machine to image the same sample with two different scanning probe microscopic techniques at the same place. A glass micropipette as a SICM probe filled with electrolyte senses ion current to feedback its position when it images samples immersed in buffer solutions. Like Scanning Tunneling Microscope operation in air, the SICM images the sample without physical contact in liquid. The SICM is particularly valuable to imaging soft biological sample, such as live cells. On the contrary, AFM is not very convenient for imaging soft biological samples due to too many interactions with samples, but it still provides invaluable data from the physical contact with the sample. No other technique other than AFM can easily measure single molecular interactions. We have successfully imaged various samples in buffer solutions using both SICM and AFM modules at the same place and will introduce comparing results in this conference for the first time. This instrument can be utilized in diverse biomedical research areas and will bring huge attention to Scanning Probe Microscopic technologies and nanobio science.

BioAFM Nanomechanical Responses of Green Tea Extract Induced Annexin-I Expression on Actin Regulation in Human Lung and Prostate Adenocarcinoma Cells in vivo

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Cancer chemoprevention is an important strategy for cancer control and one key component of these therapies is determination of surrogate end point markers which are central in predicting a subject’s risk of developing disease and monitoring the effectiveness of a particular therapy(1). Green tea, one of the most commonly used beverages around the world, is widely known for its health benefits. In conjunction with collaborators our research incorporating green tea has shown promising anticancer effects on various cancers including bladder and lung cancers(2-6). Green tea extract (GTE) and some of its major catechin components such as epigallocatechin-3-gallate (EGCG) are known to produce numerous biological activities in several cell models, including antioxidation, antiproliferation, antiangiogenesis and apoptosis(1, 7-
8). Previously we demonstrated with a human lung adenocarcinoma model that GTE induces actin remodeling. This study uses AFM to probe the potential mechanisms of the anticancer effect of GTE and identify potential biomarkers for GTE based chemoprevention trials via monitoring associated nanomechanical properties. We previously showed that actin remodeling correlated with increased cell adhesion and decreased motility(9), although the exact biomechanical mechanism remained unclear. In our current study we identify and probe a particular actin binding protein (ABP), annexin-I, as the protein target responsible for the actin remodeling effect. Using AFM we show nanomechanical responses of GTE induced annexin-I expression on actin regulation in human lung and prostate adenocarcinoma cells in vivo and further hypothesize that these protein targets might be used as potential surrogate endpoints for GTE based chemoprevention trials.

An Atomic force microscopy study of the endometrial cell surface; applications in female reproductive biology and regenerative medicine.

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Mammalian cell interactions and attachment are key processes within clinical systems and represent a challenging research area within human medicine. The endometrium, a key interface in female reproductive biology, is the site for blastocyst attachment following a complex network of hormonal and molecular signalling during ‘normal cellular function’. MUC1, a transmembrane glycoprotein and a host of adhesive proteins affect cellular adhesion and endometrial receptivity; defects in these systems lead to abnormal function. Female infertility and uterine cancer are both current and developing problem areas, approximately 1 in 6 couples are infertile with around 20% of these cases unexplained (UIF) while uterine cancer accounts for 4% of the total UK cancer figures. AFM sample preparation methods have been optimised and allow quick and simple high resolution cellular imaging at the nanoscale for comparison with Scanning electron Microscopy. Characterising cell surface differences between cell lines, biopsy and whole tissues allows novel insights into epithelial morphogenesis and will provide the platform for further adhesive and molecular cell surface research. In the case of regenerative medicine, AFM imaging has also been used to characterise biodegradable scaffolds and the proliferation of cells at their surface. Highly ordered 3D scaffolds, with micro and nanoscale pores, were produced in a range of polymers using a novel colloidal templating technique. AFM observed the remodelling of the scaffold with the development of the seeded fibroblasts. This research area has been extended by the use of force mapping, liquid imaging and the combination of AFM with confocal microscopy.
Real-Time Examination of Cell Fusion Using AFM

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Nowhere is the process of membrane fusion more accessible in an intact system than when examining myogenesis. In response to a drop in trophic factors, individual myoblasts withdraw from the cell cycle, align themselves into linear arrays and fuse their membrane to form multinucleated myotubes, the first stage in the formation of an adult muscle fiber. Importantly, the two initial conditions that must be met to enable myoblast fusion independently of protein synthesis include: 1) A rise in intracellular calcium; 2) and the existence of negatively charged phospholipids, namely phosphatidylserine, in the other leaflet of the myoblast membrane. Using an in vitro AFM approach we have been able to following the fusion events of aligned myoblasts and discern local reorganization of the membrane-associated cytoskeletal network at points of apposition in fusing cells. Specifically, tubular complexes of membrane-cytoskeleton appear shortly following alignment that connect fusing myoblasts and then grow in girth and number as fusion proceeds to completion, usually two to three hours. The Annexins are a class of protein fusion-regulators that bind phosphatidylserine in a calcium-dependent manner and have putative calcium-channel capabilities. Annexin V labels the surface of myoblasts prior to myotube formation and in some instance appears to be more highly concentrated in the tubular membrane networks that establish intracellular continuity between fusing myoblasts. We discuss the implications of these findings.

AFM EXPLORATION OF THE SURFACE OF S. EPIDERMIDIS ATCC12228 IN LIQUID WITHOUT ANY CHEMICAL OR MECHANICAL FIXATION TREATMENT

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The surprisingly high hydrophobicity of the biofilm-forming S. epidermidis ATCC12228 has been used to naturally fix it onto the surface of glass. The absence of any chemical or mechanical fixation has been decisive to provide a more realistic nano-meter scale behaviour of the surface of a gram-positive bacterium in liquid environment. The study shows how the surface of S. epidermidis exhibits both, elastic and plastic deformations and these yields to two different patterns in the adhesion force curves. These results can be very useful for revealing interesting aspects of the physical properties and structure of the cell walls.
Following the aging of RBCs by monitoring the roughness value of their plasma membrane

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We recently developed a protocol to use the roughness of the plasma membrane of RBCs as an independent morphological parameter to label a sample as a whole and to investigate the membrane-skeleton structure in healthy and pathological cells. The method is based on the collection and the analysis, performed in statistical terms, of AFM images of erythrocytes. In the present study we used the surface roughness to investigate the physiological phenomenon of the aging of RBCs. It is well-known that RBCs are unable to self-replicate. The turnover mechanism relays on the behaviour of the spleen’s macrophages which, recognising specific markers on the RBC membrane, remove from the blood circulation the aged cells. Mechanisms leading to the occurrence of an aging-marker on the membrane have been proposed and yet it seems interesting to investigate the behaviour and the possible role played by the cell membrane-skeleton. In particular, we followed the aging of RBCs by monitoring the roughness of their plasma membrane. The aging process has been accelerated and/or modulated by acting on the blood’s proteasis and by artificially consuming the erythrocytes’ ATP content. The results of such a study will be compared to the cells’ aging occurring when using the protocol of blood conservation normally employed for transfusions (addition of ACD solution, with or without DPG, in a slightly acid pH).

ON THE PARTICULARITIES OF THE MECHANICAL TRAPPING OF BACTERIAL CELLS FOR AFM ANALYSIS

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The influence of the protocol used for immobilization of bacterial cells onto surfaces is discussed, in particular, the mechanical trapping of cells onto porous filters is under revision. Two slime-producer strains of S. epidermidis are mechanically trapped onto 0.8 $\mu$m pore size filters before AFM exploration. Results show that mechanical trapping of cells induces mechanical changes on the surface walls of bacteria, and this can alter the real physico-chemical properties of the microbial surface. Such changes can be enhanced by the mechanical deformation that pores suffer during the filtration process. When the experiments are carried out with slime-producer bacteria, exopolymERIC substances tend to accumulate in their surrounding areas, making the interaction with the tip stronger at these locations.
Combined AFM and Fluorescence measurements on postnatal rat pulmonary type II cells after stimulation with ATP

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Type II cells are known to change their secretory function after stimulation of their purinergic receptors. This stimulation is leading to the exocytosis of the lamellar bodies, however, it is not yet clear how their physical properties change simultaneously. We are using Atomic Force Microscopy (AFM) in constant force mode with additional lockin modulation to record simultaneously the topography, the stiffness and the viscosity of a whole cell during the stimulation process. The monitoring of the lamellar body fusion on the same cell is done by use of FM1-43 dye in the bath solution and a fast setup configuration switch. The experiments clearly show that the physical properties of the cell are changing after ATP stimulation. The additional use of AFM lockin signal modulation is critical to access elasticity and viscosity. We can consequently present a unique set of pictures showing already described phenomena such as surfactant release (cross-checked with fluorescence images), cell swelling and most importantly, yet unknown phenomenon such as the change in stiffness. Supported by the DFG, grant D1402, the FWF, grant P15743 and the EU ("Pulmo-Net").

SENSING HYDROPHOBICITY OF FILAMENTOUS BACTERIA WITH AFM

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Certain filamentous bacteria occurring in activated sludge, causes problems with foaming and bulking at wastewater treatment plants. Foaming is believed to be due to the hydrophobicity of the filamentous bacteria leading to adsorption of air bubbles on the bacterial surface (1). Tapping mode atomic force microscopy has been proven to be a suitable method to characterize bacterial surfaces in native state without damaging the bacterial cell surface (2). The method has in this study been used to image the bacterial surface in liquid as an initial step in characterization of certain filamentous bacteria, i.e. Microthrix Parvicella and Nocardioforms. Force volume measurements with bare and functionalized tips will in further studies be used to characterize the hydrophobicity along the cell surface of the filamentous bacteria. The tips will be functionalized with different groups to investigate preferential interactions to domains of the cell surface (3 4).

Morphological changes in renal epithelial cells induced by aldosterone

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Aldosterone is known to stimulate inner medullary collecting ducts (IMCD) via the classic genomic pathway and has recently been shown to also respond in rapid, non-genomic effect. Currently we are investigating the response of mouse IMCD cells to aldosterone at different exposure times ranging from 4 minutes to 48 hours and with and without the aldosterone antagonist spironolactone. We used the atomic force microscope (AFM) to study morphological changes of the cells by imaging and measuring cell volumes. We present here preliminary findings obtained with the AFM, with data indicating cell volume increased on exposure to aldosterone. Spironolactone did not appear to inhibit the action of aldosterone on the mineralcorticoid (MR) receptor and prevent cell volume change.

Visualization of adherent Salmonella Typhimurium on inert surfaces by AFM imaging

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Fimbriae or flagella of gram-negative bacteria are often visualized by transmission electronic microscopy. This technique needs chemical fixation and dehydration of bacteria before observation and bacteria have to be detached from the surface if they are adherent. Bacterial images can be obtained rather easily with an atomic-force microscopy (AFM) without any pre-treatment of the bacteria as chemical fixation, dehydration or staining. Adherent bacteria can be visualized in situ without detachment. In this work, atomic force microscopy contact mode (PicoLE, Molecular Imaging, ScienTec, Palaiseau, France) operated under air at room temperature was used to observe adherent Salmonella to inert surfaces commonly used in food.
industries. We used cantilever (silicon nitrides gold coated oxide-sharpened, ScienTec, Palaiseau, France) with a spring constant of 0.18 N.m⁻¹. The microscope was operated at a scanning rate of 1-2 Hz in constant force mode (dc-mode). The modes used were topography and friction modes. The bacteria shape or the presence of flagella can be clearly recognized in these magnification ranges. Measurements of length and thickness of flagella can be achieved.

Detection and quantification of adherent bacteriophages by in situ AFM

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The mechanisms involve in bacteriophages adhesion to solid surfaces are poorly understood although these interfacial phenomena could be linked to high economical loss in the dairy industry. Indeed, bacteriophages of lactic acid bacteria constitute a threat during industrial milk fermentation, because infection leads to the lysis of starter cells. To have a better understanding of phage settlement on various model surfaces, we estimated their adhesion ability by in situ atomic force microscopy (AFM) and image analysis. In view of our results, it is clear that those model viral particles can adhere on processing equipment in dairy plants. These immobilized virus could therefore be protected from environmental stress i.e. hygienic procedures, and contaminate the processed goods.


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Local mechanical properties of living cells have been investigated by means of AFM, using the Digital Pulsed Force Mode (DPFM). In DPFM the sample surface is scanned by probing its local mechanical properties. At least one force curve is recorded for each point of the scanned area. Thus, more than 500,000 force curves are recorded for each single experiment. Afterwards these curves can be evaluated with respect to numerous quantities. The glass-like substrate serves as a reference material for calibration purposes. First, the force trajectories have to be corrected for the viscous drag force acting within a liquid environment. Secondly, the curves within the region of the substrate are phase corrected to compensate for signal runtimes in the setup, assuming a purely elastic response of the reference material. Finally, all the force traces are corrected using this calibration parameters. Then all data are evaluated
according to common continuum-elastic models. The results allow the determination of Young's modulus, local adhesion, and hysteretic behaviour at a high lateral resolution for the entire cell body. The measurement procedure and the described signal correction strategy of the automated data evaluation will be presented.

Scanning Conductance Microscopy of Chromosomes

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In analysing chromosomes, one of the major challenges are the sorting of the chromosomes before the actual analysis. In this work we will investigate the dielectric constant of chromosomes using an atomic force microscope (AFM) operating in dual scan mode(s?). The dielectric properties are important to know when designing integrated sorting procedures using electrical forces. The Scanning Conductance Microscopy (SCM) method is used to estimate the dielectric constant of fixed human chromosomes. The fixation method of the chromosomes gives rise to a layer of cell debris on top, which disturbs the electrical signal and a process of using pepsin has been optimised to remove this disturbance. A thorough characterisation of the setup has been necessary, as electrostatic forces are long range and background noise becomes a major issue and also an estimate of the tip and cantilever properties (tip radius, Q factor and spring constant of the cantilever) have been made by varying the tip sample distance.

AFM Investigations on Chondrocytes Subjected to Mechanical Stress

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The superficial layer of articular cartilage, lamina splendens, is a matrix rich layer that provides very low friction movement to the articular joint. Gross microscopic examinations have revealed that the integrity of lamina splendens is severely compromised in arthritic diseases. While it is well accepted that any therapeutic intervention used to repair cartilage must return the functionality of lamina splendens, neither the superficial structure of this layer of cartilage, nor the arrangement of extracellular matrix (ECM) at high magnifications is as yet investigated. In the perspective of lamina splendens’ importance for proper functionality of the joint, its structure must be understood at nanoscale level to evaluate the responses of therapeutic intervention used to treat, repair or regenerate diseased cartilage. The ultrastructure of the pericellular cartilage matrix is a composite structure, which
consists of collagen fibrils interspersed (the dispersed fiber phase) within a matrix (continuous phase) of proteoglycans secreted by the cells. The characterization of each cell, i.e., its size, shape, orientation, and distribution with respect to the articular surface and applied forces is crucial in understanding the effects of mechanical forces around a cell and how a cell responds to changes in its physical environment. The proposed hypothesis is that the molecular composition and organization of the pericellular matrix are critical factors in determining how mechanical signals are perceived by the cells (chondrocytes) and how cells respond to them.

Evaluation of photodynamic reaction on cancer cell lines by fluorescent methods and atomic force microscopy

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Photodynamic therapy (PDT) is a new treatment modality of tumours. The photochemical interactions of sensitizer, light, and molecular oxygen produce singlet oxygen and other forms of active oxygen, such as peroxide, hydroxyl radical and superoxid ion. The tumour is destroyed either by reactive singlet oxygen species (ROS), type II mechanism, and radical products, type I mechanism, generated in an energy transfer reaction. The resulting damage to organelles within malignant cells leads to tumor ablation. The cellular effects include membrane damage, mitochondrial damage and DNA damage. The promising generation of sensitizers - phthalocyanines were used to induce the photodamage. In addition, we also applied an ultrasound treatment to support the photodynamic effect. We report the sensitizer uptake, the production of reactive oxygen species and the phototoxicity on G361 melanoma cells. The semiconductor laser was used as a source for evocation of the photodynamic effect. Morphological changes in cells have been evaluated using fluorescent microscope and atomic force microscopy. The quantitative cell viability changes in relation to sensitizer concentration and irradiation doses were proved by fluoroscan. We found optimum combinations of sensitizer concentration and corresponding radiation dose which were lethal for cancer cells in tissue cultures. This work was supported by the Ministry of Education of the Czech Republic MSM 6198959216.
Examination of dentin surface using AFM

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Atomic force microscopy (AFM) as one the technique of Scanning Probe Microscopy is useful for imaging of surface structures. This method can yield 3D high-resolution topographic images of sample surfaces by using a scanning technique for conductors and insulators on atomic scale. A special construction of AFM scanner enables to follow biological samples in liquid environments. Artifacts caused by dehydration of samples are removed this way. Dentin of human teeth is a vital hydrated tissue. It is strongly sensitive to dehydration and drying that are commonly used in preparation of samples in examinations by Scanning Electron Microscopy (SEM). We describe our experience in examination of dentin surfaces of extracted human third molars using contact method of AFM under moist conditions.

An Overview of Bacterial Sterilization via Microwave Irradiation and The Effect on the Bacterial Cell Wall

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The destruction of food bacteria has been the subject of research and development in many disciplines for a very long time. Solutions have emerged from time to time but ultimately resulted in the ongoing quest for keeping abreast of new bacteria strains emanating from old. Cell membrane damage has been proposed as to the cause of cell death, and the work in progress involves the use microwaves at short time periods and high power levels, to kill the food carried bacteria, Salmonella poona, Escherichia coli and Staphylococcus aureus. Investigations carried out thus far have allowed the researchers to discover an optimum kill time and frequency for the test bacteria. The work involved controlled microwave conditions using fixed frequency generating equipment to achieve the required microwave power rate at the required time intervals and electromagnetic radiation delivery via rectangular waveguide. The use of the Bac-lite test kit was used to ascertain cell membrane damage whilst Scanning Electron Microscopy (SEM) and Atomic Force Microscopy (AFM) are being utilised to discover the anatomical and physiological effects of the Microwave Irradiation on the cell membrane.
Hyaluronan interaction with the CD44 membrane receptor locally probed on living glioma cell by AFM

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Understanding the migration process that leads glioma cells (GM) to diffuse and infiltrate the brain tissue from the primary tumour mass is of the foremost importance. Hyaluronan (HA) is a ubiquitous glycosaminoglycan of very high molecular weight largely involved in the formation of the extra-cellular matrix (ECM). Glioma cells interact with HA through CD44, a cell membrane receptor which if often up-regulated in cancer. Importantly, HA/CD44 interaction provide cell adhesion sites to the ECM, which can only allow cell progression if a proper coordination of these links is made. Using a HA modified AFM tip as a tool to locally probe single HA/CD44 interactions, we evaluated the role and impact of CD44 distribution on the surface of GM cells in the adhesion behaviour to the HA matrix. Results were compared when the receptors are either localized on microvilli uniformly distributed over the cell surface of resting cells or on membrane ruffles observed in highly motile PMA stimulated cells. Both rupture forces and mechanical profiles of the interactions lead to a cell migration model in which highly motile cells would interact lightly with HA and only at the leading edge while resting cell would have their entire cell body strongly attached to the HA. The role of the cytoskeleton in CD44 distribution and HA binding is also highlighted.

Apoptosis and Necrosis studied by Atomic Force/Lateral Force Microscopy

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Atomic Force/Lateral Force Microscopy (AFM/LFM) is an experimental technique that can provide both topographic (with nanometer scale lateral resolution) and chemical information on the cell membrane. Apoptosis and necrosis are the major cells’ death mechanisms that can be discriminated both in biochemical and morphological terms. Through a high-resolution contact-mode AFM/LFM we have studied the occurrence of these death processes in different environmental conditions and cell lines. In particular we studied the interaction of the cytotoxic metals cadmium, zinc and lead with human pancreatic cells and the selective induction of apoptosis or necrosis in human granulocytes due to the withdrawal of an essential growth factor (IL3). The results of the topographic studies evidenced the appearance of morphological markers indicating the occurrence and progression of apoptosis or necrosis-induced death. Additionally, the early appearance of strong friction contrasts have been observed on the plasma membrane of the cells which are incoming the
apoptosis pathway, while no LFM contrast was recorded on necrosis-induced cases and on healthy cells. Thus, the coupling of two surface-characterizing techniques, such as LFM detection and topographic AFM characterization, allows to distinguish between different biochemical pathways of cell degradation induced by diverse stimuli.

Ultra structure and properties of the stratum corneum: an Atomic Force Microscopy study

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Atomic Force Microscopy (AFM) extends the scope of information given by classical methods like Scanning Electron Microscopy (SEM) or Confocal Microscopy, allowing for example, a very high resolution of biological structures in ambient conditions to be reached. This is an advantage as many cosmetic properties rely on the deposition and transfer of materials onto the skin surface to repair or improve its properties. Knowledge of the local properties of skin outermost horny layer (the stratum corneum) is essential to fully understand its role. In particular, the skin’s barrier function is for a large part ascribed to adequate structure of its components. We will present some applications of AFM to the study of in vitro skin properties and in particular the local ultra structure of the stratum corneum surface and cellular constituents. This morphological analysis will be further compared with a mapping of local mechanical properties. Eventually these observations will be interpreted through well-known biological processes.
Mechanical Properties Of Mesenchymal Stem Cells and Their Lineages Using Atomic Force Microscopy

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Mesenchymal Stem Cells (MSCs) are multipotent mechanosensitive cells capable of generating multiple stromal cell lineages. We have investigated the mechanical properties and mechanoresponsiveness of MSCs on different substrates and also along different stromal lineages. Employing Atomic Force Microscopy (AFM) and live cell imaging we have established the morphology, mechanical properties, mechanosensitivity and migration characteristics of MSCs on glass and silicone substrates. Results reported a mechanosensitivity feedback loop controlled by cell substrate interactions, effecting migration, mechanoresponsiveness and mechanical structure of the cell. While studying the subcellular structure of MSCs cultured on glass, geodesic F-actin structures were observed, with their appearance correlating to time cultured on the glass substrate. Mechanical properties of these structures were calculated by dissipation mapping, indicating that mechanically geodesic F-actin differs significantly from linear F-actin. Having established the mechanical properties of unstimulated control MSCs, MSCs were treated with medium conditioned for the osteogeneic lineage. Results indicated that MSCs conditioned for osteogenesis have an increased elastic modulus compared to control MSCs. The mechanosensitivity of control MSCs was observed to be significantly higher than MSCs treated with medium conditioned for osteogenesis. While investigating the mechanical properties of control MSCs versus MSCs treated with medium conditioned for osteogenesis, variation in the mechanical properties and magnitude of mechanosensitivity was observed between animals. This illustrates that at the single cell level, genetic differences between animals can be determined. By investigating the mechanical characteristics of MSCs a greater understanding of MSCs can be established, hence greatly enhancing the field of regenerative medicine.
Nitric Oxide Signalling in Bone Cells Induced By Atomic Force Microscopy

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Bone is a self-repairing structural material, capable of adapting its mass and structure to serve its biomechanical function throughout life. Nitric Oxide (NO) is a vital signal transducer in the response of bone cells to mechanical stimulation. Employing Atomic Force Microscopy (AFM) and fluorescence microscopy, we have studied NO signalling in response to mechanical stimulation. We have observed NO responses in single osteoblasts induced by application of localised indenting forces to the osteoblast membrane. Periodic indentation (17 to 50 nN) stimulated three distinct NO responses in the indented osteoblast: (i) a rapid and sustained diffusion of NO from the perinuclear region; (ii) diffusion of NO from localised pools throughout the osteoblast and (iii) an initial increase and subsequent drop in intracellular NO. NO responses were associated with application of force to more rigid membrane sites, suggesting cytoskeletal involvement in mechanotransduction. To monitor for the creation of false positive results, independent controls working in real time with AFM data acquisition were established to monitor cell viability and fluorescence abnormalities before, during and after every experiment. By combining AFM force stimulation, measurement of local elasticity at indentation sites, and real-time monitoring of NO within single cells, we have reported that osteoblastic cells respond globally within seconds to a localised mechanical force with synthesis and release of NO from discrete regions of the cell. Correlating mechanical and biochemical signalling events, while separating responding from non-responding cells, we have come one step closer to understanding the complex nature of osteoblast response to mechanical stress.

Micro and Nano-Systems for Cellular Biomechanics

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CSEM SA Neuchâtel CH

Cell-surface interactions play a crucial role in the adhesion, motility, function, gene expression and survival of the cells. Hence, the control of the topographical and chemical structuration of the substrate interacts directly on the tissue development. Micro and nano-structuration techniques allow to create the ideal environment for tissue engineering. Tissue growth and biocompatibility studies of implants need specific characterization tools. Particularly, nanotools for micro-rheology
characterization with high throughput are particularly promising to study cause-effect phenomenon on cell adhesion. In this frame, we present different systems of micro and nano-structured surfaces designed to control osteoblast and fibroblast behaviour. The cell spreading is characterized by fluorescence microscopy to do the relation between the topographical structure of the surface and the focal points locations. We will present also the development and applications of 2D cantilever array technology for life science. The integrated system is based on passivated piezoresistive cantilever arrays. The potential of such piezoresistive cantilevers to explore biological samples has been evaluated. The mechanical properties of fibroblast cells are studied by means of force spectroscopy measurements. Such quantitative results are utmost promising for the characterization of cell mechanical properties and cell adhesion. Finally, a nanotool, based on silicon cantilevers, for attoliter volumes dispensing will be presented. In a near future, the combination of nanodispensing with arrays of cantilevers is expected to find promising applications in statistical measurements and excitation for cellular biomechanics. We thank the Nanotools team of the Institute de Microtechnology (IMT) of Neuchâtel for their collaboration.

**Roughness of the Plasma Membrane is Sensitive to the Integrity of RBCs Membrane-Skeleton: a Quantitative AFM Investigation**

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The surface roughness is an important parameter for the many applications which require a characterization and/or a comparison of surfaces. The use of such a parameter has received a new impulse from the application of SPM techniques and, in particular, of AFM which is increasingly used in biology. A novel approach to the study of RBCs based on the collection of three-dimensional high resolution AFM images and on the measure of the surface roughness of their plasma membrane is introduced. In the present study we show that the Roughness of RBCs is characteristic of the single cells composing a sample but independent from the overall geometric shape of the erythrocytes, thus providing additional information with respect to a conventional morphology study. Therefore we tested the average roughness value as a label of a whole sample on different kind of samples. Analysed data revealed that the quantitative roughness value does not change after treatment of RBCs with various commonly used fixation and staining methods. Finally, we studied cells with cytoskeletal alteration either naturally occurring or artificially induced by chemical treatments: a drastic decreasing of the average roughness value occurs. This last result explains the usefulness of a surface roughness study for ascertaining the RBCs’ membrane skeleton integrity.
AFM imaging of platelet adhesion to matrix proteins at arterial shear stress

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Vascular injury leads to disruption of the endothelial monolayer and subsequent exposure of extracellular matrix (EM) proteins. Platelet adhesion to proteins of the EM, such as collagen, fibrinogen and von Willebrand factor (vWF), is initial step of arterial thrombus formation. During adhesion, platelets become activated, degranulate and undergo profound alterations in cellular architecture. In the present study we use the Atomic Force Microscope (AFM) to obtain high resolution images of attached platelets in order to evaluate EM protein-dependent morphological changes during platelet adhesion at high shear stress. The AFM enables to study the attached platelets with nm resolution over a µm range. The studied samples consisted of isolated human platelets perfused at arterial shear rates through a flow chamber. The flow chamber is equipped with coverslips coated with collagen, fibrinogen or vWF. After perfusion the fixed cells were investigated using scanning electron microscopy (SEM) and AFM imaging. The AFM setup included an optical microscope for fast inspection of samples. Both, the adherent platelets and the different EM substrates, were successfully imaged by AFM. The images reveal detailed morphological features of platelet interaction with the protein substrate, which we were unable to detect with scanning electron microscopy. Additionally, topographic AFM-analysis of platelets showed cavities at the caudal cellular aspect, most probably resembling the localization of platelet degranulation. AFM measurements exhibit a promising way of platelet imaging. Therefore, our future goal is to analyse in depth the morphology of platelet adhesion under physiological conditions.

Adhesion Force Spectroscopy of Single Cells on Nanopatterned Substrates

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Surfaces of defined adhesion properties are required for a physical and quantitative understanding of cell adhesion in vivo. Biofunctional nanopatterns can be employed to
position adhesion ligands in a quasi-hexagonal lattice and thus allow cooperative interactions between cellular receptors to be studied. In our work, nanopatterns are used to investigate integrin-mediated cell adhesion, which is a highly complex biological process and essential for numerous cell functions. With nanopatterns, the distance between adjacent single integrin binding sites is precisely defined. Recent cell culture experiments have revealed that this distance strongly affects cell adhesion and the formation of adhesion clusters, known as focal contacts. To quantify the adhesion cluster formation for different integrin binding site spacings, cell adhesion forces have been studied by using atomic force microscopy (AFM). The experiments have demonstrated that an integrin binding site spacing of 70 nm and more prevents the cooperative formation of early adhesion clusters during the first five minutes of cell-substrate contact. In long-term adhesion studies, after several hours of cell adhesion, it turned out that the focal contact formation cooperatively increases the local adhesion strength. The experimental results were related to theoretical models and make a contribution to understanding the formation and stability of in vivo cell adhesion clusters.

A novel design of AFM probe integrated with hollow microneedle for cellular function analysis

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In order to realize cellular function analysis on a chip-base system, we have been developing a newly designed AFM probe in which a conventional sharp tip is superseded by a hollow silicon dioxide (SiO₂) microneedle connecting the root to a fluidic microchannel embedded into a silicon (Si) cantilever beam structure. The probe will be capable not only of performing AFM measurements but also of introducing desired biomolecules (nucleic acids, proteins, etc.) into living cells and the parallel extracting biomolecules expressed in the cells. The micromachining technique of a hollow SiO₂ microneedle involves photolithography and a combination of an anisotropic deep reactive ion etching (DRIE) process for producing thorough holes into a Si substrate as a needle mold followed by wet oxidation and an isotropic XeF₂ etching process for leaving a SiO₂ microneedle structure. A typical microneedle fabricated here had an inner diameter of 3 µm, an outer diameter of 5 µm, and a length of 50 µm with sharp tip shape (<1 µm). The penetration tests were conducted with a gelatin as an artificial cell, indicating that the microneedle is expected to be sufficiently stiff to penetrate living cells without fracture. Furthermore, a circular-shaped microchannel (typically 5 µm in diameter) was also successfully fabricated into Si by a combination of anisotropic DRIE and isotropic XeF₂ etching together with wet oxidation. Further development of our proposed novel AFM probes where a microneedle will be fully integrated at the end of a cantilever structure embedded a microchannel is under study.
Differential motion of intracellular organelles in response to nanonewton forces

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Mechanical stress affects and regulates many aspects of the cell, including morphology, growth, differentiation, gene expression and apoptosis. It also facilitates the way we perceive the world around us using the senses of touch and taste. In our current work we are studying how mechanical stress perturbs the intracellular structures of the cell, and how these changes induce mechanical and biochemical responses. Using various live-cell labelling techniques such as fluorescent protein fusion and in-vivo fluorescent dyes, we can label different intracellular structures such as the nucleus, mitochondria and the cytoskeleton. In order to correlate mechanical perturbations to cellular responses we used a combined fluorescence-atomic force microscope (AFM) to produce well defined mechanical perturbations and simultaneously track the real-time motion of the labelled structures. Image registration software has allowed us to begin quantifying the spatial dislocation of organelles in response to applied loads. In particular, results will be presented which demonstrate the highly dynamic and non-linear mechanical response of mitochondria in fibroblast cells. Future work will aim to understand how mechanical transduction through epithelial cells, such as skin keratinocytes and the tongue’s papillae, can affect the perception of touch and taste.

Probing Elasticity and Adhesion of Living Cells by Atomic Force Microscopy

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Atomic force microscopy (AFM) is currently used in different laboratories for either imaging or probing mechanical properties of living cells. In the contact mode AFM, the cells comply with the AFM tip shape on a contact area that depends on the cell softness, the tip geometry, the adhesion force between cell membrane and the tip, and the external force applied to the AFM probe. These parameters are crucial in imaging of the cell surface, since large deformations and tip sticking effects may arise because of large adhesion or externally applied forces. The atomic force spectroscopy experiments on living cells are also strongly affected by these parameters. The present work reports results of our AFM study on the interaction between living cells and tips of the AFM probes. We measured force-displacement curves for silicon, titanium-coated, and poly(ethylene glycol) (PEG) coated AFM tips and living Balb/c3T3 clone
A31-1-1 cells cultured on sterilized micro cover glass. While the adhesive force were very different for these tips (with the same conical geometry), the experiments allowed us to evaluate the effect of the adhesion force on the AFM indentation experiments of the living cells. We propose an indentation model for the case of relatively weak AFM tip-cell adhesive forces, when the elastic deformation of the cell cytoskeleton can be described by Sneddon’s theory and tip-cell contact area changes continuously by the change of the externally applied force.

Exploring the feasibility of the dip-pen nanolithography technique to the development of nanoscale immunosensors

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Nowadays it is clear that there is interest in the development of biosensors at the nanoscale that would allow an increase in sensitivity, speed, and reliability, and be more cost effective. One biosensing technique is immunodetection, used in genomic and proteomic microarrays. In order to increase the capabilities of this technique, one promising approach is the nanoscale patterning of biochemical functional elements. Dip Pen Nanolithography (DPN) is one capable technique to produce biochemical functional nanoscale patterns (1-3). Proteins, DNA, peptides, oligonucleotides can be directly transferred from a coated AFM probe to the substrate of interest. It is also possible to deposit smaller organic molecules which link covalently to the substrate and recognize specifically certain biomolecules. The transfer of these molecules should be faster and provide us of lower minimum features (,w20 nm), regarding their smaller sizes. This is thus the strategy we present in this work. Among the organic molecules that can be deposited by DPN technique, thiols are of special interest. They have been used in the patterning of recognition sites for proteins, enzymes, nanoparticles and RNA (4). Three different thiol derivates are proposed as "inks" to work onto gold: biotin (5), an specific receptor for avidin proteins (streptavidin, neutravidin); N-hidroxysuccinimide (NHS), an activated carboxylic acid which reacts with the free amino groups of the lysine residues of proteins; and an oligonucleotide thiol derivated which interacts with any biomolecule linked to its complementary oligonucleotide. All of them offer minimum features of about 40 nm and each one can recognise specifically a protein or other biomolecules. Multibiofunctional nanopatterns can help to distribute different proteins at the nanoscale and so to approximate the idea of a genomic/proteomic "nanoarray".

Effect of temperature on cell rheology probed by Atomic Force Microscopy

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Cytoskeleton (CSK) is a complex polymer network that regulates the structural stability of living cells. Although CSK plays a key role in many important cell functions, the mechanisms that regulate its dynamics are poorly understood. CSK dynamics exhibits a scale free behavior with a complex elastic modulus (G*) increasing with frequency as a power law. It has been suggested that CSK dynamics might be driven by activated processes. According to this hypothesis CSK rheology should exhibit a marked dependence on temperature. The aim of this work is to elucidate the effect of temperature on CSK rheology using Atomic Force Microscopy. We measured G* of A549 human alveolar epithelial cells (n = 36) with a spherical tip cantilever at 13, 21 and 37ºC. G* was measured at an operating indentation of 500 nm by superimposing small amplitude (50 nm) sinusoidal oscillations of frequencies ranging from 0.1 to 25Hz. At 37ºC, the real (storage modulus) and imaginary (loss modulus) parts of G* were 450 +/- 186 Pa (mean +/- SE) and 115 +/- 55 Pa, respectively. No significant changes with temperature were observed. This contrasts with the marked decrease in G* with temperature estimated from data reported by particle tracking rheology techniques. Our results indicate that cell mechanics is governed by passive behaviour of the viscoelastic CSK network, and that particle tracking techniques do not provide a reliable estimation of cell rheology.

MORPHOLOGICAL AND MECHANICAL CHARACTERIZATION OF MARINE DIATOM Cylindrotheca fusiformis

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This study highlights the capacity of AFM for investigating surface properties of live diatom cells. The intricate and ornate silicified cell walls of diatoms are one of the most outstanding examples of nanoscale-structured materials in nature. We studied morphological and mechanical properties of Cylindrotheca fusiformis (C.f.), which is believed to be unique because the part of its cell wall is without the shell(1). The two C.f. strains were studied: CCMP343 and a Northern Adriatic strain. The topology of intact cell in hydrated state was imaged in air using contact mode. Force curves obtained by probing the cell surface of the two morphologically different regions of the cell wall showed no significant difference in elastic modulus. Application of the greater force during repeated scanning to the both regions showed no expected effect of sweeping away organic material (2). These two findings suggest that organic material and silica are tightly bound as the organo-silica composite forming the entire cell wall of Cylindrotheca fusiformis. 1. Reimann, B.E.F., J.C. Lewin & B.E. Volcani (1965). Studies on the biochemistry and fine structure of silica shell formation in
Ultrastructural characterization of F17 fimbriae from *Escherichia coli* by AFM

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Pathogenic bacteria often adhere to eukaryotic cells by means of hairlike fibers expressed on their surfaces. These virulence organelles, termed pili or fimbriae, are used to successfully colonize target tissues in the mammalian host and to resist cleansing mechanisms that are presented by the host. Fimbriae are polymers of one single pilin protein and generally present a sticky protein, the adhesin, at their tip. Fimbriae are used to reach out to carbohydrate receptors, being glycoproteins or glycolipids on the epithelium of the eukaryotic host cells. F17 fimbriae are present on the surface of enterotoxigenic *Escherichia coli* strains causing diarrhea with high mortality rates in new born ruminants, like calves, goat kids and lambs. In this contribution, the F17 fimbriae of *E. coli* were structurally characterized using atomic force microscopy (AFM). Whole *E. coli* cells containing the total F17a gene cluster on a plasmid were adsorbed on mica and visualized using tapping mode in air. These cells were compared with cells where the usher gene was silenced. In the latter case, fimbriae were not formed and could not be detected in contrast to the former case. F17 fimbriae were isolated from the cells using shear mixing of the cell solution, and further purified using ammonium sulfate precipitation. The morphology and substructure of the fimbriae was observed using tapping mode in air. Ultrastructural characterization revealed a left-handed helix with a pitch of 20 nm. The flexibility of the isolated fimbriae has also been determined.

Effects of pore size and 3D structure of honeycomb-patterned polymer films on the initial spreading process of endothelial cells

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The mechanism that cells use to recognize micro-patterned topographies was clarified. First, 3D double-layer poly(ε-caprolactone) scaffolds equipped with honeycomb-patterned micro-pores ("honeycomb films") were prepared by simply casting a
polymer solution of water-immiscible solvent under high relative humidity of about 80% at 20°C. Then, porcine aortic endothelial cells (PAECs) were cultured on these scaffolds for 1-6 h in serum-free medium. Finally, their initial spreading process was investigated by using atomic force microscopy and confocal laser scanning microscopy. The attachment and spreading of PAECs on honeycomb films having either 6- or 16-μm pore diameters, resulted in voids within the cell cytoplasm, which correspond with the size and location of the honeycomb micropores. This is the first report of this unique morphology. The number of cells with this morphology decreased with increasing culture time. This dependence of morphology on film pore size and culture time suggests a spreading process of PAECs in which the cells spread, trying to sense suitable sites on which to adhere. Using thick filopodia, the cells spread along the rim of the film and produced pores by close contact between two spreading filopodia. Evidently, these pores became filled in during culture, presumably as the cells began to reorganize their cytoplasm. Moreover, the cells migrated into the honeycomb pores and expanded along the bottom layer through interconnecting channels of the film. In conclusion, the spreading of PAECs on a honeycomb film gives insight into the mechanism of a honeycomb film to control cellular behavior.
Ataxin-3 (AT-3) is a protein responsible for the Machado-Josephin disease, characterized by large intranuclear amyloid aggregates whose formation is strictly related to the abnormal expansion of the poly-Q tract in the protein C-terminus. In this work Atomic Force Microscopy (AFM) was exploited to investigate different humane and murine AT-3 variants: truncated at the residue 182 (Josephin domain alone), truncated at the residue 291 (without the poly-Q tract), the murine ataxin-3 carrying six consecutive glutamines. All proteins were subjected to a temperature increase from 37°C to 85°C with a rate of 1°C/min to accelerate aggregation kinetics. AFM measurements were performed on proteins deposited from solution on mica substrates. They clearly showed that the Josephin domain, the 291 residue cut and the murine Q6 protein generate aggregates constituted by skein of strictly interconnected filaments. 

These filaments are constituted from a linear assembly of spheroidal entities whose dimensions range from 2.5 nm to 9 nm in height and are consistent with the dimensions of the individual protein molecules. This fact supports the idea that the molecules act as monomers whose linear linkage leads to the formation of the observed filaments as the first aggregates in the hierarchical amyloidogenic process of ataxin-3. Our work supports the recent idea that the Josephin domain plays a predominant role in the activation of the process, while the poly-Q tract in its pathological expansions intervenes secondly triggering the growth of mature fibrils from the starting short protofilaments. We expect that AFM analysis of human healthy and pathological ataxin-3 currently under investigation should confirm the above results.
APPLICATION OF ATOMIC FORCE MICROSCOPY VISUALIZATION AND 
FORCE SPECTROSCOPY TO THE STUDY OF Aβ1-42 AGGREGATION

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One of the hallmarks of Alzheimer’s disease (AD) is the self-aggregation of the amyloid β peptide (Aβ) in extracellular amyloid fibrils. Among the different forms of Aβ, the 42-residue fragment (Aβ1-42) readily self-associates and forms nucleation centres from where fibrils can quickly grow. We have used AFM visualization in liquid environment to study in vitro the effect of carbohydrates on the fibrillogenesis of synthetic Aβ1-42. Amyloid fibrils coalesce to form larger aggregates, or senile plaques, that can be easily identified with the light microscope in preparations of AD-afflicted brains. A mechanism leading to neurodegeneration during AD is Aβ-induced neurotoxicity. There is increasing evidence that aggregation of Aβ into fibrils, and deposition of fibrils into amyloid plaques, are key steps in the onset and progression of AD. Senile plaque study has eluded so far the use of AFM techniques because plaques can not be easily purified from cerebral tissue, and their in vitro growth is difficult. Bacterial over-expression of recombinant genes can result in the aggregation of insoluble polypeptide chains as inclusion bodies (IBs). IBs mimic some of the characteristics of senile plaques and thus can be used as a model for in vitro studies. We have overexpressed a fusion of Aβ1-42 to green fluorescent protein that results in highly fluorescent IBs, and have applied AFM visualization on line to characterize the dynamics of IB removal by proteases. Force spectroscopy data have provided information about the internal structure of Aβ1-42 IBs. Future research involves single molecule force spectroscopy and AFM/fluorescence analyses.

Distribution of C12 ions induced-damages in plasmid DNA resolved by gel-electrophoresis and scanning force microscopy

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Analysis of DNA damage induced by swift ions is essential for a detailed understanding of the processes that occur in clinical cancer therapies with heavy-ions and in DNA repair. The most serious damages of DNA molecules are double-strand breaks (DSB), i.e. the rupture of both DNA strands in the range of a few base pairs and single-stand breaks (SSB), when one of the DNA strands is broken. The theoretical concept of dense track structure for heavy ions explains that DNA damages are more pronounced after irradiation with heavy ion compared to the X-rays, at the same dose. In our recent experiments scanning force microscopy (SFM) and gel-electrophoresis
have been combined for a precise analysis of damages induce in ØX174 plasmids in 20 mM Hepes solution by irradiation with X-rays and C12 ions. The percentages of plasmids with double-strand breaks (DSBs) and single-strand breaks (SSBs) have been determined as a function of radiation dose by gel-electrophoresis. The results show an increase of the percentage of DSBs after X-rays irradiation from 0% to 12.5% in the range from 0 Gy and 1 kGy, while after carbon ion irradiation a higher percentage of DSBs of 19% has been measured. For more detailed analyses SFM has been performed. The results revealed that the distribution of plasmid fragments after irradiation with C12 ions contains a significant larger amount of small fragments in the range from 50 nm to 700 nm, compared to the X-rays, while a clear reduction of large fragments has been observed. The results resolve the distribution of induced damages in plasmid DNA after irradiation with X-rays and C12 ions at low doses and support the theoretical concept of track structure for heavy ions.

**Study of the interactions between Single Walled Carbon Nanotubes (SWCNTs) and plasmid DNA**

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We propose a statistical method to verify if there’s a preferential interaction between Single Walled Carbon Nanotubes (SWCNTs) and double stranded DNA (dsDNA). This method consists in counting the number of the crossings between a SWCNT and plasmid DNA molecules (pUC-19) from a series of AFM images. Aqueous dispersions of SWCNTs with pUC-19 at different concentrations were deposited on mica and a number of AFM images were collected for each sample. We performed the statistical analysis on 2 batches: a purified CNTs dispersion in water (batch 1) and a purified CNTs suspension in O-Benzyl-L-Tyrosine aqueous solution (batch 2). O-Benzyl-L-Tyrosine is a chemically modified aminoacid able to produce stable suspensions of individual SWCNTs in water, and it is also a biocompatible agent for applications with nucleic acids. Our results show that only in the case of batch 2 a selective interaction between CNTs and DNA is found.

**PUNIAS: Protein Unfolding and Nano-Indentation Analysis Software**

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In the last fifteen years, the atomic force microscope (AFM) has emerged as a powerful tool for studying surface interactions by means of force-distance curves. For instance, single-molecule force spectroscopy experiments have given new insights into intra-
and inter-molecular forces, but have also shown variations in physical parameters of individual molecules with respect to the mean values derived from ensemble measurements. In a similar way, the mechanical properties of free standing films, micro-capsules or even biological samples has been measured using either the colloidal probe or nano-indentation force spectroscopy technique. Because of the high sensitivity of these measurements a large number of force-distance curves need to be analyzed in order to obtain accurate statistics. However, there is no software package available which allows the analysis of the force-distance curves. Thus, the files first need to be exported and manipulated with spreadsheet software. Because, of the large number of files to be analyzed, this procedure is very repetitive and time consuming. PUNIAS has been developed in this frame: it is a graphical user interface freeware allowing a fast analysis of a large amount of force-distance curves. This efficiency is reached by the use of a batch processing combined with semi-automated analysis routines which results are visualized graphically and can be modified by means of the mouse or hotkeys. The obtained results are in an ASCII form and can then easily be exploited or plotted.

**Full atom Steered Molecular Dynamics simulation of pulling rhodopsin from the membrane**

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Rhodopsin (Rh) is a prototypical G protein coupled receptor (GPCR) and only one with three-dimensional structure known. The mechanism of receptor activation appears to be conserved for all members of the GPCR superfamily so Rh may be used not only as a structural template but it is also mechanistically analogous to other GPCRs. Rhodopsin monomer (Rh) with S-S bridge unbroken was pulled out from 3-component membrane, typical for rod outer segments. During SMD simulation a harmonic potential was applied to N-terminus of Rh. The periodic box 6 nm x 9 nm x 19 nm was used. The dimension along the z-axis was long enough for a fully stretched single transmembrane helix so the simulation was divided into parts. We used pulling speed 6 nm/ns (6 m/s) and spring constant 5 N/m. Spring constant larger than used in AFM was forced by higher pulling speed. The program NAMD2 was used with full atom force field CHARMM27. There is an evident difference in height between AFM and SMD force peaks about one order of magnitude. This originates from much larger difference in cantilever speeds about seven orders of magnitude. Each new simulation started about 500 pN lower than analogous part of Rh structure in previous simulation. This is a result of nonzero friction force of unfolded fragment passing through the water. Although the speed was still several orders of magnitude larger than used in SMFS experiments there is a striking similarity of location of stable structural segments in Rh.
Polymer and Drug Adhesion onto Implants

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Biomimetic materials are often bonded systems with the surface layer consisting of polymers and/or drugs. The adhesion of this layer is crucial for the function of the compound. In the last couple of years we and others developed a technique to measure the quasi-equilibrium adhesion force of single polymers to solid substrates. We covalently attach a polymer onto a cantilever tip, bring it in contact with a substrate and measure the quasi-equilibrium force necessary to pull the polymer off the surface. With this method we already gained new insight into the electrostatic contribution of the desorption force. Here, we use and extend this method to investigate the adhesion of polymers and drugs onto stents and catheters in solvents. It is known, that the structure and hydrophobicity of the supporting material as well as the solvent play an important role for the adhesion properties of the surface layer. Our measurements are a first step towards a quick, nondestructive and easy to use method to test structured surfaces for their suitability as components of a subsequent adhesive bond in various environments. In addition, the very good agreement of our first results with MD-Simulations done in the group of Prof. Roland Netz (TUM) allows for new insights into the hydrophobic contribution to polymer adhesion on a molecular level.

DEVELOPMENT OF SINGLE MOLECULE NANOBIOSENSORS FOR THE BIODISCOVERY OF ANTIBIOTIC AND ANTIMALARIAL COMPOUNDS

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The discovery of new bioactive compounds to treat microbial infections is an ever increasing need as a result of the constant evolution of resistance by the infectious agents, notably the malaria parasite and pathogenic bacteria. We apply single molecule force spectroscopy (SMFS) to the development of nanobiosensors for the biodiscovery of new antimalarials and antibiotics. The main advantage of this approach is that by monitoring single molecule interactions novel inhibitory molecules can be detected that otherwise escape identification by current methods requiring high concentrations of the sought-after compound. Our general strategy consists on the search for inhibitors of molecular interactions that are essential for the survival of the pathogenic agents. We have selected as metabolic target a biosynthetic route that is present in the malaria parasite and in most human pathogenic bacteria, but not in vertebrates. Inhibitors of the enzymes of this metabolic pathway constitute a potential source of highly specific antimalarials and antibiotics. The chosen enzyme/substrate pairs are covalently
immobilized on the AFM tip and surface following different chemical methods with the objective of diminishing steric hindrances that can hamper the access of the substrate to the active center of the enzyme. Preliminary SMFS data indicate that binding forces between enzyme and substrate are around 200 pN, and that the interaction is abolished in the presence of soluble substrate. Our current challenge is to increase the sensitivity of SMFS-based nanobiosensors through strategies that permit the immobilization of a smaller number of enzyme molecules on the AFM tip.

**Comparison of AFM and SPR measurements of the HER2 antibody-antigen reaction**

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We have used atomic force microscopy (AFM) and surface plasmon resonance (SPR) to study the HER2 antibody-antigen reaction. HER2 protein is found in SKBR3 cancer cell, so its detection is important in breast cancer diagnosis. On a gold film evaporated on a Si wafer, we coated linker molecule, dithiobis succinimidyl propionate by self-assembly. AFM showed that the roughness of the gold surface reduced after the linker coating because of the filling effect. HER2 antibody was coated on the gold surface by dipping the substrate in a buffer solution, and then the HER2 antibody-antigen reaction was induced by dipping the HER2-coated substrate in a SKBR3 lysis buffer solution. For comparison, the HER2-coated substrate was also dipped in a H520 lysis buffer solution which did not contain HER2 protein. At each stage, the samples were examined using AFM and SPR. Morphology of the HER2-coated surface showed lumps which were thought to be antibody adsorption sites. After dipping in a SKBR3 lysis solution, we could observe lots of high protrusions on the surface, but when dipped in a H520 solution the protrusions were not observed. The SPR signal shifted in accordance with AFM measurements. Our study showed that AFM and SPR can be a good combination in detecting cancer cells.

**Evolution of amyloid peptide self-assembly: from continuous monolayer to fibrillar structures**

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Proteins are the main essential active agents in biology and their geometrical characteristics are determinant for their functioning. Changes of the tertiary structure of proteins may be at the basis of a series of diseases like Alzheimer, Mad cow, Cystic fibrosis and even some types of cancers. Fibrils of β-amyloid peptides are one of the main components of the amyloid plaques that are responsible for the disfunctioning and death of neurons in Alzheimer’s disease. Yet the mechanism and the kinetics of
the aggregation of these peptides remain uncontrolled and not well understood. With the use of atomic force microscopy we show the formation of a monolayer of peptides of β-amyloid. More than that, there are evidences of the re-assembling of this peptidic monolayer into fibrillary like structures. The continuity of this monolayer as well as the process of re-organisation is dependent on peptide concentration, adsorption time and the characteristics of the substrate (charge and hydrophilicity). These preliminary results propose a new theory of self-assembling of amyloid peptides that could be of extreme importance for the clarification of the mechanism of amyloid aggregation.

Investigation of ligand-receptor interactions via AFM influenced by force loading rate and ligand-receptor contact time

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AFM has been widely used in biological researches especially in the force measurements. However, the measured forces are subject to the experimental conditions such as the force loading rate, tip-substrate contact duration and molecular orientation, particularly in the presence of ligand-receptor interactions. The present study aims to quantify the effects of those factors on the ligand-receptor interactions. As a common issue in the infected implant biomaterials, Staphylococcus epidermidis and fibronectin (FN) were chosen as the model system. The bacteria were immobilized onto an AFM tip via our novel coating method. FN was immobilized onto different SAMs substrata. Force measurements were performed under different force loading rates and contact durations. Approach data were correlated with DLVO theory to characterize the adhesion energy barrier and the orientation effects. Retraction data were analyzed to obtain the adhesion forces, frequency and distance distribution. The adhesion forces between S. epidermidis receptors and FN ligands ranged 0.1~2.0 nN depending on the loading rates and the contact duration. Accordingly, the adhesion frequency varied in different pull-off distance regions. The pull-off distances varied from 8.31~298.37 nm. The time duration for forming a ligand-receptor interaction was estimated around 1 microsecond by conducting statistical analysis including histogram and Poisson analysis. Also the strength of a ligand-receptor interaction was derived via the greatest common divisor analysis from vast force data. This study explores the factors affecting the force measurements, which should be considered when comparing the results performed under different measurement conditions. The results also provide insightful knowledge of the fundamental biological interaction forces.
Pull-off force measurements between functionalized particle and proteins

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Atomic force microscopy (AFM) has been used to measure the strength of bonds between biological receptor molecules and their ligands in physiological conditions.[1], [2] However, roughness of the interacting surfaces is a significant factor affecting the experimentally measured pull-off forces. [3] To approach the ideal sensor configuration, PEG-linkers have been used to couple ligands to an AFM tip via long flexible spacers. [2] They facilitate the detection of single recognition events as the non-specific and specific adhesion can be distinguished from the different jump-off distances. Proteins attached to the PEG-linker can also move and orient freely about the tip and there is a better opportunity for a specific binding. The goal of this work was to investigate the binding strength of antibody molecules or FAb fragments to their specific antigen. The comparison of specific and non-specific interactions was of special interest. The used system was tested with the strongest biological and non-covalent interaction known, the streptavidin-biotin bond. A small surface concentration of bio-BSA functionalized polystyrene nanoparticles (diam. 202 nm) was distributed randomly on a polycarbonate substrate which was covered by a layer of streptavidin. AFM tips functionalized with streptavidin were used to probe the forces between biotin and streptavidin.


Construction and Imaging of DNA Nanostructures: A Combined AFM and STM approaches

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By using state-of-the-art surface science techniques such as atomic force microscope (AFM) and scanning tunneling microscope (STM), we investigate the self-assembly of artificial DNA structures and arrays into predesigned nanoarchitectures on surfaces in order to characterize and explore the basic properties of such nanostructures. DNA 3-way junctions were prepared via a precise synthetic procedure where the 3-way junctions can be assembled in two different manners, either by hybridization between complementary DNA 3-way junctions on top of each other, or by using one 3-way junction with one template, leading to the formation of DNA nanowires. Preliminary
AFM experiments recorded in air and also under liquid conditions suggest that these DNA nanowires are successfully formed with different lengths. Despite the difficulty of immobilizing individual DNA nucleobases upon their adsorption on a solid surface, we were able to mix complementary DNA bases (adenine with thymine) and (guanine with cytosine) and perform STM experiments at the liquid/solid interface, and new supramolecular nanopatterns were successfully observed that are significantly different from those of the individual nucleobases. [1, 2] Self-Consistent Charge Density-Functional based Tight-Binding (SCC-DFTB) calculations support our STM findings. Our results open attractive pathways towards host-guest complexation and molecular templating based on DNA architectures.


**Electrostatic Stretching and AFM imaging of Hyaluronic Acid Molecule**

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Hyaluronic acid (HA), which is a high-molecular-mass polysaccharide with a straight chain structure, has been suggested to exert lubricating functions in joints and tissues because of its high hydrophilicity and high shear-dependent viscoelasticity. We have studied precipitation and stretching of HA molecules on a surface of a micro chip, in particular, control of stretching direction. Silicon based micro chips with Pt/Ti thin film electrodes on SiO₂ surface were fabricated. An HA solution was dropped and air-dried on the micro chip. Square pulse voltage was applied between the electrodes during drying. After the droplet was dried, atomic force microscopic (AFM) observations for precipitated HA molecule shape were conducted. Straight string like HA molecule images were observed successfully by using an AFM tapping mode with a silicon probe of 10nm tip radius. The precipitated HA molecules were stretched and aligned toward same direction on the SiO₂ surface when the applied pulse voltage frequency was 1MHz or higher. The HA molecules seem to be loosened in the solution under high frequency electrostatic force. The stretched direction was determined only by a receding direction of the droplet edges during drying, independent of electrode shape, direction of electric field, and pulse voltage frequency. The molecules were separated into mono molecule completely during precipitation in the finest case. In addition to the AFM imaging, molecular surgery operation of the stretched HA molecule chain was also carried out successfully by using nano-scratching technique with an AFM probe tip as a cutting knife.
Mechanically functional amyloid structures in natural adhesives

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Using AFM force-extension data we have been able to provide an explanation into the mechanical design for adhesive strength and attachment of several bioadhesives at the molecular level. We recently showed that the adhesive of a green alga (Prasiola linearis) exhibited high mechanical strength and toughness due to the presence of ‘sacrificial bonds’ and ‘hidden length’ within adhesive molecules. We proposed a mechanism for its adhesive tensile strength based on protein within a pleated β-sheet amyloid structure, based not only on mechanical data, but also polarizing and confocal microscopies. We consider this quaternary protein structure, irrespective of amino acid sequence, to be non-pathological, mechanically functional amyloid. So far, we have found evidence for its presence in other permanent (unicellular and multicellular algae) and temporary (parasitic worm) bioadhesives. We have already observed differences in measured mechanical responses that we believe to be related to cross-linking, and this is being explored with Raman spectroscopy. Permanent adhesives may be cross-linked in contrast to the temporary adhesives, and this may be correlated to their specific function or the mechanical demands placed upon them (at the molecular level), in response to their natural environments. This discovery has motivated our interest towards the generic and specific nature of amyloid fibril structures. Emerging evidence exists for the occurrence of non-pathological amyloid occurring in nature, and the generic nature of the amyloid fold could provide a widespread mechanism for mechanical strength in other natural adhesives, while the attachment mechanism itself may remain species specific.


Force-distance curve treatment in DFS for multiple parallel bonds

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Dynamic Force Spectroscopy (DFS) is a technique useful to interpret the energy landscape of cellular adhesion and single molecular bonds. Using Atomic Force Microscopy (AFM), it is possible to obtain kinetic dissociation constants as well as the width of the activation energy barrier of an unbinding reaction between a receptor and a ligand. Multivalency is a molecular and cellular property that requires the binding (and unbinding) of multiple parallel bonds such as those involved in antibody-antigen or cell-cell interactions. Interpretation of experimental force-distance curves in the presence of a pulling force is not straightforward in the presence of multiple parallel
bonds. First, it is necessary to correct the applied loading rates by the loading rate experienced by the proteins chemically attached on a flat substrate. Such a correction is difficult to robotize unless the force-distance curves contain a large number of data points. It also raises a limitation in the choice of cantilever relative to the flexibility of proteins. Second, rupture force distribution in the presence of multiple parallel bonds requires the fitting of multiple Gaussian curves. These fittings allow us to obtain the most probable rupture forces (F*) that can be plotted against the logarithm of the loading rate in order to obtain the desired kinetic parameters. Another difficulty in such a plot is the statistical relevance of the fit since the greater number of population the larger experimental data points is required.

Molecular organization on a DNA chip

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Atomic Force Microscopy (AFM) presents the greatest accuracy for a direct observation of biological systems at a nanometric scale in air or in liquids. Development of dynamic modes adapted for the imaging of soft samples has significantly contributed to overcome the usual issue of damage done to these biological objects by the tip. Experiments mostly concern the observation of adsorbed biological molecules, DNA or cells to study their individual conformation or their assembly in molecular layers. Few studies relate on real biosystems with direct applications as this work concerning an AFM study of a DNA – chip does. DNA-chips are structured in an array of single-stranded DNA chains called probes, covalently bonded on a substrate, which are able to recognize complementary single-stranded DNA chains called targets, by hybridisation. Each step of preparation from probe immobilisation to target hybridisation has been characterised by AFM. In fact, the sensitivity of these objects depends on the molecular organisation of the probes on the substrate. Then, this AFM study focusses on the identification of the parameters which could influence this organisation. We studied the influence of length (25 bases or 12 bases), the sequence arrangement and the presence of a fluorescent dye at the free extremity of the probe. We showed that probes organise themselves in different structures depending on their sequence and on the presence of the dye. After hybridisation, we showed that target molecules could be individually identified. Moreover, with appropriate experimental conditions, the hybridised region along the target could be distinguished.
AFM investigations of DNA/protein complexes on mica

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Atomic force microscopy (AFM) is a widely used technique to image protein-DNA complexes on mica, and its application has now been extended to the measurements of protein-DNA binding constants and specificities. However, theses studies require imaging DNA under different physiological conditions. A better understanding of the DNA adsorption mechanism on mica should enable to adapt the buffer composition, in order to adsorb DNA, to the biological requirements like that of the ionic strength. For that purpose, we construct phase diagrams of DNA adsorption versus monovalent and multivalent salt concentrations. A model of DNA adsorption on mica is presented which shows that DNA adsorption takes place when the energy gain in counterion correlations overcomes an energy barrier. The analysis of the AFM results provides an estimation of this energy barrier. We then tackle the surface influence on protein-DNA interactions, which is critical for AFM users, under different buffer conditions. The extensively studied EcoRI-DNA complex is used as a model to provide quantitative information about the surface influence. The equilibrium binding constant of the complex is determined by AFM both at low and high ionic strengths and compared to electrophoretic mobility shift assay measurements (EMSA). Finally, we propose a two-step mechanism which describes the adsorption of the EcoRI-DNA complexes on the surface. This model could also be extended to other protein-DNA complexes.

Decreasing \textit{Escherichia coli} Adhesion Forces by the Use of Cranberry-Rich Media: Preventing Urinary Tract Infections

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Bacteria adhesion to uroepithelial cells is the first step in the development of a urinary tract infection (UTI). Previous research has suggested that compounds in cranberry juice (CJC) have the potential of decreasing this adhesion by changing the conformation of fimbriae organelles. \textit{E. coli} use these proteins, located on the surface of the bacterial cell, to adhere to receptors that are found on uroepithelial cells. Cranberry components have shown the ability to inhibit this adhesion process; however, the mechanisms of this anti-adhesion activity are not well understood. Experiments were designed to understand how adhesion is affected by cranberry juice and isolated cranberry fractions (proanthocyanidins; PACs). The P-fimbriated \textit{E. coli} strain HB101pDC1, the main cause of acute pyelonephritis, and the non-fimbriated strain HB101 were grown in the presence of 0.5 and 10 wt\% CJC or PACs for more than eight times. After the cells had reached late exponential growth phase, clean glass slides were chemically treated to bond bacterial cells to the surface. Once the bacteria had adhere, force measurements were taken in liquid and bacterial images were
captured using an atomic force microscope (AFM). E. coli HB101pDC1 that was grown in higher concentrations of cranberry juice compounds showed less adhesion forces than the bacteria that were grown in pure media that did not contain CJC or PACs. Further, adhesion forces decrease as the number of bacterial cultures increased, suggesting that the continuous intake of cranberry products might prevent significantly the development of UTIs.

Investigating the conformational properties of DNA in the presence of free and supported polycations and condensing agents by Atomic Force Microscopy

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DNA is a stiff biopolymer, with a persistent length in physiological solutions of about 55 nm. Despite its rigidity, DNA exhibits a striking capability to change its elastic properties in different environments, reaching extremely high packing ratios in viral capsids and in chromatin, achieving a five-fold reduction of its persistence length when supported on polyamine-coated surfaces [1], as well as condensing in the presence of multivalent cations [2] and of hydrophobic-hydrophilic polymers [3]. The previous examples suggest that electrostatic interactions play a major role in determining DNA rigidity. In particular, we look at the exciting possibility of tailor DNA elasticity and morphology by means of suitable interactions with charged molecules and polymers in solution and/or at interfaces. Controlling the condensation of DNA in the presence of a cell culture by means of charged polymers could be a suitable way to optimize cell transfection efficiency. It is known indeed that cells have adapted to produce several polyamines, polyl-velent cations, which can neutralize the negative charge on the DNA phosphates, softening the chain, favouring DNA aggregation and encapsulation in the cell. We will report on an atomic force microscopy study of the elastic and conformational properties of linear and circular DNA molecules in the presence of different charged polymers. We have exploited the versatility of the poly(amidoamine) family to study peptide-mimicking polymers with different polyelectrolyte structures. Special attention was given to the interaction observed with a poly(amidoamine) condensing agent, AGMA-1 [4]. Agmatine mimics the tripeptide arginin-glycin-aspartic acid (RGD), capable of reproducing the receptorial site of proteins involved in cell adhesion and it is important for the functional activity of soluble polymers as transfection agents. We have investigated the influence of buffer pH and AGMA/DNA ratio on the formation of complexes, and studied the interaction of DNA with amphoteric AGMA either in solution and supported on a surface. The morphological data obtained by AFM study have been related to the transfection efficiency of PAA-AG on NIH3T3 cells. [1] A. Podestà et
Temperature dependence of molecular interactions measured by dynamic force spectroscopy

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Molecular interactions are essential in cell biology since they are the ultimate mediators of cell adhesion. Specific ligand/receptor interactions mediate cell adhesion and are the basis of drug design. The dynamic strength of these interactions is characterized by their free energy landscape. The atomic force microscope (AFM) in dynamic force spectroscopy (DFS) mode has been shown to be an appropriate method for determining the free energy landscape of ligand/receptor complexes. DFS consists of measuring the force required to unbind single ligand/receptor bonds, i.e. rupture force (F), at different loading rates (dF/dt=rt). Rupture force spectra (F vs. rt) interpreted in terms of the Bell model enable us to determine the unbinding rate (k0) and position of the energy barrier (x) of the interaction. The streptavidin/biotin complex has been used as a model of molecular interaction and it has been widely characterized. However, the temperature dependence of the interaction strength is still unknown. In this work, AFM in DFS mode was used to characterize the streptavidin/biotin complex interaction at different temperatures, ranging from 17 to 37 ºC. The dynamic force spectra were analysed in terms of the Bell model to determine k0 and x. The suitability of the thermally activated process assumption was discussed.

Single molecule force spectroscopy probes the conformational diversity of alpha-Synuclein, an intrinsically unstructured protein

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Intrinsically unstructured proteins (IUPs) play key roles both in physiological cell function and amyloid diseases. Their structural disorder is usually depicted as the
outcome of a complex ensemble of conformers driven by a rugged energy landscape. Their folding and aggregation mechanisms remain a major unsolved problem for modern biology. In fact, IUPs often are amyloidogenic proteins. The aggregation process is linked to a conformational transition to a folded beta-sheet structure. However, no conclusive evidence has been so far provided for a long-lived beta-sheet conformation of an IUP in the absence of oligomerization. We applied for the first time single molecule mechanical unfolding by AFM to the study of human alpha-synuclein (aSyn), an IUP whose aggregation is involved in Parkinson’s disease. We were able to identify at least four distinct classes of conformers. We found that, under physiological conditions, the potentially toxic beta-structured monomeric form of aSyn exists as a stable conformer, in equilibrium with a complex ensemble of other conformers, and its population in vitro can be significantly shifted increasing the buffer concentration. This novel picture of the structural diversity of aSyn suggests that a simple population shift can trigger the aggregation of this protein. Our single molecule approach, being able to discriminate and quantify the beta-structured monomer, is a unique tool to directly test the influence of mutations and the effectiveness of therapeutic strategies upon the early steps of pathogenic assembly.

RNA and RNA-Protein Complexes studied by Atomic Force Microscopy and Force Spectroscopy

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RNA-protein complexes are crucial intermediates in the replication of viral RNA. A prominent example of such an RNA-protein complex is the so-called Rev-RRE system of the HIV virus. The RRE (Rev Response Element) is a 351 nucleotide elongated hairpin motif within the HIV RNA genome, which binds up to 10 copies of the Rev protein during the late life cycle of HIV. Formation of this complex regulates splicing and export of the HIV mRNAs and is therefore also an important possible drug target against AIDS.

We use the Rev-RRE interaction as a model system to study RNA-protein interactions at the single molecule level by the atomic force microscopy and AFM force spectroscopy. Initial studies were focused on imaging RNA and RNA-protein complexes on mica after electrostatic adsorption from buffer solutions. Further concepts of immobilization were tried. For example end-labelled RNA molecules were attached in an oriented manner on self-assembled monolayers also suitable for nanolithographic techniques like nanoshaving/ANGES. AFM force spectroscopy was used to probe unbinding forces of single specific RNA-protein complexes. For this purpose RNA mutants and oligopeptides were tethered to AFM tips and substrate surfaces via flexible linkers. Currently inhibition studies of the RNA-protein complexation are in progress.
Effect of Molecule Anchoring Positions on the Force-Extension Curves Measured in the Atomic Force Spectroscopy Experiments on Chain Molecules

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In single-molecule stretching experiments performed by atomic force microscopy (AFM), one end of a molecule is bound to the tip of an AFM probe while the other end is bound to a substrate. To do this, the molecules to be studied are deposited on a substrate where from they are picked up by the AFM tip. In some experiments, the molecules are picked up by the AFM tip from their natural substrate, as in cases of collagen from tendons and polysaccharides from a bacterial surface. In all these experiments, the AFM tip is pushed to the sample surface in order to bind the end of a molecule to its surface. Given the experimental conditions described here, it is reasonable to assume that there is a certain unknown lateral (perpendicular to the AFM tip displacement direction) distance, r, between the molecule anchoring sites on the tip and substrate, respectively. The present work gives an analysis of the consequences of this variable on the force-displacement curves measured in single-molecule stretching experiments performed by AFM. We simulated AFM force-distance curves affected by occurrence of finite values of r and then fitted these force-distance curves with the force-extension formulae predicted by freely jointed chain and worm like chain molecule models. A direct experimental evidence of the effect was obtained by simultaneously acquiring the normal and lateral forces during collagen molecule stretching.

Non-contact AFM imaging of single biomolecules in the repulsive electrical double layer regime

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We present a method to image single isolated biomolecules in aqueous media by AFM without establishing any mechanical contact between tip and sample. The biomolecules are electrostatically adsorbed onto a surface and the experimental conditions can be chosen such that a repulsive electrical double layer (REDL) force [1] exists between the tip and the surface. If the biomolecules are much smaller than typical tip radius the force on top of the molecule is still repulsive, and a load force lower than that corresponding to the jump into the mechanical contact instability can
be applied. The AFM is operated in the jumping mode (JM) [2]. JM is able to maintain constant the applied normal force while imaging, with independence of drift problems. This is a necessary condition to maintain the stability of loading forces at values lower than 100 pN. This method is applied to image various biomolecules like avidin, streptavidin and DNA characterized by different electrostatic surface potentials.


**Kinetic parameters and energy landscape of a uranyl-antibody interaction**

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Molecular recognition plays a critical role in cellular mechanisms; especially in immunology where the specific recognition of antigens by antibodies is the key to immunity. Understanding the mechanisms underlying the deleterious effect of xenobiotics at cellular and molecular levels is highly relevant in toxicology. We study a model system composed of a dicarboxilic phenanthroline acid - chelated uranyl (DCP-UO$_2$) and monoclonal antibodies raised against a DCP-UO$_2$ conjugate. Several monoclonal antibodies were selected and their dissociation constants have been determined using Dynamic Force Spectroscopy. Using Bell’s model, we analyzed kinetics parameters of the unbinding process. The energy landscape of the dissociation of the complex upon an external force revealed the presence of at least two energy barriers. Using a three-dimensional molecular model of the DCP-UO$_2$-MabU04S complex we suggest that an inner activation barrier corresponds to the rupture of chelated metal with antibody whereas an outer activation barrier corresponds to the rupture of the chelated DCP with antibody. Here we compared these results with those of a second monoclonal antibody MabU08S. Extensive force-distance rupture events analysis allows us to refine our model of the dissociation between DCP-UO$_2$ and monoclonal antibodies.

**Measurement of single fibrinogen molecule detachment force using the AFM**

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This experimental work attempts a contribution in the study of protein interaction with biomaterial surfaces by measuring the protein unbinding force. Contrary to various
other reported works, which measure the interaction between layers of adsorbed protein, we have used the AFM tip to measure the detachment force of single protein molecules from mica or glass surfaces. For this purpose, fibrinogen was covalently bound on the tip. Initially the Si3N4 probe was oxidized by short immersion in piranha solution and silanized with 3-aminopropyl(dimethylethoxysilane). Then, the homobifunctional crosslinker bis(sulfo-succinimidyl) suberate (BS3) was added to immobilize protein molecules on the tip via interaction of its sulfo-N-hydroxy-succinimide esters (one at each end) with the amino groups on the protein molecules and the functionalized probe. Single molecule interaction with the substrate was identified through the stretching pattern observed at the force curves upon retraction of the tip. Hundreds of such curves were collected at three different detachment rates - 0.5, 1 and 2 Hz – and the unbinding force as well as the separation distance were extracted from them in order to be statistically processed. The first results showed a reasonable correlation between the detachment rate and the unfolding patterns before unbinding. The authors acknowledge financial support by “Irakleitos - EPEAK II Research Programme”

[Ru(TAP)3]2+-photosensitized DNA Cleavage Studied by Atomic Force Microscopy and Gel Electrophoresis: A Comparative Study

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Topological modifications of plasmid DNA adsorbed on a variety of surfaces were investigated by atomic force microscopy (AFM). On mica modified with 3-aminopropyltriethoxysilane or poly-L-lysine, the interaction between the plasmid DNA and the surface 'freezes' the plasmid DNA conformation as deposited from solution and the AFM images resemble the projection of the three-dimensional conformation of the plasmid DNA in solution. Modified mica with low concentrations of Mg2+ leads to a decrease in the interaction strength between plasmid DNA and the substrate, and the AFM images reflect the relaxed or equilibrium conformation of the adsorbed plasmid DNA. Under these optimized deposition conditions, the topological modifications of plasmid DNA were produced under irradiation in the presence of Ru(TAP)32+, which is a non-intercalating complex, and were followed as a function of illumination time. The observed structural changes correlate well with the conversion of the covalently closed circular form (ccc form) into the open circular form (oc form), induced by a single-strand photocleavage. The AFM results obtained after fine-tuning the plasmid DNA - substrate interaction match well those observed with gel electrophoresis, indicating that under the appropriate deposition conditions, AFM is a reliable technique to investigate irradiation-induced topological changes in plasmid DNA.
FORCE SPECTROSCOPY OF LACTATE OXIDASE MONOLAYERS

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We report on the study of the nanomechanical properties of a lactate oxidase (LOx) monolayer immobilized on gold substrates by Atomic Force Microscopy techniques operating under buffer conditions. Topographical contact mode imaging evidenced the protein deformation under the applied tip load. We have performed approaching force curves with both stiff and soft cantilevers by imposing maximum loads of 1.6 nN and 400 pN, respectively. We have found that for our experimental conditions the force curves can be, in principle, well fitted by the Hertz model for both conical and spherical indenter geometries. However, as the Young’s modulus obtained for both geometries can differ appreciably, it became necessary to assess which indenter geometry was more adequate to explain the experimental data. For such purpose a systematic study of the indentation versus applied force curves obtained from both fittings for all the experimental curves was done. From this study resulted that the experimental data were better fitted by the Hertz model for a conical indenter, obtaining an average value of the Young's modulus for the LOx layer in the 0.5-0.8 GPa range. These results agreed with those obtained for LOx submonolayer deposits on mica substrates, which allows discarding any important contribution from the underlying substrate on the measured properties.

Nanomechanics of collagen fibres: Insights into the structural properties of fibres using atomic force microscopy

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Collagen is the most abundant protein in mammals. It provides a mechanical structure to our bodies, protecting and supporting the softer tissues and connecting them with the skeleton. Many of the most common skeletal diseases are related to either a loss of bone mass (both collagen and the hydroxyapatite mineral phase) such as in osteoporosis, or altered collagen structure present in the inherited disorder, osteogenesis imperfecta. Investigations of the structural behaviour of collagen matrices down to the fibrillar and molecular level are essential if findings in these diseases are to be applied to an understanding of the architecture of the normal skeleton. We present here a precursor study of native collagen (from rat tail tendons) using an atomic force microscopy based technique (nanoindentation; force mapping).
Mechanical properties and behaviour such as the evolution of Young’s modulus of single collagen fibrils were characterised. New insights into the fibril structure were also gained by stretching fibrils until rupture and performing controlled fibril nanodissection. The revealed internal structure allowed us to compare the stiffness of the fibril’s surface with the core.

**Single molecule force spectroscopy study of specific interaction between cell membrane receptor Laminin Binding Protein and different surface proteins of flaviviruses**

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Specific interactions of viral proteins with cell receptors play a crucial role in the process of penetration of virus particles into a cell and development of viral infection. Inhibition of such an interaction can prevent the entry of a virus into a cell. We applied single molecule force spectroscopy to study the specific interactions between the universal cell receptor “laminin binding protein” (LBP) and surface proteins of two flaviviruses. Two fragments of recombinant envelope protein E of West Nile Virus (WNV) were used as ligands: E1-180 fragment containing domain I and domain II, and E53-126 fragment containing domain II; the third object was the recombinant envelope protein of Tick-Borne Encephalitis Virus (TBE). In all cases a prominent specific interaction, with the average force about 100 pN has been observed for force loading rate of 60 nN/s. Specific interaction between LBP and extracellular basement membrane glycoprotein laminin was also studied. The average value of specific force recorded for this pair was about 90 pN. These single molecule force spectroscopy results are in a good agreement with the immunochemical data obtained by ELISA thus strongly confirming the hypothesis of an importance of LBP as a (co)receptor for flaviviruses attachment to the cell membrane.
Electrochemically controlled growth and dissolution of PLL/DNA multilayers for tailored DNA release studied by EC-OLWS and in-situ AFM

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Electrophoresis is the widely employed procedure for DNA and RNA transfection, nevertheless it often shows limited efficiency and it requires the use of large amount of nucleotides. Recently, our group has developed a novel method for the electrochemical dissolution of polyelectrolyte multilayers from the surface of an electrode for applications in controlled drug delivery. Nanoobjects and charged molecules, such as DNA can readily be incorporated into polyelectrolyte multilayers facilitating the surface initiated controlled release of various compounds. The aim of our project is to combine the incorporation of DNA into polyelectrolyte multilayers with the electronically controlled dissolution. The entire process of buildup and consecutive dissolution of the multilayers are monitored by Electrochemical Optical Waveguide Lightmode Spectroscopy, whereas the morphology of the film during dissociation is observed in-situ with AFM in an electrochemical liquid cell. Stable PLL/DNA multilayers are formed on substrates covered with indium tin oxide as conductive layer. We discovered that no galvanostatic dissolution occurs when the multilayers are grown at open circuit potential. On the contrary, they can be dissolved if a potential bigger than 0.2 V (versus Ag/AgCl) is applied during the build-up. The dissolution rate increases by increasing the applied potential. The underlying mechanism is still to be elucidated, nevertheless these original result may be of great consequence for the fabrication of multilayer with controlled stability.

Bacillus subtilis primosomal protein DnaD-DNA interactions
An AFM imaging and force spectrum study

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The Bacillus subtilis DnaD protein is an essential protein that has been implicated in the primosomal step of DNA replication. We have used AFM imaging and AFM-based single molecule force spectroscopy (SMFS) to investigate the DNA binding/remodeling behavior of the DnaD protein and its separate N- and C-terminal domains (Nd and Cd, respectively). We discovered that DnaD has a global DNA remodeling activity implying a more widespread role in bacterial nucleoid architecture. Full length DnaD opens up supercoiled DNA and circularizes linear double-stranded DNA molecules. In order to achieve this it untwists the DNA duplex and converts plectonemic DNA to a more paranemic form. Our results show that Nd
exhibits an oligomerization activity and Cd a DNA-binding activity and a second DNA-induced oligomerization activity. Although Cd can bind to DNA forming large nucleoprotein complexes, it does not exhibit the characteristic global DNA-remodeling activity. The presence of separate Nd does not restore this activity. Thus the global DNA remodelling activity of DnaD is the sum of separate oligomerization and DNA-binding activities residing on two distinct but linked domains.

References:
Session III: High resolution imaging

Redox proteins immobilized on carbon nanotubes: A Conductive Atomic Force Microscopy characterization at the single molecule level

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The integration between metalloproteins and single-walled carbon nanotubes (SWNTs) is an active field of research, with the goal of optimizing electronic signal transduction in advanced and highly sensitive nano-biosensors. To this aim, the immobilization strategy of the protein on the SWNT is crucial, because it determines the molecular orientation and conduction properties. It is known that metalloprotein molecules adsorbed on SWNTs via amide bonds preserve their redox functionality [1], and several metalloproteins have been imaged at the single molecule level once adsorbed on SWNTs, by means of Tapping-Mode Atomic Force Microscopy [2]. Nevertheless, these characterizations do not provide direct information on the conduction of the single molecule. By means of Conductive Atomic Force Microscopy, imaging at the single molecule level can be coupled with the investigation of single molecule conductive properties [3]. For the first time, simultaneously recorded topography and current images of single metalloproteins adsorbed on SWNTs are presented. By using different immobilization strategies, the protein-SWNT interaction is correlated with the efficiency of the transport mechanism. The I/V characteristics of single metalloprotein molecules provide information on possible conduction channels across the protein. They have been recorded as a function of the applied force load in order to establish a stable electrical contact between protein and tip. [1] J.J. Gooding et al., J. Am. Chem. Soc. 125, 9006 (2003) [2] B.R. Azamian et al., J. Am. Chem. Soc. 124, 12664 (2002) [3] L. Andolfi and S. Cannistraro, Surf. Sci. 598, 68 (2005)

Modeling of local acidosis condition: towards understanding of origin and mechanisms of cytotoxic brain edema

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Etiology of cytotoxic edema after brain lesion is still poorly understood. Clinicians are usually facing combined results of a cascade of processes triggered off by the initial impact. In this regard it is not possible to resolve each event in this cascade. In an ideal situation, in order to provide the most efficient treatment, one should have a
clear understanding which process leads to which consequences. In an effort to further understand the origin and mechanisms of cytotoxic edema, we have developed a model of isolated acidosis condition (without first initiating ischemia) by using ozone to model relevant cell condition. The model was tested on animals (rats) and Magnetic Resonance Imaging (MRI) showed condition similar to that in case of a lesion induced edema. The same approach was then used in-vitro to analyze impact of the model acidosis condition on protein structures. Albumin (specifically bovine serum albumin, BSA) was used to test the model, as it is the most abundant protein in the CSF. Atomic Force Microscopy (AFM) was used to study morphology of BSA molecules after ozone treatment. The in-vitro study demonstrated that ozone treatment results in a substantial decrease in pH down to 3.8, which subsequently triggers off unfolding of the protein molecules. This structural transformation was found to result in manifold increase in both surface area and volume of the albumin molecules.

AFM imaging and force spectroscopy: tools for studying plant surface and characterizing carbon nanotubes/bio-molecules hybrids

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In this poster, we propose to show first results about the characterization of the surface of a parasitic plant Orobanche and of complexes made of bio-molecules and carbon nanotubes (CNTs). Orobanche parasites many plants in Europe and North Africa: sunflower, oilseed rape, tobacco, tomato… In proximity of a host plant, orobanche seeds are stimulated, germinate and fix themselves on the host plant roots and develop. We are studying the physical properties of the Orobanche root surface at its first development stages. The integration of biomaterials (proteins/enzymes, antigens/antibodies, or DNA) with carbon nanotubes (CNTs) provides new hybrid systems that combine the electronic properties of CNTs with the recognition or catalytic properties of the biomaterials. Our interest is focused on the synthesis of biomaterial-CNT hybrids (non-covalent interaction) and on the characterization of the structure of these hybrids.
Scanning Probe Microscopy investigation of tumour suppressor p53 and of its complex with Azurin

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p53 is a tumor suppressor protein that plays a widely recognized role in preventing cancer development by inducing cell cycle arrest and apoptosis in response to DNA damage. p53 mutations are one of the most common genetic alterations found in human cancers. Very recently, it has been suggested that a small copper protein, Azurin from Pseudomonas aeruginosa, can enter mammalian cancer cells and form a complex with p53, contributing to its stabilization and restoring its role of “guardian of the genome” [1]. Therefore, a deeper knowledge of the molecular mechanism underlying the interaction between p53 and Azurin could open new horizons for cancer therapy. Scanning Probe Microscopies (SPM) are very advanced tools for high resolution imaging and investigating topography and molecular interactions. Indeed, they opened up many new areas of science and engineering at the atomic and molecular level. In particular, these techniques allow studying single biomacromolecules under physiological conditions. In this respect, we have used Atomic Force Microscopy (AFM) and Scanning Tunneling Microscopy (STM) to investigate the topology of p53 DNA Binding Domain (p53 DBD) immobilized directly on gold and on a glutaraldehyde-cysteamine monolayer. We have also studied the complex formation between p53 DBD and Azurin by following the variation of both volume and conductive properties. The results are discussed in connection with the physiological role of the p53 DBD-Azurin complex. [1] T. Yamada et al., PNAS 101, 4770-4775 (2004). This work has been partially supported by CNISM (Innesco-Project 2005)

Nano – Infrared AFM based spectroscopy A new diagnostic tool for Biophysicists

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Bone can be considered to be one of the best examples of composite material that nature has produced, consisting of two phases: a mineral phase (hydroxyapatite) that gives the bone its strength and resistance to deformation, and an organic phase (mainly collagen) that gives bone its flexibility. Being able to map the different region using an atomic force microscope has been a very difficult task until recently. Using the combination of atomic-force microscopy and localised infrared spectroscopy, fundamental questions that have not yet been addressed, can now be elucidated. The overall concept of our technique is to record simultaneously an image carrying both structural and chemical information (infrared) about a given sample at a scale beyond that of an optical microscope. Infrared spectroscopy enables damage
done to the collagen matrix to be clearly identified, from entire ‘tissues’ down to the single molecule level. Transitions, such as shifts in the amide I, II and III bands in the infrared spectrum can be related to the ‘gelatinisation’ of the matrix. In a proof of concept experiment, it was shown that this technique was able to discriminate between native and denatured collagen, but at the scale of an AFM probe. This ability to fingerprint the degradation state of collagen leads to studies in the field of conservation of historical documents, but as well as in case of osteoarthritis or any collagen degenerative diseases.

Kinesin tracks on single protofilaments

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The function of most enzymes involves dynamic transitions, but the small size of most proteins prohibits direct imaging of these dynamics with light microscopy. Single motor proteins of the Kinesin family can move processively along microtubules by taking hundreds of successive steps. Kinesins are responsible for intracellular transport and are among the most studied motor proteins. Fundamental questions about its stepping their functional mechanism have remained unresolved because it has been impossible to image the motors with molecular resolution while they move. We have here pushed the limits of atomic force microscopy in buffer to observe individual processive Kinesin-1 motors from Neurospora crassa in their motion along a microtubule with nanometer resolution. We specifically found that both heads of one Kinesin-1 dimer are bound for the major part of the chemical cycle time to the microtubule. Furthermore, we could unambiguously resolve that both heads bind to the same protofilament, instead of straddling two, and remain on this track when they move.

Electrical imaging of biosamples using dc and ac current-sensing atomic force microscopy

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Recent advances in the development of nanoscale biosensors have raised new challenges in understanding the fundamental electrical transport of biosamples, such as protein membranes, which demands new measurement tools and protocols. Atomic Force Microscopy (AFM) is an ideal technique towards this aim: it has nanoscale spatial resolution; it is able to work with biosamples under their physiological
environment; and it can be adapted to perform a variety of electrical and electrochemical measurements under accurate control of the force applied to the biosample. In spite of the enormous interest of electrical conductivity measurements, only a few single-point measurements on biosamples have been reported so far, obtaining current-voltage (I-V) characteristics. The fact that electrical images of biological samples have not been obtained yet is due to the difficulty of minimizing the large shear forces between the tip and the sample during the scanning in contact mode, which are destructive for the biosample. Here we present two new measurement protocols capable of mapping the dc and ac electrical transport of biological samples at the nanoscale using current-sensing AFM. While nanoscale conduction maps are obtained by dc current-sensing, ac current-sensing provides nanoscale capacitance images, offering the possibility of directly probing dielectric variations of biosamples. These techniques will provide new insight in the study of dielectric properties of biomolecules towards the development of bioelectronics applications.

An AFM study on structural and mechanical properties of native, chemically-stabilized, and drug treated pericardium tissue

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Tissues based on collagen fibrils are widely used for production of biomaterials mostly due to their advantageous mechanical properties. The properties, which are physiologically important, result from the constitution of respective collagen fibrils and the way of their arrangement in the tissue. A structural feature of the collagen fibril is striation, the so-called D-banding, which is a result of the characteristic distribution of collagen molecules along the fibril axis. The structure of the collagen fibril is stabilized by numerous intermolecular interactions, which are called the crosslinking bonding. Action of chemical agents such as preservatives or drugs may perturb the structure of collagen fibrils, which affects the mechanical properties of the tissue. Using an example of a fibrous layer of the hog pericardium tissue, which is composed mainly of collagen fibrils of type I, we present changes observed within the surface of collagen fibrils after treatment with crosslinking agent of dimethyl suberimidate (DMS) and drugs of Naproxen, Ibuprofen and acetylsalicylic acid (aspirin). The measurements were made at room temperature on samples dried in air, by employing the atomic force microscopy (AFM) Nanoscope E and Nanoscope IIIa, Veeco, operating in the contact mode. We also present results of our attempt to estimate mechanical properties of the fibrous layer of the native and the chemically and drug treated tissue. AFM images show that the treatment agents applied introduce considerable changes in surface topography, fibril thickness and fibril arrangement on the tissue surface. They also change mechanical properties of the fibrous layer.
Visualization of Straight-to-Ring transition of individual tubulin protofilaments

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We show that Atomic Force Microscopy can be used to assess the intrinsic curvature of protofilaments resulting from the microtubule lattice, and correlate this curvature with the kink between tubulin subunits. These data have allowed us to confirm the prevalent model for how microtubules are destabilized by GTP hydrolysis. Namely that GTP hydrolysis induces a kink between the subunits, thus inducing curvature in the protofilaments. This curvature works against the interprotofilament interactions, putting strain on the lattice and destabilizing it. These data also suggest the mode of action of taxol, a microtubule stabilizer. Taxol works against the tendency of the tubulin subunits to kink after GTP hydrolysis. Our data show that taxol initially prevents the GDP subunit kinking in the lattice. Perhaps most interestingly, the data show that even in the presence of taxol, there is a slow transition between the straight and curved conformations, and this analysis appears to be trapping intermediates in this process. We don’t know why there is a slow change, but the most likely explanation is that taxol slows down the straight to curved reaction. More generally, the data from taxol and MCAK (a kinesin known as a microtubule depolymerizer) suggest that one of the basic modes by which stabilizers and destabilizers affect the stability of the microtubule lattice is by stimulating or counteracting the basic tendency of the protofilament to curve. The assay that we have established in this study will allow us to test this more generally for other stabilizing and destabilizing agents.

Study of Fractal Dimension and Localization of DNA Knots by Atomic Force Microscopy

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The scaling properties of homo- or heterogeneous mixture of viral DNA knots were studied by Atomic Force Microscopy (AFM). DNA knots were adsorbed onto mica in regimes of (i) strong binding, that induces a kinetic trapping of the three-dimensional (3D) configuration, and of (ii) weak binding, which permits (partial) relaxation on the surface. The DNA contours were analyzed with a box counting algorithm, giving the
number of filled boxes as a function of the box size $L$. In (i) the radius of gyration of the adsorbed DNA knot scales with the 3D Flory exponent $\nu$ of $0.60$ within error. In (ii), we find $\nu$ of $0.66$, a value between the 3D and 2D ($\nu = 3/4$) exponents, indicating an incomplete 2D relaxation or a different polymer universality class. Evidence is also presented for the localization of knot crossings in 2D under weak adsorption conditions.

Variations in Morphology in Surfaces of Human Bone – Possible Collagen Collapse

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Understanding the structural relationships between the building blocks of bone is important for understanding the mechanical properties of the bone tissue. Human bone tissue is composite material consisting of small mineral plates embedded in a matrix of organic fibres. The size scale of the individual building blocks of bone is in the nanometer range with collagen fibrils being 100 nanometer in diameter and mineral plates being about 50 nanometer in diameter. To understand bones ability to resist fracture as a tissue, and thus its quality, one has to elucidate the intricate interplay between these building blocks. We have used the Atomic Force Microscope (AFM) as tool to elucidate this interplay in structural sense as well as in nanomechanic sense. We show detailed images of the surface from several trabecular struts in several patients. The images reveal big variances in morphology. The morphology reveals that the collagen fibrils degenerate into a lesser order. Struts from Young Patients reveal less structural degeneracy while struts from elderly patients show greater variances in morphology, suggesting that the collagen fibrils falls apart with age. The Nanomechanics of the various morphologies found is also presented. The results seems to contradict a consensus of collagen fibrils generating more internal cross-links from advanced glycation end-products (AGE), thus forming stiffer and more brittle fibrils over time. In addition we have mapped the imprint of bone remodelling, to elucidate the structural relationship between old bone and the osteoid.
Close Approximation of Two Platelet Factor 4 Tetramers by Charge Neutralization Forms the Antigens Recognized by HIT Antibodies

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Heparin-induced thrombocytopenia (HIT) is a prothrombotic drug reaction caused by antibodies that recognize positively charged platelet factor 4 (PF4), bound to the polyanion, heparin. The resulting immune complexes activate platelets. Unfractionated heparin (UFH) causes HIT more frequently than low-molecular-weight heparin (LMWH), whereas the smallest heparin-like molecule (the pentasaccharide, fondaparinux), induces anti-PF4/heparin antibodies as frequently as LMWH, but without exhibiting cross-reactivity with these antibodies. To better understand these findings, we analyzed the molecular structure of the complexes formed between PF4 and UFH, LMWH, or fondaparinux. By atomic force microscopy and photon correlation spectroscopy, we show that with any of the 3 polyanions, but in the order, UFH < LMWH < fondaparinux—PF4 forms clusters in which PF4 tetramers become closely apposed, and to which anti-PF4/heparin antibodies bind. By immunoassay, HIT antibodies bind strongly to PF4/H/PF4 complexes, but only weakly to single PF4/heparin molecules. HIT antigens are formed when charge neutralization by polyanion allows positively charged PF4 tetramers to undergo close approximation. Whereas such a model could explain why all 3 polyanions form antibodies with similar specificities, the striking differences in the relative size and amount of complexes formed likely correspond to the observed differences in immunogenicity (UFH > LMWH = fondaparinux) and clinically relevant cross-reactivity (UFH > LMWH >> fondaparinux).

Hydrophobic interactions at the three-dimensional nanoscale investigated with miniature-cantilever AFM

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Hydrophobic interactions are ubiquitous in nature. They play an important role in protein folding, self-assembly of membranes and protein-protein interactions. Nevertheless, very little is known about the exact nature of the hydrophobic force. Even something as basic as the length scale over which it acts is still under discussion. Most of the sparse experimental data comes from Surface Forces Apparatus or AFM measurements on surfaces that are homogeneous over a large scale. A complicating factor in these experiments is the occasional appearance of nanoscopic gas bubbles, that are not seen with less invasive techniques. In order to understand more about the hydrophobic interaction in more complicated geometries, we built a model system that consists of a monolayer of two different types of
molecules, that phase-separate into two-dimensional domains with a typical size of 5-50 nm. These domains present either only hydrophilic, or only hydrophobic endgroups at the interface. We investigate the interactions of these domains, immersed in pure water, with a carbon nanotube that is mounted onto an AFM tip. To optimize the force resolution without the problem of snap-in at short distances we use miniature cantilevers with 2 N/m spring constant and 1MHz resonance frequency. This allows us to present the first experimental data on hydrophobic interactions at the three-dimensional nanoscale.

High-resolution AFM/Phase Lag investigation of 20-mer oligonucleotides anchored on an unoxidized crystalline silicon surface

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Single-strand poly-thymine oligonucleotides have been anchored on a carboxylic-acid functionalized crystalline silicon (1,0,0) surface to produce a silicon-based nano-bio sensing device capable to react selectively with a specific target molecule, the Poli-adenine (Poli-A). Intermittent-contact AFM imaging and quantitative fluorescence in solution have been performed on these samples in order to optimize the preparation protocol and to fully characterize the immobilization and hybridization processes. The topography measurements and the extremely controlled preparation procedure have produced images of the single-strand oligonucleotides with a state-of-the-art lateral resolution of 8.6 nm. Moreover, phase-lag images have evidenced interesting non-topographic structures on the functionalized substrates that have been interpreted as areas of different molecule self-orientation. In conclusion, the coupling between Tapping-mode AFM, Phase-lag imaging and quantitative fluorescence have proved useful for the characterization of DNA-based proto-biosensors allowing to bridge the gap between the macroscopic functional behaviour of the device and the microscopic molecular-molecular recognition.

ENHANCED COMPOSITIONAL SENSITIVITY IN ATOMIC FORCE MICROSCOPY BY EXCITACIÓN OF THE FIRST TWO FLEXURAL MODES

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We demonstrate that the compositional sensitivity of an atomic force microscope is enhanced by the simultaneous excitation of its first two normal eigenmodes. The coupling of excited modes by the probe-surface force interaction
enables to map compositional changes in several conjugated molecular materials with a phase shift sensitivity about one order of magnitude higher than the one achieved in amplitude modulation AFM1,2. This technique has been used for high resolution imaging of organic molecules, as T6 and TTF, and also antibodies in two different forms: IgG (trimers) and IgM (pentamers).

3. S. V. Patil et al. PNAS, (submitted)

Escherichia coli FtsZ filament structure and dynamics studied with atomic force microscopy

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FtsZ, the prokaryotic homologue of tubulin, is an essential cell division protein. In the cell it localizes at the center forming a ring that constricts during division. In vitro it binds and hydrolyzes GTP, and polymerizes in a GTP-dependent manner. We have used atomic force microscopy (AFM) to study the structure and dynamics of FtsZ polymer assembly on a mica surface under buffer solution. The polymers were highly dynamic and flexible, and continuously rearranged over the surface. End-to-end joining of filaments and depolymerization from internal zones were observed, suggesting that fragmentation and reannealing may contribute significantly to the dynamics of FtsZ assembly. The shape evolution of the restructured polymers manifested a strong inherent tendency to curve. Polymers formed in the presence of non-hydrolysable nucleotide analogues or in the presence of GDP and AlF3 were structurally similar but showed a slower dynamic behaviour. Theoretical modelling indicates that lateral interactions between neighbouring filaments and the presence of a preferred angle between monomers are sufficient to account for the structures observed, suggesting that lateral interactions could be relevant for the function of the FtsZ filaments during cell constriction. Since mutations that affect the lateral interaction between filaments have been described as being non-functional, we explore the structure and dynamic behaviour of some of these mutants on a mica surface in order to associate functionally relevant interactions with the in vitro structure and dynamic of the filaments on mica observed with AFM.
Ultrastructure and polymorphism of amyloid fibrils

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Amyloid fibrils are self-assembled, beta-sheet-rich superstructures of peptides or proteins. Although these aggregates have first been found in connection with protein-misfolding diseases, such as Alzheimer's or Parkinson's disease, there is evidence that the ability to form fibrils is a thermodynamic property of any polypeptide chain rather than a result of specific, disease-related amino-acid sequences. Fibrils can easily be formed in-vitro from disease- and non-disease-related proteins, which can serve as model systems to study the morphological and physico-chemical properties of amyloid fibrils. Here, we present AFM-investigations of the ultrastructural and physical properties of the peptide TTR105-115, which forms well-defined fibrils of ca 10nm diameter. Fibrils showed typical polymorphism, which is indicative of the different strains often observed in protein-misfolding diseases. They could be chemically dissected into smaller protofilaments, which were studied at high resolution by AFM. Furthermore, it has been found that fibril adsorption on surfaces is influenced by electrostatic surface properties and can be controlled by appropriate choice of solvent conditions.

Global architecture of full length human poly(A)-specific exoribonuclease, PARN, revealed by AFM in liquid

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AFM imaging has been routinely focused on objects of ~100 nm or greater. The aim of this work was to learn a shape and dimensions of a single protein molecule in solution with a resolution below 10 nm. PARN, poly(A)-specific exoribonuclease, is a large dimeric protein responsible for turnover of eukaryotic mRNA. Deadenylation of the 3’ poly(A)-tail by PARN is the initial and rate-limiting step in the mRNA decay. Disturbance of mRNA turnover leads to cancer, inflammation, Alzheimer disease, and influences embryogenesis. PARN activity is regulated by the mRNA 5’ cap. A fragmentary crystal structure of PARN without one functional domain, RRM, was published recently. Two remaining domains, nuclease and R3H, resembled a see-horse shape, with an empty space in the middle. The structure neither explained the poly(A)-specificity nor contained the cap-binding site. Hence, learning an overall...
appearance of full length PARN was highly desired. Imaging was performed in a contact mode, using sharp tips (rc of ~2 and 20 nm) on cantilevers with soft spring constants (0.006 to 0.05 N/m). Collected images show that a single PARN molecule has a compact, ellipsoidal shape, with dimensions of 10.87 +/- 0.19, 7.56 +/- 0.17, 4.56 +/- 0.31 nm, and a volume of 196 +/- 15 nm3. These results suggest that the R3H domain is moved toward the nuclease of the same subunit and the RRM fills the central empty space. Thus, high resolution imaging provided an insight into a global architecture of PARN. Supported by Polish Ministry of Science 2P04A03328

AFM characterization of DNA-biosensor nanofilms adsorbed onto charged surfaces

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In a health perspective the need for the analysis of gene sequences, oxidative damage to DNA and the understanding of DNA interactions with molecules or ions led to the development of electrochemical DNA-biosensors. The detection of chemical compounds that cause irreversible damage to DNA is very important, as they may lead to hereditary or carcinogenic diseases. The electrochemical DNA-biosensor is a complementary tool for the study of biomolecular interaction mechanisms of compounds with DNA, enabling the screening and evaluation of the effect caused to DNA by health hazardous compounds and oxidising substances. A critical issue in the development of an electrochemical DNA-biosensor is the sensor material and the degree of surface coverage. AFM was used to characterize the process of adsorption of DNA on a highly oriented pyrolytic graphite (HOPG) electrode surface using different concentrations of DNA and adsorption procedures [1-3]. Reactions with chemical substances cause changes in the structure of DNA and the base sequence, leading to perturbations in DNA replication. The electrochemical DNA-biosensor provides very relevant information because the mechanisms of DNA-drug interactions at charged interfaces mimic better the in vivo DNA-drug complex situation, where it is expected that DNA will be in close contact with charged phospholipid membranes and proteins, rather than when the interaction is in solution.

AFM imaging of heterologous membrane proteins expressed in Xenopus laevis oocytes

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Xenopus laevis (X. laevis) oocyte represents a well-known, high efficient model system for the heterologous expression of plasma membrane proteins [1]. Recently, atomic force microscopy (AFM) has been proved to be a useful tool for studying membrane proteins in native environment [2]. In this study, the first reproducible AFM images of eukaryotic plasma membrane proteins starting from X. laevis oocyte membranes purified by ultracentrifugation on sucrose gradients were obtained. The morpho-dimensional analysis of these proteins confirmed previously published AFM data [3] obtained preparing samples using different protocols. The average diameter and height of the protein complexes resulted of 23.3 nm (S.E. = 0.3 nm; n = 200) and 1.5 nm (S.E. = 0.1 nm; n = 90) respectively. Besides, protein complexes densely packed and arranged in an ordered way according to two different patterns, hexagonal and square packings, were visualized. These ordered membrane domains were processed and analysed by Fast Fourier Transform to study the protein assembly and their spatial organization. Electron microscopy imaging allowed to validate AFM results with a different technique. Finally, the first data of an AFM study of rat gamma-aminobutyric acid transporter (rGAT-1) expressed on X. laevis oocyte plasma membrane are reported. In particular, the rGAT-1 proteins were visualized as oligomers in accordance with recent physiological and crystallographic studies [1, 4]. Future developments will be addressed to perform AFM study of the oocyte plasma membrane expressing rGAT-1 operating in single molecule force spectroscopy mode [5].

Supramolecular polymers based on proteins and DNA for conformational sensitive biosensors

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In order to implement a new generation of biosensor based on the detection of conformational changes of biomolecules, supramolecular self-assembled scaffolds were created by genetic and protein engineering, immobilized on a functionalised Si/SiO₂ substrates and characterized by Atomic Force Microscopy. Each scaffold is made by polymerization of monomers constituted of cytochrome b₅ protein linked to an oligonucleotide. Membrane anchor of native human cytochrome b₅ was removed by genetic engineering to obtain a soluble form. A His-tag sequence was added to the truncated C-terminus and a unique cystein residue was introduced by directed mutagenesis on a surface loop. The thiol-extremity was coupled through a synthetic bifunctional linker to different types of oligonucleotides bearing an amino group terminated extension at their 3’-end. Resulting PDNAs were synthesized and purified to homogeneity at mg scale. Thus polymeric chains (200 nm length) of double strands DNA bears regularly spaced (40 nm) proteins able to be anchored at the surface. Different monomers were linked together using fragment of complementary oligonucleotides, leading to supramolecular polymer. The polymers were immobilised onto a Si/SiO₂ substrates modified with a silane and bearing a nickel layer. AFM images were performed on this surface in the dynamical mode in soft and hard tapping conditions. Images revealed the presence of the polymeric chains, of 200 nm long, lying on the surface which became entangled at some places. In hard tapping conditions, some hillocks attributed to the presence of the b₅ protein were observed along isolated polymer on the phase images.

Interchromatidal central ridge in early metaphasic human chromosome one by AFM

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The structure of GTG stained early metaphase human chromosomes was analyzed by AFM in AC mode. Longitudinal height measurements for the early sister chromatids and the middle of chromosomes showed a central ridge that was further characterized by transversal height measurements. The heterochromatic regions displayed a higher level of transversal symmetry, while the euchromatic ones presented several peaks across the transversal height measurements, supporting evidence for interchromatid interactions prior to disjunction. We suggest that this central ridge could point out a transitional arrangement of the early metaphase chromosome towards the typical disjunction of the sister chromatids.
Fast force mapping using small cantilevers

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Force mapping allows the acquisition of locally resolved, quantitative material properties such as stiffness and adhesion, simultaneously with sample topography. By using 12-micron-long, high-resonant-frequency cantilevers in a special AFM, we reduced the force map acquisition time in liquid by two orders of magnitude, as compared to conventional AFM. We thereby recorded high-speed image sequences of topography and stiffness of organelles during their enzymatic digestion. To further reduce the acquisition time, we have developed a theory that strips off the hydrodynamic drag of the cantilever from the force curves, thereby revealing the bare tip-sample forces. This theory automatically adapts to changing experimental conditions. It is therefore well suited for high-throughput reconstruction of true tip-sample forces and even allows extracting viscous surface forces that are usually covered by the hydrodynamic drag in the liquid. Using small cantilevers together with this theory, we reconstructed tip-sample forces up to a vertical speed of 10,000 microns per second.

DETAILED MORPHOLOGICAL ANALYSIS OF ALPHA-SYNUCLEIN AGGREGATION INTERMEDIATES BY ATOMIC FORCE MICROSCOPY

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Protein misfolding and aggregation is associated with a variety of diseases, including several neurodegenerative disorders. High concentrations of fibrillar aggregates of the natively unfolded protein α-synuclein are found in the brain of Parkinson’s patients. A relation between the formation of fibrillar α-synuclein aggregates and loss of neurological function has been proposed, although the exact mechanism is not yet known. The aggregation process is thought to take place in a nucleation dependent manner, from monomeric folding intermediates to nucleation centers, protofibrillar structures and mature fibrils. We applied high-resolution atomic force microscopy to study the detailed morphology of the various species formed during α-synuclein aggregation at different sample conditions, including different initial protein concentrations and agitation methods. We also monitored the kinetics of aggregation by measuring the enhancement of Thioflavin T fluorescence. Agitation was seen to influence aggregation kinetics and fibril length. Many fibrils showed a periodicity perpendicular to the fibril length. In the longitudinal direction we also observed fibril sections with distinct differences in height. We propose that the fibril formation process may rather be based on the assembly of smaller fibril segments than on intertwining of fibrillar structures, suggesting that the previously described hierarchical assembly model, that proposes intertwining of protofilaments and protofibrils, may not be fully applicable. Knowledge on the mechanism of fibril
assembly, possibly a multi-pathway process fundamental to many protein misfolding diseases, is important in view of the development of means for therapeutic intervention.

Surface Diffusion Controls the Growth Mechanism of Triosephosphate Isomerase Crystals

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In the protein crystallization process, a growth unit has two possible molecular pathways it can follow from solution to the crystal bulk, namely the process of direct incorporation from solution or the process of surface diffusion preceded by surface adsorption. We use real time in situ atomic force microscopy to monitor the molecular processes that govern the crystallization of the protein triosephosphate isomerase. With this technique, we study the step edge dynamics on a molecular scale. We conclude that step reorganization as a result of line diffusion has a negligible effect on step dynamics. Therefore, step displacements are attributed to the exchange of growth units with the surrounding phases, i.e. the terrace and the solution. Recorded high resolution atomic force microscopy images allow us to identify the dominating growth units as single and double unit cells. From the statistics of molecular attachment and detachment from the step, we conclude that the incorporation of growth units occurs through surface diffusion. Additionally, in the supersaturation range of ~1.5 to 6, normal growth is dominated by the two-dimensional nucleation of triangular islands. The step edges of these nuclei have equal step formation energy.

Atomic force microscopy study of photodynamic effect on cancer cell lines using liposomes for photosensitizer delivery

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Atomic force microscopy (AFM) has been used to image the morphology of developing tumorous cells and their processes. However, it is frequently reported that prior fixation is required for reliable imaging of cells with lower adhesive properties. Scanning of these objects is limited by construction of atomic force microscopy
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(AFM), therefore we replenished the apparatus with an inverse optical microscope. In addition, a liquid scanner gave us possibility to study cells in vitro. The aim of the presented study is picture of cell surface in liquid surroundings of nutrient media and in air. In our work we used cell line G361 and A549 as biological materials. We imaged the cancer cells before and after photodynamic effect (PDE). PDE of photosensitisers ZnTPPS4 and CIAIPcS2 incorporated in liposomes was induced by an efficient LED source. We also sonicated the cell samples by ultrasonic therapeutic device to improve the effectiveness of PDE. After sonication and irradiation the cells were scanned in air and in liquid. Our results show images of the cell lines before and after PDE including the both types of imaging the topography and the phase image. The treatment of the cells with the photosensitisers leads the loss of surface rigidity and eventually to dramatic changes of the cells shape. Individual cells before PDE were characterized by smooth surface without protrusion on the whole surface. This work was supported by the Grant Project MSM 6198959216.

AFM images of modified DNA strands

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Nanoscale imaging of drug-induced alterations in Mycobacterium bovis BCG

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Progress in understanding the structure-function relationships of the mycobacterial cell wall has been hampered by its complex architecture as well as by the lack of sensitive, high resolution probing techniques. For the first time, we used atomic force microscopy (AFM) to image the surface topography of hydrated Mycobacterium bovis BCG cells and to investigate the influence of antimycobacterial drugs on the cell wall architecture. We first studied the effect of ethambutol, an antibiotic that inhibits the synthesis of cell envelope polysaccharides. While untreated cells showed a very smooth and homogeneous surface morphology, incubation of cells in the presence of ethambutol caused dramatic changes of the fine surface structure. At 4 µg/ml, the drug created concentric striations at the cell surface and disrupted a 8 nm thick cell wall layer, attributed to the outer electron-opaque layer usually seen by electron microscopy, while at 10 µg/ml an underlying 12 nm thick layer reflecting the thick electron-transparent layer was also altered. We are currently extending this study to drugs that target other components of the mycobacterial cell wall, including ethionamide and isoniazid which affect lipids and streptomycine that inhibits the synthesis of proteins. These non-invasive ultrastructural investigations provide novel information on the macromolecular architecture of the mycobacterial envelope as well as into the destructuring effects of antibiotics.
Session IV
Model membranes and protein-membrane interactions

AFM studies of SNAREs interactions during membrane fusion and fission

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SNAREs are considered the minimal machinery for membrane fusion in vitro. Liposomes reconstituted with SNARE proteins have been used to investigate membrane fusion. We employed the atomic force microscope to measure fusion forces of two apposing lipid bilayers and rupture forces associated with the dissociation of v- and t-SNARE complexes during approach and retraction, respectively. Egg phosphatidylcholine bilayers containing v-SNAREs (synaptobrevin 2) or binary t-SNAREs (syntaxin 1 and SNAP 25) were formed by lipid vesicle adsorption onto opposite glass dishes and glass microbeads attached to cantilever tips. Fusion and rupture forces increased with loading rate (LR). The dynamic force spectrum (DFS) of the fusion process revealed a single linear loading regime in presence/absence of SNAREs. This indicates that membrane fusion is governed by one energy barrier under the current conditions. In the presence of SNAREs, the width of the energy barrier increased by ~3 fold compared to that in egg PC alone, whereas the activation potential was unchanged. This suggests that interaction of cognate v- and t-SNAREs facilitated membrane fusion by reducing the slope of the energy barrier. Alternatively, the DFS of the SNARE complex dissociation revealed two loading regimes, suggesting the presence of two energy barriers during dissociation of v-SNARE/t-SNARE complexes. The inner (high LRs) and outer (low LRs) barrier widths were 0.39Å and 2.9Å, and dissociation rate constants were 15.5 s⁻¹ and 0.3 s⁻¹, respectively. These energy barrier parameters suggest that under pulling force, dissociation of the v-SNARE/t-SNARE complex is effectively dominated by the inner barrier.
Nanoscale interactions between tilted peptides and supported lipid bilayers

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Tilted peptides are hydrophobic peptides known to insert in lipid bilayers with an oblique orientation, thereby destabilizing membranes and facilitating membrane fusion processes. Despite the vast body of literature that has accumulated on tilted peptides in the past decade, information on the forces that drive their interaction with lipid membranes is dramatically lacking. Here, we used atomic force microscopy (AFM) to explore the nanoscale interaction forces between the simian immunodeficiency virus (SIV) peptide and supported bilayers composed of various lipids (dipalmitoylphosphatidylcholine, dioleoylphosphatidylcholine, dioleoylphosphatidic acid and dipalmitoylphosphatidylethanolamine). To detect single molecule interactions, histidine-tagged peptides were attached onto AFM tips terminated with nitrolotriacetate and tri(ethylene glycol) groups. This coupling approach offers several advantages: optimal exposure of the C-terminal hydrophobic domain, high mobility of the attached peptides, nonspecific adsorption minimized and low coupling density. AFM force-distance curves recorded between the SIV-tip and the bilayers did not show any unbinding events, whatever the nature of the lipid investigated. To explain this unexpected behavior, we suggest a mechanism in which lipids are pulled out from the bilayer during the first retraction due to strong interaction with the SIV tip, which is consistent with the relative low force needed to extract lipids from supported bilayers (20-50 pN). We conclude that while AFM tips modified with hydrophobic peptides have a strong potential in biophysics, they are not appropriate for measuring forces on supported lipid bilayers. Suggestions are made to circumvent this limitation in future studies.

AFM analysis of Starfish footprint adhesives

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Starfish (Echinodermata, Asteroidea) use their tube feet for activities such as attachment to the substratum, locomotion, handling of food and burrow-building. The tube feet are specialized organs that produce adhesive secretions. The locomotory adhesive is the most interesting but it is still not very clearly recognized. All temporary adhesives are by their nature cyclical and must be followed by de-adhesion and yet while the starfish literature abounds with information on adhesion the subject of de-adhesion has only recently been discussed. The de-adhesive aspects of such temporary interactions are obviously important and as necessary as the adhesive ones.
and are fundamental to understanding many biological systems. To understand the process of locomotion in starfish, we will analyse the secretion footprint (histochemically and biochemically). The footprint of the starfish consists of a sponge-like material deposited as a thin layer on the substratum. Although the diameter of the footprint is easily measured after histochemical staining of the adhesive material, the thickness of the footprint is difficult to estimate. By using the surface-imaging tool AFM to analyse the footprint we can provide a three dimensional image of the surface ultrastructure with molecular resolution in real time, under physiological conditions and minimal sample preparation. AFM can help to probe the physical properties, such as the molecular interactions, surface hydrophobicity and surface charges of the starfish footprint adhesives.

**Nanocharacterization of a Cr(VI)-Sensitive Layer Electrolyte-Membrane-Insulator-Semiconductor Sensor by Atomic Force Microscopy**

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The health of workers in many industries is at risk through exposure to hexavalent chromium (Cr[VI]) compounds. Airborne Cr(VI) has been identified as a known human respiratory carcinogen, and analytical methods for the measurement of this species in workplace aerosols are desired. Aside from carcinogenic effects, some Cr(VI) compounds have also been found to act as contact allergens. Hexavalent chromium exposures in the workplace have been associated with numerous industries. So, there is a strong desire on the part of the occupational and environmental health community to have the capability to accurately measure airborne Cr(VI) species at trace levels. Hence, in recent years the development of improved analytical methods for the determination of Cr(VI) has been a subject of significant interest. In this work, we characterize by atomic force microscopy (AFM) a novel Electrolyte-Membrane-Insulator-Semiconductor (EMIS) structure sensitive to Cr(VI). Two molecules, tributylphosphate (TBP;PO(C4H9O)3) and tri-n-octylphosphine oxide (TOPO; C24H51OP), have been used as the sensing ionophores for Cr(VI) due to their high sensitivity. The developed device has been characterized by C-V and by AFM to study the sensitivity and the conformational topography of the two polymeric membranes. Adhesion force measurements were also performed to study and compare the properties of adhesion of the two selective membranes. We conclude from our results that the differences in the conformational topography are due to variations in the microstructural properties of the two ionophores. Adhesion force studies probe the exposition of different functional groups on the surface of both membranes.
The Complex Film Structure and Properties of Human Saliva

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Films formed from saliva on surfaces are important for maintenance of oral health and integrity by protection against chemical and/or biological agents. The aim of the present study was to investigate adsorbed amounts, thickness and the structure of films formed from human whole saliva on various model surfaces by means of in situ ellipsometry, neutron reflectivity and atomic force microscopy. The results showed that the salivary film morphology strongly depend on the type of surface used. In general, the film can be modelled by two layers: an inner and dense thin region which forms a uniform layer, and an outer, more diffuse and thicker region that protrudes towards the bulk of the solution. The film morphology described a uniformly covering dense layer and a second outer layer containing polydisperse adsorbed macromolecules or aggregates. The density, size and height of the large aggregates strongly depend on the type of substrate used. Further AFM studies in terms of interaction forces and frictions were performed to further understand the properties of these systems.

Supported bilayer membrane domains at the Nanoscale

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Studying biological structures and organization of cell membranes is one of the major challenges for understanding cell’s behaviour in response to their external environment. Studying these structures in vitro needs artificial reconstitution of supported lipidic bilayers, mimetic of the membrane of living organisms. This assembly coupled with imagery tools like AFM in liquid media opens new ways for studying natural spatial arrangement and interactions of membranes proteins. In this perspective, we are developing the technique of micro contact printing “µcp” in order to elaborate domains of supported bilayer membrane at the nanoscale. Properties of these domains are currently analyzed and will be followed by the investigation of proteins incorporated in these membranes. The technology we are developing is inspired by the pioneering works of Boxer et Al [1]. µcp is used as a nanoscale lithography method enabling the deposition of bio molecules along patterns of arbitrary shapes. In our works BSA proteins are patterned by µcp on a cleaned glass slides and patterns down to the submicronic scale are routinely produced. These BSA
features serves as a barriers for the fusion of liposome’s and subsequently proteoliposomes rendering possible the elaboration of domains of supported lipidic membranes of arbitrary shape and dimensions. We will concentrate our presentation on the AFM characterization of this process, from the BSA patterns to the supported bilayer membranes partitioned into sub-micronic patches. This work was supported in part by the EC-funded project NaPa and by the ANR (French Research National Agency) funded project FlaNaMo [1] Langmuir 2000, 16, 6774-6776, Steven G. Boxer

STRUCTURAL ANALYSIS BY ATOMIC FORCE MICROSCOPY OF COMPRESSED PULMONARY SURFACTANT FILMS

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Pulmonary surfactant is a lipid-protein complex synthesized and secreted by the alveolar epithelium of mammalian lungs. The saturated phospholipid DPPC is the major component and the responsible of surfactant main function: reduces the surface tension of the thin aqueous layer covering the alveolus to near 0 mN/m, to prevent the alveolar collapse during expiration. Nevertheless, transference of DPPC from the lamellar structures, were surfactant is stored, into the aqueous interface is not easy due to the physical properties of this lipid. Other components, as unsaturated phospholipids, cholesterol and the hydrophobic surfactant proteins SP-B and SP-C, are proposed as modulators of the rheological and structural properties of surfactant in order to facilitate the adsorption and recycling of surface active species during the breathing cycle. Atomic force microscopy has been used to analyse the structure of Langmuir-Blodgett films transferred from films of native and cholesterol-depleted pulmonary surfactant preparations. The compression-driven isotherms of these surfactant films show a first plateau occurring at surface pressures about 45 mN/m, previous to the collapse plateau of the films occurring at ~70 mN/m (almost 0 mN/m surface tension). The films contain condensed microdomains at pressures prior to the first exclusion plateau indicating lateral segregation of material into the interfacial plane. AFM allows observation of the presence of condensed nanodomains and extruded areas protruding out from the interface. The presence of these three-dimensional structures have been interpreted in terms of partial exclusion of minor surfactant components from the compressed interface to improve stability of the films at pressures close to 70 mN/m.
Atomic Force Microscopy with ultrasound excitation

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AFM-based procedures that rely on the excitation of surface ultrasonic vibration have already demonstrated their ability to render information about nanoscale dynamic materials properties such as elasticity, viscoelasticity, adhesion and friction. Techniques such as Acoustic-AFM [1] or SLAM (Scanning Local Acceleration Microscopy) [2] monitor the cantilever vibration linearly induced through the tip-sample contact when the tip is in contact with a vibrating surface typically excited at frequencies of some tens of MHz. In Ultrasonic Force Microscopy (UFM) the surface ultrasonic vibration is detected via the "ultrasonic force" [3] that originates due to the non-linearity of the tip-sample interaction. Based on the nonlinear detection of ultrasound, novel ultrasonic AFM-based techniques such as Heterodyne Force Microscopy (HFM) [4] and Mechanical-Diode Mode Ultrasonic Friction Force Microscopy (MD-UFFM) [5] have been recently proposed. Experiments from our lab performed on mica demonstrate the nonlinear detection of surface ultrasonic vibration with an AFM cantilever tip operating in liquid environments. In the poster I will describe the techniques of UFM, HFM and MD-UFFM, discuss the possibilities to implement them in liquids, and the opportunities for their application in biology.


Biolubrication of mucin studied by Friction force microscopy

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Colloidal Probe Friction Force Microscopy has been used to study the lubricating ability of various mucins in vivo. Mucin is well known to provide a lubricating and protective role for many areas in the body including eyes, mouth, and gut. The study reveals that the adhesion and frictional properties of mucin is intrinsically linked to complexation with other lower molecular weight biomolecules. Excitingly it is shown to be possible to tune the frictional behaviour of adsorbed mucin layers. This study has direct relevance for application in new bio-functional materials for implants and in particular for contact lenses.
**Atomic force microscopy as a tool for imaging Salmonella biofilm formation**

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Bacteria tend to form sessile communities on surfaces, where the cells are embedded in a matrix of extracellular substances. These so-called biofilms provoke major concern in industry and in medicine. In order to prevent and to treat biofilms, it is necessary to get a detailed understanding of biofilm formation at the molecular level. In this work we have established a model system based on atomic force microscopy in combination with light microscopy, which allowed us to follow Salmonella biofilm development at the morphological level with high resolution over time. Biofilms were allowed to grow on mica mineral surfaces that were submerged in Petri dishes with standing cultures of Salmonella enterica serovar Typhimurium. After 3 h, 4 h, 8 h and 24 h the biofilm covered mica chips were rinsed with water, air-dried and analyzed by AFM and light microscopy. Our results showed that the bacteria undergo dramatic changes in colony and individual cell morphology during biofilm growth. Beside differences in cell and colony size as well as colony topography, we also noted that the bacteria expressed flagella, the locomotor system of bacteria, and other extracellular substances, such as pili, curli and cellulose, in a growth-phase-dependent manner. By using strains, mutated in these surface components, we were able to investigate their roles in biofilm development. We found that curli and cellulose were necessary for the formation of a confluent biofilm after 24 h, but not for the initial attachment events within the first 8 hours of biofilm formation.

**Lipid Layers Assembled on Multilayer Polyelectrolyte Supports—Studies by Colloidal Force Spectroscopy**

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The interaction between lipid layers supported by polyelectrolyte multilayer cushions has been studied by colloidal force spectroscopy (CFS). In a typical experiment a colloidal probe engineered with a layer-by-layer film and a lipid bilayer on top is approached to a planar surface coated in a symmetrical way. Charged lipid layers composed of mixtures of DMPC and sphingosine typically show repulsion upon approaching and almost no adhesion in the retraction curves. Lipid layers composed of DPPC, DMPC, DOPC, or POPC present approaching curves characterized by
almost no repulsion and the presence of kinks of a few nanometers in width, reflecting
either fusion processes involving vesicle membranes or the penetration of the polymer
blobs into or through the phospholipid layer. Adsorbed vesicles can be identified by
CFS. Retracting curves show polymer stretching demonstrating thus that either the
lipid coverage was incomplete or the polyelectrolyte multilayers made contact as a
result of penetration or fusion. These phenomena are largely absent when charged
phospholipids were employed. In addition pulling of lipid tethers was found. The
assembly of phospholipids on polymer cushions was also followed with the quartz
crystal microbalance with dissipation, which allowed us to distinguish between
deposited lipid bilayers and a layer of adsorbed vesicles. Inhomogeneities in the
zwitterionic lipid coverage on colloidal particles have also been confirmed by
confocal laser scanning microscopy.

Continuous Planar Phospholipid Bilayer Supported on Porous
Silicon Thin Film Reflector

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Reconstituting artificial membranes for in vitro studies of cell barrier mechanisms and
properties is of major interest in biology. Here, artificial membranes supported on
porous silicon photonic crystal reflectors are prepared and investigated. The materials
are of interest for label-free probing of supported membrane events such as protein
binding, molecular recognition, and transport. The porous silicon substrates are
prepared as multilayered films consisting of a periodically varying porosity, with pore
dimensions of a few nanometers in size. Planar phospholipid bilayers are deposited on
the topmost surface of the oxidized hydrophilic mesoporous silicon films. Atomic
force microscopy provides evidence of continuous bilayer deposition at the surface,
and optical measurements indicate that the lipids do not significantly infiltrate the
porous region. The presence of the supported bilayer does not obstruct the optical
spectrum from the porous silicon layer, suggesting that the composite structures can
act as effective optical biosensors.
Improving AFM microbial cell nanomanipulation by the use of a nanohandling microrobot

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There are two principal problems when using the AFM for biological nanomanipulation. The first one is the lack of visual information during the nanomanipulation experience. The second one is the use of the same tip for imaging and manipulating the microbial cell. The use of non-suitable tip for imaging and tip contamination during the manipulation cause loss of resolution in the images acquired before and after each nanomanipulation. The lack of visual information can be partially solved by the use of virtual imaging and force feedback techniques. The use of a nanomanipulation microrobot equipped with a self-sensing AFM tip (piezoresistive cantilever) to work in cooperation with the AFM microscope is also proposed. The whole system will have the benefits of using the right tip for imaging the sample with the AFM and the best tip to perform the nanomanipulation with the robot. The system also allows simultaneous measurements (electrical, mechanical and thermal conduction) in different points of the sample. Different implementations on the AFM robot have been performed. A 6DOF robot with 12mm/6° travel and 2 nm resolution in closed loop using a piezoresistive cantilever from KLA Tecnor supplier and a robot based on linear micropositioning axis from SmarAct with 7 mm travel, a step size of 50 nm and a subnanometer resolution have been developed. First results show the ability of these robots to perform AFM based mechanical nanomanipulation with a resolution of 2 nm.

Langmuir-Blodgett models of the bacterial membrane studied by surface probe microscopy

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1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl-sn-phosphoglycerol phospholipids are the main constituents of the bacterial membrane of gram negative species such Escherichia Coli (1). POPE appears to be involved in processes of biological relevance such as folding and activity of transmembrane proteins (2) and has a definite role in the formation of pores across the membranes most likely because of its ability to form inverted hexagonal phases (HII) (3). It is also involved in the formation of segregated domains when mixed with cardiolipin (CL) (4) and phosphaditylglycerol (5). In this communication Langmuir-Blodgett (LBs) films, mono- and bilayers of these phospholipid species were transferred at different surface pressure onto mica and imaged through Atomic Force Microscopy (AFM). Differences in topography and nanomechanical properties of
these LBs were observed when temperature and ionic strength of the system was changed. The observations of definite regions suggest the existence of domains which differ in height that indicates the presence of different ordered states in these samples. Thus, for LBs with phospholipid mixtures it has been also seen differences in height suggesting the presence of laterally segregated domains that differ in phospholipid composition.


IV-15

FORMATION OF SUPPORTED LIPID MEMBRANES FROM BIOTINYLATED LIPOSOMES ONTO DIFFERENT STREPTAVIDIN-COATED SOLID SUPPORTS

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Supported phospholipid bilayer (SPB) formation from biotinylated liposomes has been examined by dissipative QCM (quartz crystal microbalance based on impedance analysis) and AFM (atomic force microscopy) techniques on various surfaces. With a view to construct a model for a biological membrane by fusion of proteoliposomes, unfavorable interactions of transmembrane proteins with the solid substrate should be avoided. In our approaches the surface was first functionalized by streptavidin. Then the biotinylated liposomes were anchored onto the streptavidin molecules attached to the solid support. The first approach was to adsorb streptavidin directly onto the hydrophobic substrate (polypropylene or polystyrene) after which the fusion of biotinylated vesicles on that surface was done. AFM results showed a 6 to 8 nm thick continuous bilayer formation revealed by the holes in the structure. Although almost all the 100 nm diameter vesicles had ruptured, some of those were intact and had only flattened due to adhesion with the surface. The second method used was to agglutinate streptavidin to SPB formed from biotinylated L-alpha-Phosphatidylcholine vesicles onto the silicon dioxide. [1] Streptavidin allows subsequent tethering of biotinylated vesicles. Several parameters, of which liposome and solution composition have

Kinetics and mechanistic studies of self-assembled monolayer of a series of thiolipids by using AFM

Muhammad Raza Shah, Ingo Koper

Max Planck Institute for Polymer Research Mainz, Germany

Tethered monolayer and bilayer lipid membranes have been developed in the past decade as a model system of the biological membrane. They provide a fluid, stable, and electrically sealing platform for the study of membrane related processes, specifically, the function of incorporated membrane proteins. We have used AFM to study the growth process of eight self-assembled monolayer of thiolipids, i.e. (2,3-di-o-phytanyl-sn-glycerol-1-tetraethylene glycol-D,L-α-lipoic acid ester lipid (DPTL), 2,3-di-o-phytanyl-sn-glycerol-1-hexaethylene glycol-D,L-α-lipoic acid ester lipid (DPHL), 2,3-di-o-phytanyl-sn-glycerol-1-octaethylene glycol-D,L-α-lipoic acid ester lipid (DPTL), 2,3-di-o-phytanyl-sn-glycerol-1-tetradecaethylene glycol-D,L-α-lipoic acid ester lipid (DPTDL), 2,3-di-o-phytanyl-sn-glycerol-1-tetraethylene glycol-thiol (DPTT), 2,3-di-o-phytanyl-sn-glycerol-1-hexaethylene glycol-thiol (DPHT), 2,3-di-o-phytanyl-sn-glycerol-1-hexaethylene glycol-D,L-α-Di-lipoic acid ester lipid (DPHDL), mono-phytanyl-sn-glycerol-1-tetraethylene glycol-D,L-α-lipoic acid ester lipid (MPTL). AFM images (performed in liquid cell or air) of samples immersed in ethanol solution for varying exposure times showed that before forming a complete monolayer the thiolipids molecules aggregated in the form of small islands, in which 2D aggregates of adsorbate molecules nucleate, grow, coalesce, etc. on the substrate. With the proceeding of immersion, these islands gradually grew and merged into larger patches. Finally, a close-packed film with uniform appearance was formed. The kinetics of the self-assembly process also depends on the chain length of the thiolipids. Longer-chained thiolipids, such as DPTDL, formed complete SAMs more rapidly than did shorter-chained thiols, such as DPTL. The rate of self-assembly was also found to be dependent on the anchor group, like the self-assembly of DPTL was
found faster than DPTT, probably due to the more susceptibility of DPTT toward oxidation to form sulphoxide. A very interesting behavior was shown by self-diluted DPHDL. Fastest self-assembly is shown by MPTL dictating the role of phytanyl group.

**Nanoscale Visualization of Extracellular Polymer Release by Marine Diatom**

*Cylindrotheca fusiformis*

**Vesna Svetličić**, Vera Žutić, Tea Mišić, Amela Hozić, Richard Gordon
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2) University of Manitoba, Winnipeg, Canada

AFM has already proven useful as a technique for investigating structural and mechanical properties of the diatom extracellular polymer substance of which polysaccharides are the primary component. Extracellular polymer substance is defined as any macromolecule secreted external to the cell wall that may stay attached or be released to the environment. Direct visualization of extracellular polymer molecules and of their supramolecular organization has not been achieved before. We selected *Cylindrotheca fusiformis* (the planktonic, raphid, motile marine diatom) to investigate extracellular polymers at the time of their release and their capacity for supramolecular organization using experimental procedure adopted for AFM imaging of the marine gel phase. *Cylindrotheca* spp. are considered the main exudates producers in the shallow northern Adriatic Sea and may be associated with the formation of an enigmatic gel phase in this marine system. We show that the carboxylated and sulfonated polysaccharides produced by single *Cylindrotheca fusiformis* cell form a nanoscale structured material. The structure consists of a web or tangle of strings up to 900 nm in length that are 0.7 to 0.9 nm thick, interconnecting beads 2.5 to 12 nm in diameter. The beads may have a protein core that allows efficient intracellular packing of negatively charged polysaccharide fibrils and may serve the diatoms as nucleation centers for silica precipitation when excreted.

**ZipA reconstituted in planar lipid bilayers: its effect on bacterial FtsZ polymerization**

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Bacterial cell division requires the assembly of a proteolipid complex at the midcell division site called the septosome. Two of the initial components of this septal complex in *Escherichia coli* are the membrane protein ZipA and the soluble protein FtsZ. This 40 kD soluble protein with GTPase activity polymerizes on the inner side of the cytoplasmic membrane during bacterial cell division forming a dynamic ring that is essential for the recruitment of other proteins. It is thought to be anchored to the membrane through its interaction with ZipA. Our aim is to study the structure and dynamic behaviour of FtsZ polymers formed on a surface in vitro in a similar environment as that found in the cell. For that purpose we reconstitute ZipA in planar lipid bilayers and use atomic force microscopy to characterize the shape and dynamic
behaviour of E. coli FtsZ protein filaments formed on the surface. We find that FtsZ shows no affinity for lipid bilayers of different composition, but is able to attach and polymerize on a lipid surface in the presence of oriented ZipA reconstituted in bilayers prepared with E. coli lipid extract.

**Detergent-mediated reconstitution of membrane proteins in planar lipid bilayers**

Ellen Verheyen, Frits Flesch, Rene van Nostrum Bart Gadella Wim Hennink

Utrecht University

Supported lipid-protein bilayers are widely used to study membrane proteins in their native environment. The building up of membranes on glass and the subsequent insertion of proteins, however, are poorly understood. We developed a method to prepare a supported lipid-protein bilayer on glass, based on detergent mediated reconstitution of membrane proteins in a preformed bilayer. Lipid bilayers were formed by spontaneous fusion of vesicles onto the glass support (96 wells format). Next, proteins were allowed to insert by incubating the bilayer with a protein-detergent solution (pmoles of protein and detergent far below the CMC). Finally, the detergent was removed by rinsing with buffer. Epifluorescence microscopy (fluorescent dye-lipid probe) showed that the lipid bilayers were homogenous. Total phospholipid content (colorimetric determined) showed the presence of a single bilayer covering the glass surface. Embedded proteins were investigated by AFM. Height measurements of the extramembranous domains protruding from the bilayer demonstrated that proteins were indeed incorporated in the lipid bilayer. An ELISA-like assay was designed to confirm these findings. Detergent mediated reconstitution of membrane proteins in a preformed bilayer is a simple and versatile method, by which membrane proteins can be successfully reconstituted into lipid bilayers, within two hours, using only as little as 1 pmol protein. This array approach can facilitate High-Throughput-Screening methods, e.g. to identify membrane proteins as potential drug targets and assess the interaction between drug target and drug delivery system.

List of participants
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