Imaging Individual Biomolecules on Surfaces

Molecular Structure from High Resolution Scanning Probe Microscopy

Scanning tunneling microscopy produces high resolution images of surfaces and their adsorbates, ultimately reaching atomic resolution. Despite this impressive performance, the scope of this method is greatly limited by sample preparation. STM samples are prepared in ultrahigh vacuum, because they have to be chemically pure at the atomic level. Using electrospray ion beam deposition (ES-IBD), a preparative mass-spectrometry method based on soft-ionization, mass-selected molecular ion beams, and their soft-landing UHV we recently extended the reach of STM towards bio- and macro- molecules. Our work shows that STM and ES-IBD are an ideal match, because the precise chemical information of the preparative mass spectrometry perfectly complements the high resolution structural information.

Imaging Molecules by STM

Due to its ability to image individual molecules and their assemblies at sub-nanometer resolution and simultaneously extract spectroscopic information, scanning probe microscopy earned a central role in molecular nanoscience. In particular scanning tunneling microscopy (STM) imaging has led to a deep understanding of molecular interactions at surfaces where complex behavior is emerging from relatively simple building blocks. Unraveling such complexity would be of greatest interest for the investigation of complex, biological molecules, however, until recently, STM was only applied to small organic molecules, whereas biological molecules were out of reach due to the limitations imposed by the sample preparation method: For STM imaging, molecules are evaporated in ultrahigh vacuum (UHV) onto atomically flat metal crystal surfaces.

Despite their intuitive look, obtaining high quality, meaningful STM micrographs of molecules is far from trivial. An image typically is created by recording the height of the tip as it is scanned over a surface while the tunneling current is held constant by a feedback loop varying this height. This generates maps that can be interpreted as topographs of constant density of states. In them the actual height information is
therefore convoluted with the properties of the electronic structure. It is this highly complex relationship between physical reality and imaged quantity that makes STM images often difficult to interpret despite the high spatial resolution.

Therefore, more than for any other microscopy method, the performance of an STM critically depends on the quality of the sample. Due to the extremely high resolution even the smallest contamination appears in the image and cannot be easily distinguished from the molecule of interest since the chemical information in the DOS is far too convoluted. As a consequence, STM imaging is performed in ultrahigh vacuum where a crystal surface stays atomically clean for the time of the experiment and the molecules are evaporated by thermal evaporation which purifies the molecular adsorbate through specific evaporation/sublimation temperatures.

**Electrospray Ion Beam Deposition (ES-IBD)**

To make biomolecules and other non-volatile compounds accessible for STM, or more generally for any UHV-based analytical method, we developed electrospray ion beam deposition (ES-IBD), a preparative mass spectrometry method, which is based on the soft ionization (electrospray ionization, ESI) and controlled, soft- or reactive landing [1]. The purity of the ion beam is ensured by mass-filtering the molecular ion beam before deposition (fig. 1). Because ESI takes place under ambient conditions, six differential pumping stages are needed to transmit the ion beam from air to UHV. The use of ion beams allows a high level of control over the deposition process: integrated time-of-flight mass spectrometry combined with a continuous beam quadrupole mass filter ensures the chemical purity of the beam, current monitoring allows to quantitatively control the coverage and the kinetic energy of the beam can be measured and adjusted to control the interaction with the surface.
Samples are prepared and transferred in situ. Once a sample returns to the STM after deposition, images can be taken at room- or cryogenic temperature (10 - 40K), the latter to suppress surface mobility. In addition, we developed a system of versatile UHV vacuum suitcases that allow us to transfer samples to any other UHV instrument, even over extended distances [2].

The universality of ESI, which can ionize a huge variety of molecules as long as they are soluble and at least slightly polar, has made ESI mass spectrometry the prime tool for organic/biological molecular identification. Similarly here, ESI allows for a huge variety of molecules that can now be investigated on surfaces: proteins, peptides, saccharides, lipids and surfactants, dye molecules, coordination complexes, polymers, and a large variety of large synthetic molecules. In this framework, the self-assembly of proteins and peptides is of particular interest. These biological sequence controlled polymers, fabricated via a universal synthesis, are the prime example for the powerful principle that is self-assembly in biology. Proteins reach highly complex structures of specific functionality via folding along a pathway, which is programmed into their sequence alongside their functionality. Understanding this intricate scheme at the atomic level and eventually being able to use this principle in technological applications could be extremely beneficial.

**Results and Discussion**

Using ES-IBD, the investigation of proteins with STM is now possible at approximately single amino acid resolution, as illustrated in figure 2. The inset shows the entire strand of an unfolded protein (BSA, approx. 66500 Da) of 80 nm. The resolution of the structural details in the high magnification image corresponds to the size of one amino acid. Currently it is not possible to identify the amino acids chemically, however, we are able to observe and manipulate their behavior as polymers. Unfolded proteins are visible as meandering strands, like those shown in figure 2, when they are immobilized on the surface upon landing. Depending on their charge state they adopt a straight or a more compact conformation, for high and low charge states respectively. If the unfolded proteins are given the freedom to diffuse, for instance by deposition on a weakly interacting surface such as Au(111), they fold back in two dimensions into a compact low energy conformation, a patch of random shape. We cannot expect that a protein sequence, which evolved to be folded in aqueous environment, will be folded in a specific conformation on a surface in vacuum, because the interaction strengths have changed. However, we find that, in principle, folding in vacuum is possible, but it occurs in two dimensions due to the strong adsorption and it forms different motifs.
Treating the protein in solution gently, in particular by avoiding the use of organic solvent, allows to ionize the proteins in their native conformation. Depositing folded proteins leads to the observation of globular structures on the surface by STM, however, without resolving any specific submolecular features. Recently, using this capability, ES-IBD was used as sample preparation method in conjunction with the newly developed method of low energy electron holography, a destruction-free microscopy method that implements Denis Gabor’s original idea of a holographic electron microscope [3]. Therein, the folded proteins are deposited on ultrapure freestanding graphene where they can be imaged individually. In a similar fashion, ES-IBD could be used for other analysis methods that require the chemically pure deposition of native proteins.

Other than proteins, peptides are much shorter amino acid sequences. This limits their complexity significantly, while they still should possess many of the essential properties of long polypeptide strands. Using the natural peptides Angiotensin I and II (At-I, At-II) and Bradykinin (BK) we were able to demonstrate this [4, 5]. Angiotensin I formed ordered structures in rows and dimers, which were altered significantly when changing the sequence slightly using Angiotensin II, which only differs by two amino acids. Large, highly ordered, chiral hexagonal networks of Angiotensin were observed on Au(111) surfaces. Within these structures the At-II molecules were arranged in dimers, three of which connected in vertices to form the network. While the Angiotensin molecules are relatively stiff, BK, a nine amino acid peptide has considerable degrees of conformational freedom. On a strongly interacting surface we consequently find many conformations of the immobilized molecules on the surface. Given freedom to diffuse, the proteins in the STM images show only one folded dimer conformation, evidence that two-dimensional folding has occurred.

The high degree of ordering in the peptide assemblies of BK and At-II arise because two distinct binding motifs are active. We identify these motifs with the help of theoretical investigations based on molecular dynamics simulations and DFT based STM simulations. Together with the modelling, we can assign the amino acids to the images and determine the binding motives. We further noticed a separation of unipolar and polar groups in the molecule, similar yet inverted to the biological counterpart.

**Conclusion**
The examples given here summarize finished projects with proteins and peptides on the ES-IBD setup. These projects are currently extended towards metal–peptide coordination and the use of tunneling spectroscopy to identify amino acids. Also other biological and synthetic molecules are currently intensely investigated. ES-
IBD can handle DNA and sugar very well and also working with synthetic molecules is very well possible in most cases. Not mentioned here are the experiments of chemical surface coating using the collision energy, a parameter which is freely tunable in ES-IBD. This universality in control and molecules makes ES-IBD a potentially very important deposition method and consequently currently several groups are setting up similar experiments.

References
[2] In collaboration with Ferrovac. See also www.ferrovac.ch

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