The background of the entire cover is a high-contrast, black and white scanning probe microscopy image showing a complex, porous, and textured surface. The texture consists of irregular, interconnected shapes and voids, creating a rough, crystalline appearance. In the top right corner, the Wiley logo is partially visible, showing the letters 'WILEY' in a serif font.

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Procedures in Scanning Probe Microscopies

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Module 7.1.4

A general method for immobilizing biomolecules on gold for SPM in aqueous buffers

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One of the conditions to be fulfilled for SPM imaging of macromolecules in aqueous solutions is that they should be firmly anchored on appropriate substrates and thus not be displaced by the tip while scanning. Physical adsorption provides in general insufficient immobilization at the liquid-substrate interface and may lead to denaturation of samples. Therefore, covalent linking (or bonding of comparable strength) to a surface is in principle preferable.

Suitable substrates should be flat, *i.e.*, featureless over large areas and should allow covalent fixation of the objects. Mere electrostatic interactions, for example, would severely limit the conditions under which biological objects can be eventually investigated (*e.g.*, only at low ionic strength or within a limited pH range). A variety of crystalline or amorphous substrates have been used to date, having different chemi- or physisorption properties. The substrates mostly used are mica, glass, silicon wafers, highly oriented pyrolytic graphite (HOPG), and gold. Thin gold films are a promising substrate: they are inert against O₂, yet they can form very stable covalent Au thiolate bonds and they are easy to prepare (see also module 7.1.3 and [3]).

Biomolecules may bind to gold surfaces without additional treatment (*e.g.*, via pre-existing thiol groups, or physisorption), or with introducing (extra) thiol groups (*e.g.*, DNA [4]). A more reproducible and reliable way of immobilizing molecules on gold surfaces is the formation of derivatized self-assembled monolayers (SAM) onto these ultraflat Au(111) surfaces with subsequent covalent anchoring of biological macromolecules. Hydrophobic thiols (*e.g.*, dodecanethiol) easily form close-packed monolayers covalently bound on Au(111) surfaces with a commensurate ($\sqrt{3} \times \sqrt{3}$) $R30^\circ$ overlayer structure (see also module 5.3.1 and 5.3.2), which are further stabilized by their lateral hydrophobic interactions. If the ω -substituent is a highly reactive group, it can act as the anchoring site, docking proteins onto the monolayer carpet. As a general method for protein immobilization on gold surfaces we present here one of the ω -functionalized SAMs, which have been developed in our lab [5, 6]. It is based on dithio*bis*(succinimidyl undecanoate) (DSU) (Fig. 1), which is homologous to the commercially available dithio*bis*(succinimidyl propionate) (DSP), but exhibits better properties for monolayer formation due to its elongated methylene chain. STM and SFM images of the NHS-terminated SAMs exhibit the characteristic depressions (shown in Fig. 3). The N-hydroxysuccinimidyl (NHS) ester groups, which are exposed at the liquid-solid interface, react with accessible α -amine groups of proteins and with the ϵ -amines of lysines under very mild conditions (pH 7-9) to form stable amide (peptide) bonds. Figure 4 shows three examples of biomolecules immobilized by the method described here and imaged under physiological conditions with a commercially available SFM.

Procedure

Immobilization of proteins on gold using dithiobis(succinimidylundecanoate) (DSU)

The following method is a generalized protocol for coupling a protein onto NHS-activated gold surfaces for SPM use and is based on the following steps (Fig. 2 shows a general set-up):

1. Preparation of gold films by depositing gold on freshly cleaved preheated mica (see also module 7.1.3) or onto a chromium coated cantilever.
2. Formation of the NHS-terminated SAM using DSU.
3. Immobilization of the protein onto the bioreactive SAM.
4. SFM measurement (preferably in aqueous solution).

MATERIALS

- Gold substrates ($\leq 1 \text{ cm}^2$) with adequate flatness, *e.g.* TSG described under module 7.1.3.
- Dithiobis(succinimidylundecanoate) (DSU): 1 mM in 1,4-dioxane or acetone. This compound is not yet commercially available, *i.e.* synthesis (or collaboration) is obligatory. In brief, the synthesis of DSU is achieved by coupling N-hydroxysuccinimide to the corresponding dicarboxylic acid using dicyclohexylcarbodiimide (DCC/HOSu-method). For details see [6].

Note: Attempts to replace DSU with dithiobis(succinimidyl propionate) (DSP), which is commercially available (Pierce, Sigma), failed in most cases for SPM purposes.

- 1,4-dioxane and acetone
- Protein approx. 1 μg per ml in coupling buffer.
- Coupling buffer: *e.g.* phosphate, bicarbonate/carbonate, borate or HEPES buffer at concentrations between 50 and 200 mM and pH 7-8. Use highly purified water (*e.g.* NANOpure[®] (Barnstead, Dubuque, IA) or (SKAN, Basle, Switzerland)) or filter the buffer solution through a 0.1 μm -membrane (*e.g.* Gelman Super Acrodisc[®], Gelman Sciences, Ann Arbor, MI). Do not use Tris or glycine buffers since they contain primary amines.
- Piece of parafilm[®] and a Petri dish for the immobilization step.

EQUIPMENT REQUIREMENTS

- High vacuum coating system for thermal evaporation of gold with integrated quartz crystal deposition controller and substrate heater. (Vacuum during deposition $< 2,66 \times 10^{-4} \text{ Pa}$). For preparation of ultraflat template-stripped gold, see module 7.1.3.
- Ambient SFM with fluid cell for measurement in liquids.
- Equipment for synthesis of DSU.

SAMPLE PREPARATION

Preparation of gold surfaces:

- Deposit by thermal evaporation a 200 nm thick gold layer on a freshly cleaved preheated (300 °C, overnight) mica sheet. According to the procedure described in module 7.1.3, ultraflat gold can be easily obtained. These TSG surfaces are highly recommended for imaging of objects having unfavourable length:width ratios.
- Cut the gold deposited mica sheet in pieces of approx. 1 cm².
- For TSG use the THF stripping process described under module 7.1.3.
- Use the gold samples immediately to avoid particle contamination.

Note: For cantilever modification coat the lever with 2 nm chromium by electron-beam evaporation and then with 30 nm gold.

Formation of the NHS-SAM:

- Incubate the gold platelet for 30 min - 2 h at room temperature in 2-3 ml of 1 mM DSU in dry 1,4-dioxane or acetone (632 µg per ml). Do not use methanol to avoid transesterification ! The platelet can be stored in the solution of DSU in 1,4-dioxane for days if necessary.
- Rinse the NHS-terminated monolayer thoroughly with 3 - 5 ml of first 1,4-dioxane and then acetone (not water !).
- Dry the sample under a stream of nitrogen and use it immediately for the immobilization step.

Note: Use the same protocol for activation of gold-coated SFM tips.

Protein immobilization and SFM imaging:

- Prepare a protein solution of 0.01 to 10 µg per ml in an amine-free coupling buffer system. Despite its high coupling efficiency, hydrolysis of the NHS ester is a major competing reaction, resulting in carboxylate groups, which can lead to unspecific binding and unpredictable results during scanning. To decrease the rate of hydrolysis buffers with pH values lower than 8.0 are recommended.
- Place 50 µl of the protein solution on a piece of parafilm[®] and put the NHS-activated gold platelet upside down onto the drop. Use a Petri dish equipped with a wet paper as a hood to prevent evaporation.
- Rinse the platelet after 1 hour at room temperature (now carrying the covalently immobilized biomolecules) with 2 ml buffer solution (if desired, quench the excess active groups by incubating the platelet in 100 mM Tris-buffer pH 8.0 for 1 h).
- Place the sample into the fluid cell of the microscope. Do not allow to dry !
- Image acquisition should be done in any scanning probe microscope in aqueous solution and with lowest forces possible.

CONCLUSION

The presented procedure paves the way for SPM investigations of amino-group containing biomolecules (*e.g.* phospholipids, amino-sugars, peptides, proteins) in aqueous buffers. Covalent anchoring with this NHS-terminated SAM is simple, rapid and reproducible. It has been used for SFM imaging and also for measurement of interaction forces after binding ligands or effectors on NHS-activated gold-coated SFM tips and/or onto the gold substrates. This method is also recommended for biological applications with tapping-mode or non-contact-mode in fluids.

References

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Figures

Figure 1 Structural formula of dithiobis(succinimidylundecanoate) (DSU)

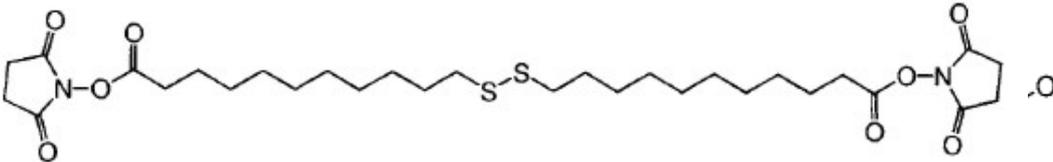


Figure 2 A schematic view of the experimental set-up for SPM imaging in aqueous buffers (not to scale). (Some of) the amino groups of the biomolecule have reacted with the NHS-activated carboxylic acid groups at the water-solid interface. The monolayer is covalently bound via S-Au bonds to the gold surface. The Au(111) film is supported by either a mica platelet, or the Si-epo-tek® 377 platelet of TSG (see module 7.1.3) or a chromium interlayer on glass coverslips or SFM-cantilevers, respectively.

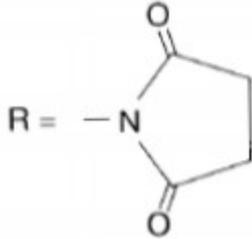
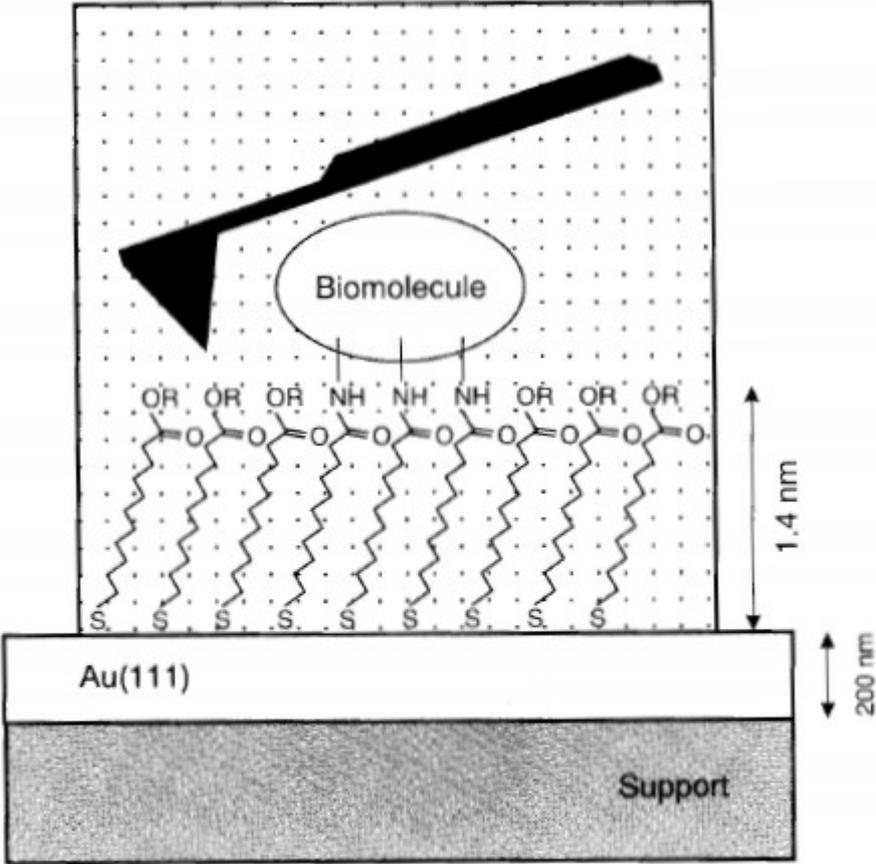


Figure 3a STM topview of the NHS-terminated SAM (2 pA, 1.2 V); z-range 2 nm. The black arrow indicates the characteristic depressions due to an etching process of the underlying gold surface. The white arrow shows a hole in the gold film.

Figure 3b SFM topview of the NHS-terminated SAM; z-range 5 nm. The black arrow indicates the characteristic depressions due to an etching process of the underlying gold surface.

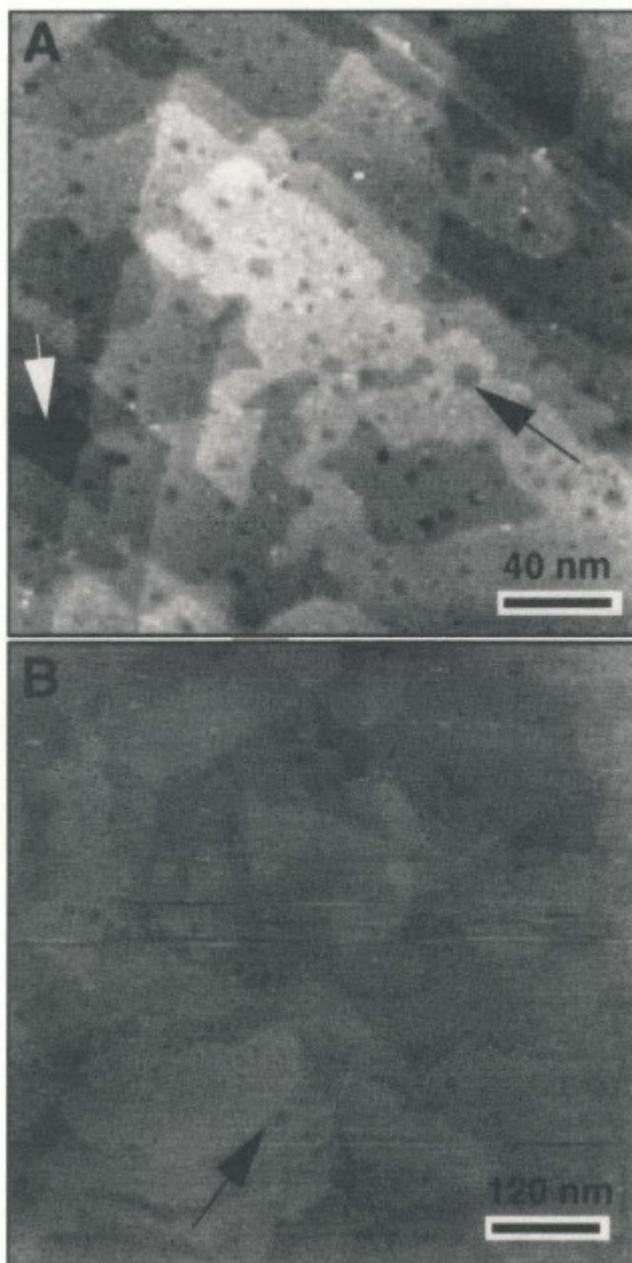


Figure 4a-c SFM images of proteins immobilized on the NHS-terminated SAM (using DSU on ultraflat template-stripped gold). The measurement was performed in phosphate buffers in the contact-mode. 4a1-a4: SFM topview of the "basket-like" structures of in-situ disassembled clathrin cages. For details see [5]. 4b: SFM topview of a three-legged clathrin protomer (triskelia). For details see [5]. 4c: SFM topview of a single collagen type V molecule.

