Метод на Атомно-силова микроскопия (AFM): Принцип на действие, модификации на метода за изследване на разнообразни физико-химични процеси в течни среди

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- **History and Principle of AFM**
- **AFM as a tool for studying the enzyme kinetics**
- **Experimental techniques- Sample preparation and scanning**
- **Results**
История на методиката

Heinrich Rohrer

Gerd Binning

- A sharp probe is scanned over a surface and its motion is monitored.
- An image is constructed from multiple profiles.
FIGURE 1-1 In the AFM, a sharp probe is scanned across a surface, left, and by monitoring the motion of the probe from each pass across the surface, a 2-D line profile is generated. Then the line profiles are combined to create a three dimensional image of the surface, right.
Principle of Atomic Force Microscope (AFM)
4.1 Topography Modes

FIGURE 4-4 Potential energy diagram of a probe and sample. The attractive potential is caused by the capillary forces from surface contamination.

4.1.1 Contact Modes

FIGURE 4-5 Left: Potential diagram showing the region of the probe while scanning in contact mode. Right: In contact mode the probe glides over the surface.
The forces applied to the surface by the probe in contact mode are given by Hooke's law:

\[ F = -kD \]

- **\( F \)** = force
- **\( k \)** = force constant
- **\( D \)** = Deflection distance

**FIGURE 4-6** Contact mode images: Left: Bits on a compact disk. Center: Image of a metal surface. Right: Nano-particles on a surface.
4.1.2 Vibrating Modes

**FIGURE 4-7** Left: Potential diagram showing the motion of the probe in vibrating mode. Right: The probe vibrates as it scans across a surface.
As illustrated in Figure 4-8, the cantilever can be excited with a piezoelectric ceramic. The cantilevers have natural resonant frequency $\omega_0$ given by:

$$\omega_0 = c\sqrt{k}$$

$c =$ proportionality constant
$k =$ force constant
FIGURE 2-5 Block diagram showing the components in an AFM. The image is created by monitoring the voltage that drives the Z piezoelectric ceramic.
2.2.2 Force Sensors

Methods for measuring the deflection of a cantilever in an AFM

**Scanning tunneling microscope:**
In the original AFM built in 1985 a scanning tunneling microscope tip was used to measure the motion of a cantilever. Although this technique was viable, implementation and operation were very difficult.

**Interferometer:**
A Michelson interferometer can be adapted to measure the deflection of a cantilever in an AFM. Although very sensitive, the interferometer was not successful because of fringe hopping. That is, the probe could jump between interference fringes while scanning.
Methods for measuring the deflection of a cantilever in an AFM

Crystal Oscillator: A piezoelectric crystal such as quartz can be used to measure the force between a probe and a surface. If the probe mounted on the crystal is vibrated and positioned close to a surface, the interaction of the probe and surface will cause a change in the vibration. This change is proportional to force.

Piezo-resistive Cantilevers: A cantilever can be fabricated that has a small piezo-resistive element in it that changes resistance if the cantilever bends. This type of sensor is viable, but very difficult to manufacture in appropriate quantities.
Crystal Force Sensors

**FIGURE 2-18** Left: Crystal force sensor and electronics. Right: SEM image of a crystal force sensor. The cantilever is visible at the left end of the crystal tine.
FIGURE 2-17 Left: Illustration of the light lever AFM force sensor. In the LL-AFM sensor the end of the cantilever bends and thus the angle between the probe and sample changes as the cantilever bends. Right: It is possible to fabricate cantilevers with force constants as small as 0.1 nN/nm.
2.5 LL-AFM Cantilevers and Probes

FIGURE 2-35 Illustration of an AFM cantilever/probe/substrate created by micromachining of Si or SiN. All commercially available probes have substrates with the same dimension.

SEM micrographs of a square-pyramidal PECVD Si$_3$N$_4$ tip (a), a square pyramidal etched singlecrystal silicon tip (b), and a three-sided pyramidal natural diamond tip (c).
Basic Geometry of Cantilever and Probes

**FIGURE 2-37** SiN cantilevers are typically triangular with two arms meeting at an apex. The probe on SiN probes are typically pyramidal and appear hollow at the top. (Top) Si cantilevers are typically rectangular and the probes tend to have a triangular shape to them. Si probes are crystalline and are prone to chipping and breaking if they crash into a surface. (Bottom)
**Specialty Coatings/Configurations**

**FIGURE 2-38** This silicon probe has a 300 nm latex sphere glued to its apex.

**Sharpened Probes**

FIGURE 2-41 If the probe geometry is described as an upside down triangle, it is simple to remove the effect of the probe from an image of a nanoparticle. The actual diameter of the nanoparticle is calculated using the equation at the right. Rarely is a probe in the shape of an upside down triangle.

\[ r_c = r \left\{ \cos \theta_c + \sqrt{\cos^2 \theta_c + (1 + \sin \theta_c) \left[ -1 + \left( \frac{\tan \theta_c}{\cos \theta_c} + \tan^2 \theta_c \right) \right]} \right\} \]
A force/distance (F/D) curve is a measure of the forces on the probe as a function of the distance of the probe from a sample’s surface.

**FIGURE 4-36** The force distance curve is measured by moving the probe at the end of a cantilever toward a surface (red and black traces at the top). The letters A-F indicate the position of the probe as it moves toward and away from the surface.
Stiffness (Young's modulus)

Adhesion

Single force

Force volume
Fig. 1. Experimental setup of Atomic force microscope liquid cell. A. Principle scheme of liquid cell. B. Photography of the setup
Enzymes:

• Are proteins
• Have high specificity
• Catalysts
• Substrate organization
Lipases
Sample preparation

**Langmuir-Blogette technique**
- Lipid monolayer
  - Pressure barrier
- Hydrophobic sidegroups
- Hydrophilic sidegroups

**Sample-Lipid Double Layer**
- Sample transfer into the AFM liquid cell

**Obtaining real time AFM images**

![AFM images](image-url)
AFM images of a time sequence of the PLA$_2$ hydrolysis of supported DPPC bilayer on mica
(A) Image prior to PLA$_2$ injection, and (B) 8 min., (C) 14 min., (D) 17 min., respectively, after
PLA$_2$ injection.

(E) Analysis of the complete image series of Figure 2A (including frames not shown in
the figure). The graph shows the hydrolysed area as functions of time. The lag-burst
kinetics is clearly illustrated as the sudden change between 8 and 14 minutes.

Nielsen, L.K., Risbo, J., Callisen T.H, T. Bjørnholm, Lag-burst
kinetics in phospholipase A$_2$ hydrolysis of DPPC bilayers
visualized by atomic force microscopy, *Biochim. Biophys. Acta*
1420, (1999), 266-271
Schematic illustration of the transfer of the mixed monolayers to a solid support. First a monolayer of DPPC and products were transferred to the solid support, and then a double layer was formed on the down stroke of the support. (B) In the AFM, the bilayer was hydrolyzed by phospholipase A2 (shown as its crystal structure), which created additional product molecules. The structure of the bilayer was observed by the AFM. Especially the height difference between the top of the bilayer and the substrate and between the top and product regions can be seen in the images.
AFM images of a time sequence of HLL hydrolysis of supported DPPC/DPG bilayer on mica obtained from collapsed vesicles

(A) Image prior to HLL injection, and (B) 4 min., (C) 30 min., (D) 130 min., respectively, after HLL injection. (E) Analysis of the complete image series of Figure 4A (including frames not shown in the figure). The graph shows the hydrolysed area as functions of time.

Slope = $2 \times 10^7$ Lipid molecules/min
Balashev, K. Bjørnholm, T. et al.
Synergetic action of PLA$_2$ and HLL on bilayer lipid substrates studied by Atomic Force microscopy (to be submitted)

**Fig 2.** AFM images of a time sequence of HLL hydrolysis of supported DPPC/MOG bilayer on mica. (A) image (15*15μm²) of the doublelayer incubated in water for 2 hours, but prior to HLL injection. Inset shows the hole 35Å deep. And (B) 4 min., (C) 14 min., (D) 28 min., (E) 52 min., (F) 108 min., (G) 158 min. (H) 240 min., respectively, after HLL injection.
Possible molecular mechanism of enzyme action

(A) Enzyme binding

(B) Enzyme activation

(C) Enzyme hydrolysis

(D) Passive action

DPPC exposes hydrophilic head group to solvent and forms 3D aggregates, micelles etc.
Fig. 4. Witnessing the stochastic nature of single enzyme action and crossing the regime to multiple enzyme action. AFM images of the scans taken before (t=0) and immediately after HLL injection (t=3 min) Enzyme concentrations are -2.5 nM, C, D-15 nM, E, F-45 nM, respectively. Top 4 are 15*15μm²; bottom 2 are 11, 5*11,5μm². Graphs G-H show the growth of individual holes obtained from analysis of image subsequences. The slope equals $k_{init}$, while the intersection equals the initial circumference of the hole.
Synergetic action of phospholipase A2 and *Humicola lanuginosa* lipase
AFM images obtained after the vipoxin injection showing the appearance of small depression areas in the bilayer (A) 30 min after the vipoxin injection. The inserts show a software. Zoom of the boxed area with one of several small depression area observed with a typical depth of 0.3–0.5 nm (the z scale has been extended to clearly show the depression).
The change of the hydrolyzed area versus time for the two morphological types of bilayer areas. The two regimes of hydrolysis (lag phase and burst) are easily distinguishable. In the time interval 0–2000 s (lag phase) the initial slope of curve 1 is bigger than the slope of curve 2.
Images of 3D crystals formed within the lipid bilayer defect during the enzyme hydrolysis obtained approximately (A–C) 4000 s and (D–F) 6000 s after the enzyme injection into the AFM liquid cell.
Two typical curves showing the crystals volume increase versus time. Data points are extracted from two time-course sets of images corresponded for the two morphological types of bilayer areas. Dotted curve with squares corresponds to a bilayer with initially small depression like defects while the solid line with circle points corresponds to a bilayer with initially big convex defects.
Proteases

How HIV Protease Works

- Cutting Sites
- Viral Polyprotein (Inactive)
- Protease
- Polyprotein is cut by protease into individual proteins
- Active proteins

How HIV Protease Inhibitors Inhibit Viral Replication

- HIV protease recognizes a complementary shape on polyprotein and cuts protein. Fits like “Lock and Key”
- Inhibitor mimics cutting site and binds to protease but cannot be cut thereby inactivating protease
Combined studies of substrate-enzyme interactions by SPR and AFM

Results presented at Biacore Symposium 2002, Chicago (Illinois, USA), May 5-8, 2002

- **Surface plasmon resonance (SPR)** - changes in *binding* detected optically via a coupling to the refractive index of material passing over the active surface

- **Atomic force microscopy (AFM)** - *topology* is mapped by a nanometer-size “pick-up” which is scanned over the surface
Adsorption of a 1 mg/mL protein substrate and change in adsorbed mass upon injection of 80 nM protease
In search for the substrate for protease action

Imaging blood spin coated on mica

Possible substrate for protease obtained from spin coated blood?

Spin coated blood film
2 um x 2 um, 5 nm deep hole scraped by the AFM tip
Imiging protease in action

Before enzyme injection  Immediatly after enzyme injection  1h after enzyme injection
Imaging Albumine on mica in buffer pH7 undergoing Savinase action
Images 5 μm×5 μm showing topography changes of the BSA monolayer in the time course of the enzyme hydrolysis: (A) before Savinase injection into the liquid cell. A defect with a rectangular shape with size 1 μm×1 μm formed by scratching the surface using the AFM tip; (B) 10 min and (C) 25 min after the enzyme injection. (D) Bigger scan 8 μm× 8 μm made 50 min after enzyme injection as a check for mechanical changes inserted by the tip on the sample. (E) Cross sections of the three images (A–C) (black lines through the defect) presented at the same graph.