Scanning probe microscopies (AFM, Conductive-AFM, STM) applied to the integration of biomolecules in hybrid systems for biosensing

Salvatore Cannistraro
Research activities

Scanning Probe Nanoscopies & Spectroscopies

Surface-Enhanced Raman Spectroscopy

Modelling & Molecular Dynamics

Surface Plasmons Resonance

Biosensing & Bioelectronics

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Outline

Biorecognition can be exploited to overcome the current resolution limits in medical diagnostics and environmental monitoring.

Metalloproteins are optimal candidates as transducers and biorecognition elements in nanobiosensor devices.

Scanning probe techniques allow single-molecule characterization.

We study the influence of the immobilization strategy on protein morphology, conduction properties, and biorecognition efficiency.
Biosensing

Current resolution limit (ELISA): $10^{-10} \text{ M, } 10^{13} \text{ molecules}$

Ideal goal for early diagnosis: detection of a single molecule

Main limit: NOISE

Concrete goal: resolution limit to $10^{-18} \text{ M, } 10^5 \text{ molecules}$

Such result can be achieved by improving biosensors with:

Molecular biorecognition exploitation: one signal per event

Nanostructures conjugation: noise lowering and signal enhancement
Biorecognition-based Biosensors

- Reduced dimensions
- Small recognition volume
- High speed of response
- Low cost
- High resolution
- Low noise
- Signal enhancement

Immobilization strategy
- Functionality preservation
- High charge transport efficiency
- Proper orientation
- Flexibility
Redox Metalloproteins

- One way, directional, fast, and single electron exchange
- Energy transport in biology (photosynthesis, respiration...)

$\rightarrow$ conjugation of a metal electrode with biological material
Immobilization strategies on Au(111): chemisorption via specific functional group

- Yeast Cytochrome c
- Poplar Plastocyanin

Optimal electron transfer distance: 1.6 nm

- Oriented immobilization
- Good electrical contact
  - But...
  - No flexibility
  - Denaturation may occur

Single free Cys insertion (PCSH)
Disulphide bridge insertion (PCSS)

L. Andolfi et al., Arch. Biochem. Biophys. 399, (2002) 81
Immobilization strategies on Au(111): optimized organic linkers and nanostructures

- Flexibility
- Preserved functionality
- Noise lowering
- Signal enhancement


Redox Metalloproteins

Copper Proteins

Azurin

Heme Proteins

Cytochrome

3-4 nm
Atomic Force Microscopy (AFM)

- 0.1 nm vertical resolution, pN sensitivity
  - Single molecule resolution
  - Without any labelling
  - In near-physiological conditions
  - Even at work

But lateral tip convolution
Some applications of AFM

- Imaging & topography
- Atomic (dynamic) force spectroscopy (AFS): biorecognition
- Nanomechanics of cells & polymers
- Unfolding of proteins & nucleic acids.
Tapping Mode AFM in fluid of metalloproteins on Au(111)

**Azurin**


**PCSS**

A. R. Bizzarri et al., CHEMPHYSCHEM 4, (2003) 1189

**Yeast cytochrome c**

B. Bonanni et al., CHEMPHYSCHEM 4, (2003) 1183

Measured mean height lower than the crystallographic size

**Azurin**

- Measured mean height lower than the crystallographic size
- \( h_0 = 2.3 \text{ nm} \)
- \( \sigma = 0.5 \text{ nm} \)

**PCSS**

- Measured mean height lower than the crystallographic size
- \( h_0 = 2.3 \text{ nm} \)
- \( \sigma = 0.5 \text{ nm} \)

**Yeast cytochrome c**

- Measured mean height lower than the crystallographic size
- \( h_0 = 2.6 \text{ nm} \)
- \( \sigma = 0.7 \text{ nm} \)
Tapping Mode AFM in fluid of metalloproteins on modified Au(111)

Measured mean height similar to the crystallographic size

Conductive Properties?

Conductive AFM

I-V curves and current imaging through a physical electric contact
Relation between topography and current images emerging from image subtraction

I-V curves as a function of the applied load

Current in the nA range, increasing with the force load.

Role of the linking spacers?
Small organic molecules: no reproducible results.
Metallic nanomaterials: conduction enhancement.

I. Delfino et al.,

L. Andolfi et al.,
Ycc/Au - C-AFM

In Azoto
V = -1.5 V

Topography
Friction
Current
**SWNTs: General Remarks**

- \((m,m)\) \(m-n = 3N\) metallic
- \((m,n)\) otherwise semiconducting

- Diameter: 1-100 nm.
- Length: 10 nm - up to several \(\mu\)m.
- Young’s modulus 1 TPa (Aluminium ~ 700 GPa)
- Tensile strength > 60 GPa (Steel ~ 2 GPa)
- Conductivity ~ \(10^{10}\) A/cm\(^2\) (Copper \(10^6\) A/cm\(^2\))
- Flexible
- Field-emitter

- Young's Modulus 1 TPa
- Tensile Strength > 60 GPa
- Conductivity ~ \(10^{10}\) A/cm\(^2\)
SWNTs deposited on Au(111) surface

Cytochrome-coated metallic SWNTs on Au(111)

Proteins covalently linked to functionalized SWNTs can be imaged in current images.

C. Baldacchini et al.,
Cytochrome-coated metallic SWNTs on Au(111)

Metallic SWNTs, used as linking spacer, enhance the coupling between the protein and the gold electrode.

Integration of Au nanoparticles with Azurin on Au(111)

Integration of Au nanoparticles with Azurin on Au(111)

Current-voltage response

Azurin/Au(111): to register a current signal, a force load of **14 nN** is needed

AuNP/Azurin/Au(111): an intense current response is recorded at **3 nN** of force load

Gold NPs enhance the coupling between the protein and the metallic tip, and more reproducible electrical contacts are achieved.

L. Andolfi et al., submitted
Scanning Tunnelling Microscopy

- 0.01 nm lateral resolution
- Observation of system density of states
  But vertical size influenced by conductivity

I-V curves and current imaging through a tunneling gap
By placing a piece of metal close to another as shown in the scheme, a finite square barrier can be created. The probability for the electrons at the Fermi energy to tunnel through the barrier is proportional to $e^{-d}$ where $d$ is the distance separating the two pieces of metal.
Electron tunneling

Quantum mechanics predicts an exponential decaying solution for the electron wave function in the barrier. For a rectangular barrier we get:

\[ \Psi(d) = \Psi(0) e^{-kd} \]

where \( k \) is:

\[ k = \sqrt{\frac{2m(\Phi - E)}{\hbar}} \]

\( E \) is the electron energy
\( m \) is the electron mass
\( \hbar \) = Plank's constant
\( \Phi \) = the height of energy barrier

When a small bias voltage is applied between the sample and the tip, the overlapping of the electron wavefunction permits quantum mechanical tunneling and a current (I) will flow across the vacuum gap (d).

\[ I \propto e^{-2kd} \]
According to quantum mechanics, electrons on a surface behave both as particles and waves. One result of this is that electrons behave like an "electron cloud" at the surface of a material which is schematically represented below:

What does a STM Measures?

\[ I \propto e^{-2kz} \]
Scanning Tunnelling Microscopy

STM Basics

Tunneling current
\[ I \sim e^{kx} \]
VERY sensitive to changes in \( z \)!

Atomic scale

Bias polarity determines current direction
STM of metalloproteins on Au(111)

0.01 nm lateral resolution

but...

measured molecular height ≤ 1nm


Azurin


PCSS

L. Andolfi et al., CHEMPHYSCHEM 4, (2003) 1183

YCC
Scanning Tunneling Spectroscopy

Single protein tunneling conduction

Molecular rectification

Azurin
Au(111)

STM of metalloproteins immobilised via linkers

Azurin/Au(111)

Azurin on gold modified by sulfhydryl terminated alkanethiol monolayer

Molecular height

0.5 ± 0.1 nm

1.8 ± 0.4 nm


Recovering of the real topographic height: increased conductivity!
STM of metalloproteins immobilised via SWNTs

YCC height on Au(111): 0.3-0.5 nm

YCC height on MAL-SWNTs: \(1.7 \pm 1.0\) nm

Recovering of the real topographic height: increased conductivity!

C. Baldacchini et al.
Single-molecule biosensors

- Reduced dimensions
- Small recognition volume
- High speed of response
- Low cost
- High resolution
- Low noise
- Signal enhancement

- Metalloprotein

- Molecular recognition element

- Marker

- Detectors

- Transducer

- Biologic recognition event

- Electronic signal

- Biorecognition

- Single molecule recognition
- Specificity
- High efficiency
Biorecognition refers to highly specific interactions between two biological molecules, exhibiting unambiguous one-to-one complementarity.

Biorecognition is involved in many important biological processes, including genome replication and transcription, enzymatic activity, immune response, cellular signalling, ...
Force Spectroscopy
Force Spectroscopy

1. Measurements of the cantilever deflection $S$ at the single rupture event
2. Calculation of the single rupture force $F$ by applying the Hooke’s Law
3. Iteration of the force-distance cycle
4. Construction of the unbinding force distribution
5. Estimation of the most probable rupture force: $F_{\text{unb}}$

$S \cdot k = F$ \hspace{1cm} \textit{Hooke’s Law}

\textbf{Histograms:} Heterogeneity, Multiple events, Conformational changes
**Force curves: selection**

No event

Specific unbinding event

Multiple unbinding events: the last one, starting and ending at zero deflection, can be accepted

Non-specific adhesion

Specific unbinding event

Multiple unbinding events: zero deflection is ambiguously defined, thus the last event can be discarded
Bell-Evans Model

The unbinding force depends on the loading rate, which is the rate at which the external force is applied to the complex while pulling the proteins: \( r = \dot{f} \)

**Bell-Evans model**: linear dependence of the unbinding force on the logarithm of the loading rate

\[
F_{\text{unb}} = \frac{k_B T}{x_\beta} \ln \left( \frac{r x_\beta}{k_{\text{off}} k_B T} \right)
\]

- \( x_\beta \) = length scale of the energy barrier
- \( k_{\text{off}} \) = dissociation rate constant

Slope and intercept are connected to \( k_{\text{off}} \) and \( x_\beta \)
The biomolecule partners must be bound to tip and substrate by covalent bonds, which are stronger than their interaction within the complex.
Strong binding, controlled orientation, and re-orientational freedom, to favour the biorecognition process, can be assured by functionalized linking spacers (PEG, cysteamine + gluteraldheide,..)

- Partner interaction sites involved in the complex formation must be available for the biorecognition.
Docking predicts the structure of a complex starting from the structures of the components.

Rigid-body docking algorithm performing an exhaustive six-dimensional search in the translational and rotational space between the two molecules.

The obtained configurations are ranked based on different criteria, such as:
- Pairwise shape complementarity
- Desolvation energy
- Electrostatics

The predicted configuration (“best complex”) can be exploited to develop optimized immobilization strategies possibly with a defined orientation, leaving the interacting regions available for the complex formation.
Unbinding frequency should decrease when the binding sites are occupied. A significant reduction after blocking indicates the formation of a specific complex.
Biological interest: **electron transfer interaction** involved in the nitrate respiration of bacterium *Pseudomonas aeruginosa.*

**First AFS study on an electron transfer complex**

**Docking simulations:** best complex from close contact between the hydrophobic regions of the two proteins

**Immobilization strategies:**
- PEG molecules for flexible linking of cytochrome to the tip, targeting –NH₂;
- oriented azurin bonding to the Au (**electr sens**) substrate via disulphide bridge, with or without spacers.

**AFS results:**
- Single energy barrier;
- \( k_{\text{off}} \) values consistent with a **transient complex:** 7 and 14 s\(^{-1}\);
- immobilization via organic spacers increases the binding efficiency

[Bonanni et al., BJ 89, 2783 (2005) and JPCB 110, 14574 (2006)]
p53 is an important tumour-suppressor

Mdm2 is the main down-regulator of p53, binding its N-terminal region (inf. from parts)
Single energy barrier

**Oriented immobilization:** $k_{\text{off}} = (0.09 \pm 0.03) \text{ s}^{-1}$  $\tau_{\text{off}} = (11.1) \text{ s}$  
[Taranta et al., JMR 21, 63 (2008)]

**Random immobilization:** $k_{\text{off}} = (2.5 \pm 0.6) \text{ s}^{-1}$  $\tau_{\text{off}} = (0.4) \text{ s}$

Value comparable to that of the Mdm2-p53 complex (transient character)  
[Funari et al., JMR (2009)]
AZURIN has an anticancer role. It forms a complex with p53 and stabilizes it. Since Mdm2/p53 complex Azurin/p53 complex

Question: can azurin stabilize p53, by competing with Mdm2 for the same binding site?


Blocking control experiments

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<thead>
<tr>
<th>Mdm2</th>
<th>p53</th>
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<tr>
<td>Au</td>
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Before blocking = 15%

After blocking = 5%

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<tr>
<th>AZ</th>
<th>p53</th>
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<tbody>
<tr>
<td>Au</td>
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Before blocking = 14%

After blocking = 6%
Competitive blocking experiments
p53/Mdm2/azurin interaction

NO competition for the same site

- Azurin does not compete with Mdm2 for the same binding site
- Conclusive indication of a TERNARY COMPLEX FORMATION

Interference with the UBIQUITINATION PROCESS

G. Funari et al., J.Mol.Rec. 2010
Conclusions

Metalloproteins can be used as nanosized transducers and biorecognition elements in biosensors, and the **immobilization strategy** on the electrodes is a crucial task.

**Immobilizing** metalloproteins to the electrodes **via organic linkers** allows:

- recovering of the crystallographic size **(TM-AFM)**
- increased conduction properties **(STM)**
- higher biorecognition ability **(Force Spectroscopy)**

The use of **metallic linkers** (mSWNTs or AuNP) increases the electrical coupling between metalloproteins and electrodes **(C-AFM)**.
Dynamic Force Spectroscopy and Biomolecular Recognition
Editors: Anna Rita Bizzarri, Salvatore Cannistraro

http://www.crcpress.com/product/isbn/978143986237

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Thank you for your attention
Electron Tunnelling

Without applied bias

\[ I_{\text{tunnelling}} \propto V_{\text{bias}} e^{-2kz} \]

\[ k \approx \sqrt{\frac{2m \Phi}{\hbar}} \]

\( \Phi \) is the local barrier height or the average of tip and sample workfunctions

\( m \) electron mass

With applied bias
Scanning Probe Microscopies at the Biophysics and Nanoscience Centre

Nanoscope IIIA, Digital Instrument

XE-100, PSIA Co.

PicoLE 5100, Agilent

PicoSPM, Molecular Imaging