CONSEJO SUPERIOR DE INVESTIGACIONES CIENTIFICAS
CSIC

NANOSCALE OPTIMIZATION OF THE DET PROCESS IN BIOCATHODES LOADED WITH MULTICOPPER OXIDASES

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ICP-CSIC MALMÖ UNIV.

IARIA, ICQNM 2013. August 27th

INSTITUTO DE CATÁLISIS:

Ingeniería de procesos

Estructura y Reactividad

Catálisis Aplicada

Biocatálisis

- Biocatalysis Department
  - Study biocatalytic processes
  - Inspiration source for new non-biological catalysts
  - Integration with heterogeneous catalysis yielding hybrid processes
  - Soft chemistry: enantioselective synthesis
  - Green chemistry: biological catalysts
  - Bioremediation
  - Bioelectrocatalysis: biofuel cells and bio production of fuels
  - Electrochemical biosensors

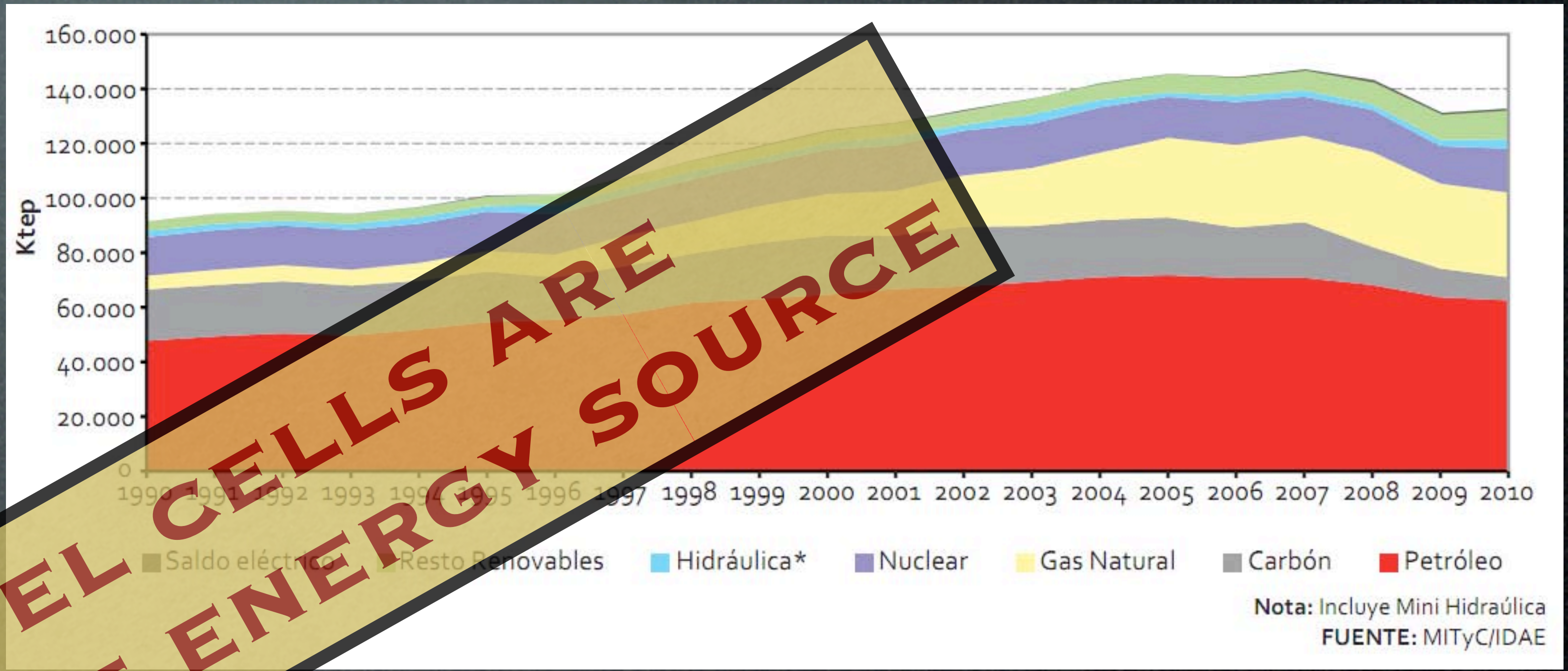
Outline

- Motivation
- Laccase and its interaction with surfaces
- Improvement of bioelectrodes based on C
- Overcoming the Laccase-gold problem
- Approaching physiological conditions: pH and Chloride resistance, alternative biocatalysts
- Directed evolution

Energy based on fossil fuels: non sustainable

- National consume of Energy > 90% fossil and nuclear fuels.
- Non renewable energy, imported, pollution.
- Prediction of Hubbert: fossil fuel shortage.
- Increase of the price and derivatives.
- High environmental impact.
- Oil is far away: expensive transport, possible ecological

ENZYME FUEL CELLS ARE AN ALTERNATIVE ENERGY SOURCE



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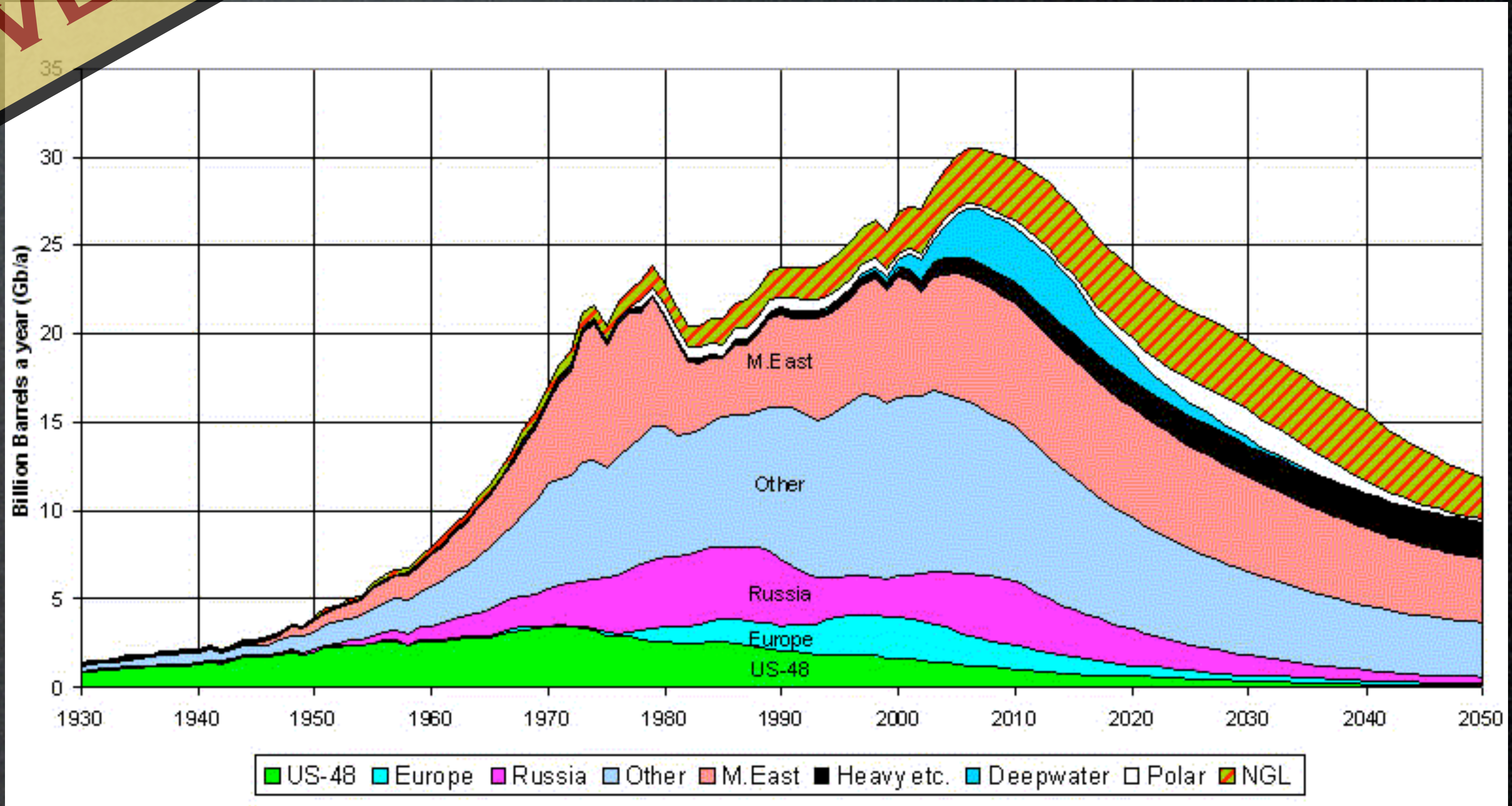
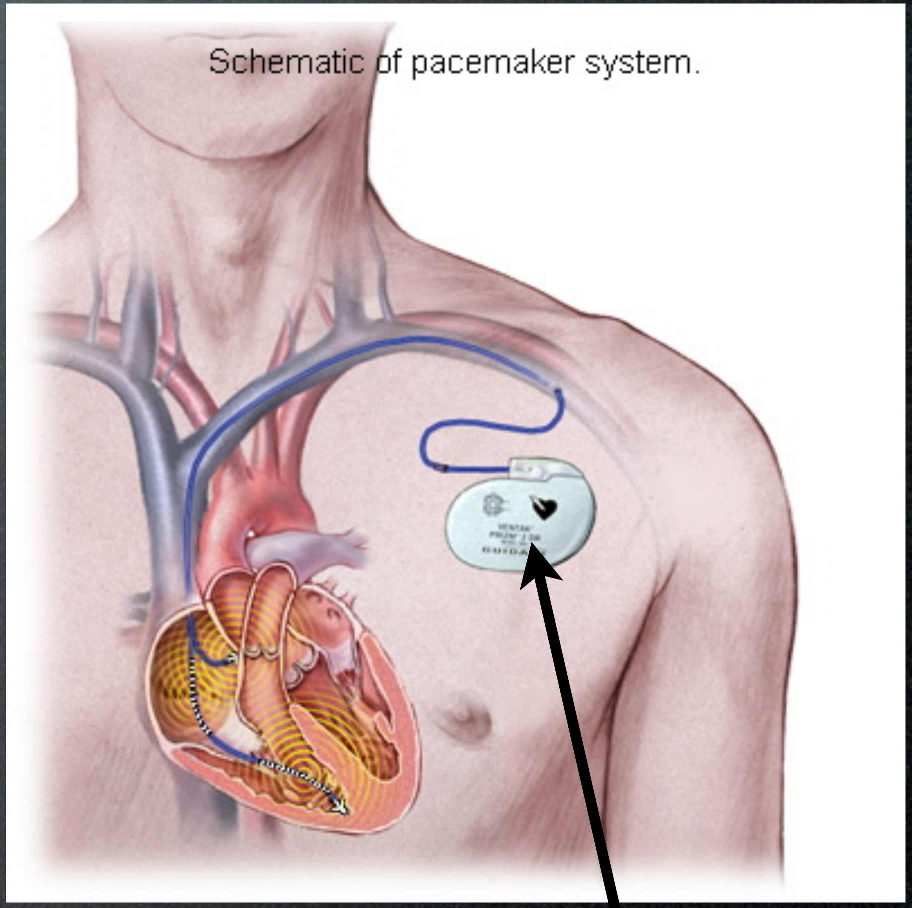


Nowadays possibilities for enzyme biofuel cells

- Currently biofuel cells do not produce high amounts of energy.
- Applications:
  - Micro implantable devices for biological tissues or blood
  - Fuels or oxidants from environment (juices, waste water)
  - Portable Source of energy for MEMS or self standing microchips.



pacemaker powered by biofuel cell

3D-Nanobiodevice (7th FP-NMP)

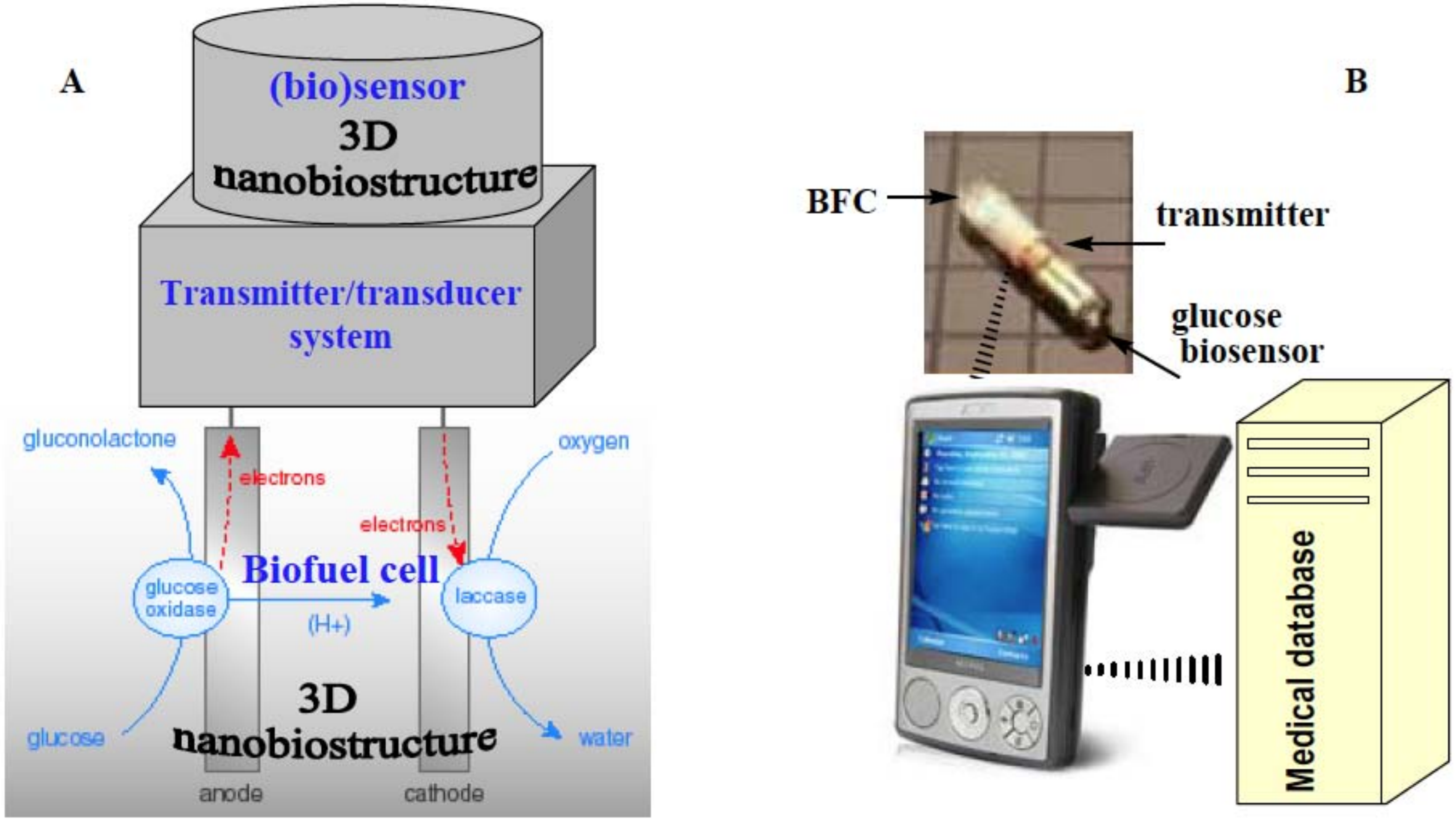
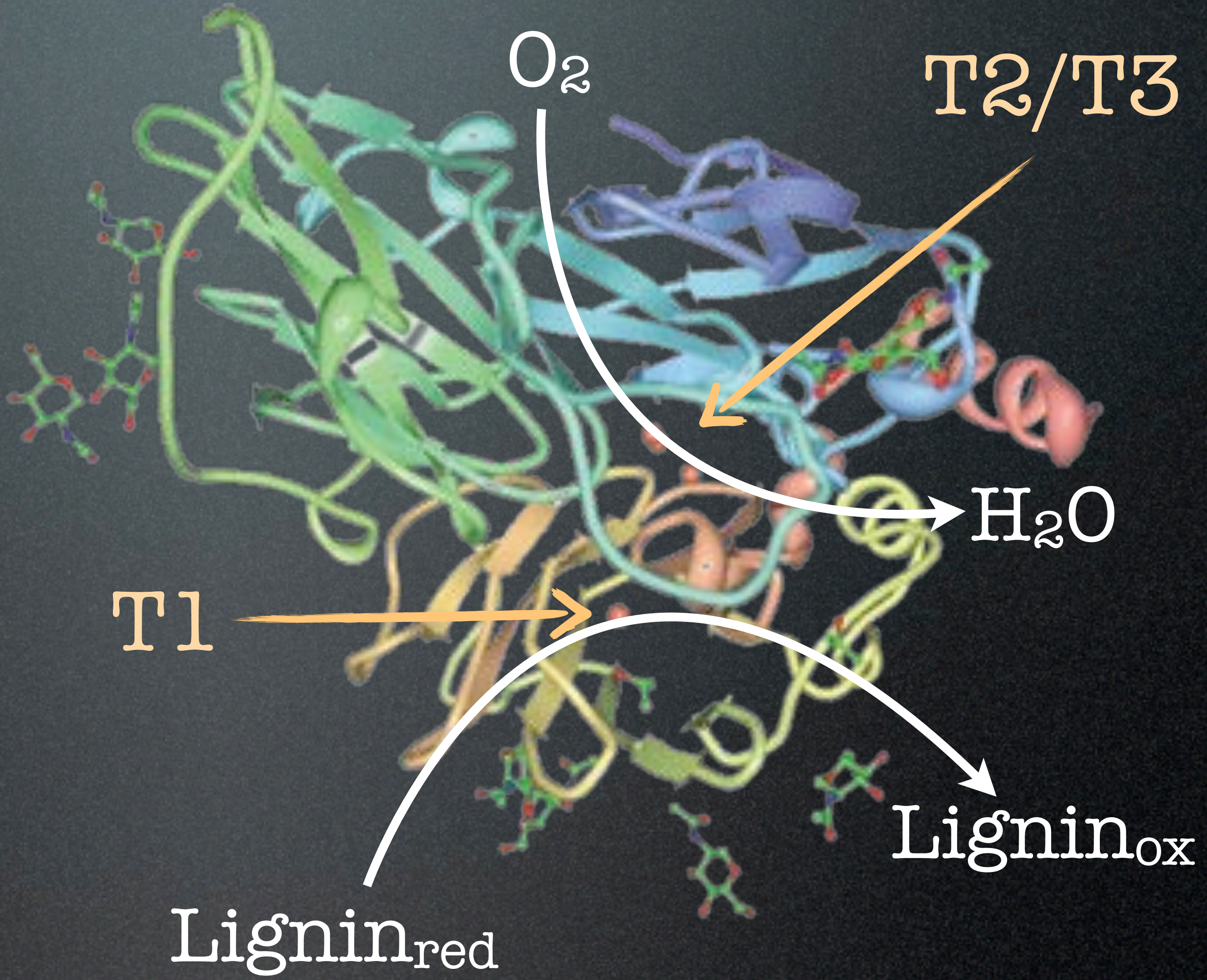


Fig. 1. (A) Principal scheme of a self-contained bioelectronic device for biomedical application. (B) Self-contained biodevice for glucose monitoring in blood, plasma, and saliva

- Build a bioimplantable device that can work in different physiological environments.
- Develop conductive nanoarchitechtures to assemble 3D bioelectrocatalytic structures with high efficiency towards biofuel cells and biosensors.
- Wire redox enzymes (glucose, O_2) to the 3D structures to generate energy inside physiological environments.
- **Work area for ICP bioelectrocatalysis group: Strategies for the immobilization of Laccases and Bilirubin Oxidases to obtain high DET.**

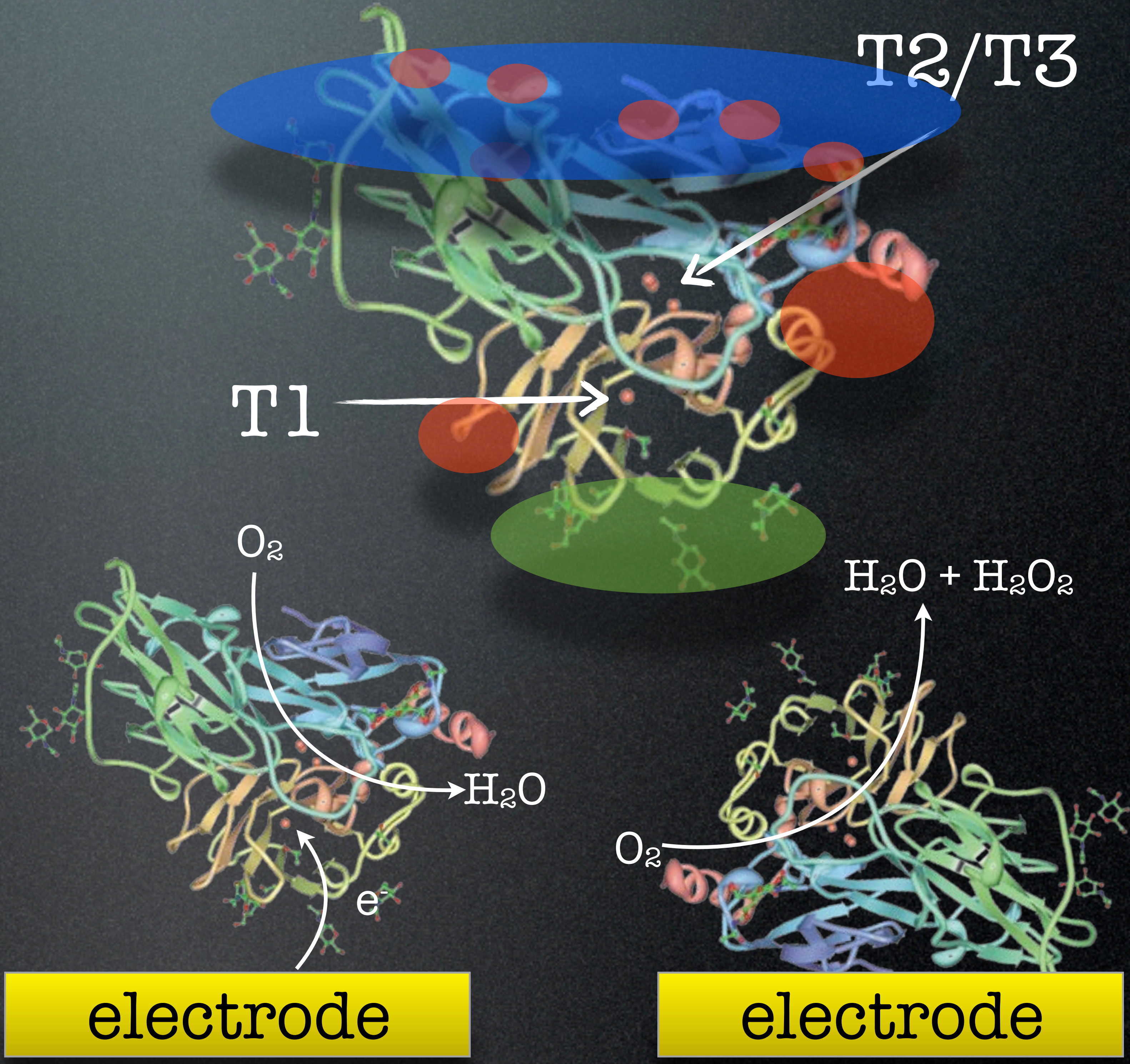
Trametes Hirsuta laccase: Structure

- Enzyme sources: Fungal (high potential) or Tree (low potential)
- Redox enzyme. Function:
  - Oxidizes polyphenols, lignin, and other substrates.
  - Reduces O_2 to H_2O.
- Active site: 4 Cu cations distributed as:
  - 1 Cu, T1 site, substrate oxidation,
  - 3 · Cu cluster, T2/T3 site, O_2 reduction.
  - Intramol. bridge, histidine-cysteine.
- Applications:
  - Direct reduction of O_2 to H_2O, O_2 detection and quantification.
  - Waste treatment, water cleaning, oxidation of phenol-derived hazards.



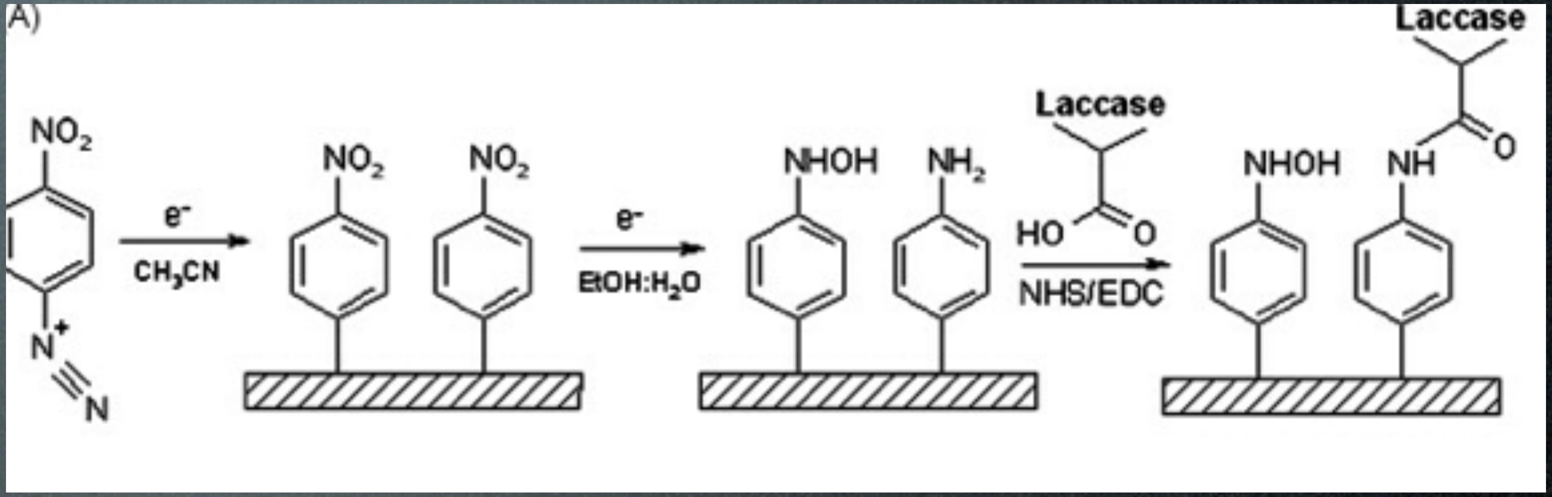
Trametes Hirsuta laccase: oriented immobilization

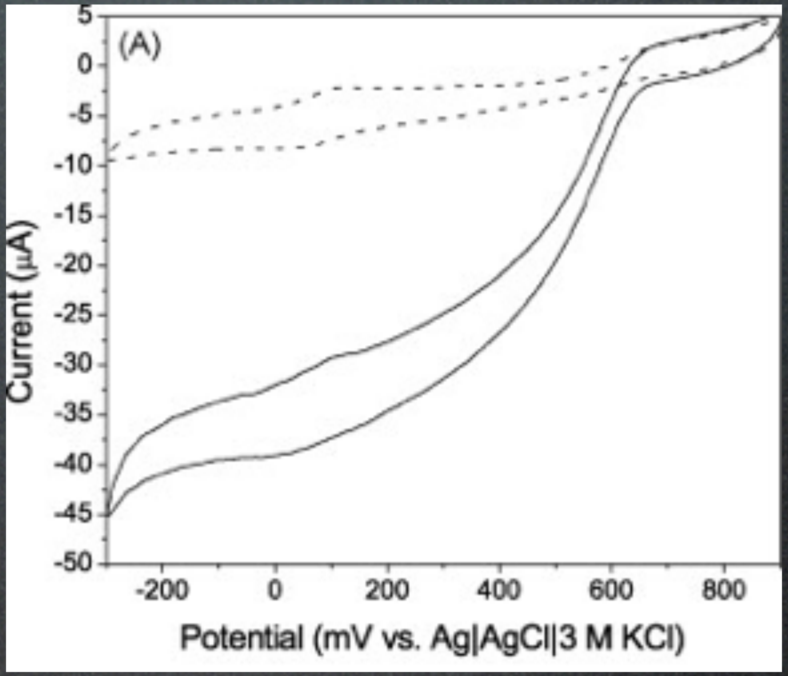
- Goal: DET via Cu T1 site.
- Challenge: avoid O_2 shortcut
- Enzyme's appropriate residues: Carboxylic groups (carbodiimide coupling), sugar residues (Schiff's base bond).
- Enzyme's orientation hindering groups: Amino region, carboxylic groups.
- Goal 1: Achieve Oriented immobilization of laccase on electrodes.
- Goal2: Improve performance under physiological-like conditions.

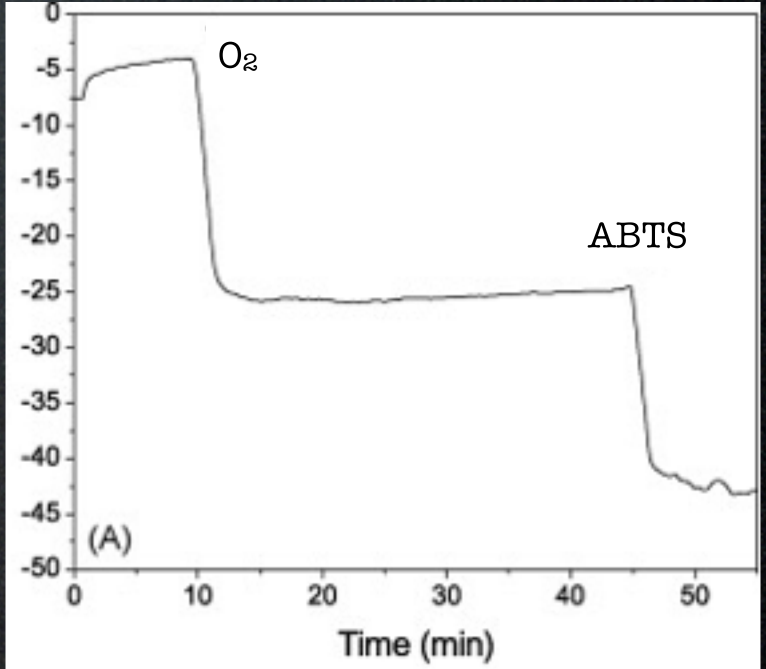


Prime Approach: Graphite Electrodes

- Graphite surface resembles laccase natural substrate, lignin.
- Functionalization of graphite yields modified electrodes suitable for covalent attachment of Laccase.
- High O_2 electroreduction current (~200 µA/cm^2), biofuel cells need higher current density.
- MET pretty high: needs for better laccase orienting.
- Possible improvement routes:
    - Nanostructured electrodes
    - 2-step laccase immobilization to orient it on the electrode



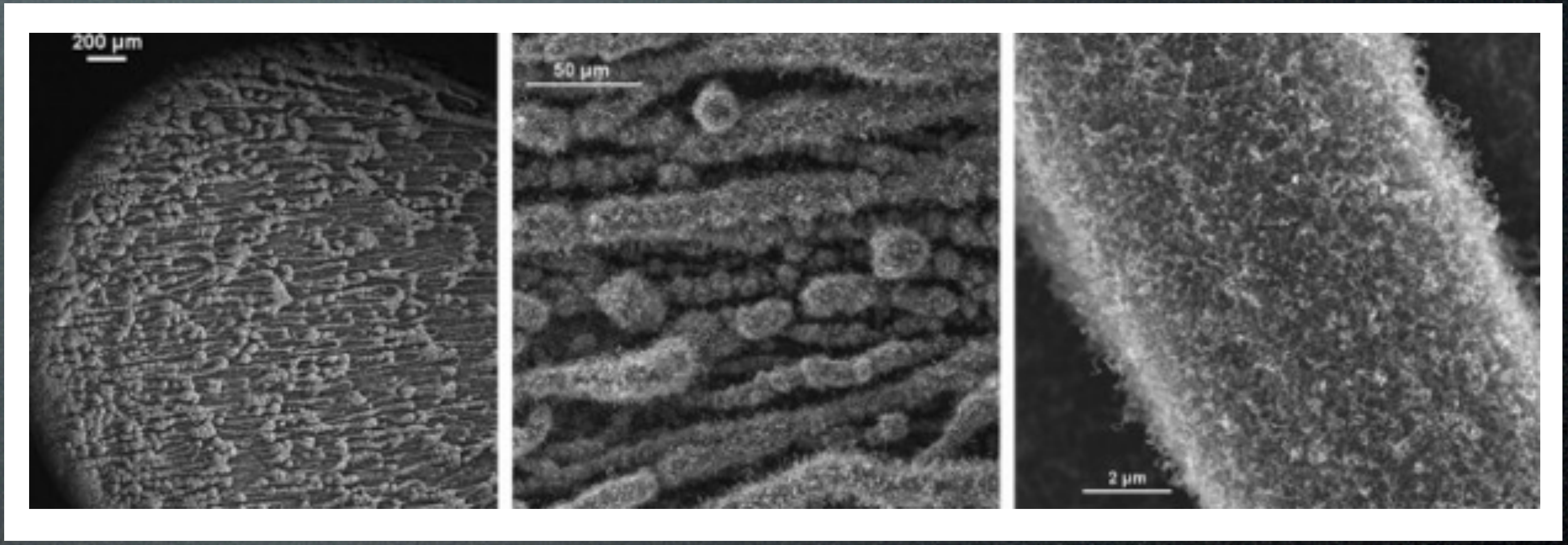


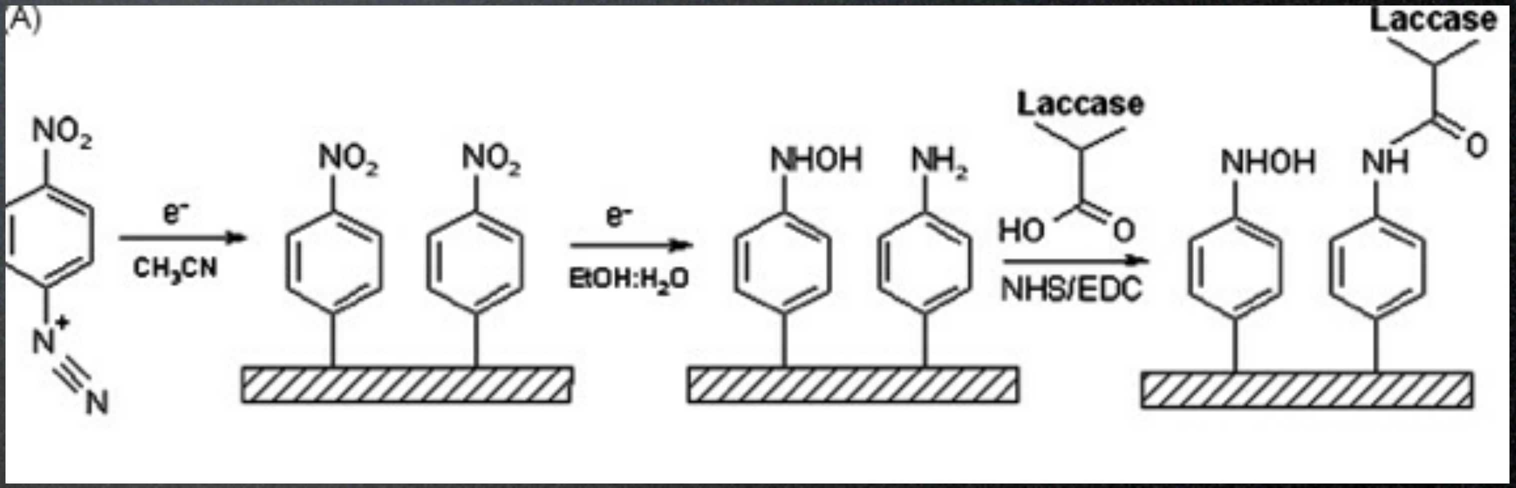


Vaz-Dominguez, C.; Campuzano, S.; Rüdiger, O.; Pita, M.; Gorbacheva, M.; Shleev, S.; Fernandez, V.M.; De Lacey, A.L. Biosensors and Bioelectronics 24 (2008) 531-537

CNT-CMF Graphite electrodes for laccase immobilization

- CNT-CMF hierarchically nanostructured electrodes.
- 4-nitrophenyldiazonium salt was used for electrode modification.

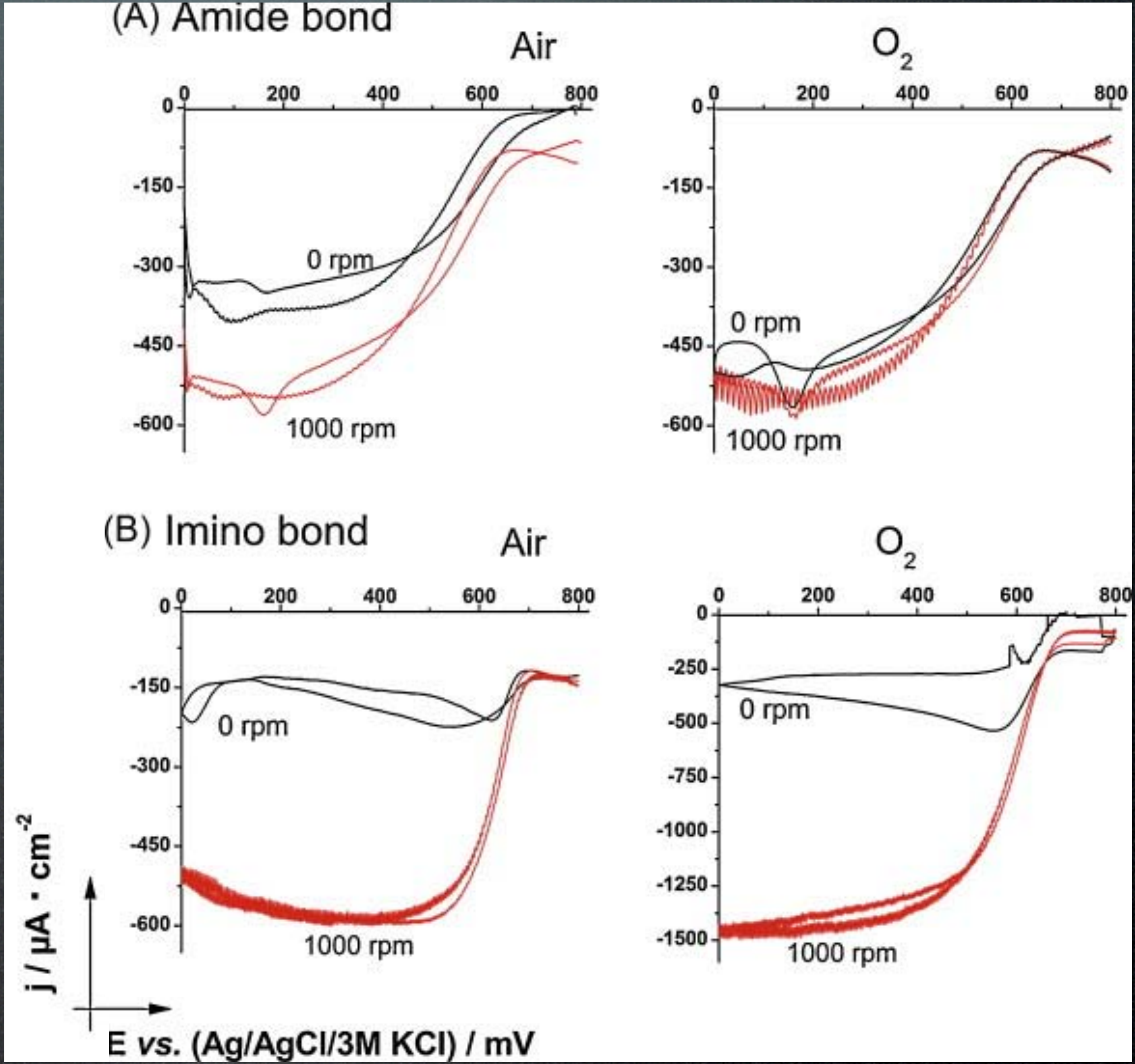




Gutierrez-Sanchez, C.; Jia, W.; Beyl, Y.; Pita, M.; Schuhmann, W.; De Lacey, A.L.; Stoica, L. Electrochimica Acta, 2012, 82, 218-223

CNT-CMF Graphite electrodes for laccase immobilization

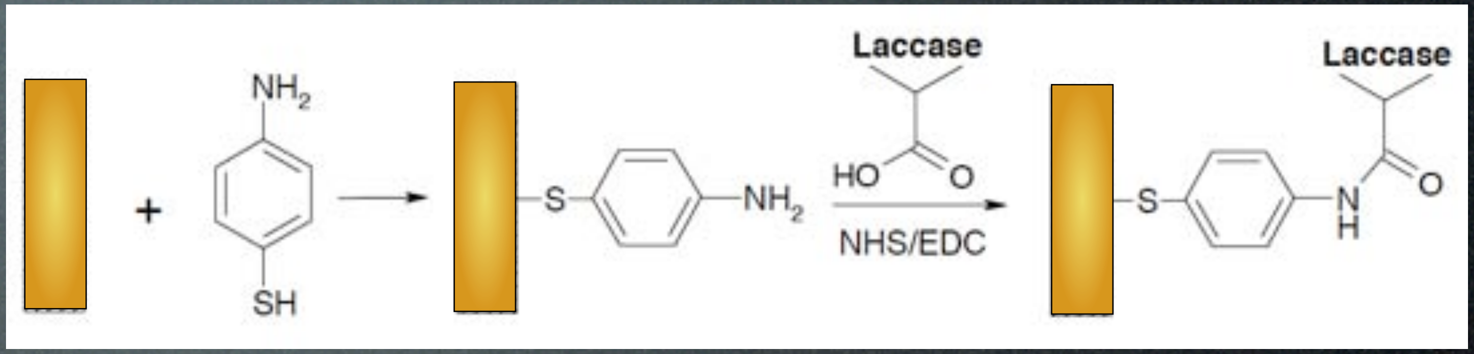
- CNT-CMF hierarchically nanostructured electrodes.
- 4-nitrophenyldiazonium salt was used for electrode modification.
- Laccase immobilized via **(A) Amide bond** formation or via **(B) imino bond** by sugar oxidation to aldehyde and Schiff base reaction.
- High current density values:
    - Effect of O_2 saturation
    - Effect of electrode rotation

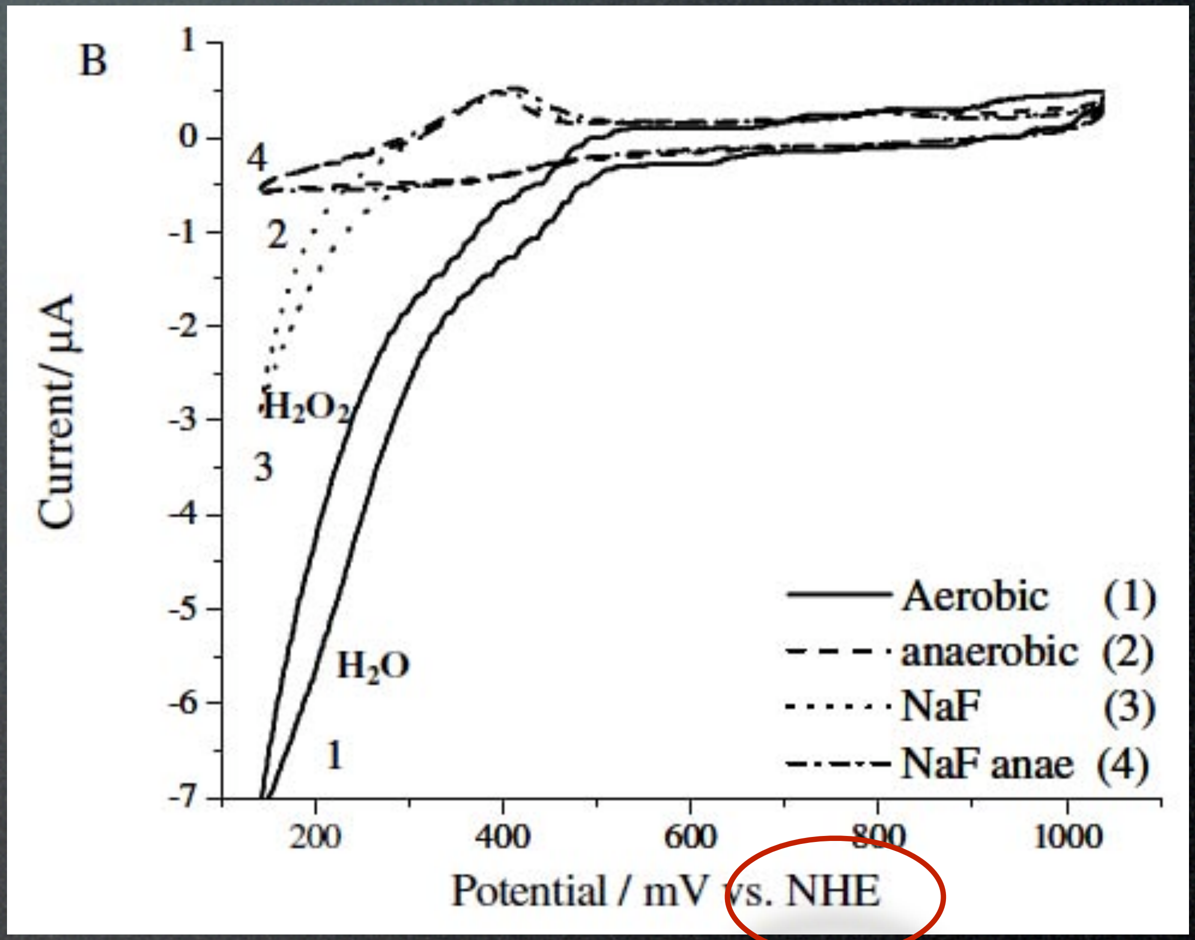


Gutierrez-Sanchez, C.; Jia, W.; Beyl, Y.; Pita, M.; Schuhmann, W.; De Lacey, A.L.; Stoica, L. Electrochimica Acta, 2012, 82, 218-223

Prime approach: SAM modified gold electrodes

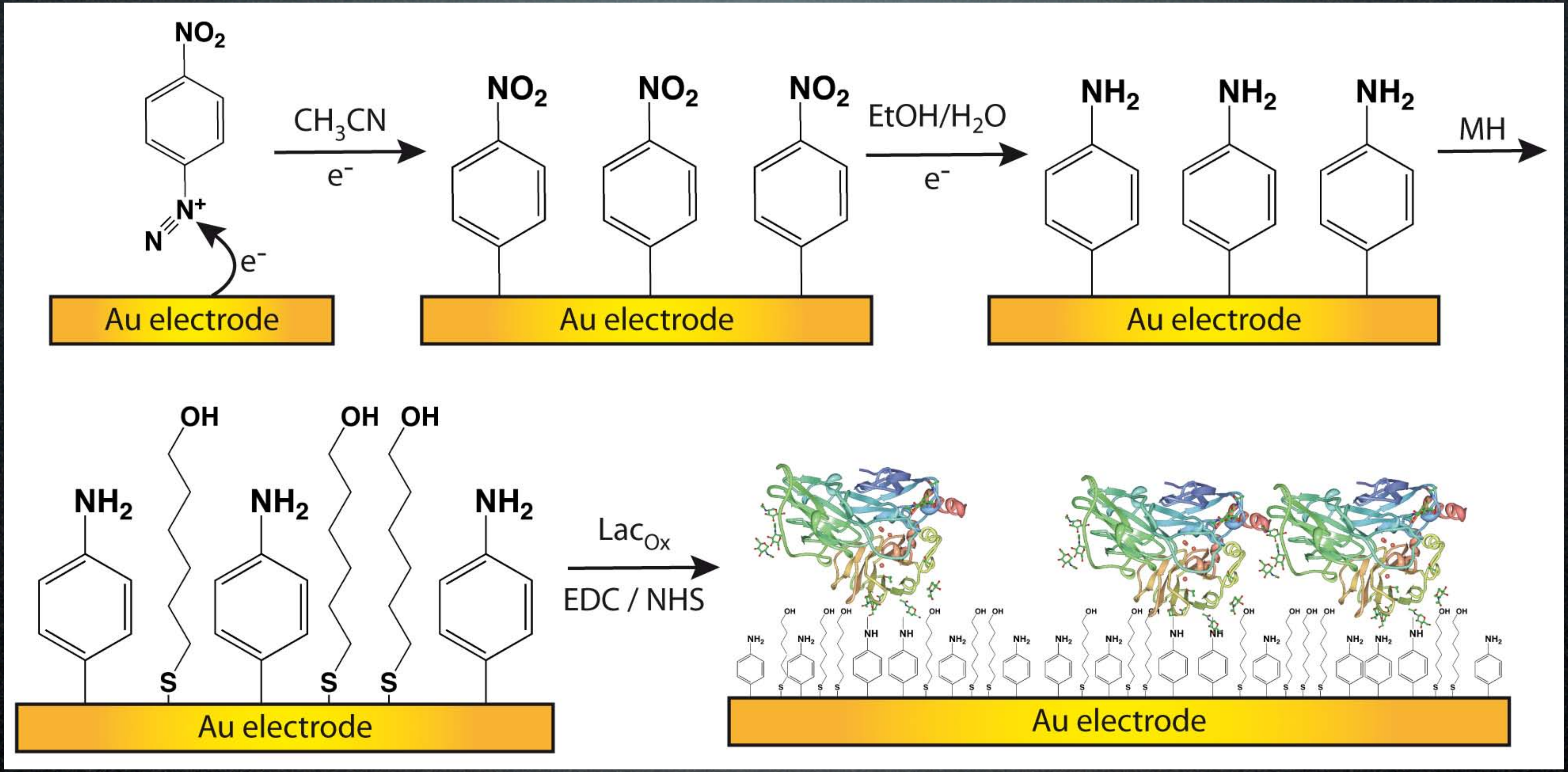
- SAMs of thiols for modifying gold to attach laccase.
- **4-aminothiophenol** yields a SAM alike LDG modification.
- Laccase show scarce 4 e$^-$ DET and higher proportion of 2 e$^-$ process.
- Production of H_2O_2.
- Laccase is not oriented. Possible reasons:
    - Excess of amino groups
    - Mobility of thiols on Au surface





Pita, M.; Shleev, S.; Ruzgas, T.; Fernandez V.M.; Yaropolov, A.I.; Gorton, L. Electrochem. Commun., 2006, 747-753

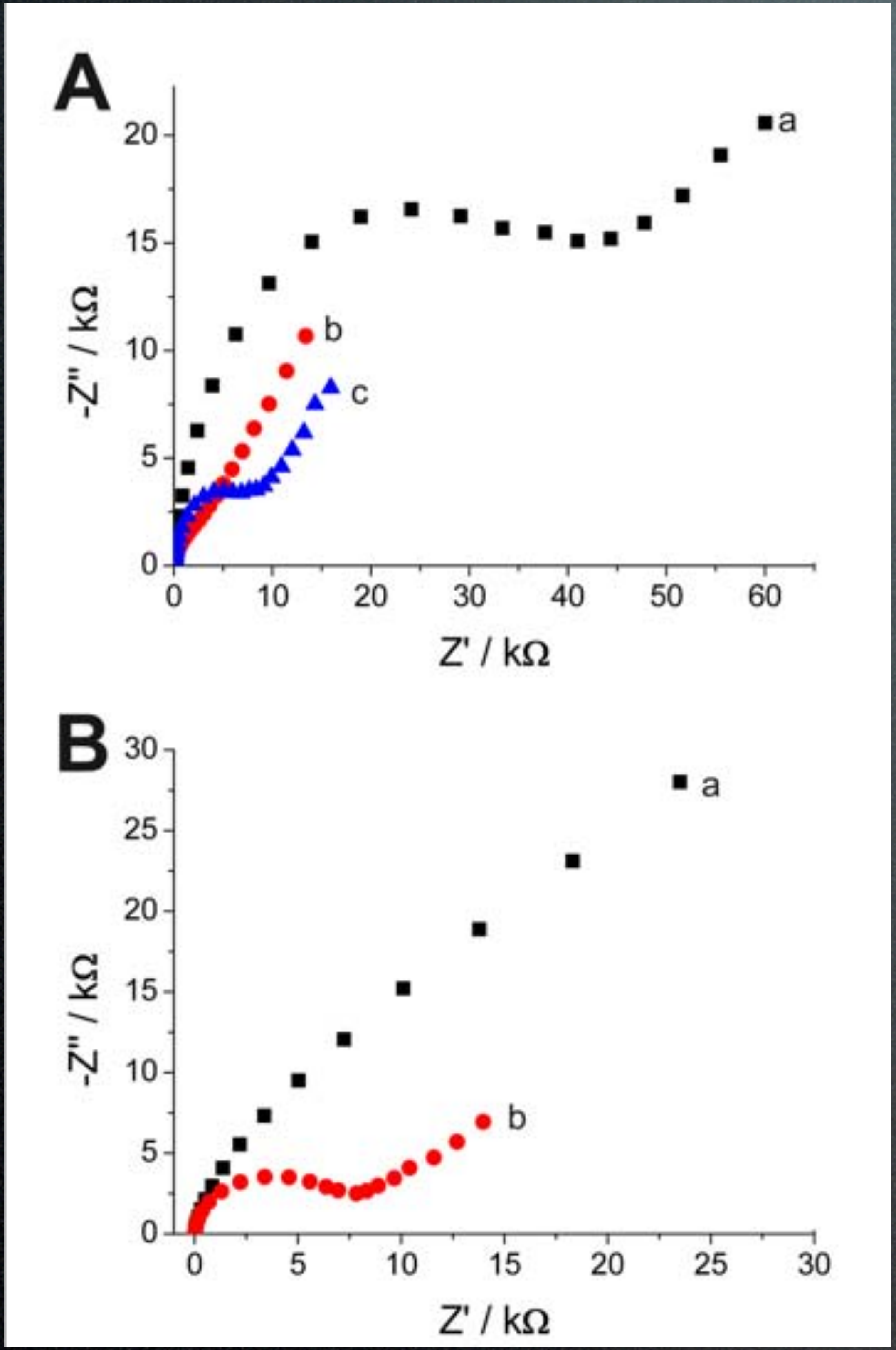
High DET on laccase-gold electrodes

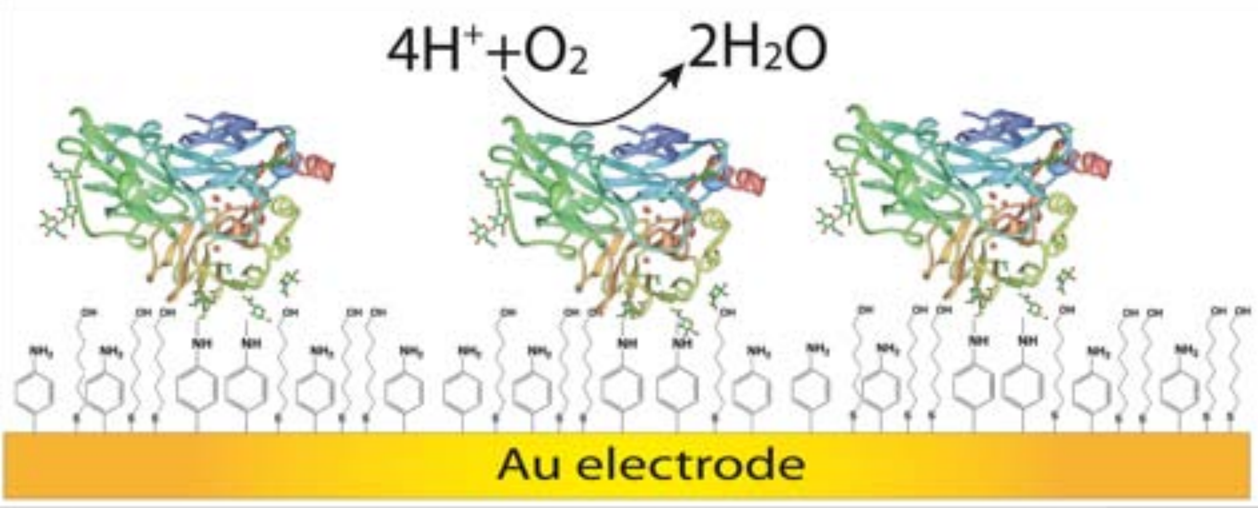


- Strategy: 1. Diazonium salt reduction on gold electrodes, 2. reduction nitro-to-amino
- Surface blotting with thiols. Molecules used: **6-mercapto-1-hexanol**, 3-mercaptopropionic acid, cistamine, 2-mercapto-1-ethanol, thioctic acid.
- Laccase orientation: 2-step strategy. (a) Laccase sugars' oxidation to aldehyde and further amine Schiff's base formation. (b) Amide bonding catalyzed by EDC-NHS.

Pita, M.; Gutierrez-Sanchez, C.; Olea, D.; Velez, M.; Garcia-Diego, C.; Shleev, S.; Fernandez, V.M.; De Lacey, A.L. J. Phys. Chem. C, 2011, 115, 13420-13428

Mixed monolayer characterization





Impedance Spectroscopy

Electrochemical Probe: $[Fe(CN)_6]^{3-/4-}$

(A) Frequency responses:

a) Nitroaryl layer

b) aminoaryl layer

c) aminoaryl + MH SAM layers

(B) Frequency responses:

a) Bare gold

b) MH SAM

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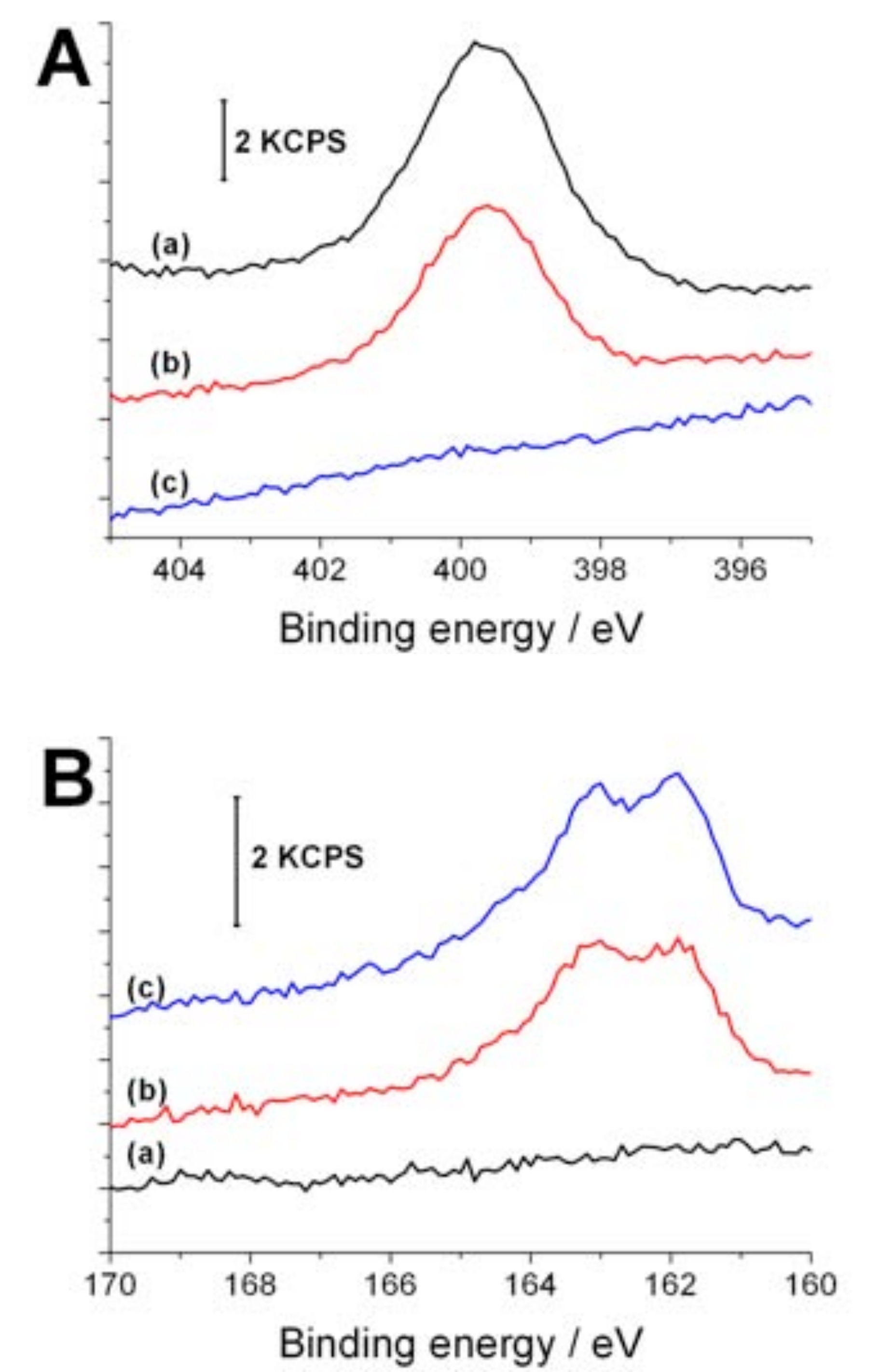
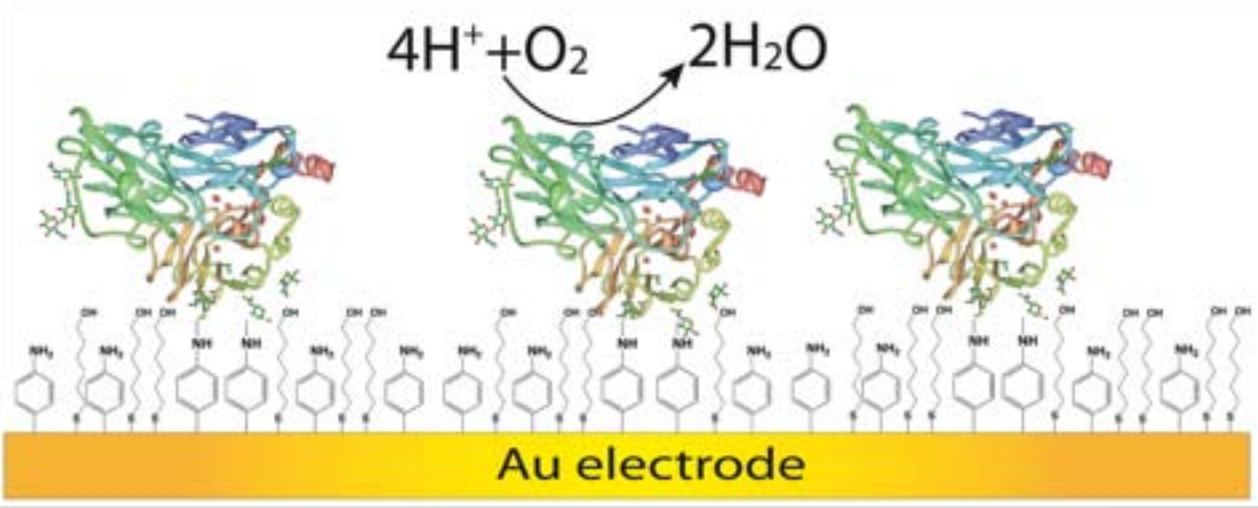




X-ray Photoemission Spectroscopy

Search for signals that determine presence of $-NH_2$ and S-

(A) Amine core:

a) aminoaryl layer

b) aminoaryl + MH SAM layers

c) MH SAM

(B) Thiol core:

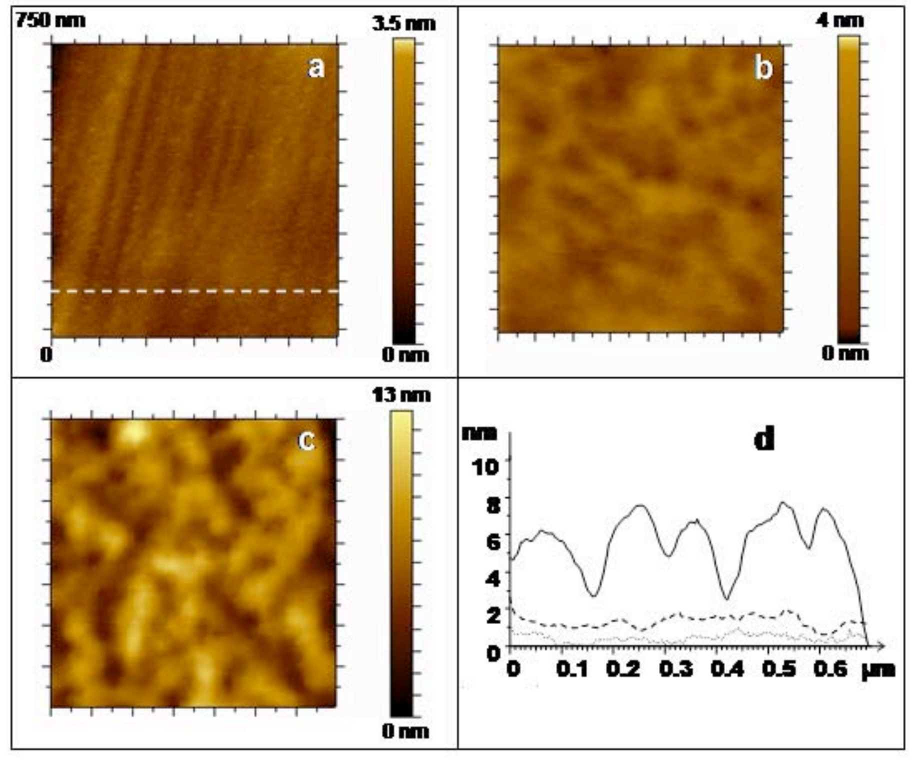
a) aminoaryl layer

b) aminoaryl + MH SAM layers

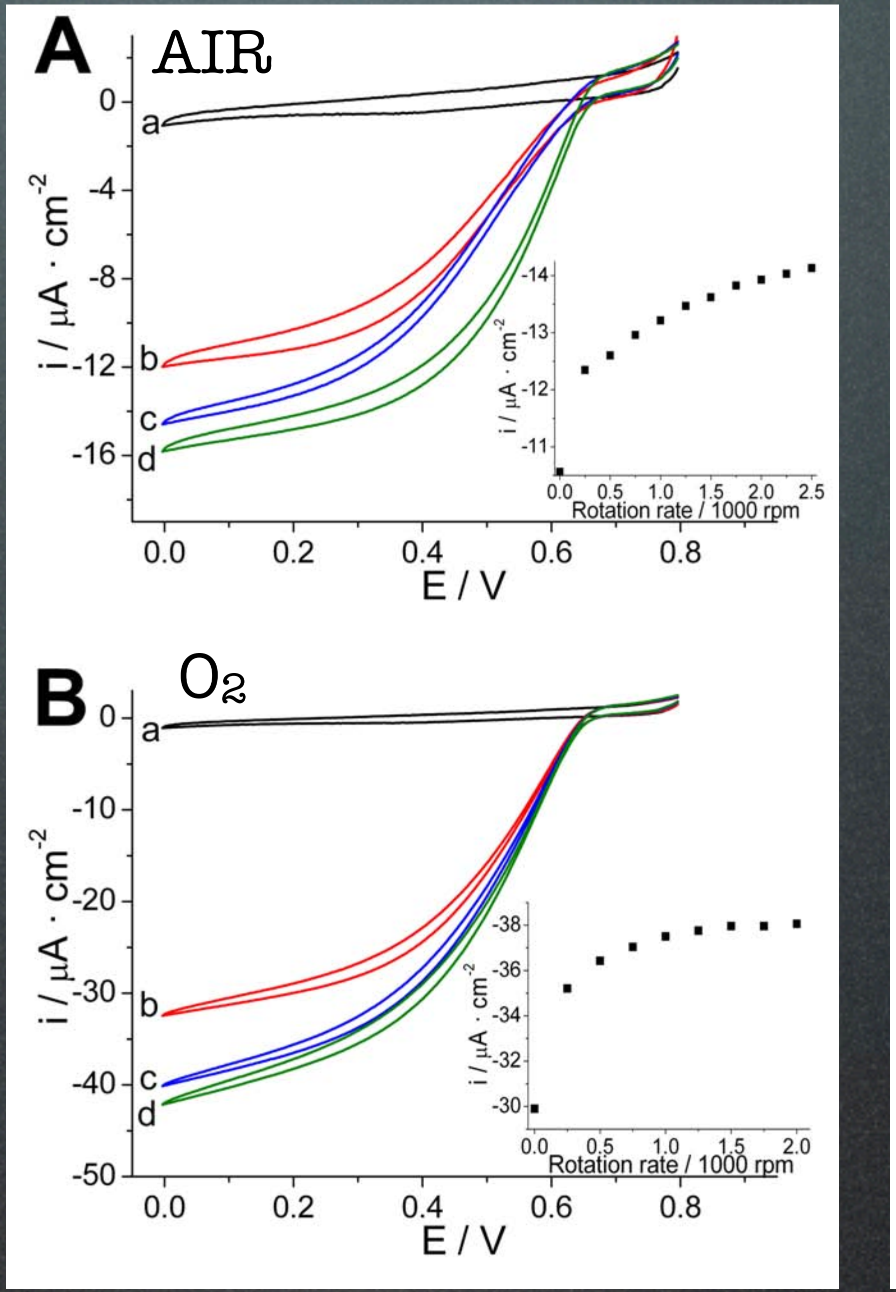
c) MH SAM

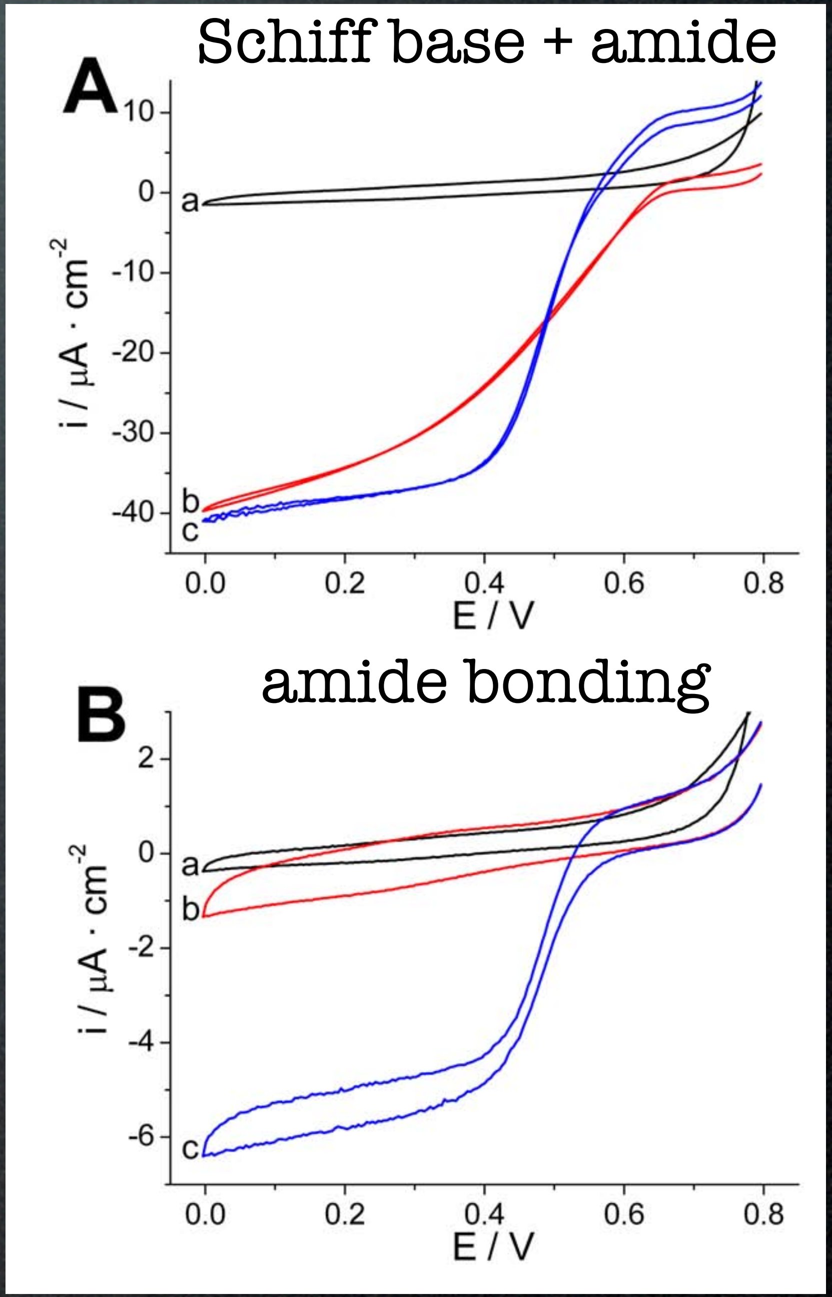
Pita, M.; Gutierrez-Sanchez, C.; Olea, D.; Velez, M.; Garcia-Diego, C.; Shleev, S.; Fernandez, V.M.; De Lacey, A.L. J. Phys. Chem. C, 2011, 115, 13420-13428

Results on Gold-Dz Laccase electrodes



AFM: (a) bare gold (b) aminophenyl-MH gold (c) Laccase gold



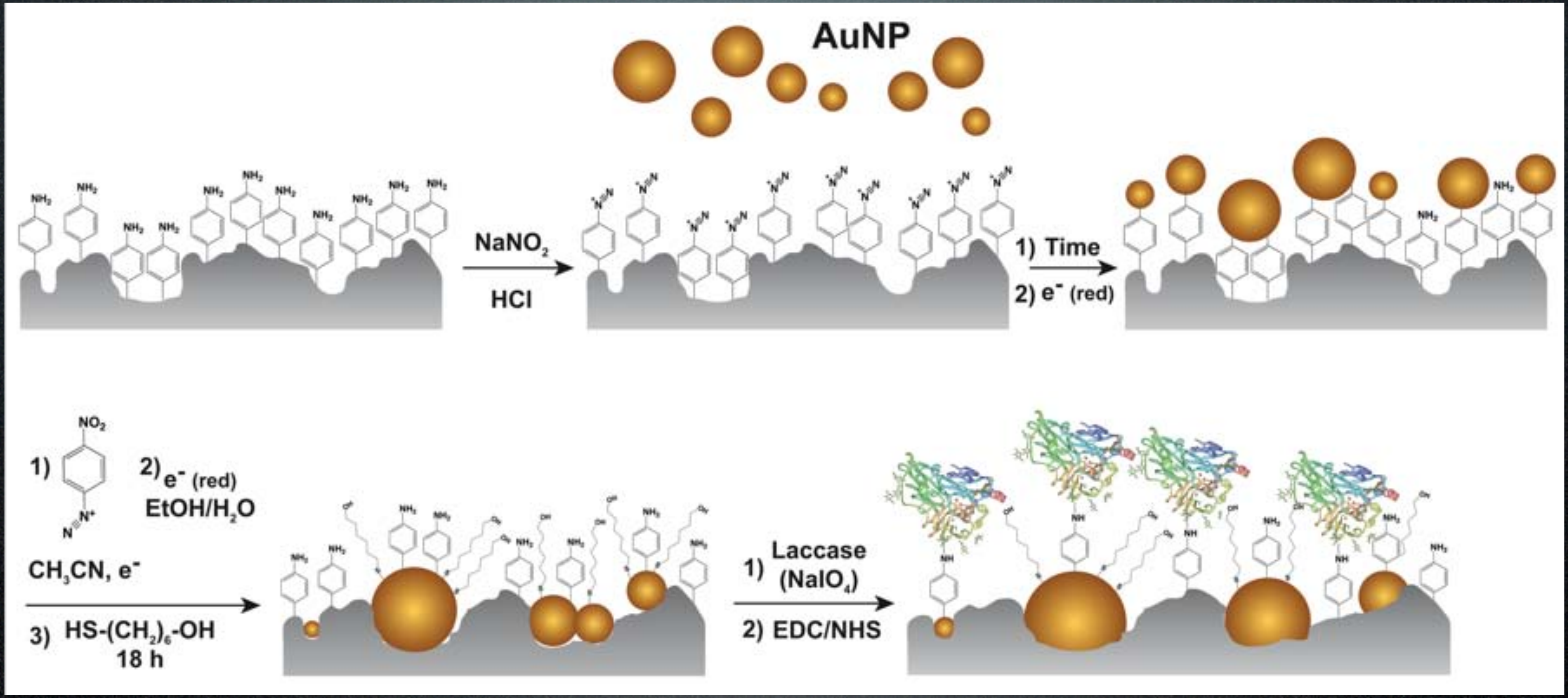


Laccase electrode **response vs. Ag/AgCl** (A) air, (B) Oxygen sat. Increased rotation rates are shown.

Laccase electrode **response vs. Ag/AgCl**(A) MET (blue) vs DET (red) for 2-step laccase immobilization (B) MET and DET amide immobilization

Pita, M.; Gutierrez-Sanchez, C.; Olea, D.; Velez, M.; Garcia-Diego, C.; Shleev, S.; Fernandez, V.M.; De Lacey, A.L. J. Phys. Chem. C, 2011, 115, 13420-13428

AuNP-Graphite



- Covalent attachment of AuNP to functionalized LDG electrodes.

- AuNP size, time of contact electrode-particle, further modification

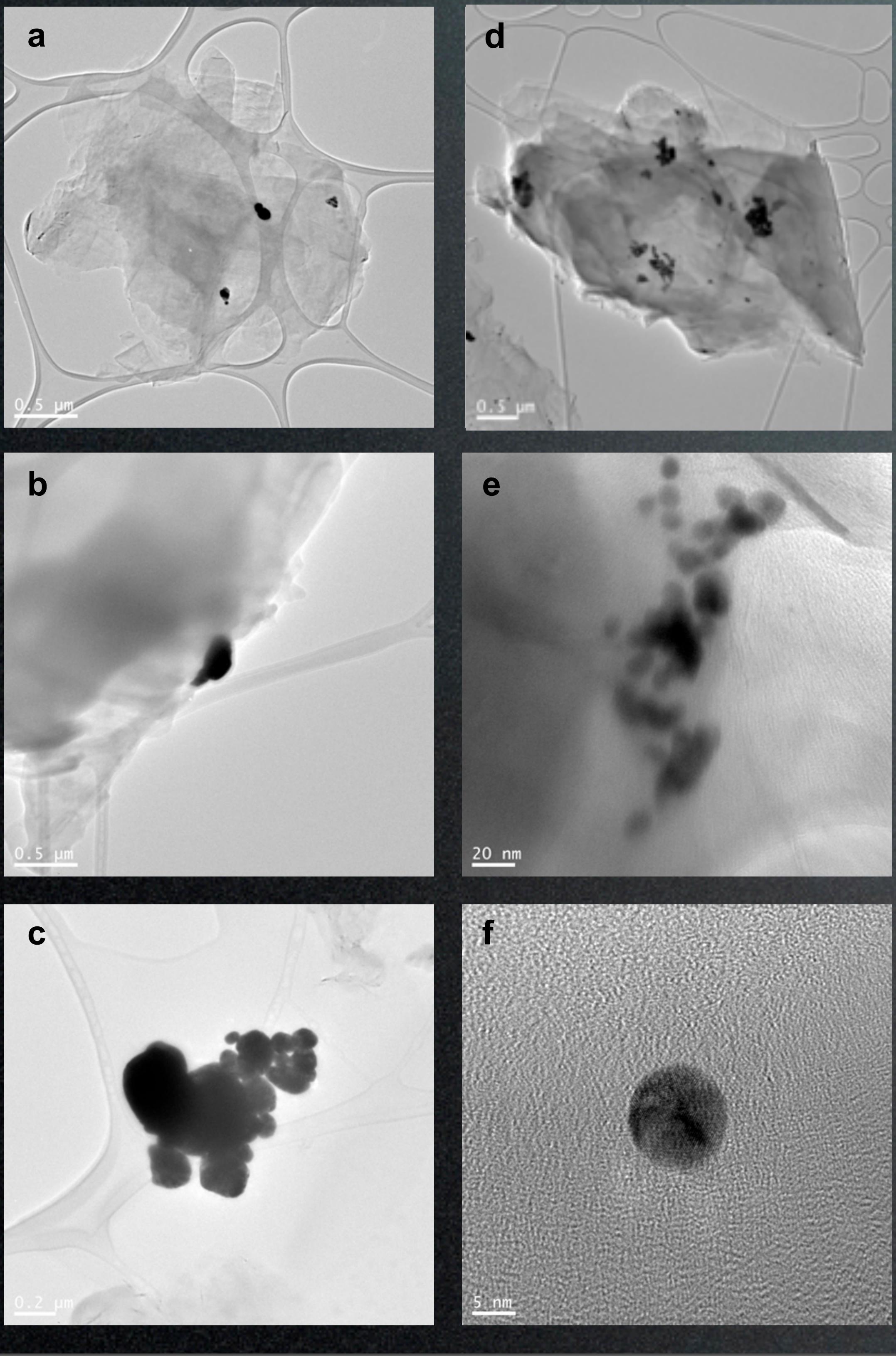
- Enhance of electron transfer electrode-laccase

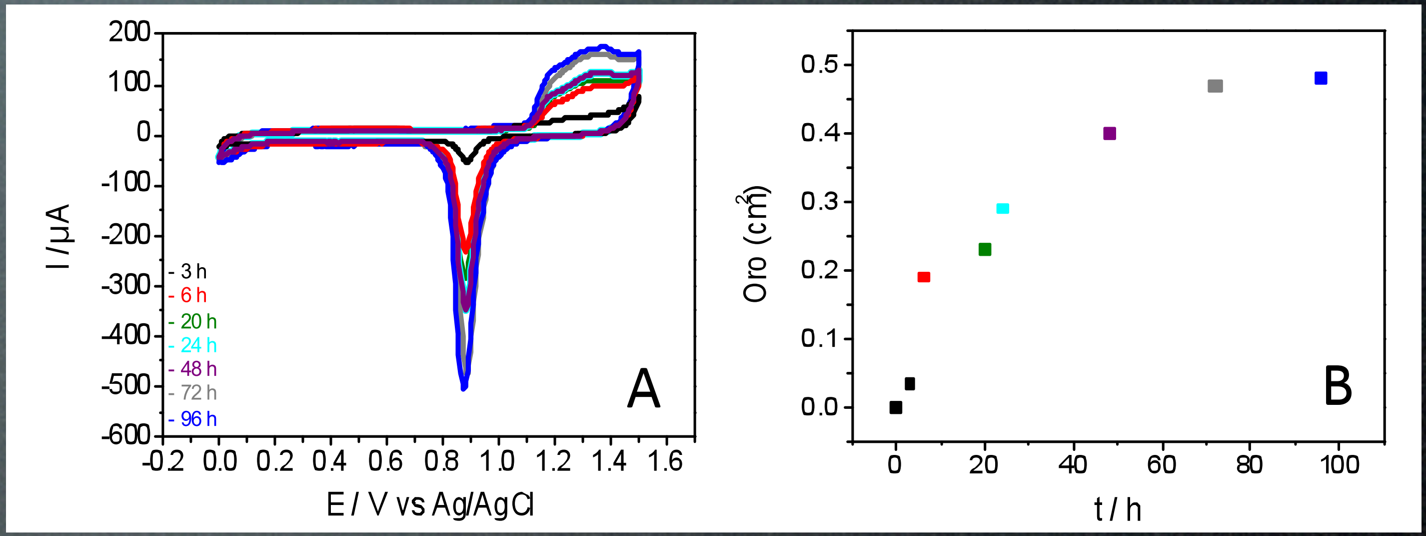
Gutierrez-Sanchez, C.; Pita, M.; Shleev, S.; De Lacey, A.L. J. Am. Chem. Soc. 2012, 134,017212-17220.

Characterization of hybrid electrodes

Adsorbed AuNP

Covalent AuNP



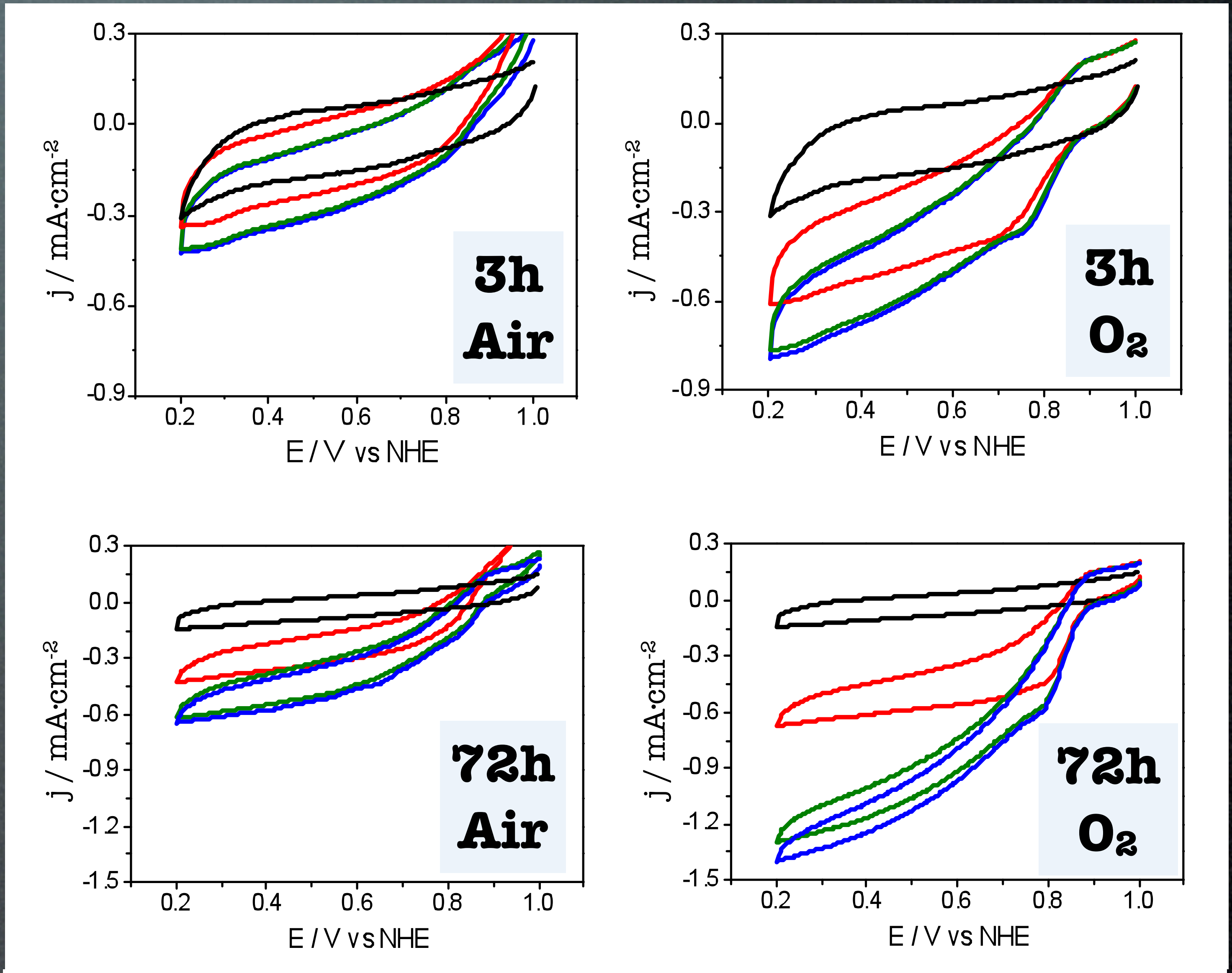


- CV of LDG-AuNP in H_2SO_4 gives Au electroreduction signal.
  - Incubation time (3h - 3d) yields an increase of the amount of AuNP.
- Chips of LDG modified with AuNP were observed by TEM
  - More AuNP when covalently bonded
  - Lesser electro-alloy Au growth

Gutierrez-Sanchez, C.; Pita, M.; Shleev, S.; De Lacey, A.L. J. Am. Chem. Soc. 2012, 134,017212-17220.

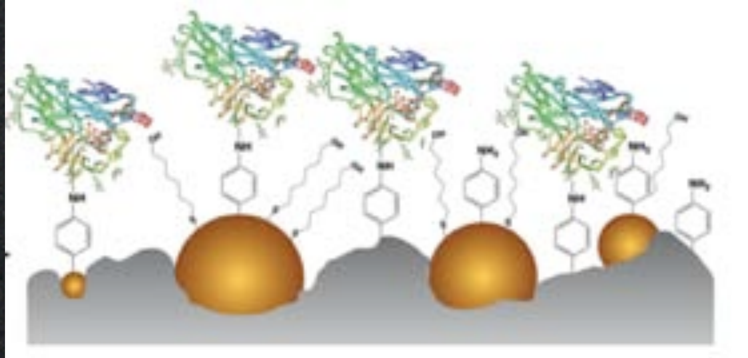
Laccase-Au-LDG: AuNP deposition **Time**

- Higher presence of AuNP yields higher current densities
- Optimal conditions: Two electrocatalytic processes are observed!!
  - Sharp initial electroreduction which **does not appear in experiments without AuNP**
  - second mass-transfer dependent process, mainly observed when rotating



Black: F^- (blank). **Red**: 0 rpm.
Green: 500 rpm. **Blue**: 1000 rpm

	3 hours	72 hours
0,8 V vs NHE	-(265±3) μA/cm²	-(580±10) μA/cm²
0,4 V vs NHE	-(500±10) μA/cm²	-(1070±20) μA/cm²



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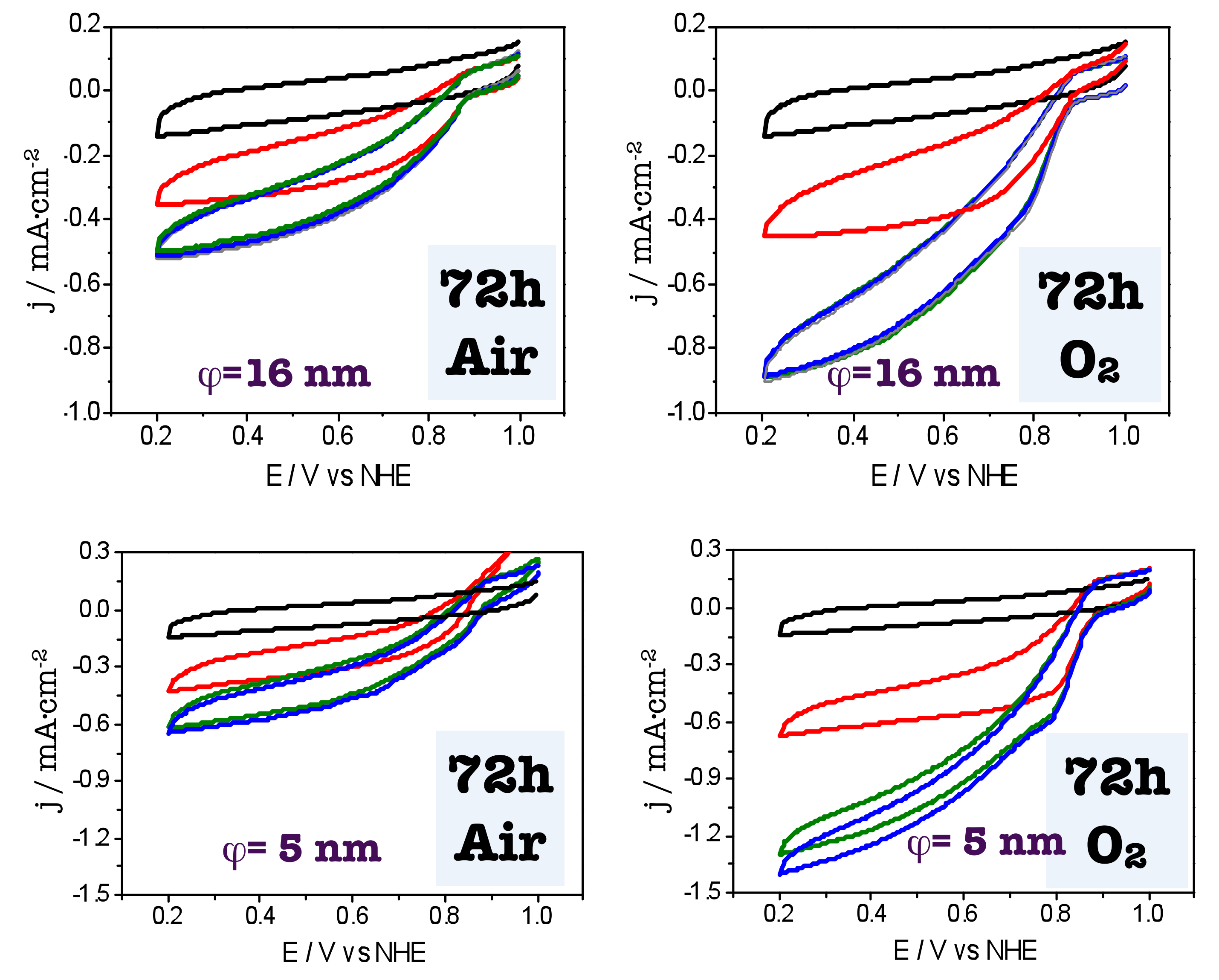
Laccase-Au-LDG: Size of nanoparticles

- Use of φ=16 nm AuNP does not enhance the electrode performance
  - Match size with laccase (~5 nm^3) gives slightly better connection: higher curvature.
  - Smaller particles bring higher current at lower potential



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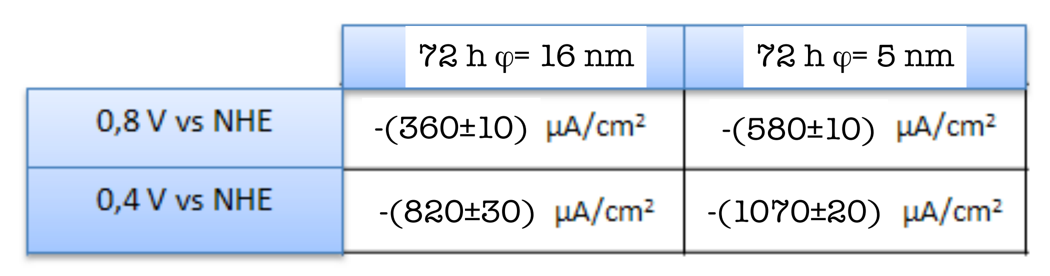
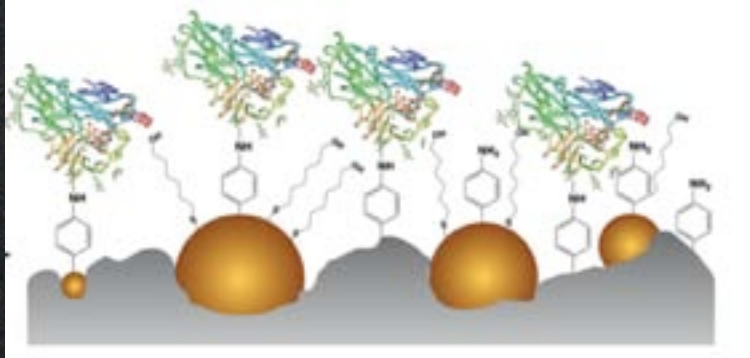


	72 h φ= 16 nm	72 h φ= 5 nm
0,8 V vs NHE	-(360±10) µA/cm^2	-(580±10) µA/cm^2
0,4 V vs NHE	-(820±30) µA/cm^2	-(1070±20) µA/cm^2



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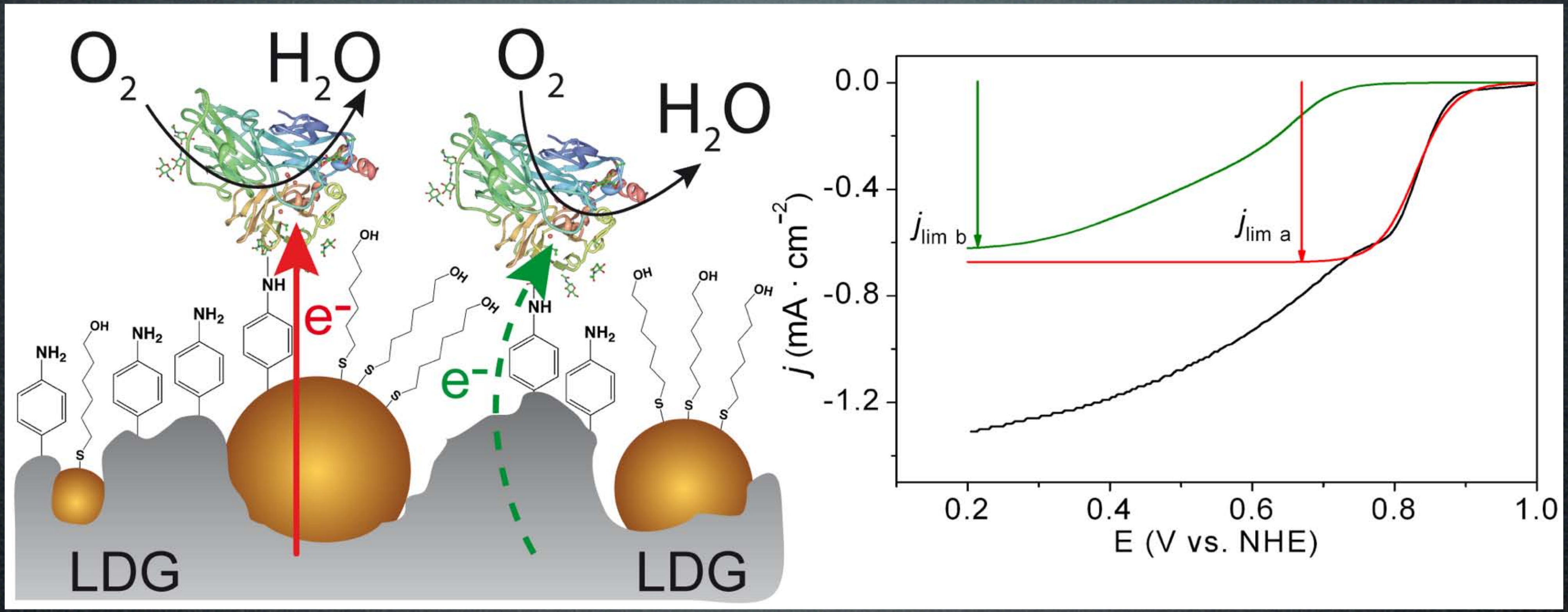
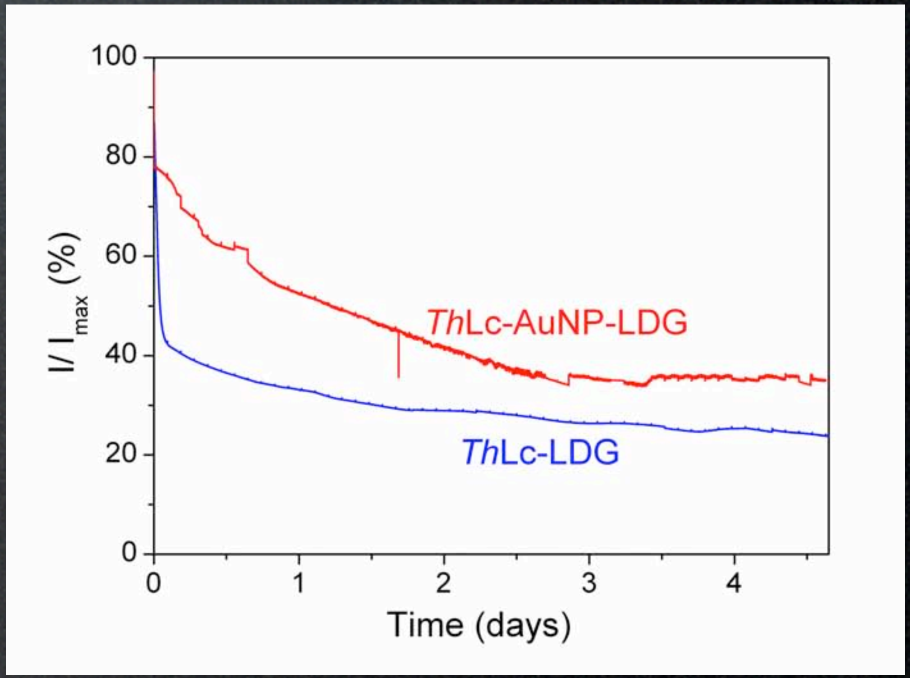
Laccase-Au-LDG: Electronic bridges



- Two electrochemical processes:
  - AuNP (5nm)-wired laccase: Nernstian process E_a= 0.83V vs. NHE and $j_{lim\ a}$ = -0.697 mA·cm^{-2}.
  - LDG-wired laccase: Leger-distribution process Eb=-0.67V and $j_{lim\ b}$ = -0.628 mA·cm^{-2}.
- Higher Operational stability than LDG-laccase



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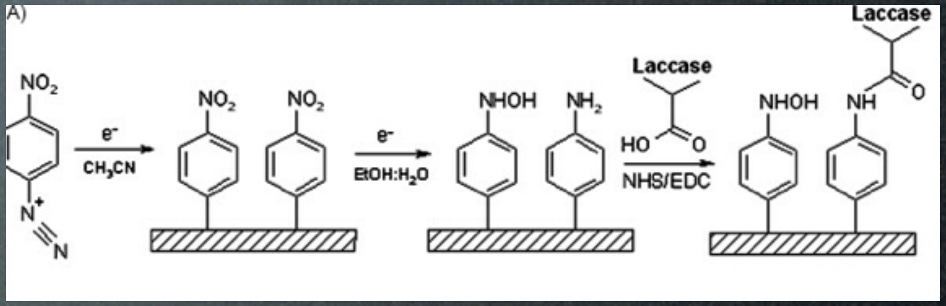
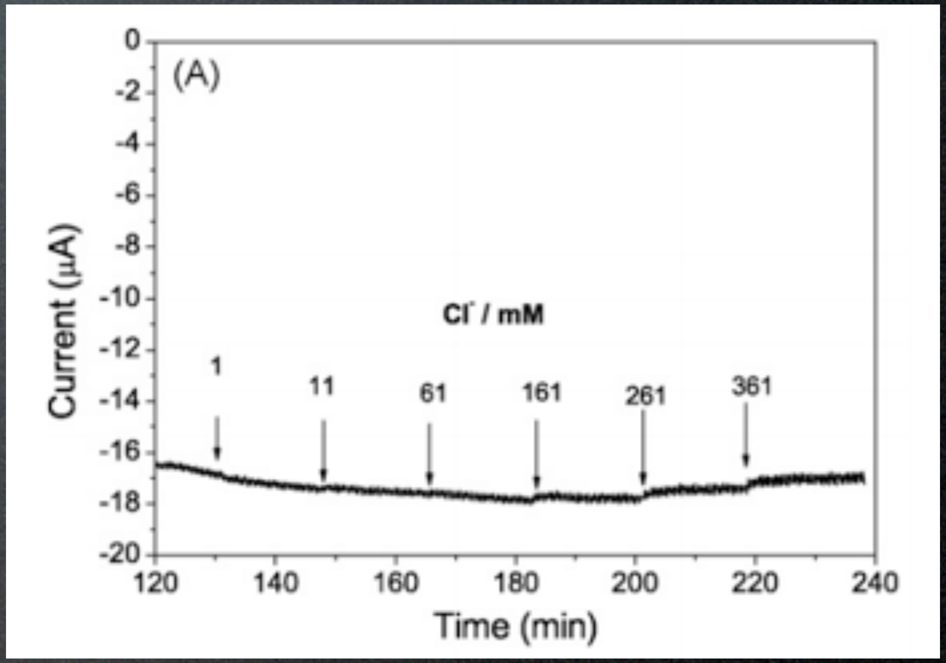
Chloride inhibition

- Chloride is a laccase inhibitor present in most biofluids, binding T1 site. Immobilization seeks to minimize this drawback.



- Chloride effect:
- **LDG amide**: negligible $[Cl^-]$ = 150 mM



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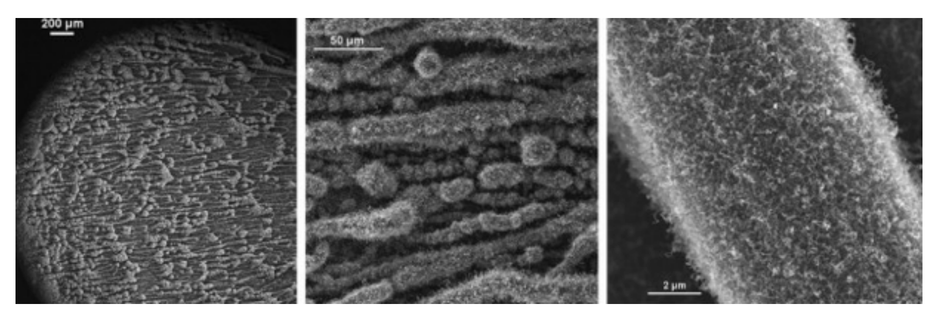

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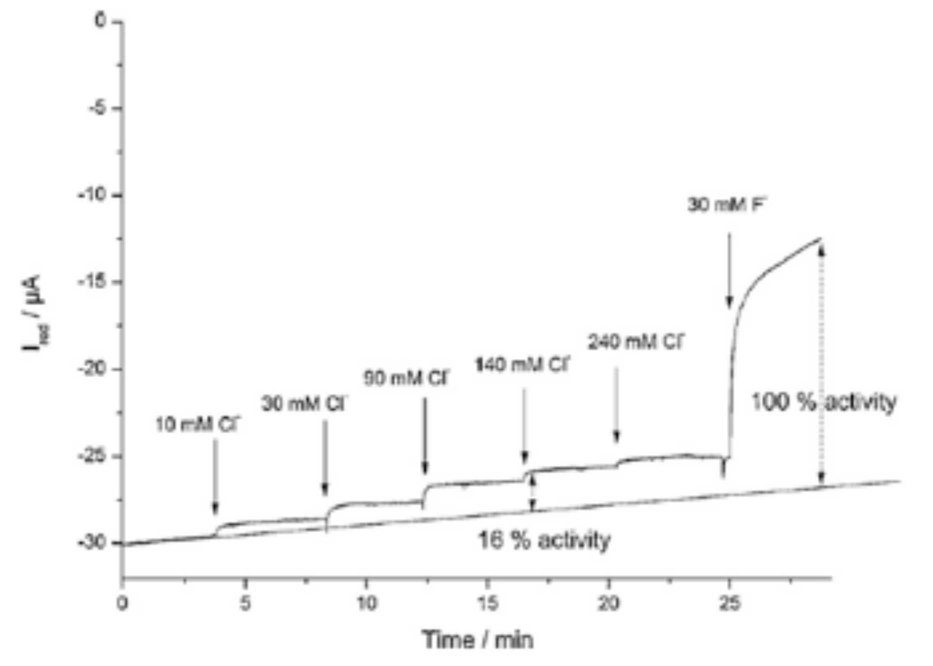
- **CNT/CMF**: -16% $[Cl^-]$ = 140 mM



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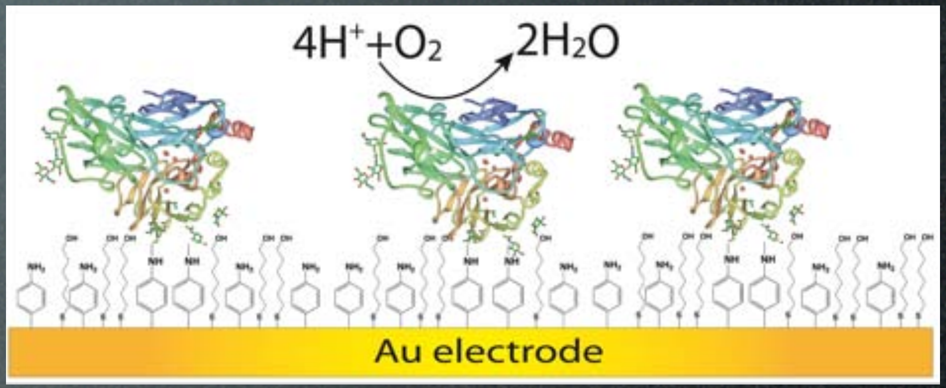

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- **Gold**: -58% [Cl$^-$] = 28 mM

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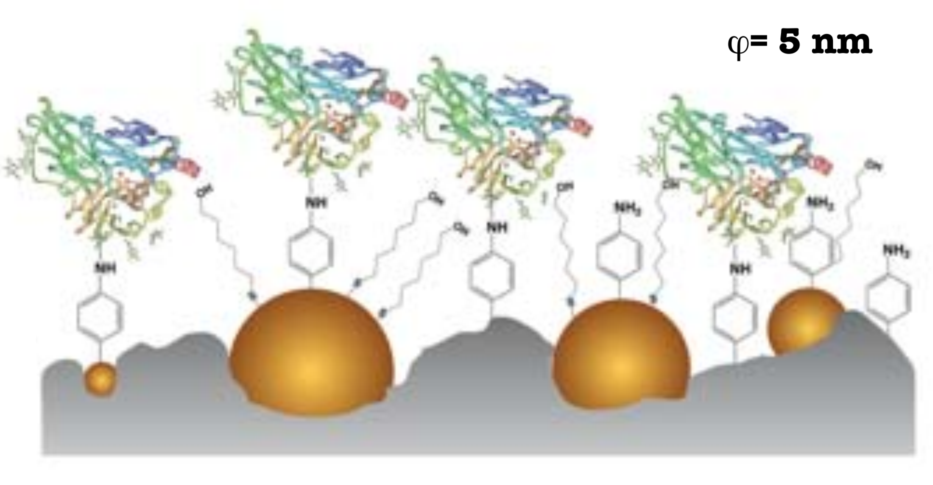
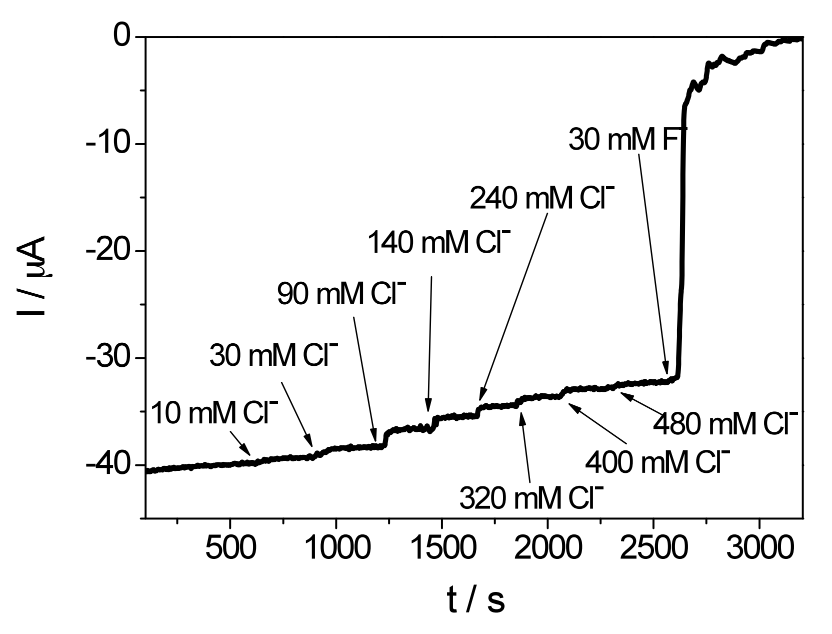
- **LDG-AuNP 5 nm**: -16% [Cl$^-$] = 140 mM





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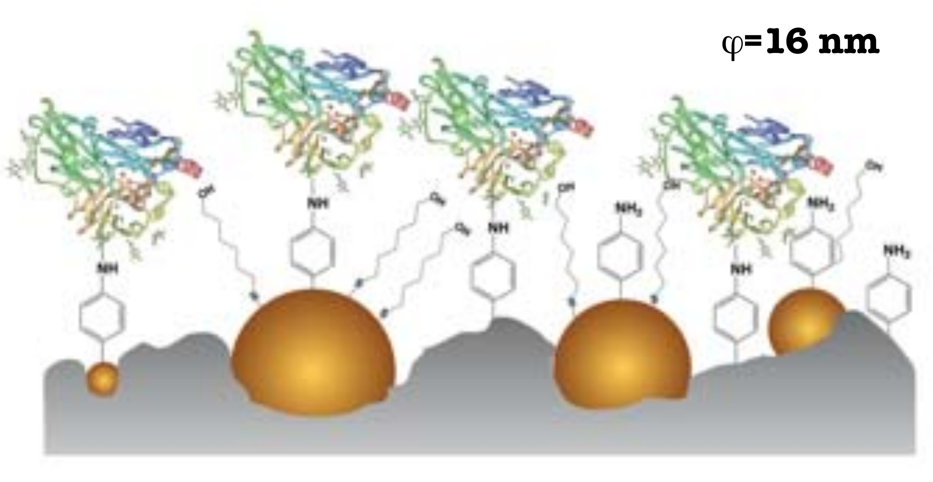

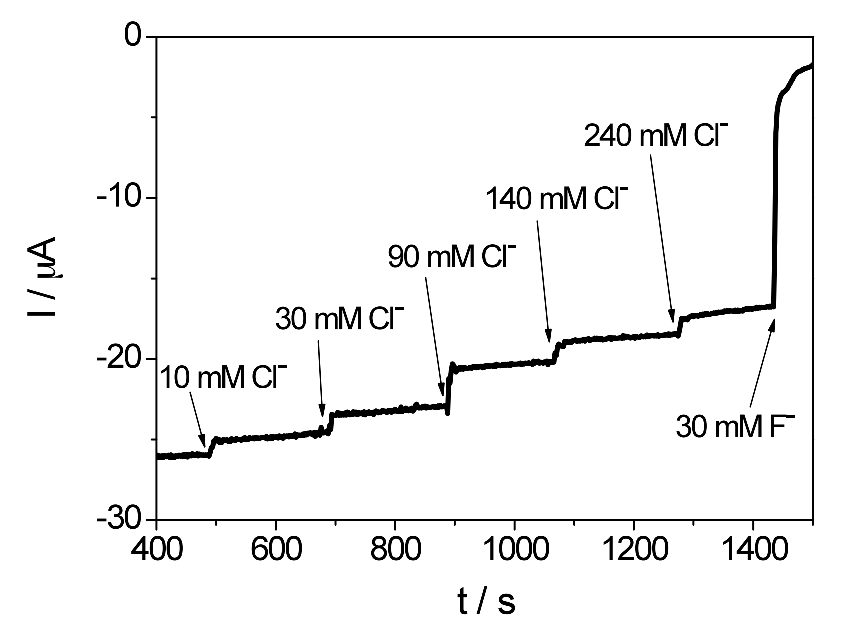

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- **LDG-AuNP 5 nm**: -16% $[Cl^-]$ = 140 mM
- **LDG-AuNP 16 nm**: -25% $[Cl^-]$ = 140 mM

Approach to physiological pH conditions

- Laccase optimal working pH is acidic: activity at neutral pH is negligible.
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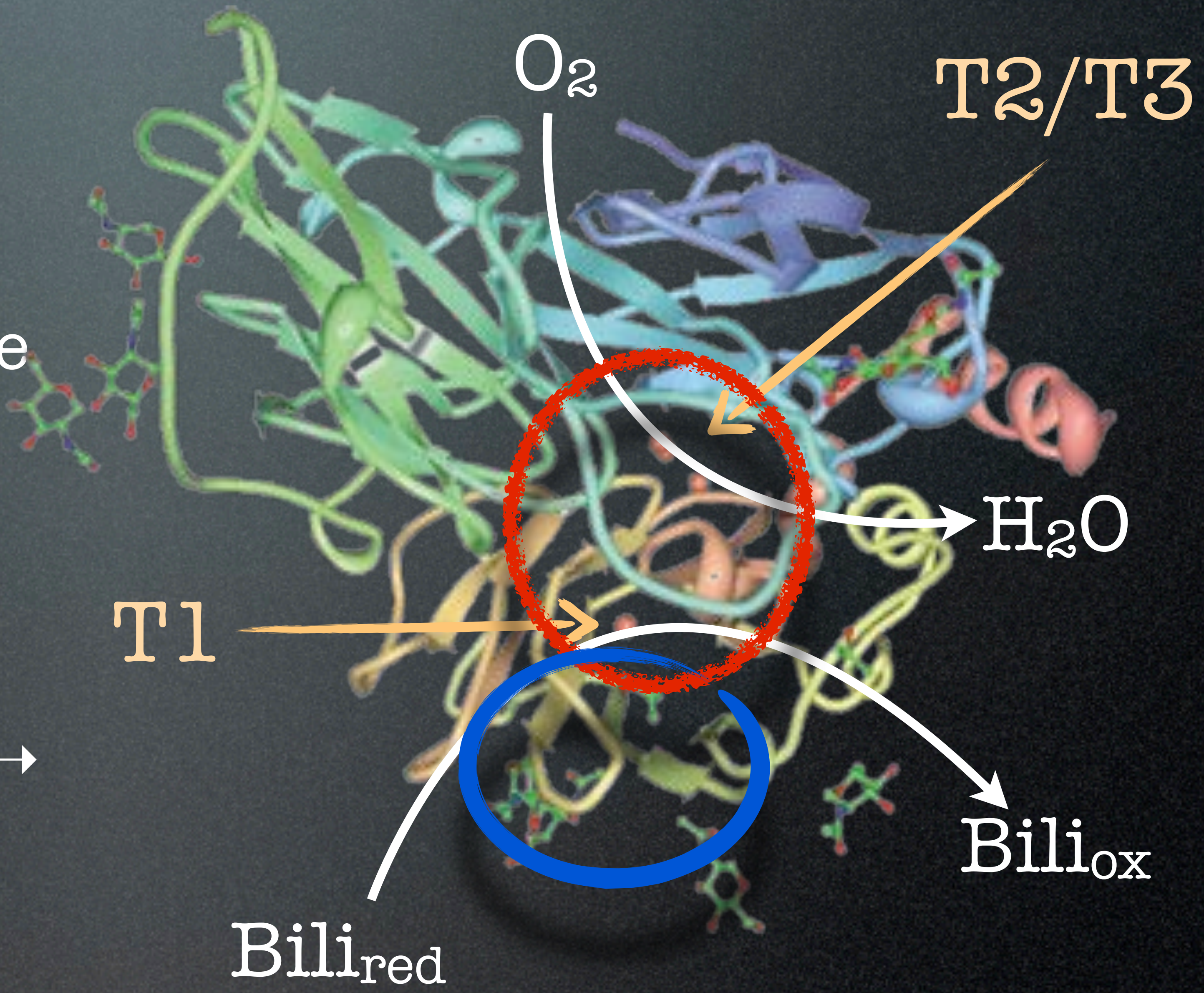
2. Transform Local Environment
3. Different catalyst (BOx)

Bilirubin Oxidase (BOx)

- Multicopper oxidase with T1 and T2/T3 active site (≈ Laccase).
- Different coordination sphere for the Cu sites:
    - Enhance Cl- resistance
    - Lowers T1 redox potential
- Natural substrate: bilirubin → Changes in T1 access channel.



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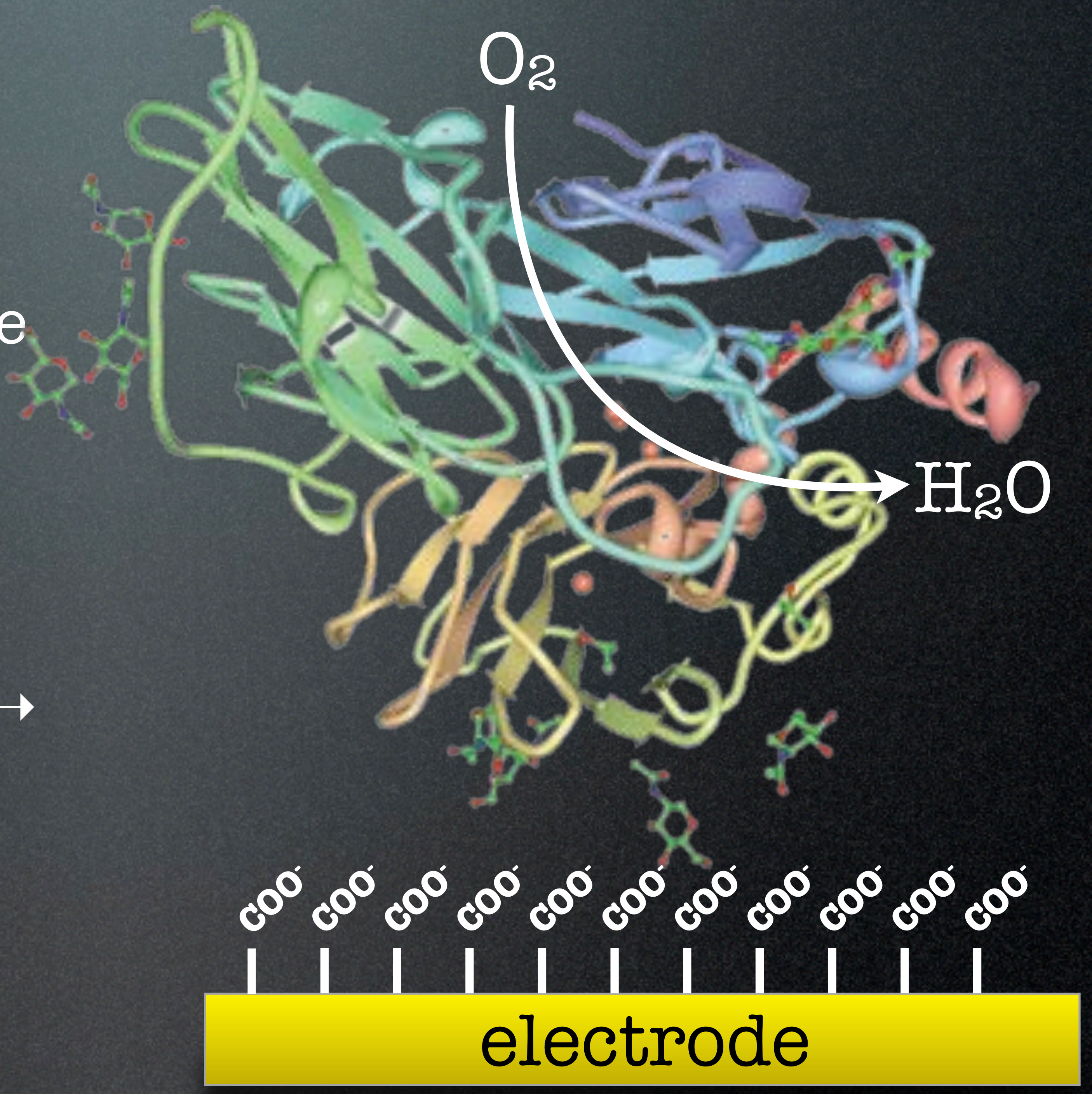
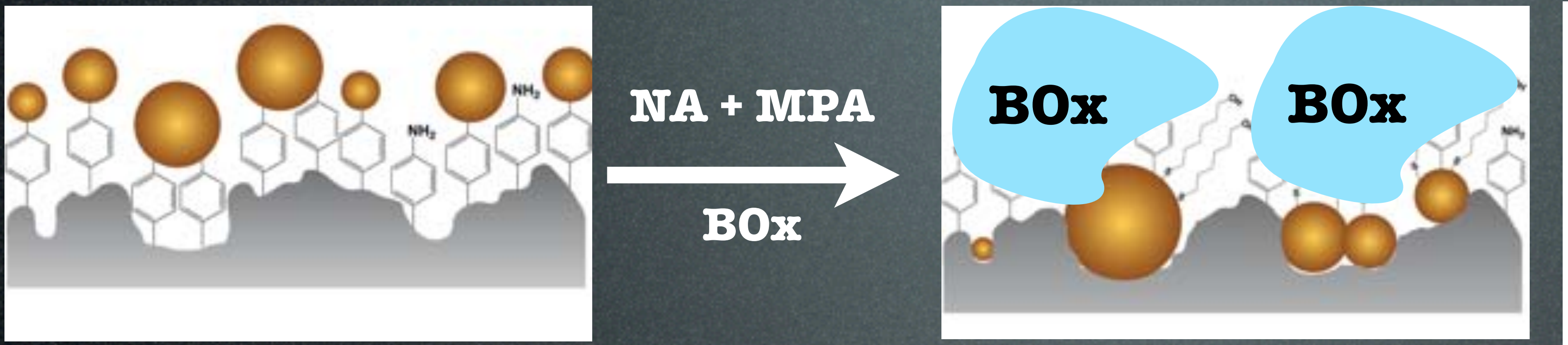
  - Lowers T1 redox potential
- Natural substrate: bilirubin → Changes in T1 access channel.
- Different immobilization strategy: negatively charged electroactive surface.



BOx biocathode



- LDG electrode loaded with AuNP.
- Mixed monolayer: (1) diazotation of 6-amino-2- naphtoic acid (2) mercaptopropionic acid.
- Oriented BOx performs in phosphate and serum-mimic buffer.
- Potential given: 200 mV lower than laccase.

Gutierrez-Sanchez, C.; Pita, M.; Toscano, M.D.; De Lacey, A.L. Electroanalysis 2013, 25 (6), 1359-1362.

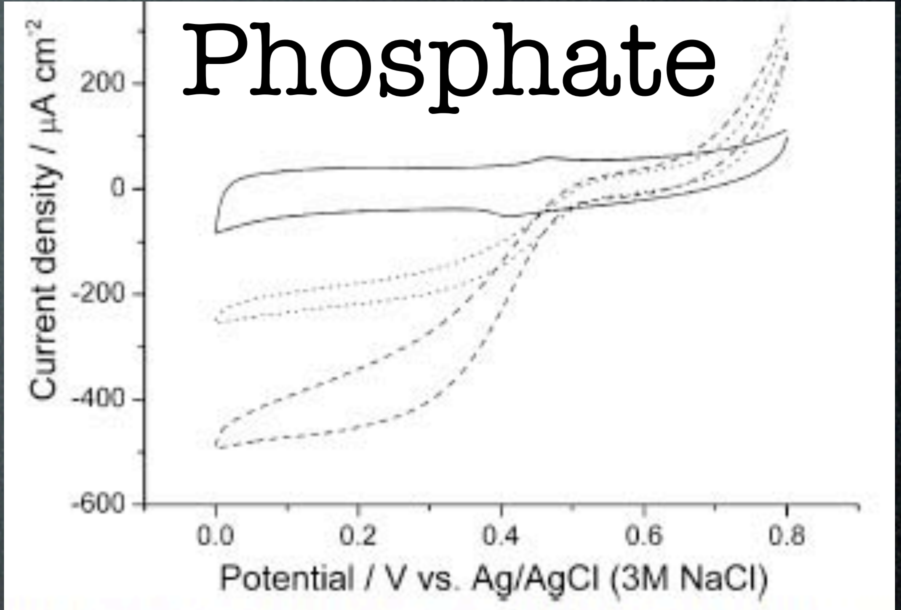


Fig. 2. Cyclic voltammograms of a BOx-AuNp-LDG electrode in phosphate buffer 100 mM, pH 7.4 at 10 mV s^{-1} scan rate and 500 rpm: under air (dotted line); 1 atm of O_2 (dashed line); background signal after inhibition with 30 mM NaF (solid line).

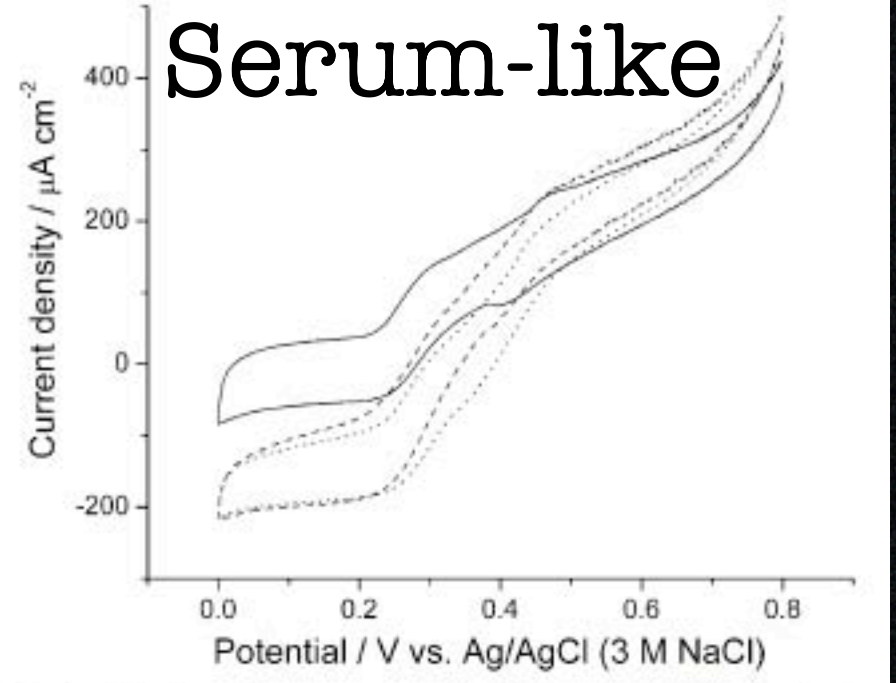
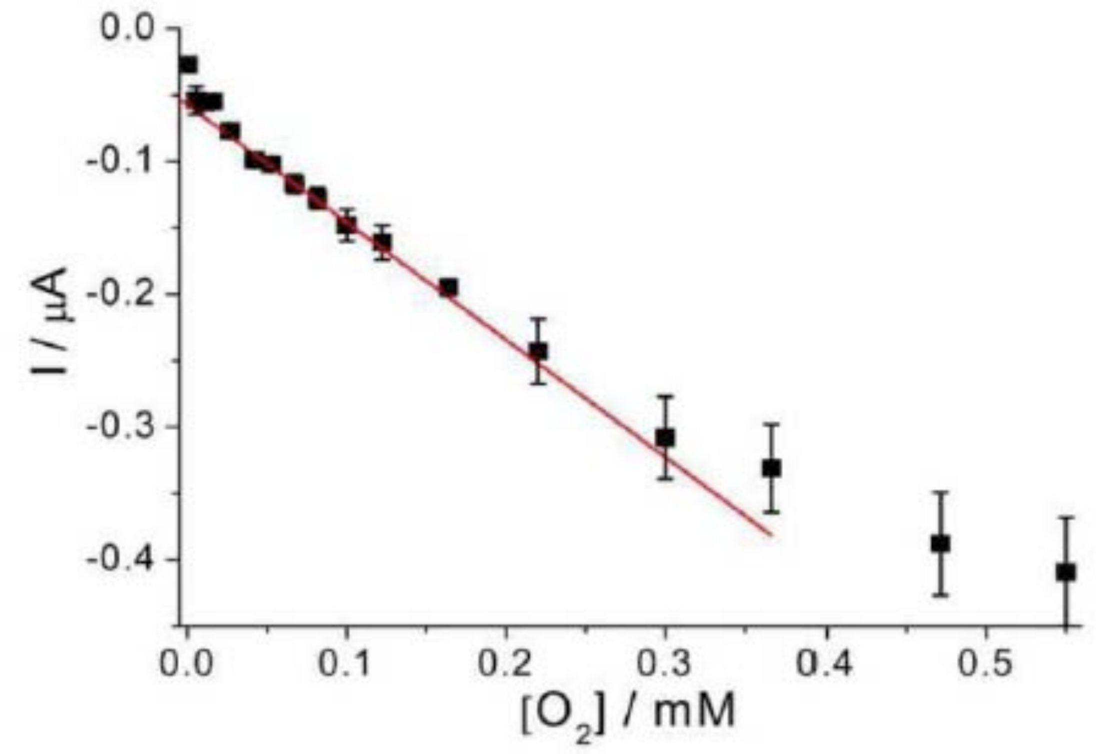
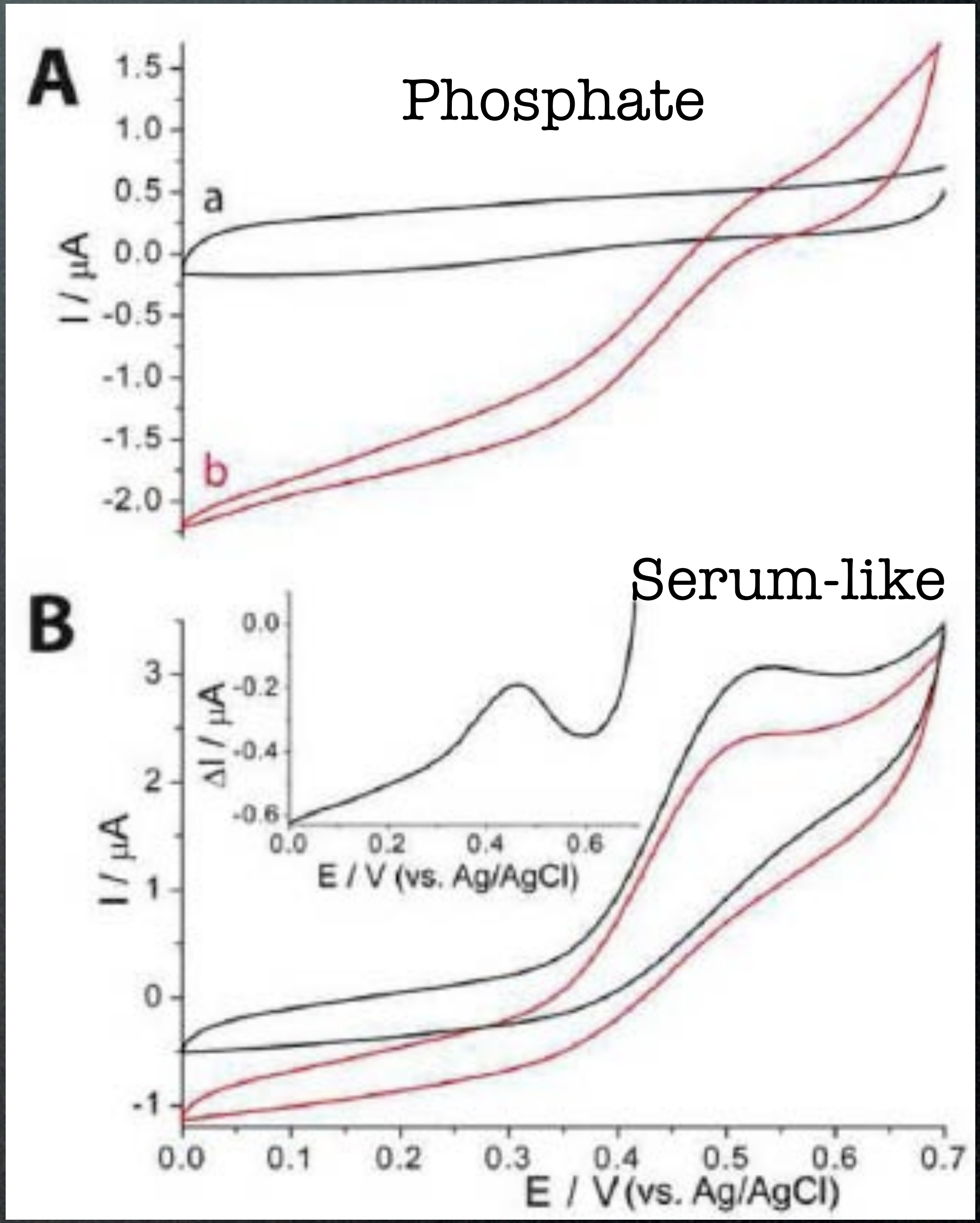


Fig. 3. Cyclic voltammograms of a BOx-AuNp-LDG electrode in serum mimic buffer at 10 mV s^{-1} scan rate and 500 rpm electrode rotation: under air (dotted line); 1 atm of O_2 (dashed line); background signal after inhibition with 30 mM NaF (solid line).

BOx biosensor



$I\ (mA) = -0.054 - (0.9 \pm 0.1)\ [O_2]\ (mM)$



- Au electrode enhanced with AuNP.
- Mixed monolayer: (1) diazotation of 6-amino-2-naphtoic acid (2) mercaptopropionic acid.
- Oriented BOx performs in phosphate and serum-mimic buffer.
- Measure O_2 at +100 mV vs. Ag/AgCl from 6 to 300 µM

Pita, M; Gutierrez-Sanchez, C.; Toscano, M.D.; Shleev, S.; De Lacey, A.L. Bioelectrochemistry 2013, in press.

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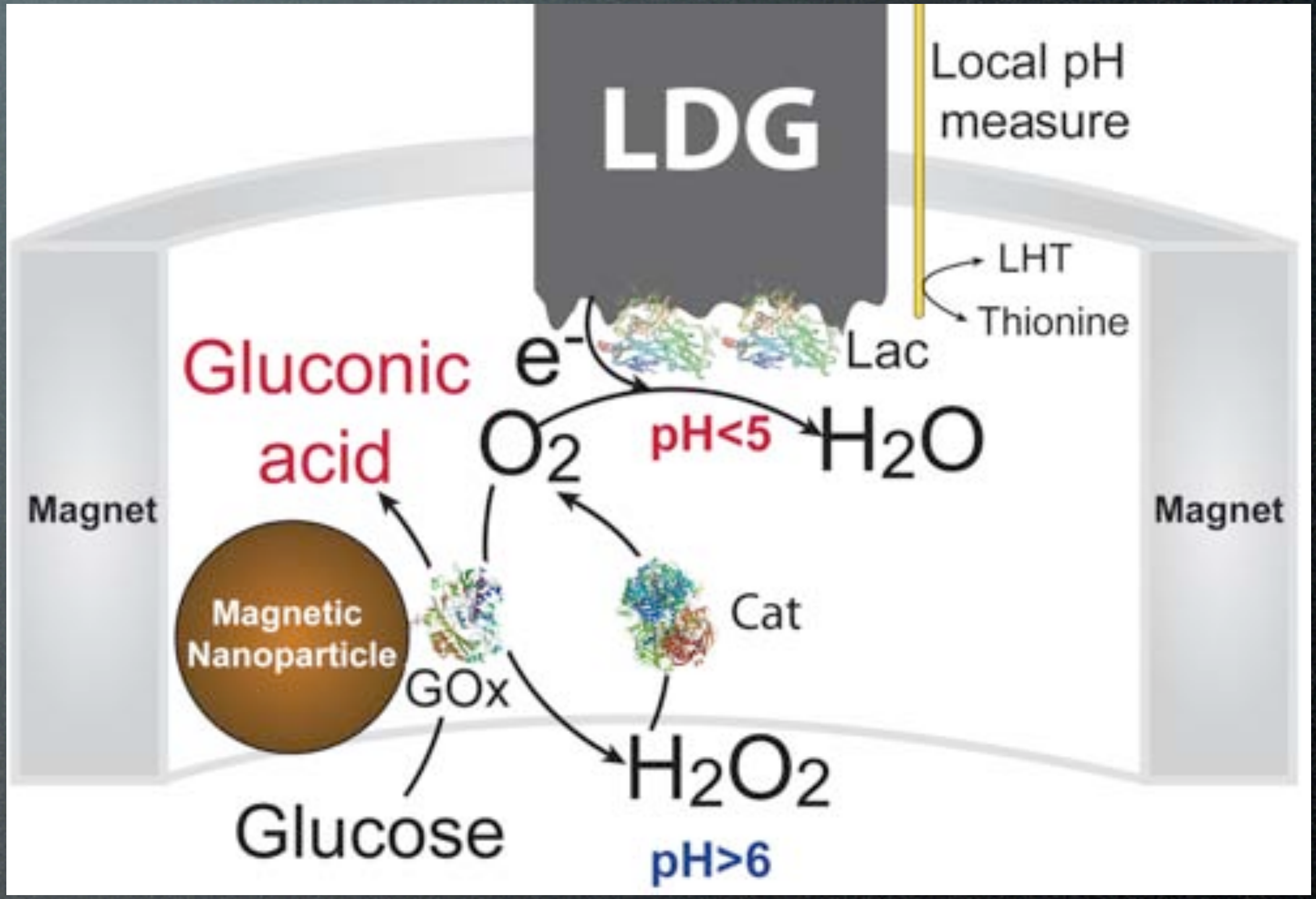
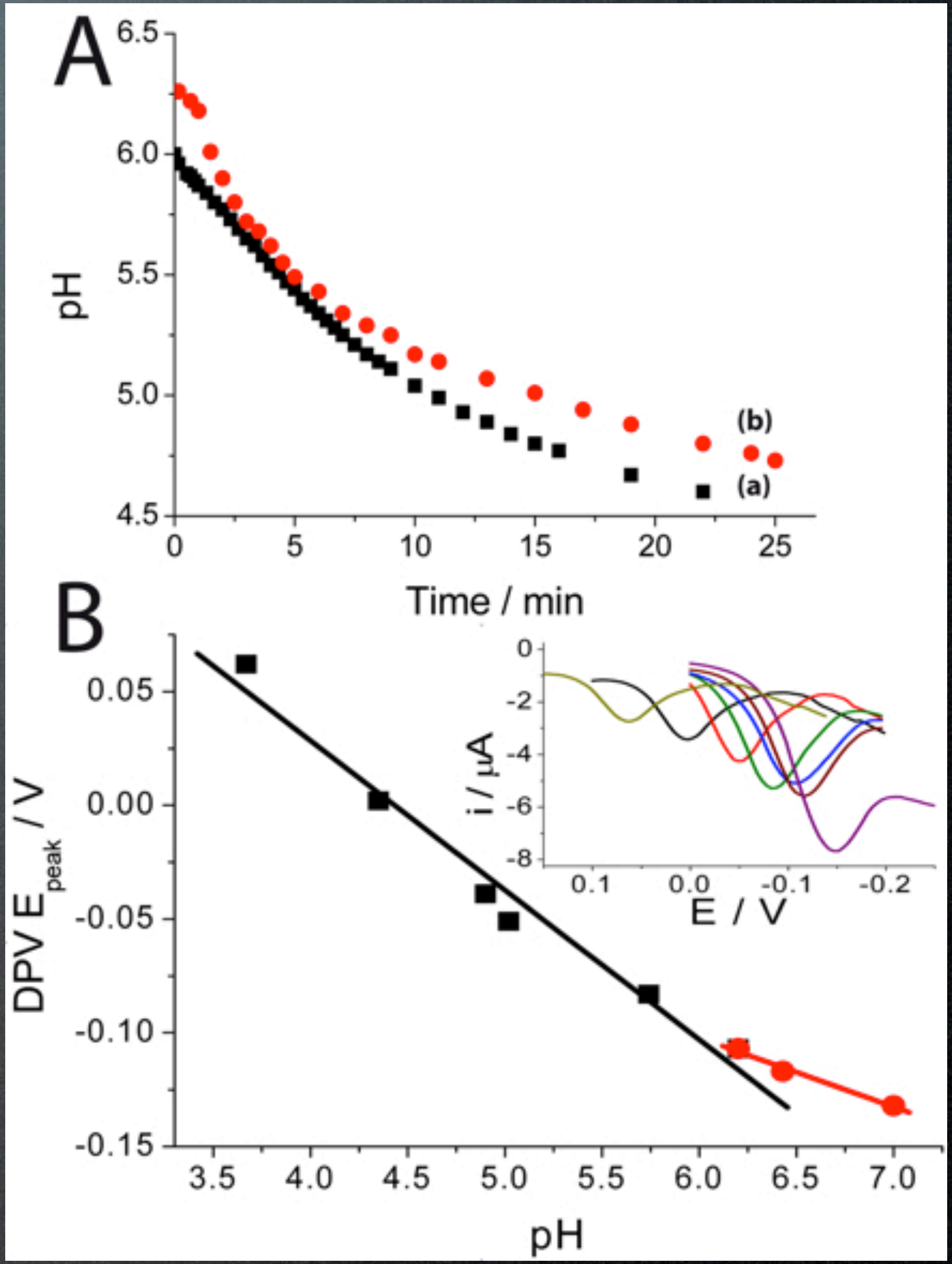
Local pH acidification

- Generate in the electrode surround a local pH more acid than bulk pH
- Collect magnetic nanoparticles loaded with GOx in the electrode with a magnet: produce gluconic acid only close to the electrode.
- Competition for O_2: add catalase
- Local pH measured with thionin (pH-sensitive probe) and a separated gold electrode



Clot, S.; Gutierrez-Sanchez, C.; Shleev, S.; De Lacey, A.L.; Pita, M. Electrochem. Commun. 2012, 18, 37 - 40

System characterization and results



(A) ΔpH vs. t. (a) GOx in solution. (b) attached to magnetic nanoparticles.

(B) Titration of thionin vs. pH. Thionin starts to deprotonats at pH>6, thus responding to two different straight lines. Inset: DPV measures for the titration.

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