PROTEIN-FILM VOLTAMMETRY-ELECTROCHEMICAL SPECTROSCOPY FOR PROBING THE REDOX FEATURES OF BIOCATALYSTS

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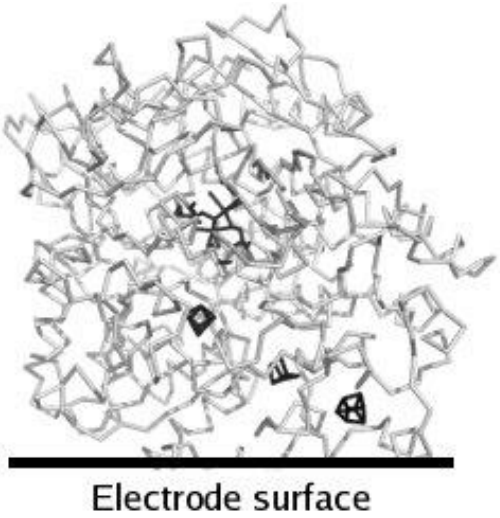
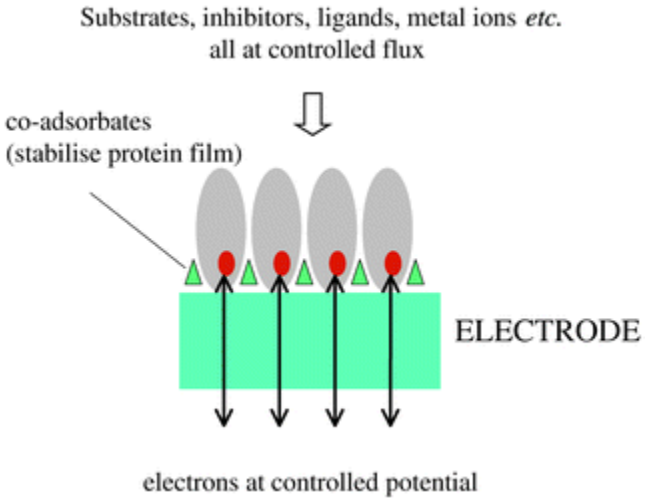




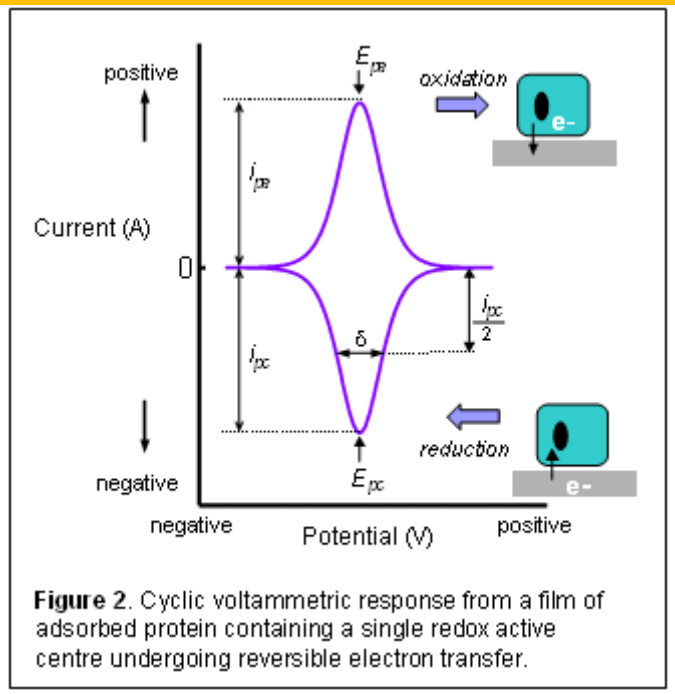
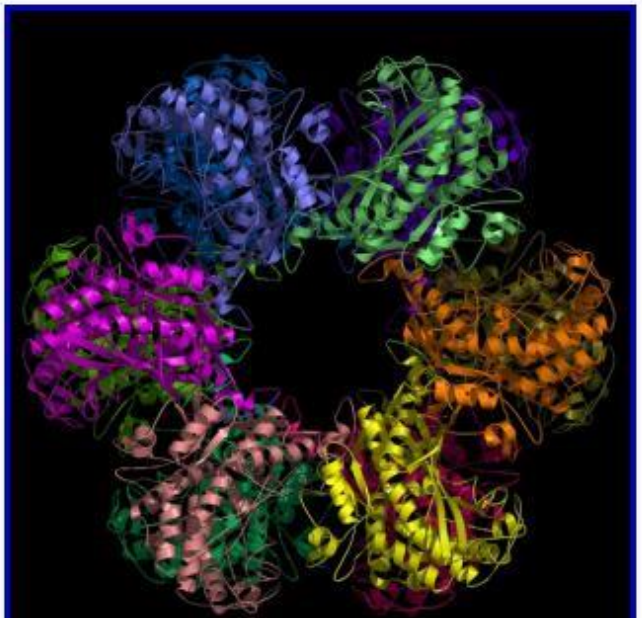
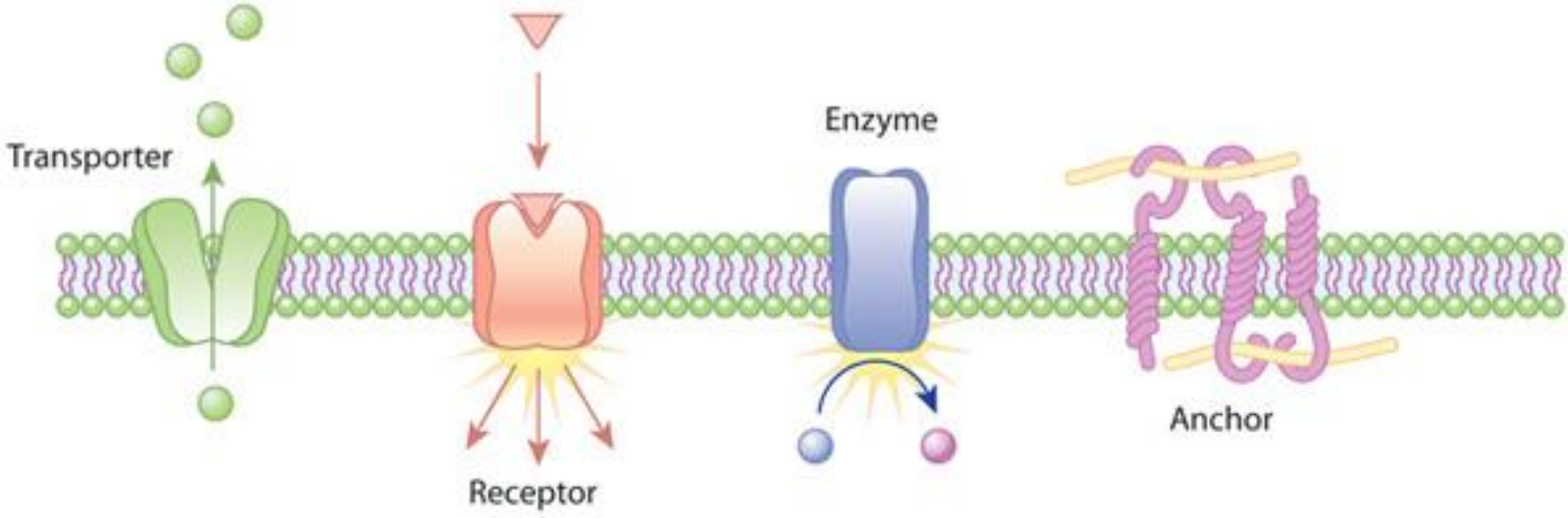


Figure 2. Cyclic voltammetric response from a film of adsorbed protein containing a single redox active centre undergoing reversible electron transfer.

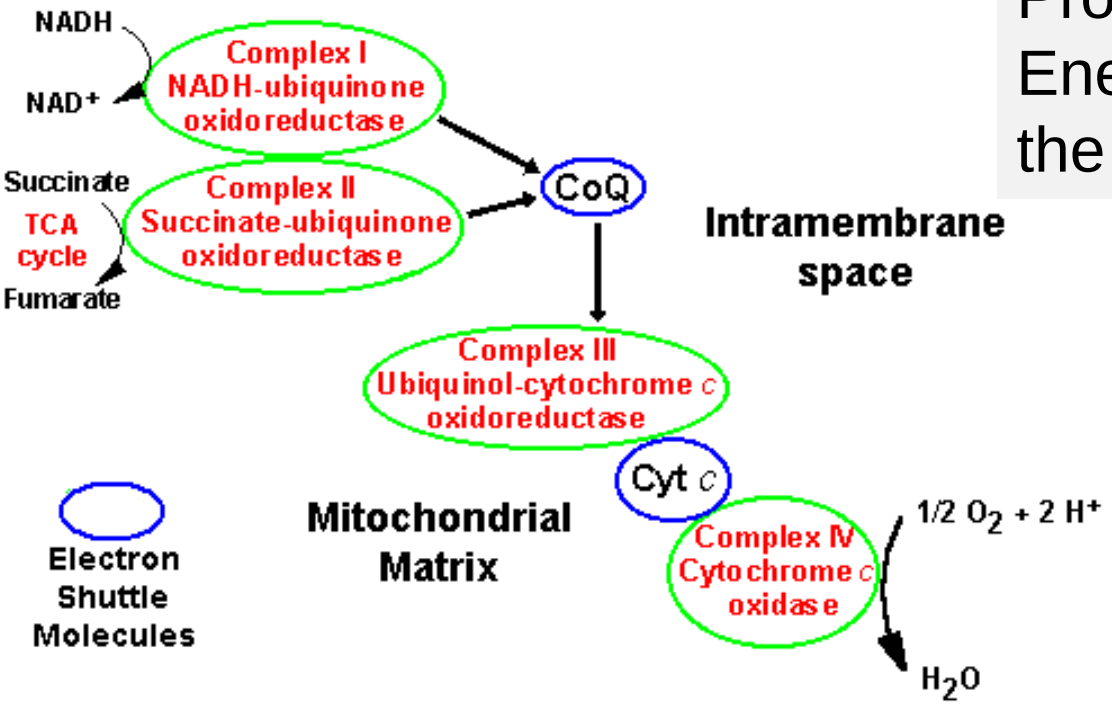
Protein functions

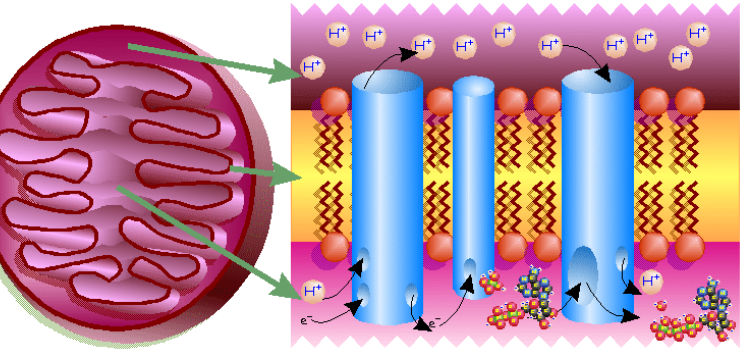
- Structural – muscle, skin, hair
- Signalling – insulin, growth hormone, EPO
- Catalysts – enzymes
- Immunity – antibodies
- Regulation – DNA-binding proteins
- Poisons – toxins in snakes/spiders etc
- Transport – **hemoglobin**

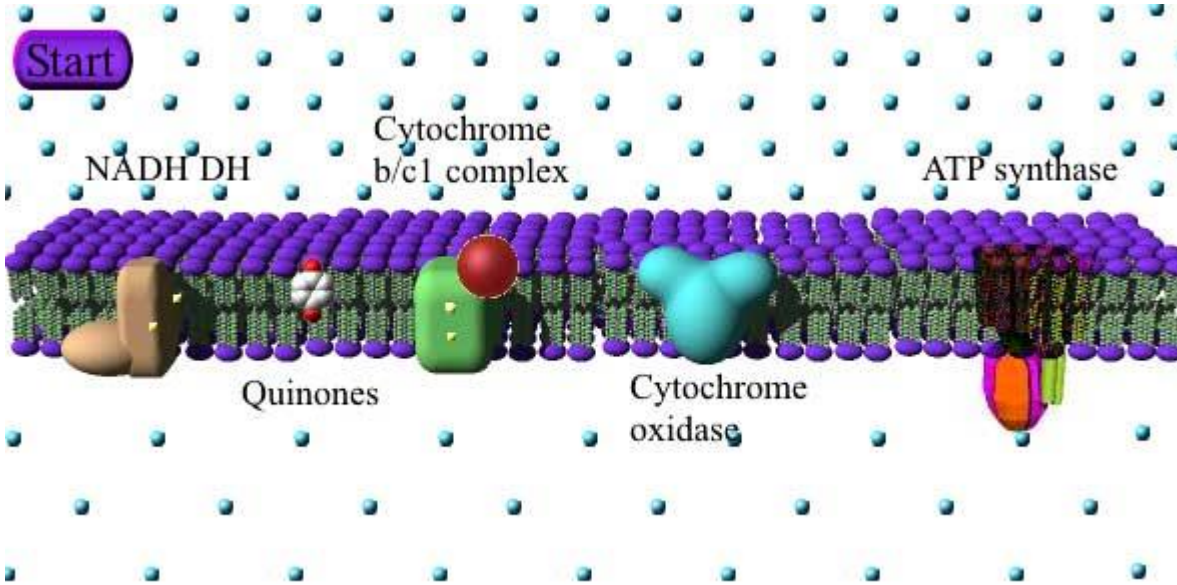




Proteins play crucial role in Energy conversion and the ATP synthesis

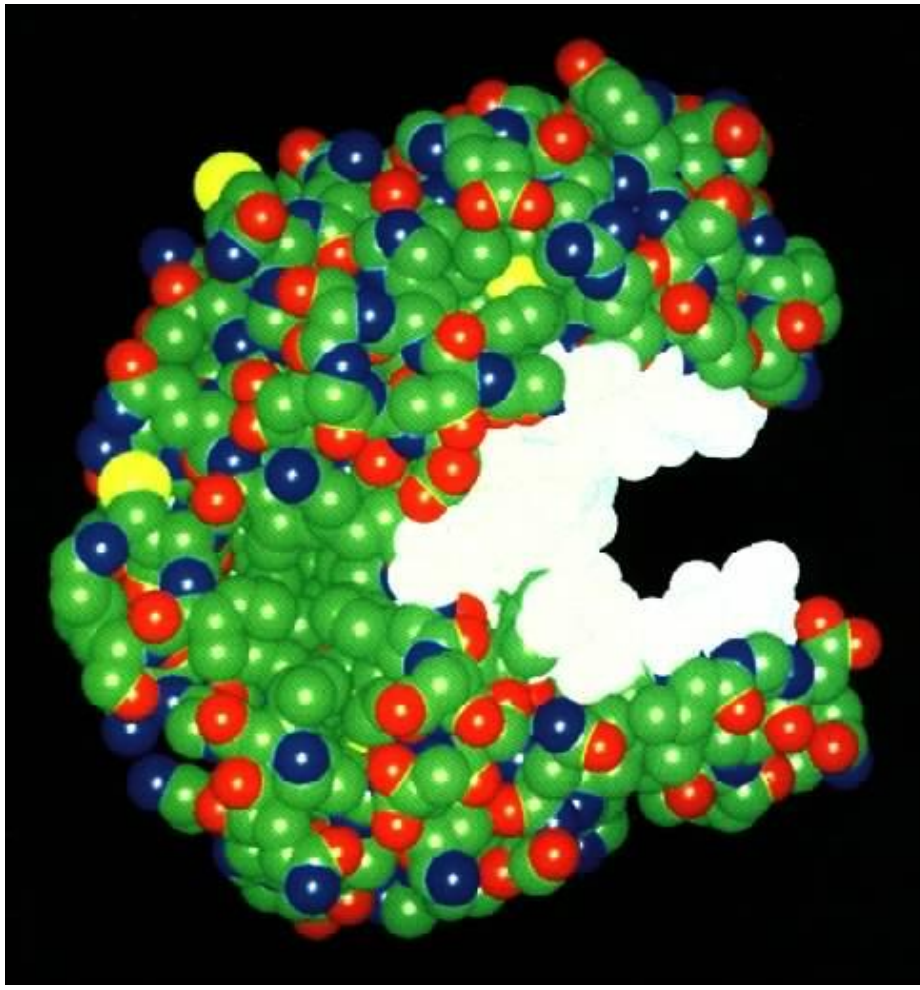






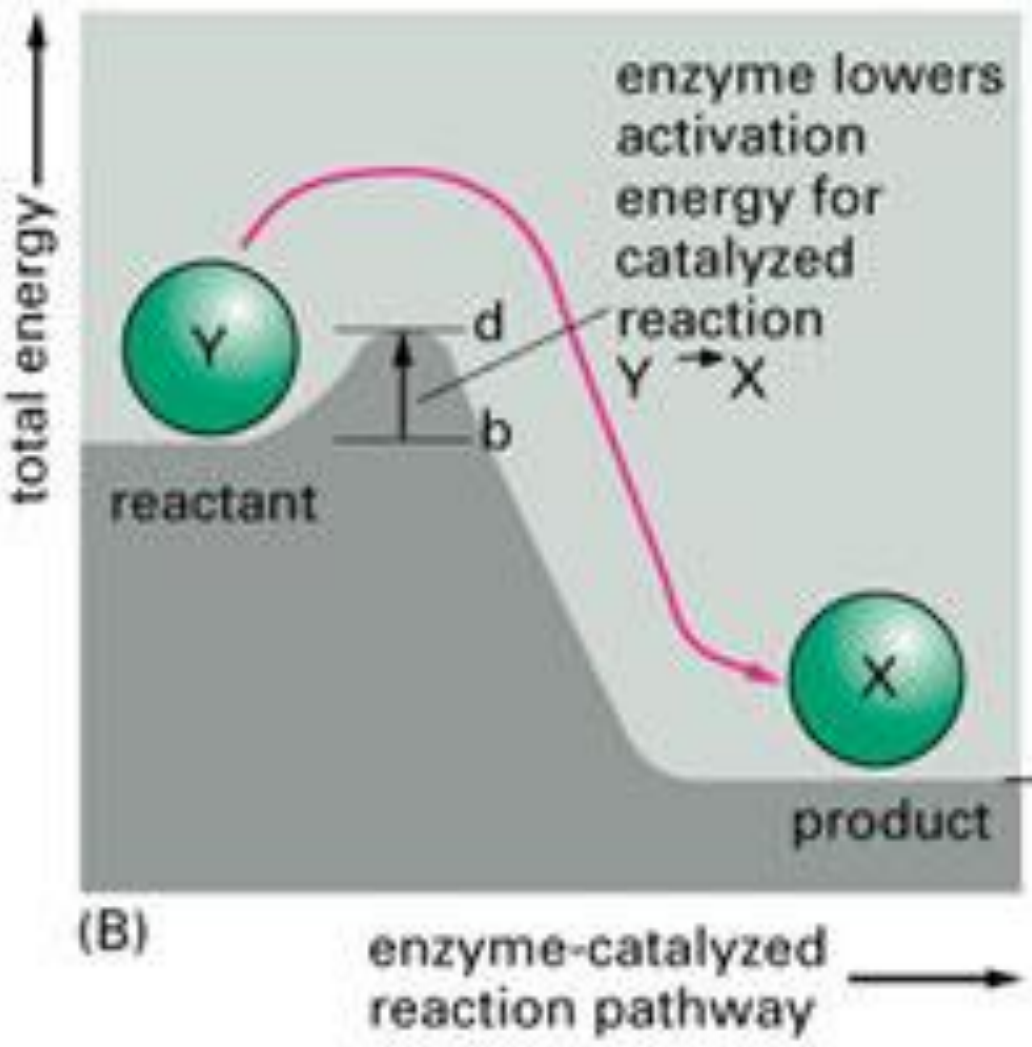
Special group of Proteins are the Enzymes

- Almost all enzymes are **Proteins** (tertiary and quaternary structures)
- Act as **Catalyst** to accelerates a reaction
- **Not permanently** changed in the process

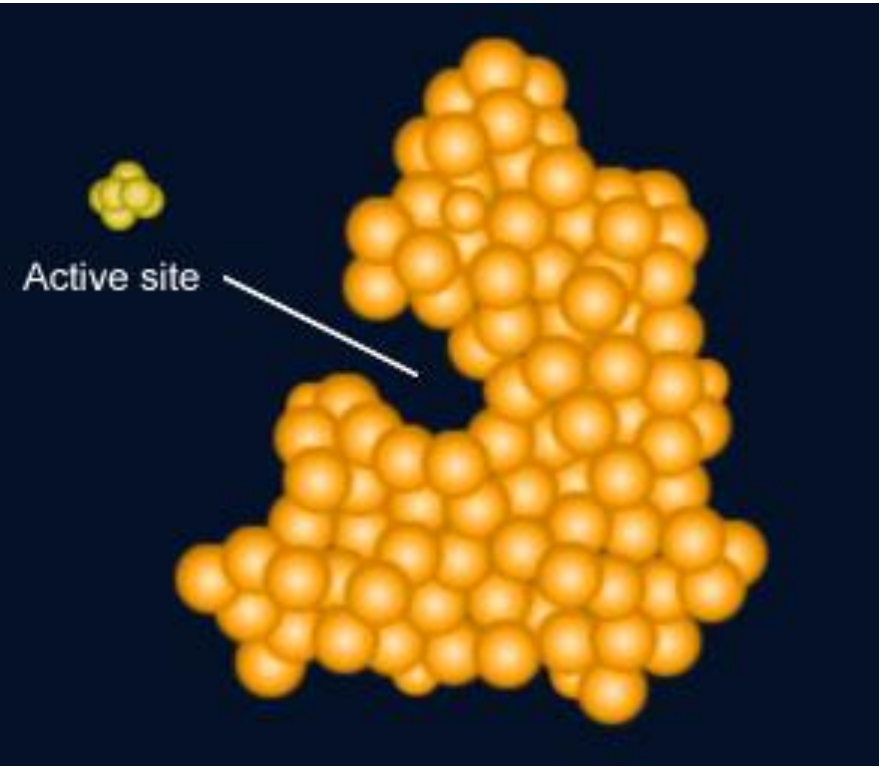


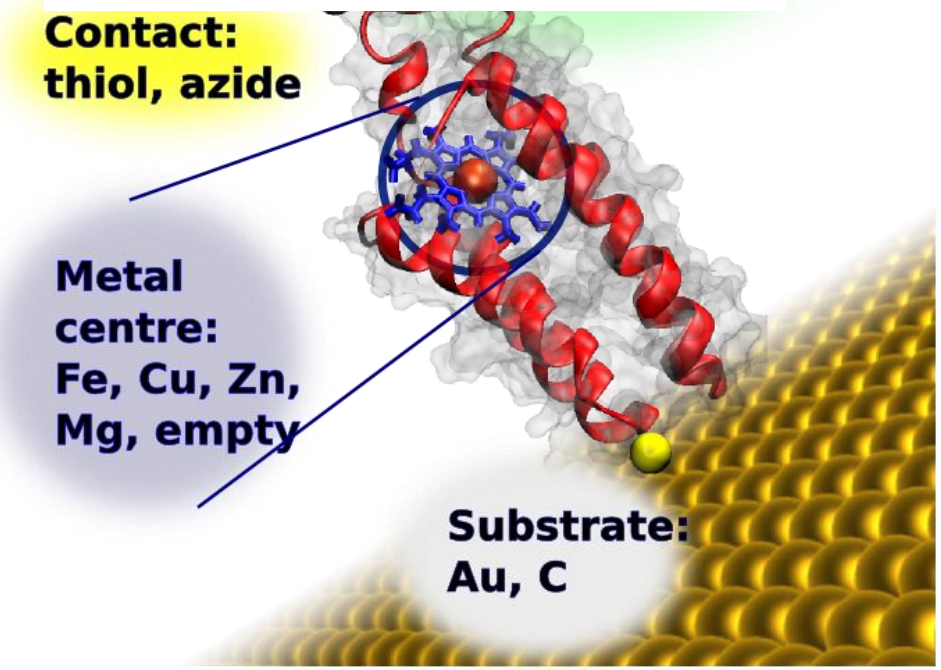
Enzymes work by

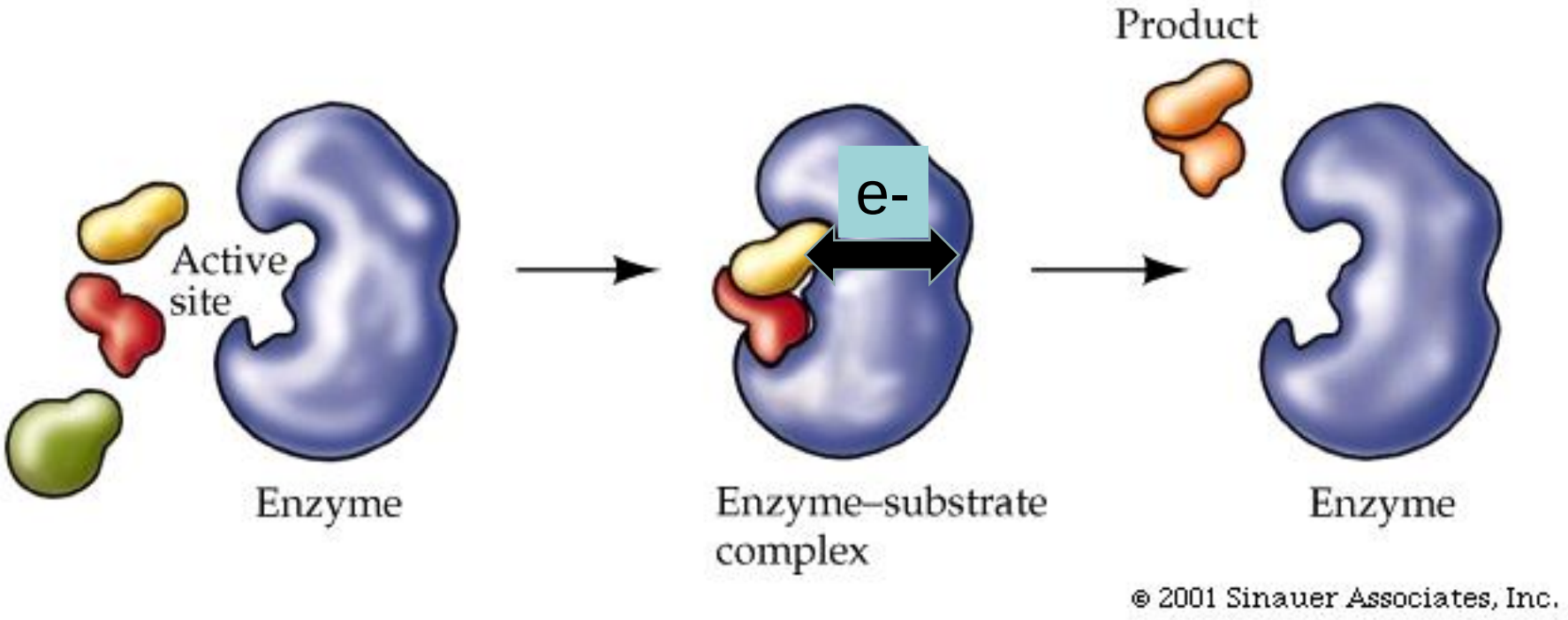
weakening chemical bonds of the Substrates (reactants) which lowers activation energy



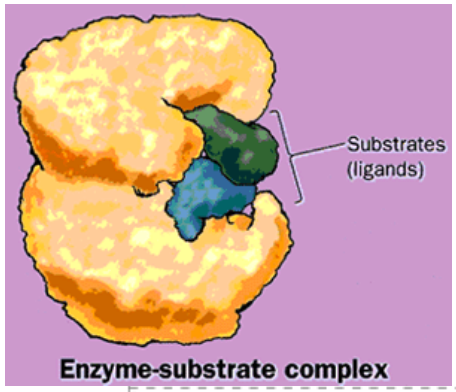
Many of the natural enzymes contain a ***redox-active center*** that exchanges electrons with a specific **substrate**

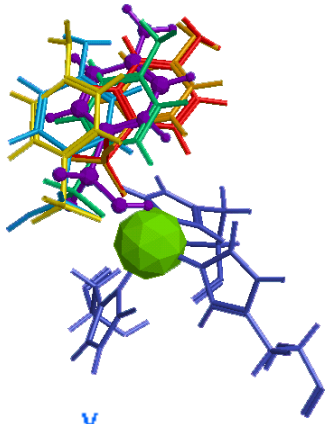




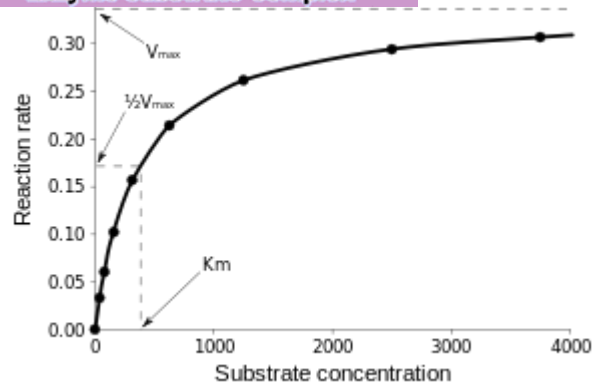


If we get insight into the Enzyme-Substrate electron-exchange reaction, than we can get access to valuable thermodynamic and kinetic parameters relevant to the enzymatic reaction studied

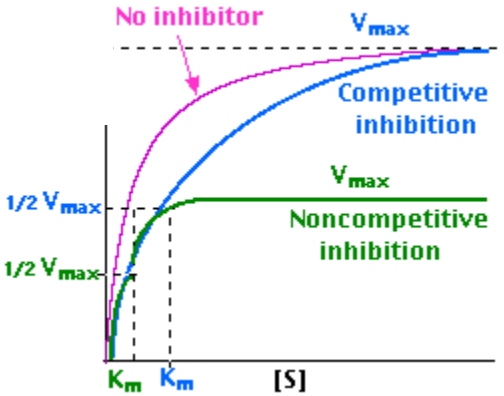




$$E + S \underset{k-1}{\overset{k+1}{\rightleftharpoons}} ES \underset{k-2}{\overset{k+2}{\rightleftharpoons}} E + P$$



$$v_1 = \frac{V_{max}[S]}{\{K_m + [S]\}}$$



We can get access to:

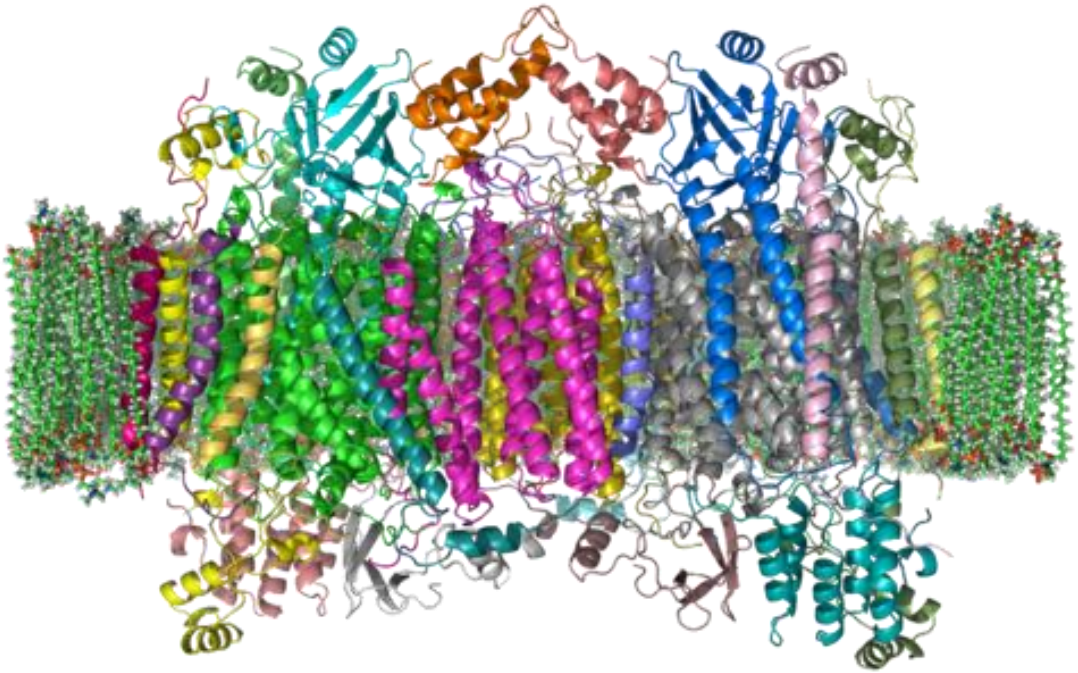
-Michaelis constant, relevant thermodynamics and kinetics parameters
-order of the reaction
-conditions affecting the enzymatic reaction
-possible inhibitors
-specificity of the enzymatic reaction
-effects of inhibitors…
-CREATING ENERGY CONVERSION SYSTEMS!!!
-…

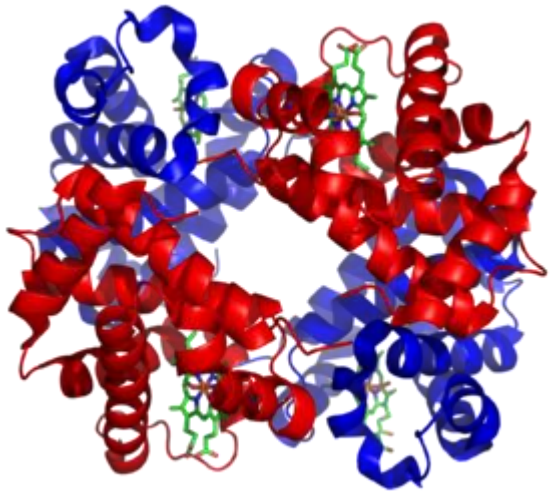
Whenever we want to study the **redox chemistry** of the ENZYMES we meet **big troubles**.

Performing electrochemistry on such bulky molecules is not an easy task

Various hindrances appear, mainly linked to the POOR WATER SOLUBILIT and INSTABILITY OF THE PROTEINS.

Physical phenomena-**adsorption, precipitation**… **limit significantly the performances of the electrochemical methods applied**





A NEW APPROACH emerged recently to study the features of the Redox enzymes.
The method is called-***PROTEIN-FILM VOLTAMMETRY*** (PFV)

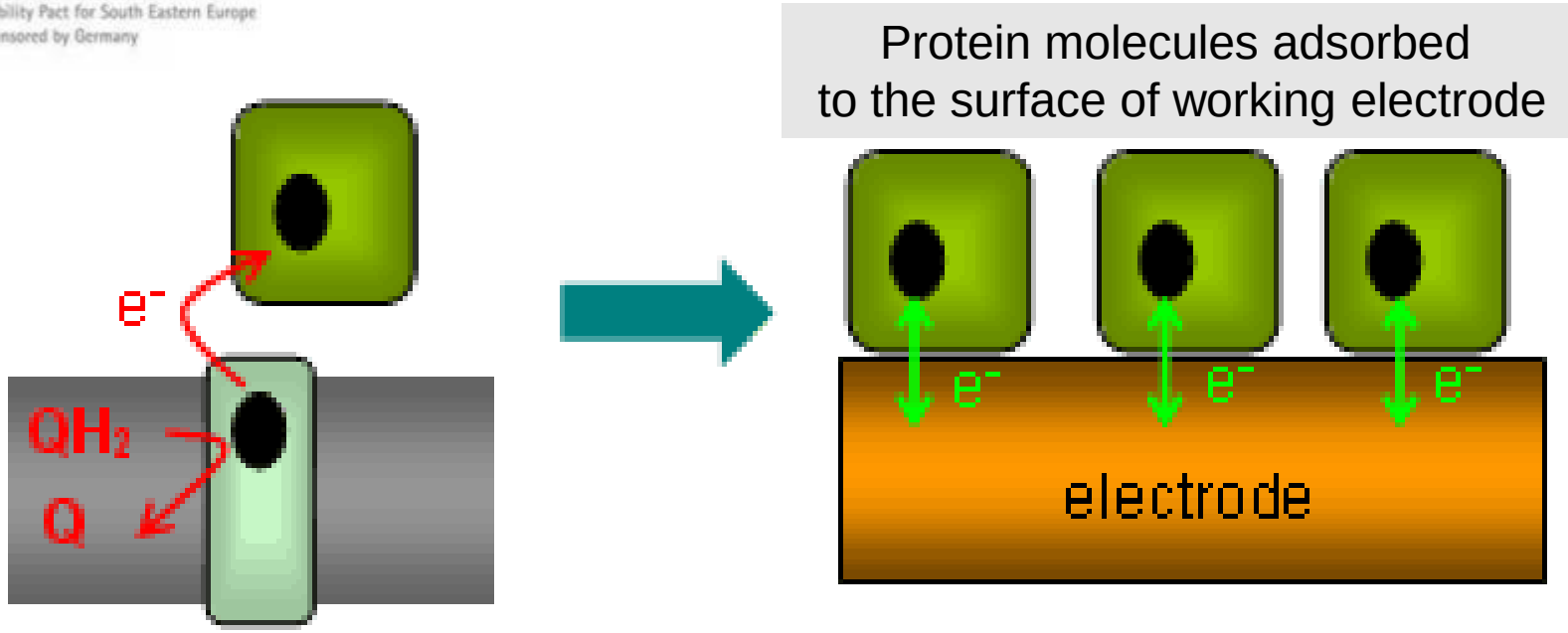
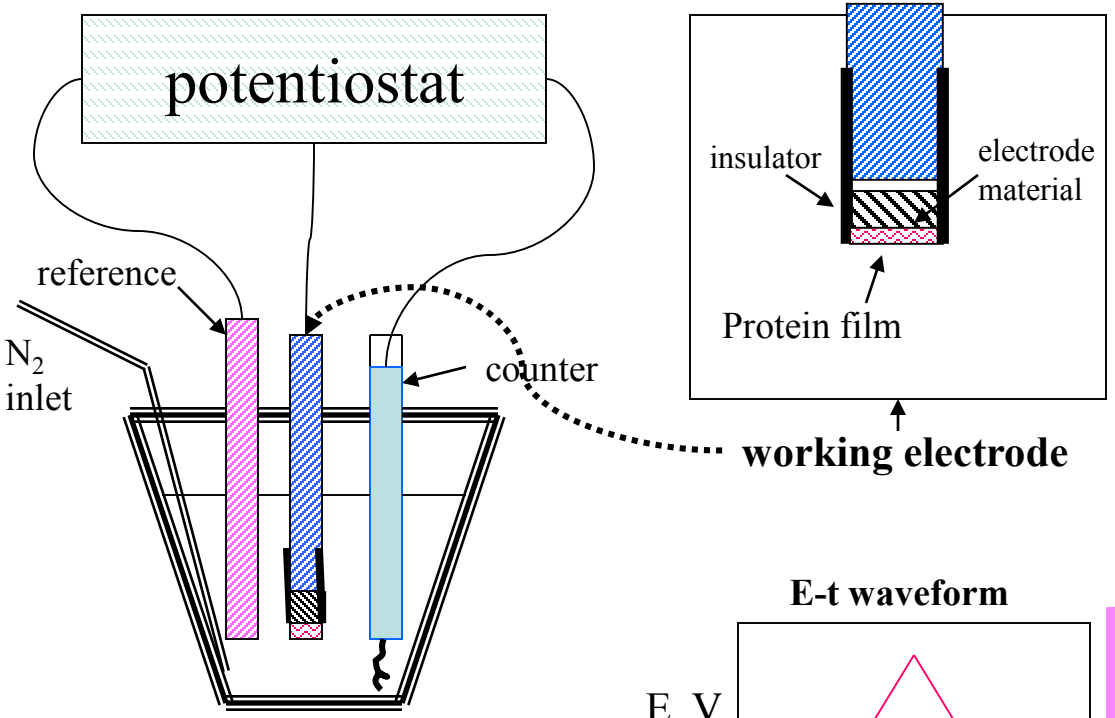
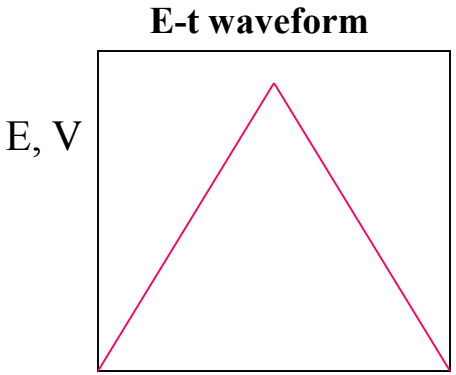


Figure 1. The PFV concept. An electrode takes the role of redox partner to a protein of interest adsorbed on its surface.

EQUIPMENT FOR PFV



Electrochemical cell



Cyclic voltammetry

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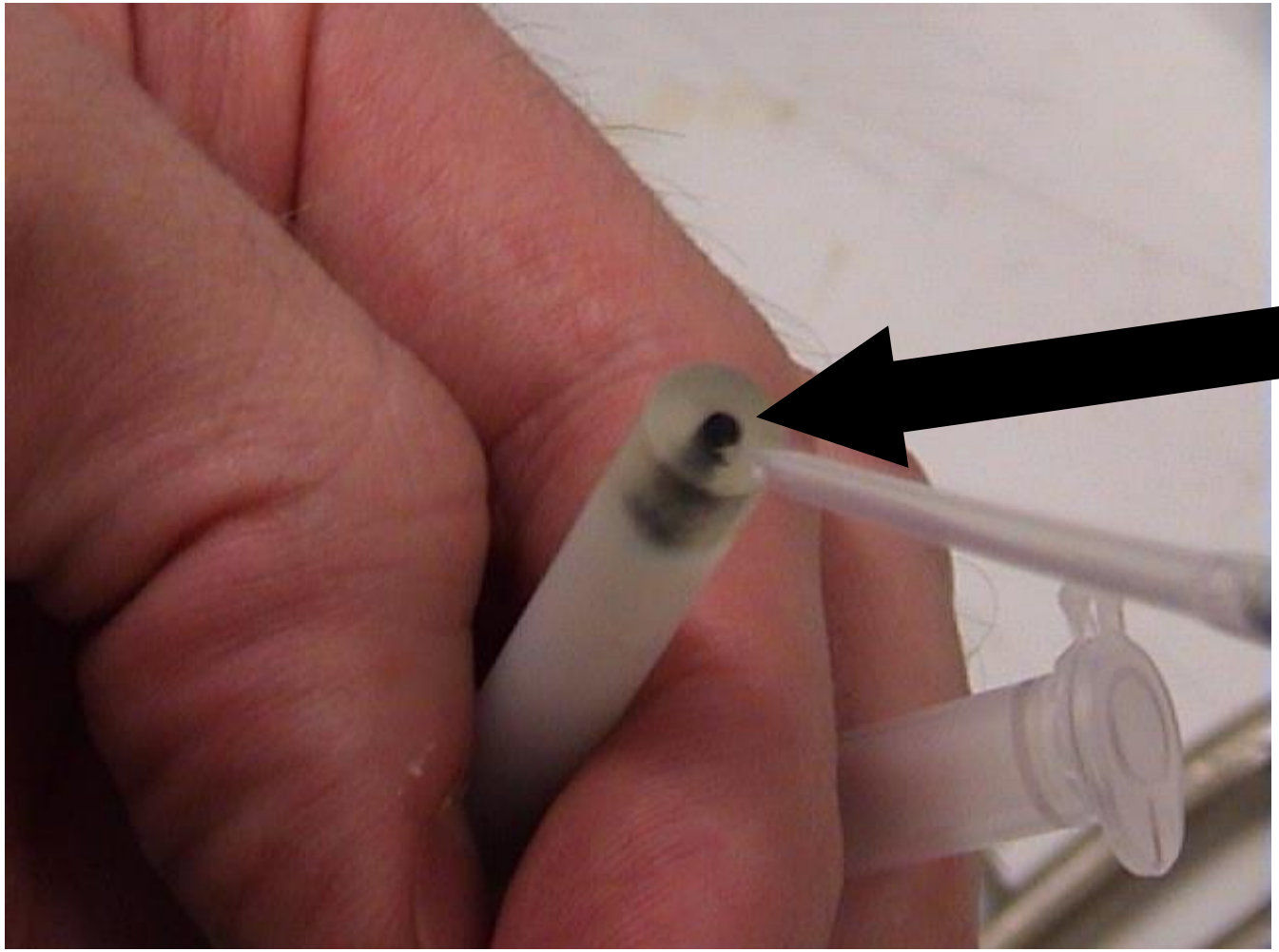
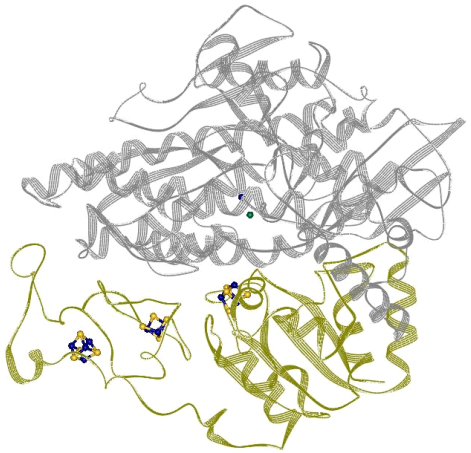
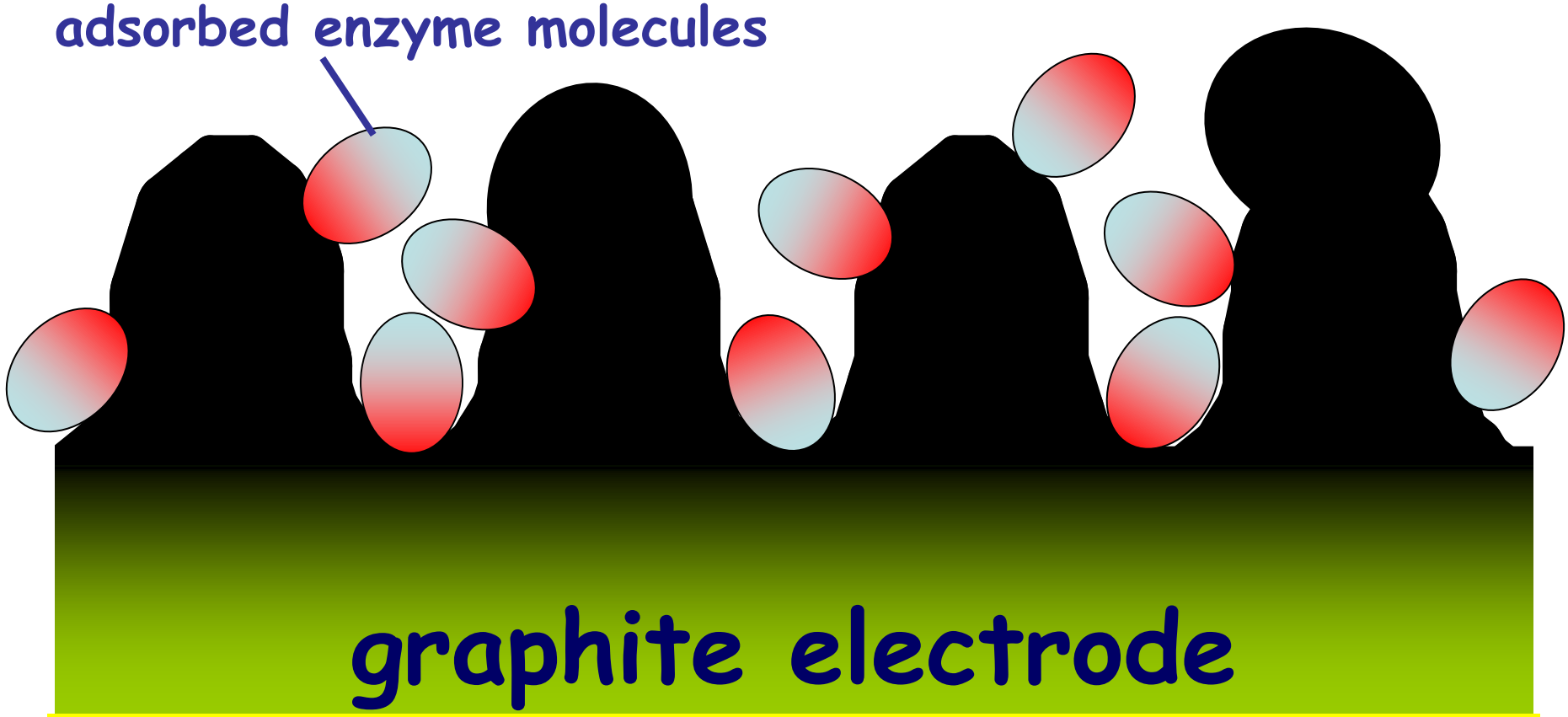
Protocol of performing PFV EXPERIMENTS:
Enzyme is adsorbed on working (commonly Graphite) Electrode
LESS THAN 10 FEMTOMOLE OF ENZYME is addressed, and numerous consecutive experiments can be conducted on same sample.





solution



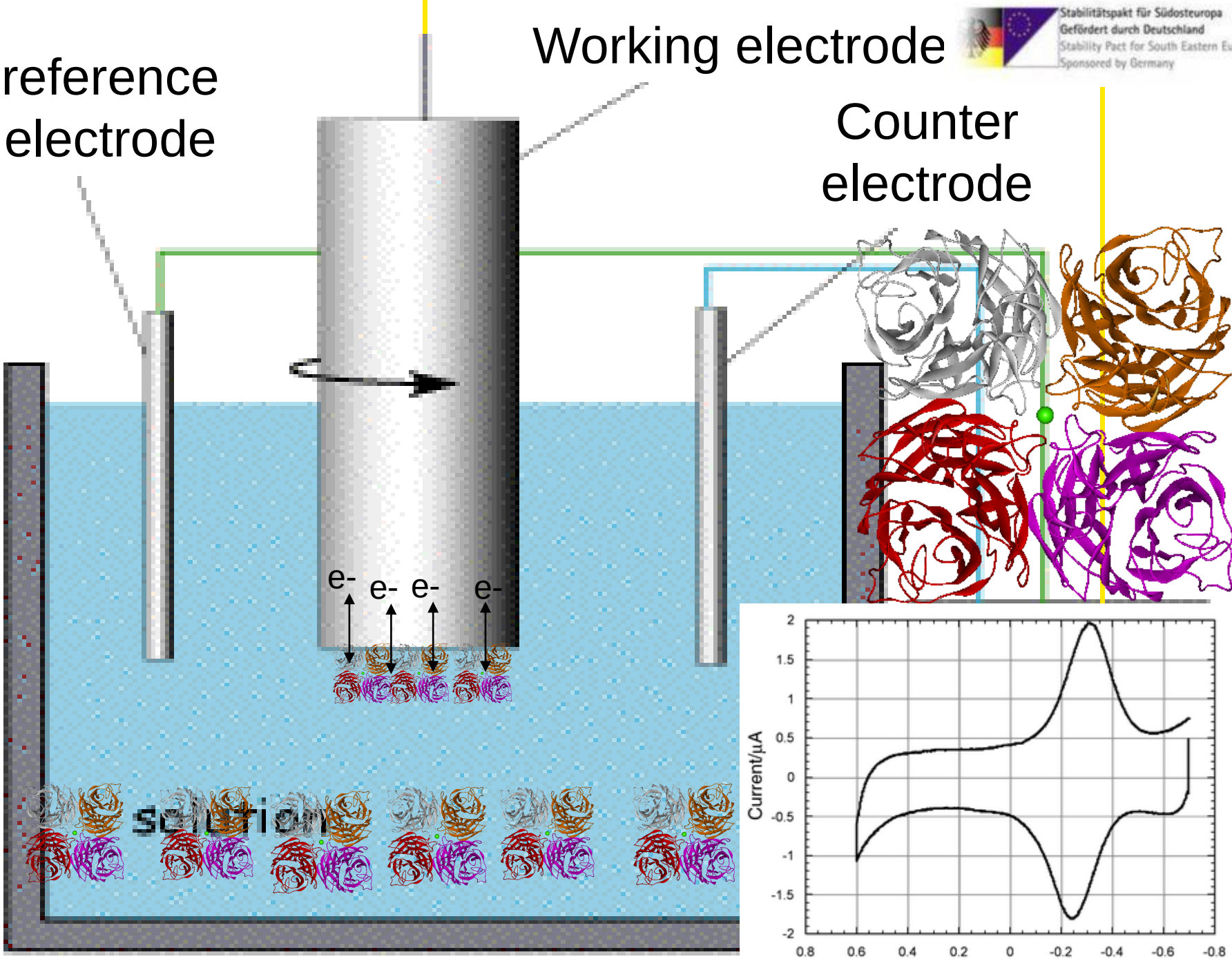
ANOTHER APPROACH:

self-assembling (adsorption) of the enzymes from the
Water solution to the electrode surface (mainly graphite electrode)

reference electrode

Working electrode

Counter electrode



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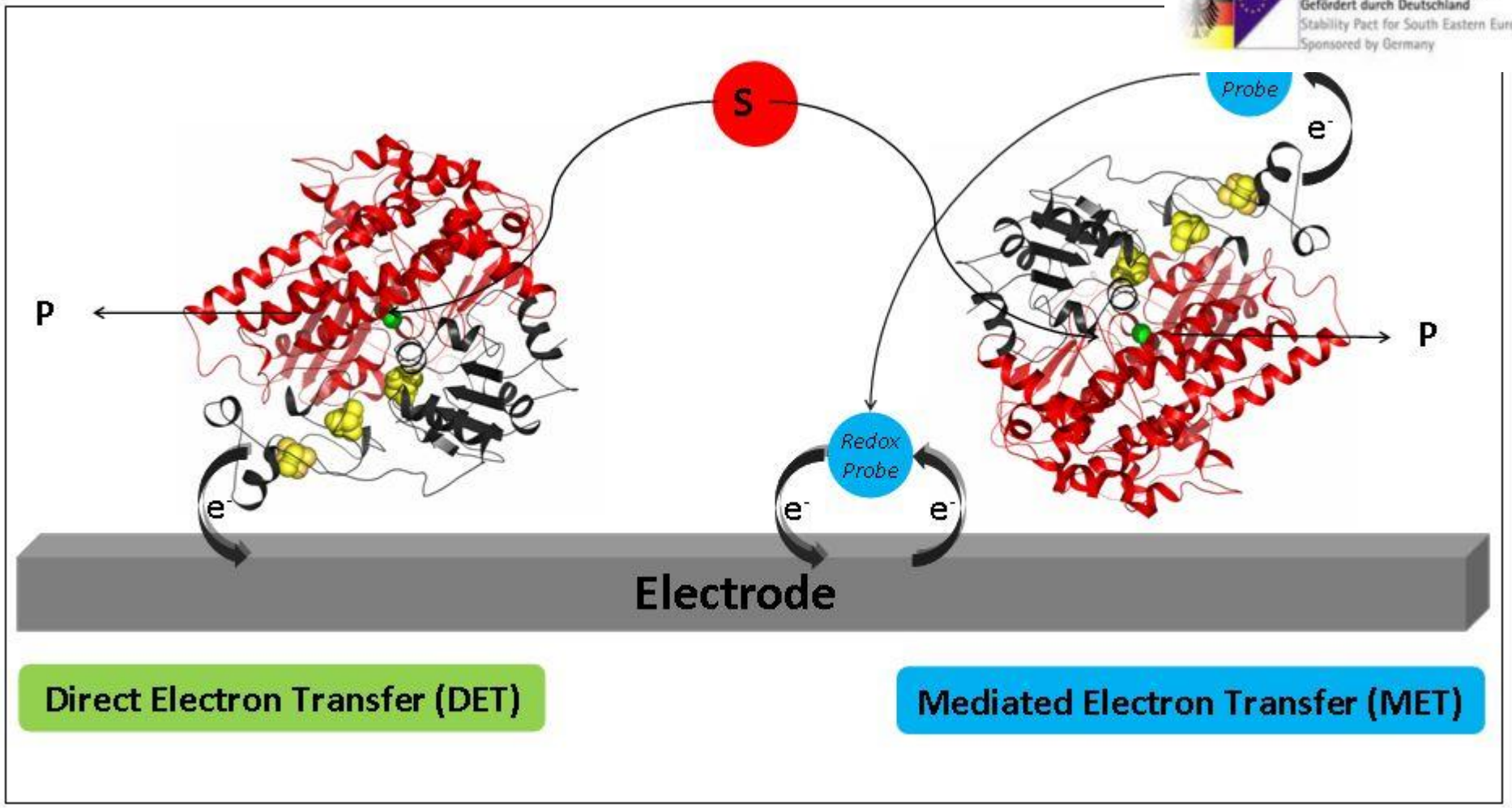
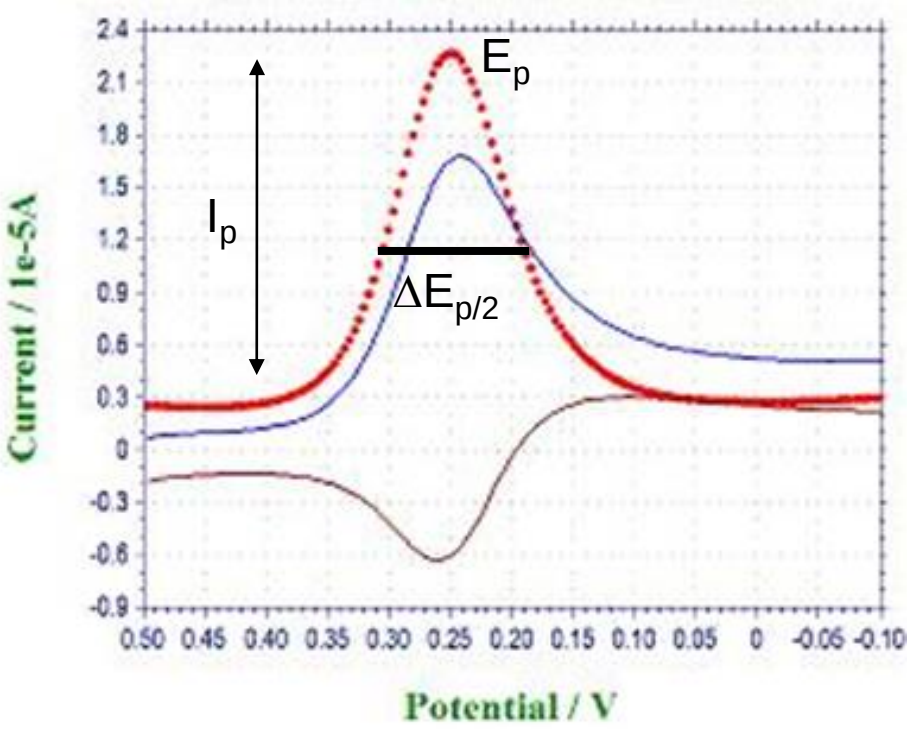
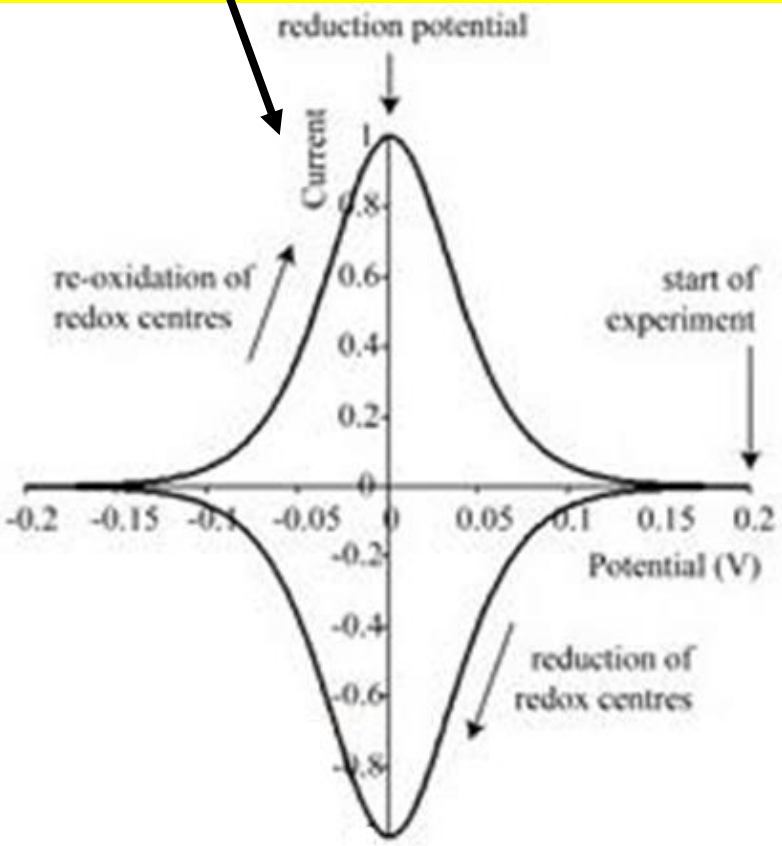


Scenarios for achieving electron transfers between the working electrode and the redox protein

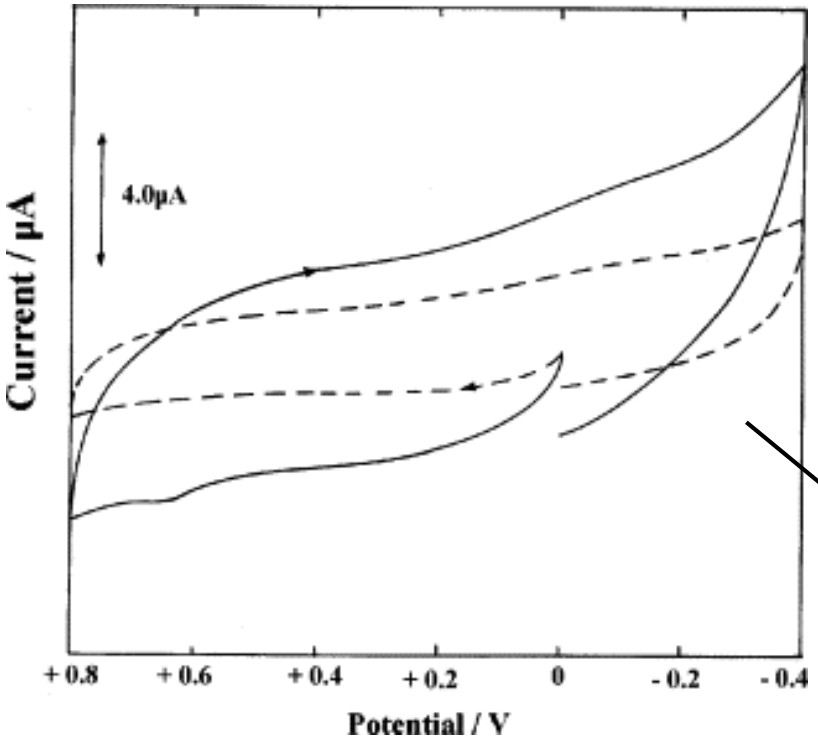
As an instrumental output we get a CYCLIC (or SQUARE-WAVE-SW) voltammogram typical for ***surface confined*** redox processes.

The features of the voltammograms:
(***mid-peak potential E_p, peak-to-peak separation, peak current I_p, half-peak width*** $\Delta E_{p/2}$)
hide valuable set of kinetic and thermodynamic parameters of the redox enzyme studied

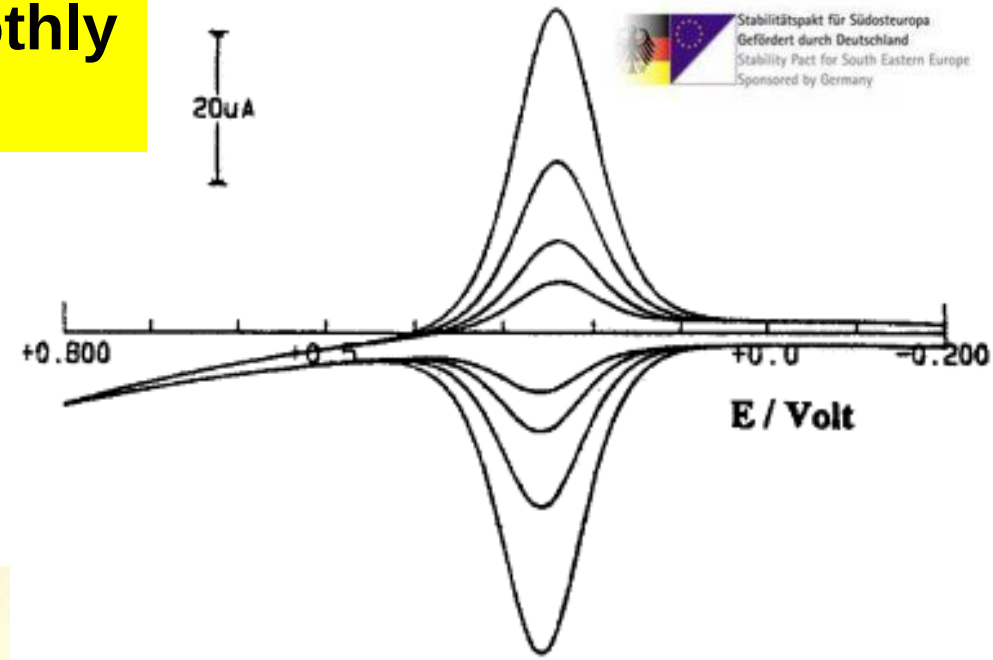




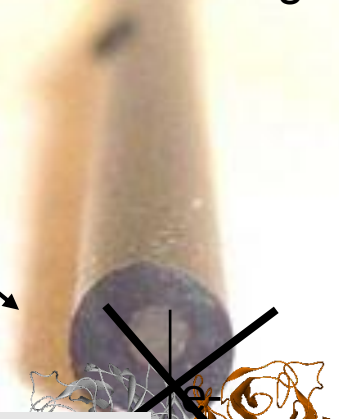
Does everything go so smoothly In PFV methodology?



A type of "**poor**" voltammogram of a protein



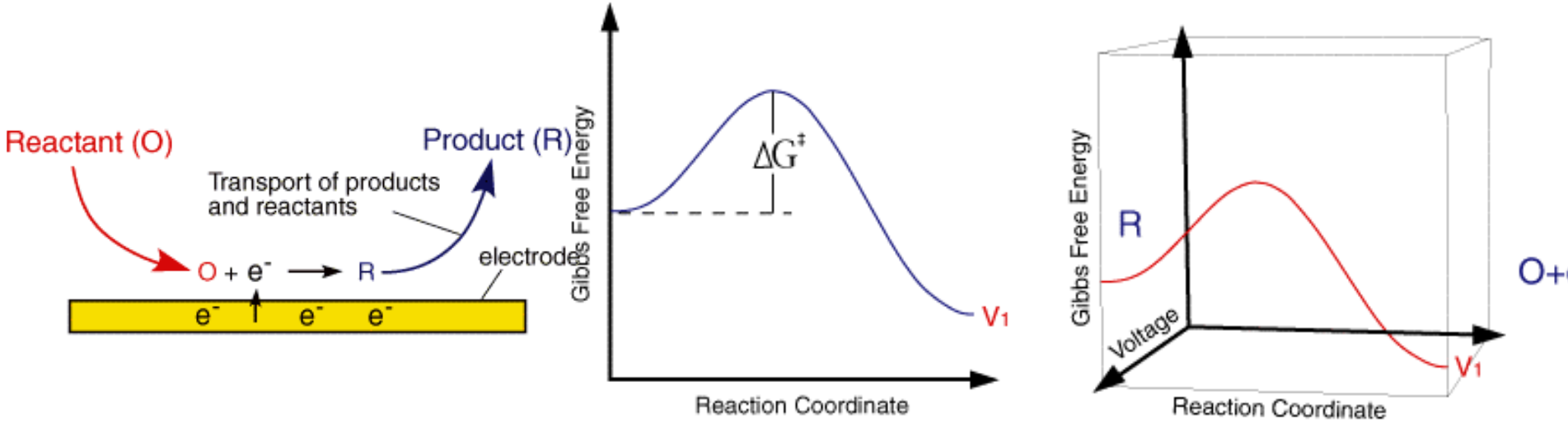
A type of "nice" voltammogram of a protein



The hindrances appear mainly due to the INSULATING PROPERTIES OF THE BULKY PROTEIN moiety that hinders the electron transfer between the electrode and the redox center of the protein studies

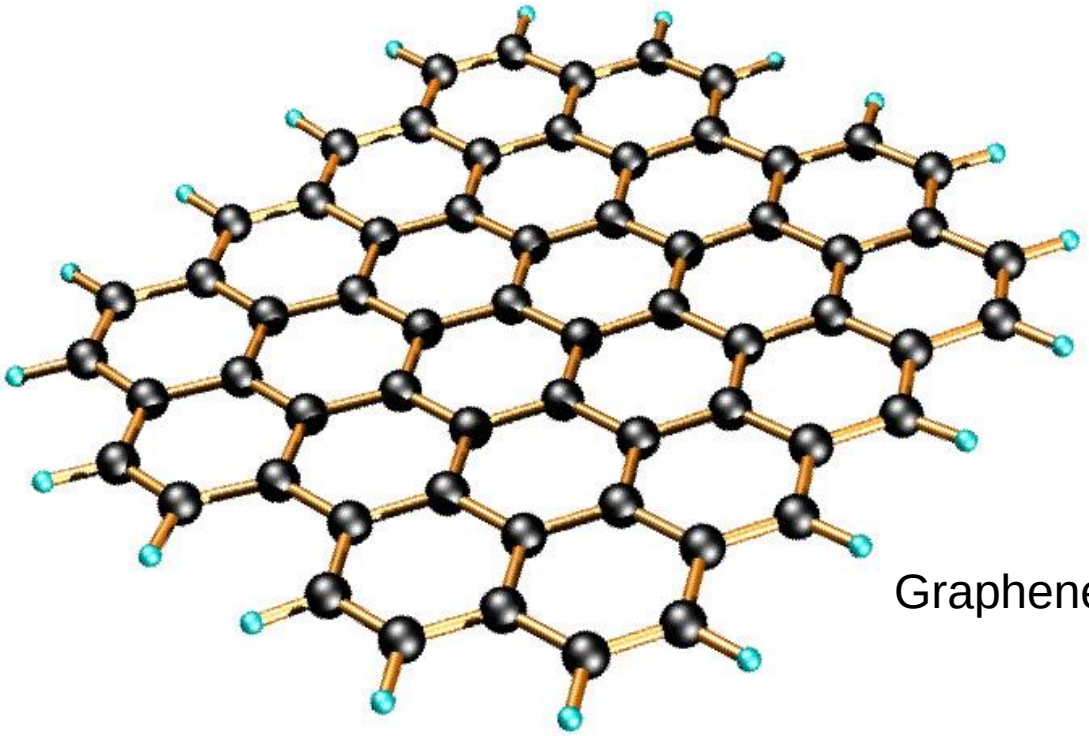
In order to overcome this problem, and to facilitate the electron transfer between the electrode and the redox protein, one usually plays around with the electrode material or with ***modification of the electrode surface***

***1. First choice*: To test electrodes made from various Materials having much ordered structure and much Better conductivity than the common electrodes such as glassy carbon electrode or some other Carbon-type electrodes**

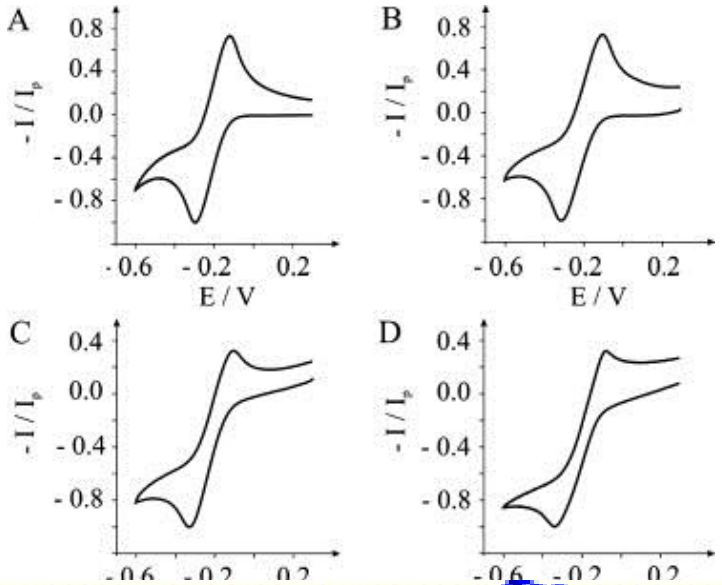


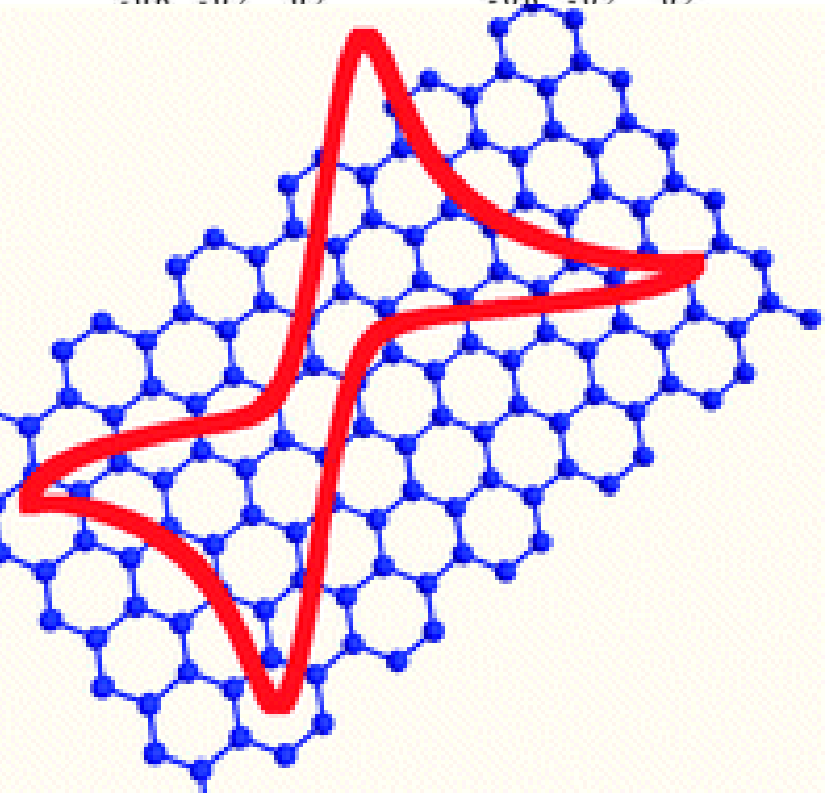
In the last few years, **GRAPHENE** emerged as a very promising material for designing electrode materials

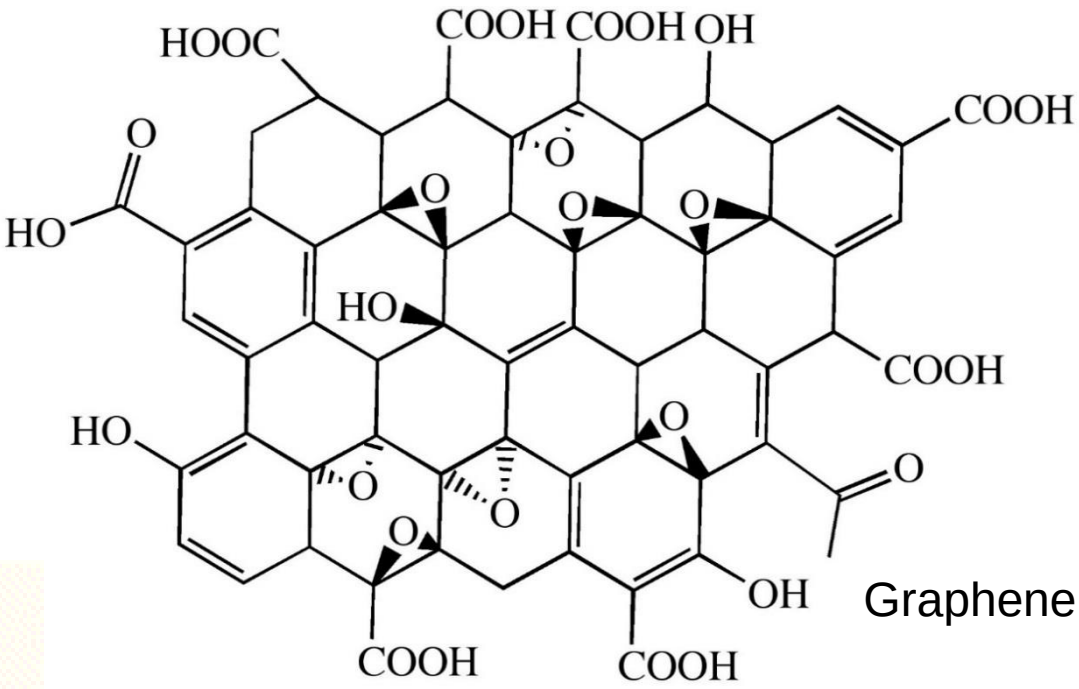
Its has very good electrical conductivity, a big surface area that allows various functional groups to be attached on it



Graphene







Graphene

Graphene exhibits excellent electron transfer promoting ability for some enzymes and excellent catalytic behavior toward small biomolecules such as H_2O_2, , NADH, which makes graphene extremely attractive for *enzyme-based biosensors*, e.g. glucose biosensors and ethanol biosensors

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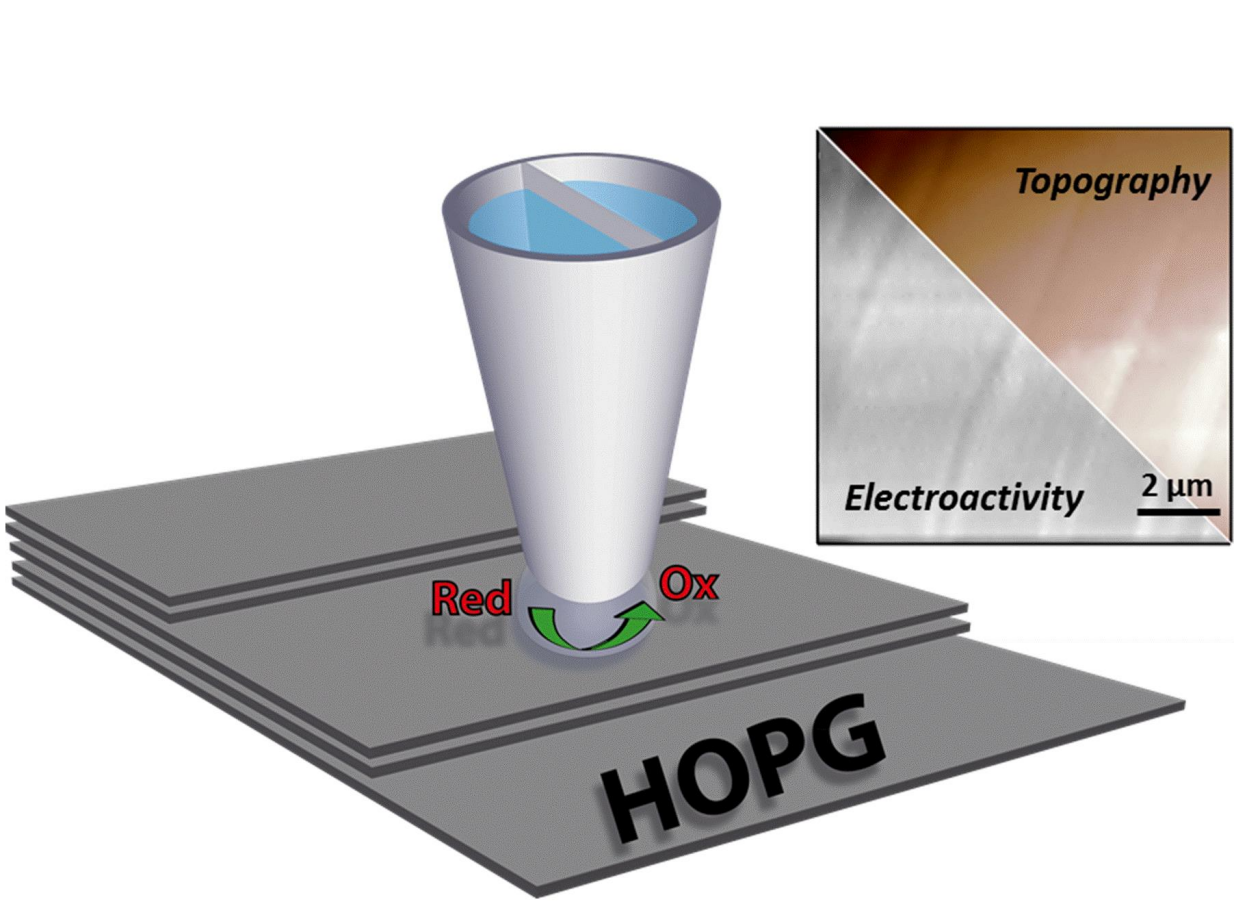
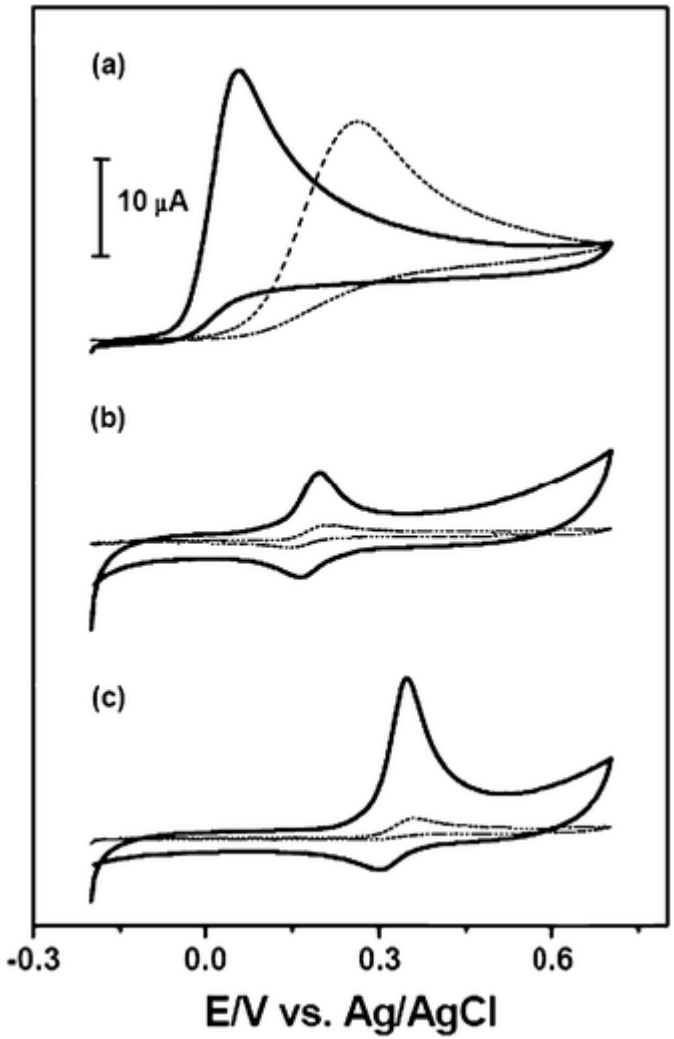
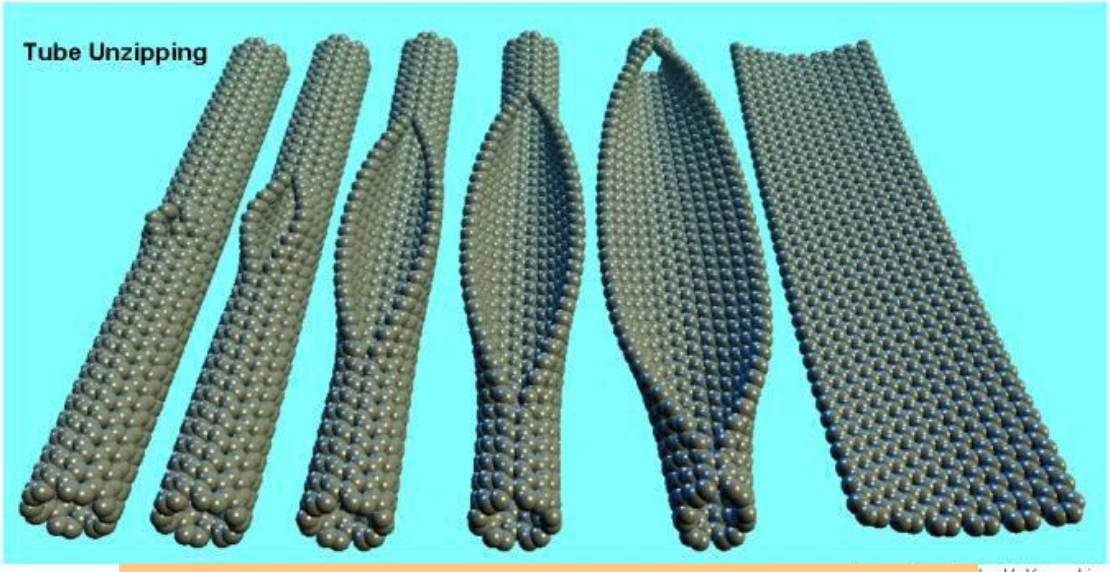
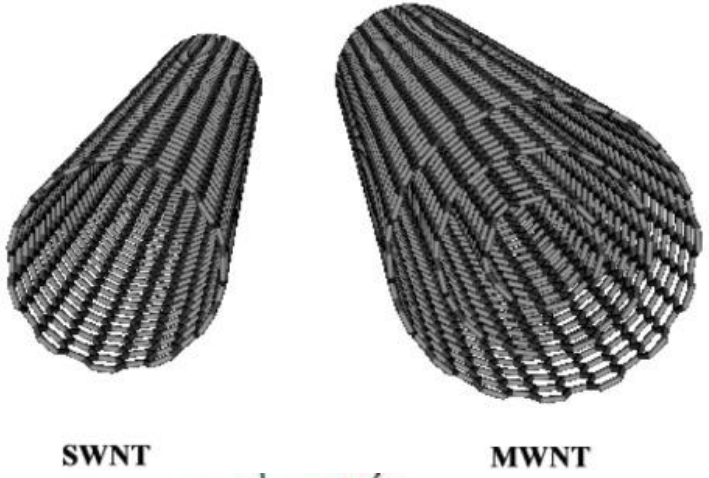


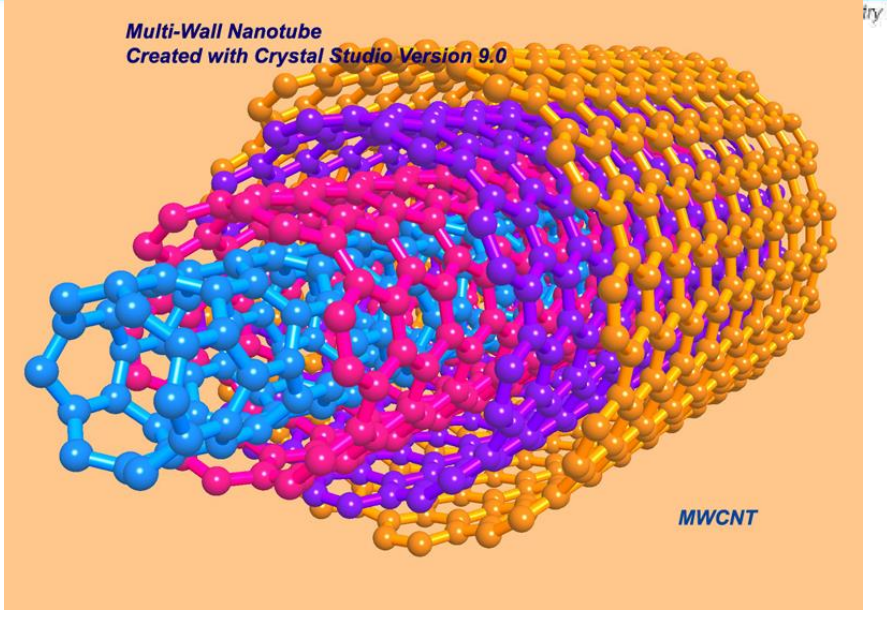


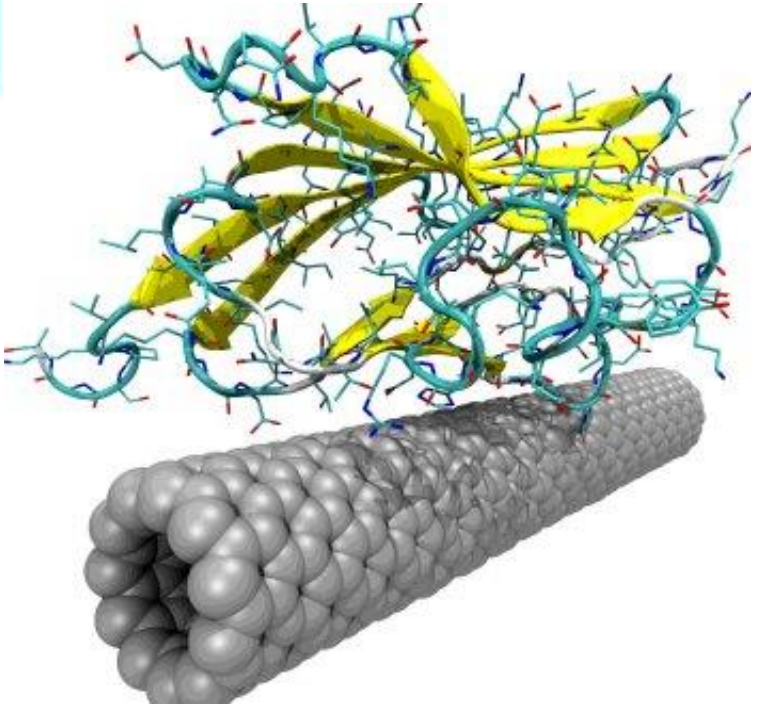
Another promising electrode material is the
Highly Oriented Pyrolitic Graphite (HOPG)

NANOPARTICLES (especially carbon nanotubes) are one of the most excited choices
for modifying the electrode materials









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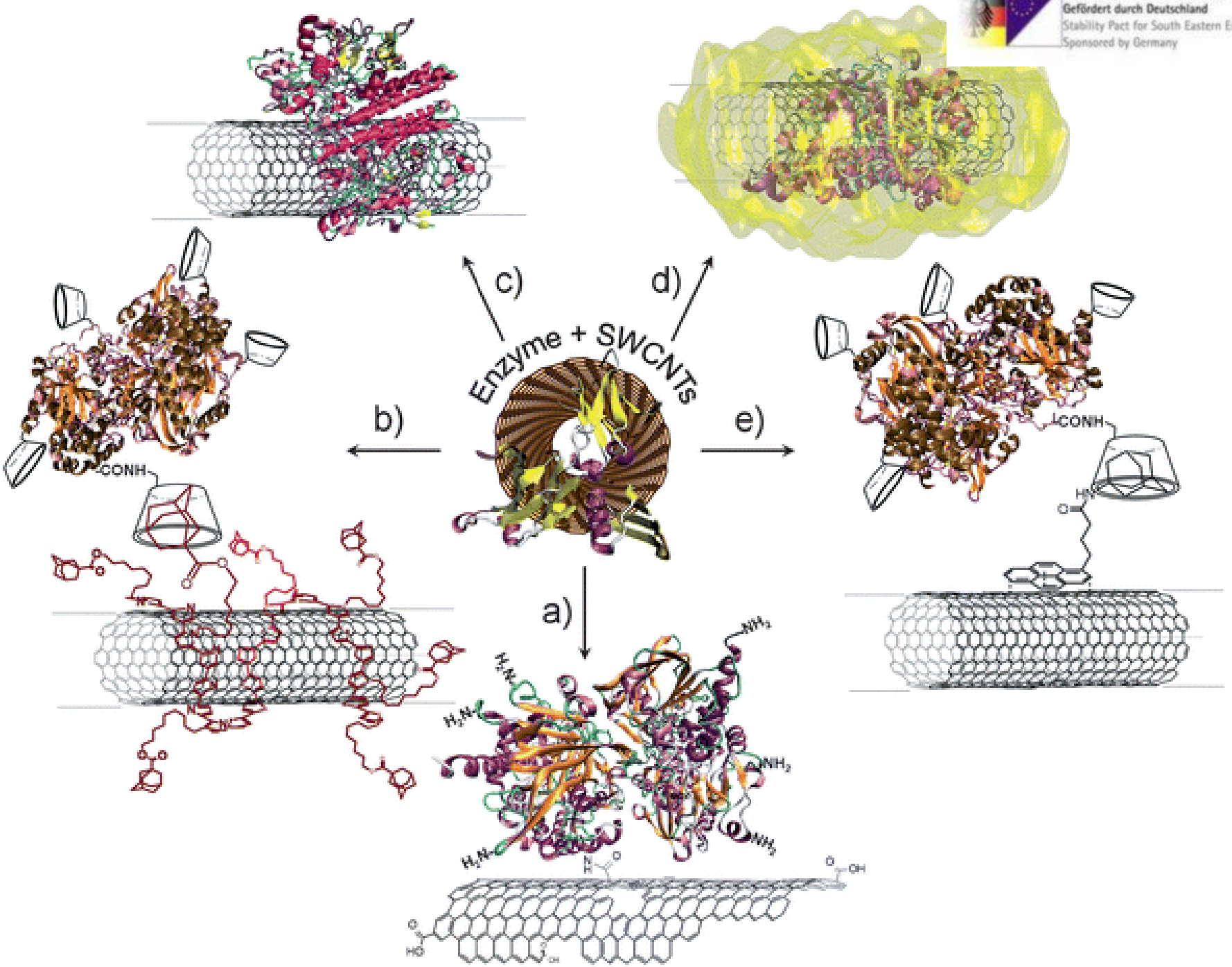

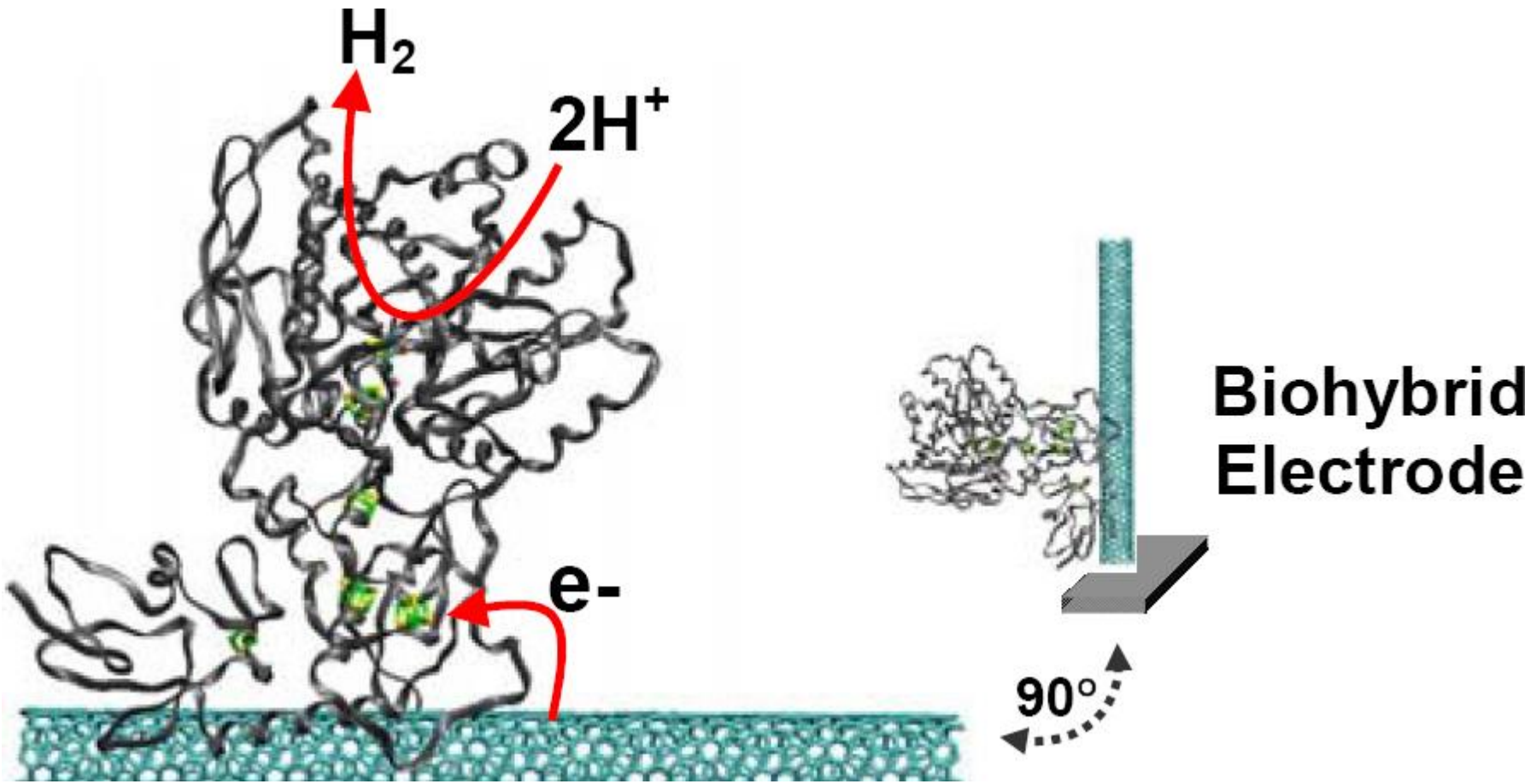

By attaching a given protein on the surface of Carbon Nanotubes modified-electrode we get so-called BIOHYBRID ELECTRODES-especially useful for studying the Redox enzymes

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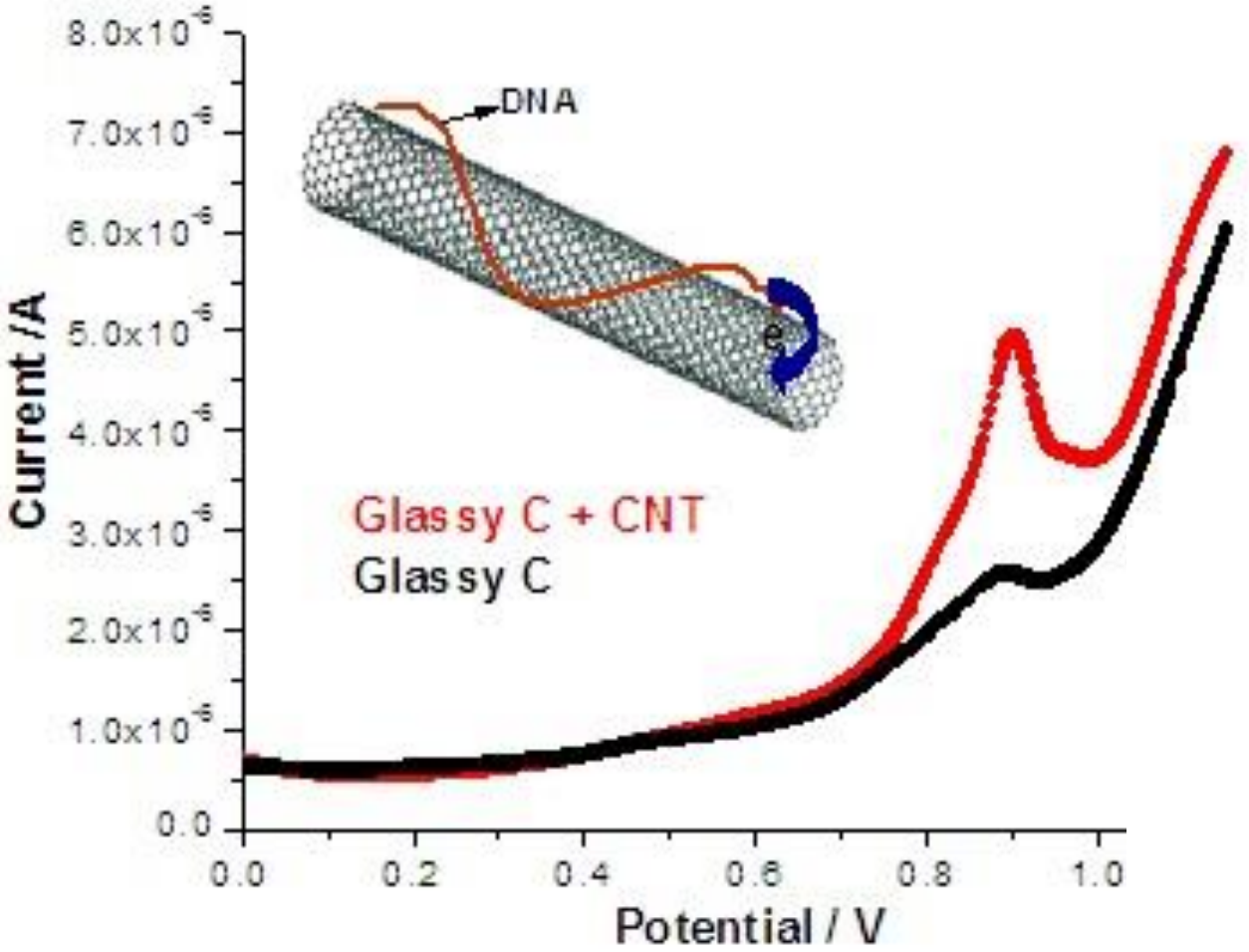

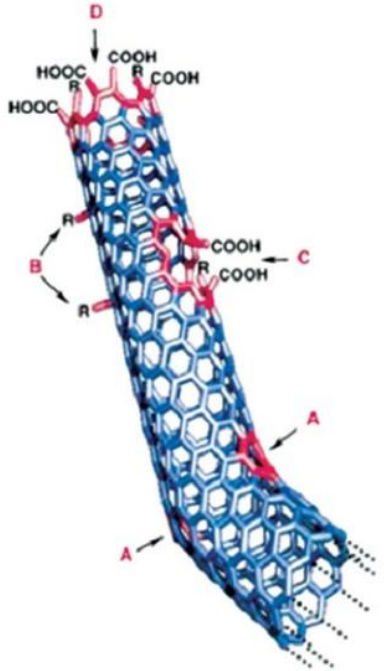

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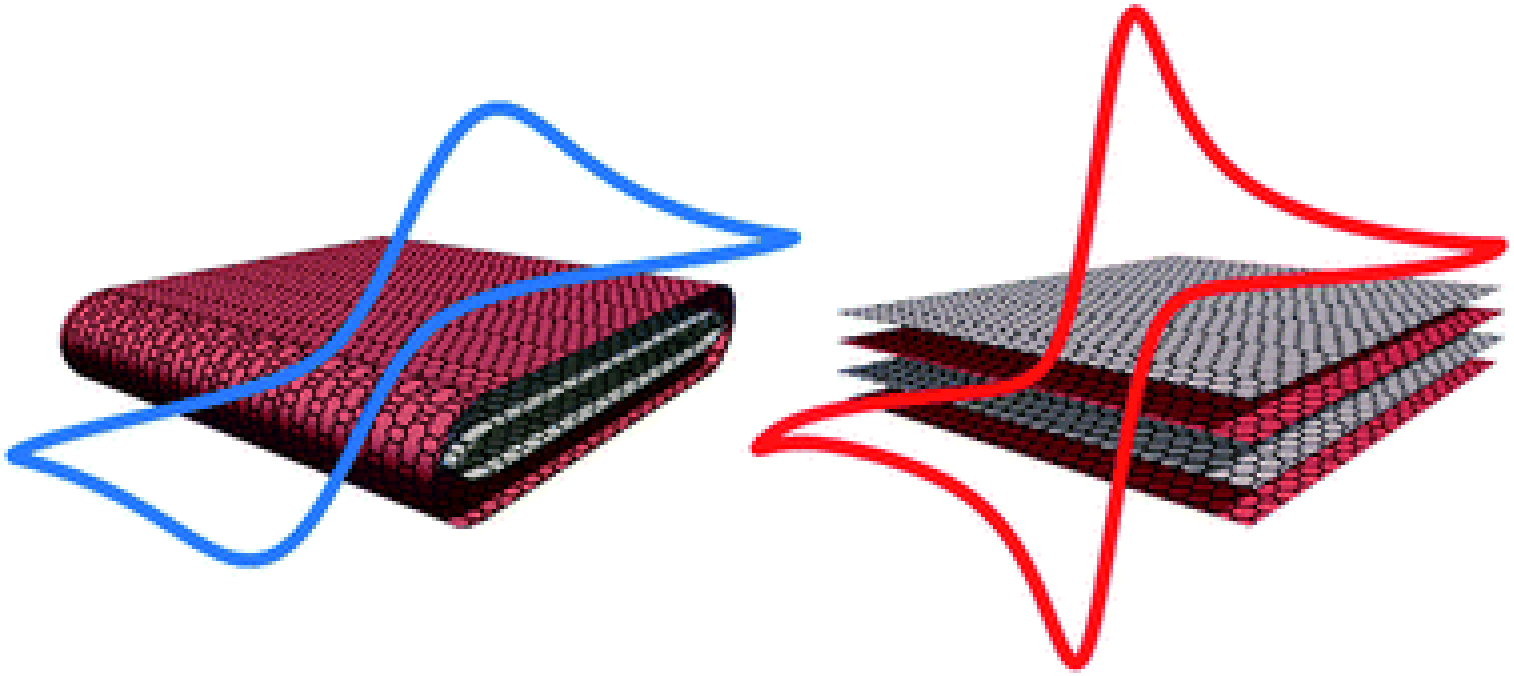
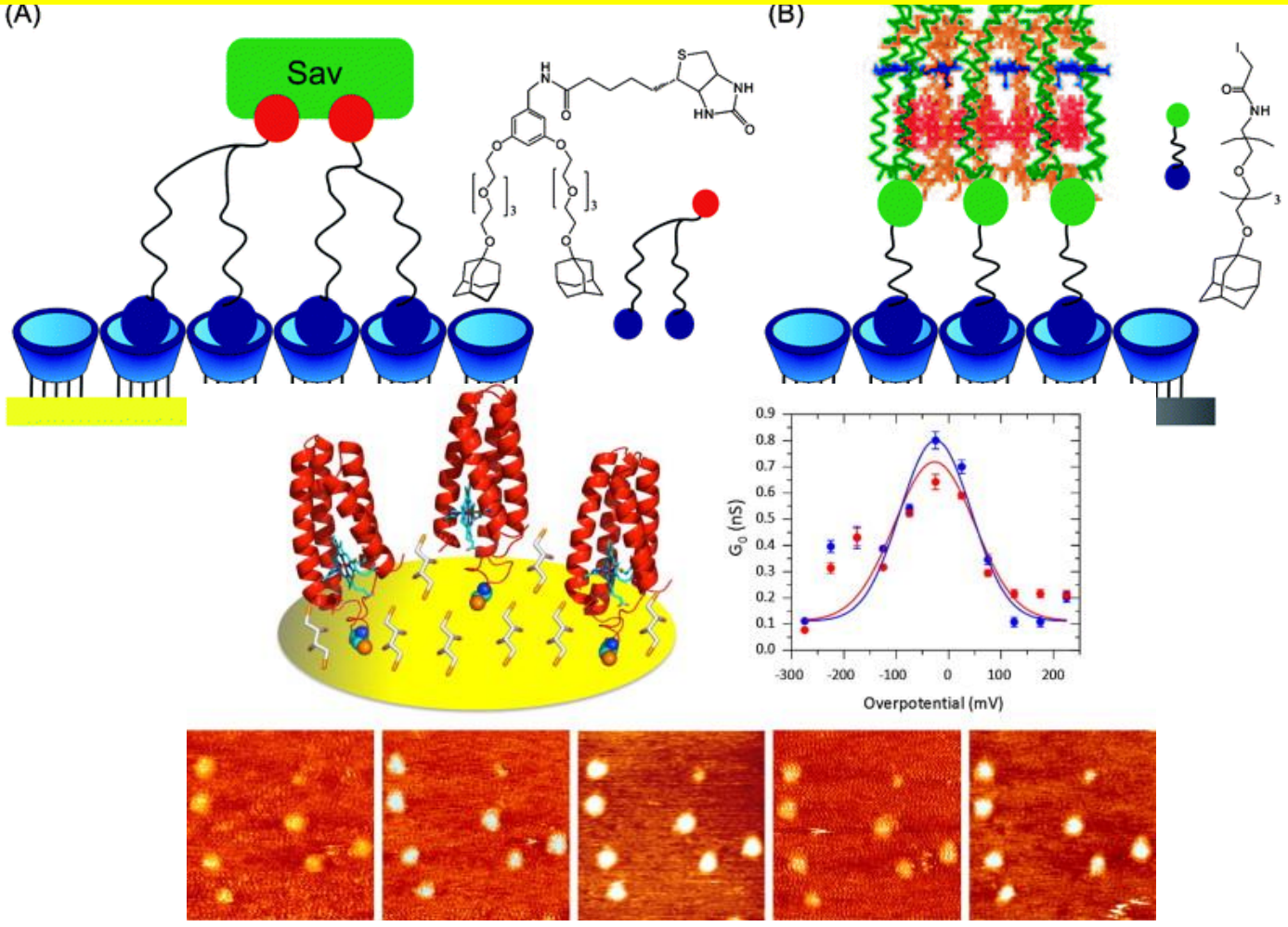
Especially attractive in the last few years are
THE GRAPHENE-BASED NANO-MATERIALS

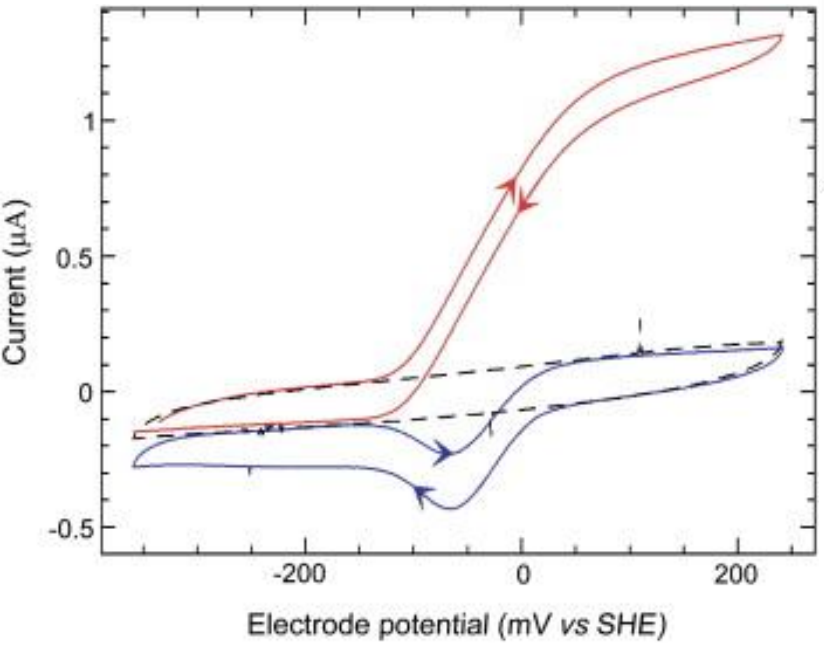


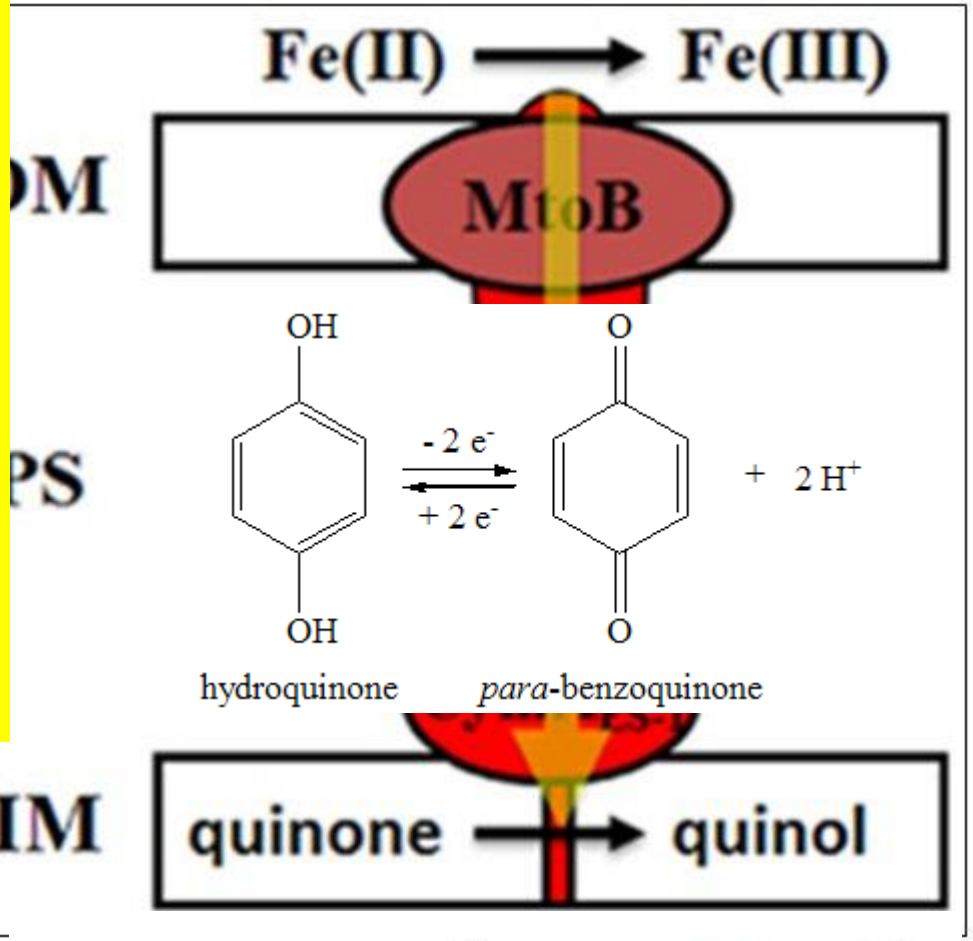
LINKERS-BASED PROTEIN-FILM VOLTAMMETRY

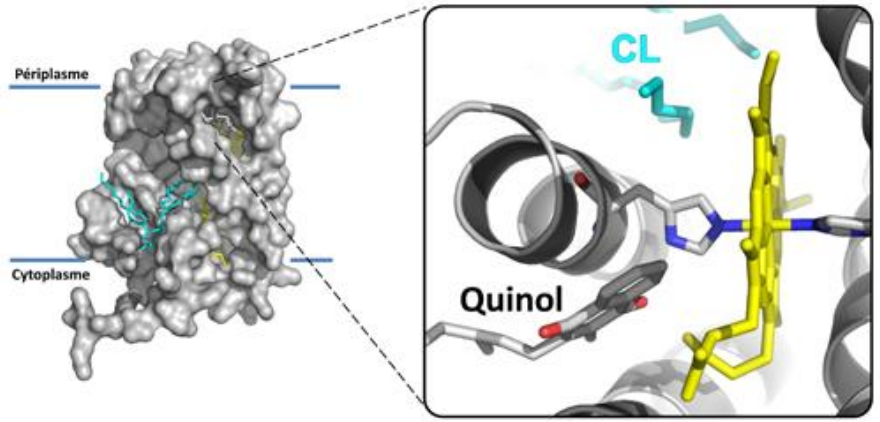
LINKERS **- small lipophilic or amphiphilic compounds adsorbed on the surface of the working electrode, serving as *docking sites* for the redox enzymes**



Especially interesting linkers are those containing *Quinone-quinol* moieties due to its reversible Redox chemistry and due to its *S-H binding activities* that allows MANY S-H (THIOL-CONTAINING) proteins to dock on it







APPLICATIONS of PFV

What kind of redox proteins can be studied with PFV?

-Hydrogenases

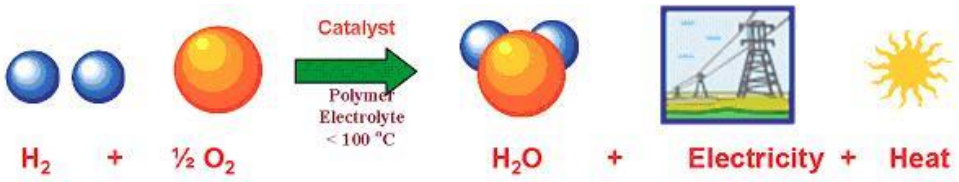
-Peroxidases

-Heam-containing proteins (catalase, hemoglobin, Myoglobin, Cytochrome P450…

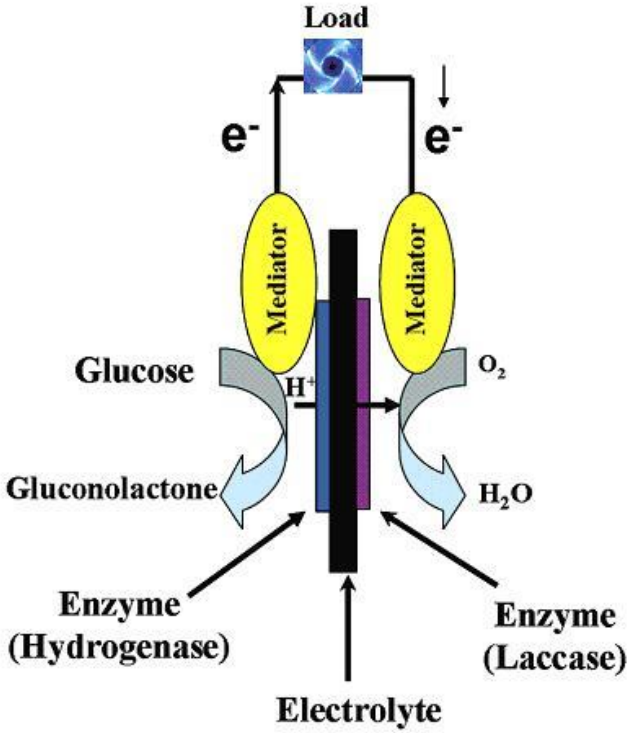
-Enzymes with quinone moieties…

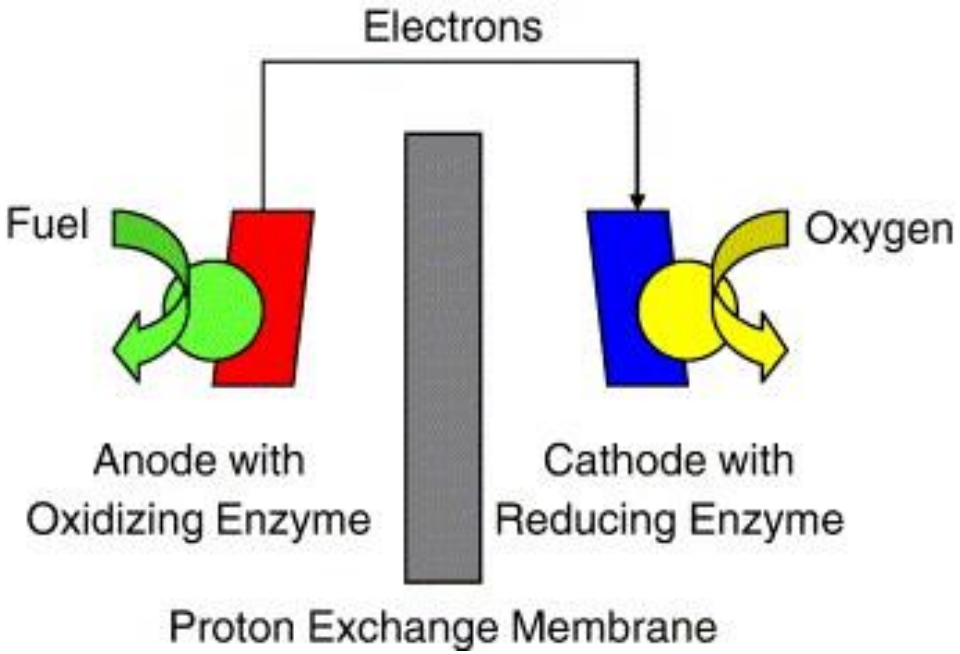
1. Obtaining Energy by using PFV methodology

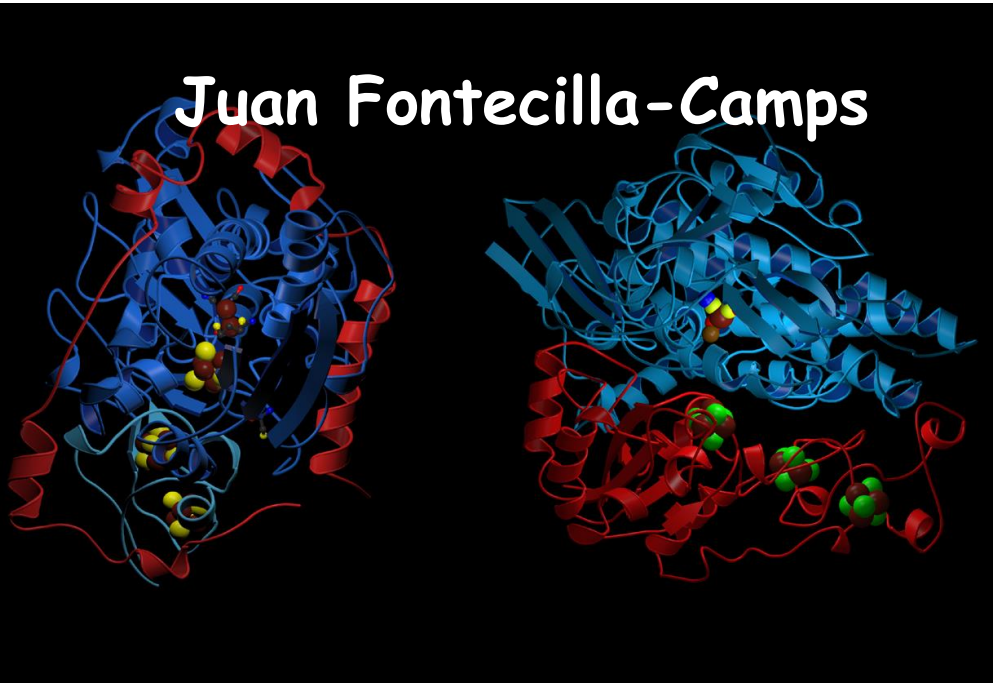
(a)



(b)





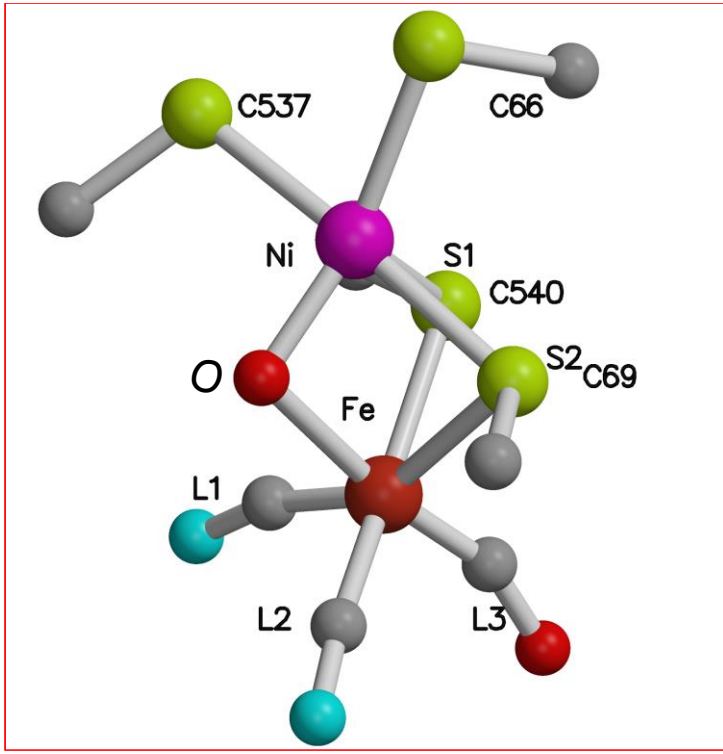


Ni-Fe hydrogenase is a type of hydrogenase that is an oxidative enzyme that activates reversibly molecular hydrogen in prokaryotes

$$H_2 \rightleftharpoons 2H^+ + 2e^-$$

Active site of

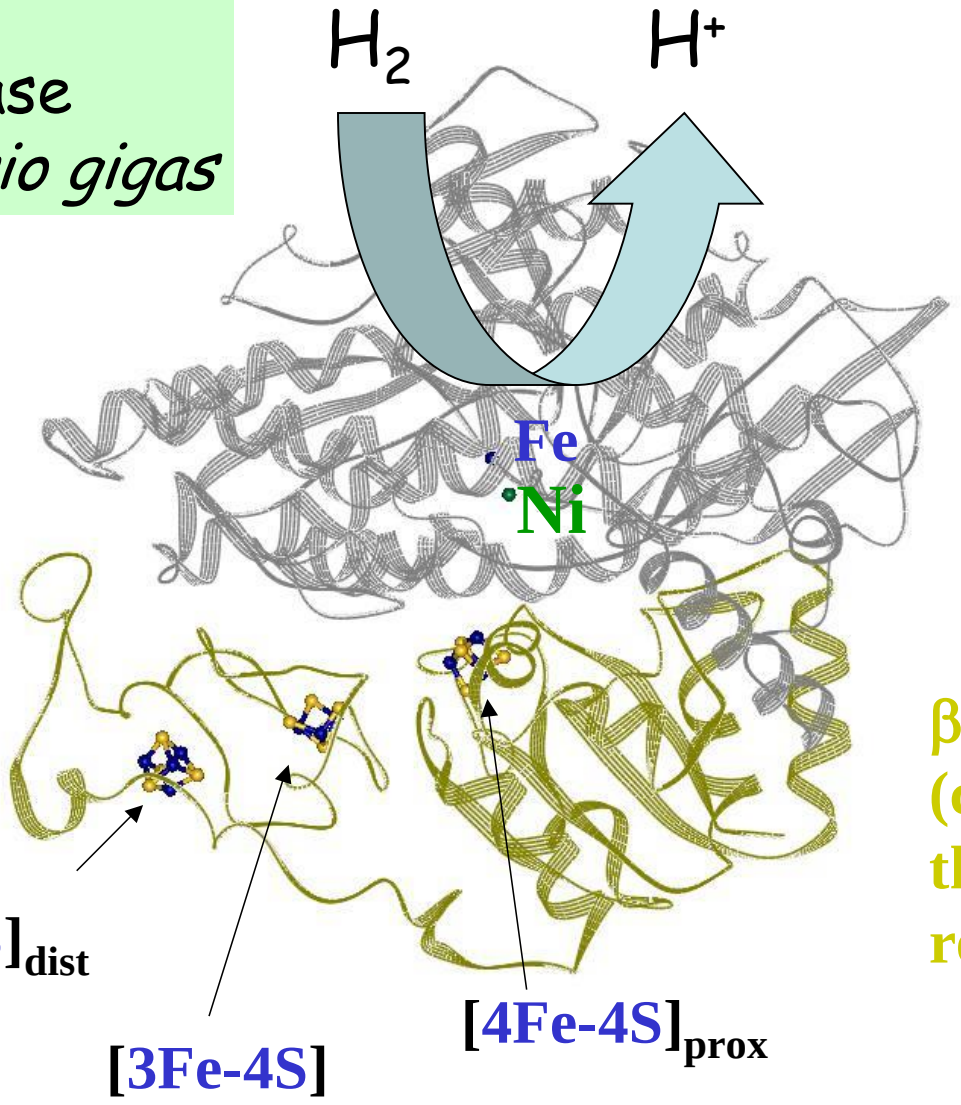
[NiFe]-hydrogenase



Fe-only hydrogenase NiFe-hydrogenase

An additional O-ligand is present in inactive states

Structure of [NiFe]-hydrogenase from *Desulfovibrio gigas*



α-Subunit (contains the active site)

β-Subunit (contains the electron relay)

Other [NiFe]-hydrogenases have similar sequences or spectroscopic properties

$H_2(g) + O_2(g) \rightarrow H_2O\,(liq)$ $\Delta H = -286$ kJ/mol

specific enthalpy -143 kJ/gram H_2

Hydrogen is the fuel for the future!!!

NASA uses hydrogen fuel
to launch the space shuttles.

The future....fuel cells with cheap, inexpensive specific electrocatalysts, perhaps without a membrane ?

$H_2(g) + O_2(g) \rightarrow H_2O$ (liq) $\Delta H = -286$ kJ/mol

specific enthalpy -143 kJ/gram H_2

Ideas from Nature

H_2

O_2

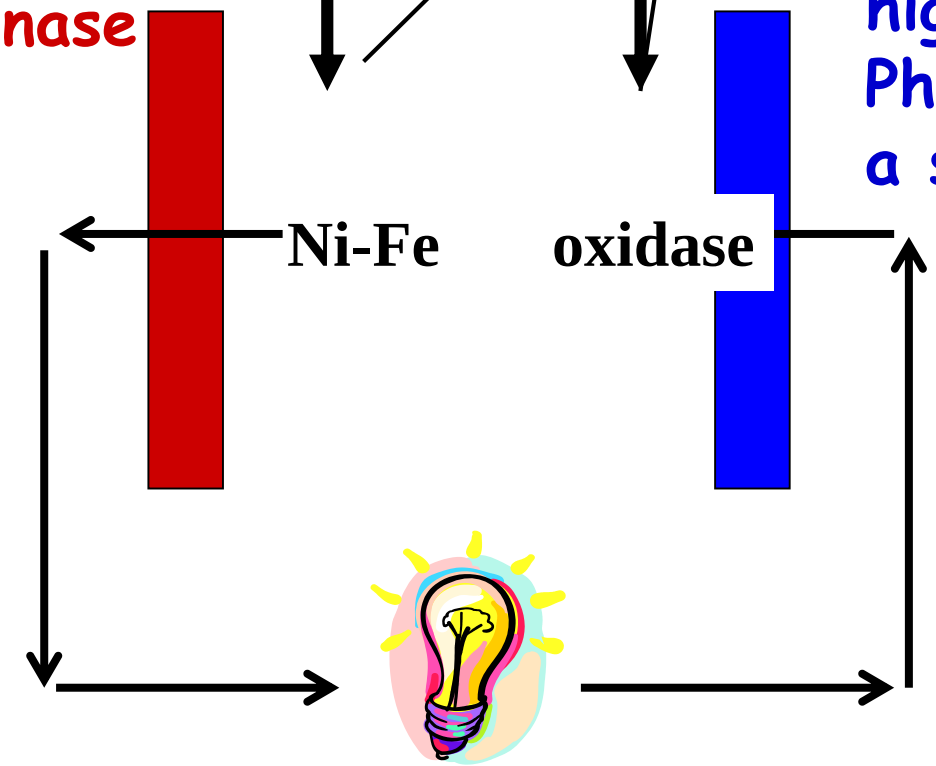
ANODE

Hydrogenase

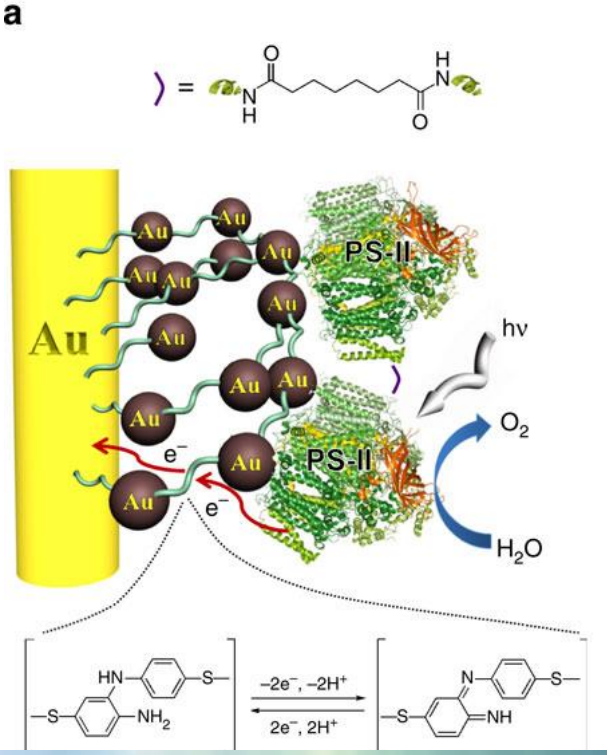
CATHODE

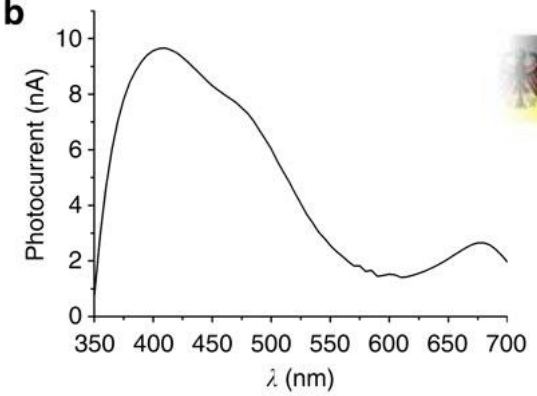
high E oxidases ?

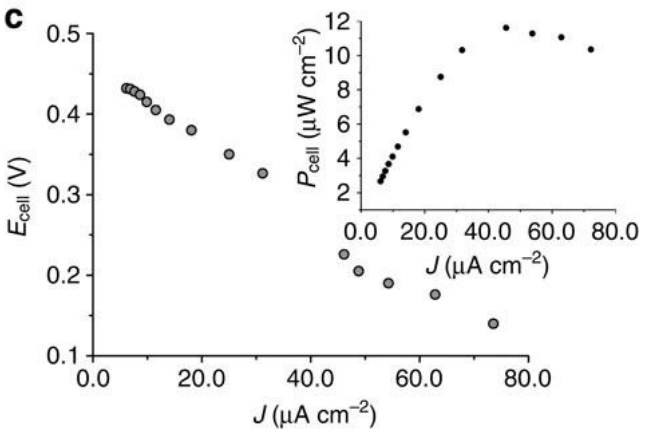
Photosystem II is a suitable candidate

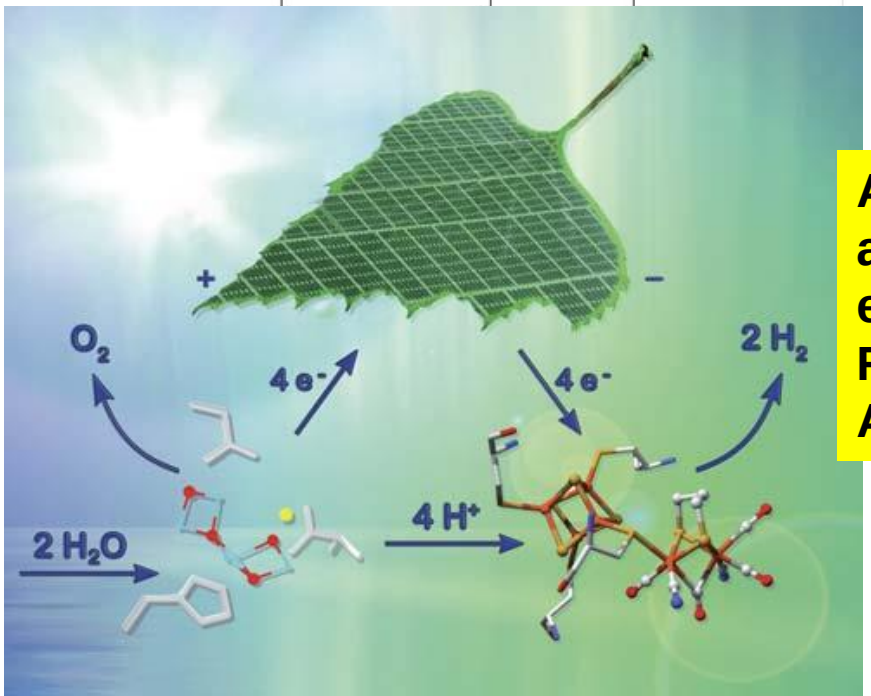


Power ?





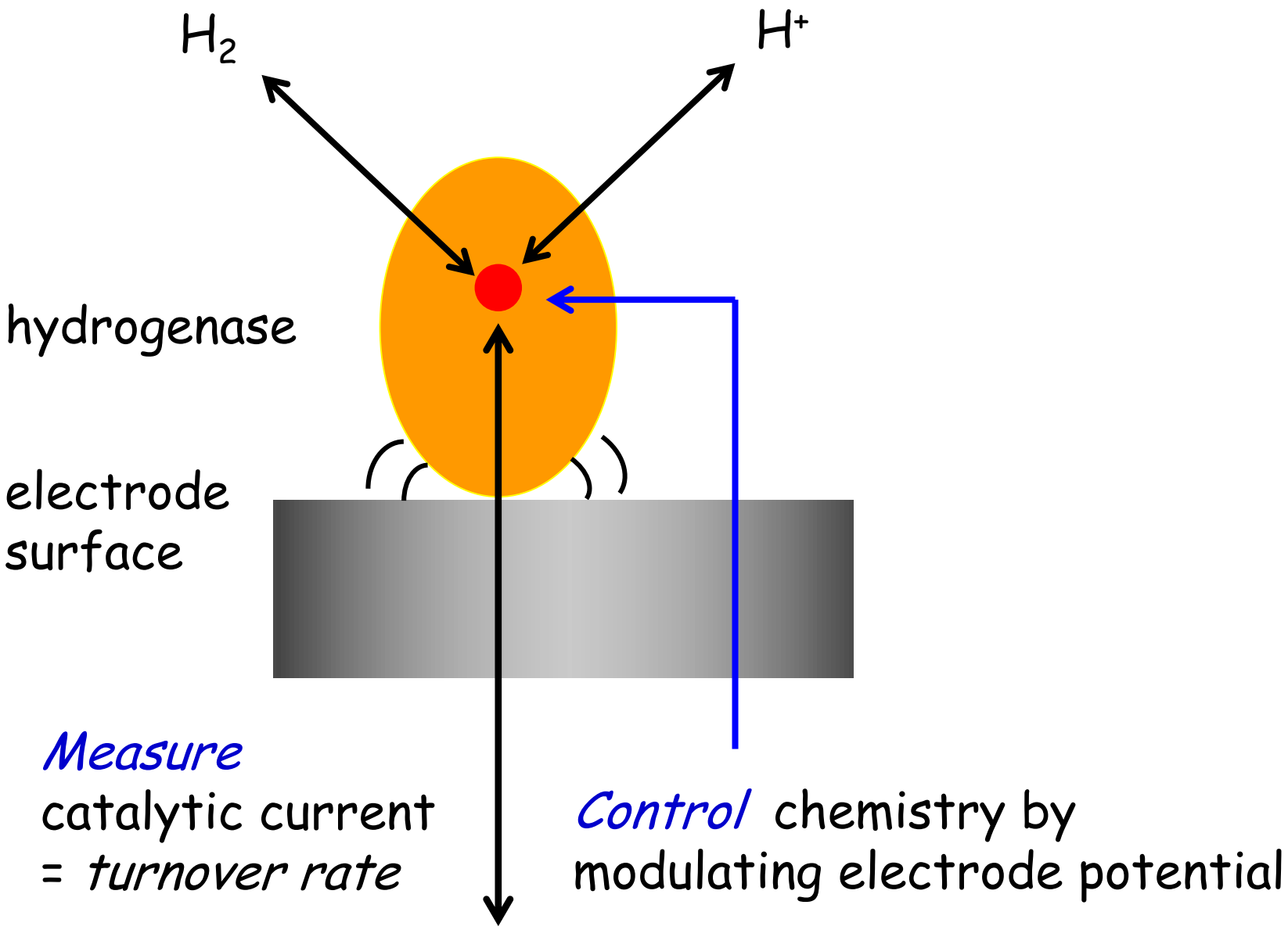




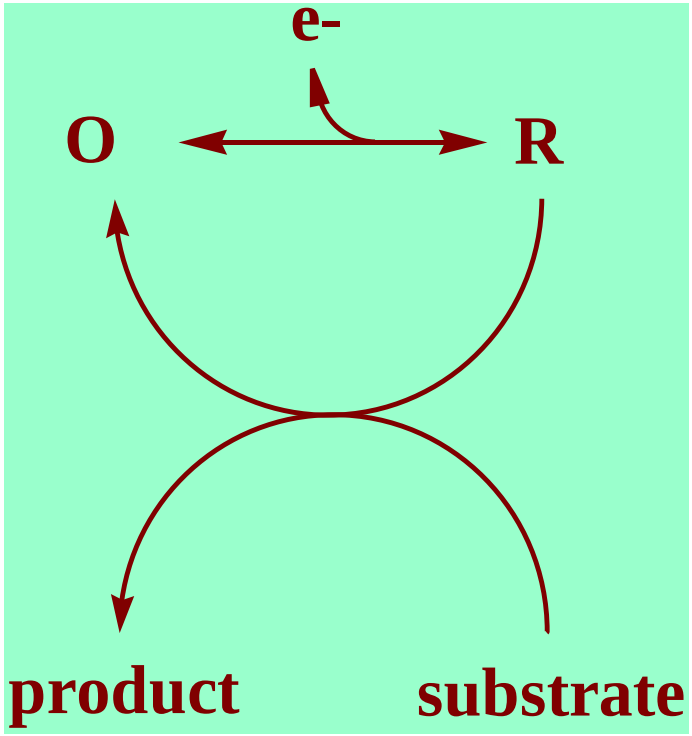
An interesting scenario for obtaining O2 at the anode for getting energy by electrochemical enzymatic Reaction is via the Photosystem II (PS II) And Hydrogenases redox transofrmation

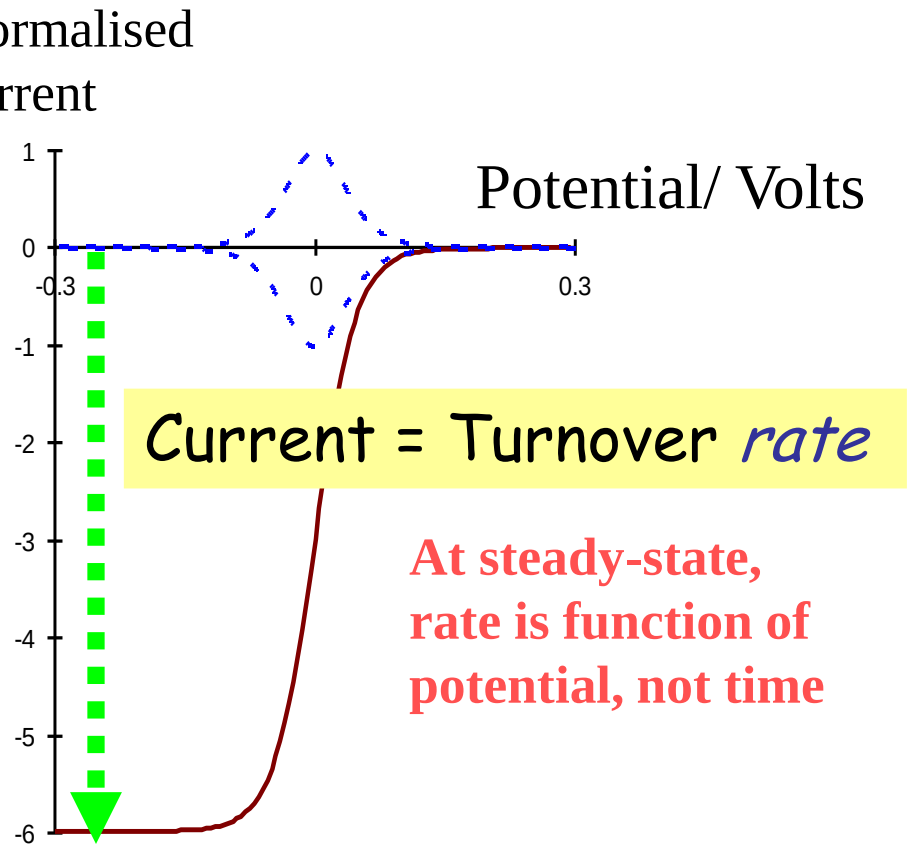
Photosystem II (or **water-plastoquinone oxidoreductase**)

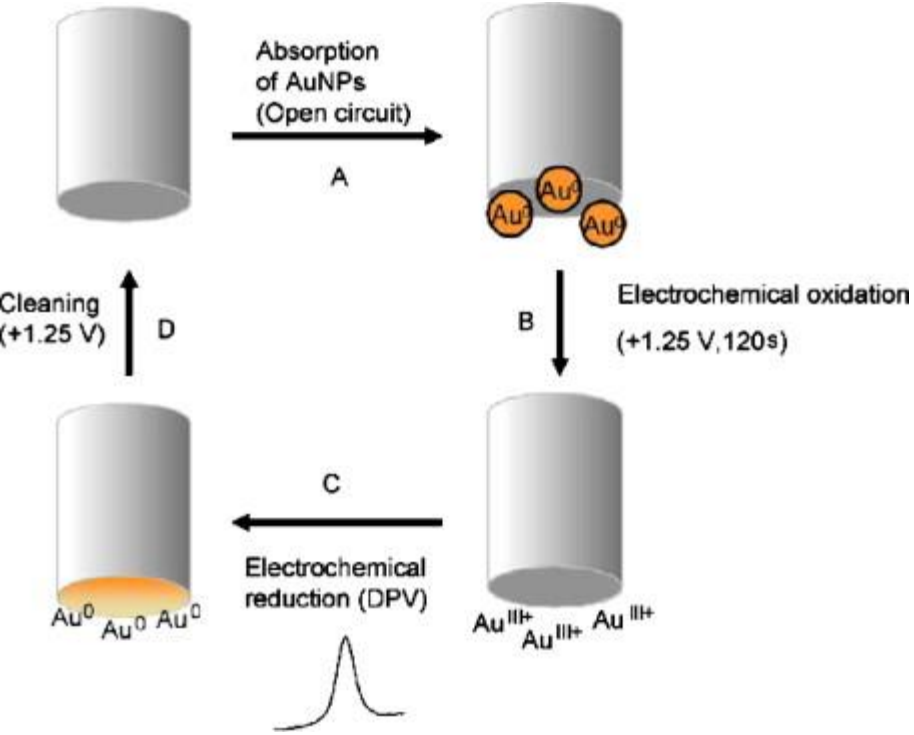
Investigating hydrogenases by protein film voltammetry

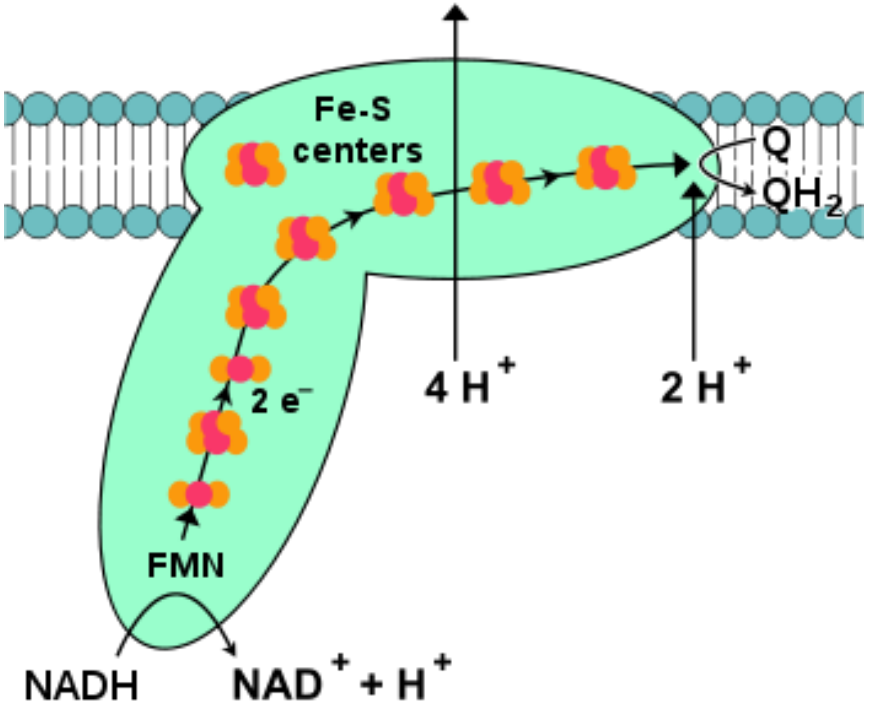


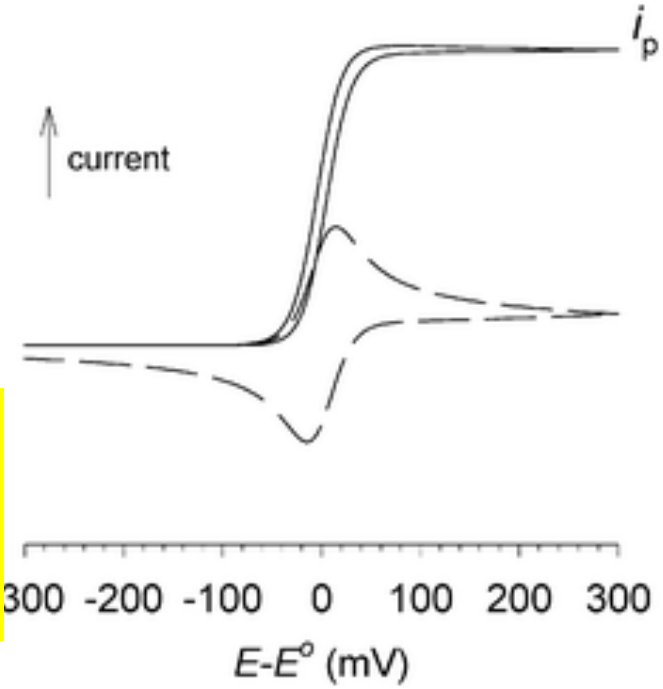
Protein Film Voltammetry: Catalytic action can produce a large current with characteristic dependence on potential





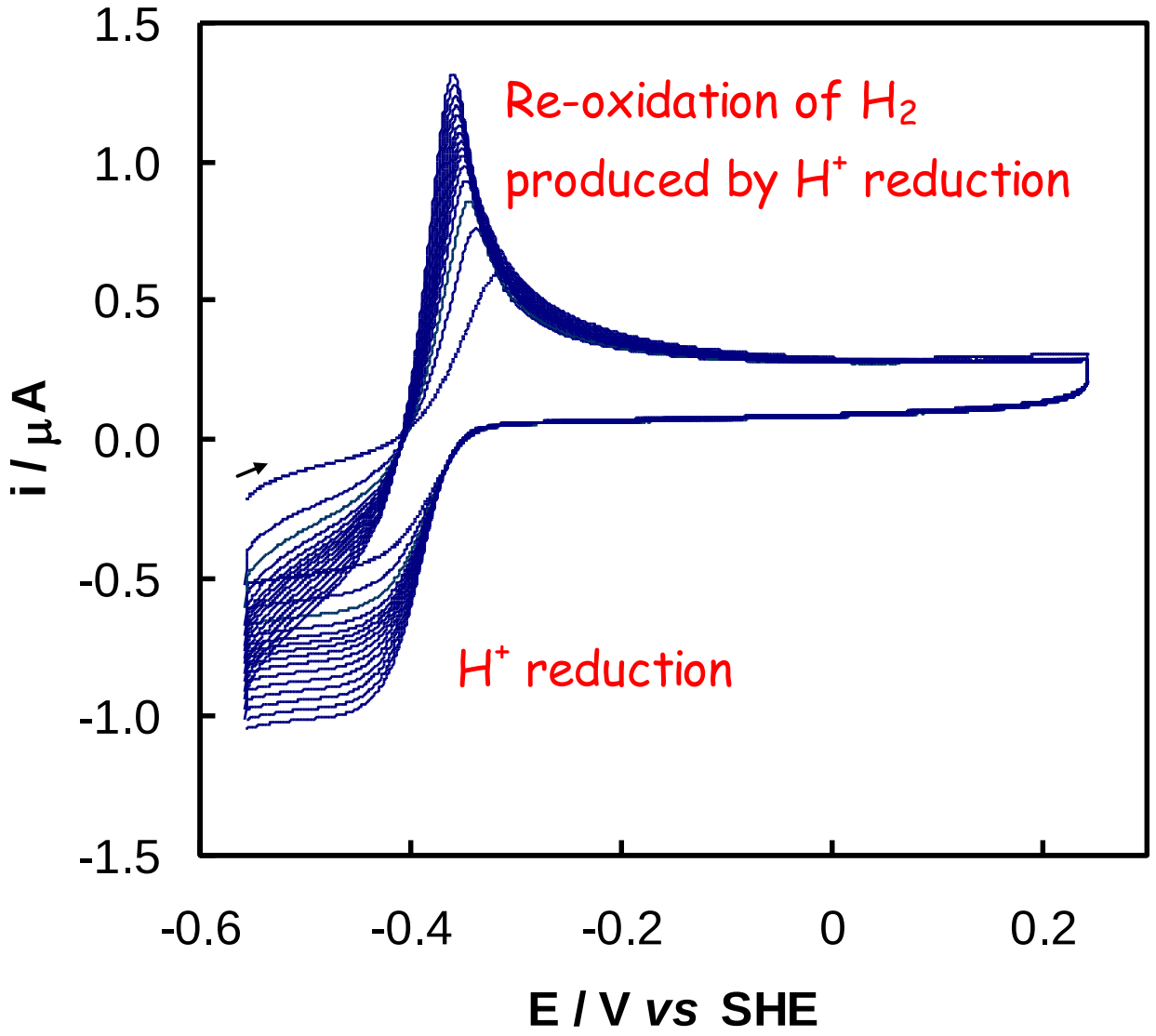






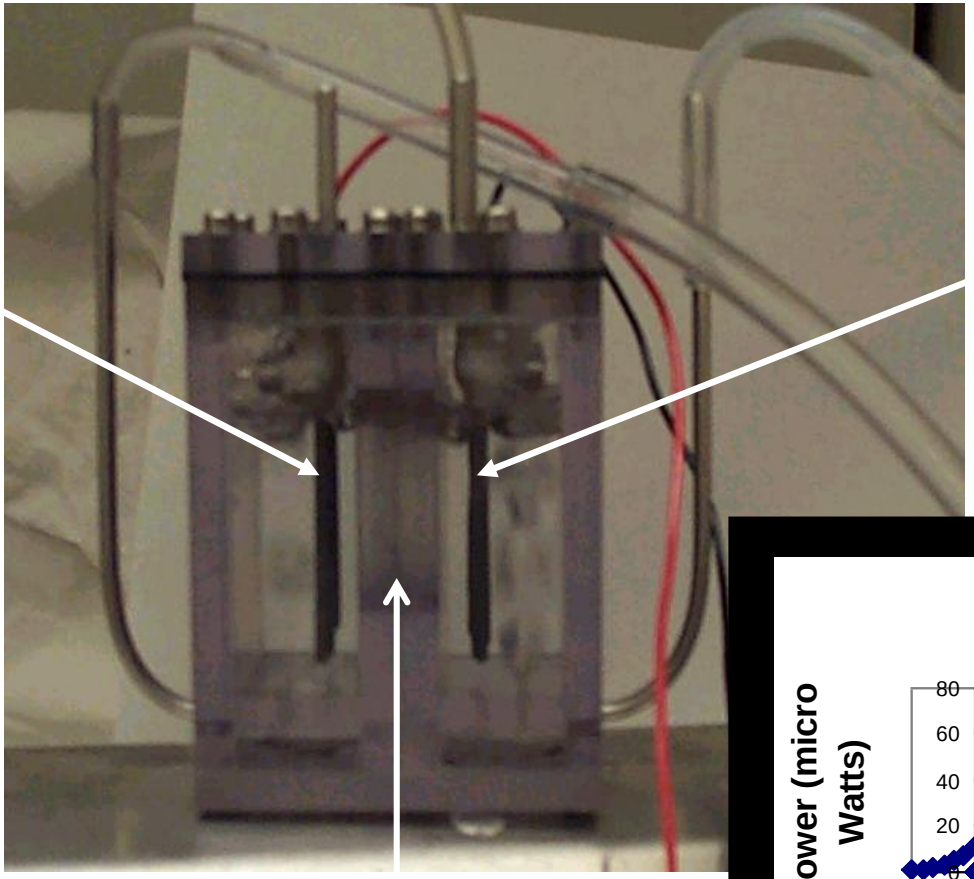
Gold electrode can also be suitable to studying Hyrdogenases with Fe-S clusters due to the covalent binding between gold with SH groups

Preparing the film: Stationary PGE electrode is potential-cycled in dilute H2ase solution (< 1 μM) (in this case *D.gigas* NiFe enzyme)



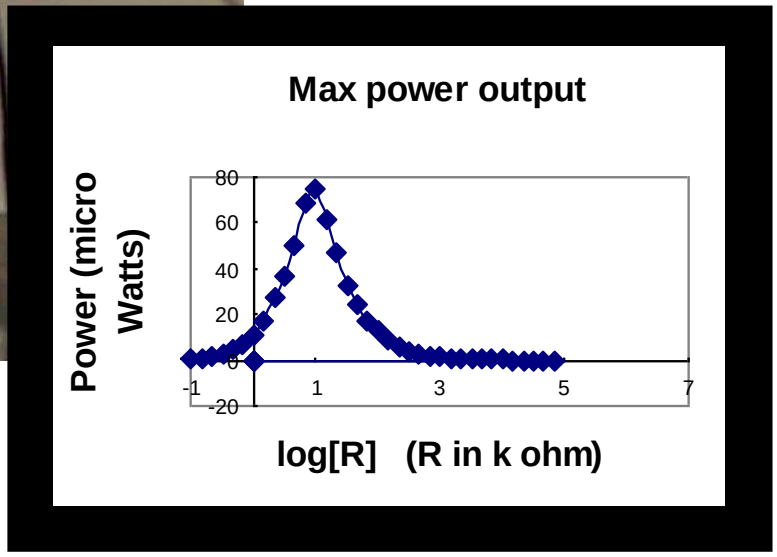
'100%- Bio' hydrogen fuel cell : no chemical catalysts

laccase (Cu enzyme) on PGE electrode



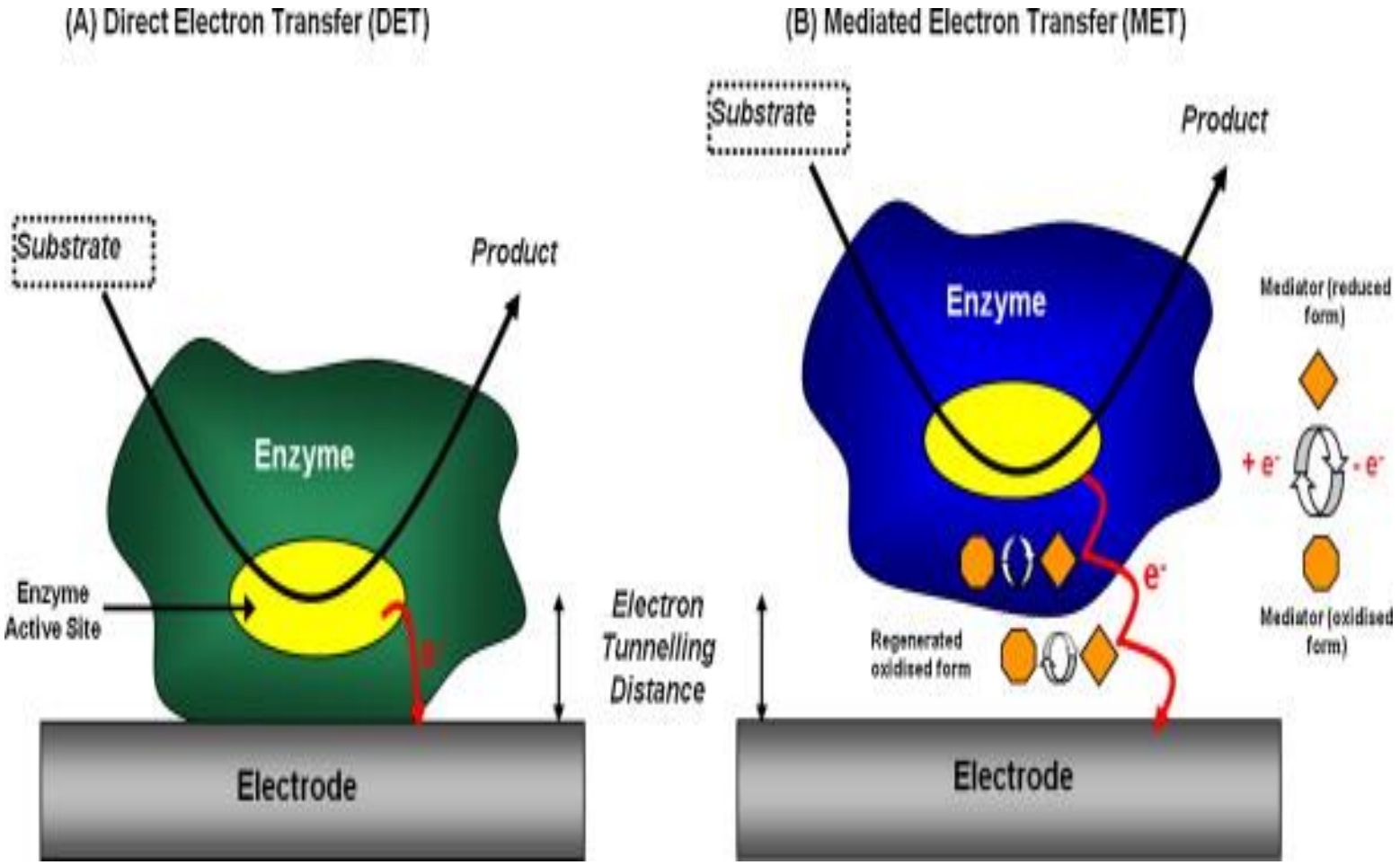
H_2ase (NiFe enzyme) on PGE electrode

Nafion membrane

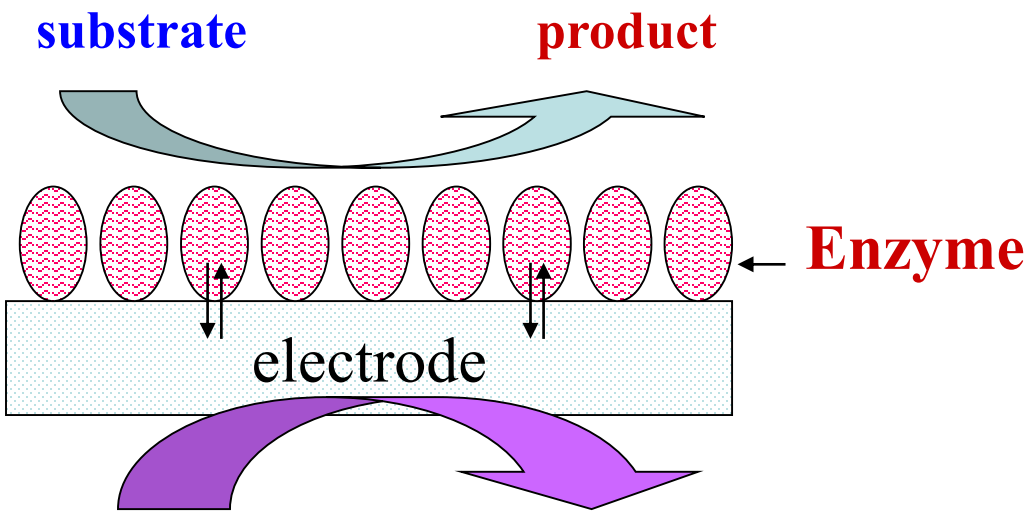


2. Designing Bio-sensors by using PFV

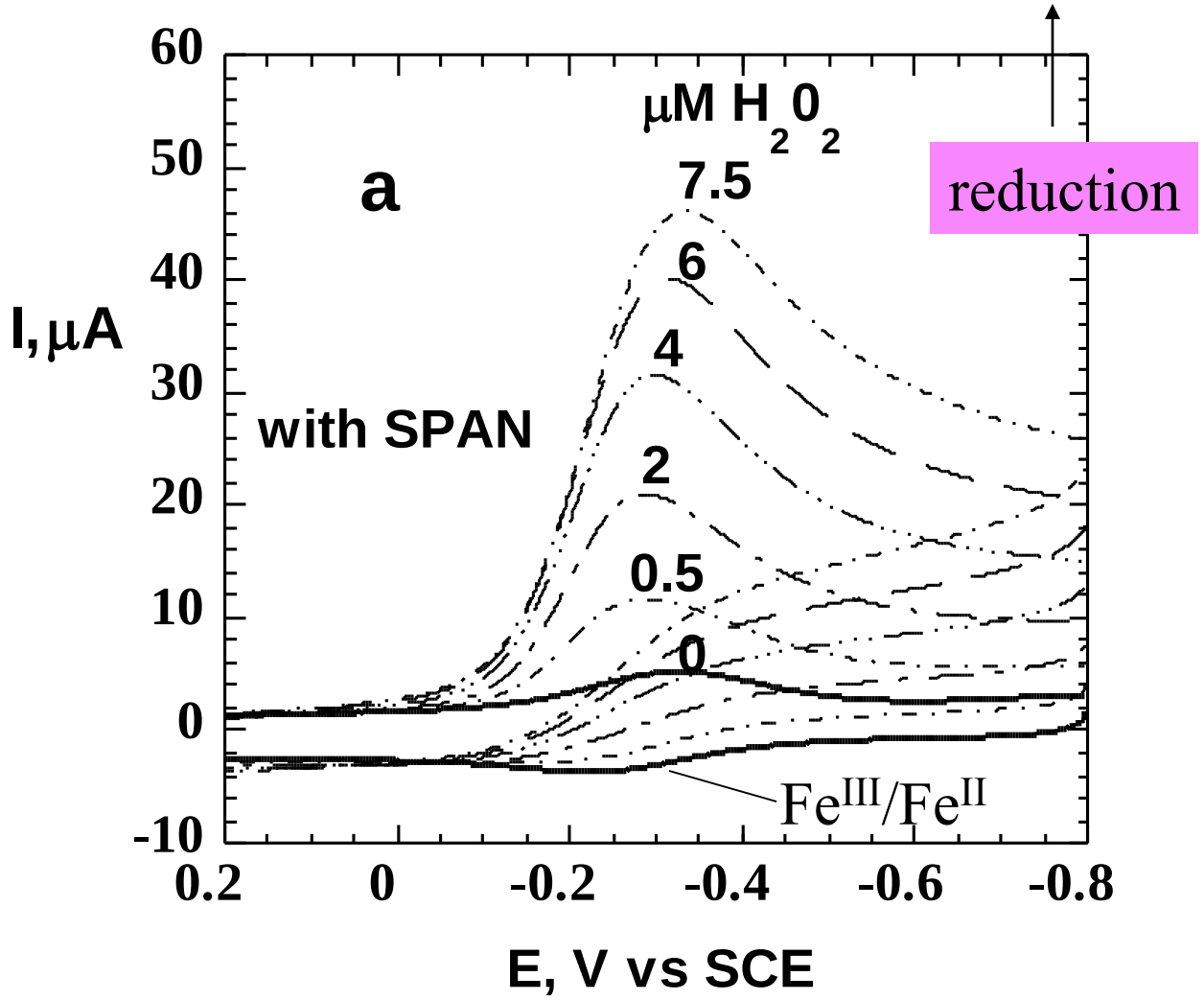
-Principles of working:

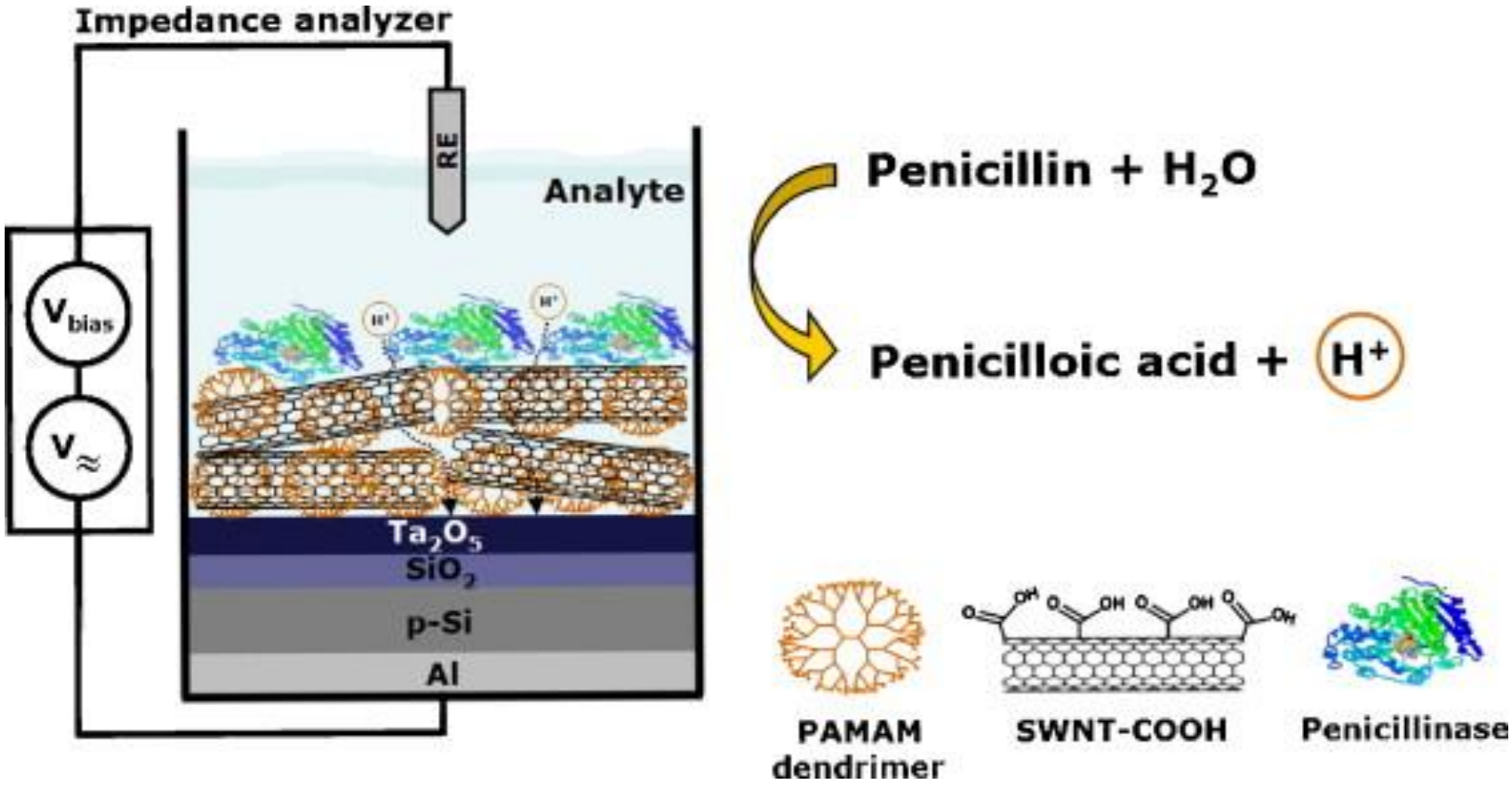


Principle of Electrochemical Biosensors in PFV



Catalytic regenerative mechanism in PFV

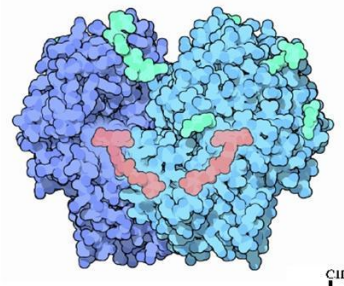




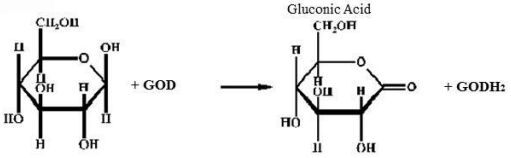
Biosensor based on PFV for penicillin detection

Glucose Biosensor based on pFV

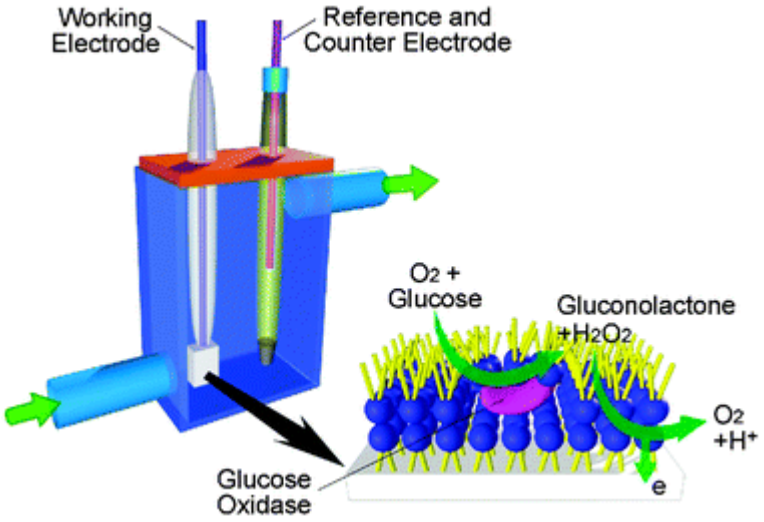
Glucose Oxidase

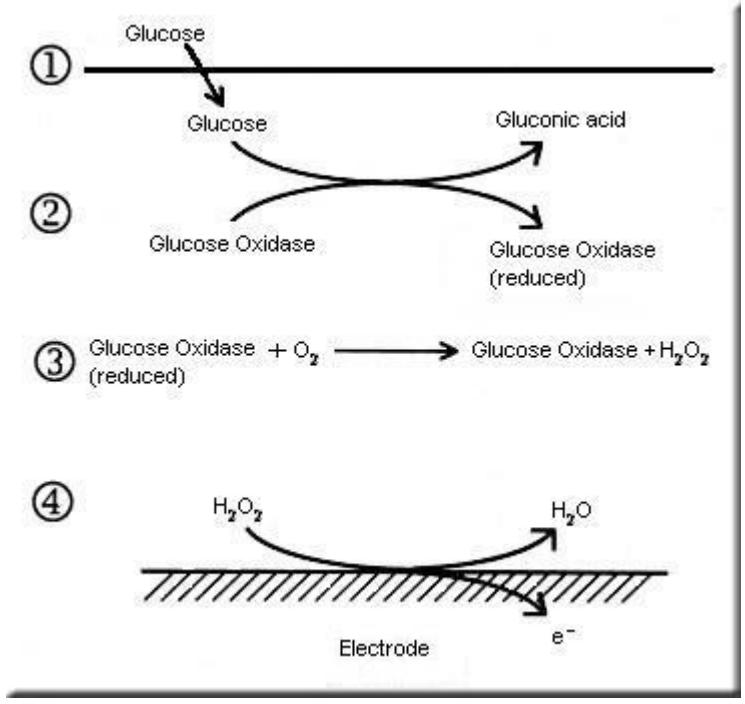


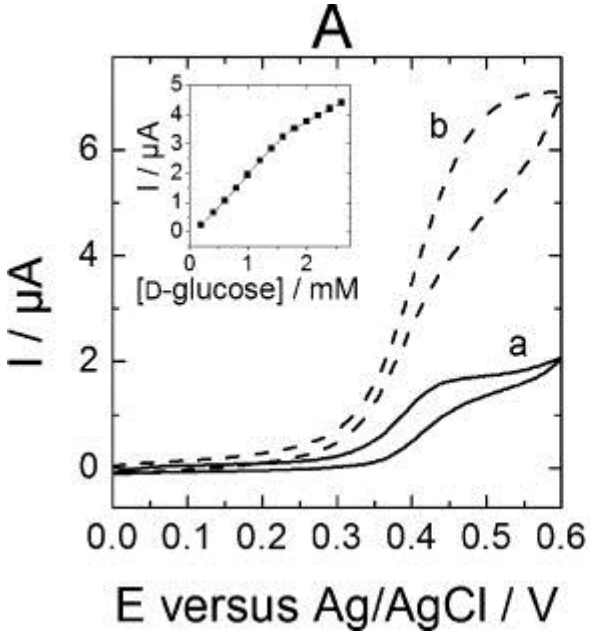
- Small stable enzyme
- Oxidizes glucose into glucolactone
- Converts O_2 to H_2O_2in the process
- Found in honey
  - Natural preservative
  - H_2O_2 kills bacteria

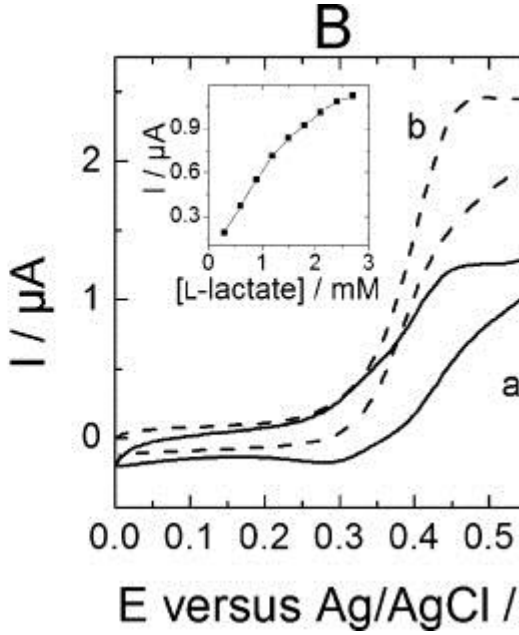


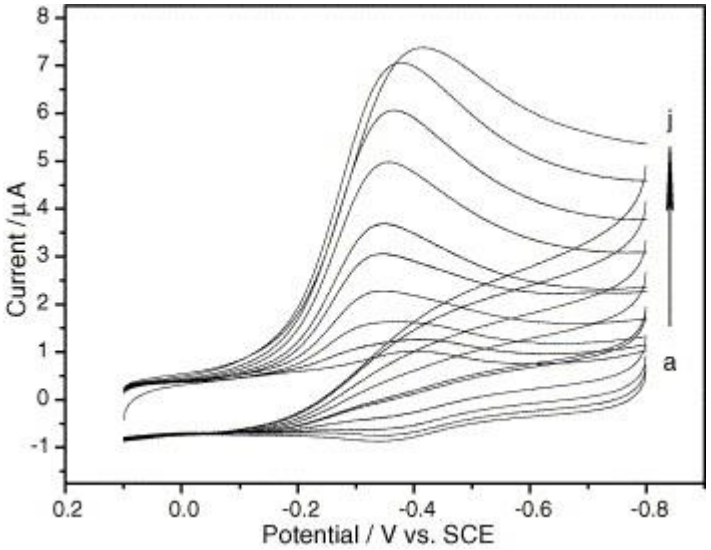
$GODH_2 + O_2 \rightarrow GOD + H_2O_2$

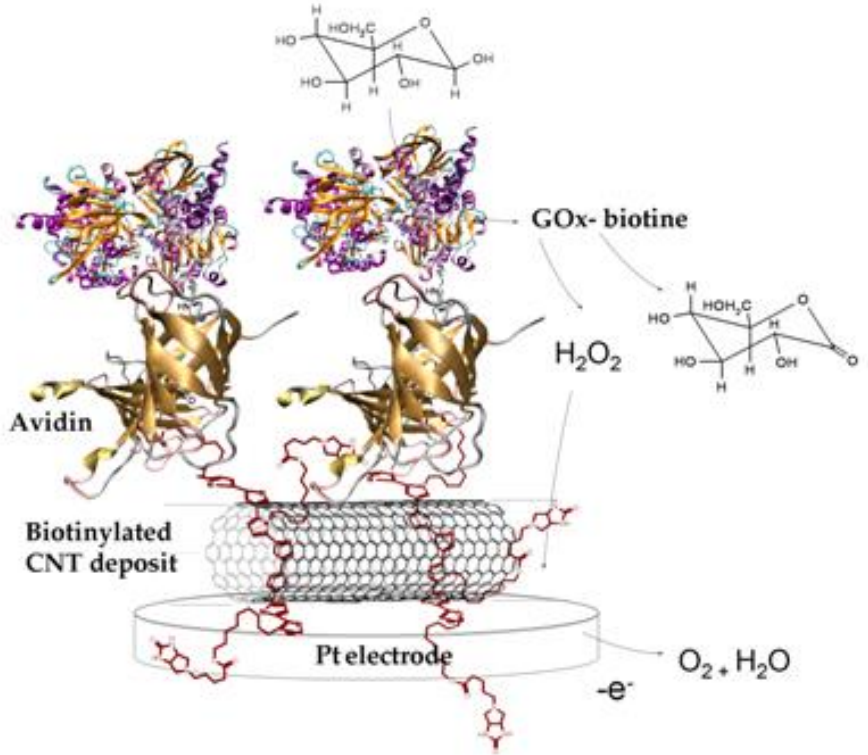




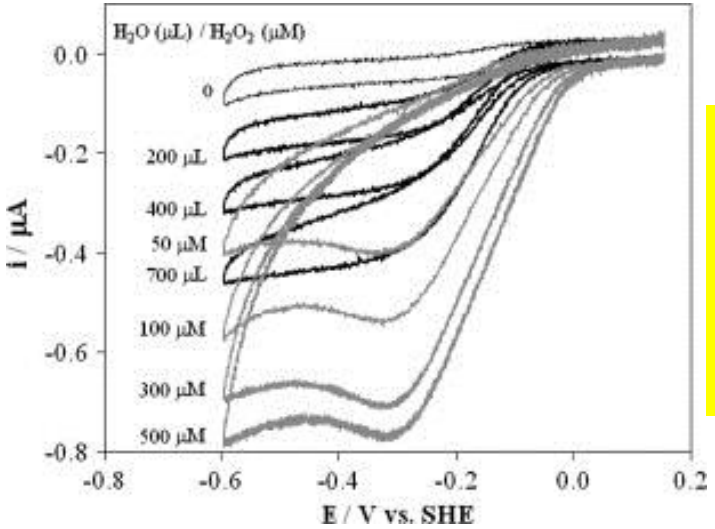




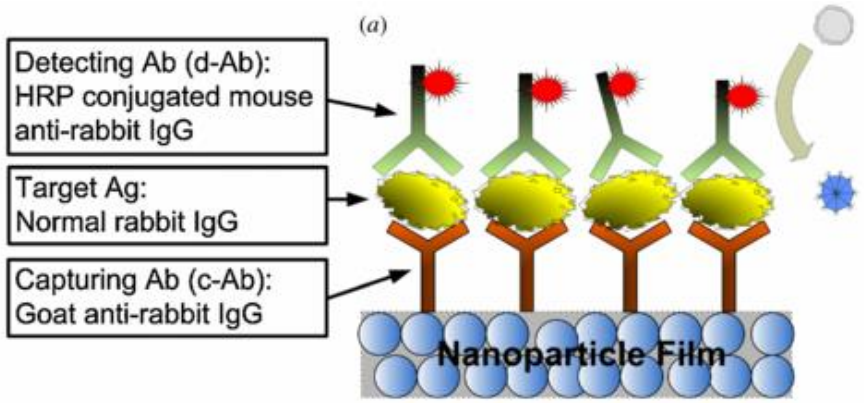


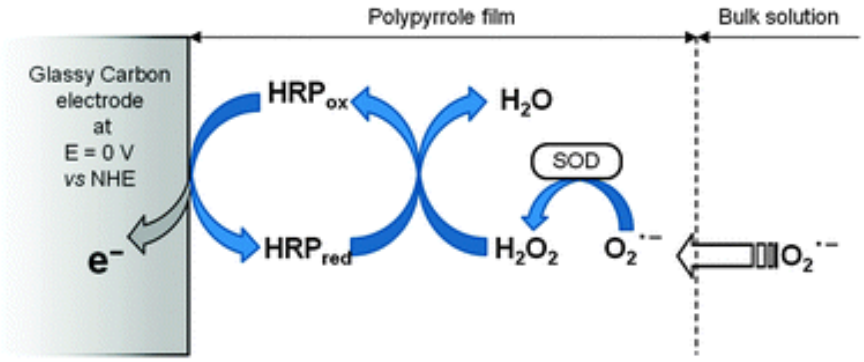


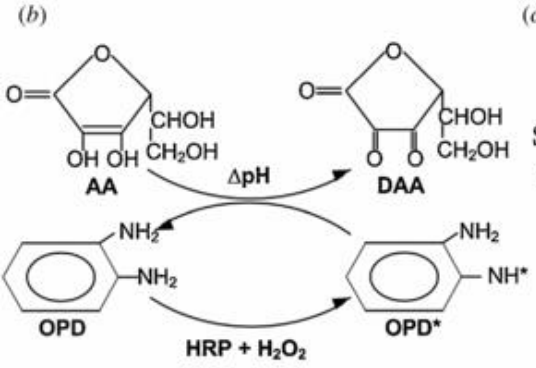
H_2O_2 biosensor based on
Redox reaction of Protein Avidin

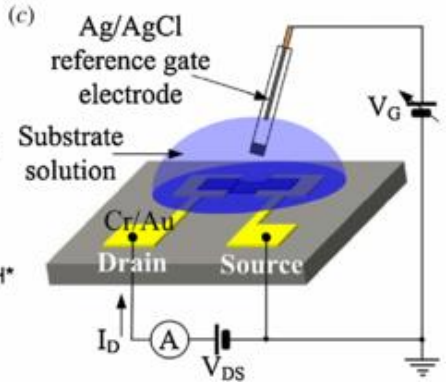


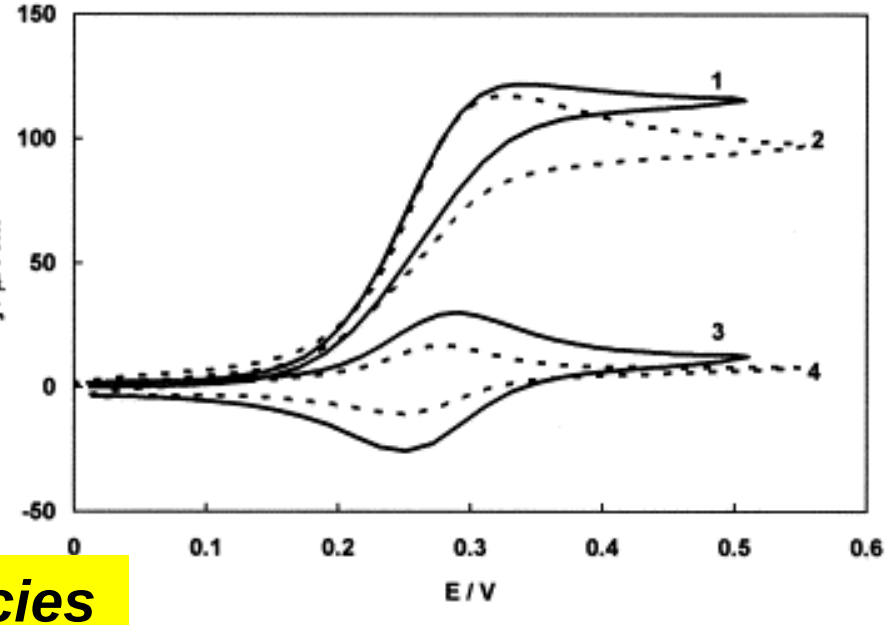
On some electrodes Detection of Hydrogen peroxide is also possible without mediator







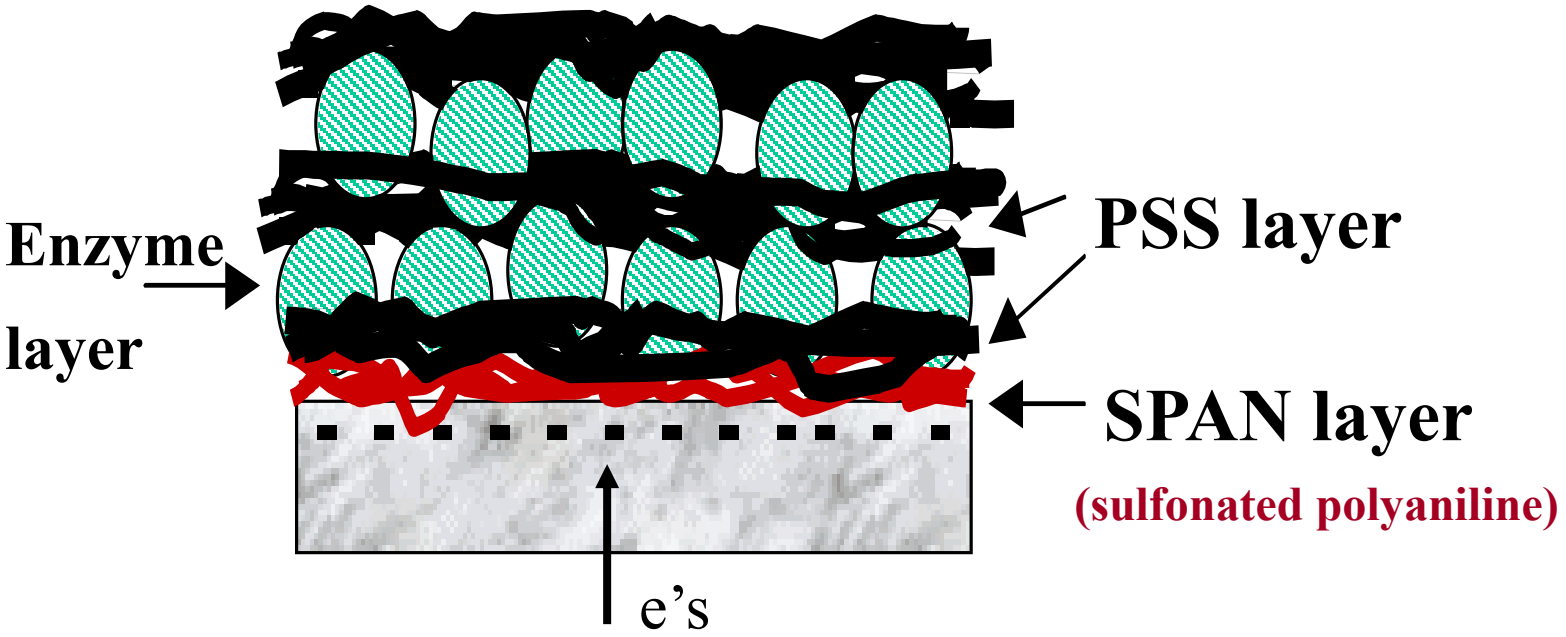




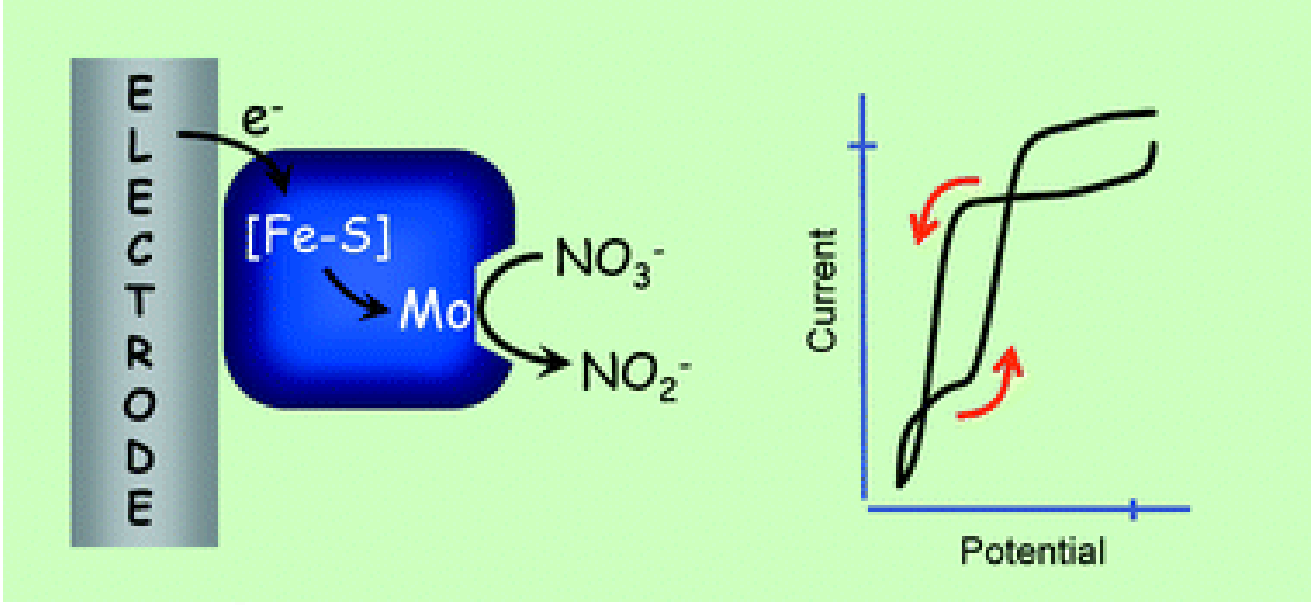
Detection of ***Reactive Oxygen species***
by
PFV set-up with Horseradish peroxidase

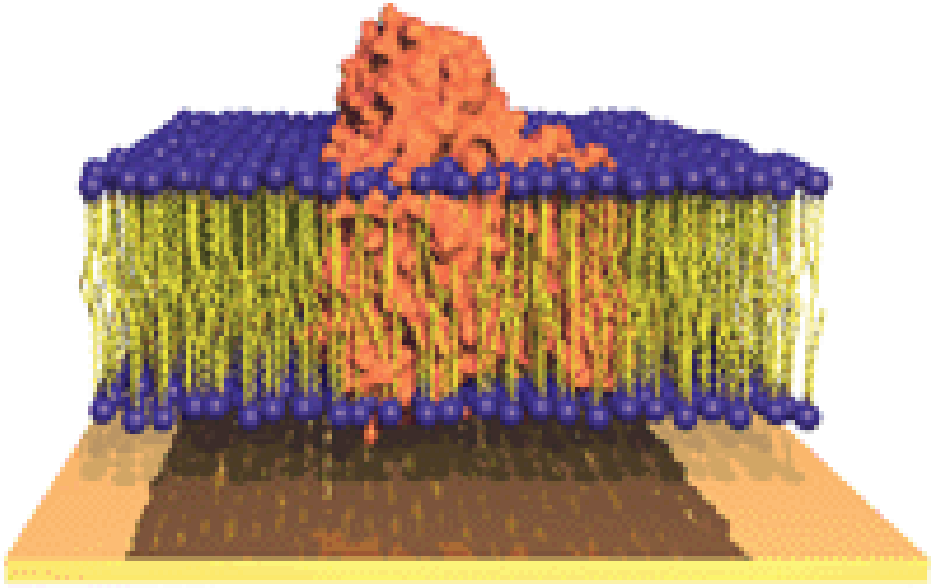
Detection of hydrogen peroxide

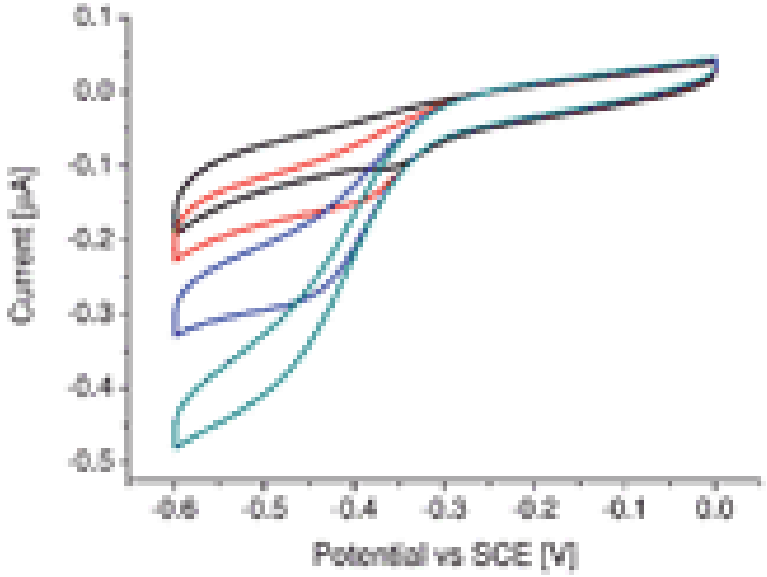
Conductive polymers efficiently wire peroxidase enzymes to graphite



Anal. Chem., 2003, *75*, 4565-4571.

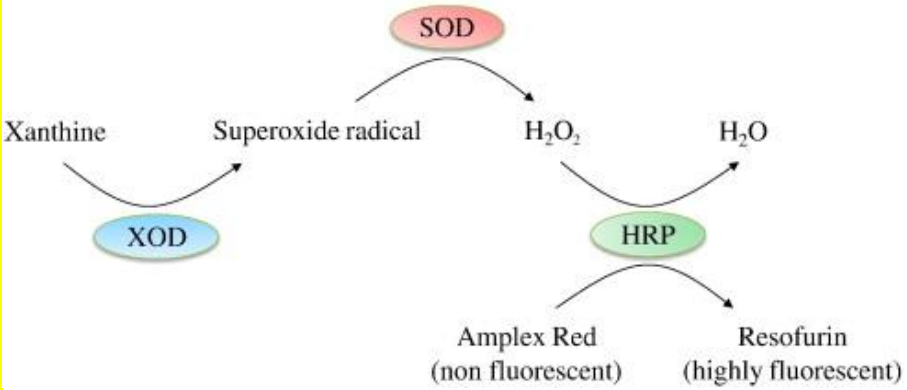






Detection of Nitrites/Nitrates anions by PFV set-up

In most of the PFV studies, the authors have explored **haem-containing proteins as catalase, hemoglobin and myoglobin**, **cytochrome P450** and horseradish peroxidase as platforms **for the detection of oxygen, reactive-oxygen species, hydrogen peroxide, trichloroacetic acid, nitrites…**



the enzymes used as a platform for ROS detection are sensitive to rather big concentrations of the substrates (i.e., the enzyme sensors can work only in the concentration regions of ROS of over 50 μM), which make their use for the direct detection of ROS in the cells quite limited.

THEORY OF SOME COMMON REDOX REACTIONS IN PFV

The theory of PFV
almost fully complies with ***the theory of surface confined redox reactions***

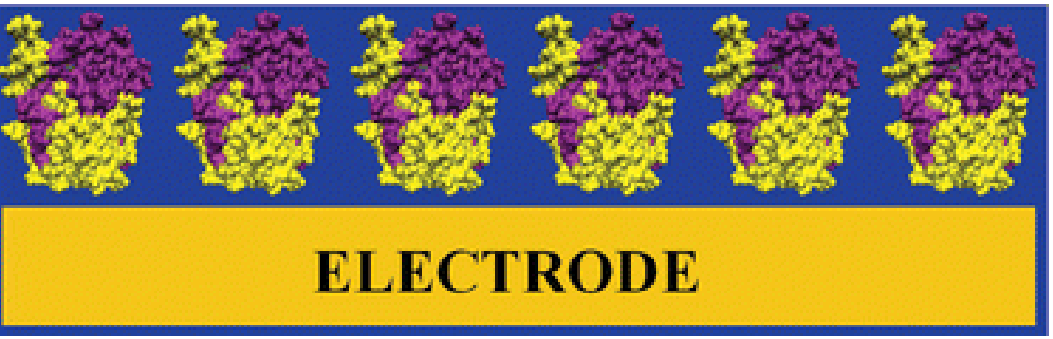
By making the theoretical models in PFV we get insights into the

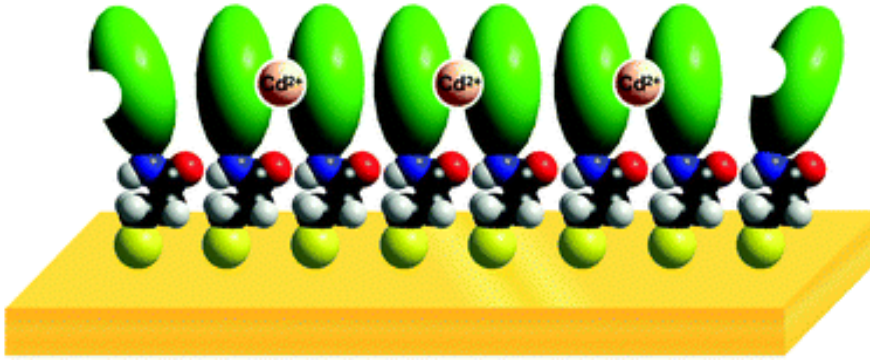
-redox mechanism of a given enzyme studied

-thermodynamics and kinetic parameters relevant to the electron transfer reaction

- thermodynamics and kinetic parameters relevant to the eventual chemical reactions

…





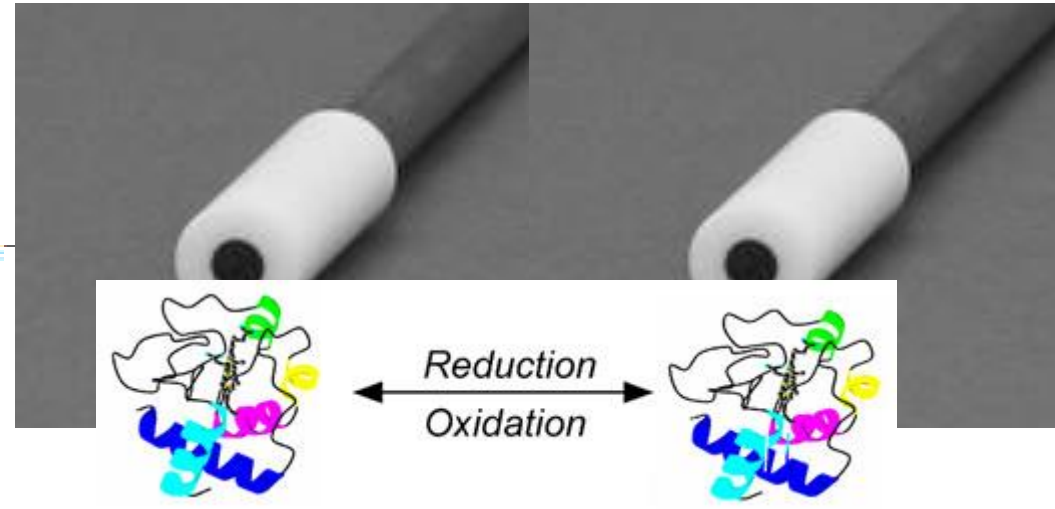
Simple surface electrode reaction

$$R_{(ads)} \overset{k_{sur},\,\alpha_a}{\rightleftarrows} O_{(ads)} + ne^-$$

C_rE mechanism

$$Y_{(ads)} \rightleftarrows R_{(ads)};\ R_{(ads)} \rightleftarrows O_{(ads)} + ne^-$$

Keq.



EC_i mechanism

$$R_{(ads)} \rightleftarrows O_{(ads)} + ne^-;\ O_{(ads)} \xrightarrow{k_f} Y_{(ads)}$$

EC' catalytic mechanism

$$R_{(ads)} \rightleftarrows O_{(ads)} + ne^-;\ O_{(ads)} \xrightarrow{k_f} R_{(ads)}$$

Surface redox reaction- Reactant and the product of the redox reaction remain **firmly Adsorbed** on the electrode surface- ***no diffusion*** effects are considered

Two-step mechanism (EE)

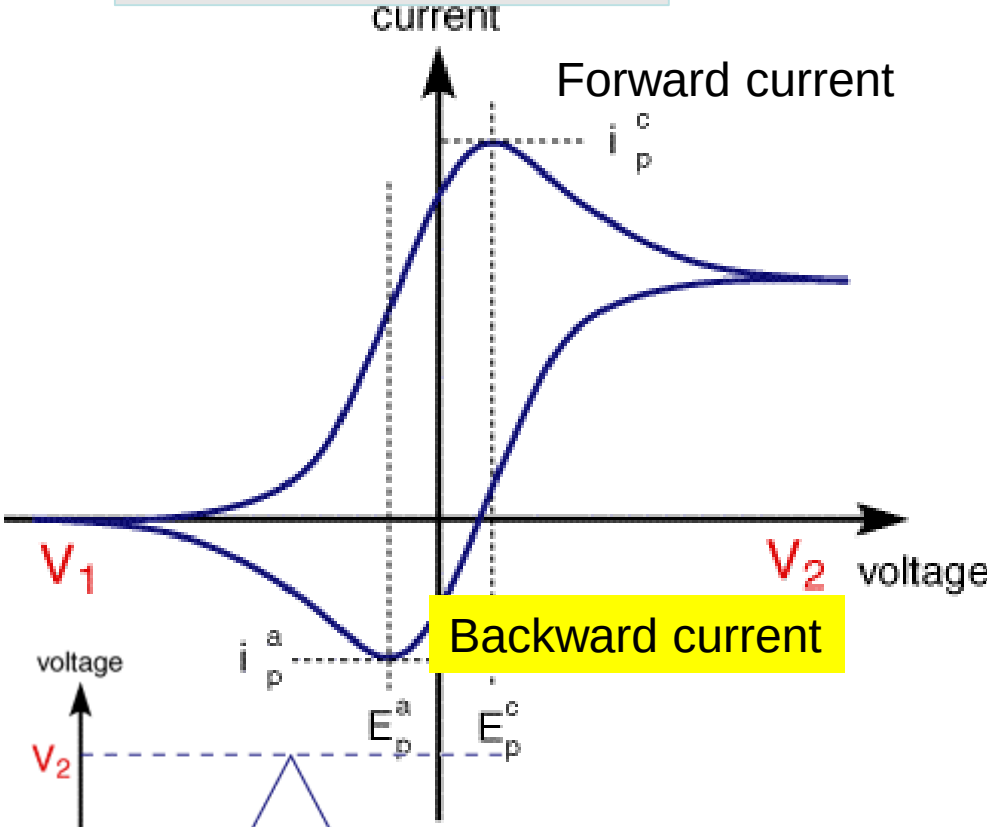
$$A_{(ads)} \rightleftarrows B_{(ads)} + n_1e^- \rightleftarrows C_{(ads)} + n_2e^-$$

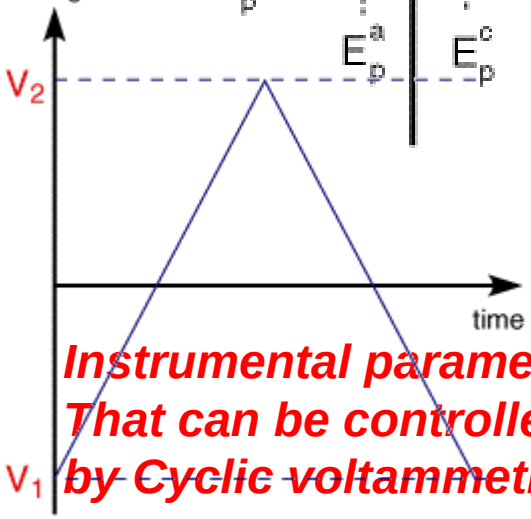
ECE mechanism

$$R1_{(ads)} \rightleftarrows O1_{(ads)} + ne^-;\ O1_{(ads)} \xrightarrow{k_f} R2_{(ads)}$$

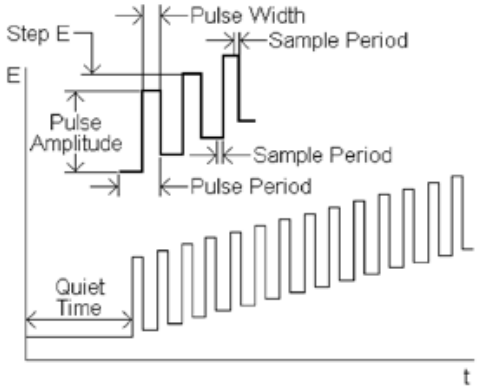
$$R2_{(ads)} \rightleftarrows O2_{(ads)} + ne^-$$

Cyclic voltammogram



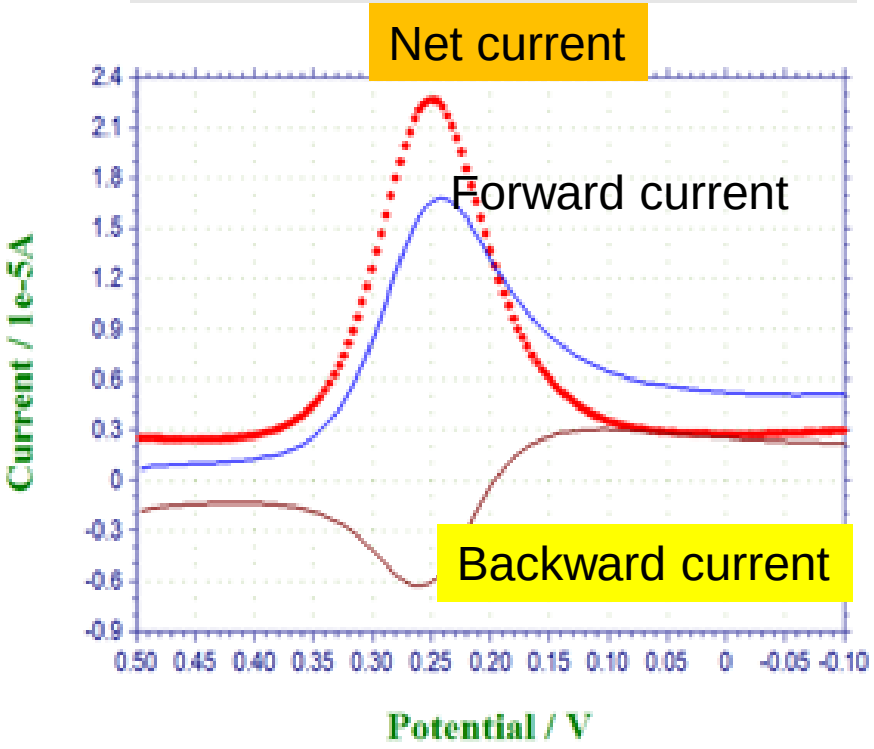


Instrumental parameters That can be controlled by Cyclic voltammetry



-scan rate

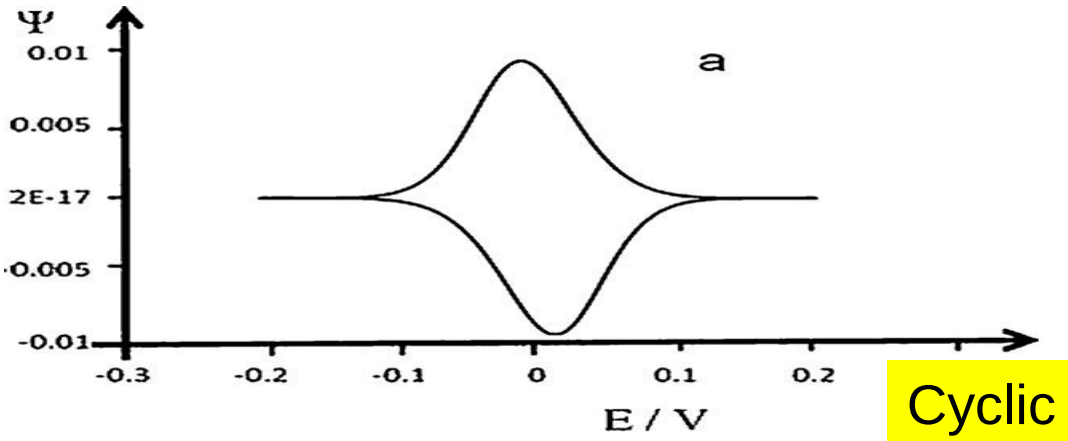
Square-wave voltammogram

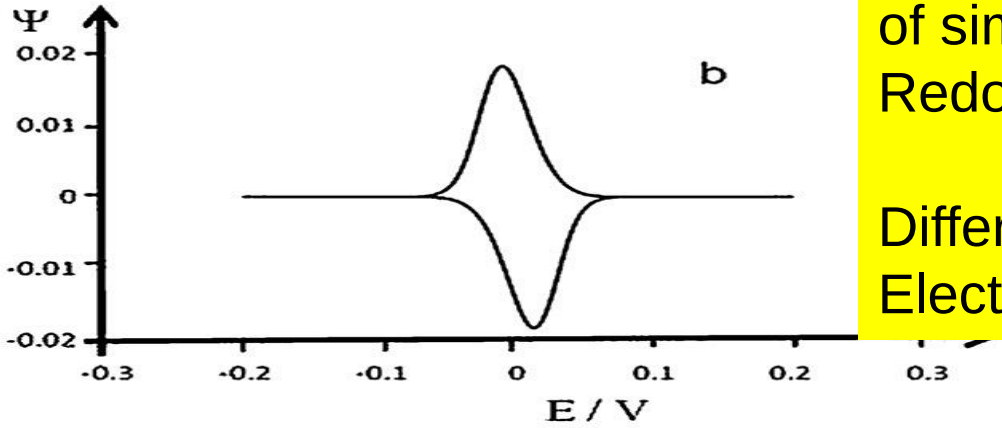


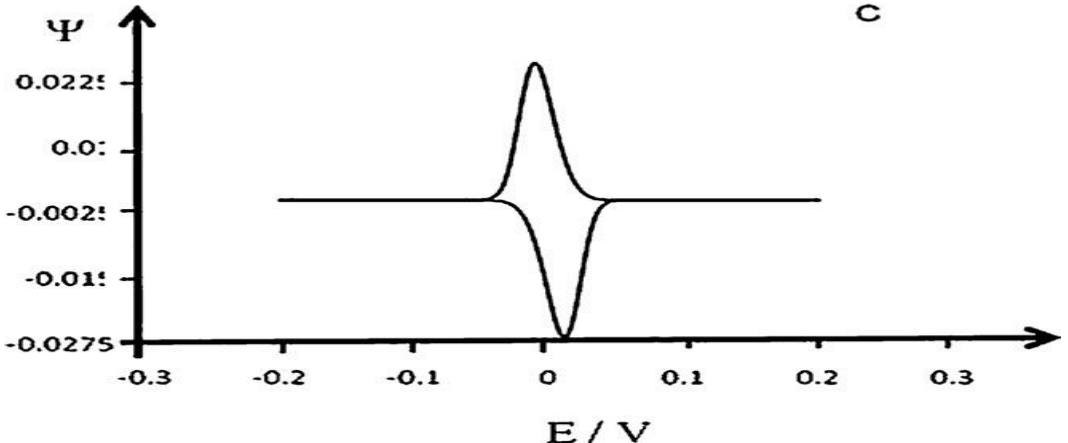
Instrumental parameters That can be controlled by square-wave voltammetry

-**frequency** (analogue to scan rate)

-**SW amplitude**





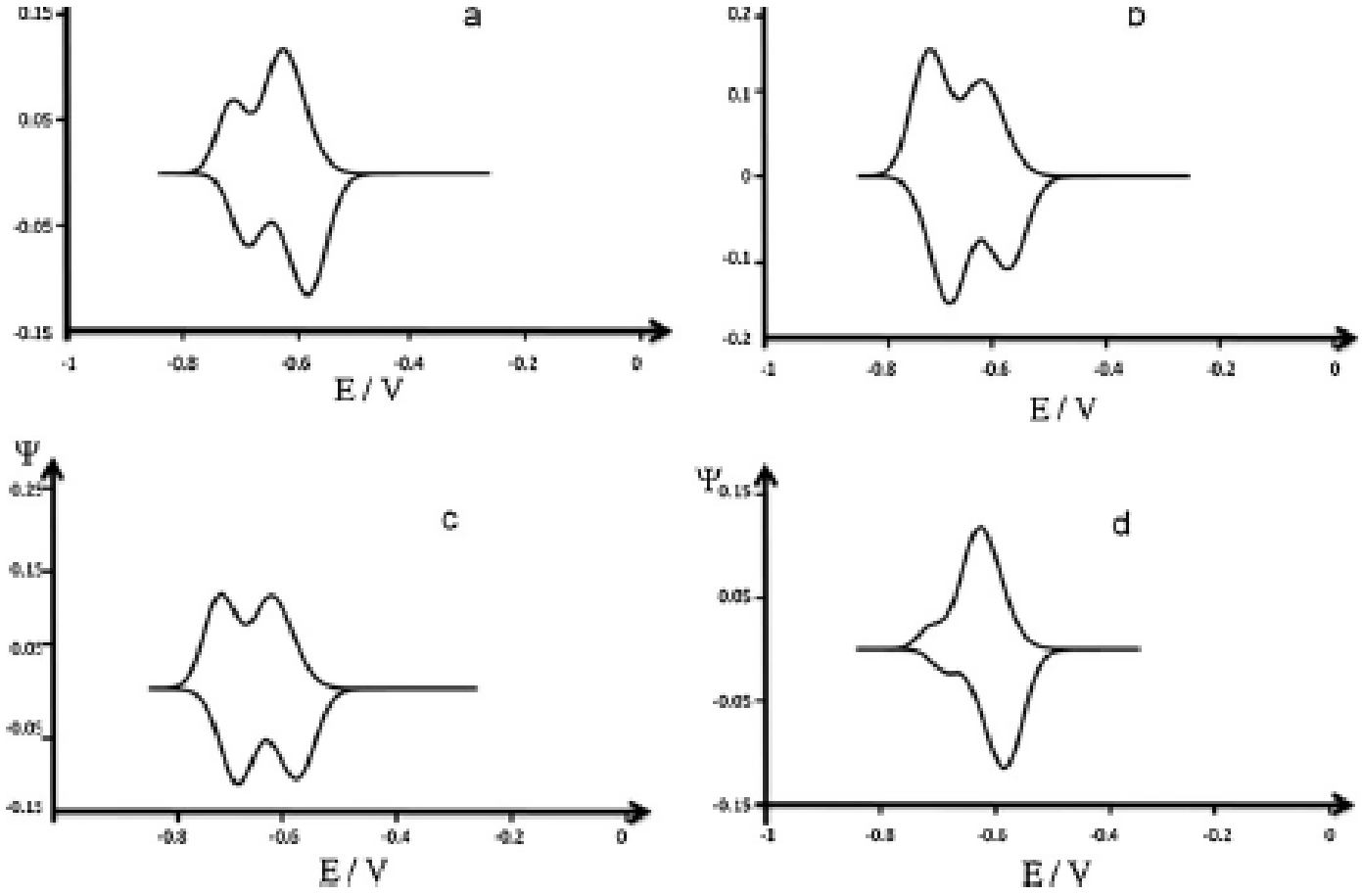


Cyclic voltammograms of simple surface Redox reaction:

Different number od Electrons exchanges

Two-step mechanism (EE)

$$A_{(ads)} \rightleftarrows B_{(ads)} + n_1 e^- \rightleftarrows C_{(ads)} + n_2 e^-$$



Cyclic voltammograms of an EE Mechanism in PFV

Effect of different kinetics of both redox steps

This mechanism holds for PFV in which the redox center of the enzyme is some multivalent cation (Mo, V, Cr, Cu,

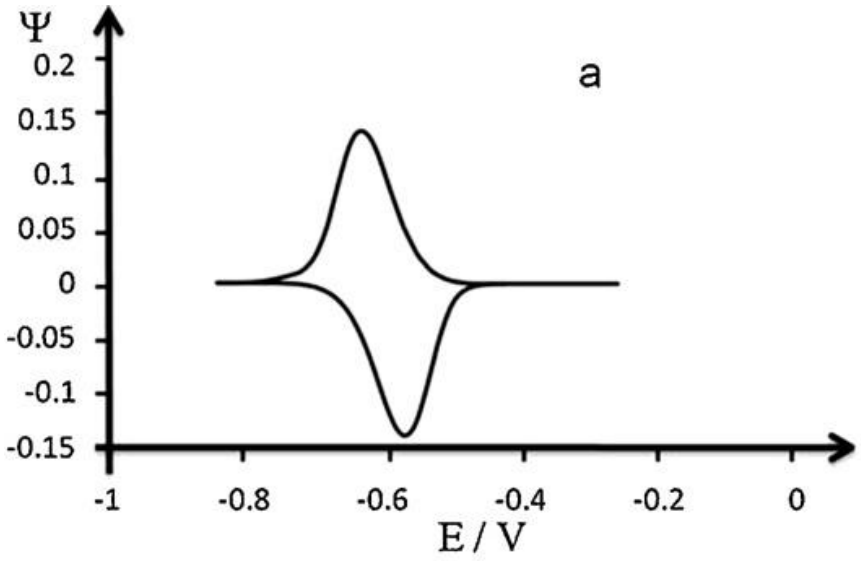
Cyclic voltammetry

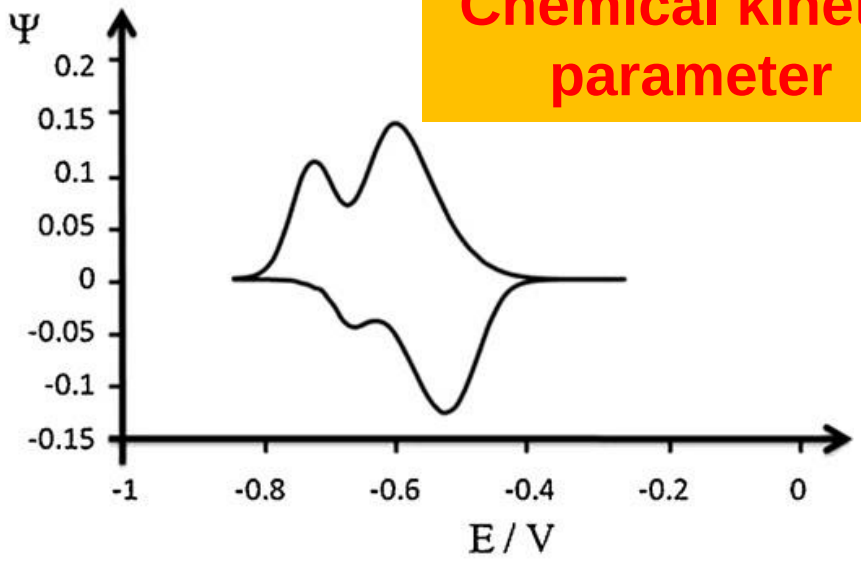
Effect of the Chemical kinetic parameter

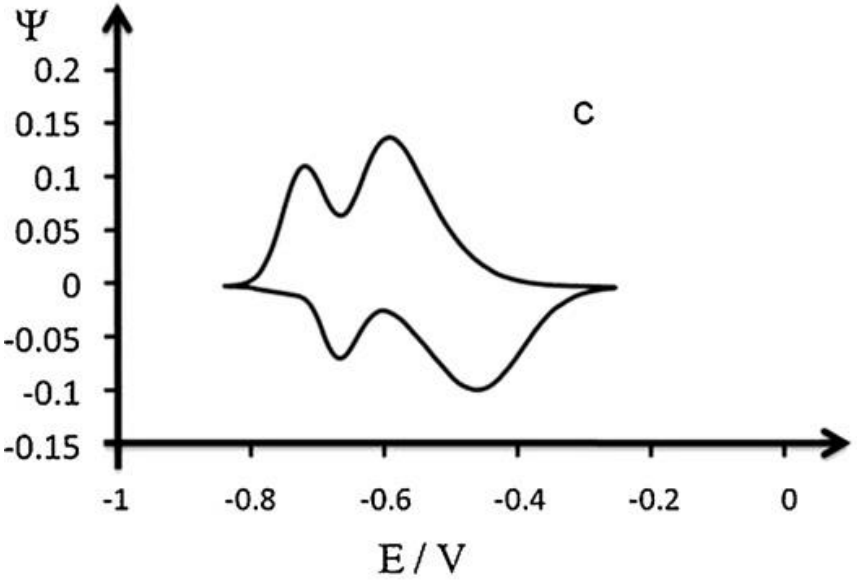
ECE mechanism

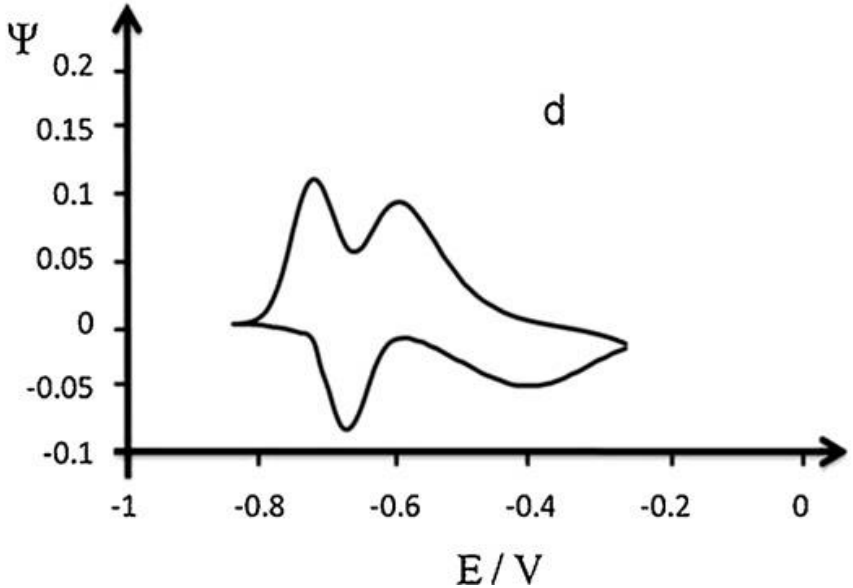
$$R1_{(ads)} \rightleftarrows O1_{(ads)} + ne^- ; \quad O1_{(ads)} \xrightarrow{k_f} R2_{(ads)}$$

$$R2_{(ads)} \rightleftarrows O2_{(ads)} + ne^-$$



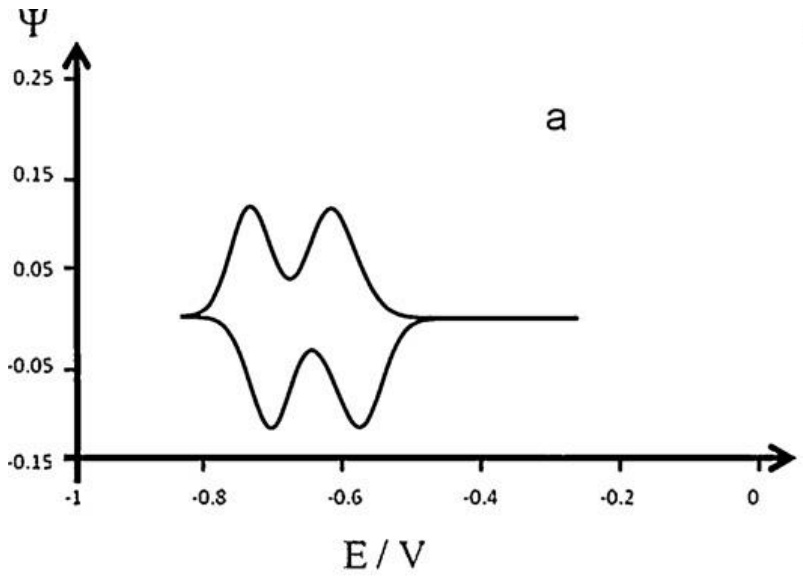


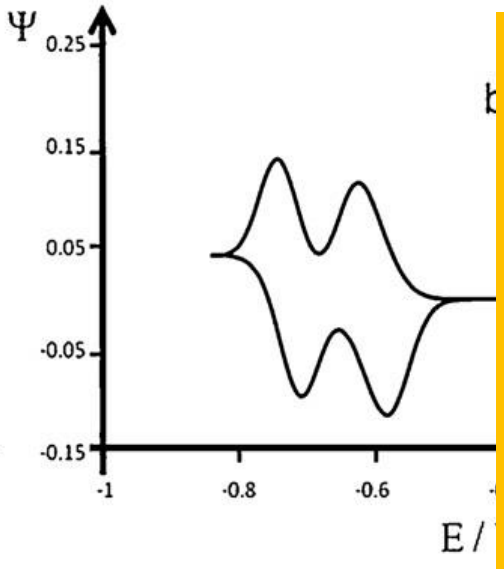


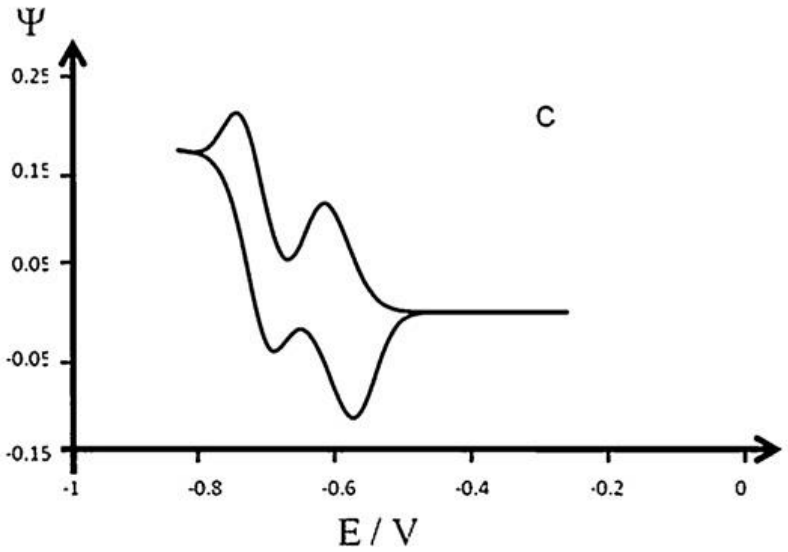


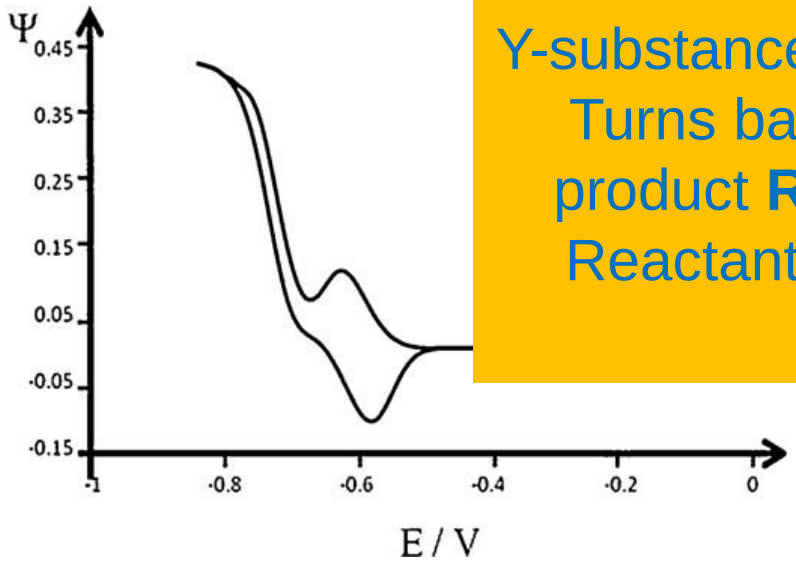
EEC' catalytic mechanism

A(ads) +ne- $\leftrightarrow$ $R_{(ads)} \rightleftharpoons O_{(ads)} + ne^-$; $O_{(ads)} \xrightarrow[+Y]{k_f} R_{(ads)}$





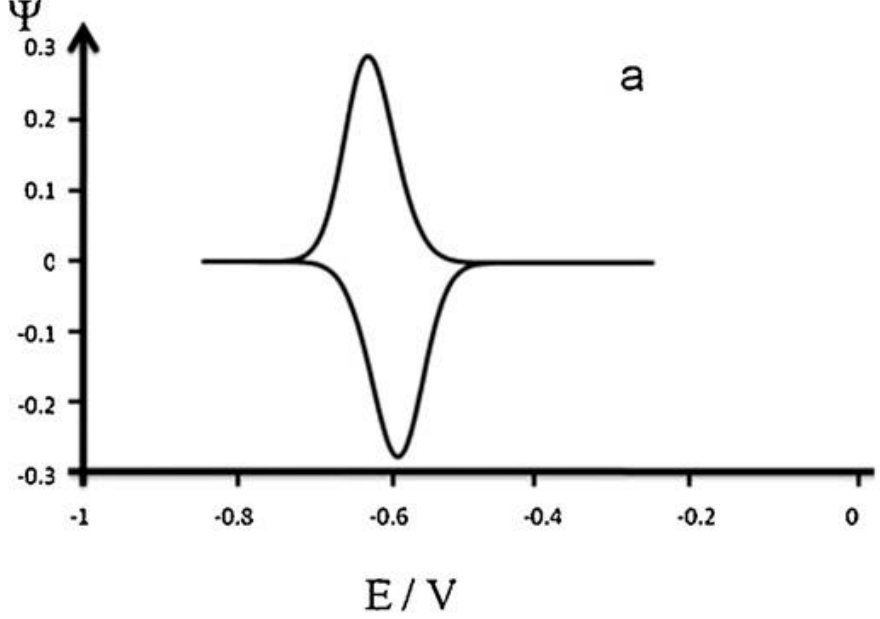


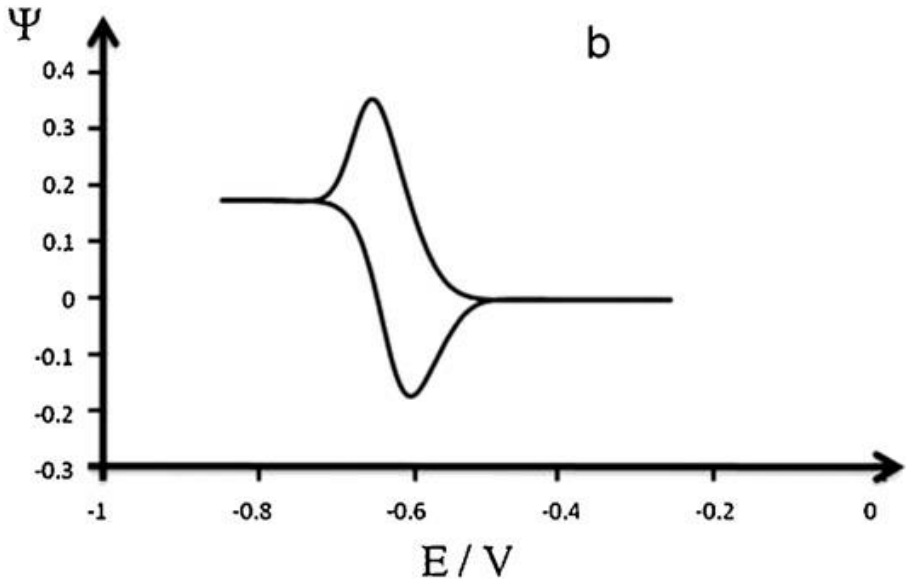


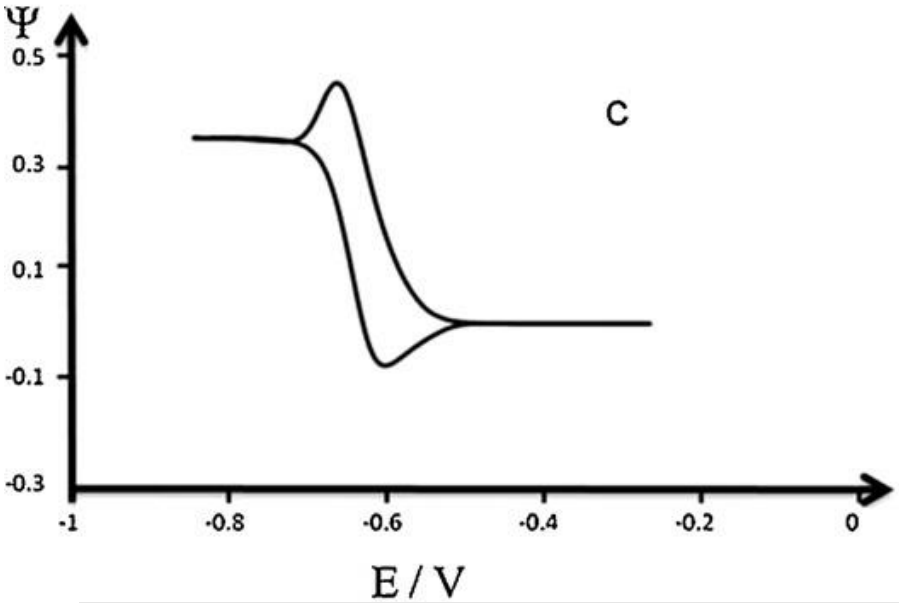
Cyclic voltammetry

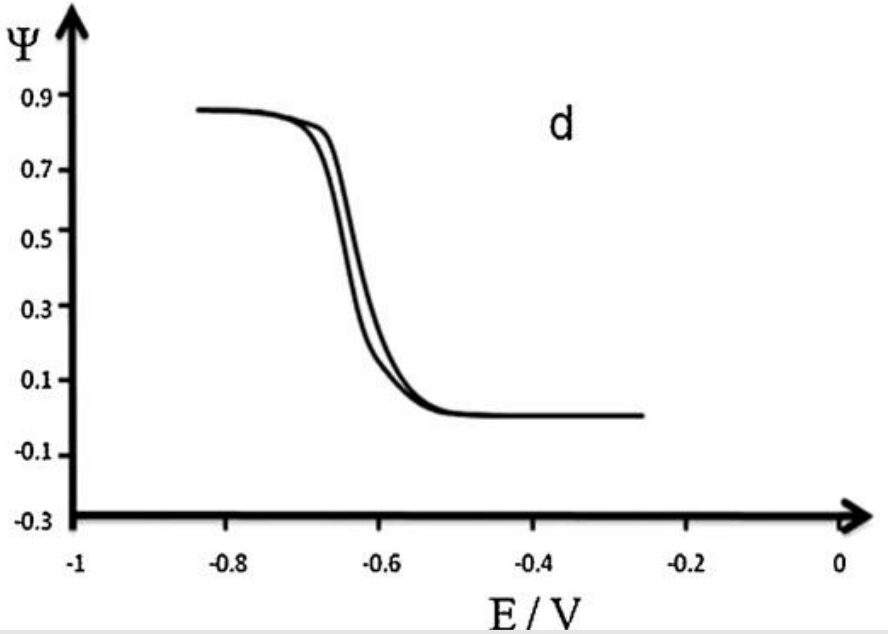
Effect of the Chemical catalytic Parameter

Y-substance that Turns back product **R** to Reactant **O**

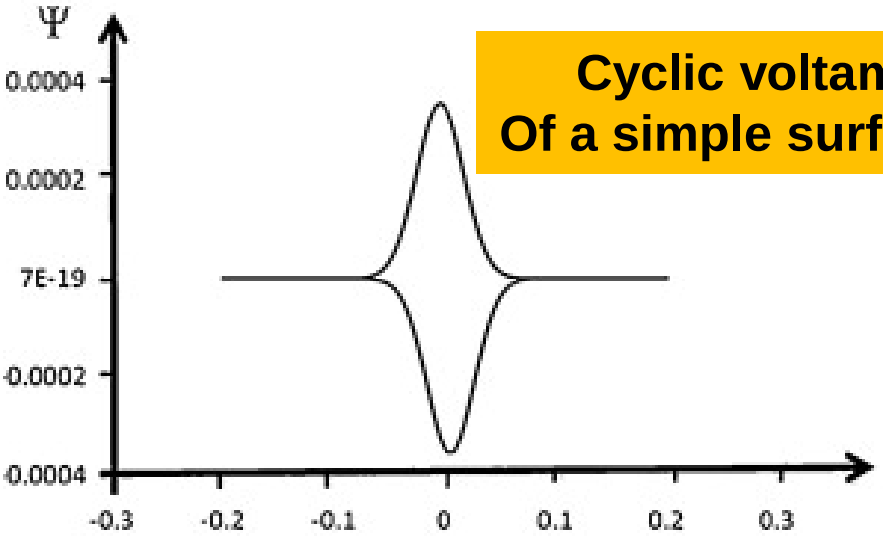






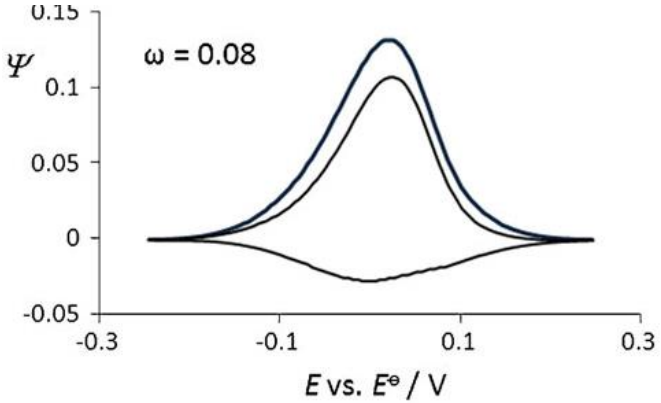


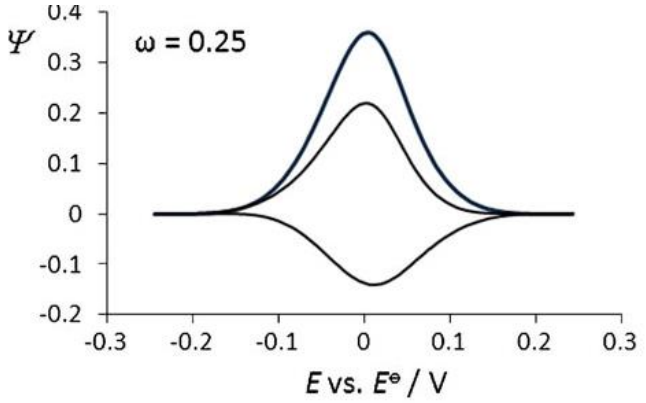
Theoretical cyclic voltammograms of an EC' (catalytic regenerative reaction) Obtained by increasing concentration of the substrate

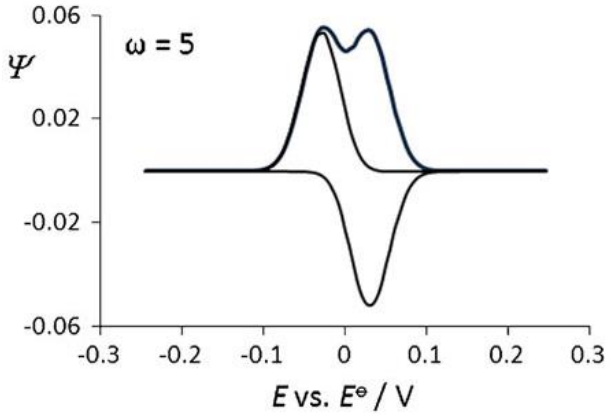


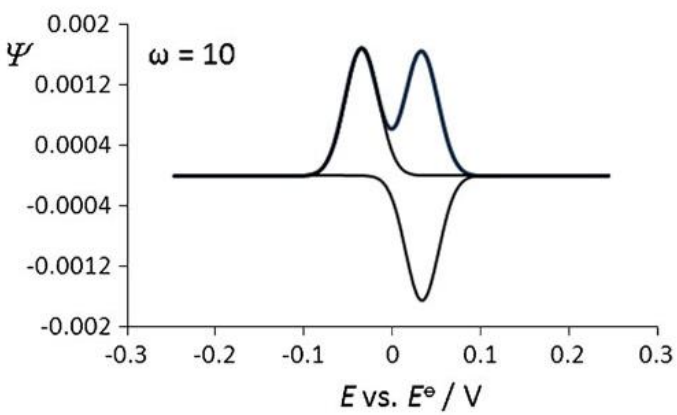
Cyclic voltammogram
Of a simple surface reaction

Effect of the standard rate constant of electron transfer on the voltammetric features in *Square-wave voltammetry* (SWV) of simple Surface redox reaction



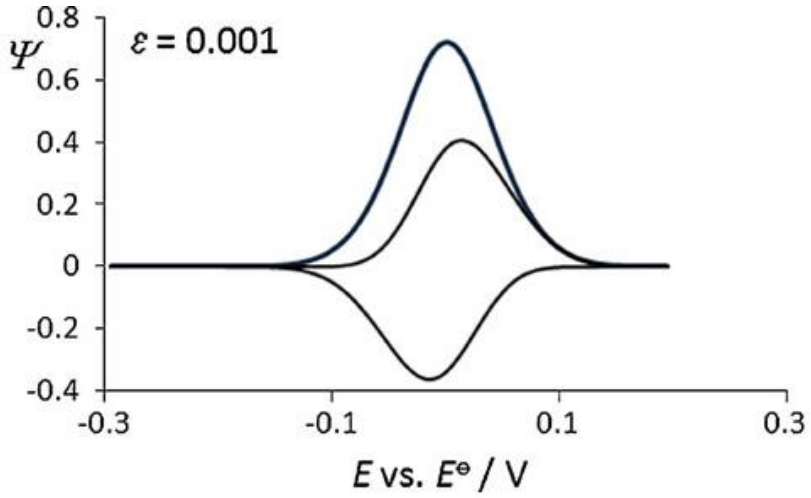


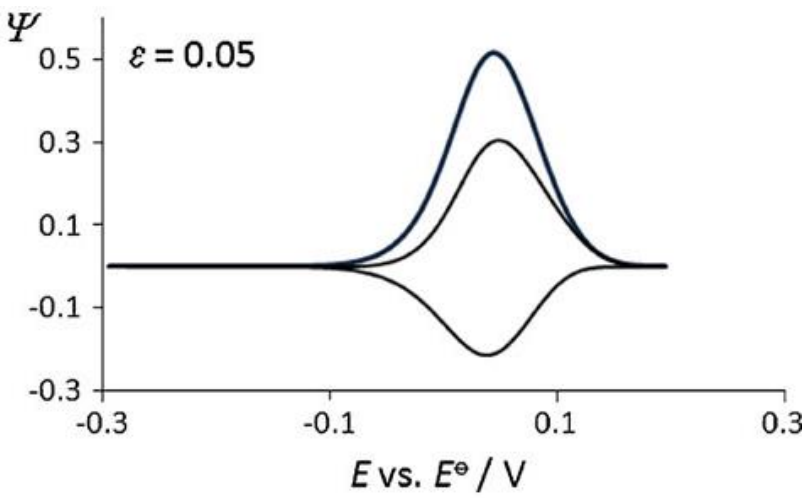


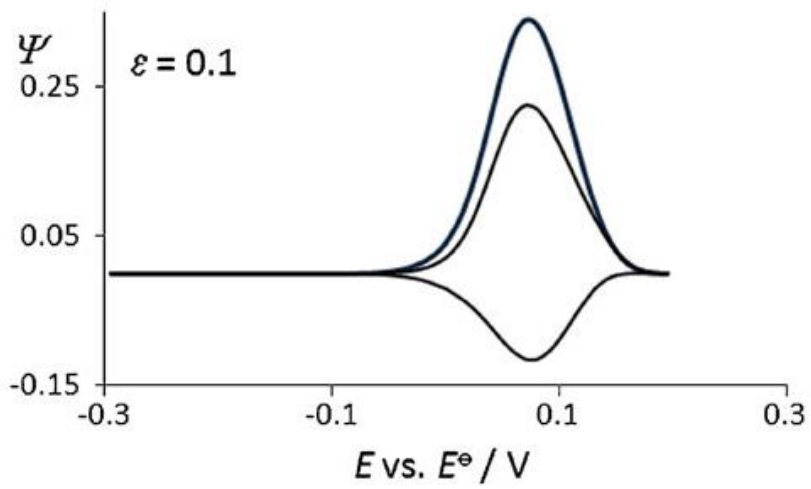


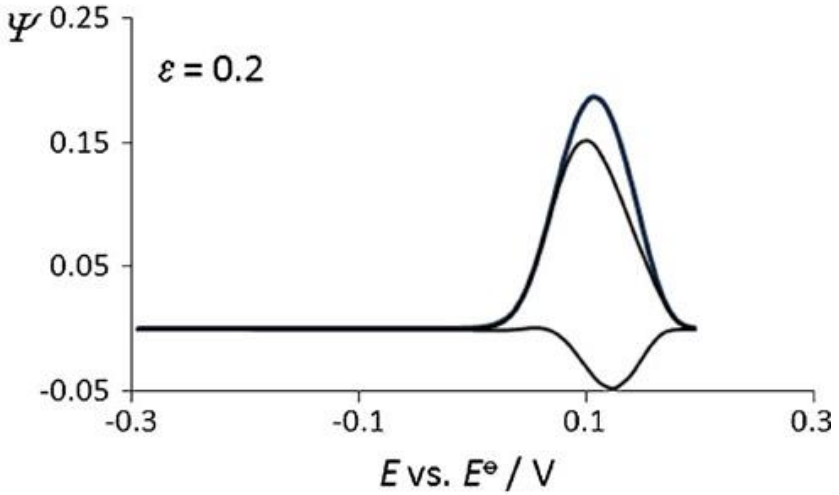
EC_i mechanism

$$R_{(ads)} \rightleftarrows O_{(ads)} + ne^-; O_{(ads)} \xrightarrow{k_f} Y_{(ads)}$$







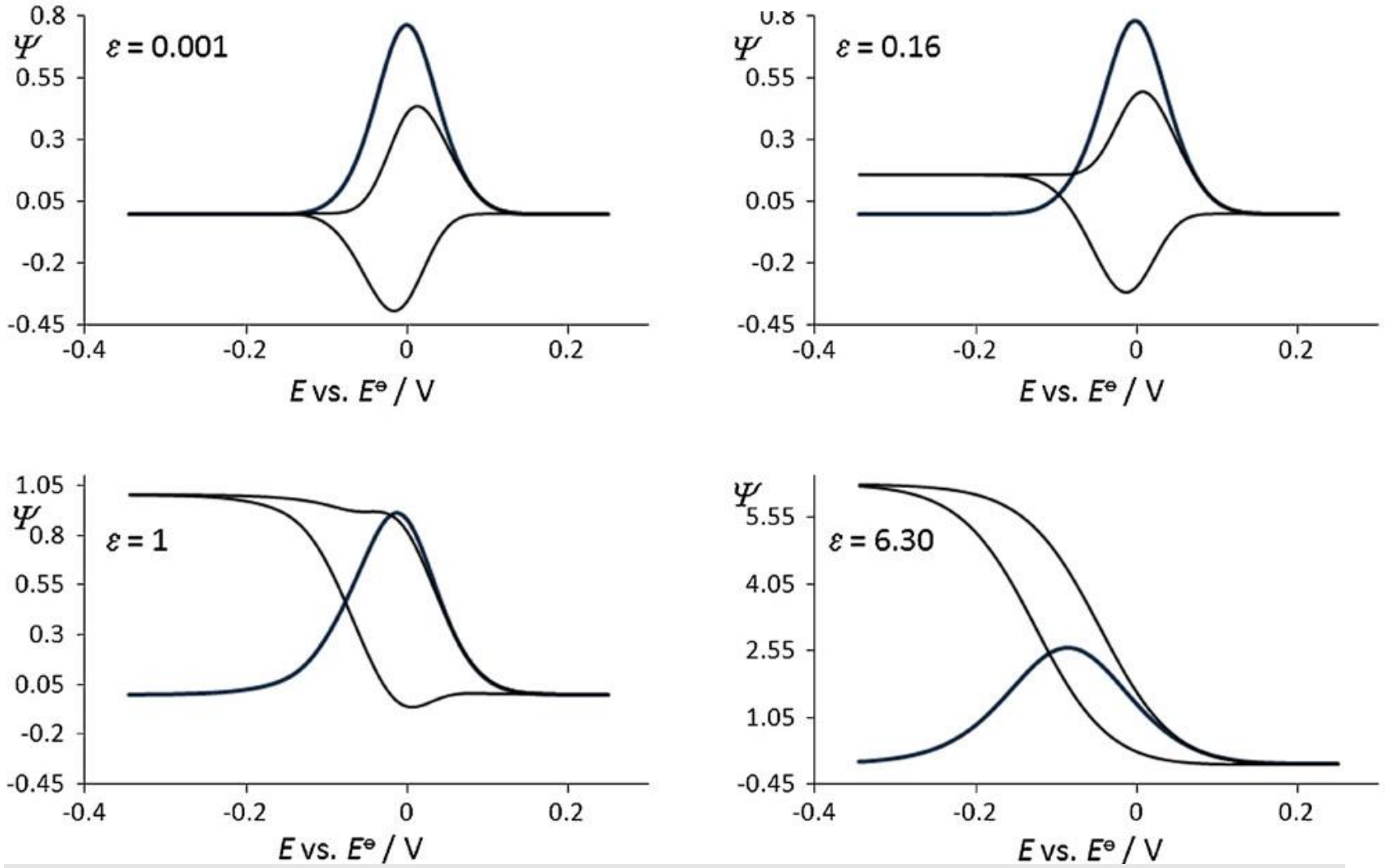


Effect of the ***chemical parameter*** to the features of the SW voltammograms by the ***EC*** (electrochemical-chemical) surface redox reaction

EC' catalytic mechanism

$$R_{(ads)} \rightleftarrows O_{(ads)} + ne^-; O_{(ads)} \xrightarrow{k_f} R_{(ads)}$$

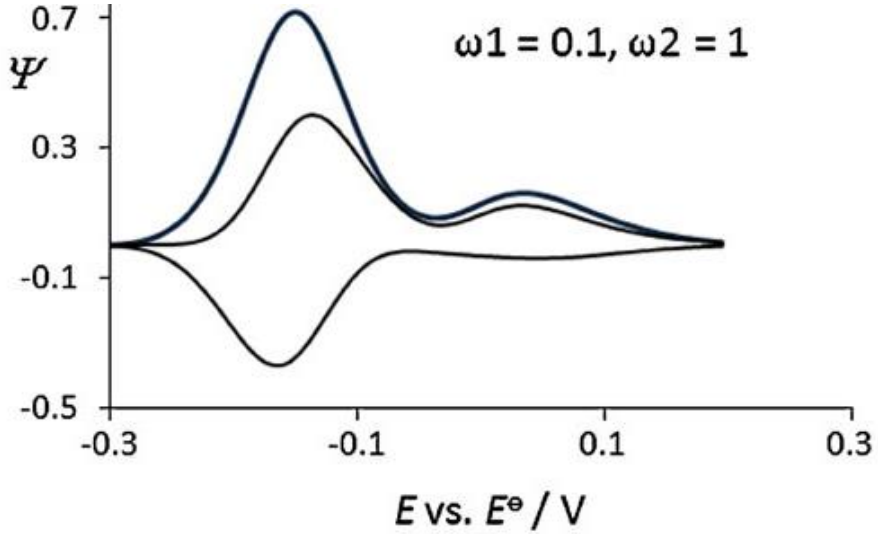
d

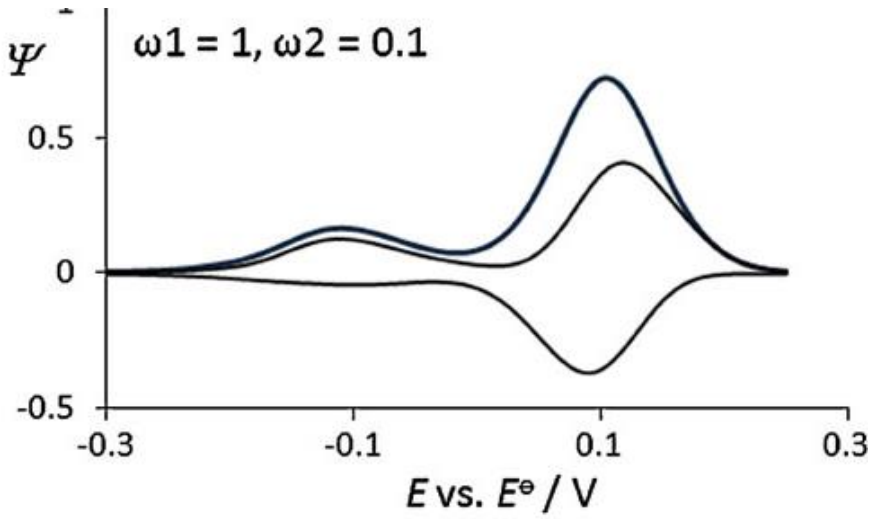


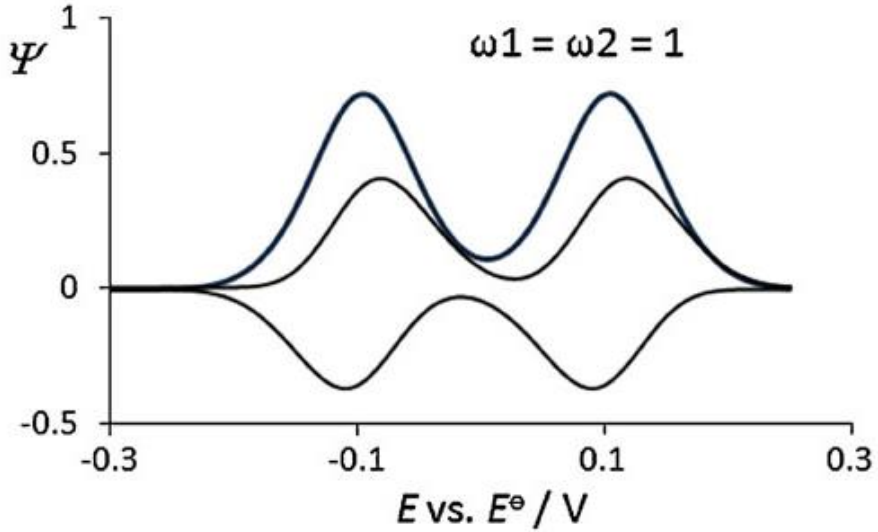
Effect of the ***catalytic parameter*** to the features of the SW voltammograms by the ***EC' (catalytic regenerative)*** surface redox reaction

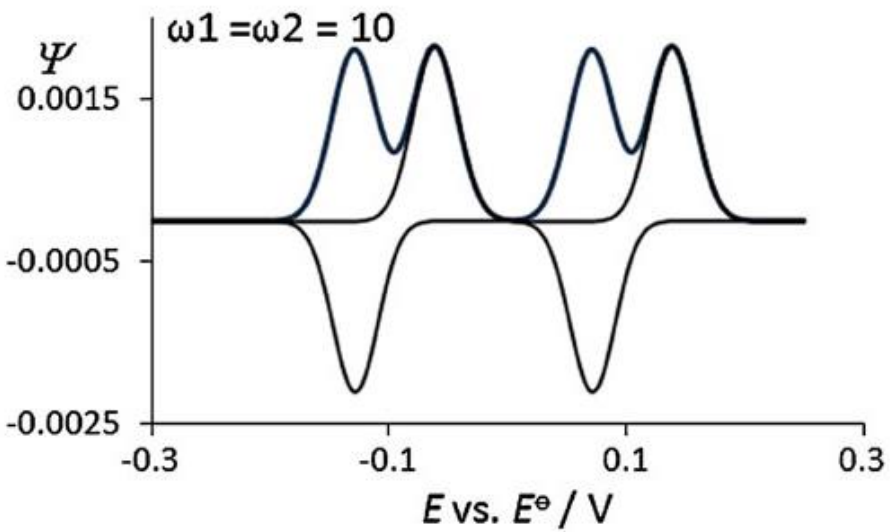
Two-step mechanism (EE)

$$A_{(ads)} \rightleftarrows B_{(ads)} + n_1 e^- \rightleftarrows C_{(ads)} + n_2 e^-$$



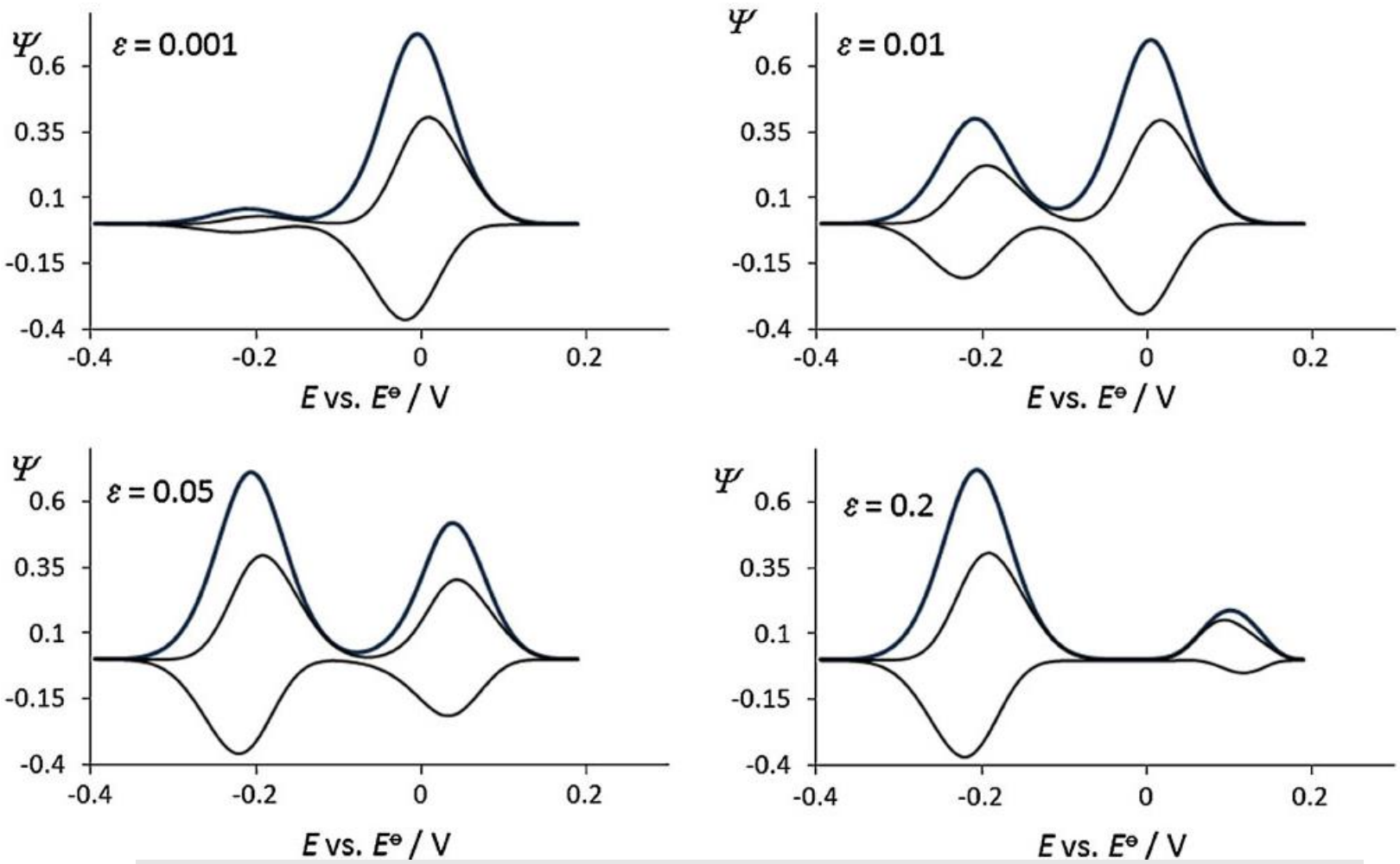






Effect of the ***kinetic parameters of both electron transfer steps*** to the features of the SW voltammograms by the ***EE(two-step) surface redox reaction***

E-C-E Reaction mechanism-
two electron transfer steps coupled by a chemical reaction in SWV

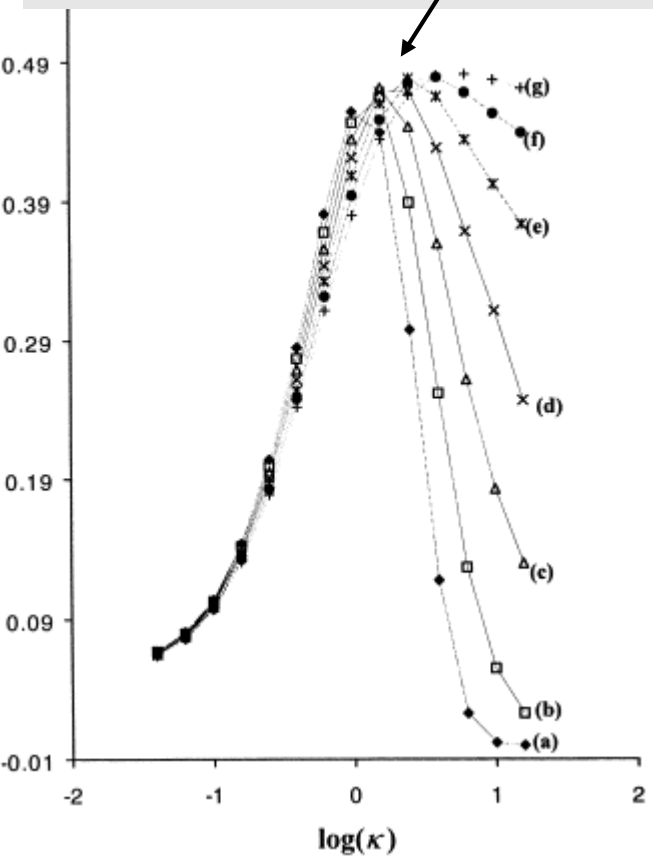


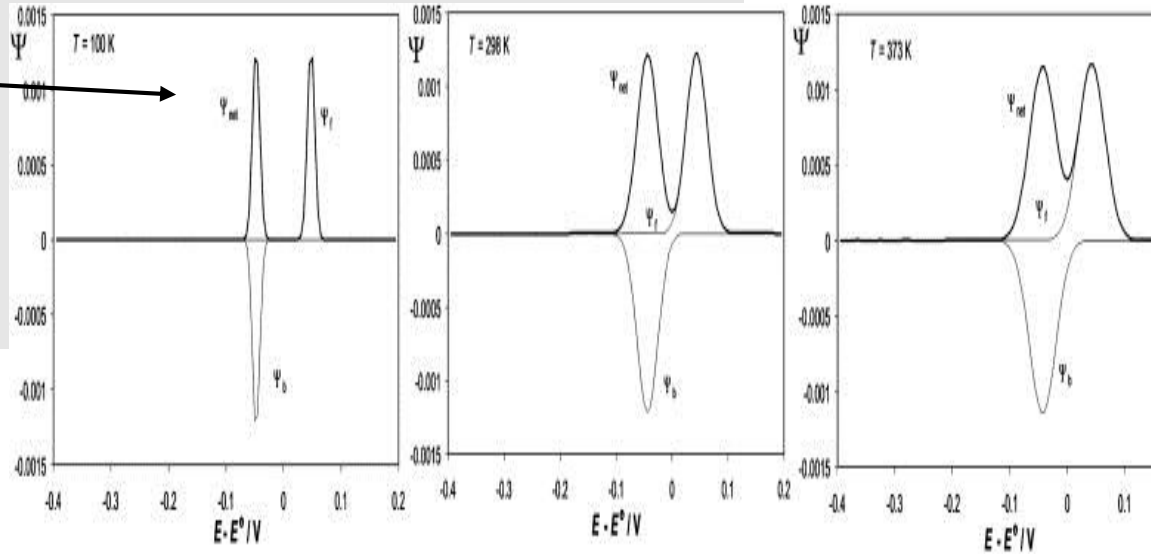
Effect of the ***chemical kinetic parameter (ε)***
to the features of the SW
voltammograms by the ***ECE(two-step) surface redox reaction***

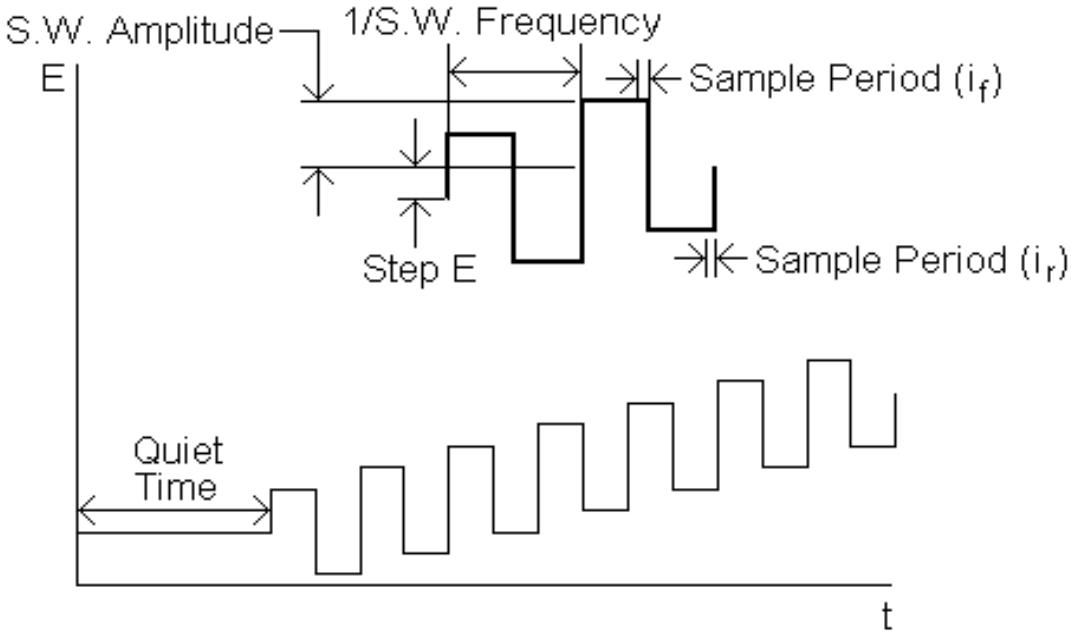
Methods to determine the kinetics od the electron transfer step in square-wave voltammetry

splitted SW voltammograms

-quasireversible maximum







Outlooks for the future of the Protein-film voltammetry

-**challenges** that remain:

to find **suitable electrode material** for many proteins

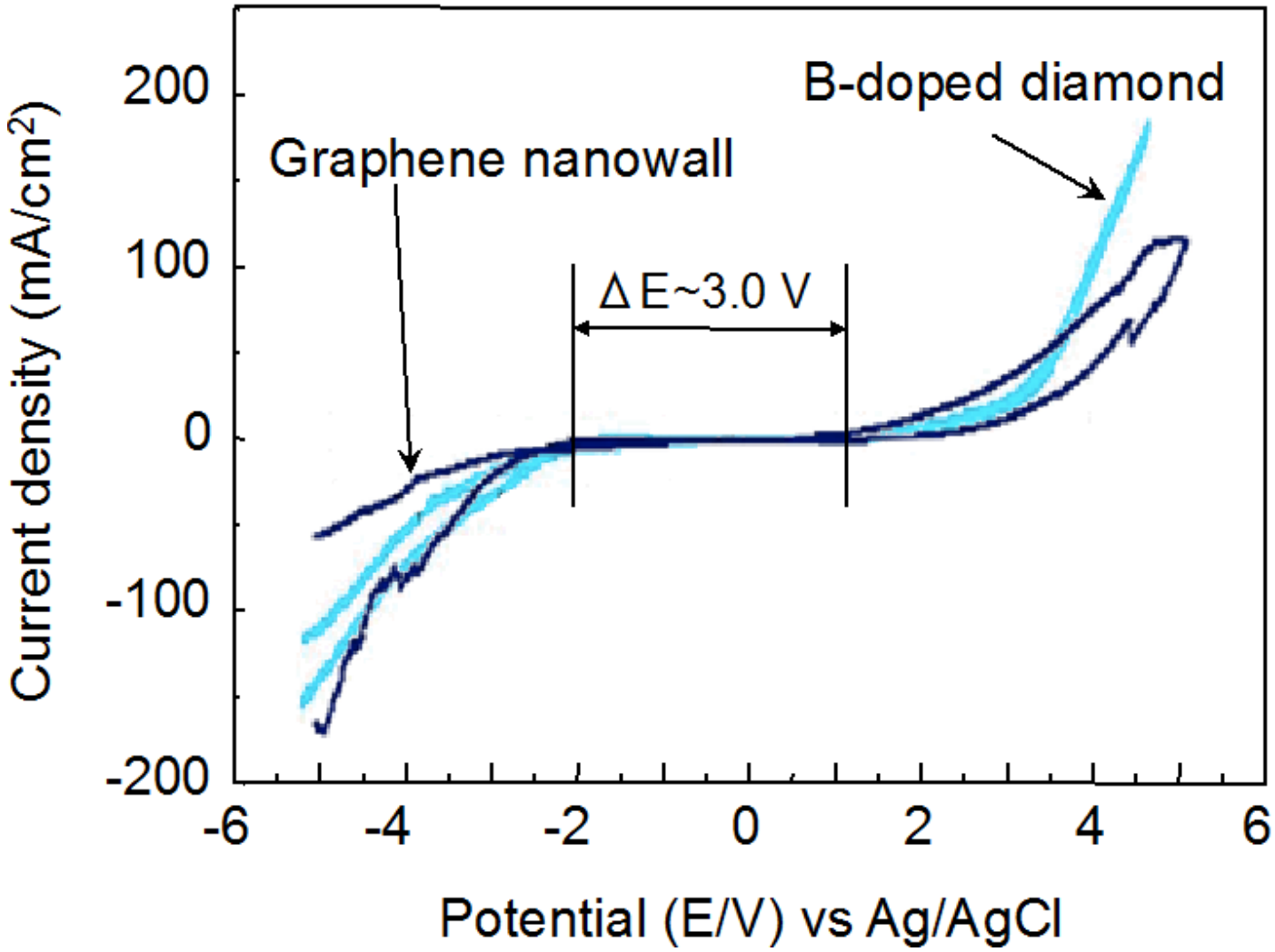
to **overcome insulating protein features** of many proteins

to **enlarge potential window** available

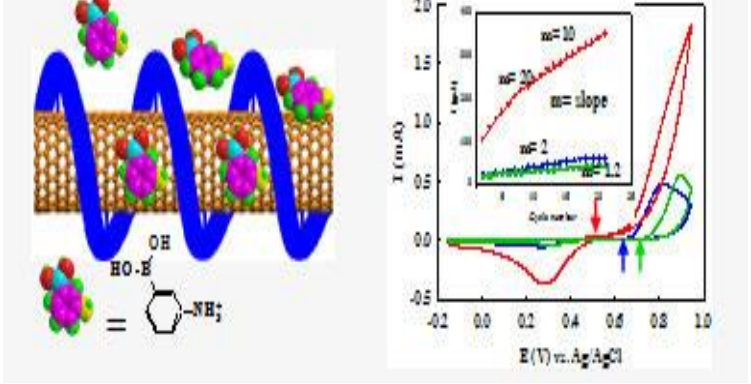
new strategies for studying novel proteins
(up to now, about 80 different proteins are studied
by PFV methodology)

Designing **new types of Nanoparticles-inevitable for PFV**

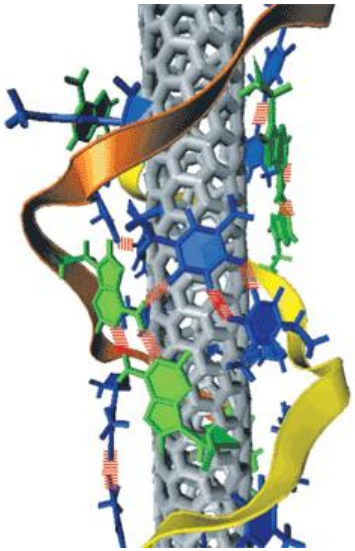
Designing reliable **biosensors**
- Designing Energy-conversion systems based on PFV

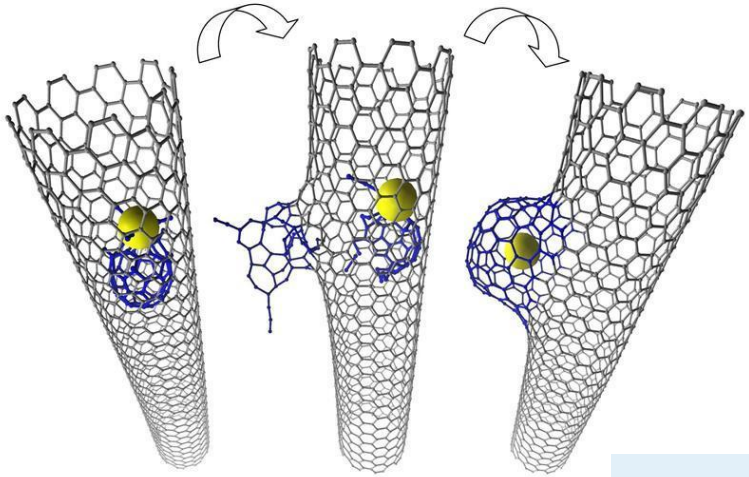


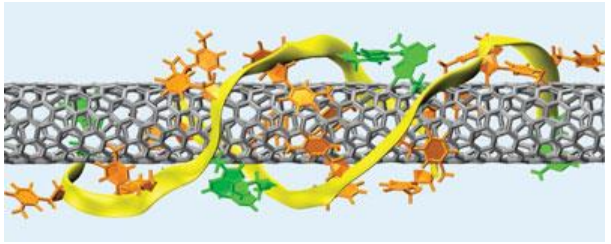
Potential window of some common electrodes in used in PFV



Carbon nanotubes







Relevant Literature about Recent Theories in PFV

1. Rubin Gulaboski, Ivan Bogeski, Valentin Mirčeski, Stephanie Saul, Bastian Pasieka, Haleh H. Haeri, Marina Stefova, Jasmina Petreska Stanoeva, *Saša Mitrev*, Markus Hoth, Reinhard Kappl, "Hydroxylated derivatives of dimethoxy-1,4-benzoquinone as redox switchable earth-alkaline metal ligands and radical scavengers" *Nature Scientific Reports*, 3 (2013) 1-8,

l

2.Rubin Gulaboski, Valentin Mirceski, Ivan Bogeski, Markus Hoth, Protein film voltammetry: electrochemical enzymatic spectroscopy. A review on recent progress„ *Journal of Solid State Electrochemistry* 16 (2012) 2315-2328.

3. R. Gulaboski, P. Kokoskarova, S. Mitrev, Theoretical aspects of several successive two-step redox mechanisms in protein-film cyclic staircase voltammetry“ *Electrochimica Acta* 69 (2012) 86-9

4. Ivan Bogeski, Rubin Gulaboski*, Reinhard Kappl, Valentin Mirceski, Marina Stefova, Jasmina Petreska, Markus Hoth, „Calcium Binding and Transport by Coenzyme Q„ *Journal of the American Chemical Society* 133 (2011) 9293-9303

5. R. Gulaboski, M. Lovric, V. Mirceski, I. Bogeski, M. Hoth, Protein-film voltammetry: a theoretical study of the temperature effect using square-wave voltammetry., *Biophys. Chem.*137 (2008) 49-55.

6. R. Gulaboski, M. Lovric, V. Mirceski, I. Bogeski, M. Hoth, A new rapid and simple method to determine the kinetics of electrode reactions of biologically relevant compounds from the half-peak width of the square-wave voltammograms., *Biophys. Chem.* 138 (2008) 130-137.

7. Rubin Gulaboski, Ljupco Mihajlov, "Catalytic mechanism in successive two-step protein-film voltammetry- Theoretical study in square-wave voltammetry", Biophysical Chemistry 155 (2011) 1-9.

8. R. Gulaboski, Surface ECE mechanism in protein film voltammetry—a theoretical study under conditions of square-wave voltammetry, *J. Solid State Electrochem.* 13 (2009) 1015-1024

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-Prof. Ivan Bogeski (Homburg, Germany)

-Prof. Reinhard Kappl (Homburg, Germany)

Prof. Milivoj Lovric (Croatia)

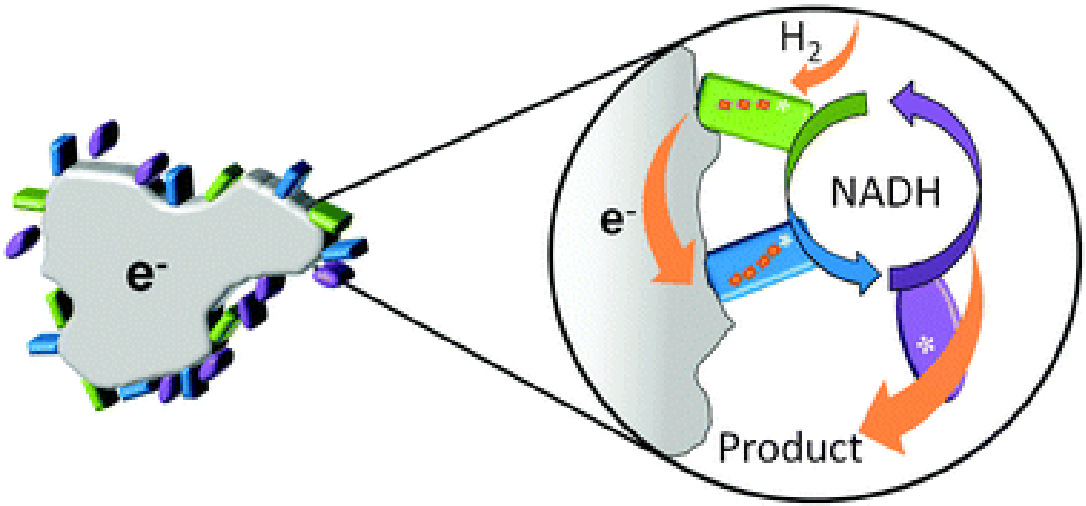
Prof. Carlos Pereira (Portugal)

Prof. Natalia Cordeiro (Portugal)

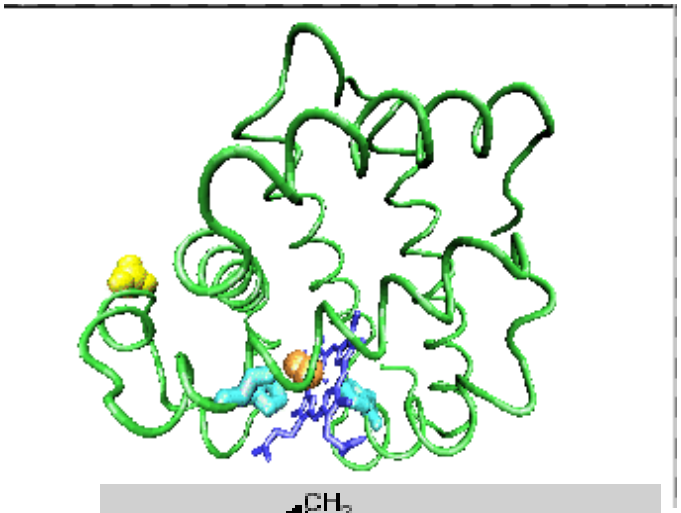
Prof. Sasa Mitrev (Macedonia)

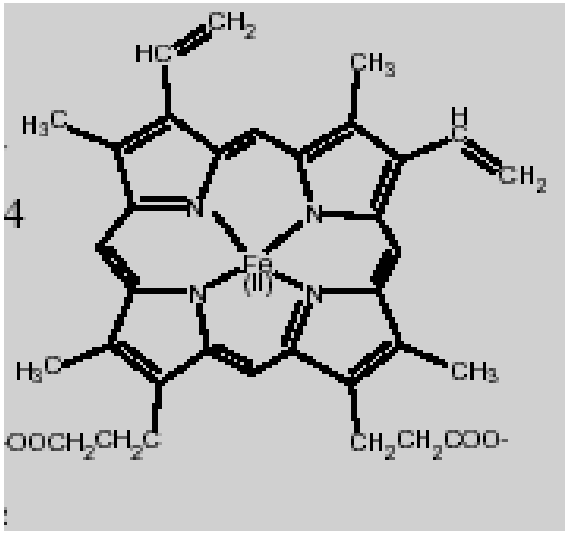
…

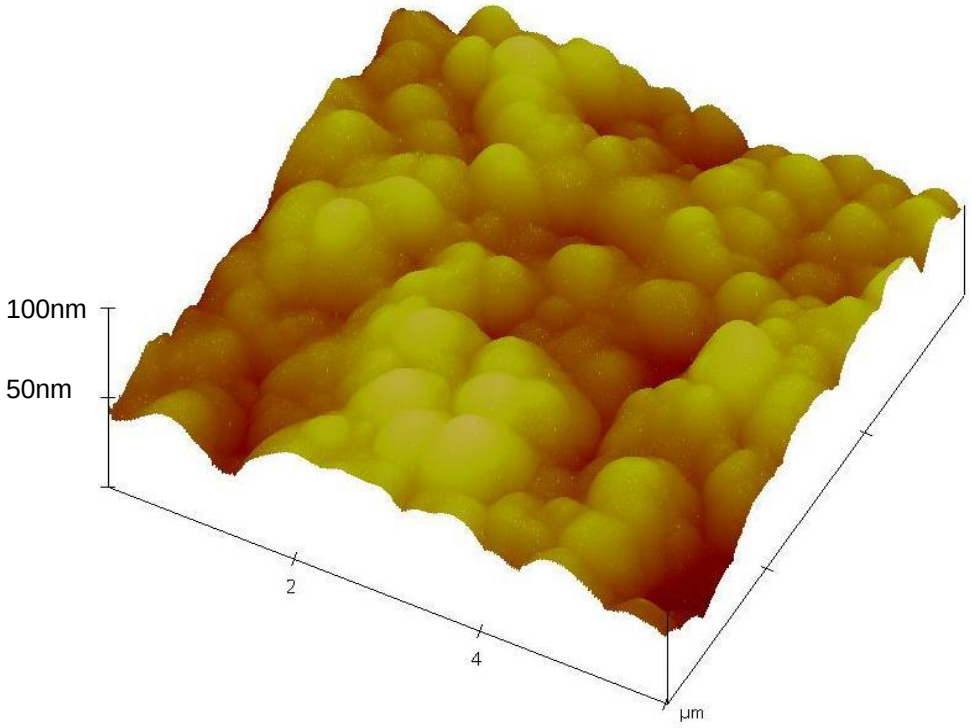
Stabilitätspakt für Südosteuropa
Gefördert durch Deutschland
Stability Pact for South Eastern Europe
Sponsored by Germany


e-
H2
e-
NADH
Product

Horseradish Peroxidase (HRP)







Tapping mode atomic force microscopy (AFM) image of HRP film