Electrochemically-assisted protein crystallisation: applications to biosensors

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OUTLINE OF THE TALK

1. State-of-the-art of the protein crystallisation electrochemical-based approaches.

2. Coupling Dynamic Light Scattering Methods to Electrochemistry for Protein Crystallisation.

3. Monitoring the crystal growth efficiency, and electrocrystallisation processes via EC-AFM.

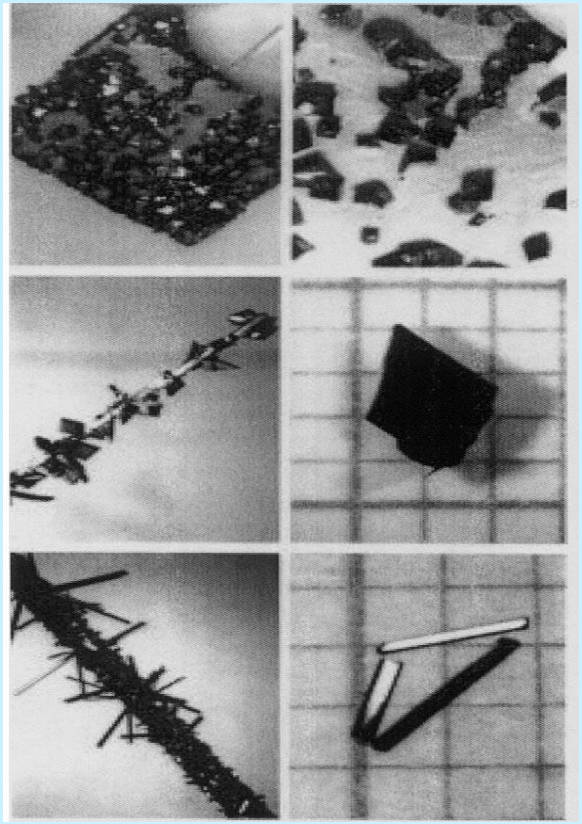
4. Crystalline quality via kinetics control and some applications to the development of protein-based biosensors.

5. Conclusions

I. State-of-the-art

Some inorganics obtained by electrocrystallisation

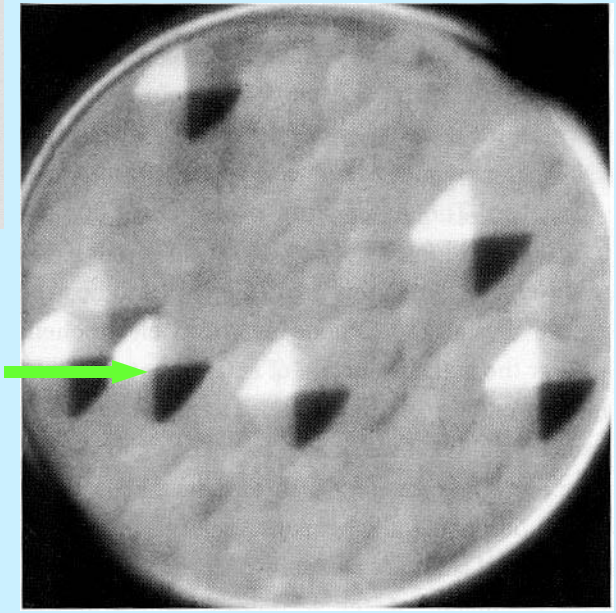
(The term '**electrocrystallization**' was coined by Fischer in the 1940s)

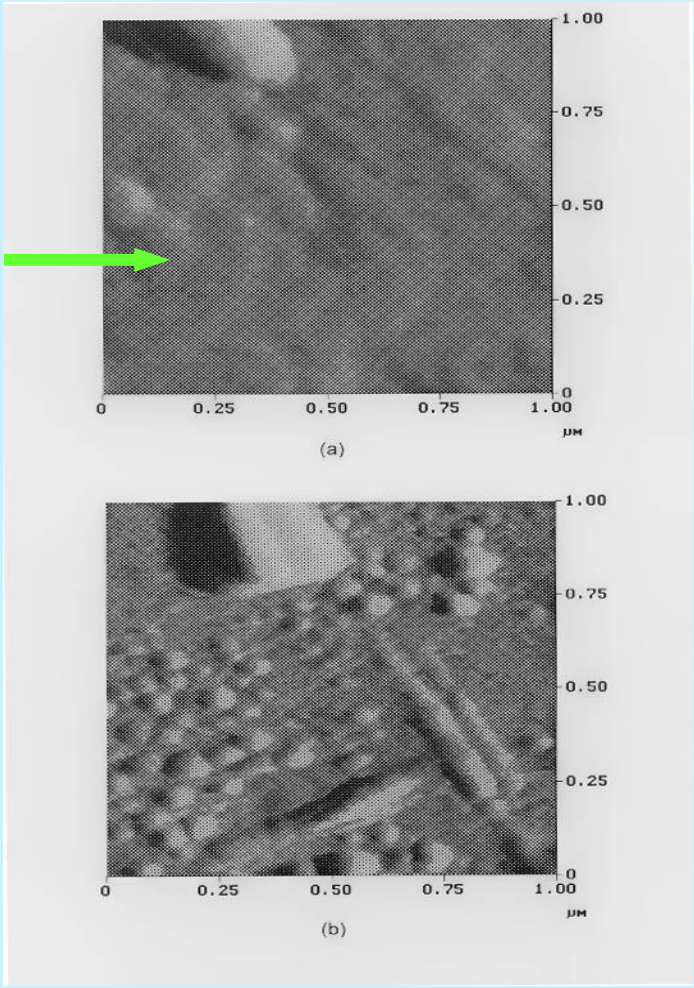


Silver monocrystals for materials science investigations

1991 *J. Phys. D: Appl. Phys.* **24** 217.

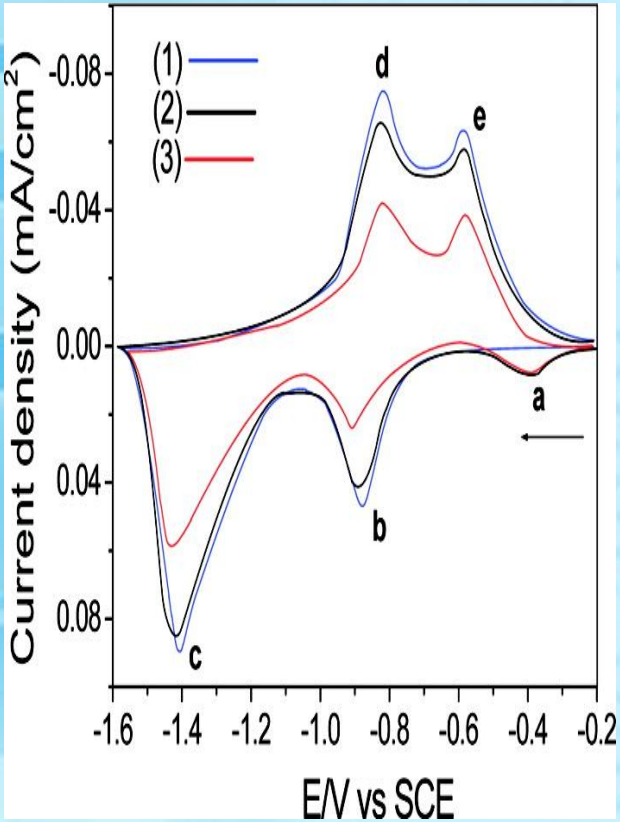
Layers of superconductor $YBa_2Cu_3O_{7-\delta}$, before and after the electrodeposit

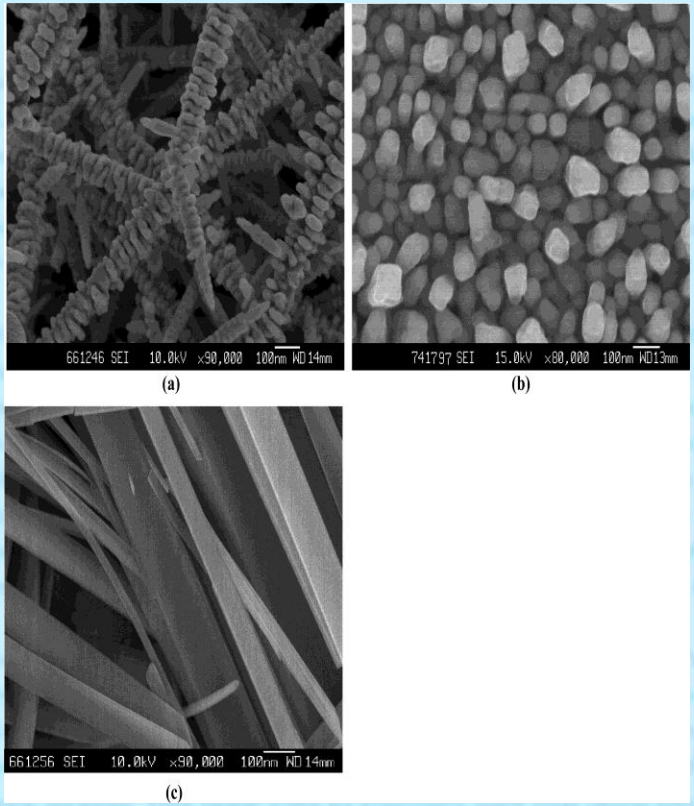




Reference: Applied Superconductivity 19, No. 3 (2009) 3451-3454.

ZnCd Oxides





Cyclic voltammograms of redox processes in (1) the solution of 0.1 M ZnCl2 + 0.03 M CdCl2 + 0.1 M KCl (no oxygen); (2) the oxygen saturated solution of 0.1 M ZnCl2 + 0.03 M CdCl2 + 0.1 M KCl; (3) the oxygen saturated solution of 0.05 M ZnCl2 + 0.01 M CdCl2 + 0.1 M KCl at 90 °C.

SEM images of **(a)** the hierarchical Zn*x*Cd1−*x*O nanoparticle aggregates prepared in a solution of 0.1 M ZnCl2 + 0.03 M CdCl2 + 0.1 M KCl with a current density of 0.5 mA/cm^2; **(b)** out-of-order Zn1−*x*Cd*x*O nanoparticles prepared with a current density of 1.0 mA/cm^2. **(c)** Zn*x*Cd1−*x*O nanorods prepared in a solution of 0.1 M ZnCl2 + 0.03 M CdCl2 + 0.1 M KCl with a current density of 0.05 mA/cm^2 at 90 °C.

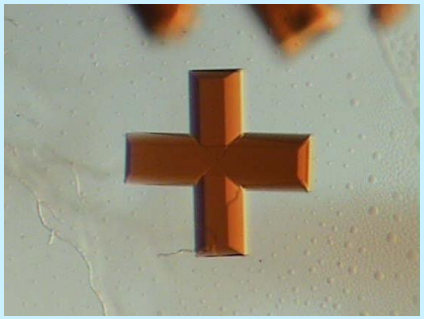
Published in: Gao-Ren Li; Qiong Bu; Fu-Lin Zheng; Cheng-Yong Su; Ye-Xiang Tong; *Cryst. Growth Des.* **2009,** 9, 1538-1545.
DOI: 10.1021/cg800496d

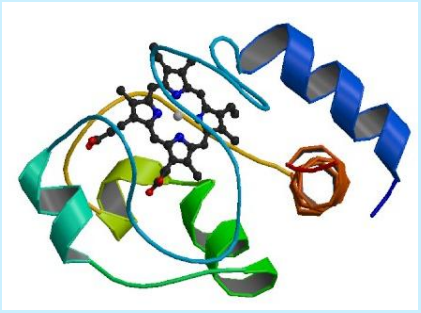
ELECTROCHEMICALLY-ASSISTED PROTEIN CRYSTALLISATION

3D structure determination of proteins

Monocrystal

Protein Purification





Crystallization Techniques

X Ray Crystallography

Limiting process

Applications

Physical parameters used to induce the nucleation process:

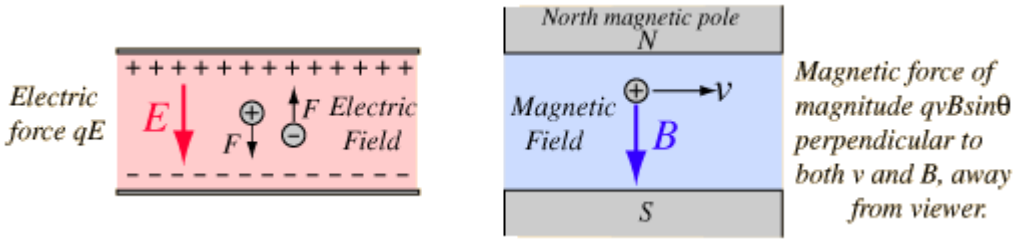
Lorentz Force Law

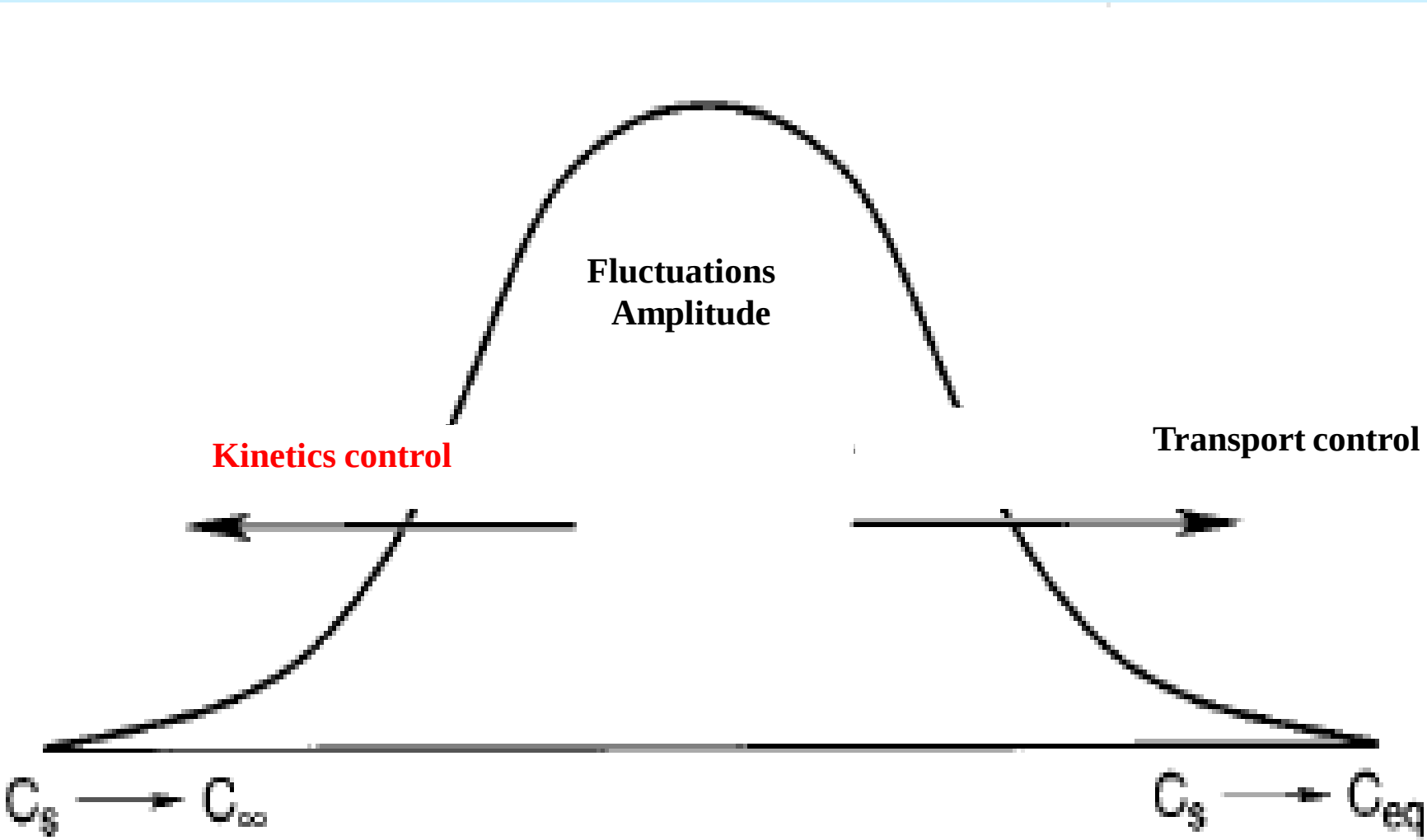
Both the electric field and magnetic field can be defined from the Lorentz force law:

$$\vec{F} = q\vec{E} + q\vec{v} \times \vec{B}$$

Electric force — Magnetic force

The electric force is straightforward, being in the direction of the electric field if the charge q is positive, but the direction of the magnetic part of the force is given by the right hand rule.





REFERENCE: G. Foffi et al., Phys. Rev. E65, 31407-17.

ELECTROCHEMICALLY-ASSISTED PROTEIN CRYSTALLISATION

External parameters that affect the crystallisation

Magnetic force

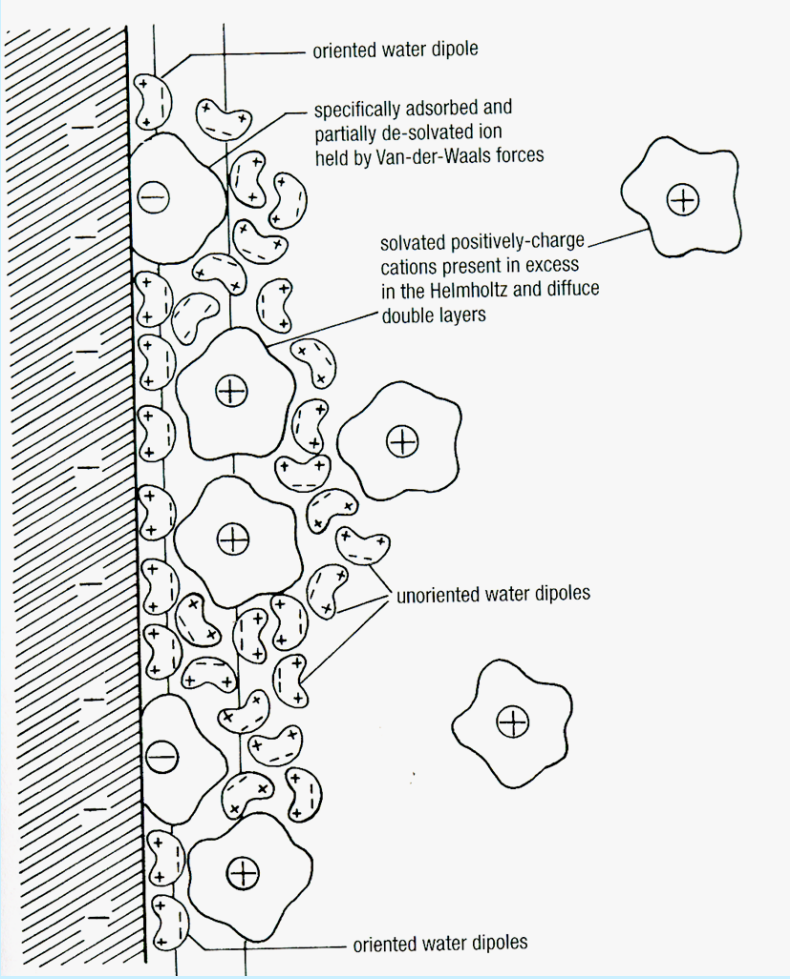
Gravity force

Pressure

} Crystal Quality

Electrochemical principles and theory

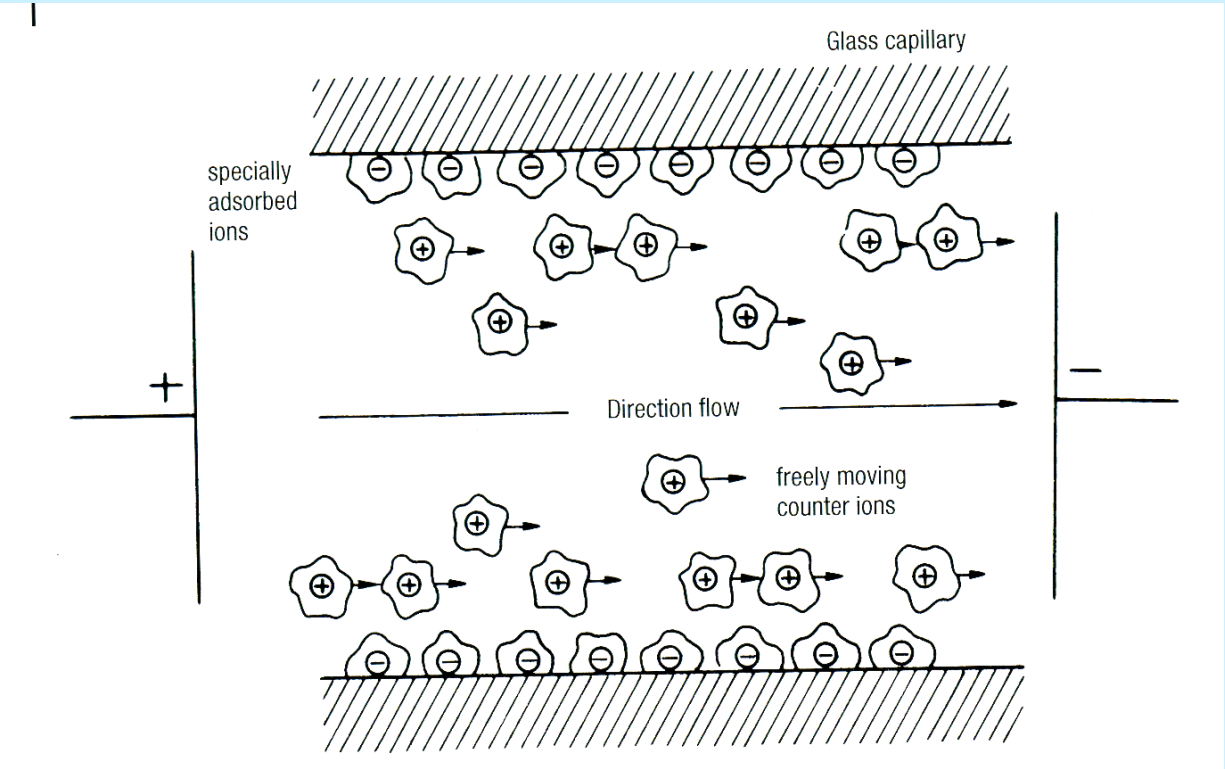
Internal electrical field on a small electrochemical cell



The electrical field generates movement of ions in solution throught the attraction and repulsion (Coulombic) interactions

Electrochemical-assisted protein crystal growth

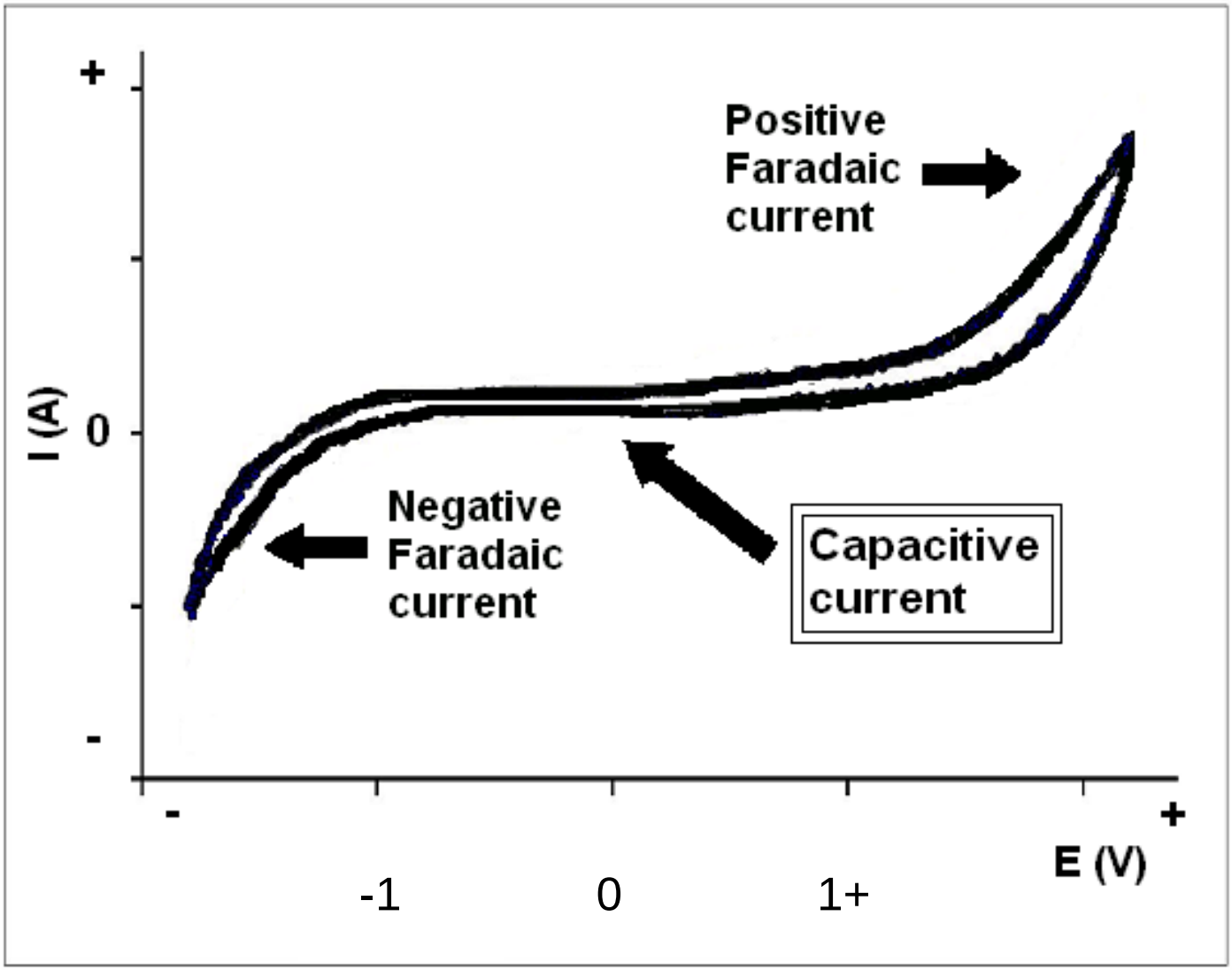
Internal electrical field on a small electrochemical cell



The electromigration phenomena produces selectivity of ions and molecules

Proteins can be viewed as a large ion (migration phenomena)

Fundamentals of the method…



Electrochemically-assisted protein crystal growth

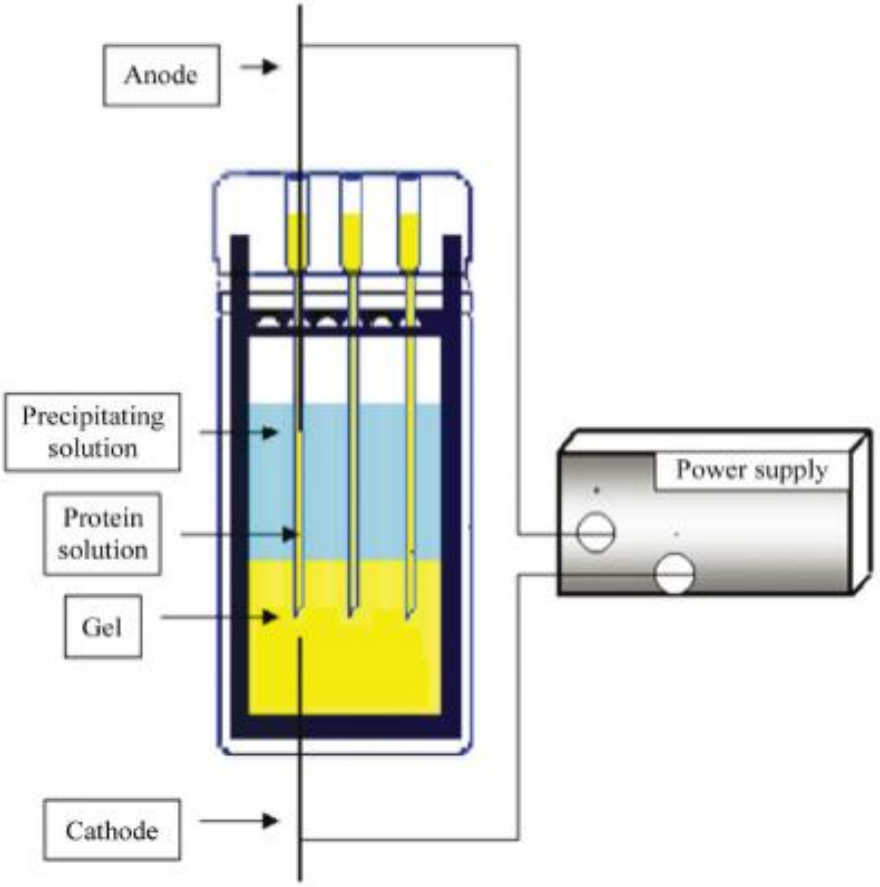


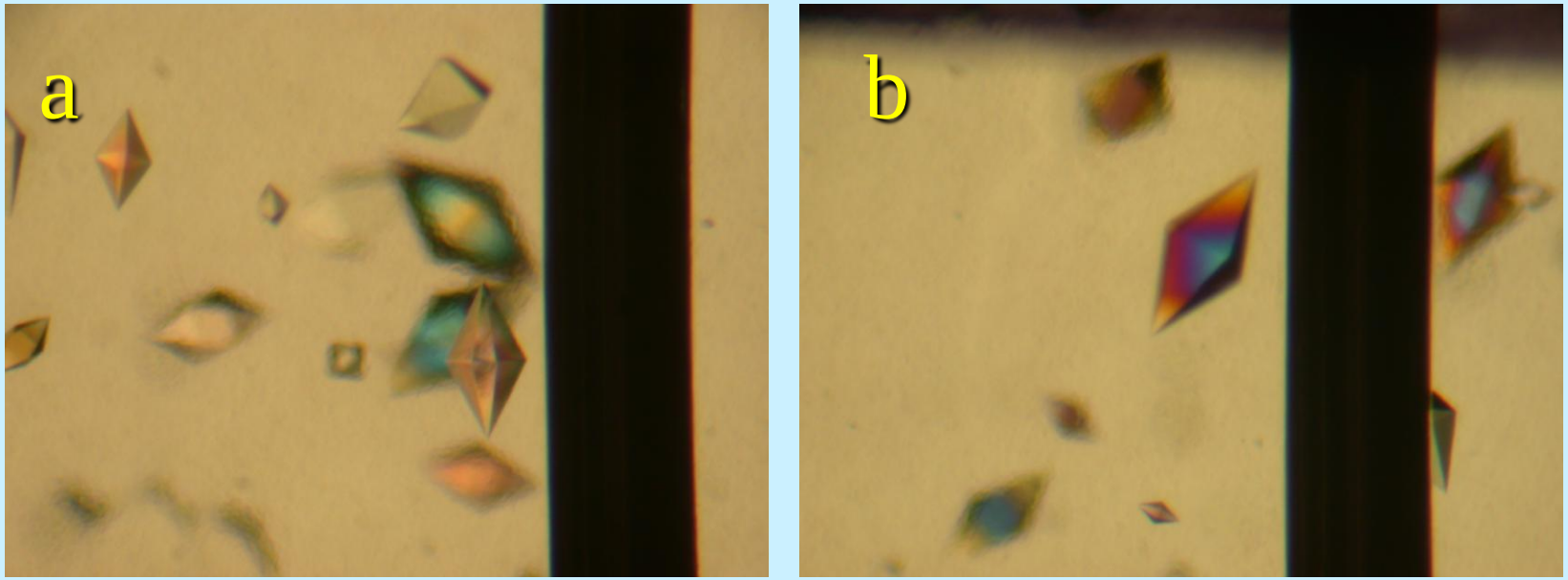
Figure 1
Experimental setup of the crystallization cell connected to a power supply (galvanostat).

Adapted GAME Method

Lysozyme (40 mg/mL)
Silicate HydroGel 6%
NaCl (40 mg/mL)
Acetates Buffer (0.2M)
pH = 4.5
T =18°C
I = 2 μA

Galvanostat PAR 175

Application of $\vec{E}_{internal}$ to thaumatin from Taumatoccoccus danielli



CONTROL

ANODE

300 mμ

Electrochemically-assisted Protein Crystal Growth

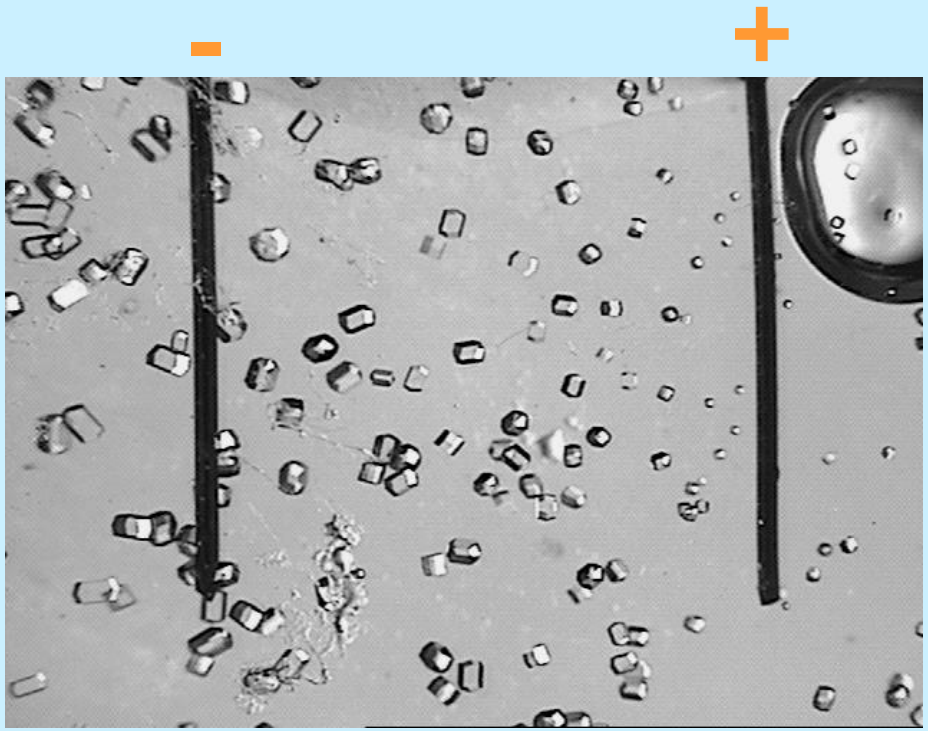
X-ray data statistics for the thaumatin crystals.

	Control	Electhau1	Electhau2
Data collection			
Space group	$P4_12_12$	$P4_12_12$	$P4_12_12$
Unit-cell parameters (Å)			
a	58.664	58.647	58.633
b	58.664	58.647	58.6337
c	151.391	151.390	151.427
Resolution range (Å)	15.0–2.0	15.0–2.0	15.0–2.0
No. of reflections	135685	134546	105150
No. of unique reflections	18694	19702	19204
$I/\sigma(I)$	9.0	3.6	7.8
Completeness	99.2	98.8	96.5
R_{merge} (%)	0.053	0.112	0.059
Structure refinement			
R/R_{free}	0.177/0.205	0.192/0.224	0.173/0.187
Average *B* factor	23.4	24.2	22.6
R.m.s. deviations			
Bonds (Å)	0.005	0.005	0.005
Angles (°)	1.3	1.3	1.3
Torsions (°)	25.3	25.2	25.4
No. of non-H atoms			
Protein	1545	1546	1548
Ligand	10	10	10
Water	93	97	98

N, Mirkin, et al., *Acta Crystal*. D59, 2003, 1533-1538.

ELECTROCHEMICALLY-ASSISTED PROTEIN CRYSTALLISATION

Lysozyme crystallization at 2μA during 48 h



With Electrochemistry

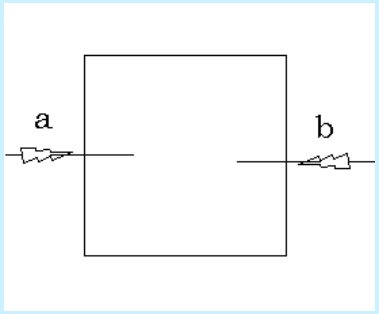
a) Faster than the control experiment

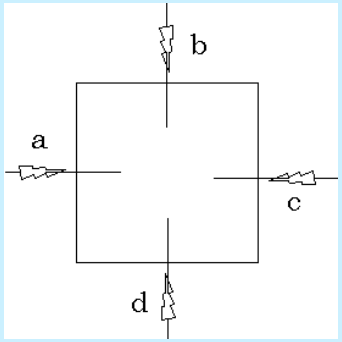
b) Crystallisation towards the cathode

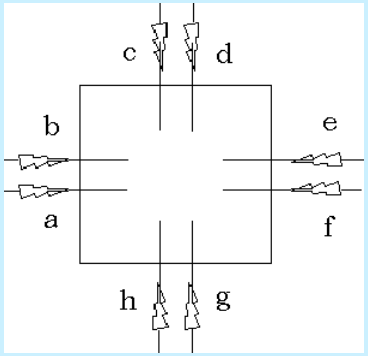
[Lysozyme] = 40mg/mL, at I = 2μA, [NaCl] = 40 mg/mL, [Agar Gel] = 0.066% w/v in Acetate buffer pH=4.5 at 18°C.

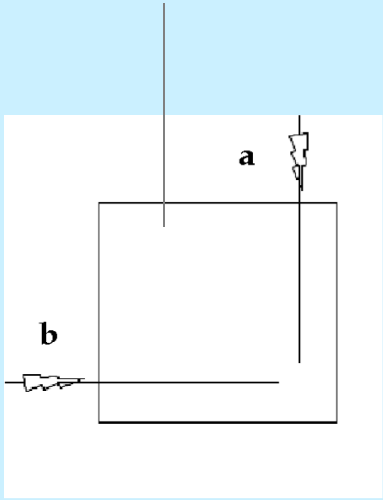
Electrochemically-assisted protein crystal growth

Study of the cell geometry influence in a batch galvanostatic cell



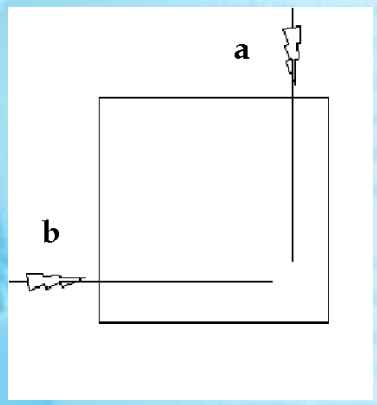


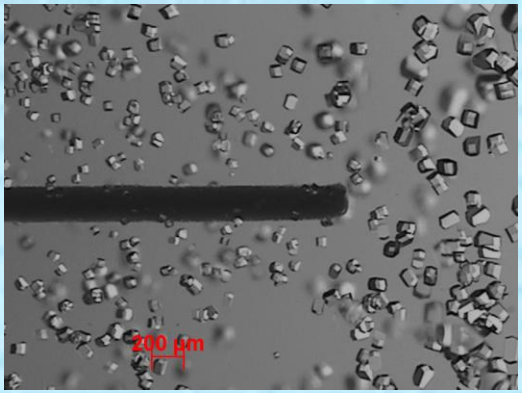


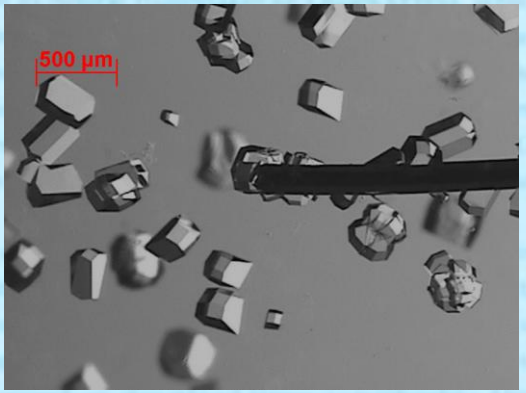


E, Nieto, B. Frontana, A.Moreno, *J. Crystal Growth* 75 (2005) 1442-1453.

BIOELECTROCRYSTALLISATION BEHAVIOUR OF LYSOZYME UNDER CON TROLLED POTENTIAL







Possible reduction reactions

$2H_3O^+ + 2e^- \rightarrow H_2 + 2H_2O$

$O_2 + 2e^- + 2H^+ \rightarrow H_2O_2$

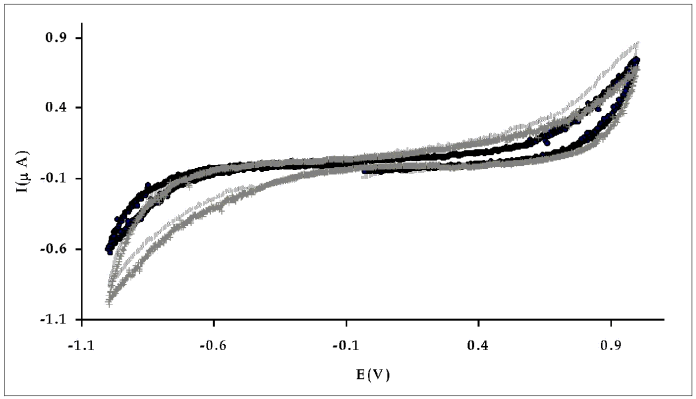
$H_2O_2 + 2e^- + 2H^+ \rightarrow 2H_2O$

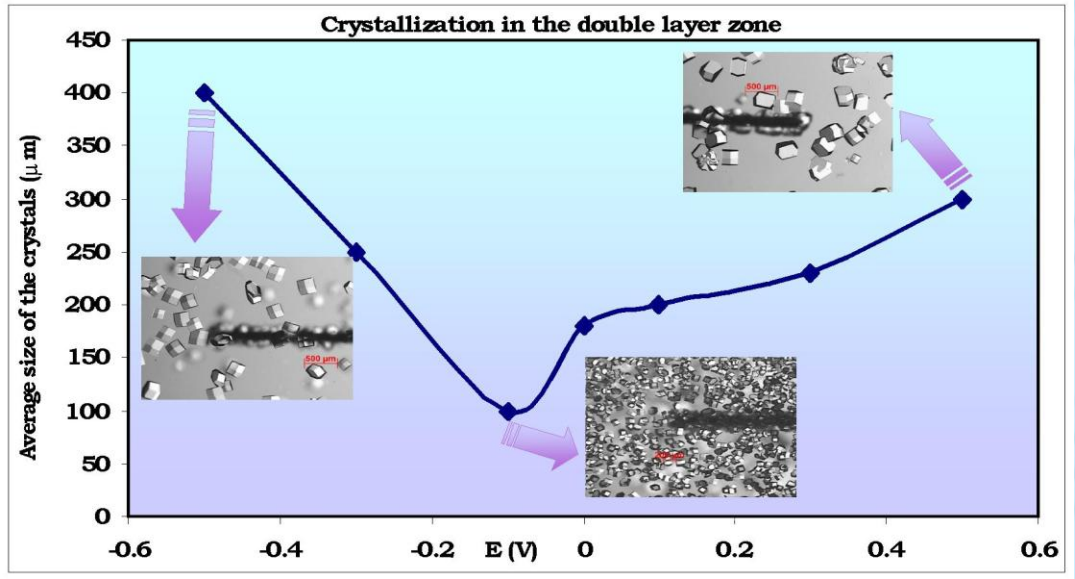
Possible oxidation reactions

$2H_2O - 4e^- \rightarrow O_2 + 4H^+$

$4Pt + O_2 \rightarrow 2Pt_2O$

$2Cl^- - 2e^- \rightarrow Cl_2$





E. Nieto Mendoza et al. Journal of Crystal Growth 275 (2005) 1443-1452

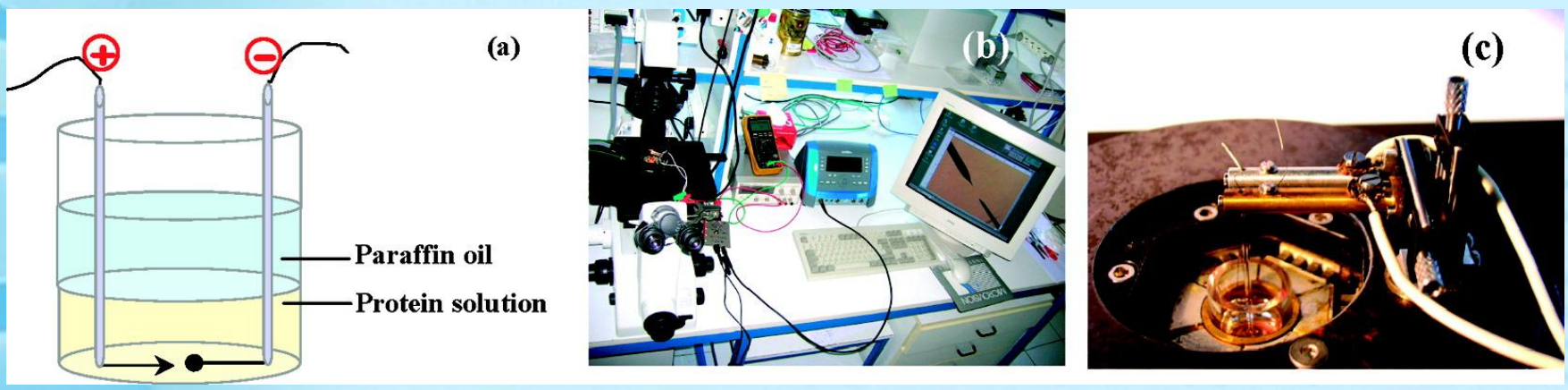


Figure 1. (a) Scheme of the crystallization cell with the two electrodes, (b) image of the whole experimental setup, and (c) *Y*,*Z* positioning stage for the electrodes.

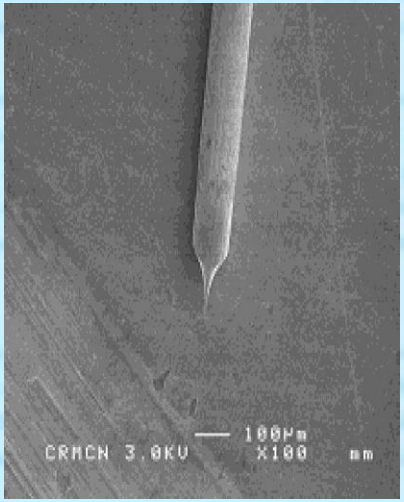


Figure 2. Scanning electron microscopy (JEOL 6320F) image of the tip of the sharp electrode.

Published in: Zoubida Hammadi; Jean-Pierre Astier; Roger Morin; Stphane Veesler; *Crystal Growth & Design* **2007,** 7, 1472-1475.
DOI: 10.1021/cg070108r

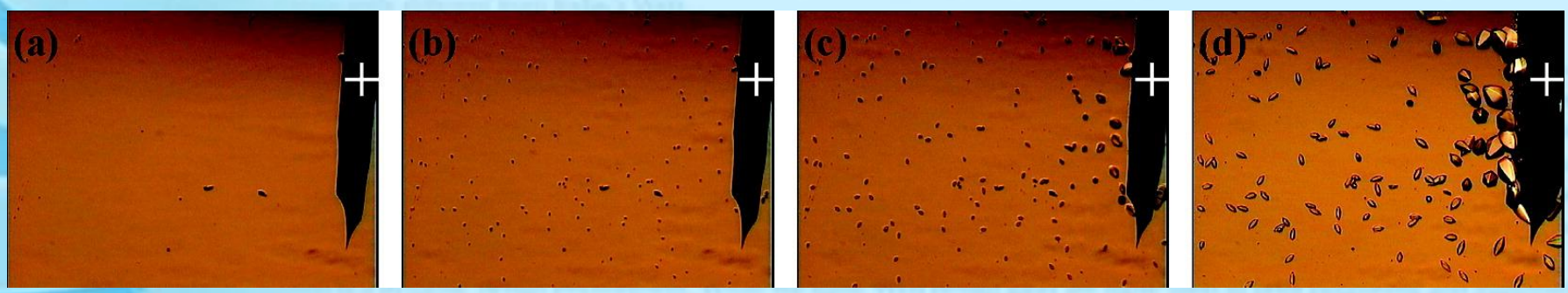


Figure 3 In situ observations under optical microscopy of BPTI crystallization at 20 C with a direct voltage of 0.785 V (experiment 1), with a time of (a) 0, (b) 7 , (c) 11, and (d) 20 h. As reference, the W-electrode wire diameter is 125 m (the + sign indicates the anode).

Figure 4 BPTI crystals obtained at 20 C with a direct voltage of 0.785 V (experiment 1) after 24 h at the (a) cathode and (b) anode. As reference, the W-electrode wire diameter is 125 m (the + sign indicates the anode).

Published in: Zoubida Hammadi; Jean-Pierre Astier; Roger Morin; Stphane Veesler; *Crystal Growth & Design* **2007,** 7, 1472-1475.
DOI: 10.1021/cg070108r

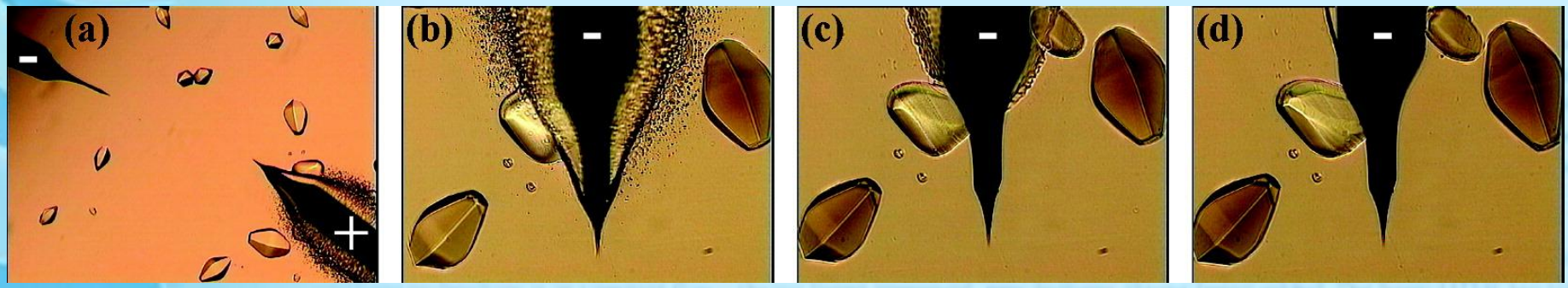


Figure 5 In situ observations under optical microscopy of BPTI crystallization at 20 C with a direct voltage of 0.785 V (experiment 2), at (a) *t* = 18 h and after inverting the electrode polarity at times of (b) 0, (c) 3.5, and (d) 9 h. As reference, the W-electrode wire diameter is 125 m (the + sign indicates the anode).

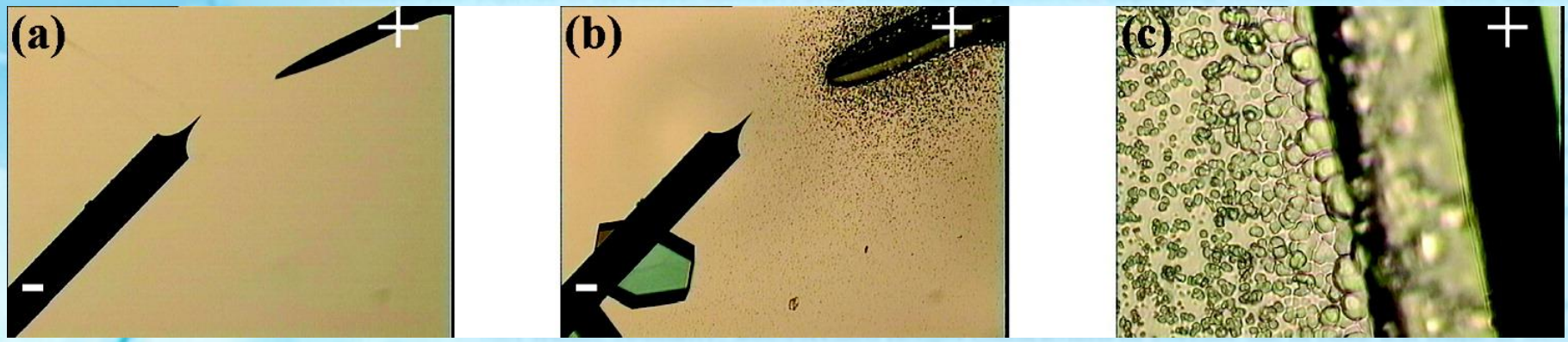
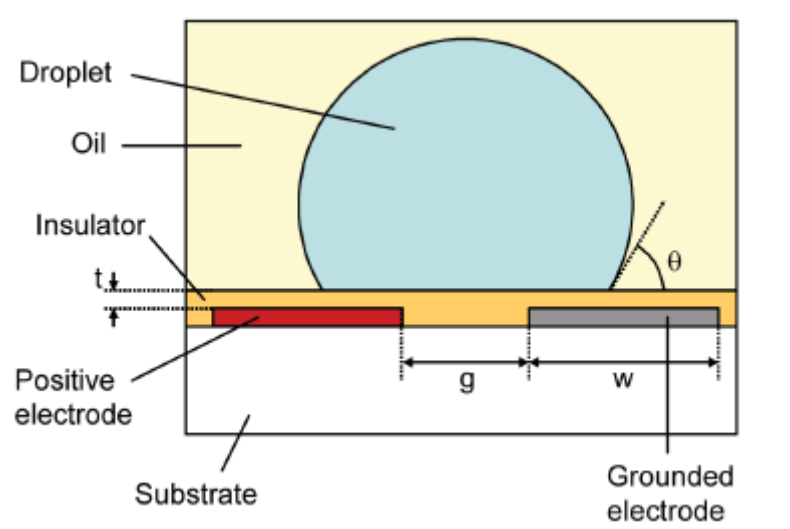


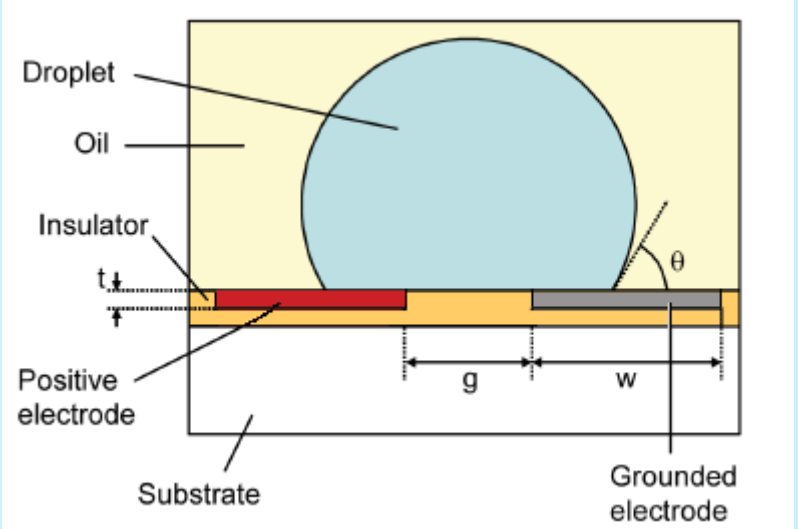
Figure 6 In situ observations under optical microscopy of lysozyme crystallization at 20 C (lysozyme 25 mg/mL in 0.7 M of NaCl and 50 mM of sodium acetate buffer solution at pH 4.5) with a direct voltage of 0.9 V (experiment 2), at times pf (a) 0 and (b) 12 h, and (c) a zoom of the layer formed at the anode. As reference, the W-electrode wire diameter is 125 m (the + sign indicates the anode).

28/05/2012
Published in: Zoubida Hammadi; Jean-Pierre Astier; Roger Morin; Stphane Veesler; *Crystal Growth & Design* **2007,** 7, 1472-1475.
DOI: 10.1021/cg070108r

Electrochemically-assisted protein crystal growth

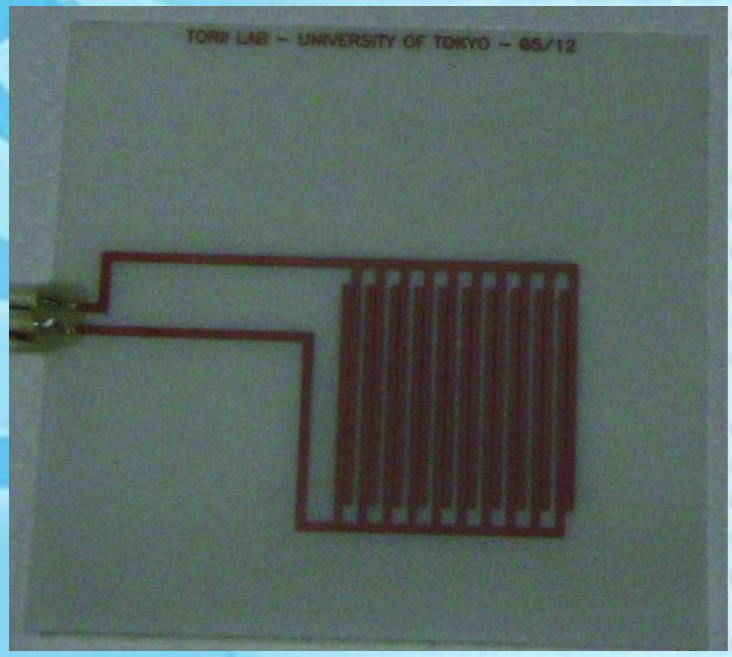
External electric field





External field: the electrodes do not touch the solution Internal field: the electrodes are immersed in the solution.

Frontana-Uribe, Bernardo & Moreno, Abel: Review on Electrochemically Assisted Protein Crystallization and Related Methods. Crystal Growth and Design 8 (2008) 4194-4199.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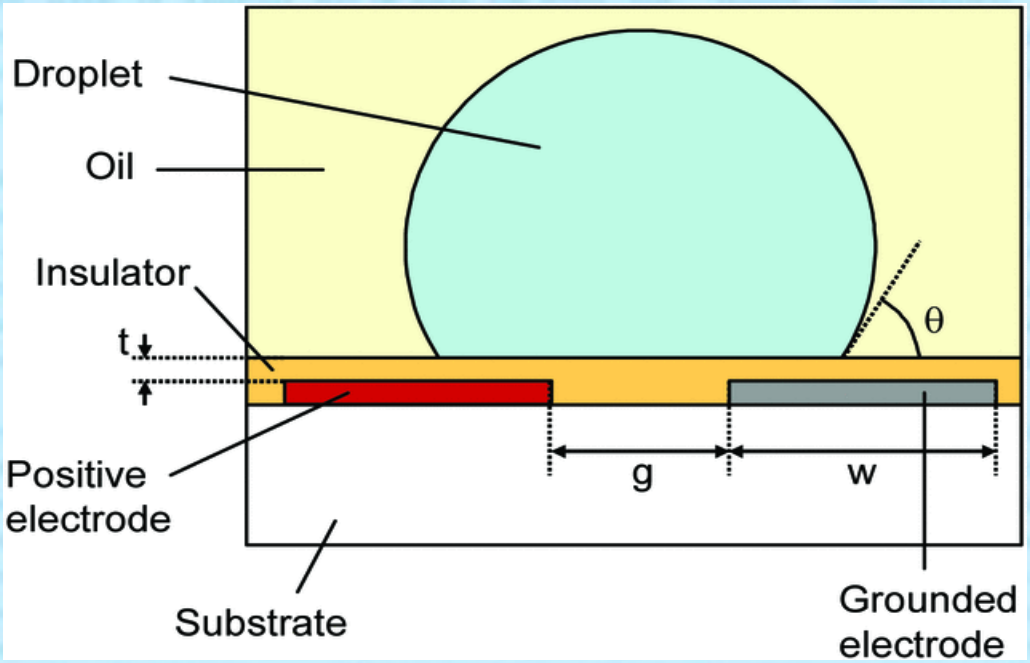
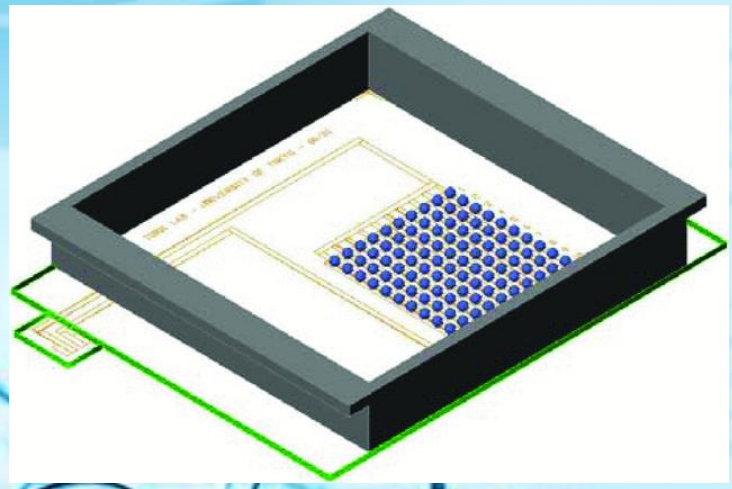
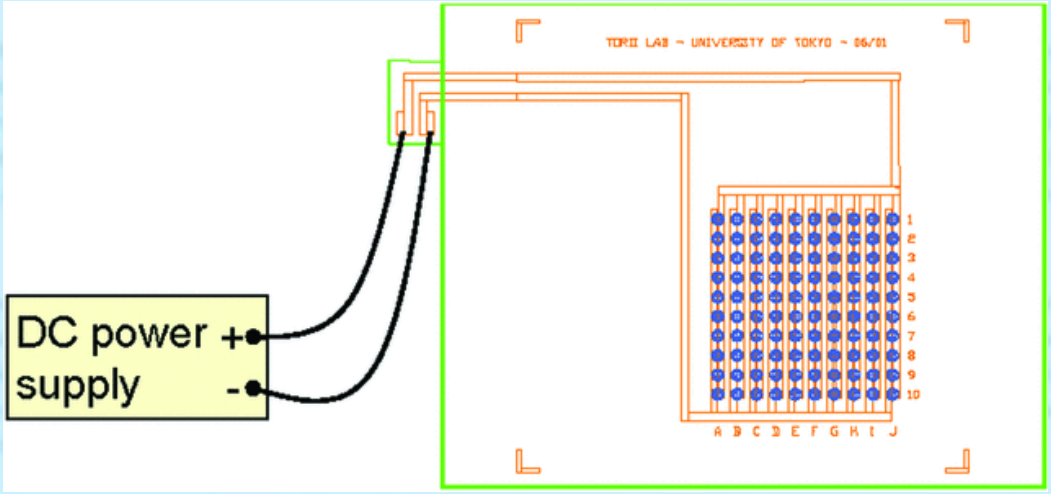
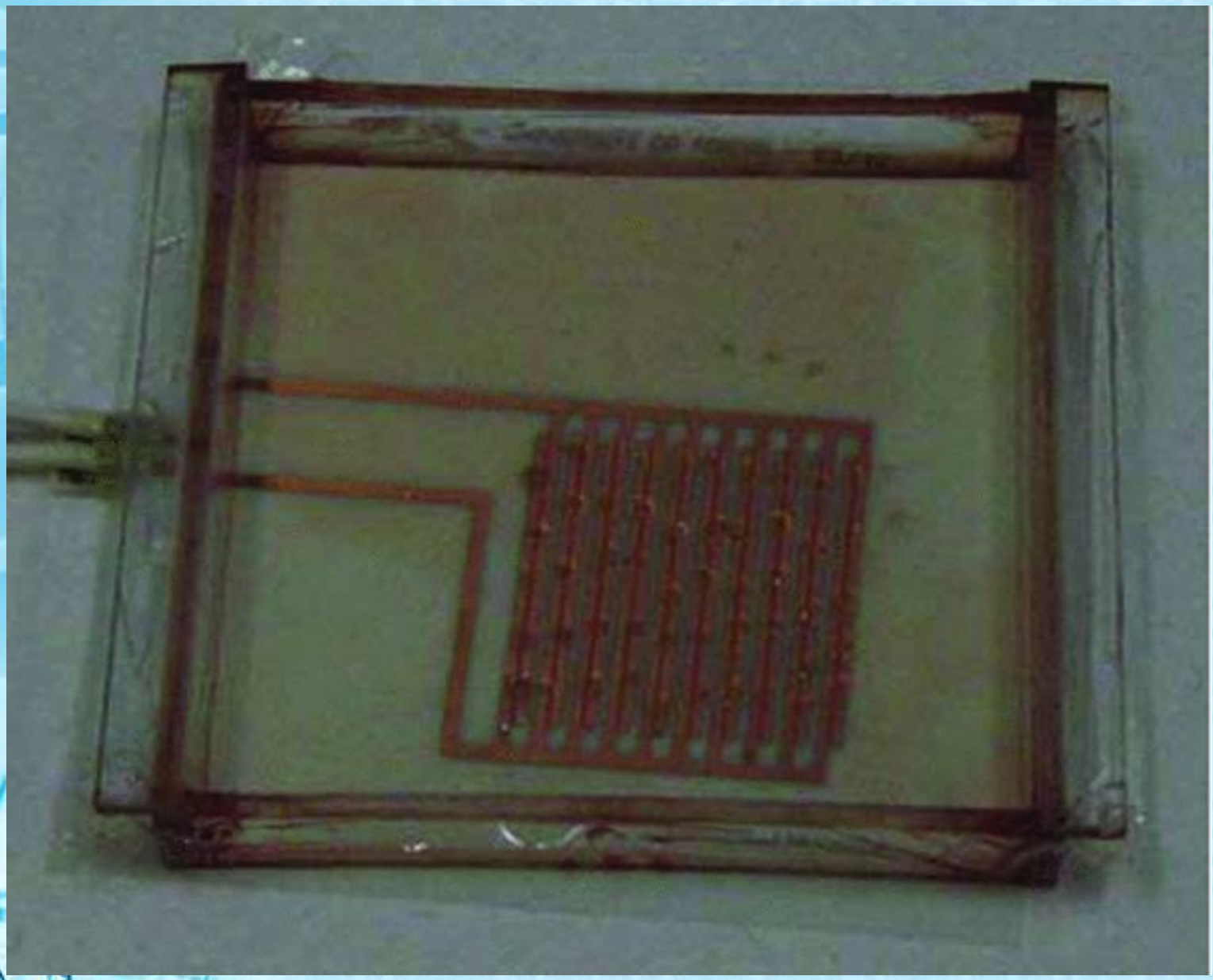

Droplet
Oil
Insulator
t
θ
Positive electrode
g
w
Substrate
Grounded electrode




DC power supply
+
-



Journal of Applied Crystallography 40 (2007) 199-201.

New approaches based on the same idea…

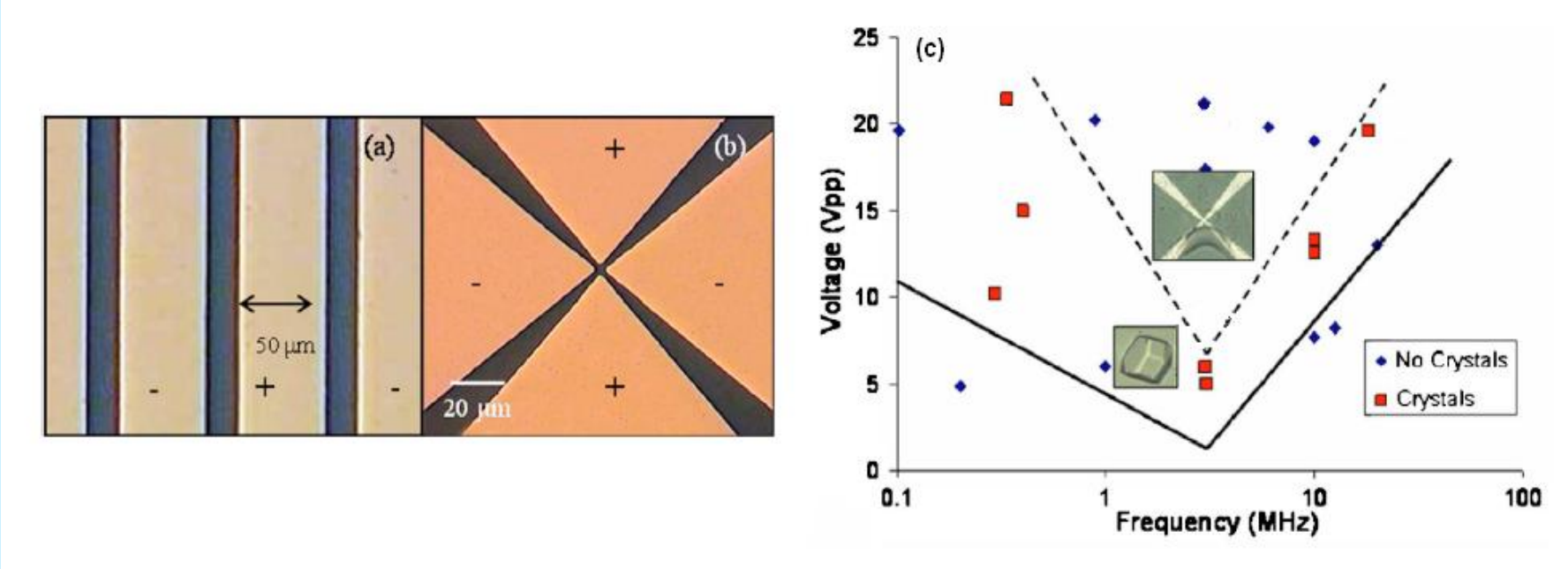


Figure 1 (a) Parallel electrodes cell with opposite polarities; (b) quadrupole electrode cell with opposite polarities; and (c) lysozyme solutions under different ac fields that were monitored for 24 h for indications of nucleation. Conditions where crystals were seen are marked by the red squares and conditions where no nucleation sites were seen are marked with a blue diamond **(Reference: How D. Applied Phys. Letters 92 (2008) 223902).**

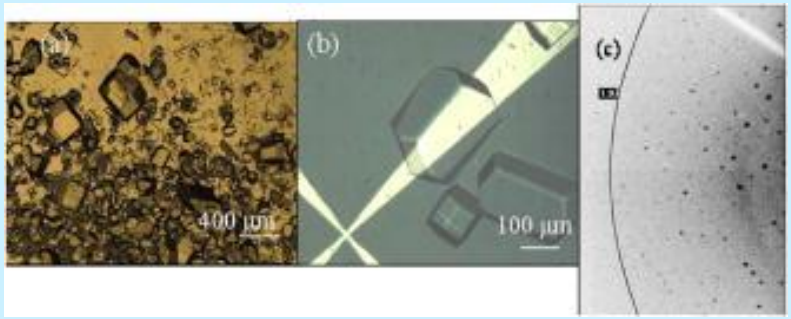
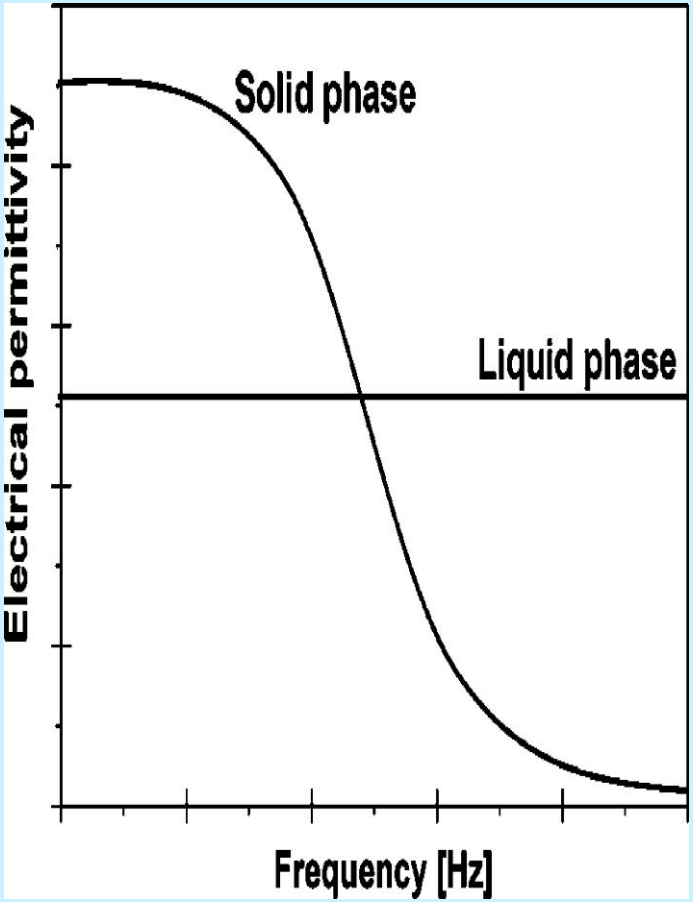


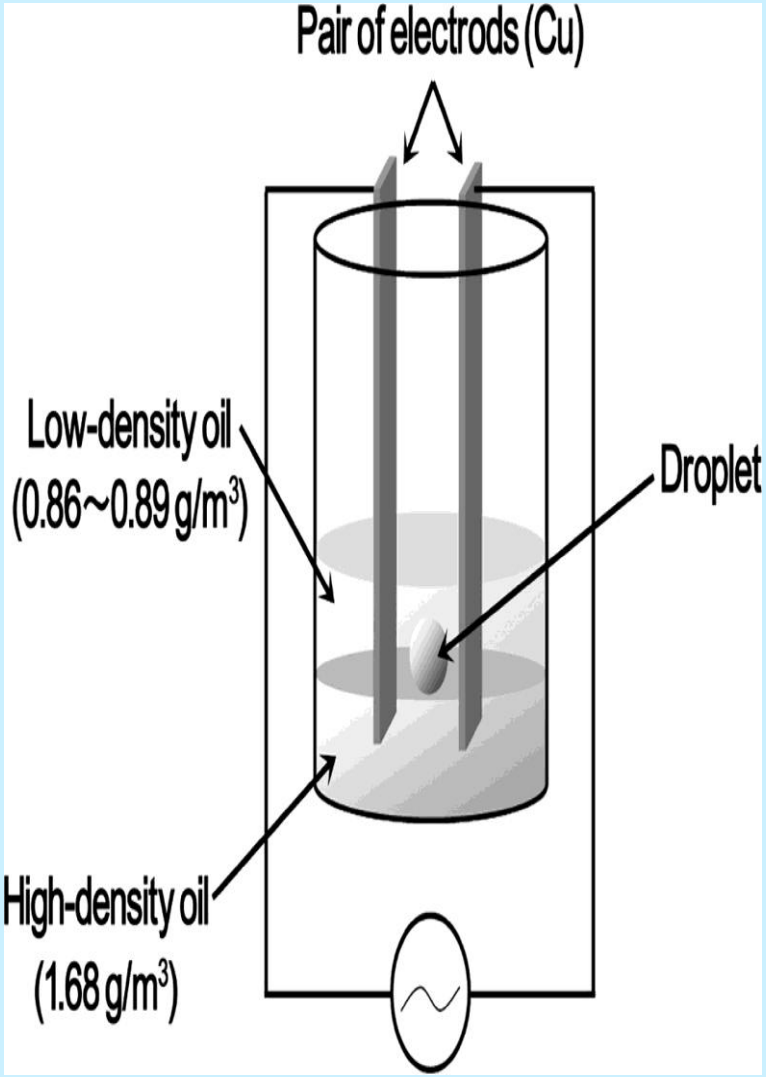
FIG. 3. Colour online (a) Uncontrollable nucleation of lysozyme with a wide size distribution in the absence of an electric field. b) Lysozyme crystals under an ac field of 8 Vpp and 3 MHz point D in Fig. 2 for 72 h. c) Diffraction pattern of the lysozyme crystals grown under an ac field.

Crystal Growth under oil, and alternant electric field



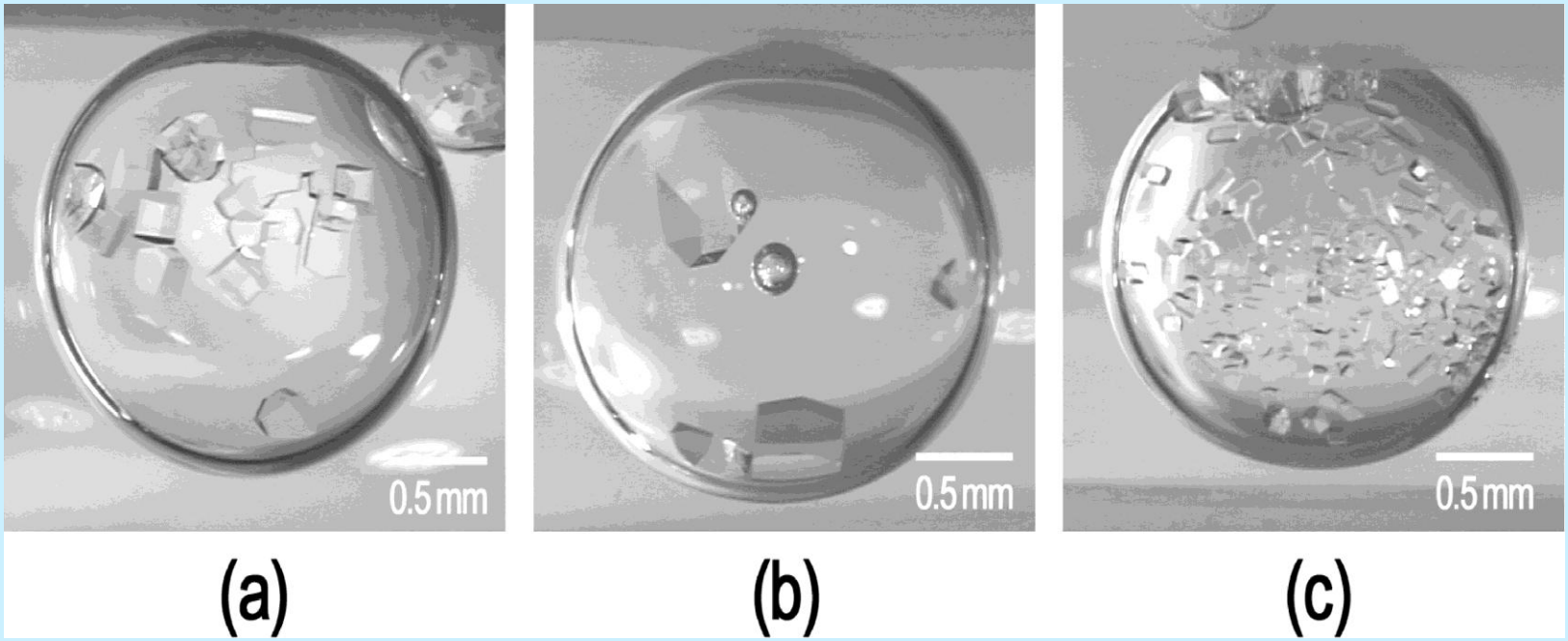
Schematic diagram of the predicted dependence of the dielectric permittivity for protein solution and protein crystals on the imposed frequency.

Published in: H. Koizumi; K. Fujiwara; S. Uda; *Cryst. Growth Des.* Article ASAP
DOI: 10.1021/cg801315p



Schematic illustration of the “containerless” batch arrangements with electrodes on both sides of a protein droplet.

Published in: H. Koizumi; K. Fujiwara; S. Uda; *Cryst. Growth Des.* Article ASAP
DOI: 10.1021/cg801315p

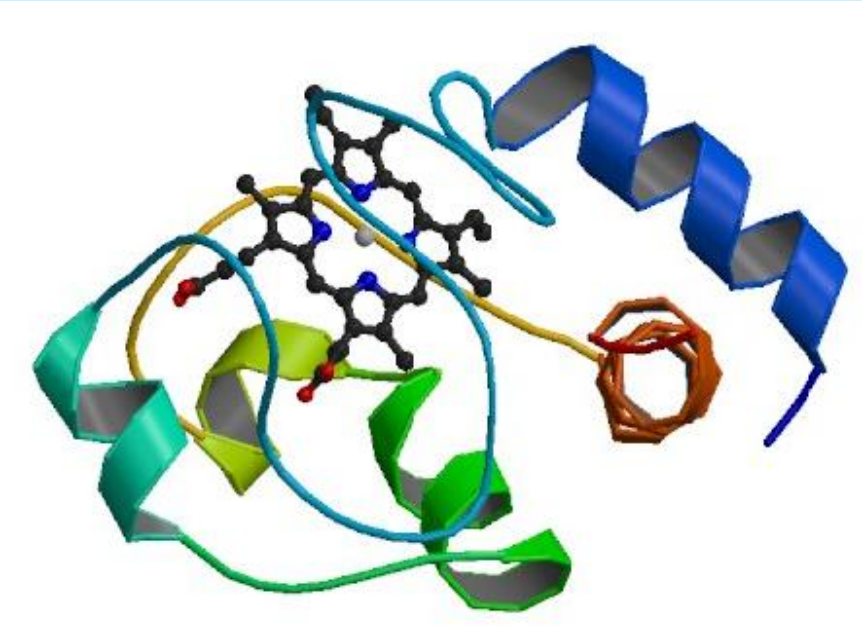


HEW lysozyme crystals in drops nucleated in the presence of (a) no electric field, (b) an electric field at 500 kHz, and (c) an electric field at 1 MHz using $NiCl_2$ as a precipitant.

Published in: H. Koizumi; K. Fujiwara; S. Uda; *Cryst. Growth Des.* Article ASAP
DOI: 10.1021/cg801315p

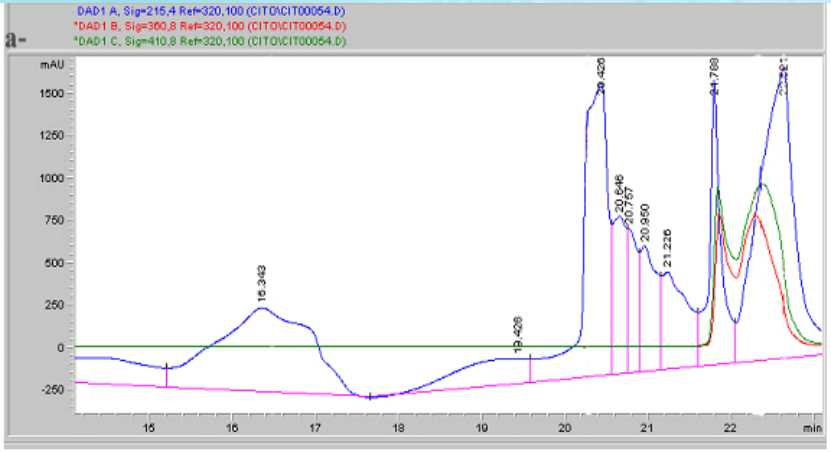
ELECTROCHEMICALLY-ASSISTED PROTEIN CRYSTAL GROWTH

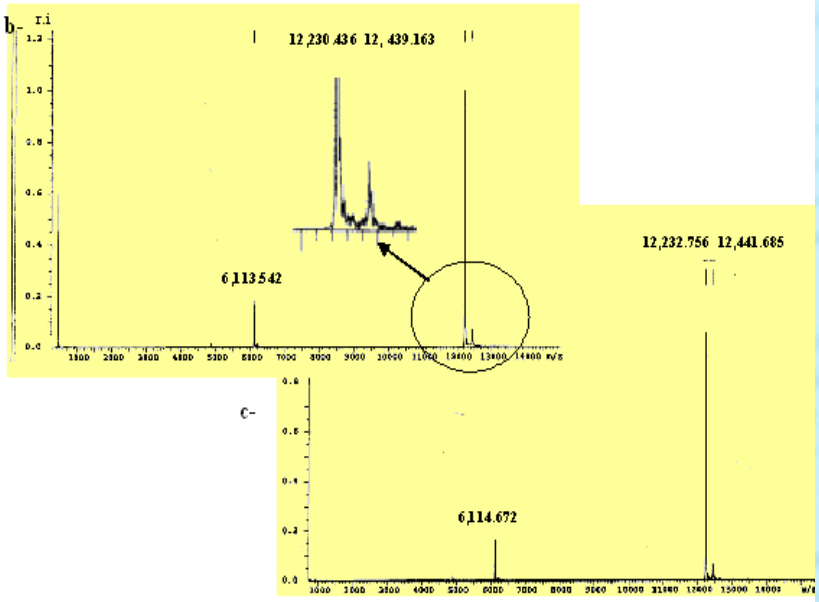
Cytochrome C

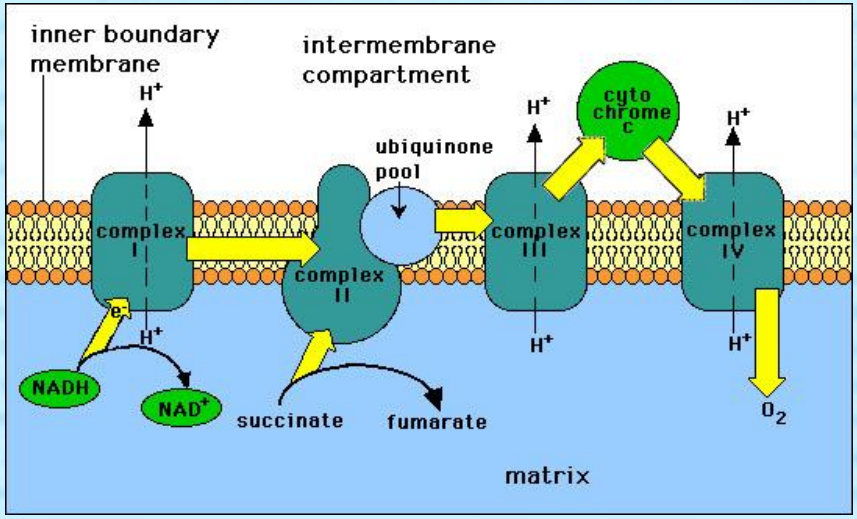


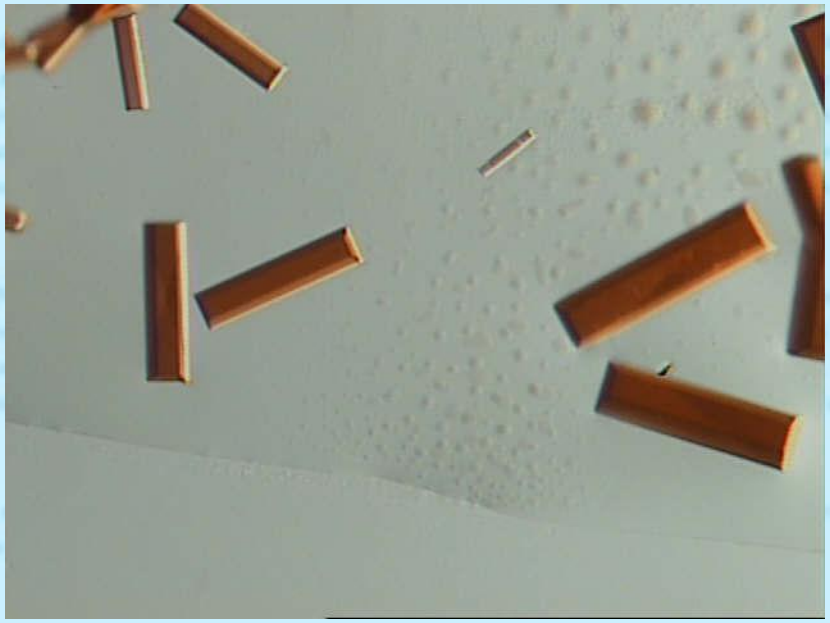
- It is a redox protein
- One of the most electrochemically studied proteins.
- Involved in the electron-transfer of aerobic and anaerobic respiration.
- Four isoforms are mixed in the commercial source

Cytochrome C characterization





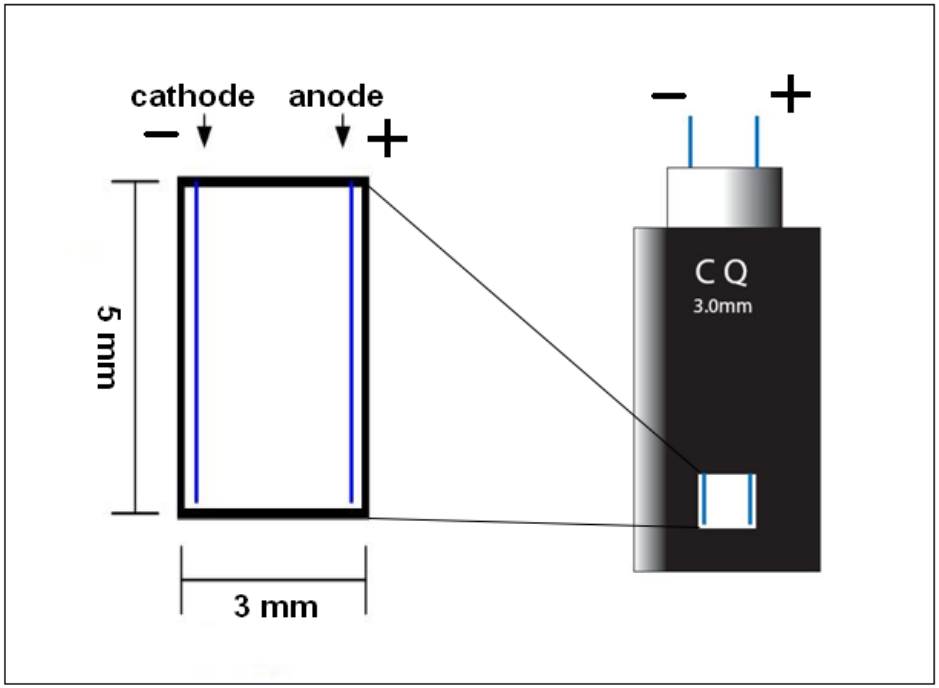




II. Coupling Dynamic Light Scattering (DLS) Methods to Electrochemistry for Protein Crystallisation

ELECTROCHEMICALLY-ASSISTED PROTEIN CRYSTAL GROWTH

DLS electrochemistry coupled technique



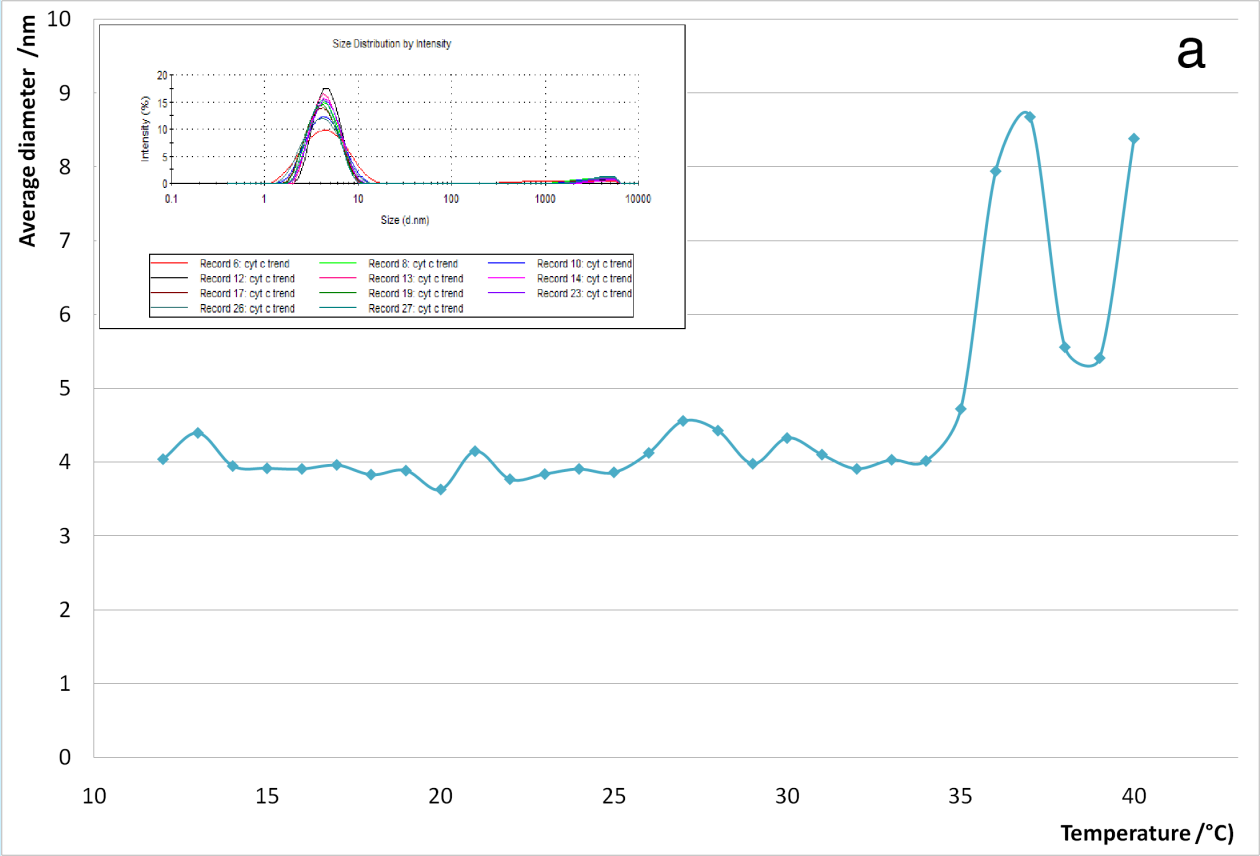
DLS-Echem Cell

1. **In situ monitoring of All chemical species**
2. **Very small volume only 12 μL**

EQUIPMENT AND EXPERIMENTAL SET-UP

ELECTROCHEMICALLY-ASSISTED PROTEIN CRYSTAL GROWTH

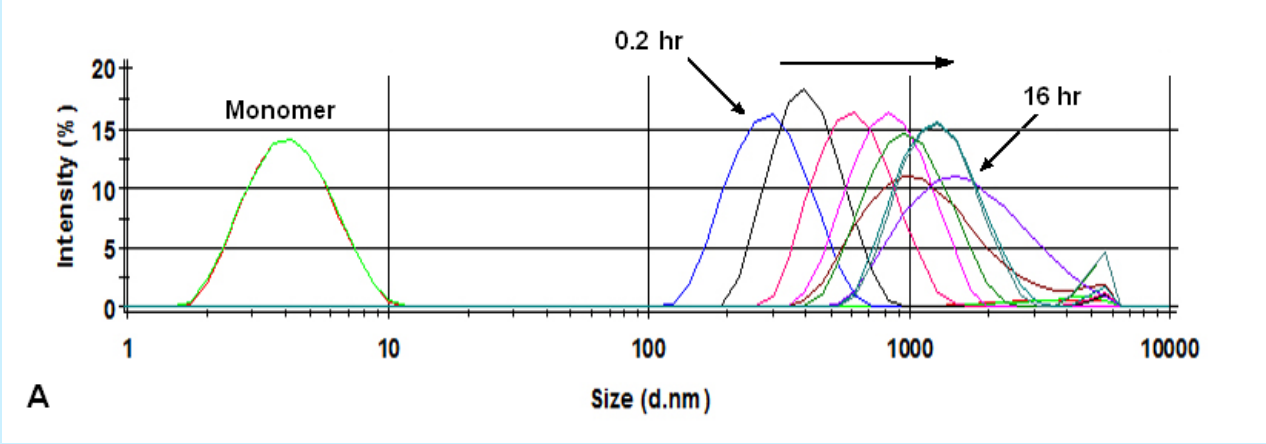
Cytochrome C

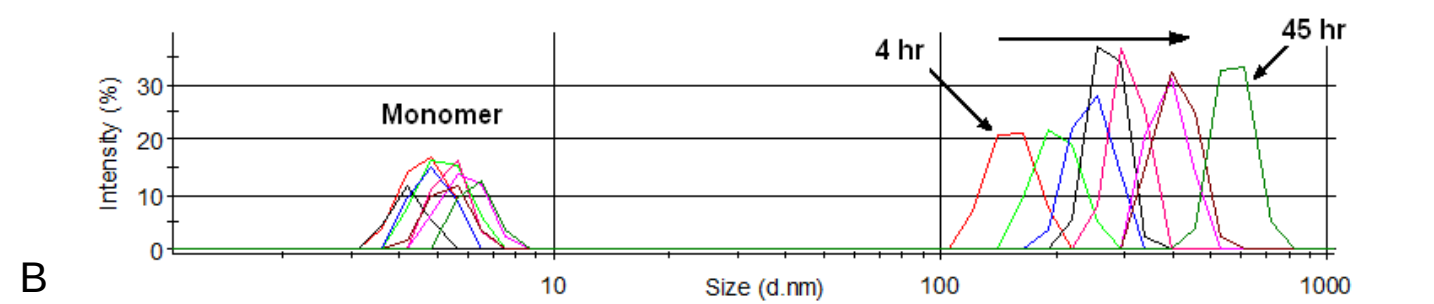


Average size distribution of the protein versus temperature (the inset shows the monodispersity of the protein) dissolved in buffer phosphate solution (Na_2HPO_4 /NaH_2PO_4) 100 mM, pH 7.0.

ELECTROCHEMICALLY-ASSISTED PROTEIN CRYSTAL GROWTH

Cytochrome C: DLS coupled to Electrochemistry

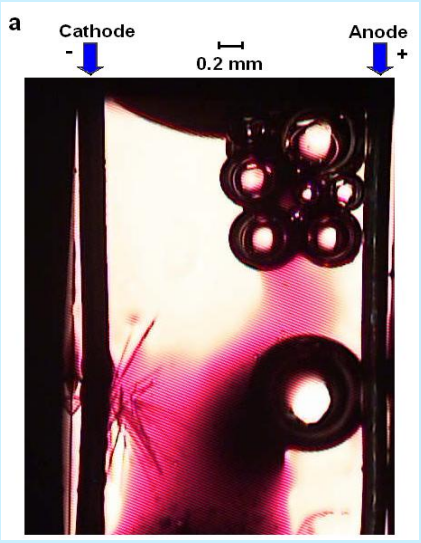


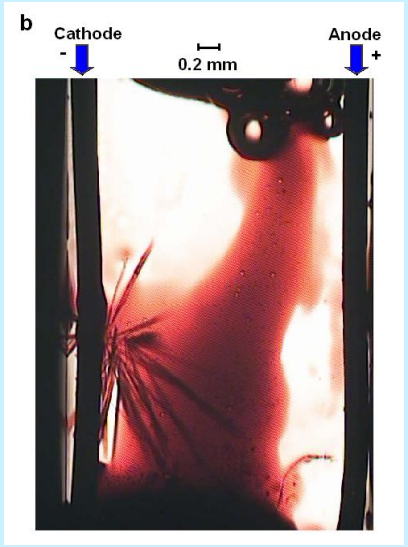


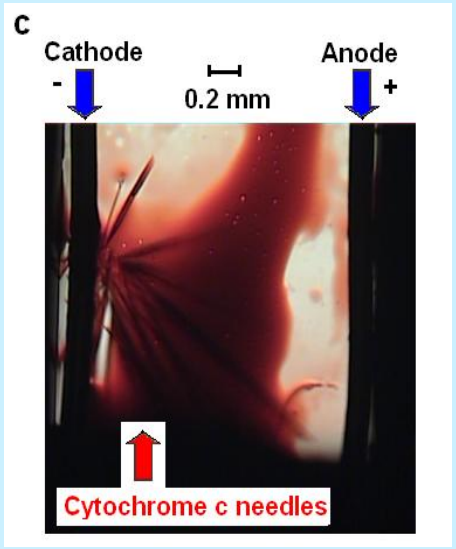
DLS experiments using 50 μL of 1:1 mixture of cytochrome c solution (62 mg/mL) and PEG 1000 (50-60% w/v), both dissolved in buffer phosphate solution (Na_2HPO_4 /NaH_2PO_4) 100 mM, pH 7.0. a) without electrical current b) in the presence of an electrical current of 0.8 μA

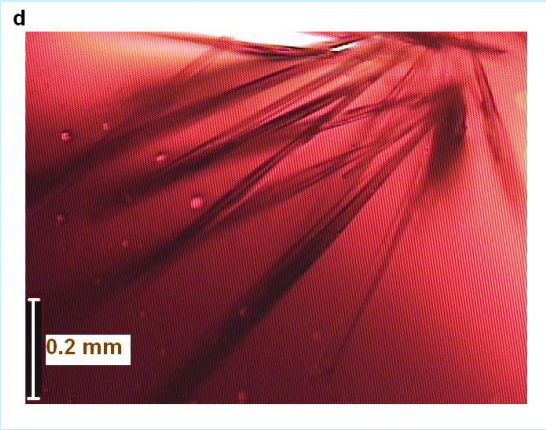
ELECTROCHEMICALLY-ASSISTED PROTEIN CRYSTALLISATION

Cytochrome C









Electrochemically assisted crystallisation of cytochrome c in a DLS cell at 10 °C at 0.9 μA. a) after 3 days experiment. b) After 5 days of crystal growth. c) Cytochrome c needle-like crystals after 8 days of experiment. d) Close-up of the needles shown in (c).

III. Crystalline efficiency and redox-properties of crystals

ELECTROCHEMICALLY-ASSISTED PROTEIN CRYSTAL GROWTH

Cytochrome C

Crystalline quality in classical vapour diffusion technique and microseeding

Table I
Data Collec

Space grou	
Unit cell par	
Resolution (Å)	
Number of r	
asymmetr	
Number of r	
I/σ (I)	
Completenes	
R_{merge} (%)	
R_{factor} (%)	
R_{free} (%)	
B_{factor} avera	
Bond length	
Bond angles	
Dihedral ang	
Number of non-H protein atoms	875
Number of water molecules	161
Ligands or ions	43

45 working days plus isoforms separation (15 days) = 60 days

Proteins 70 (2008) 83-92.

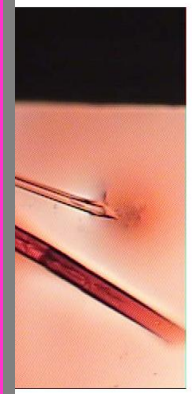
ELECTROCHEMICALLY-ASSISTED PROTEIN CRYSTAL GROWTH

Cytochrome C

Crystalline quality in electrochemically-assisted technique

Data collection parameters		
Wavelength		
Detector-Sa		
Number of I		
Exposure ti		
Oscillation		
Space group		
Unit Cell (Å		
Resolution I		
I/σI		
R merge* (%		
Average mo		
Total reflections	139522	382292
Unique Reflections	26865 (1067)	60854 (2718)
Multiplicity	5.9 (4.0)	6.3 (3.9)

5 working days without isoforms separation



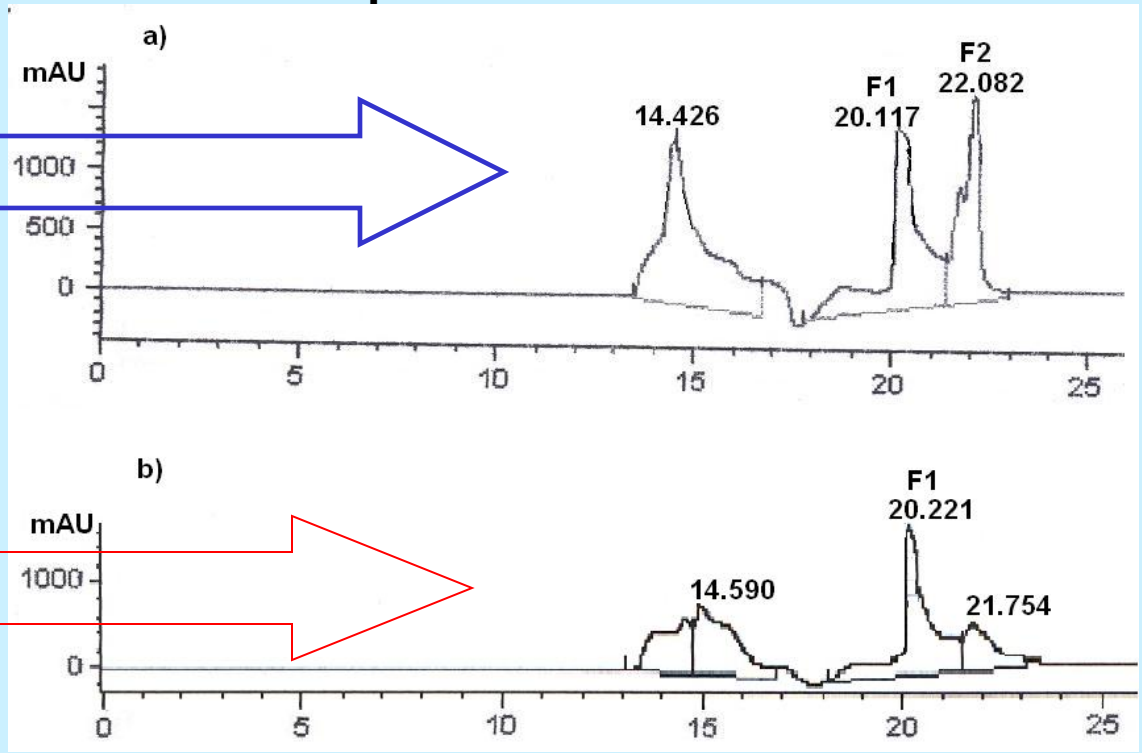
Y. Pérez, et al., *Cryst. Growth & Design* 8 (2008) 2493-2496

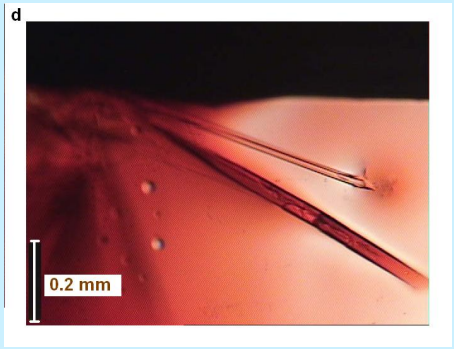
ELECTROCHEMICALLY-ASSISTED PROTEIN CRYSTAL GROWTH

Cytochrome C

Efficiency in the overall process no isoform separation

Commercial cyt-c





Y. Pérez, et al., *Cryst. Growth & Design 8 (2008) 2493-2496.*

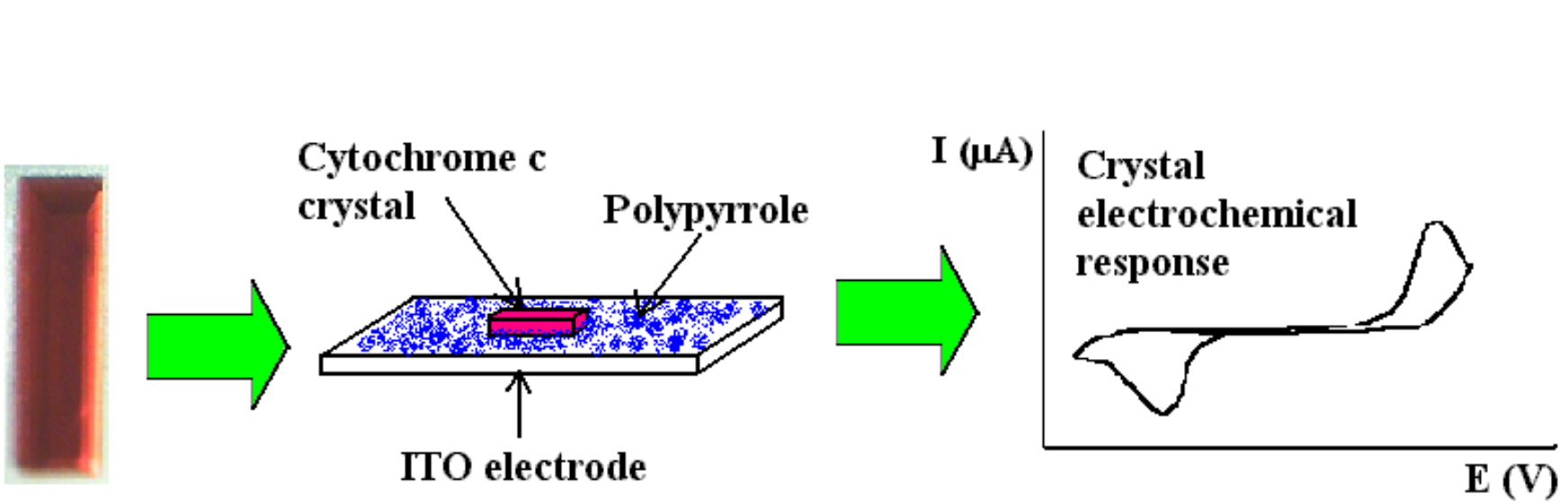
Frontana-Uribe, Bernardo & Moreno, Abel. Crystal Growth and Design 8 (2008) 4194-4199.

Are these crystals redox electroactive in solid state?

ELECTROCHEMICALLY-ASSISTED PROTEIN CRYSTAL GROWTH

Cytochrome C

Are the crystals redox electroactive in solid state?



Francisco Acosta, Désir Eid, Liliana Marín-García, Bernardo A. Frontana-Uribe, and **Abel Moreno** "From Cytochrome C Crystals to a Solid-State Electron-Transfer Device". Crystal Growth and Design **7** (2007) 2187-2191.

Atomic Force Microscopy

(Structural topography)

+

Electrochemical techniques

(Cyclic voltammetry)

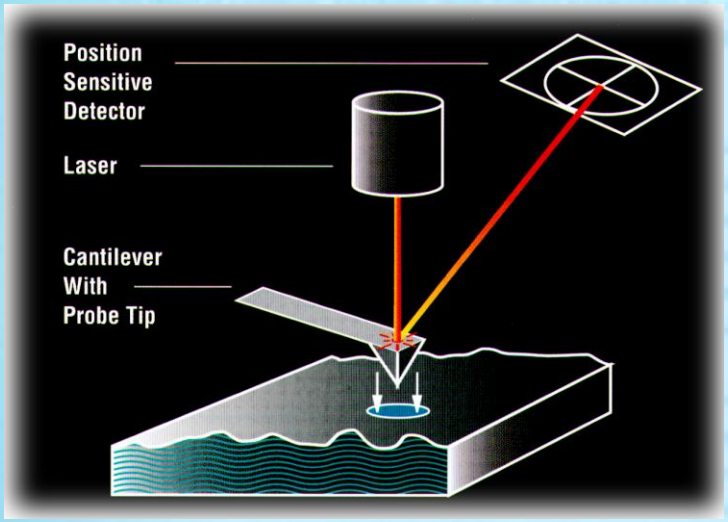
MECHANISMS OF CRYSTAL GROWTH

&

BIOSENSORS DEVELOPMENT

Electrochemistry and Atomic Force Microscopy

Fluid Cell for electrochemical research



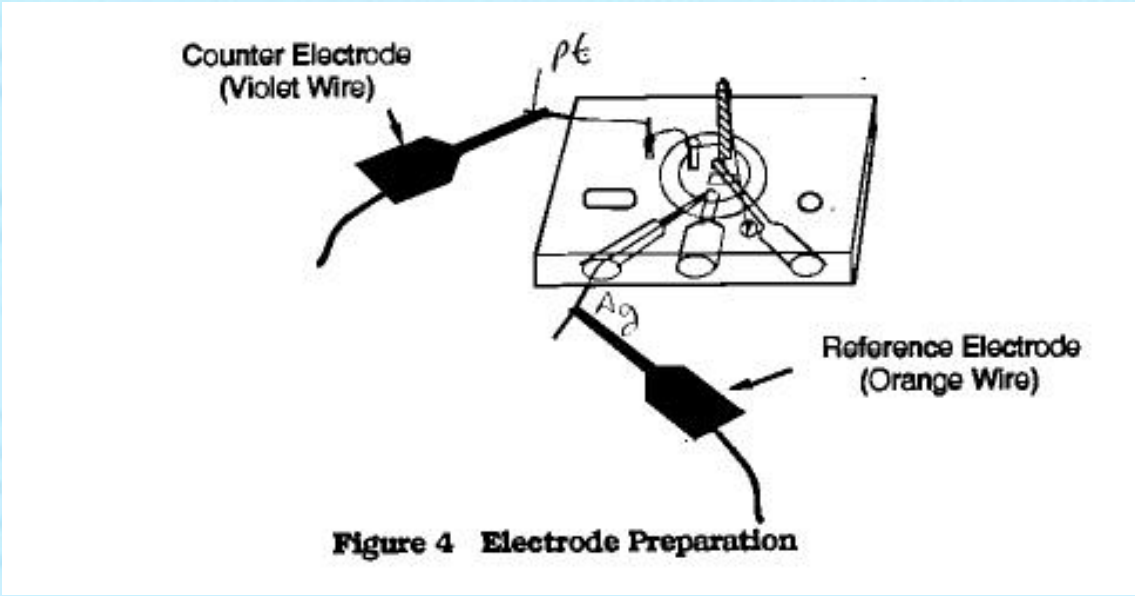
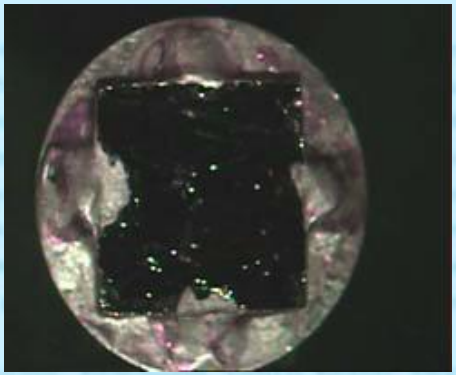


Figure 4 Electrode Preparation

Electrochemistry AFM

The experimental set- up



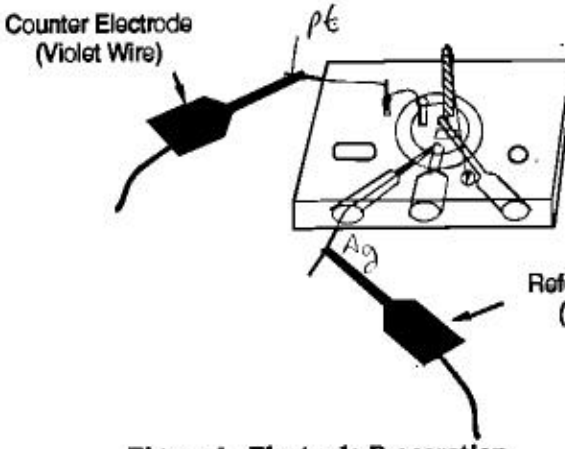
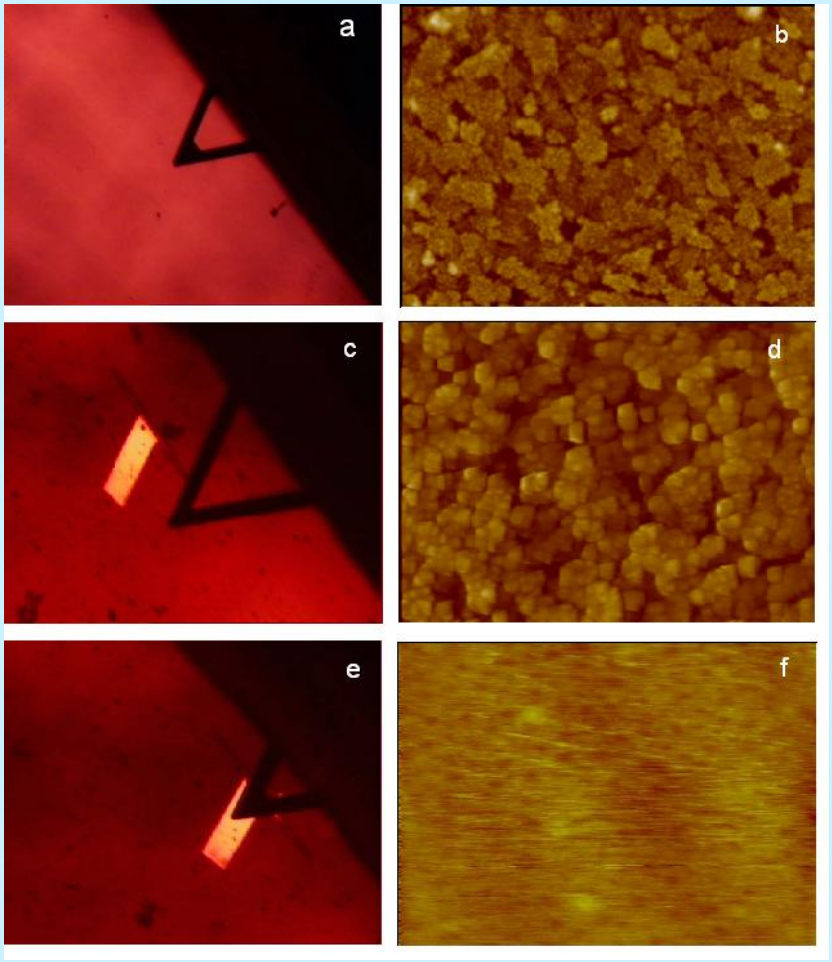


Figure 4 Electrode Preparation

Electrochemically-assisted protein crystal growth



Cytochrome C

AFM micrographs 2.5 x 2.5 microns image

a) - b) Surface of the ITO electrode;

c) - d) Polypyrrole surface

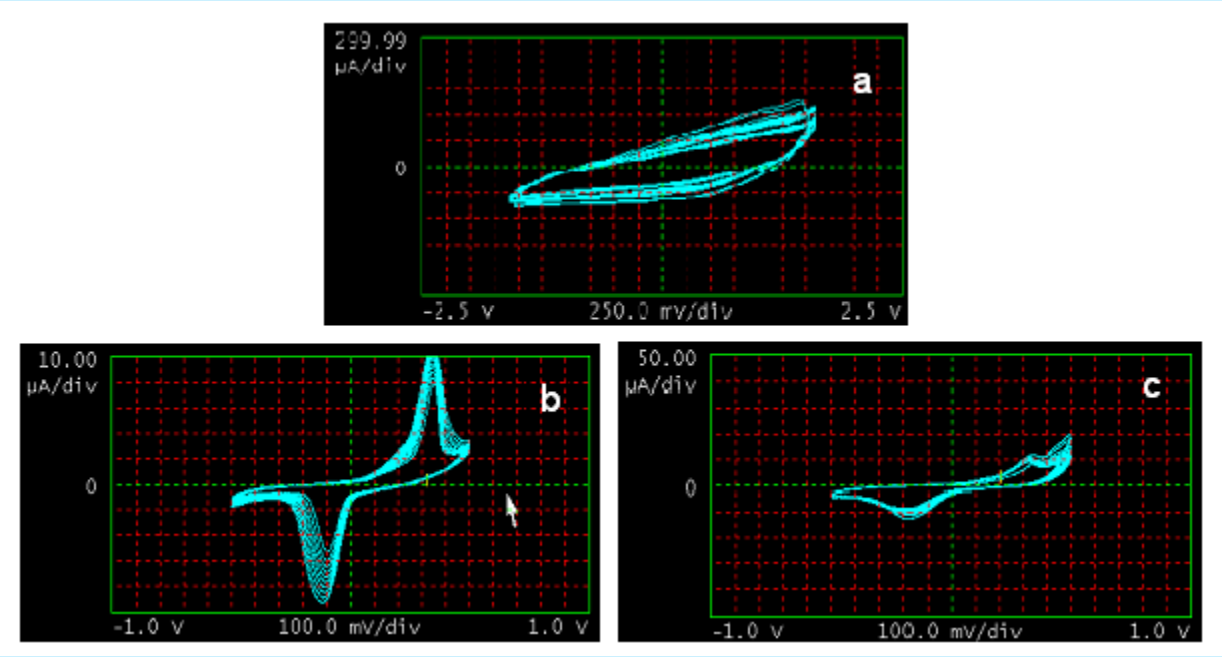
e) - f) After pyrrole polymerisation the crystal surface was scanned.

Francisco Acosta, Désir Eid, Liliana Marín-García, Bernardo A. Frontana-Uribe, and **Abel Moreno** "From Cytochrome C Crystals to a Solid-State Electron-Transfer Device". Crystal Growth and Design **7** (2007) 2187-2191.

ELECTROCHEMICALLY-ASSISTED PROTEIN CRYSTAL GROWTH

Cytochrome C

Are the crystals redox electroactive in solid state?



CV in-situ AFM

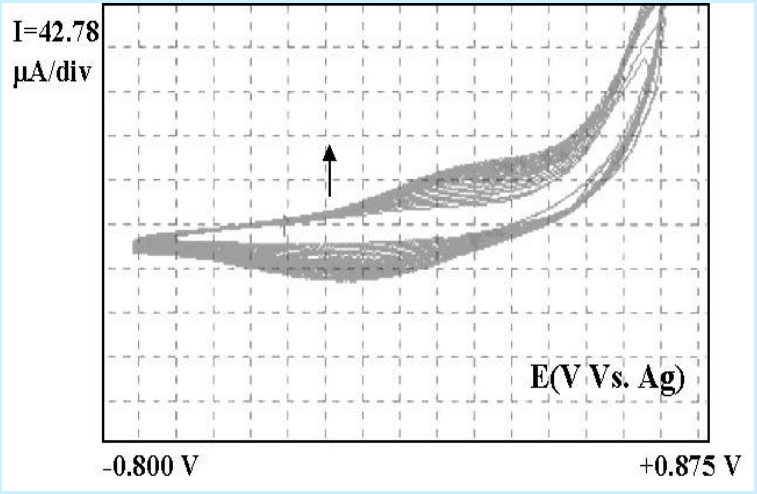
F. Acosta, et al., *Crystal Growth & Design* 2007, 2187-2191.

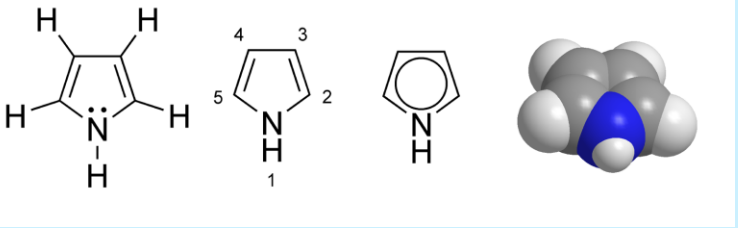
a) Polypyrrole (Ppy) layer's voltammogram (control) on ITO

b) Signal of cytochrome c adsorbed by evaporation on ITO

c) Voltammogram of cytochrome c crystals fixed by using Ppy

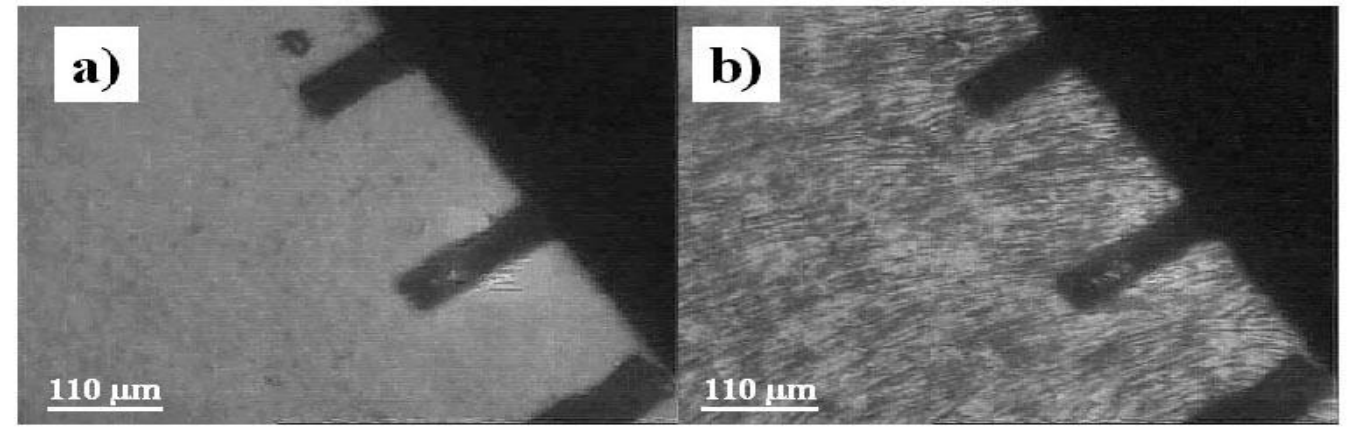
Electrochemically-assisted protein crystallisation: a case study for Catalase's immobilisation for AFM investigations.

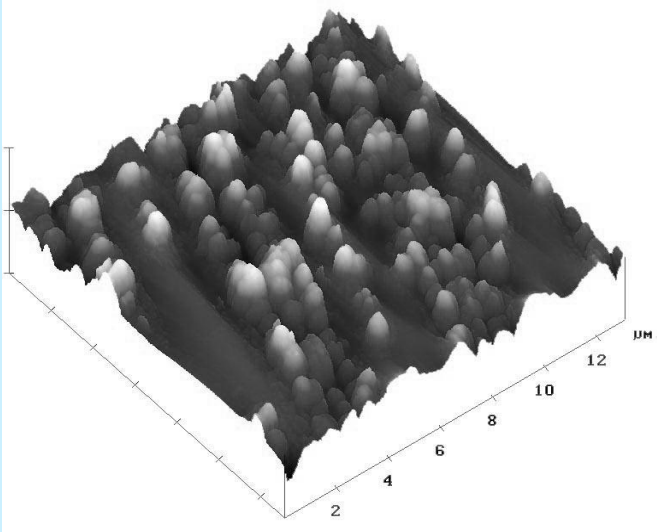


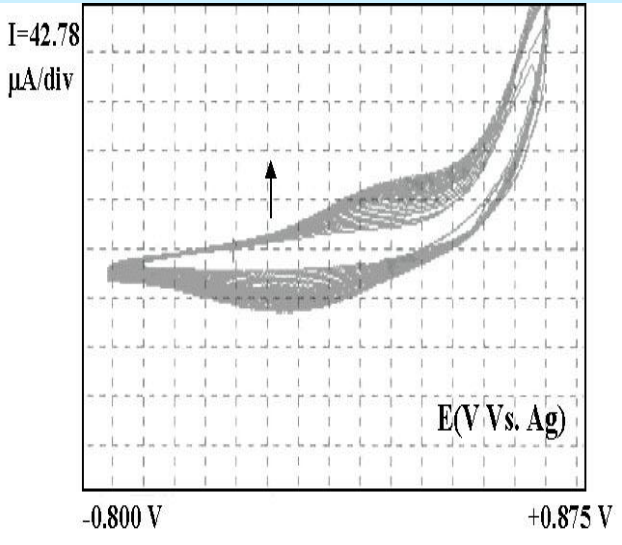


Crystals are immobilised with ppy films. An equilibrated droplet of the crystallisation conditions, with crystals of catalase, were mounted on the HOPG surface previous to the injection of an aqueous solution of 0.77 M pyrrole (py)/0.34 M $LiClO_4$ in the fluid cell of the EC-AFM.

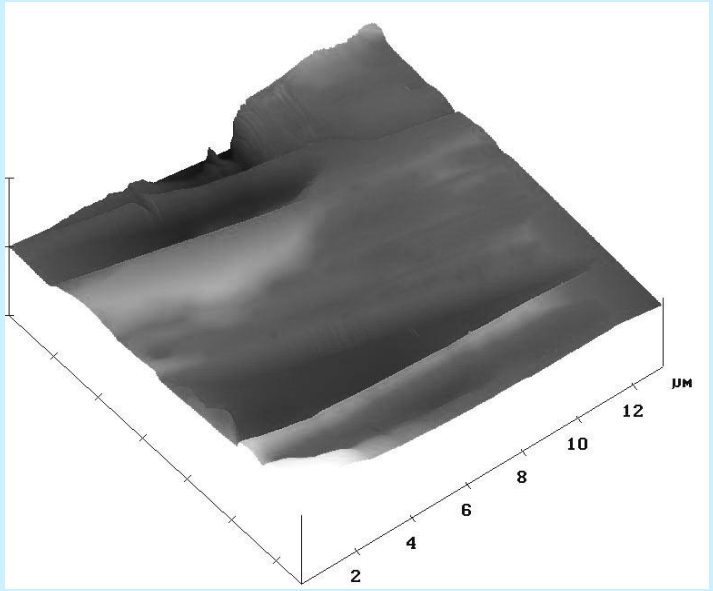
PYRROLE ⟶ Conductive polymer

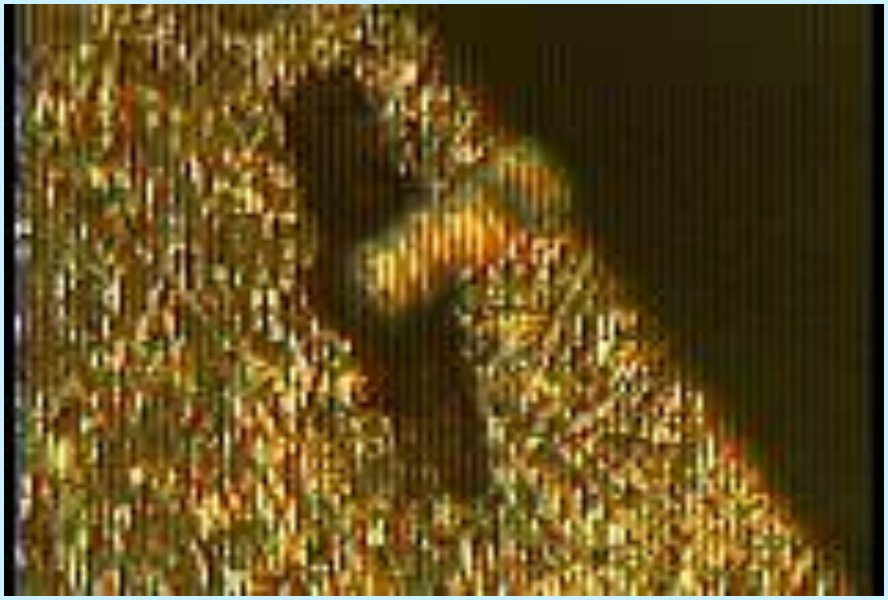




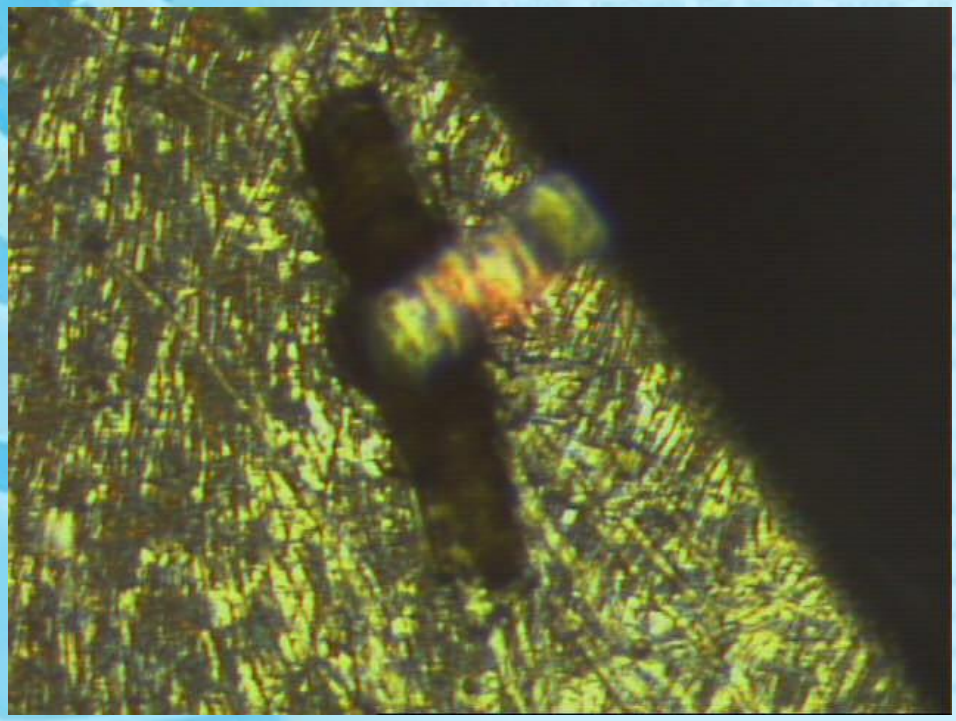


Polypyrrole and protein surface characterization

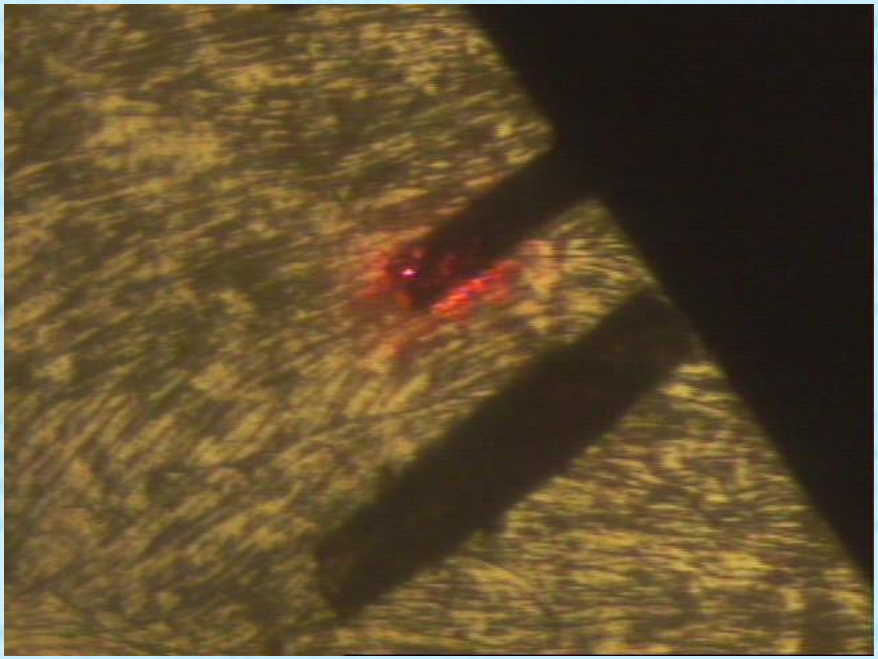




T. Hernández-Pérez, N. Mirkin, **A. Moreno** & M. R ivera "*In situ* Immobilization of catalase monocrystals on HOPG by the voltammetric growth of polypyrrole films for AFM investigations". ***Electrochemical and Solid-State Letters* 5**, No. 8 (2002) E37-E39.



Is the protein well supported on the surface by this chemical glue?

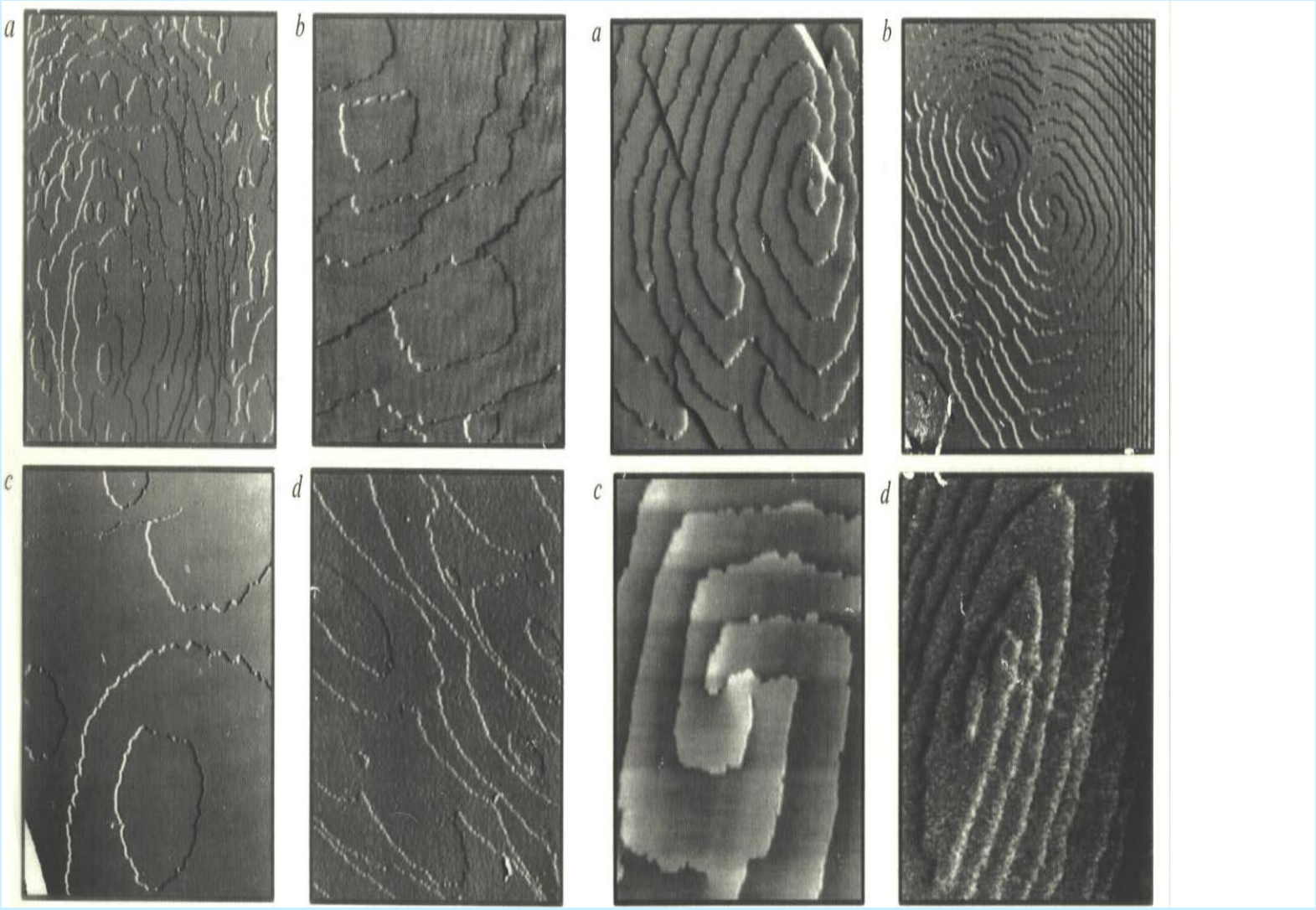


Scanning on the surface and pushing the crystals with the cantilever.

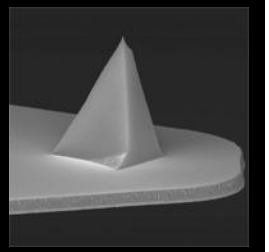
IV. Applications to Protein Crystal Growth and Biosensors

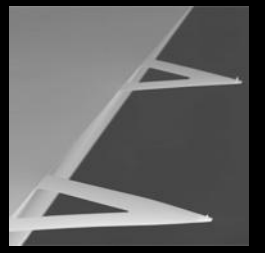
- Mechanisms of crystal growth
- 2D images of high resolution
- Protein-based biosensors

Different images of mechanisms of crystal growth observed by atomic force microscopy



High resolution of 2D images of protein surfaces via AFM





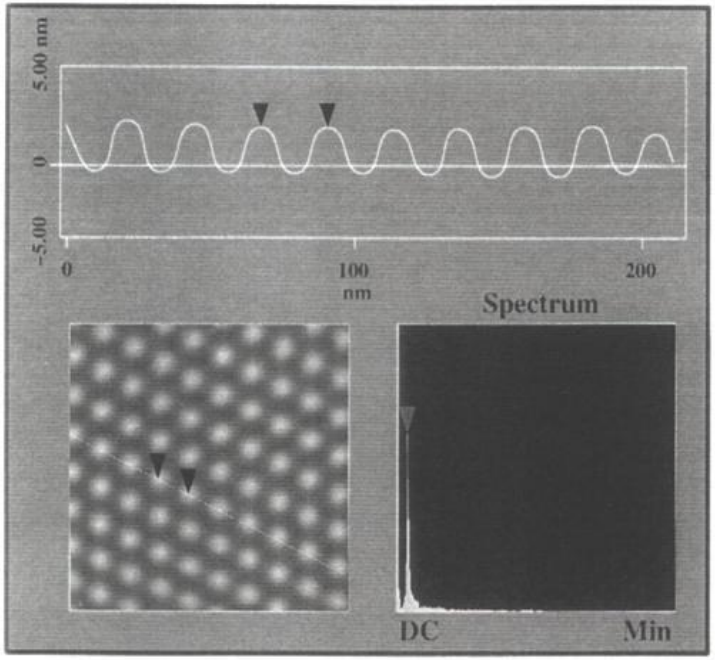


FIGURE 1 At lower left is a Fourier-filtered image of an area (190 × 190 nm^2) on the surface of an STMV crystal. The line drawn along a row of virus particles is reproduced at the top, with height variation added, showing, in turn, the periodicity of the surface. The two daggers can be specified by the AFM operator and distances immediately displayed. The power spectrum of the periodic array is shown at the lower right.

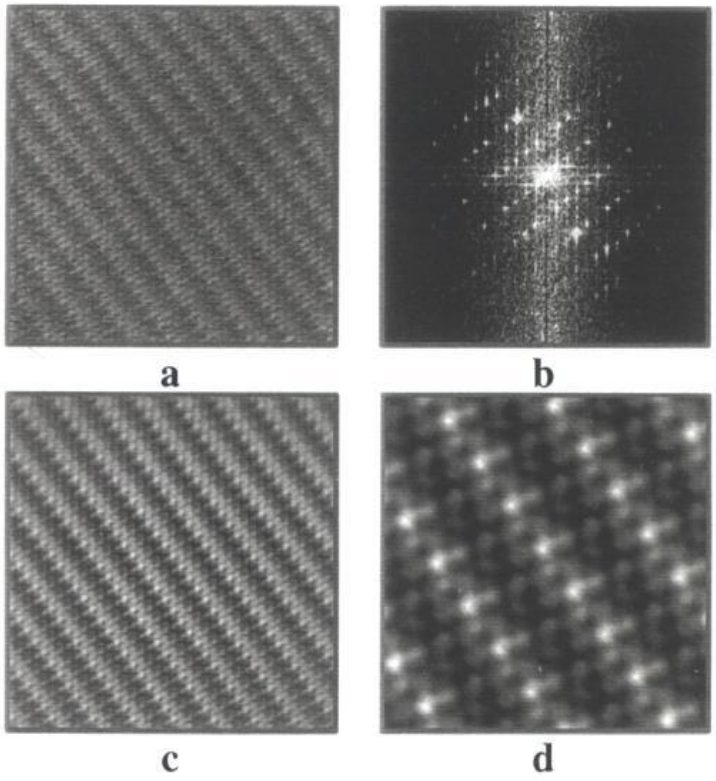
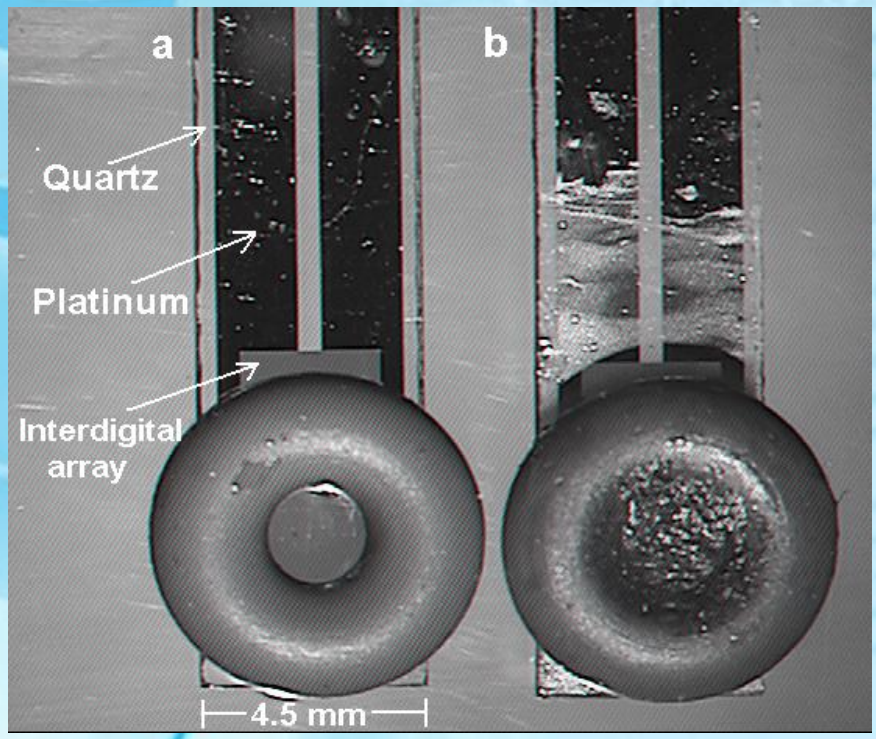


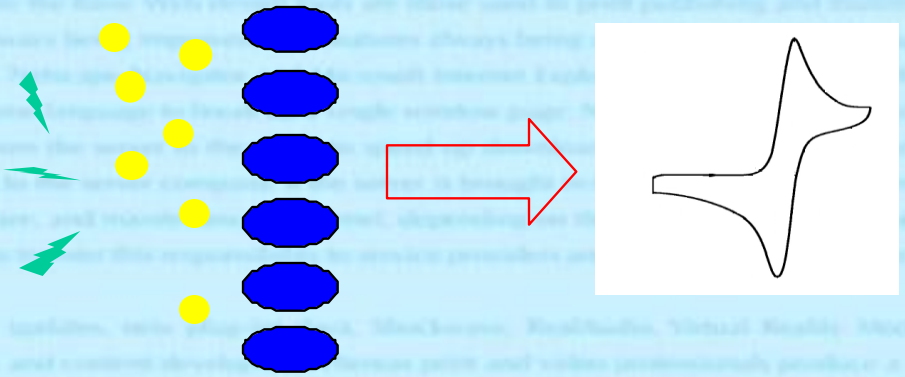
FIGURE 2 (*a*) A 200 × 200 nm^2 scan area image of the (100) face of a hexagonal crystal of fungal lipase, with space group $P6_1$ (or $P6_5$) and cell dimensions $a = b = 142.9$ Å, and $c = 80.4$ Å. The lattice, and even individual molecules, are clearly evident in this raw image. (*b*) Fourier transform (diffraction pattern) of the raw image, with a resolution of ~12–14 Å. It was subsequently filtered using the program ICE, and the Fourier-filtered image obtained from *a* is presented in *c*. (*d*) Fourier-filtered image (5 × 5 unit cells) of the lipase crystal. Light features are above and dark features below the mean plane of the crystal surface.

This method will probably help in getting 2D high resolution structures, and 3D structures by modelling in the near future.

Biosensors' design

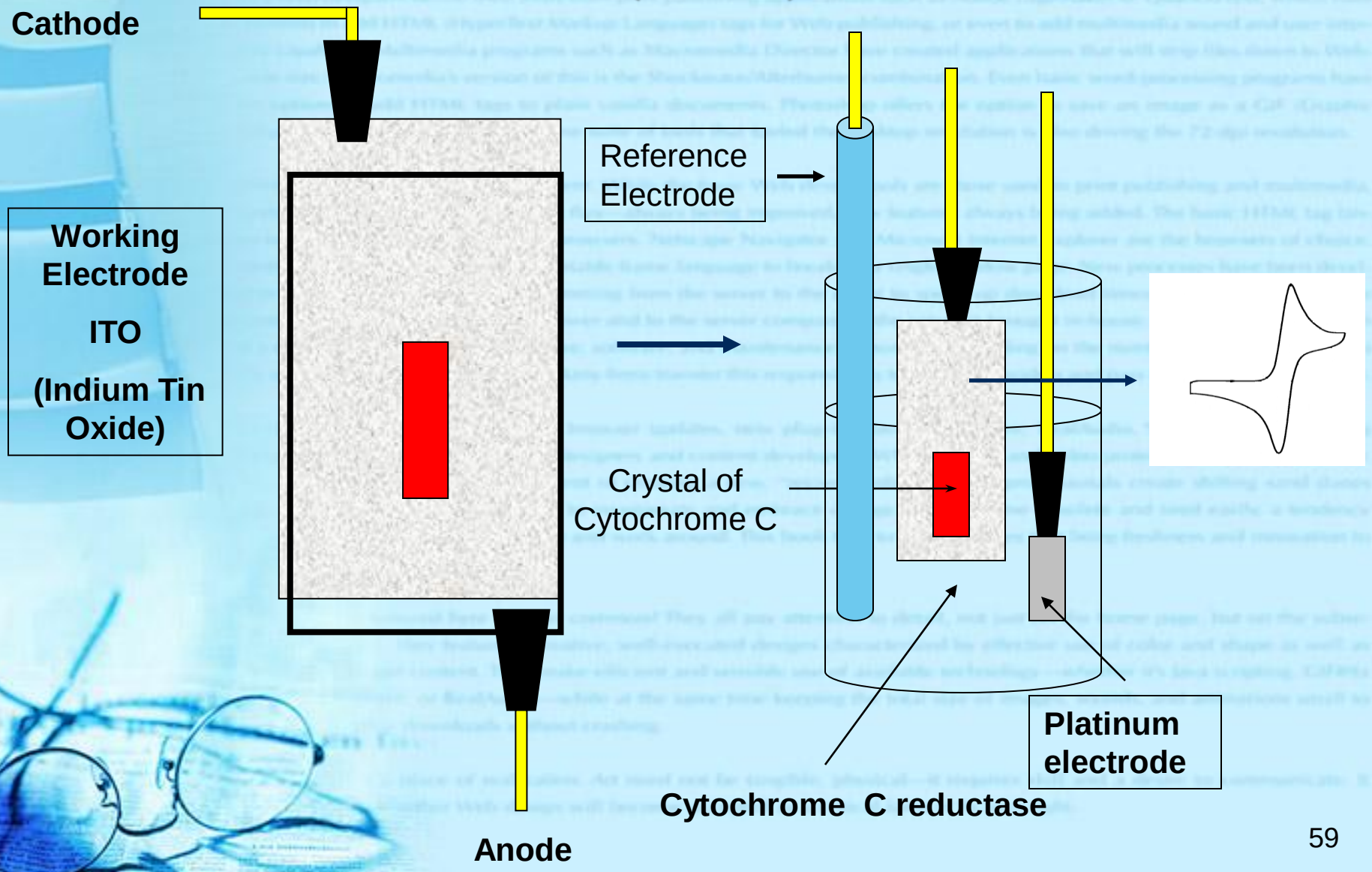
…Carbonates Biosensor...



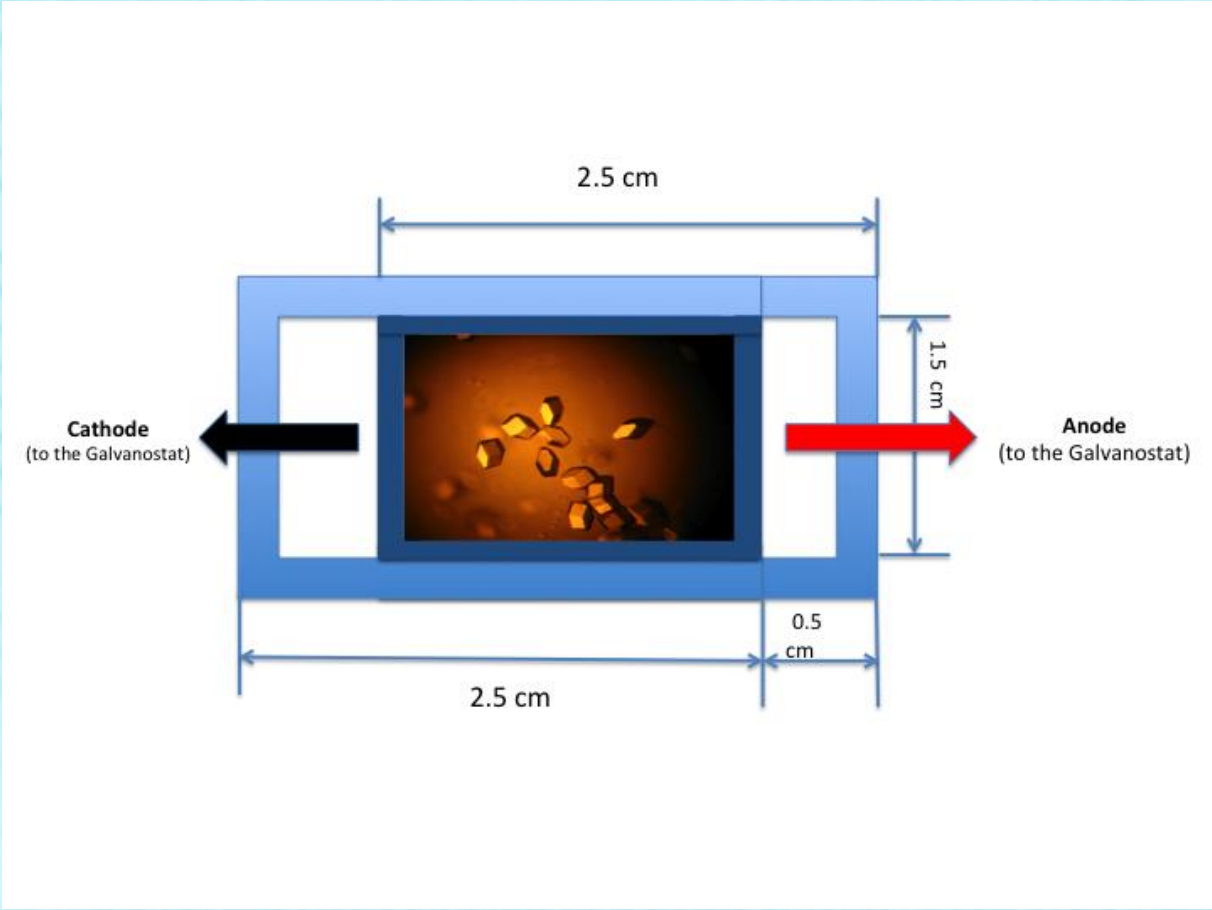


Liliana Marín-García, et al., Crystal Growth and Design 8 (2008) 1340-1345

Perspectives for a Solid-State Electron-Transfer Biosensor (SS-ETB)

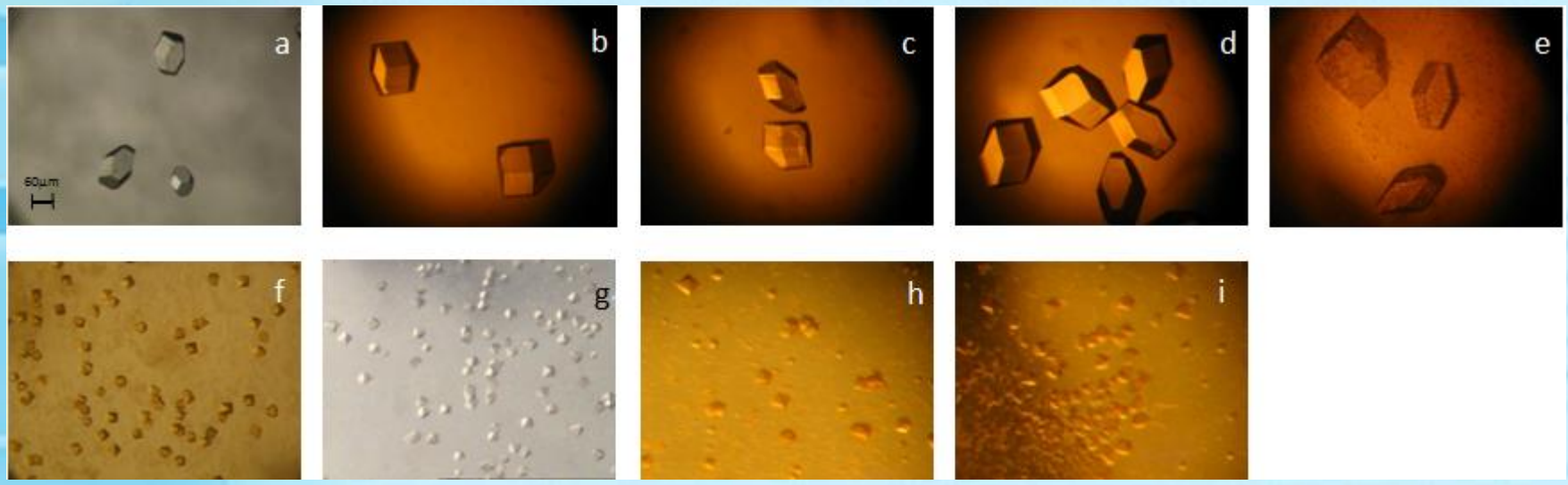


Protein crystallization cell

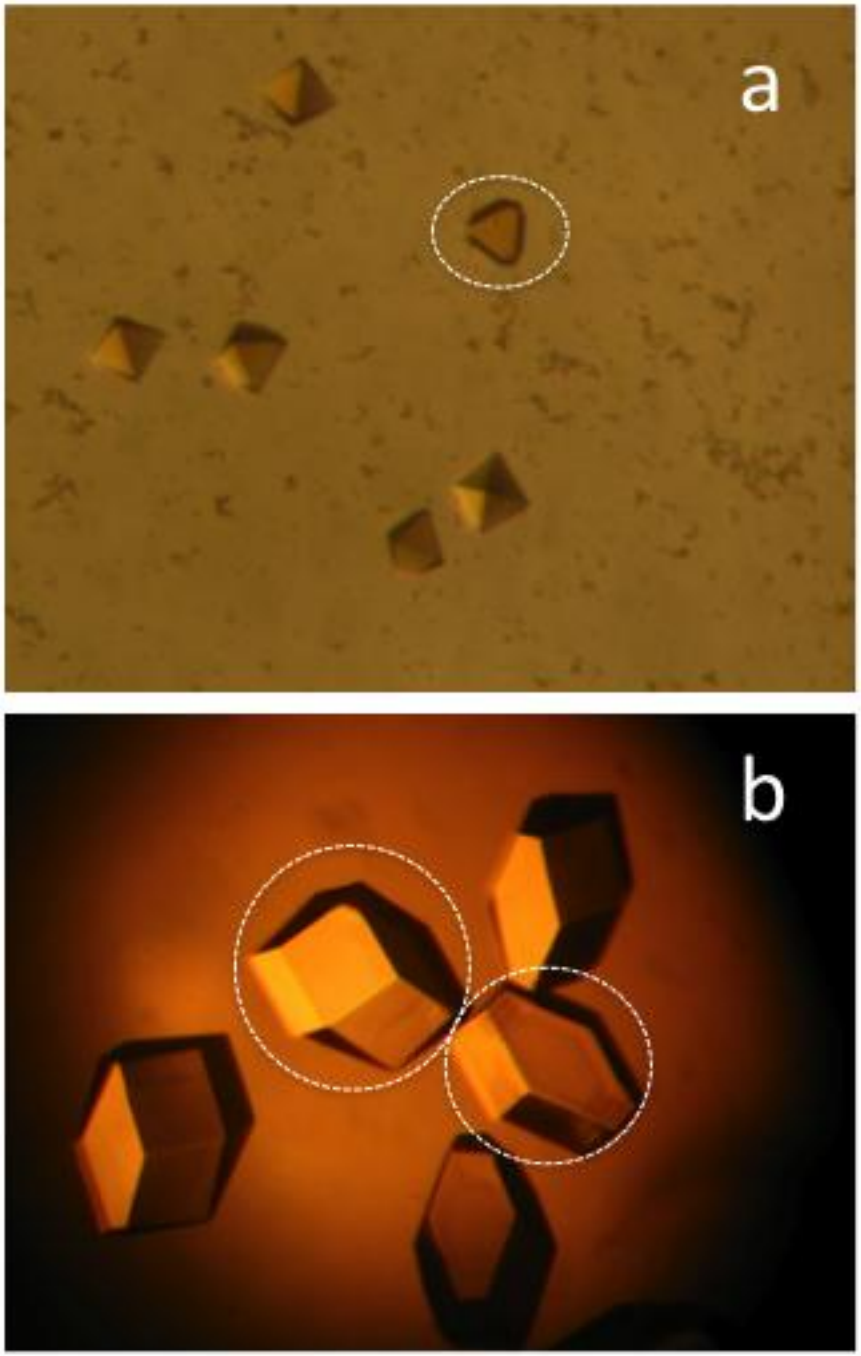


Gabriela Gil-Alvaradejo, Rayana, R. Ruiz-Arellano, Christopher Owen, Adela Rodriguez-Romero, Enrique Rudiño-Piñera, Moriamou K. Antwi, Vivian Stojanoff & **Abel Moreno** "Novel Protein Crystal Growth Electrochemical Cell for Applications in X-Ray Diffraction and Atomic

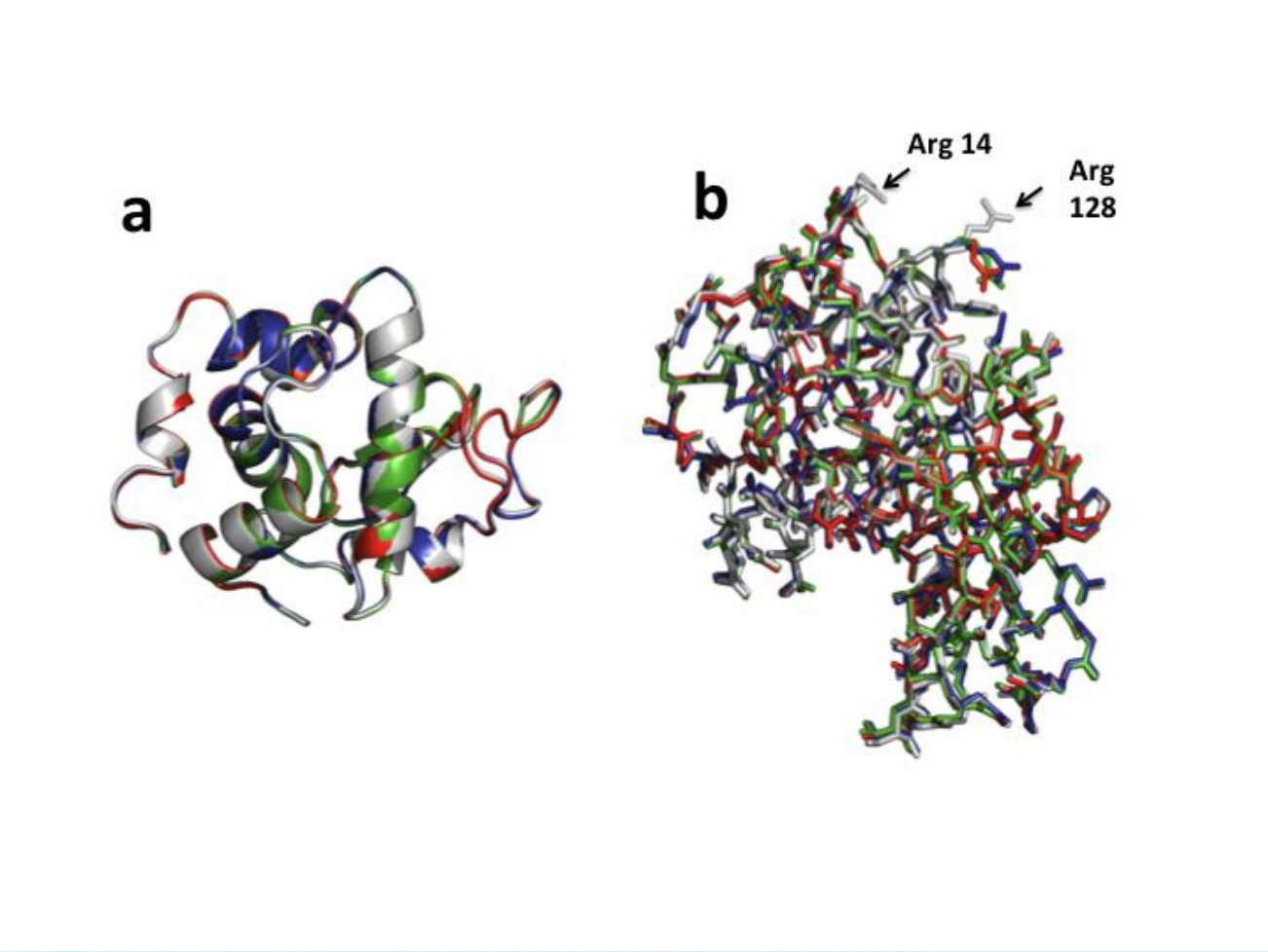
Force Microscopy" Crystal Growth and Design 11 (2011) 3917-3922.



Gabriela Gil-Alvaradejo, Rayana, R. Ruiz-Arellano, Christopher Owen, Adela Rodriguez-Romero, Enrique Rudiño-Piñera, Moriamou K. Antwi, Vivian Stojanoff & **Abel Moreno** "Novel Protein Crystal Growth Electrochemical Cell for Applications in X-Ray Diffraction and Atomic Force Microscopy" Crystal Growth and Design 11 (2011) 3917-3922.


a
b

Protein structures comparison



	Control	2 µA	4 µA	6 µA
Space Group	F432	F432	F432	F432
Unit cell dimensions	181.77	181.84	182.38	182.00
Mosaicity	0.530	0.308	0.675	0.308
Resolution Range	25.00 - 2.27 (2.31 – 2.27)	25.00 – 1.97 (2.00 – 1.97)	25.00 – 2.24 (2.28 -2.24)	25.00 – 2.07 (2.11 – 2.07)
Total number of reflections	2804198	1189907	720448	2228266
Number of unique reflections	12400	18819	13091	16282
Average redundancy	20.6 (21.5)	11.7 (12.1)	5.0 (5.1)	20.8 (20.9)
% Completeness	100.0 (100.0)	99.9 (100.0)	99.3 (99.1)	100.0 (100.0)
Rmerge	0.204	0.173	0.134	0.235
<I/σ(I)>	25.0 (2.88)	21.2 (2.03)	13.7 (2.07)	16.7 (1.94)

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CONCLUSIONS

- The electrochemically-assisted protein crystallisation (not electrocrystallisation) is a useful tool to control the nucleation, and the induction time for protein crystallisation process.

- The use of electrochemistry and AFM to grow *In situ* single crystals is a new possibility to develop the biosensors of the near future.

- All is electricity: inside the cell, outside of the cell, the human body works based on electrons & the chemical reactions, etc.

Acknowledgments

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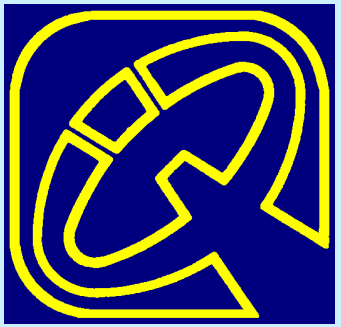
Dr. Nurit Mirkin, Hunter College New York , USA.

Dr. Margarita Rivera, Institute of PhysicsUNAM (Mexico)



THANK YOU SO MUCH!