



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 ZnxCd1-xO 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/cm²; (b) out-of-order Zn1-xCdxO nanoparticles prepared with a current density of 1.0 mA/cm². (c) ZnxCd1-xO 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/cm² at 90 °C.

Published in: Gao-Ren Li; Qiong Bu; Fu-Lin 28/05/240ng2Yong Su; Ye-Xiang Tong; *Cryst. Growth Des.* 2009, 9, 1538-1545. DOI: 10.1021/cg800496d Copyright © 2009 American Chemical Society



ELECTROCHEMICALLY-ASSISTED PROTEIN CRYSTALLISATION

3D structure determination of proteins



Physical parameters used to induce the nucleation process:

Lorentz Force Law

Both the <u>electric field</u> and <u>magnetic field</u> can be defined from the Lorentz force law:

$$F = q \underbrace{E}_{\text{Electric}} + q \underbrace{v x}_{\text{Magnetic}} B$$

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 <u>right hand rule</u>.





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 $T = 18^{\circ}C$ $I = 2 \mu A$

Galvanostat PAR 175

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



CONTROL

ANODE

- **300 m**μ



Electrochemically-assisted Protein Crystal Growth

X-ray data statistics for the thaumatin crystals.

	Control	Electhau1	Electhau2
Data collection			
Space group	P4.2.2	P4.2.2	P4.2.2
Unit cell parameters (Å)	1 41212	1 41212	1 -1212
oniteen parameters (A)	58 664	58 647	58 633
<i>u</i>	50.004	59 6 47	59,6227
D	38.004	58.04/	38.0337
	151.391	151.390	151.427
Resolution range (A)	15.0 - 2.0	15.0-2.0	15.0 - 2.0
No. of reflections	132082	134540	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 _{merse} (%)	0.053	0.112	0.059
Structure refinement			
R/R _{tree}	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 $2\mu A$ during 48 h



With Electrochemistry

a) Faster than the
control experiment

b) Crystallisation

towards the cathode

[Lysozyme] = 40mg/mL, at I = 2μ A, [NaCI] = 40mg/mL, [Agar GeI] = 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









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 28/05/2012 Copyright © 2007 American Chemical Society



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-Pier 8 A Grand 2007 Morin; Stphane Veesler; *Crystal Growth & Design* 2007, 7, 1472-1475. DOI: 10.1021/cg070108r Copyright © 2007 American Chemical Society his particular the borner "arear" or "share apt" will be assid. The b





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).

Published in: Zoubida Hammadi; Jean-Pier 28 A Grand Published in: Zoubida Hammadi; Jean-Pier 2007, 7, 1472-1475. DOI: 10.1021/cg070108r Copyright © 2007 American Chemical Society



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.





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 Copyright © 2009 American Chemical Society





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 Copyright © 2009 American Chemical Society





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 NiCl₂ as a precipitant.

Published in: H. Koizumi; K. Fujiwara; S. Uda; *Cryst. Growth Des.* Article ASAP DOI: 10.1021/cg801315p Copyright © 2009 American Chemical Society



ELECTROCHEMICALLY-ASSISTED PROTEIN CRYSTAL GROWTH

Cytochrome C



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

Cytochrome C characterization



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

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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 (Na₂HPO₄ /NaH₂PO₄) 100 mM, pH 7.0.



ELECTROCHEMICALLY-ASSISTED PROTEIN CRYSTAL GROWTH

Cytochrome C: DLS coupled to Electrochemistry



DLS experiments using 50 μ L 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 μ A



ELECTROCHEMICALLY-ASSISTED PROTEIN CRYSTALLISATION

Cytochrome C



Electrochemically assisted crystallisation of cytochrome c in a DLS cell at 10 °C at 0.9 μ A. 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 redoxproperties of crystals



ELECTROCHEMICALLY-ASSISTED PROTEIN CRYSTAL GROWTH Cytochrome C

Crystalline quality in classical vapour diffusion technique and microseeding

Table I		
Data Collec		<u> </u>
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Space group		
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R _{free} (%)		
B _{factor} avera		
Bond length	n = n n	Oavs
Bond angles		
Dihedral and	075	
Number of non-H protein atoms	875	Proteins 70 (2008)
ligands or ions	101	83-92
Liganus or ions	40	



ELECTROCHEMICALLY-ASSISTED PROTEIN CRYSTAL GROWTH Cytochrome C

Crystalline quality in electrochemically-assisted technique

	Data colle	ection parameters	a		_
Wavelength Detector-Sai Number of I Exposure th Oscillation 1 Space group Unit Cell (Å	5 w with	orki out	ing d isof	lays orms	
Resolution I Completene I/σI R merge* (% Average mo Total reflections	S	sepa	ratio	n	
Unique Reflections Multiplicity		26865 (1067) 5.9 (4.0)	60854 (2718) 6.3 (3.9)		

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.

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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





Electrochemistry AFM

The experimental set- up













Electrochemically-assisted protein crystal growth





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?



a) Polypyrrole (Ppy) layer's voltammogram (control) on ITOb) 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 LiClO₄ in the fluid cell of the EC-AFM.



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

AFM of Protein Crystals



Kuznetsov et al.





2359

FIGURE 2 (a) A 200 × 200 nm² scan area image of the (100) face of a hexagonal crystal of fungal lipase, with space group P6₁ (or P6₅) 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 $\sim 12-14$ 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.

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Biosensors' design

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... Carbonates Biosensor...

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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 .

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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.

Protein structures comparison

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Arg 14

b

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	Control	2 μΑ	4 μΑ	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	25.00 - 1.97	25.00 - 2.24	25.00 - 2.07
	(2.31 – 2.27)	(2.00 – 1.97)	(2.28 - 2.24)	(2.11 – 2.07)
Total number of reflections	2804198	1189907	720448	2228266
Number of unique reflections	12400	18819	13091	16282
Average	20.6	11.7	5.0	20.8
redundancy	(21.5)	(12.1)	(5.1)	(20.9)
% Completeness	100.0	99.9	99.3	100.0
	(100.0)	(100.0)	(99.1)	(100.0)
Rmerge	0.204	0.173	0.134	0.235
	25.0	21.2	13.7	16.7
~1/0(1) ~	(2.88)	(2.03)	(2.07)	(1.94)

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.

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.

Dr. Gen Sazaki, Tohoku University. Sendai (JAPAN)

Dr. Bernardo Frontana Uribe, IQ UNAM Mexico.

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

Dr. Margarita Rivera, Institute of PhysicsUNAM (Mexico)

THANK YOU SO MUCH!