Progress in Biophysics and Molecular Biology 101 (2009) 38–44

Contents lists available at ScienceDirect

Progress in Biophysics and Molecular Biology

journal homepage: www.elsevier.com/locate/pbiomolbio

New approaches on crystallization under electric fields

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ARTICLE INFO

Article history:
Available online 16 December 2009

Keywords:
Biocrystallization
Nucleation
Growth from solutions
External field
Electric field

ABSTRACT

This review presents the state of the art in protein crystallization, nucleation and growth under electric fields. Both external and internal applications of Direct Current (DC) and Alternative Current (AC) experiments are discussed. It is shown that competing effects account for the decreased nucleation time and number of crystals observed yielding larger and sometimes better quality crystals.

1. Introduction

The aim of this review of crystallization under electric fields is to provide biocrystallographers who intend to tackle crystallization with a theoretical basis and practical examples of the effect of electric fields on protein nucleation and growth. In crystallization from solution there are at least two different successive problems to solve. First, after crystallization conditions have been determined, the nucleation rate must be controlled in order to obtain only a few high-quality single crystals near the equilibrium state, thus controlling growth. This latter task is extremely challenging because growth of crystals is optimal in the metastable zone, at low supersaturation, where nucleation is kinetically inactive (by definition). Thus, the challenge for biocrystallographs is to separate nucleation and growth phases. In a recent review Chayen (2005) reports methods for improving crystal size and quality by separating nucleation and growth phases, for instance seeding techniques (Boistelle et al., 1992; Stura and Wilson, 1991), temperature monitoring (Astier and Veesler, 2008; Dematteis and Feigelson, 1993; Heinrichs et al., 1992; Jones et al., 2001; Rosenberger et al., 1993), dilution techniques and light-scattering techniques (D'arcy, 1994).

An alternative solution is to induce crystallization from metastable solutions, using external fields: magnetic, electric, ultrasonic or electromagnetic.

In this review we present recent advances in experimental methods for improving protein crystallization by control of nucleation and growth using an electric field. In the literature this topic was firstly reviewed by Al-Haq et al. (2007b) and discussed by Frontana-Uribe and Moreno (2008).

The paper is organized as follows. The second section recalls the historical and theoretical background. In the third section we present experimental examples from the literature. In the fourth section we comment on these examples. Conclusions are drawn in the last section.

2. Historical and theoretical background

Surprisingly, it is only recently that the effects of electric fields on crystallization in solution have been studied other than for electrocrystallization, which does not concern us here. Apart from a theoretical paper by Kashchiev (1972) and an experimental paper by Chin et al. (1976) presenting the coupling of zone electrophoresis and membrane dialysis little was written about this subject until the work of the Aubry group in Nancy (Taleb et al., 1999). From this date, two approaches have been encountered in the literature: application of (1) an external DC electric field: groups of Aubry (Charron et al., 2003; Taleb et al., 2001; Taleb et al., 1999), Nanev (Nanev and Penkova, 2001, 2002; Penkova et al., 2005) and Al-Haq (Al-Haq et al., 2007a) and (2) an internal DC electric field: groups of Moreno (Frontana-Uribe and Moreno, 2008; Mirkin et al., 2003; Moreno and Sazaki, 2004; Nieto-Mendoza et al., 2005; Perez et al., 2008; Sazaki et al., 2004) and Veesler (Hammadi et al., 2007; Hammadi et al., 2009). Recently Chang (Hou and Chang, 2008) and Koizumi (Koizumi et al., 2009) have investigated the effects of an AC electric field. Some of these authors report an improvement in crystal quality due to the use of an internal or external electric field.

The effect of an external electric field on the Gibbs free energy of formation of a cluster of size x ($\Delta G(x)$, x number of atoms in the cluster) was treated theoretically by Kashchiev (1972):

$$\Delta G(x) = \Delta G_0(x) + \Delta W_E(x) \quad (1)$$

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doi:10.1016/j.pbiomolbio.2009.12.005

where $\Delta G_0(x)$ is the usual Gibbs free energy in the absence of an electric field (E) and $\Delta W_E(x)$ is the free energy change due to the electric field. Finally, after further simplification we get:

$$\Delta W_E(x) = -k_B T c E^2 x \quad (2)$$

where k_B is the Boltzmann constant, c a constant in which the term $(1 - \varepsilon_c/\varepsilon_m)$ appears (where ε_c and ε_m are the dielectric constants of the new and the old phase) and E the uniform electric field. Finally $\Delta G(x)$ becomes:

$$\Delta G(x) = x k_B T s_0 - x k_B T c E^2 \quad (3)$$

where $s_0 = \mathrm{Ln}\beta$ with $\beta = C/C_0$ is the supersaturation, C_0 is the solubility and C the solution concentration.

$$\Delta G(x) = x k_B T (s_0 + s_E) \quad (4)$$

where $s_E = -cE^2$, the sum $s_0 + s_E$ may be considered as an effective supersaturation.

According to the sign of $(1 - \varepsilon_c/\varepsilon_m)$ the nucleation work may be either reduced (for $\varepsilon_c < \varepsilon_m$) or enhanced (for $\varepsilon_c > \varepsilon_m$) by the electric field. Note that reduction in nucleation work is associated with increased nucleation frequency. Isard (1977) added to this analysis and obtained $\Delta W_E(x) < 0$ for $\varepsilon_c > \varepsilon_m$, thus nucleation is enhanced by the field in this case, contrary to Kaishchiev.

Recently, Koizumi et al. (2009) has discussed these calculations in the light of introducing AC. According to these authors there is a possibility of a reverse in the dielectric constant between liquid and solid, at frequency < 500 kHz $\varepsilon_c > \varepsilon_m$ and at frequency > 500 kHz $\varepsilon_c < \varepsilon_m$. This means it is possible to enhance or diminish the nucleation frequency according to the external AC electric field frequency applied.

3. Experimental background: DC and AC electric fields

3.1. External DC electric field

As pointed out in the previous section two main approaches are encountered in the literature: application of an external and of an internal DC electric field.

In 1976 Chin et al. (1976) crystallized Estradiol 17β-dehydrogenase from human placenta by a new technique they called electrophoresis diffusion. They also suggested that this technique is applicable to the crystallization of other proteins that have so far resisted crystallization by conventional methods. The principle of the experiment consists in placing the buffered protein solution in an electrophoresis tube closed by dialysis membranes (Molecular Weight Cut Off 18 kDa) at both ends (Fig. 1). The tube was placed in a gel electrophoresis apparatus filled with buffer. Potential of 100–300 kV was applied for 24–48 h causing protein to concentrate at the bottom of the tube, so that opalescence was observed. This fraction of the solution was withdrawn and stocked at 4 °C overnight; subsequent observation revealed the presence of protein crystals. Note that the authors did not specify whether crystals were present in the opalescent phase. The authors explained their results from a semi-quantitative application of the second Fick's law, where a driving force due to a uniform electric field applied and using the Boltzmann law for the protein–concentration profile in the tube C(y) gives:

$$C(y) = bLCe^{-by} \quad (5)$$

where the membrane surface has coordinate $y = 0$, $b = EeZ/k_BT$ with eZ the protein charge at the pH of the experiment, L the tube length and C the initial concentration. They calculated for $E = 1500$ V/m, $e = 1.6 \times 10^{-19}$C, $Z = 10$ and $L = 0.1$ m that all the

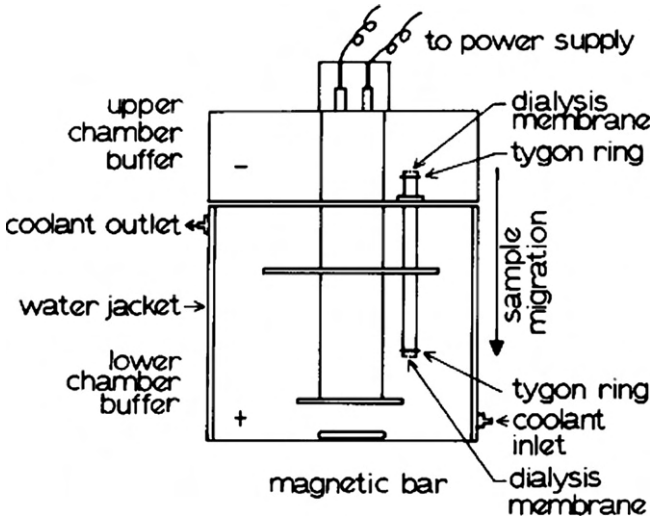

Fig. 1. Electrophoresis diffusion set-up of Chin et al. (1976), with permission of J. Biol. Chem.

protein concentrates on a layer <19 μm at the bottom of the tube on the dialysis membrane, in qualitative good agreement with the opalescence observed in the vicinity of the membrane.

Taleb et al. (1999) designed two simple devices, to study the effect of the DC external electric field on protein crystallization, from the usual hanging and sitting drop set-up used by biocrystallographers (Fig. 2a and b). They studied the nucleation rate of protein (lysozyme) solution drops placed between two flat electrodes outside the crystallizer under an electric field of 2000 V/cm; the experiments were performed in a room 20 ± 0.5 °C. They observed that the drops expanded until they touched the cathode and then continued to expand towards the anode. Crystallization then occurred near the cathode due to the positive charge of lysozyme at the pH of the experiment, with fewer but larger crystals under the electric field (Fig. 2c and d). In a later study, Taleb et al. (2001) measured the lysozyme concentration gradient due to the external electric field at different NaCl concentrations from 0 to 1 M and at pH 4.5 and 7.5 respectively. The highest concentration was observed near the cathode and the effect was less marked at pH 4.5 and at higher NaCl concentration. pH 4.5 and 7.5 correspond to 11 and 3 positive charges respectively (Ries-Kautt and Ducruix, 1999), thus the higher the charge the greater the electric field effect. They also found that at 1.5 M NaCl the external electric field cannot counteract the charge screening effect of the salt. Moreover, they confirmed, not surprisingly, that the external electric field does not modify protein crystal solubility. The effect of the external electric field is only kinetic via the creation of a concentration gradient between the electrodes. Interestingly, they observed an improvement in the quality of the crystals nucleated and grown in an external electric field of 750 V/cm. Lastly, they developed a device derived from the standard Linbro plate in order to use the external electric field as a parameter in the initial screening of crystallization conditions (Charron et al., 2003). With the same aim, a device was developed by Al-Haq et al. (2007a) to perform crystallization under an external electric field with an array of droplets.

The Nanev group (Nanev and Penkova, 2001, 2002), using a DC external electric field (1500 V/cm), studied its impact as a function of temperature on lysozyme crystallization using an all-glass quasi-two-dimensional cell (Fig. 3) with glass-plate distances from 20 to 500 μm. The advantages of this set-up are that it allows easy

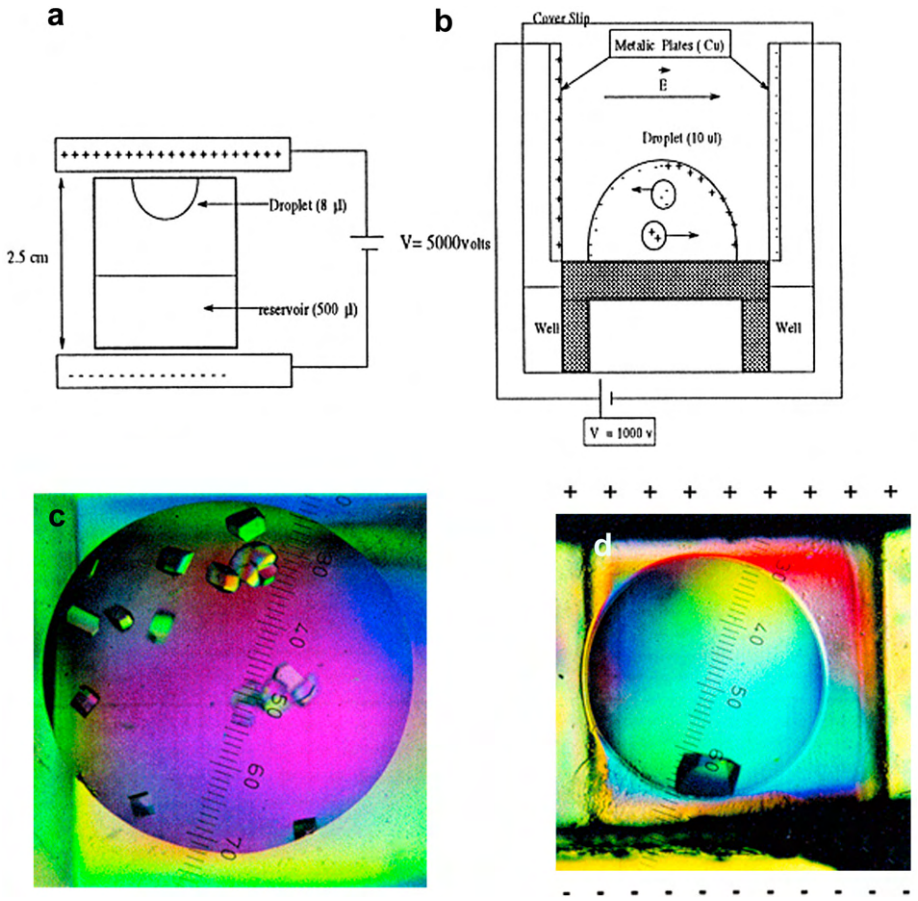

Fig. 2. The two crystallization devices developed by Taleb et al. (1999): (a) the whole Linbro plate is placed between two metal plates connected to a power supply, (b) the droplet is directly placed between two metal plates connected to a power supply; crystals form in the drop: (c) without electric field and (d) under electric field, with permission of J. of Crystal Growth. Elsevier.

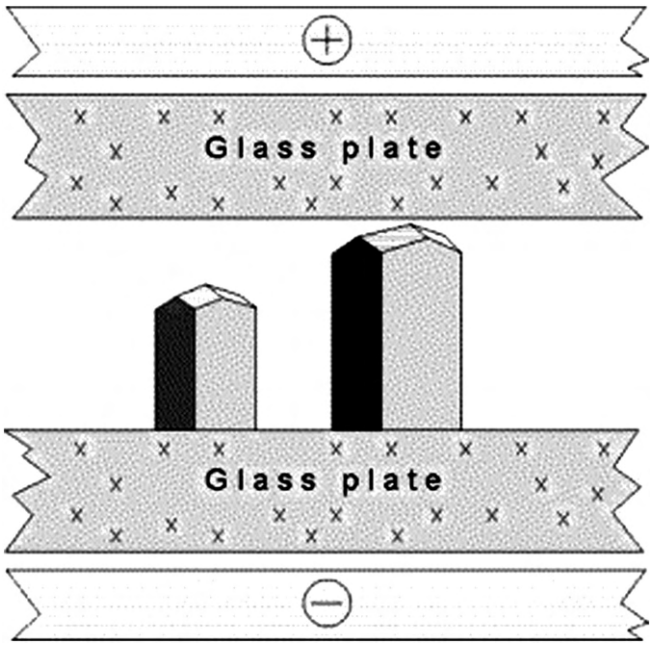

Fig. 3. Schematic representation of the growth cell of Nanev and Penkova (2002), together with the two electrodes and two HEWL crystals oriented along c-axis, with permission of Colloids and Surfaces A: Physicochemical and Engineering Aspects. Elsevier.

temperature control, observation under a microscope and reduced convection.

On the basis of the Taleb results on a concentration gradient in the cell, they confirmed that nucleation occurs predominantly at the cathode and that crystals grow faster at the cathode. They also concluded that the electric field has a greater impact on nucleation rate and induction time when temperature is lower. They tried to calculate the concentration using an equation similar to equation (5), but due to the external application of the electric field, it is difficult to estimate the part of the electric field which actually reaches the thin solution layer at the cathode. However a qualitative estimation showed that a small part of the electric field can create approximately a three-fold increase in local protein concentration. Moreover, they observed that, at low temperatures (below 5–7 °C), an electric field increases the number of crystals nucleated and they observed a preferential orientation along the crystallographic c-axis. The authors interpreted this finding by the anisotropy of charge distribution on the lysozyme molecule, creating a “north pole” and an orientation along the c-axis in the electric field. They concluded that there are three effects to consider with an electric field: electromigration, thermal motion and gravity. In a recent paper Penkova et al. (2005) used a sitting drop set-up equipped with external electrodes to show that an electric field causes solution stirring at rates that increase with field strength: they spoke of an electrically-driven solution flow. Moreover they found a direct correlation between solution flow-rate and nucleation rate.

3.2. Internal DC electric field

In the following section we present experiments conducted at low applied direct current in the μA range in order to avoid water electrolysis (discussed below).

The Moreno group (Mirkin et al., 2003) modified the gel-acupuncture set-up developed in Granada (Garcia-Ruiz and Moreno, 1994) in order to apply the electric field via electrodes (Pt and graphite) inside the medium of crystallization (Fig. 4). Mirkin observed a decrease in induction time and in number of crystals appearing, in agreement with Taleb's observation for both lysozyme and thaumatin. Crystals appeared in both cases at the anode. They also compared thaumatin crystal structures obtained with and without electric fields and found no structural difference.

Moreover, using a new growth cell set-up with parallel electrodes (Fig. 5) Moreno and Sazaki (2004) observed a faster nucleation of lysozyme in supersaturated solutions and in gel, fewer but larger crystals, crystals at the cathode, and an amorphous phase forming around the anode. This amorphous precipitation was also observed by Penkova (Nanev and Penkova, 2001; Penkova et al., 2005) with solutions of ferritin and apoferritin.

It should be noted that nucleation control is better achieved in gel than in solution, as shown in Fig. 6. They discussed three possible explanations: (1) electromigration, causing an increase both in protein concentration at the anode and in chloride concentration at the cathode, which may explain the precipitation; (2) redox reactions on the electrode surface, but this was rejected since direct current applied was low and there was no apparent gas generation and (3) influence of the electric potential generated by the electric field on the crystallization driving force, i.e. the chemical potential. Indeed, they calculated an increase in the chemical potential at the anode and a decrease at the cathode.

A precise investigation was performed by Nieto-Mendoza et al. (2005) using different electrode geometries in the growth cell. They used a pH indicator to show clearly that there is no pH variation during the experiment, as also observed by Hammadi et al. (2007).

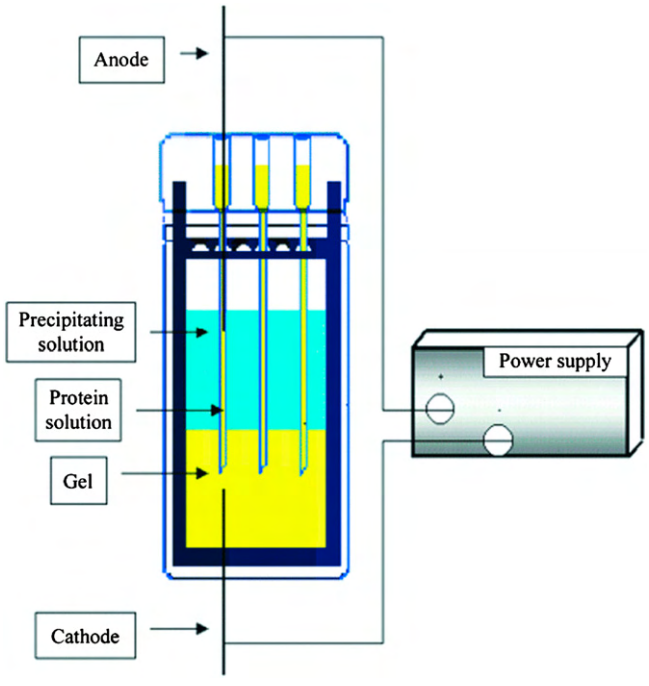

Fig. 4. Modified gel-acupuncture set-up of Mirkin et al. (2003), with permission of Acta Cryst. Wiley.

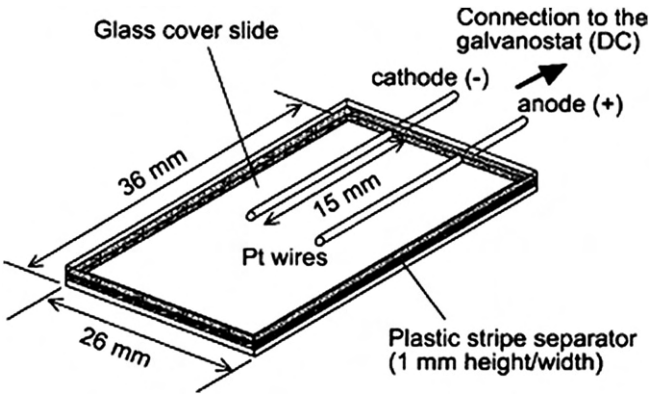

Fig. 5. Experimental set-up of the crystallization cell with parallel electrodes, 0.2 mm diameter (Moreno and Sazaki, 2004), with permission of J. of Crystal Growth. Elsevier.

From cyclic voltammograms (I–V curves) obtained with and without lysozyme in solutions they showed that at low potential $-0.75\ V < V < +0.75\ V$ there is a plateau at 0 μA, meaning there is no electrochemical reaction; these values of potential correspond to the values in their crystallization experiments.

The Veesler group used sharp electrodes to investigate the effects of a DC internal electric field on BPTI and lysozyme nucleation (Hammadi et al., 2007). They developed a temperature-controlled set-up for in-situ investigation of the effects of localized voltage on the nucleation and growth of proteins in the metastable zone (Fig. 7). One of the electrodes of the crystallization cell was sharp; because of the nanometer size of its tip, intense electric fields, a large field gradient and high current density were encountered near the tip and depending on its polarity, nucleation near the tip was observed. For BPTI, crystals and a gel-like phase (dense and rich in protein) due to a liquid–liquid phase separation (LLPS) appeared at the cathode (Fig. 8). Interestingly, when the electrode polarity was reversed the dense phase dissolved and crystals grew confirming the fact that the dense phase is metastable. Moreover there was a crystal-size gradient: crystals were larger in the vicinity of the anode because the growth kinetic is larger in the vicinity of the anode than at mid-distance of anode and cathode (Revalor et al. submitted for publication). When the experiments were conducted with lysozyme, crystals were located near the anode and the dense phase nucleates on the cathode.

Since it was previously noted that at the electrode tip the electrical current effect is so intense that crystallization is not favored (Nieto-Mendoza et al., 2005), Hammadi performed experiments in agarose gel in order to counteract convection and favor nucleation in the vicinity of the tip apex (Fig. 9) (Hammadi et al., 2009). Creating localized fields and fluxes and preventing convection led to a spatial and temporal control of the nucleation event. Moreover, by coating the anode with wax, the authors were able to concentrate the current lines at the tip apex, leading to very high local current density, giving rise to a localized gelation due to LLPS.

3.3. AC electric field

To date there are only two papers dealing with crystallization under AC electric field, probably because it introduces a supplementary parameter, i.e. the current frequency. The first advantage of using an alternative current is that it limits Faradaic reaction at the electrodes and thus makes it possible to work at higher electric field values. In their paper, Hou and Chang (2008) applied an internal AC electric field to lysozyme solutions and observed 3

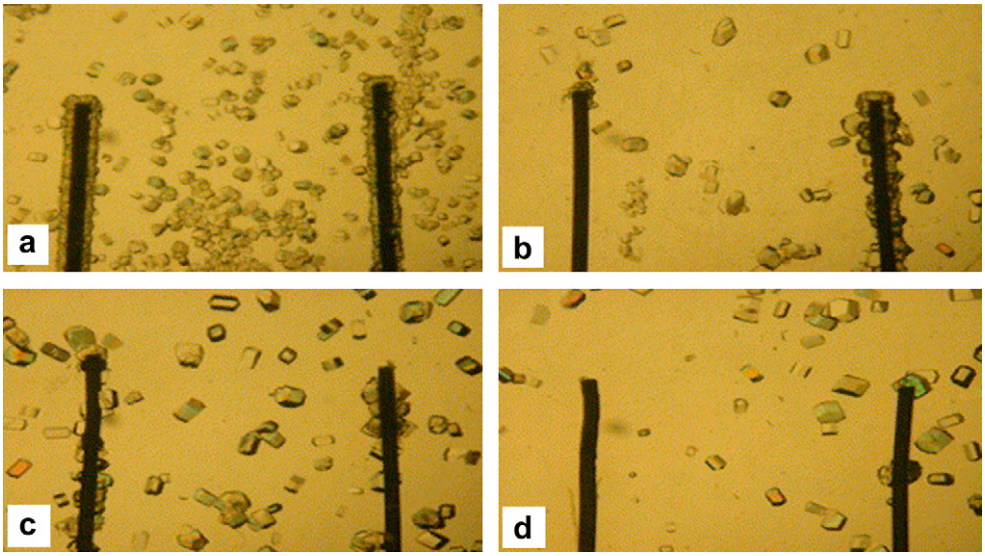

Fig. 6. Images from (Moreno and Sazaki, 2004), (a) Corresponds to the experiment in solution without current, (b) with current, (c) growth in gels without current, and (d) growth in gels in the presence of a constant current, with permission of J. of Crystal Growth. Elsevier.

zones according to the potential and its frequency values: zone 1 no crystals, zone 2 some nice single crystals and zone 3 formation of a gel (probably due to LLPS). They also claimed that the best crystals are obtained when nucleated and grown from the gel.

In another paper, Koizumi et al. (2009) were able to control increase or decrease in nucleation rate by applying an external AC electric field with an appropriate frequency. The authors reconsidered the thermodynamic treatment of the effect of an external electric field on the chemical potential (see Section 2). Therefore, they performed the experiments at different current frequencies and obtain good agreement with the theory.

4. Discussion

In the experimental examples presented in this review the authors were able to decrease the nucleation time and the number of nucleated crystals, thus obtaining larger crystals and sometimes improving crystal quality. In most cases, nucleation occurred in the metastable zone. This is in agreement with the discussions by Voss (1996) and Oxtoby (2002), who highlighted the implications of an external field for crystal growth in solution and postulated two effects on the structure of the supersaturated solutions: molecular orientation and density fluctuation.

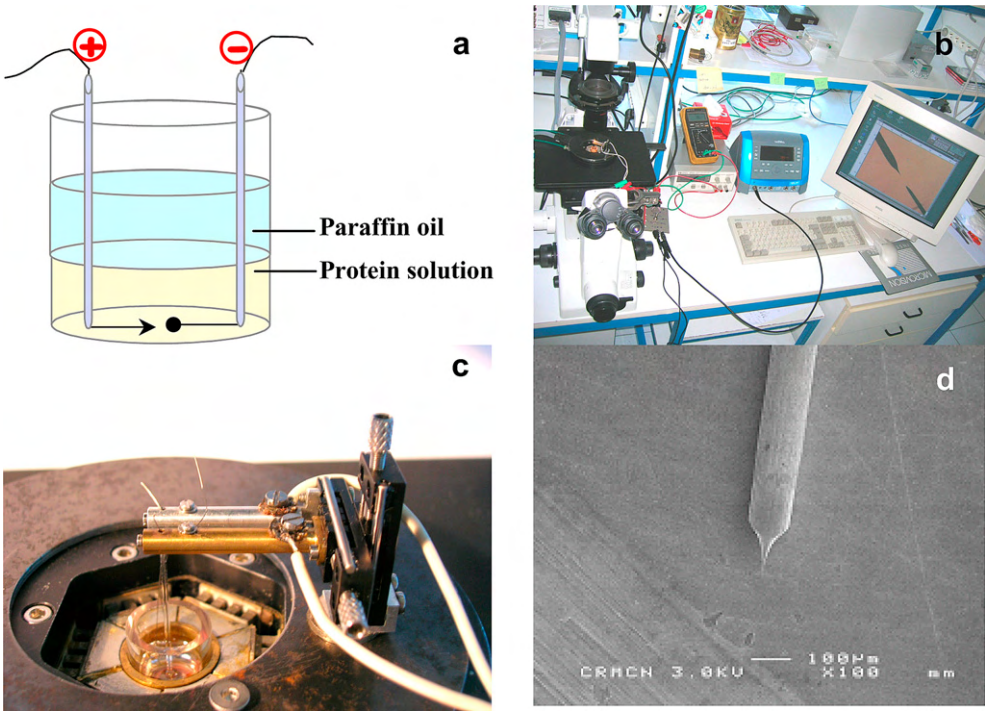

Fig. 7. (a) Crystallization cell with the 2 electrodes, (b) whole experimental set-up, (c) Y, Z positioning stage for the electrodes and (d) sharp tip of the electrode (Hammadi et al., 2007) with permission of Crystal Growth and Design. American Chemical Society.

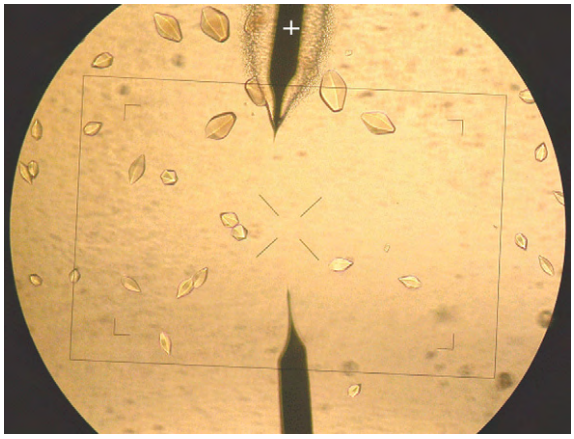

Fig. 8. In-situ observations under optical microscopy of BPTI crystallization at 20 °C with direct voltage of 0.785 V at t = 18 h As reference the W-electrode wire diameter is 125 μm (+indicates the anode).

In the interpretation of experimental results, locally increased concentration of protein and anions or cations was proposed to explain the location of the nucleation at one electrode (density fluctuation). Moreover, the Nanev group suggested that for lysozyme molecules are oriented along the c-axis in the electric field (molecular orientation).

In practice different effects are superimposed: Faradaic reaction, electromigration, influence of E on the chemical potential, thermal motion and gravity. Note that a study by Sazaki et al. (2004) was devoted to the Lorentz force or coupling effect of electric and magnetic fields, which seems to have a positive synergistic effect on crystallization.

Faradaic reaction occurring at the surface of an electrode is undesired because of its negative impact on the solution composition, on pH and on protein stability. Thus it is necessary to control the potential during the experiments at values below the threshold for electrochemical reactions ensuring reproducibility and quality. There are two ways of estimating this threshold: first, observing the formation of gas bubbles when V is increasing or second, measuring I–V curves or voltammograms. In practice, the first is easy to perform; but the second provides more precise electrochemical information on the protein in solution. In Fig. 10, we present such curves for three solutions: without protein, with protein, here BPTI,

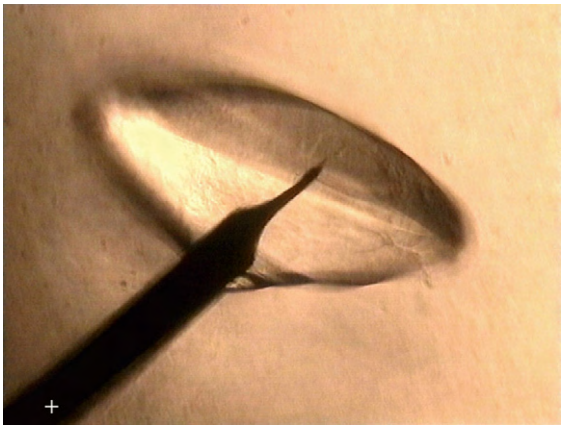

Fig. 9. BPTI (20 mg/mL – NaCl 1.6 M) nucleation in the vicinity of the tip in 0.5% agarose gel (0.8 V – 0.74 μA). As reference the W-electrode wire diameter is 125 μm (+indicates the anode), (Hammadi et al., 2009) with permission of Crystal Growth and Design. American Chemical Society.

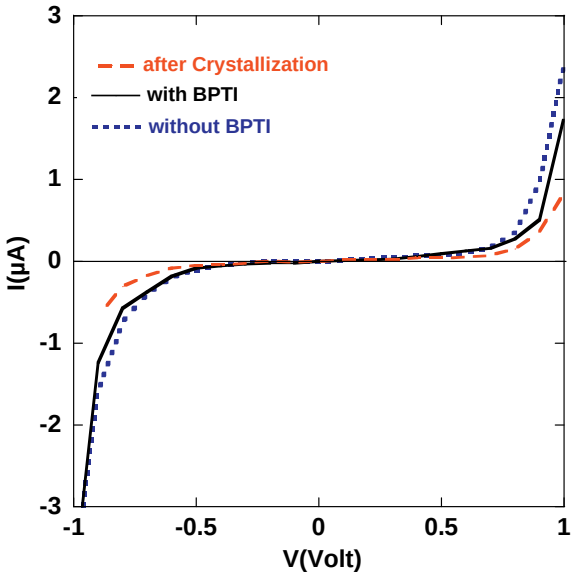

Fig. 10. Different I–V curves for NaCl 1.6 M solutions at pH 4.5, without BPTI, 20 mg/mL and after crystallization.

prior to the experiment and with protein after crystallization. These kinds of curves were previously discussed in Section 3.2 and they clearly provide the experimentalist with data from which to design an experiment. In the case of Fig. 10, in the range $-0.9\ V < V < +0.9\ V$ there is no electrochemical reaction and therefore these potentials define a good experimental range.

In practice all the possible effects are in competition. A positively charged protein in solution presents at the cathode: increasing protein concentration and decreasing anion concentration, for instance Cl^-. Thus, for most proteins, solubility increases. The competition between these two opposing effects leads to a variation in the local chemical potential which is not easy to predict. In addition we need to consider the effect of electric fields on the chemical potential.

At the anode, protein concentration decreases, anion concentration increases and thus, the solubility decreases. Here again we need to consider the effect of electric fields on the chemical potential.

Finally, these effects are difficult to predict “ab-initio”. Experimentally gelation or LLPS is always observed at the cathode both for BPTI and lysozyme, where chloride ions concentrate. Lysozyme crystals appear at the anode, (except in one experiment in gel where lysozyme and thaumatin crystals appear at the cathode, Mirkin et al., 2003), and BPTI crystals appear at the cathode. All these proteins are positively charged at the experimental pH. These results are in agreement with the two-step nucleation mechanism (Kashchiev et al., 2005; Ten Wolde and Frenkel, 1997; Vekilov, 2004), where a density fluctuation is followed by a structural fluctuation. In some cases crystals are nucleating and growing from the dense phase (Hou and Chang, 2008; Vivares et al., 2005) but not always (Grouazel et al., 2006). Dixit and Zukoski (2003) discuss this competition between gelation and crystallization in a theoretical paper. Moreover, an electric field was used recently to generate droplets of high-concentration protein of attoliter volume by LLPS (Shah et al., 2009).

5. Conclusions and perspectives

In the experimental examples presented in this review the authors were able to decrease the nucleation time and the number

of nucleated crystals, thus obtaining larger crystals and sometimes improving the crystal quality. In most cases, nucleation occurred in the metastable zone. In practice, the effects are increased when the ionic strength is reduced and/or pH is removed from the pI of the protein. It is also important to avoid electrolysis; this can easily be checked by observing the formation of gas bubbles due to electrolysis when V is increasing.

For the moment there are many fundamental studies with applications to protein crystallization which clearly shown the advantages for inducing crystallization of an electric field applied to a crystallizing solution. To date, there have been few attempts to propose an easy to use set-up for biocrystallographs, and this would be a valuable contribution to future study.

Acknowledgements

The authors thank M. Sweetko for English revision.

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