An electrically assisted device for protein crystallization in a vapor-diffusion setup

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A new easy-to-use device has been designed and implemented for electric field-induced protein crystallization in a vapor-diffusion configuration. The device not only controls crystal nucleation by means of the electrical current, but also favors crystal growth owing to its vapor-diffusion setup. Crystallization was conducted in the presence of an internal electric field and direct current. The proteins investigated were lysozyme, as model protein, and 2TEL–lysozyme (a synthetic protein consisting of two tandem alpha helix motifs connected to a lysozyme moiety). Lysozyme crystals that grew attached to the cathode were larger than those grown attached to the anode or in the absence of an electric current. On the other hand, crystals of 2TEL–lysozyme qualitatively showed a better X-ray diffraction pattern when grown in the presence of an electric current.

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1. Introduction

X-ray crystallography is the most used technique for obtaining the three-dimensional structure of proteins. In addition, neutron crystallography is a powerful complementary technique used to establish the location and orientation of deuterium atoms in proteins and gain mechanistic insights (Munshi *et al.*, 2012; Myles *et al.*, 2012). However, the success rate of newly solved structures represents only 15–20% of the purified protein targets (<http://targetdb-dev.rutgers.edu/TargetDB-dev/index.html>), and there are only 38 structures deposited in the PDB obtained by neutron crystallography. Large high-quality protein crystals are necessary for neutron crystallography and recommended for ordinary X-ray crystallography. In order to produce such crystals, several strategies have been developed that focus on engineering the target protein or modifying the crystallization method. Regarding the crystallization method, novel strategies such as the application of magnetic fields (Sazaki, 2009; Surade *et al.*, 2009), internal or external electric fields (Espinoza-Montero *et al.*, 2013; Hammadi & Veesler, 2009; Mirkin *et al.*, 2003), and a combination of electric and magnetic fields (Sazaki *et al.*, 2004) have been successfully used to enhance the quality of protein crystals (Candoni *et al.*, 2012). Previous reports using crystallization of proteins under the influence of electric fields all involve crystallization in batch configuration. Additionally, advances in recombinant DNA technology combined with bioinformatic analysis of the primary sequence of the target protein allow the investigator to rationally design protein constructs with better chances of crystallizing (Dale *et al.*, 2003; Derewenda, 2010).

In this report we present the design and implementation of a sitting-drop vapor-diffusion crystallization device in the presence of a direct electrical current. The device consists of an easy-to-use setup for biocrystallographers that does not require sophisticated materials for its construction. The device is made of two communicated rubber chambers sealed by two indium tin oxide glasses used as electrodes. Two protein crystals were grown in this device: the classical control protein lysozyme, and the more challenging protein 2TEL–lysozyme (2TEL–Lys). 2TEL–Lys is a synthetic protein formed by a 2TEL module fused to the T4 bacteriophage lysozyme (Nauli *et al.*, 2007).

Qualitatively, crystals of the 2TEL–Lys protein grown by vapor diffusion and in the presence of an electrical current were of higher quality than those obtained by the classical vapor-diffusion method, as judged by their diffraction patterns whose spots were less elongated. On the other hand, lysozyme crystals grown attached to the cathode in the presence of an electric current were significantly larger in size than the control ones, while those grown on the anode showed no difference.

2. Materials and methods

The crystal growth device consisted of two polished conductive ITO (indium tin oxide) electrode glasses whose conductive coated surfaces were placed inward and facing parallel to each other. The dimensions of the glass pieces were 3.0×2.2 cm with a resistance ranging from 4 to 8 Ω (Delta Technologies, USA). A rubber material shaped such that it contained two intercommunicated reservoirs held the ITO glasses together. The ITO electrodes were displaced by 0.5 cm relative to each other, to provide the appropriate connection area with the electric alligator clips between the galvanostat (VIMAR FCC-17) and the electrodes. The device was sealed with nail polish and silicon glue. One of the reservoirs had a volume capacity of approximately 50 μ l, while the other reservoir could hold up to 100 μ l (Fig. 1). The smaller reservoir was filled with the crystallization mixture (equal volumes of protein plus precipitating agent) and the larger one with precipitating agent only. One of the faces of the ITO glass constituting the large reservoir was covered with nail polish to prevent the flow of electric current and thus avoid attachment of the precipitant agent molecules. The proteins used for the experiments were hen egg white lysozyme (Seikagaku Corporation, code 100940) and 2TEL–Lys. The protein 2TEL–Lys was expressed and purified as previously described by Nauli *et al.* (2007). During nucleation, the device was connected to a galvanostat programmed to apply a direct current of 2 μ A at a constant temperature of 291 K (T-incubator GE model Profile). The current was switched off after 48 h for the lysozyme experiments and after

Figure 1

Experimental design of the electrical device used for protein crystallization using a vapor-diffusion setup. (a) Frontal view. (b) Lateral view. Dark grey (dark blue in the electronic version of the journal): rear ITO glass; light grey (light blue): front ITO glass; black: rubber compartments.

12 h for the 2TEL-Lys protein experiments. Crystal growth was monitored for a further one–two weeks. The crystallization conditions for lysozyme consisted of a 60 mg ml^{-1} protein concentration with 200 mM Na acetate buffer pH 4.4, 20% NaCl as precipitating agent. The concentration of 2TEL-Lys protein used for crystallization was 4.5 mg ml^{-1} and the corresponding precipitating agent was 100 mM sodium citrate pH 6.2, 4.8 M NH_4NO_3 . Control crystals for the 2TEL-Lys protein were obtained using the same precipitating agent but in a hanging-drop vapor-diffusion setup. Lysozyme control crystals were grown in a sitting-drop vapor-diffusion setup inside the crystal growth device but in the absence of electric current. The lysozyme crystal size was measured along the longest axis and the data from three independent experiments were analyzed using a Student's *t* test for unpaired data with unequal variances. 2TEL-Lys crystals obtained from the same drop as the control and electric field conditions were analyzed using a Rigaku/MSD Micromax-007 X-ray generator (copper anode) with an R-AXIS-IV⁺⁺ detector at a wavelength of $1.54 \text{ \AA}$. The crystals were cryo-protected by adding equal volumes of 50% glycerol to the reservoir solution and plunged into liquid nitrogen prior to irradiation.

3. Results and discussion

In a previous publication we reported the design of a crystallization cell suitable for growing protein crystals under the influence of a direct electrical current in a batch method (Gil-Alvarado *et al.*, 2011). However, batch crystallization conditions are not available for all proteins, nor are they easy to interconvert between different crystallization methods. We reengineered the electrically assisted crystal growth device so that it could be used in a sitting-drop and vapor-diffusion configuration, and thus impact a wider range of protein crystal-

lization experiments. The device can be easily scaled up to grow larger crystals. Besides controlling crystal nucleation, this cell has the additional advantage that once the electric current is switched off it can be kept for several days, allowing the protein crystals to grow by vapor diffusion until they reach their maximum size. Two proteins, lysozyme and 2TEL-Lys, were used as model proteins to test the aforementioned device.

Characteristic lysozyme crystals obtained in the presence and absence of electric current are shown in Fig. 2. The presence of electric current influenced the resulting number of crystals. While a large number of crystals (430) were observed in the absence of an electric field, there were at least fifty times fewer crystals when grown in the presence of an electric current (Fig. 2). Though fewer in number, the crystals grown attached to the cathode in the presence of an electric current were significantly larger [$t(24) = -4.21$; $p = 0.0003$] than those grown in its absence. On the other hand, there was not a significant difference in the size of the crystals grown attached to the anode [$t(7) = 1.67$; $p = 0.13$] compared to the control ones (Fig. 2). The lysozyme molecules were positively charged because the pH of the precipitating agent was lower than the pI of the protein. Because of charge repulsion, it is no surprise that the electric current had a profound impact on lysozyme crystals growing on the cathode but not on those growing on the anode. No new crystals appeared once the electrical current was turned off, suggesting that the nucleation process had ceased by that time. Once the electric current was turned off, the growth process of lysozyme crystals comprised three distinctive phases: an initial equilibration period ranging from zero to two days (1), a crystal growth period lasting six days (2) and a final depletion stage (3) corresponding to the last eight to 14 days (Fig. 3b). During the equilibration phase, the concentration of the precipitating agent equilibrated against that of the reservoir solution until it reached supersaturation. The already formed crystals remained stable in the solution and grew further until the experiment reached the end of the process. Lysozyme crystals grew $3.5 \mu\text{m}$ in six days along the small axis of face (100), suggesting that on average 12 molecules of lysozyme were incorporated per hour per face onto the protein crystal after the onset of the equilibration stage (Fig. 3).

Additionally, we tested the virtues of the crystallization device described in this contribution with the test case protein 2TEL-Lys. The 2TEL module consists of two tandem copies of the sterile alpha motif domain from the protein translocation ETS leukemia (TEL)

Figure 2

Comparison of the lysozyme crystals obtained in the absence (control) and in the presence of an electric current. Scale bar = $300 \mu\text{m}$.

Figure 3

Lysozyme crystal growth behavior. (a) Crystals obtained after applying a 2 μ A direct current. (b) Lysozyme crystal growth curve of face (100). Growth stages correspond to (1) equilibration, (2) crystal growth and (3) depletion. Scale bar = 300 μ m.

fused to the target protein. The 2TEL module has been used as carrier protein to encourage crystal lattice formation when fused to proteins that will not otherwise crystallize on their own: with the disadvantage that evidence suggests its crystals are highly mosaic (Nauli *et al.*, 2007). 2TEL–Lys crystals grown in the presence of an electric current were qualitatively better than those obtained by the conventional vapor-diffusion method (control crystals). Crystals obtained in the presence of an electrical current had regular borders (data not shown) and their diffraction patterns consisted of a larger number of well defined signals (Fig. 4). These results suggested that the control crystals consisted of more than one crystalline network and that the use of an electric field improved the crystal packing. Future experiments will follow to evaluate the enhancement of the crystal quality in detail. Furthermore, 2TEL–Lys crystals grown in the presence of a direct electrical current of 2 μ A grew four times quicker than the control ones (one week compared to a month, respectively). Crystals grew attached to the cathode (negatively charged electrode) because at pH 6.2 (pH of the precipitating agent) the 2TEL–Lys molecules are positively charged.

4. Conclusions

A novel protein crystallization device coupling the use of an electrical current with a vapor-diffusion setup was implemented. This device allowed us to separate two difficult issues in protein crystallization: nucleation and crystal growth. At the beginning of the crystallization

experiment, the influence of the electric field favored the formation of a small number of nuclei, and once the current was switched off the crystals grew through the vapor-diffusion phenomenon. Protein crystals were obtained in shorter times and were larger in size when grown in the presence of an electric current compared to those obtained in its absence. We hope that in the near future the crystallization growth cell presented in this manuscript can be routinely used in the laboratory for growing crystals.

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Figure 4

Comparison of the X-ray diffraction patterns of the 2TEL–Lys protein crystals. (a) Control without electric field and (b) 2 μ A electric field.