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A supersaturation wave of protein crystallization

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Abstract

A microgravity protein crystallization experiment is described in which the existence of a supersaturation wave traveling across a diffusion-reaction system is experimentally demonstrated for the first time. The self-organized dynamics of the experimental setup were used to implement a crystallization technique able to search automatically through the crystallization parameter space for optimum nucleation and growth conditions. The crystals obtained by this automatic optimization produced the highest quality X-ray diffraction data ever collected from the model protein used in the experiment.

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1. Fundaments and motivation of the work

Crystallization in diffusion-reaction media is a highly non-linear process in which the local concentration of the chemical species controls both the mass transport (through the concentration gradient) and the precipitation (through the local supersaturation). In these systems, the relaxation of concentration gradients produces local increases in supersaturation. If the critical supersaturation is reached at one such point, nucleation occurs, which depletes one or more of the reactants from solution thus increasing the concentration gradient. In this way, a self-organized system is established in which the kinetics of mass transport and precipitation interplay to produce a rich landscape of spatio-temporal patterns. The best known of such systems is the one producing rhythmically distributed crystals in the form of discrete bands, that is, Liesegang rings [1,2]. In the patterns, the initial conditions (and the geometry of the system) select the kind of crystal distribution obtained: from no crystals at all to a single jumble of microcrystals or amorphous precipitate as well as other more interesting patterns like rhythmic bands or single crystals more or less evenly distributed. In the latter two cases, as a result of the dynamics of the pattern formation, the crystals forming the newer bands are of larger size and better crystallinity. As suggested by Ostwald [3,4], and later elaborated through numerical simulation [5] and experimental

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inference [6], these crystal distributions (in position, size and crystallinity) can be explained as due to a supersaturation wave traveling across the system from the collective effect of different local precipitation events at different values of supersaturation, although alternative explanations have been also offered [7–9].

The properties of these systems are of great interest to pattern formation and crystal growth scientists. Two kinds of difficulties have been found in working with practical implementations of counter-diffusion crystal growth techniques able to produce, in a single experiment, crystals nucleated and grown at different supersaturation and supersaturation rate values, automatically screening in doing so for the best crystallization conditions. First, theoretical knowledge of the system dynamics is not complete and has not been experimentally validated. The experimental details not included in the theoretical model may limit their suitability and predictability for a given chemical system, making difficult the preparation of experiments. Second, crystals of most interesting compounds can be obtained using simpler steady state techniques. Counter-diffusion growth techniques would be applicable only to substances reluctant to crystallize for which the size and/or quality of crystals must be optimized for some reason. In this paper we report the results from a microgravity crystal growth experiment showing both, an experimental validation of the traveling wave genetic hypothesis and the application of non-equilibrium counter-diffusion techniques to one of the most challenging problems in crystal growth: the production of crystals of biological macromolecules having a size and quality optimized for X-ray structural studies.

Biological macromolecules are currently crystallized by evaporation or batch methods due to their solubility dependence on the ionic strength of the solution (tuned by the addition of a precipitating agent). In practice, crystallization conditions for proteins, viruses and nucleic acids are sought nowadays mainly by a trial and error approach involving thousands of experiments [10–13]. By applying the fundamentals of non-equilibrium crystallization to large macromolecules this time consuming search can be minimized. We implemented that non-equilibrium crystallization technique by allowing the precipitating agent and the protein solutions to counterdiffuse one against the other (see Fig. 1). Four requirements need to be fulfilled to exploit this technique for crystallization purposes:

(a) The critical supersaturation for precipitation must be reached. A phase diagram for the protein used (lysozyme) has been obtained by gathering literature data and measuring solubility in different conditions [14]
(b) One of the reactants must invade (i.e. diffuse much faster than) the other one. This is required to produce nucleation events at different parts of the system and, therefore, to produce an spatial pattern. Because of the difference between the diffusion constants of the precipitating agent (usually a small molecule such as a salt with $D \approx 10^{-5}\,\mathrm{cm}^2/\mathrm{s}$ and of the large protein molecules (typically $D \approx 10^{-6}\,\mathrm{cm}^2/\mathrm{s}$), this simple arrangement immediately behaves in this way.
(c) Convective fluid motion and particle sedimentation must be avoided by ensuring a diffusive environment. This can be achieved using microgravity conditions or by gels (or viscous materials). In order to use a chemically clean diffusive mass transport scenario (without interaction with foreign materials such as gels), we proposed to perform the experiment under microgravity conditions, which was conducted during the STS-95 mission of the NASA Space Shuttle program.
(d) There must be room for the precipitation pattern to develop in space and time. As all microgravity protein crystal growth facilities use short growth reactors (typically 5 mm long) we designed a new reactor with a long crystallization chamber (70 mm long) built ad-hoc to fit into the Advanced Protein Crystallisation Facility (APCF) [15].

2. Experimental procedure

The free interface diffusion (FID-XL) reactor designed for this experiment consists of two

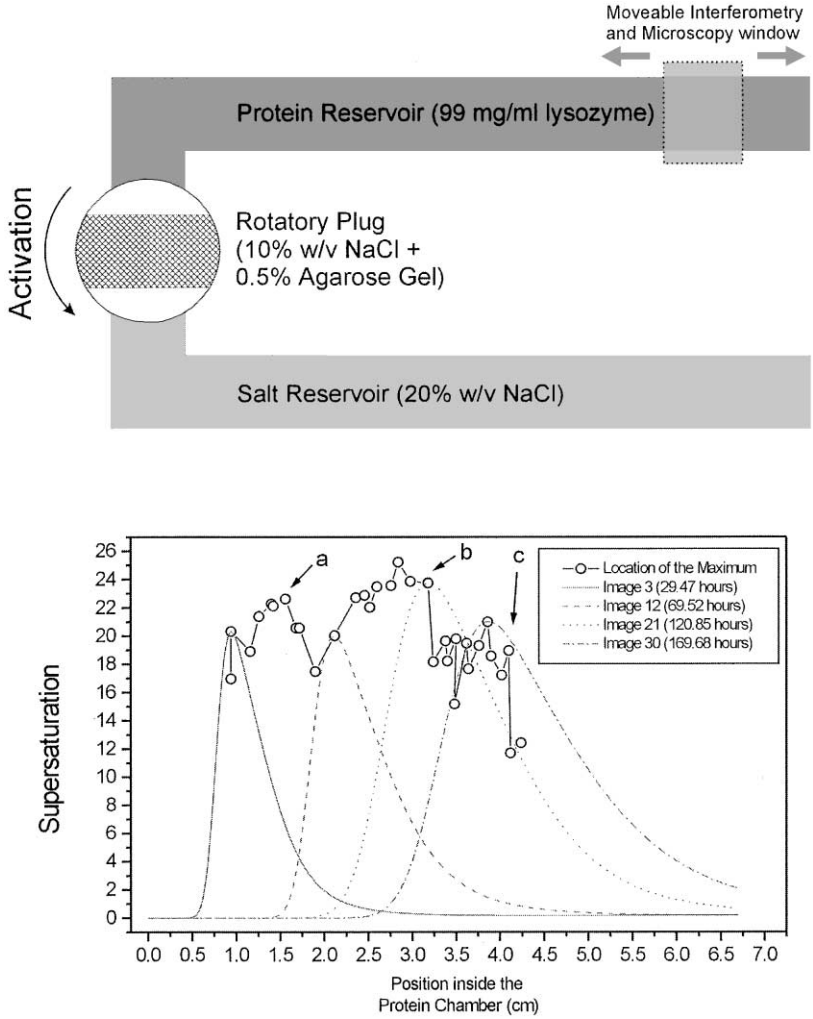

Fig. 1. Experimental setup and time evolution of supersaturation. Top: The FID-XL reactor designed for this experiment consists of two chambers containing the protein solution and the precipitating agent solution connected by a plug filled with agarose gel. Once under microgravity conditions, the plug is rotated to connect the protein with the precipitating agent solutions. Then, they start to diffuse one against the other. Because of the difference between their diffusivity values, the salt invades the protein chamber and a wave of supersaturation moves across the protein chamber. Bottom: Actual development in time and space of the supersaturation values obtained by interferometric analysis. Open circles indicate the position of the maximum supersaturation at different times. The wave-like supersaturation distribution along the protein chamber is shown as curves for four different times.

chambers containing the protein solution and the precipitating agent solution connected by a plug filled with agarose gel. The starting chemical conditions are shown in Fig. 1. A Mach–Zehnder interferometer monitored the development in time of the supersaturation and a time elapsed video microscope recorded the crystal nucleation and growth. The experiment was performed at $20 \pm 0.1^{\circ}C$ inside the thermostated APCF box. Hen egg white lysozyme from Seikagaku (ref: 100940; lot no. E96302) was used as purchased without further purification. The crystallographic analysis was performed at the beamline BW7B of the EMBL-DESY facility (Hamburg).

3. Results and discussion

Unlike classical crystallization methods, in counter-diffusion experiments it is important to set the initial conditions to force the first precipitation event to occur far from equilibrium. High supersaturation is created immediately after the opening of the plug separating the reactor chambers. The first precipitate is, therefore, a jumble of small and low quality crystals (even an amorphous phase may form, depending on the initial conditions) at the interface between the rotatory plug and the protein solution (Fig. 2). Its formation depletes the concentration of protein in the neighboring zones. As the salt diffuses ahead through the long protein chamber, a new precipitation event takes place, this time at lower supersaturation and at slower rate of development of supersaturation. Iteration of this process provokes subsequent nucleation events along the protein chamber at progressively lower supersaturation, producing less crystals of larger size and higher quality. As shown in Fig. 1, the maximum of supersaturation advances as a wave across the protein chamber at decreasing velocity. Its amplitude first increases and later decreases with time as it moves ahead, a pattern characteristic of the coupling between counter-diffusion and precipitation [16]. The width of the wave increases with time while its velocity decreases, which explains the larger size of the crystals formed at later precipitation events: after a single crystal nucleates in a given location, its final size is a function of the residence time inside the moving wave, i.e., it depends on the width of the wave and how fast it moves across the protein chamber. Therefore, unlike classical protein crystallization methods, this non-equilibrium technique explores in one single experiment a large number of crystallization conditions. And what is more important, such exploration is carried out most of the experimental time from high to low supersaturation and from fast to slow rate of development of supersaturation (Fig. 1). Considering that there is a direct relationship between crystal growth rate and crystal quality [17,18] this automatic search should yield better crystals as the experiment goes on.

Two different kinds of gravity fluctuations are observed in every microgravity experiment in a shuttle: a low amplitude and quasi-permanent residual acceleration due to the relative position of the experimental box and the center of gravity of the spaceship and other high amplitude and sometimes large fluctuations (g-jitters) due to sudden maneuvers. The STS-95 mission of the Space Shuttle, where the experiment was carried out, was very jarring because of satellite release and recovery maneuvers. These g-jitters had a clear effect on the location of the growing crystals

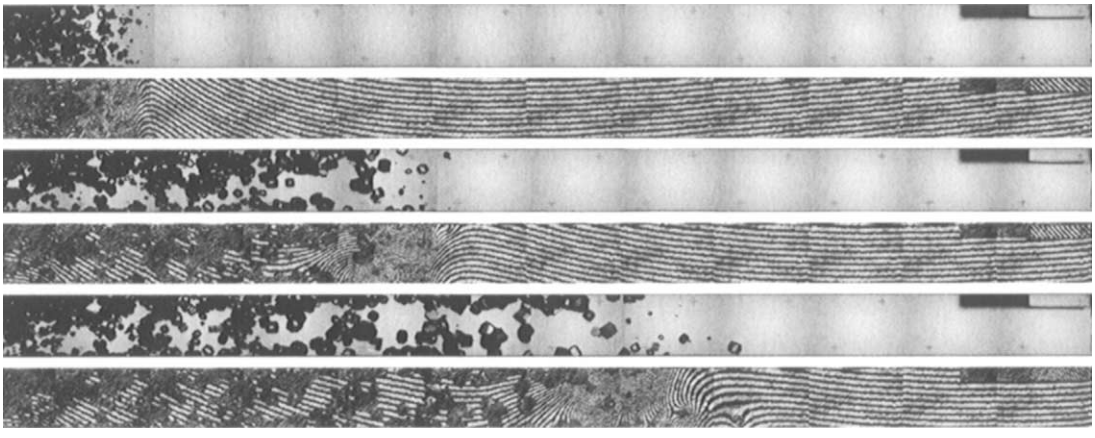

Fig. 2. Video and interferometric images composed from the pictures collected during the microgravity experiment. From top to bottom, video image after 27 h, interferogram after 26 h, video image after 106 h, interferogram after 104 h, video image after 199 h, interferogram after 202 h.

but fortunately, the component of the motion in the direction of the precipitation front was slow compared to the velocity of the precipitation front. However, three perturbations of the trend, labeled in Fig. 1, correspond to fast crystal motion correlated to the release (a) and rendezvous (b) of the Spartan satellite and to the reaction control system hot fire (c). Although the crystallization system was able to absorb these three intense perturbations, they provoked an overall acceleration of the traveling wave. Slow movement of crystals due to residual acceleration was also observed but also with a small component (about 25 μm/h) in the direction of the precipitation front and, hence, did not affect the overall trend of the chemical pattern, which moves at a faster velocity (about 225 μm/h).

As expected from previous computer simulations [14], banded structures were not observed to form. As in the case of Liesegang's patterns, discrete precipitation bands are only obtained from a window of initial conditions producing small supersaturation gradients. In chemical reactions forming Liesegang's bands the two counter-diffusing reactants are consumed, and the byproducts of the reaction affect the kinetics of the main precipitation reaction [19]. However, protein molecules crystallize due to a change in solubility provoked by the counter-diffusing precipitating agent. The concentration of precipitating agent is not depleted because its molecules are not included into the growing crystal (or more exactly they are at a negligible concentration) so the same reactant gradients produce larger supersaturation gradients than in usual diffusion-reaction systems, which in practice narrows the window of initial conditions able to produce discrete bands. As the main difference between our experimental setup with proteins and that of classical Liesegang's patterns is of a chemical nature, the existence of a supersaturation wave, for the first time observed in this work, supports Ostwald's approach to this type of disequilibrium patterns.

As shown in Figs. 1 and 2, the supersaturation wave only explored 42 mm out of the 71 mm long protein chamber, due to the short duration time of the STS-95 mission (8 days). It prevented the full exploitation of our crystallization technique in terms of obtaining crystals of the highest quality. Although this fact was expected from computer simulations, the cost of the reactor construction and the intended use in longer future missions, made advisable to design a reactor longer than the one needed for this particular mission.

The goal of this experiment being the understanding of the physics of the supersaturation wave, we selected values of the initial concentrations to provoke a fast moving precipitation front. These concentrations are higher than those suggested by our previous computer simulations to fully explore the possibilities of the technique. Nevertheless, we analyzed the quality of some of the obtained crystals by X-ray diffraction.

As shown in Fig. 3, our tetragonal lysozyme crystals diffract up to 0.94 Å, a value much better than the one (1.33 Å) corresponding to the best X-ray diffraction data set from tetragonal lysozyme crystals reported in literature [20]. This result could be optimized with longer experimental time and with a less bumpy flight history. In addition, the model protein used for this test is thought to grow under a surface kinetic regime [21], while the technique is expected to work better when the overall crystal growth process is governed by transport kinetics. Even for these less favorable cases, growing large crystals by this technique under microgravity will permit them to reach a

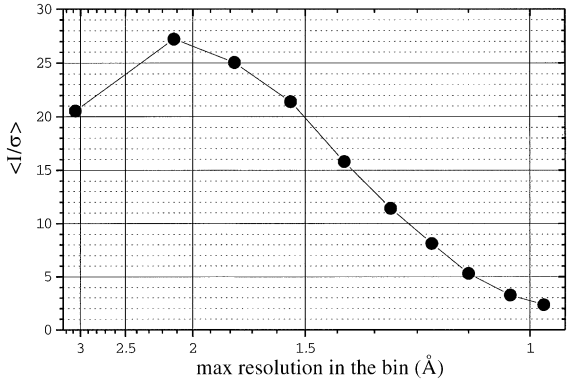

Fig. 3. Crystallographic evaluation of the grown crystals showing signal to noise ratio as a function of the resolution. Crystals diffract up to 0.94 Å (completeness of 98.4% in the 0.94–25 Å resolution shell).

size-dependent transition to mass transport kinetics. It is expected that the outer part of these large crystals in combination with X-ray microbeam techniques will yield diffraction data of the highest quality, due to the demonstrated relationship between growth rate and crystal quality [17,18]

From an applied point of view, our results show that non-equilibrium crystallization techniques are the simplest way to search automatically for the set of crystallization conditions assuring the highest crystal quality. It should be noted that this counter-diffusion crystallization experiment has no exact on-ground equivalent. Certainly, under terrestrial conditions we can reduce convective transport by lowering the ratio between buoyancy-driven convection and diffusion, for instance by decreasing the characteristic length of the system [22], i.e., using capillaries [23,24] or gels [25–27]. However, convective flow cannot be completely avoided inside X-ray capillaries of 0.2 mm inner diameter (the minimum crystal size for significant X-ray data collection of macromolecular crystals at conventional sources) and, with gels, the gel polymeric network may in some cases interact chemically with the protein molecules [28,29]. Nevertheless, the nowadays scarce flight opportunities, the existence of g-jitters and relevant values of residual accelerations on board the Shuttle [30] and the complex logistics associated with space experiments, makes it worth to develop research studies devoted to investigate in these on-ground analogs.

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