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The prospects of protein nanocrystallography

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The miniaturization of protein crystallography's experimental method has several advantages. Firstly, it reduces the amount of protein required for identifying crystallization conditions, allowing crystallographic studies of rare natural proteins and complexes. Secondly, higher levels of supersaturation can be obtained in very small volumes, allowing the exploration of additional crystallization conditions. Thirdly, there are indications that protein crystals grown in very small volumes may be better ordered. Fourthly, miniaturization and automation go hand in hand, opening the prospects of easier and more reproducible experimentation. Progress in the development of nanocrystallography is discussed and the remaining bottlenecks are highlighted.

1. Introduction

The rate-limiting step in structure determination by protein crystallography is the identification of crystallization conditions. Important parameters include protein concentration, pH and type of buffer, ionic strength and species, type and concentration of precipitant, temperature, presence and concentration of surfactant molecules and other additives (*e.g.* cofactors, inhibitors).

The crystallization conditions of proteins cannot be predicted and although general strategies are available, these by no means guarantee success. The general method for screening of crystallization conditions is fairly simple: create a supersaturated solution and if the conditions are right, crystallites will eventually appear. There are several approaches to creating a supersaturated solution. The most common are vapour diffusion and dialysis methods, in which protein solutions are slowly brought to equilibrium with a solution that has a higher concentration of precipitant. Batch crystallization has also achieved popularity. There are many excellent reviews of the most important current crystallization strategies (*e.g.* Ducruix & Giegé, 1999).

Often, most of the sample is used for determining crystallization conditions and *not* for the actual diffraction experiment. It is obvious that testing more conditions with a given amount of protein increases the chances of identifying crystallization conditions. Indeed, the accuracy, speed and ease of available microdispensing methods often determine the volume of protein crystallization experiments. With the availability of systems that can dispense picolitre volumes, like piezo- or bubble-technology inkjet printers, screening crystallization conditions in nanolitre to picolitre volumes is coming within view (Stevens, 2000; Kuil *et al.*, 2002). Special attention needs to be given to dispensing highly viscous fluids

such as concentrated PEG solutions, or fluids with a low surface tension. Furthermore, evaporation has to be controlled and finally the relationship between volume and the number of crystal nucleation sites forming in a droplet has to be taken into account.

The volume of a crystallization experiment has not generally been appreciated as an important parameter in crystal growth. However, it does have an effect on nucleation. The nucleation rate J of a given mother liquor can be expressed as (Veesler & Boistelle, 1999; Auer & Frenkel, 2001)

$$J = (\kappa/q^{1/2}) \exp(-q\Delta G/k_{\rm B}T),$$

where J is the nucleation rate, expressed as the number of nuclei formed per unit volume of mother liquor per second, κ is an empirical kinetic factor, determined by the frequency per unit volume with which a molecule is hopping on and off a crystal nucleus, q equals 1 when nucleation is homogeneous and is between 0 and 1 when nucleation is heterogeneous and takes place on a non-protein surface, ΔG is the free energy required to assemble a stable crystal nucleus that will grow from its monomeric constituents, $k_{\rm B}$ is Boltzmann's constant and T is the absolute temperature.

From the expression of the nucleation rate, it is obvious that the volume of the mother liquor determines the average incubation time before the first stable nucleus forms spontaneously in the bulk solution. However, when nucleation is heterogeneous and crystals primarily nucleate on impurities, the average incubation time is proportional to the total surface of the nucleation substrate contacting the mother liquor. In this case, only the volume of mother liquor around the nucleating substrate has to be taken into account. It therefore appears that one cannot drastically reduce the volume of a crystallization experiment without paying a price. If crystallization is homogeneous, it will take longer for crystal nuclei to form or one has to raise the supersaturation. If crystallization is heterogeneous, care must be taken that each trial contains at least one heterologous nucleation substrate.

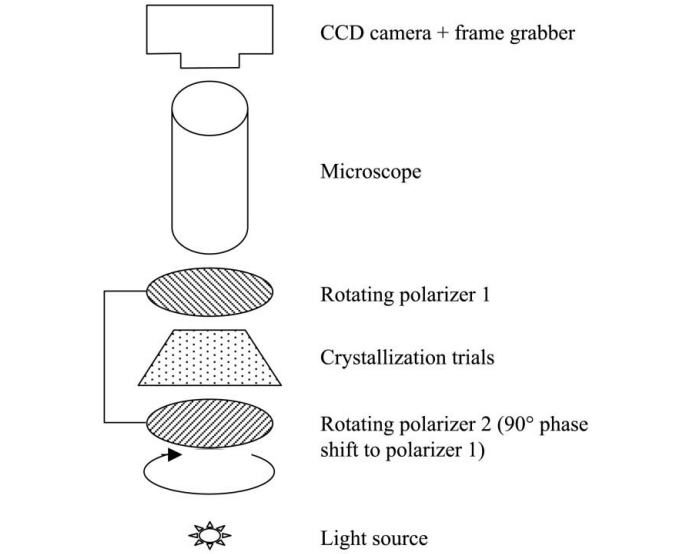

Figure 1
(*a*) Schematic diagram of the automatic setup for crystal detection by birefringence. Crystalline material is identified by rotating both the polarizing filters simultaneously, making birefringent material light up and extinguish. (*b*) Crystalline material shows up as light patches in an otherwise dark background using the setup described in (*a*). Here, 70 different wells, each containing 10 nl of mother liquor, are simultaneously evaluated for crystal growth. The evaluation time is less than 1 min. Wherever crystalline material is present the polarized light is repolarized, so it is no longer extinguished by the analysing polarization filter. The setup easily detects crystals smaller than 10 µm in size.

2. Results

2.1. Crystallizing protein in nanolitre volumes

As a substrate for batch crystallization in nanolitre volumes, we manufactured well plates from an epoxy resin using a brass mould of our own design. Each of the wells has a maximum capacity of about 250 nl. The well plates are optically inactive: they do not polarize light, which is an advantage for detecting crystals by birefringence. We adapted a piezoelectric dispensing system from MicroDrop GmbH (essentially as described in Schober *et al.*, 1993) to dispense individual crystallization setups with total volumes ranging from 1 to 250 nl. Essentially this is a drop-on-demand system, dispensing droplets with a volume of 200–500 pl (depending on the viscosity of the fluid and the pulse shape on the crystal). By programming the pulse shape on the piezoelectric crystal, we can reproducibly dispense solutions with a viscosity up to that of a 20% PEG 4000 solution. Higher concentrations can be achieved by evaporating water out of the droplet prior to adding protein. Fluids with a low surface tension tend to 'creep' round the nozzle of the dispenser and are therefore more difficult to dispense accurately. Essentially the same procedure as for highly viscous fluids may be followed for non-volatile components: a diluted solution is dispensed and allowed to dry.

We tested the system with systematic crystallization screens, varying the pH and precipitant concentration, of lysozyme [10 mg ml^{-1} in various buffers, pH 4.0–9.4, 3–10%(*w*/*v*) sodium chloride] and glucose isomerase (30 mg ml^{-1} in 1.7–

2.5 *M* ammonium acetate pH 6.5–9). Prior to setting up the crystallizations, all solutions were filtered with a low-protein-binding membrane with a nominal cutoff of 0.2 µm. Evaporation of the mother liquor during dispensing was prevented by cooling the well plates. After dispensing had finished, the droplets were covered with a layer of oil as in the commonly used batch method of crystallization (Chayen *et al.*, 1992). In all cases, we could reproduce in very small volumes (1–20 nl) crystallization also observed in volumes of 1 µl and up.

2.1.1. Detecting crystals by birefringence. When many crystallization conditions are to be tested, an automated method for crystal detection becomes essential. Ideally, one would like to identify even very small crystallites and crystalline precipitates. We use birefringence as a signature of crystallinity. An obvious requirement for such a setup is that the well plates containing the crystallizing solutions are optically inert, hence our use of epoxy resin rather than pressed plastics for the manufacture of such well plates. A diagram of our setup is shown in Fig. 1(*a*). Because the amount of extinction of polarized light is not only determined by the symmetry but also by the orientation of the crystal, we rotate the polarizing and analysing filters simultaneously, whilst integrating the amount of transmitted light using a CCD camera. Since this setup measures the transmittance of polarized light, there is no need for extensive image processing, including edge detection, of high-resolution images of individual crystals (Fig. 1*b*). The setup allows us to test many samples simultaneously. A further advantage is that we can discriminate between crystalline and non-crystalline precipitates, as individual crystallites do not have to be resolved optically. The disadvantage is that this method will miss certain crystals: crystals with cubic symmetry are not birefringent, whilst crystals of some of the lower symmetry space groups are also not birefringent in special orientations.

We believe that detecting crystals by birefringence is more effective than other techniques such as edge detection. For example, we routinely scan 70 different cystallization setups simultaneously and identify any crystalline material therein within 3 min. The major advantage of crystal detection by birefringence compared with edge detection is that only changes in transmitted light need to be monitored.

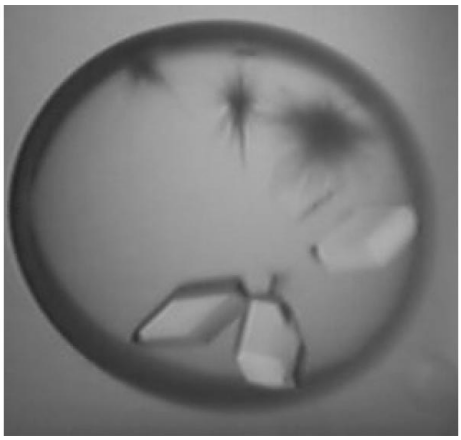

Figure 2
Two morphologies of lysozyme crystals growing in 64 nl mother liquor containing 10 mg ml^{-1} hen egg-white lysozyme in 20 m*M* sodium acetate pH 4.6, 1 *M* NaCl.

2.2. Volume dependency of crystal nucleation

We tested the crystallization of lysozyme in various submicrolitre volumes to establish whether we could differentiate between homogeneous and heterogeneous nucleation. Firstly, we prepared a fresh mother liquor of 10 mg ml^{-1} hen egg-white lysozyme in 20 m*M* sodium acetate pH 4.6, 1 *M* NaCl (this solution will produce lysozyme crystals in about 24 h). Immediately after its preparation, we filtered the mother liquor using a low-protein-binding membrane with a nominal cutoff of 0.2 µm. The filtered solution was quickly transferred to the nanodispenser and dispensed as droplets of about 350 pl into volumes ranging from 1 to 250 nl in 16-fold. After dispensing, paraffin oil was layered over the droplets. Dispensing 144 individual experiments took less than 5 min. Within 24 h crystals appeared in all the droplets, independent of the volume. We observed two different crystal habits, which seldomly (<1%) grew simultaneously in the same droplet (see Fig. 2 for an exception). The needle-shaped crystals were more prominent in small volumes, whilst the more bulky tetragonal crystals were more prominent in larger volumes. We scored the number of crystals per droplet and averaged the results of the 16 experiments to obtain good statistics. The results are shown in Fig. 3.

For the bulky tetragonal crystals, there is a linear relationship between volume and the number of nucleation events leading to a macroscopic crystal. The slope of the graph in Fig. 3(*a*) suggests that the nucleation rate of this particular mother liquor is of the order of one nucleation event per 10^{-1} mm^3 per 24 h. However, since we scored nucleation events by the appearance of macroscopic crystals after 24 h, this is likely to be an underestimation of the nucleation rate: a growing crystal will deplete the mother liquor, reducing the concentration of protein and thereby decreasing the nucleation rate. This is reflected by the extrapolated offset of 0.5 macroscopic crystals at zero volume in the graph. Given the linear relationship between the volume of the droplet and the number of the tetragonal crystals, we assume that these crystals nucleate homogeneously in the bulk of the mother liquor.

The needle-shaped lysozyme crystals grow in clusters radiating from a central core. Because of their shape, we refer to them as 'sea urchins'. The clusters are rarely observed in larger volumes, yet predominate in smaller volumes (Fig. 3*b*). We established that they are lysozyme crystals and that their space group very likely differs from that of the bulky tetragonal crystals: seeding the needle-shaped crystals into a similar mother liquor with a volume of about 50 µl yields similar needle-shaped crystals. Without seeding, only the bulky crystals grow. The needle-shaped crystals do not grow large enough to characterize them by diffraction.

Our observations suggest that the needle-shaped crystals nucleate heterogeneously on the surface of the mother liquor,

at the oil–water interface. Given our experimental results, we estimate the heterogeneous nucleation rate to be of the order of one nucleation event per 0.1 mm^2 per 24 h. However, in order to explain the correlation between the frequency of appearance of the needle-shaped crystals and the surface-to-volume ratio, we have to assume that the needle-shaped crystals grow more slowly than the bulky tetragonal crystals and that both crystal forms compete for protein during their growth. Alternatively, the needle-shaped crystals may predominately nucleate at higher pressures (R. Giegé, personal communication): owing to the surface tension, the pressure inside a droplet is inversely proportional to its radius according to

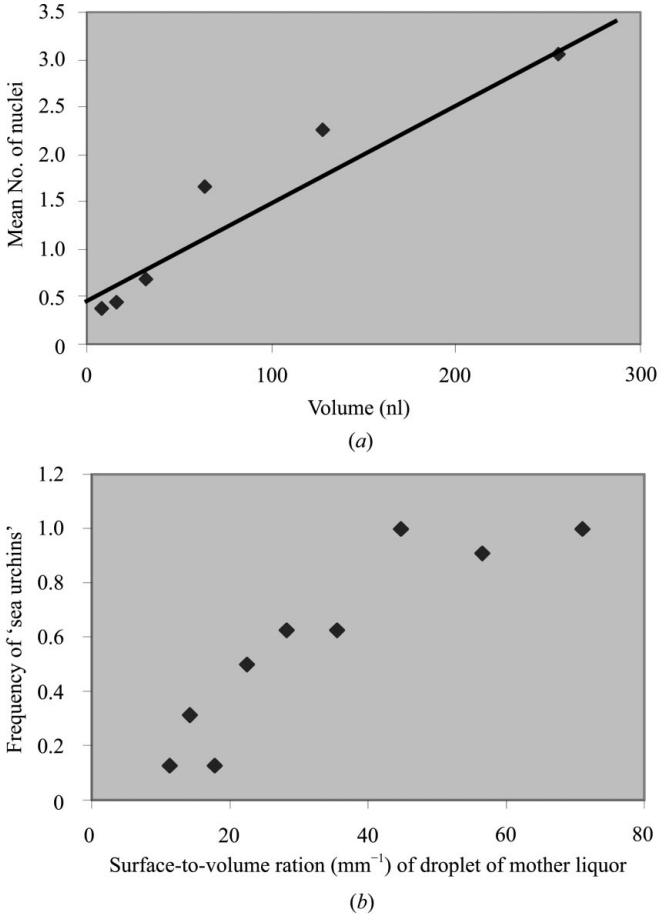

Figure 3
(*a*) 144 crystallization experiments were set up using a mother liquor containing 10 mg ml^{-1} hen egg-white lysozyme in 20 m*M* sodium acetate pH 4.6, 1 *M* NaCl, in volumes of 1, 2, 4, 8, 16, 32, 64, 128 and 256 nl (all in 16-fold). In the smaller volumes (1–8 nl) needle-shaped crystals predominated (see also Fig. 2), whereas in the larger volumes bulky tetragonal crystals appeared. The graph shows the average number of tetragonal crystals per droplet that appeared after 24 h as a function of the volume of the droplet. The straight line is a linear fit: (number of crystals) = 0.5 + 0.011 nl^{-1} × (volume of droplet), with a correlation coefficient of 0.95. (*b*) In the experiment described in (*a*), needle-shaped crystals appeared predominantly in the smaller volumes. The frequency with which these crystals appeared within 24 h is plotted as a function of the surface-to-volume ratio of the droplets, assuming a spherical shape. Note that in the larger surface-to-volume ratios (corresponding to the 1, 2 and 4 nl droplets) we still always found crystals, but these were mostly of the 'sea-urchin' type.

$$\Delta p = \sigma/r,$$

where Δp is the pressure inside the droplet, σ is the surface tension and r is the radius of the droplet.

3. Discussion

3.1. Disadvantages of nanocrystallography

We have demonstrated the viability of nanocrystallogenesis, but there are still improvements that can be made to the system. For nanodispensing, the use of electrospray as opposed to piezo-dispensing systems is attractive. The disadvantages of piezo-dispensing do not apply to dispensing by electrospray technologies: the viscosity of the fluid to be dispensed or the presence of detergents is virtually irrelevant for electrospray. However, there are currently no commercial dispensing stations that make use of electrospray. We are currently developing a prototype. Fig. 4 summarizes a comparison between the setup for piezo-dispensing and electrospray.

The crystals that are produced by nanocrystallogenesis may be too small for diffraction experiments unless a microfocus synchrotron beamline is available. We anticipate that in the near future crystals grown in small volumes will mainly be used as seedlings in larger volumes of similar or slightly less supersaturated mother liquors. Perhaps it will become possible to determine crystal structures straight from submicrometre crystals by electron diffraction, since electrons interact more strongly with matter than high-energy X-rays, whilst inducing less damage in biological material. For electron diffraction to become an alternative to X-ray diffraction, several problems will have to be solved, such as multiple scattering and the determination of crystal thickness. Using an energy filter to remove inelastically scattered electrons was shown to significantly improve the data for protein crystals with a thickness of up to 750 Å (Yonekura *et al.*, 2002).

It takes longer for crystals to appear in small volumes if nucleation is homogeneous. In order to increase the nucleation rate, the level of supersaturation of the mother liquor has to be increased. If crystallization conditions are to be identified in volumes of 1 nl rather than in 1 µl, the nucleation rate has to be boosted 1000-fold. Assuming classical nucleation theory (*e.g.* Veesler & Boistelle, 1999), the relationship between nucleation rates and levels of supersaturation can be derived as

$$\ln(J_1/J_2) = C(\ln^{-2}\beta_1 - \ln^{-2}\beta_2),$$

where J_n is the nucleation rate at a given supersaturation β_n, β_n is the level of supersaturation, expressed as the ratio between the actual protein concentration and the protein concentration of a saturated solution, β = [protein]/[protein]$_{\rm saturated}$ (for crystallizing protein solutions β is usually in the range 2–15) and

$$C = 16\pi V^2\gamma^3/3(k_{\rm B}T)^2,$$

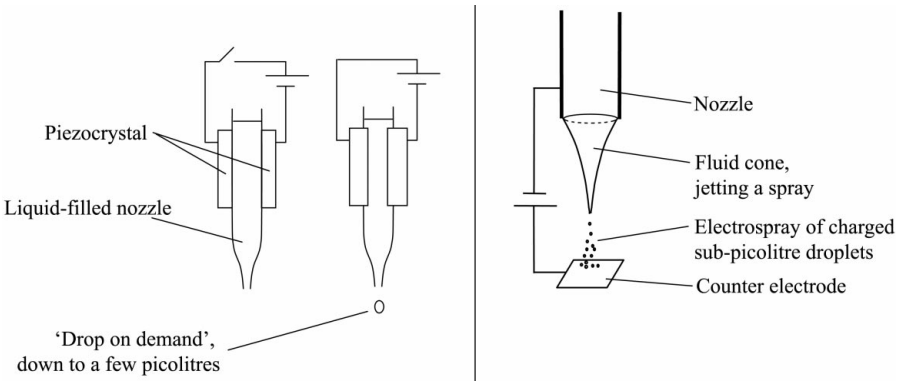

Figure 4
Comparison of nanodispensing by piezotechnology (left) and electrospray (right). In piezotechnology, a piezocrystal changes its shape when a voltage is applied. This change in shape creates a pressure difference, either by opening a valve leading out of a pressurized container or (as indicated above) by pushing against the fluid. The pressure wave creates a drop on demand. Because it is a ballistic technique, it is strongly affected by the viscosity of the fluid. Electrospray uses a very different principle: an electric potential difference pulls droplets out of a fluid body. It is hardly affected by viscosity or surface tension (see also Hartman *et al.*, 1999).

where V is the volume of the protein and γ is the surface free energy of the nucleus with respect to the solution (usually ranging between 1 and 10 mJ m^{-2}).

If C is large enough, even a relatively small increase in supersaturation will have a marked effect on the nucleation rate. For example, the nucleation rate at room temperature of a protein of about 40 kDa and with a γ of 5 mJ m^{-2} would be increased 1000-fold if its level of supersaturation were raised from 5 to 5.15. However, a similar protein with a γ of 2 mJ m^{-2} would require a rise in supersaturation from 5 to 12.5 to achieve a 1000-fold increase in nucleation rate. Reducing the solubility of the protein by changing the physicochemical characteristics of the solvent increases the magnitude of γ, which will boost the nucleation rate.

Many proteins nucleate heterologously on solid supports, or on air–water or oil–water interfaces. For these cases the above limitations are less relevant, as long as such heterologous interfaces are present.

3.2. Advantages of nanocrystallography

The major immediate benefit of nanocrystallogenesis is that it minimizes the amount of protein required to identify crystallization conditions. This may make it more feasible to determine structures of scarce proteins and protein complexes. Furthermore, the automation required for nanocrystallogenesis also increases the throughput of experimentation.

We demonstrate that a mother liquor may produce different crystal forms in small volumes. In the case of lysozyme, the new crystal form is less useful for structure analysis, but for other proteins the reverse may be the case. As the needle-shaped crystals remain too small for diffraction, we cannot be certain if they are an unknown crystal form or if they are the orthorhombic form known to grow in bulk at 313 K in the same mother liquor. The specific appearance of this crystal in small volumes may be the combined effect of more rapid heterologous nucleation at the mother liquor–oil interface and slower growth compared with the tetragonal lysozyme crystals. Alternatively, the increased pressure inside a nanolitre-sized droplet owing to the surface tension may induce a different crystal form (R. Giegé, personal communication).

When protein crystals grow from larger volume mother liquors, density and temperature-driven convection can compromise crystal quality, hence the interest in crystallizing under microgravity conditions or in gels. Both reduce gravity-induced convection upon depletion of protein during crystal growth (*e.g.* Wardell *et al.*, 1997; Robert *et al.*, 1999). Growing crystals in very small volumes is another alternative: the shorter distances in nanolitre-sized droplets reduce the magnitude of the concentration gradients that result from the accretion of molecules on the growing crystal, reducing local gravity-induced convection. We have anecdotal evidence that crystals grown in small volumes may indeed be somewhat better ordered, but more study is required.

4. Conclusions

Nano-crystallogenesis is becoming a robust means of identifying crystallization conditions and producing seedlings for macroscopic crystals required for standard structure determination by X-ray crystallography. Several inconveniences still exist, mainly concerning the reproducible dispensing and handling of very small volumes. Using the tiny crystals grown in nanolitre volumes for diffraction studies may require the availability of microfocus beamlines. Beam damage will however remain a problem and if crystals are submicrometre sized then electron diffraction may be an attractive alternative in view of the reduced beam damage of electrons compared with X-rays. Small volumes will sustain a supersaturated solution for longer periods if nucleation is homogeneous. As a result, a given mother liquor may grow different crystal forms depending on its volume. On the basis of this observation, we cannot exclude the possibility that certain proteins will only nucleate in very small volumes and hence only be found in nanolitre volumes. Protein crystallography is on its way to incorporate nanotechnology.

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