CRYSTALLIZATION IN GELS

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Crystallization of Macromolecules in Silica Gels

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Abstract

Procedures are described for the crystallization of proteins, nucleic acids and viruses in a silica-gel matrix using otherwise standard reagents and conditions. Methods are given based on both vapor diffusion in a sitting drop and liquid–liquid diffusion. Using a variety of macromolecules our results suggest that the gel matrix suppresses nucleation, reduces the rate of growth, and generally leads to larger, higher-quality crystals of enhanced stability. Presumably these effects arise from the decreased mobility of the macromolecules and their flux at the crystal surface during growth.

Introduction

Kinetic factors that characterize the crystallization of macromolecules can have profound effects on outcome. This is easily seen in everyday experience. Different methods of crystallizing the same protein or nucleic acid, vapor diffusion, dialysis, or batch, may yield entirely different quantities and qualities of crystals, or different crystal habits, even though the final state of the mother liquor may be the same in all cases. With a single technique, rapid equilibration may yield entirely different products than if equilibration is slow. This reflects the magnitude of the activation barrier for crystal nucleation, rates of transport of nutrient to the growing crystal, probability of incorporation of individual molecules into the crystal lattice, and rates at which supersaturation varies during the course of an experiment.

Physical properties of the mother liquor, such as viscosity, may directly affect several of these processes, particularly the transport of nutrient or the rise in supersaturation due to the transport of precipitant through the liquid phase. Thus, changes in the physical character of the medium in which equilibration is proceeding and crystallization occurring can prove a useful tool for altering the size, quality or habits of crystals.

Gels have been employed in crystallization from an aqueous medium for many years, particularly for the crystallization of conventional inorganic compounds (Henisch, 1988; Sharbaugh & Sharbaugh, 1989). More recently, attempts have been made, with some success (Miller, He & Carter, 1992; Robert & Lefaucheux, 1988; Robert, Provost & Lefaucheux, 1993; Garcia-Ruiz, 1993), to extend the use of gels to the crystallization of proteins as well. Indeed a number of protein crystals have been grown using gels composed of agarose and polyacrylamide, common media used as well in biochemical electrophoretic procedures.

We have investigated protein and virus crystal growth in several types of gel matrix and found one in particular that yields, in our hands, better and more reproducible results than others. In particular, crystallization with this gel as the medium appears to result in fewer but generally larger, single crystals. The gel we find most effective is in fact silica gel, a popular gel matrix in the past used for the crystallization of conventional inorganic molecules.

Because of the labile nature of macromolecules, however, standard methods are somewhat inappropriate and it was necessary to modify composition and procedures for compatibility with proteins and nucleic acids. Here we describe several protocols for macromolecular crystal growth in silica-gel media and present some results we have obtained for a variety of samples that include proteins, tRNA and one small virus.

Materials and methods

Vapor diffusion in a sitting drop

(*a*) Mix equal volumes (generally 0.5–1.0 ml) of 0.48 *M* acetic acid with 2.7%(*v*/*v*) sodium silicate (Aldrich, No. 33,844-3) and vortex for 30 s. The resultant gel prepared in this way will have a final pH of 7 and will polymerize within 15 min.

(*b*) 5 μl drops (other volumes may be substituted as desired) of the gel are dispensed by micropipette onto Micro-Bridges (Hampton Research, Riverside, CA) in Linbro tissue culture plates (Hampton Research) or onto the posts of Cryschem crystallization plates (Charles Supper Company, Natick, MA). The plates are then sealed with glass or plastic cover

slips or transparent tape. The gel will polymerize within 5 min, but it should be allowed to stabilize or 'cure' for about 1 h.

(*c*) Pipette 5 μl of the chosen precipitant solution directly onto the gel. Seal the plates as before and allow the precipitant solution to completely diffuse into the gel. Diffusion time will vary with the precipitant composition but typically requires 1 h. The concentration of the precipitant should be less than that which would produce precipitation of the macromolecule when mixed in equal amounts. In effect, what is being established here is the initial precipitant concentration prior to equilibration against the reservoir.

(*d*) Pipette 10 μl of the macromolecule solution onto the surface of the precipitant-imbibed gel, seal the plate as before, and allow the gel to completely absorb the protein, virus, or nucleic acid solution. This will, in general, require about 4 h.

(*e*) Pipette 1.0 ml of reservoir (precipitant) solution into the reservoir wells of Linbro trays, or 0.7 ml of reservoir solution into wells of Cryschem plates. Seal with cover slips or clear tape.

(*f*) Store the plate as you would a routine sitting- or hanging-drop crystallization experiment. The gel is stable between 277 and 310 K.

Liquid–gel diffusion crystallization

(*a*) This procedure is based on a 384-well Macro-Store plate (Hampton Research) although appropriate substitutions of alternate plasticware are acceptable.

(*b*) Mix equal volumes (generally 0.5–1.0 ml) 0.480 *M* acetic acid with 2.7% sodium silicate and vortex for 30 s. The resultant gel, prepared in this way, will have a final pH of 7.

(*c*) Dispense 10 μl of the gel solution into each well of the Macro-Store plate. In general this is best done using a fine, sequencing gel, micropipette tip (*e.g.* Fisher No. 05-541-8). Allow the gel to polymerize and then stabilize ('cure') for about 1 h.

(*d*) Pipette 10 μl of the protein, nucleic acid, or virus solution on the surface of the 'cured' gel and allow it to be completely absorbed into the gel matrix. This generally requires 1–4 h.

(*e*) Pipette 10–20 μl of an appropriate precipitating solution on the surface of the macromolecule-saturated gel. Seal the plate with clear sealing tape as you would a standard Cryschem plate.

(*f*) Store the plate as you would a routine sitting- or hanging-drop crystallization experiment between 277 and 310 K.

Liquid–liquid diffusion across a gel boundary

(*a*) The procedure is based upon use of a glass Caraway or Natelson tube (used for clinical blood sampling, Fisher Nos. 02-668-30 and 02-688-15) or any capillary of 0.5–2 mm inside diameter open at both ends.

(*b*) Mix equal volumes (generally 0.5–1.0 ml) of 0.48 *M* acetic acid with 2.7% sodium silicate and vortex for 30 s. The resultant gel, prepared in this way, will have a final pH of 7.

(*c*) Using syringe or sequencing gel, micropipette tip, pipette 10 μl (somewhat smaller volumes for capillaries, to give about a 2 mm partition), into the midsection of a Caraway or Natelson tube. Allow the gel to polymerize for about 5 min and stabilize ('cure') for about 1 h.

(*d*) Pipette 10 μl (appropriately smaller volumes for capillaries) of the macromolecule solution into the space on one side of the polymerized gel. Be certain there are no air spaces or bubbles between the macromolecule solution and the gel surface.

(*e*) Pipette 10 μl of the appropriate precipitant solution on the opposite side of the gel, again being certain that no air spaces or bubbles are interposed. The two ends of the Natelson or Caraway tube or capillary are then sealed with melted paraffin, beeswax, or some other material to prevent evaporation.

(*f*) Store the Caraway tube or capillary as you would any routine crystallization experiment. The gel is stable between 277 and 310 K.

Macromolecules

Experiments were performed using a variety of macromolecule samples, including one virus and one transfer RNA. These proteins included lysozyme (Sigma), *Thaumatococcus danielli* thaumatin (Ko, Day, Greenwood & McPherson, 1994) (Sigma), rabbit muscle lactate dehydrogenase (Sigma), papain from papaya (Sigma), horse hemoglobin (Sigma), bovine pancreas ribonuclease A (Sigma), leupeptin (Bachem), leutenizing hormone α subunit, leutenizing hormone β subunit, edestin (Sigma), DPDPE (Multiple Peptide Systems), pyruvate kinase chicken muscle (Sigma), protease P6911 (Sigma), sweet potato β-amylase (Sigma) (Cudney & McPherson, 1993), canavalin (Ko, Ng & McPherson, 1993; Ng, Ko & McPherson, 1993), and bovine liver catalase (Sigma). Crystallization conditions were in most cases identified using the Crystal Screen (Hampton Research). The virus sample was satellite tobacco mosaic virus (Larson *et al.*, 1993) and the nucleic acid was yeast phenylalanine tRNA (Sigma). Very similar results were observed for virtually all of the macromolecule samples. Commerical sources for the macromolecules were generally used with the exception of STMV, canavalin and the subunits of LH which were produced in this laboratory by procedures previously described.

Results

Methods described above were compatible with precipitant solutions that included ammonium sulfate, sodium citrate, sodium acetate, sodium phosphate and a wide range of other salts tested. There was no difficulty with most organic precipitants or polymeric organic precipitants including MPD, hexanediol, or PEG 400 to PEG 20 000. In addition, volatile organic solvents such as ethanol or propanol were similarly acceptable. A wide range of buffers, extending over the range 4.0–9.0, was used without incident and included acetate, citrate, cacodylate, HEPES, Tris, Bicine and MES. In addition, we found the procedures entirely compatible with the inclusion of detergents such as BOG as well as numerous others.

A number of qualitative observations were made, generally consistent from macromolecule sample to sample.

(*a*) The onset of 'visible crystal nuclei' as visualized under a microscope of 200-fold magnification was in general delayed by several days to several weeks depending on the macromolecule sample as compared with an equivalent experiment conducted in the absence of gel matrix. This was observed over the temperature range 277–295 K. It was generally found that the appearance of crystals, or the lag in time compared with equivalent non-gel experiments, was correlated to the size of the macromolecule.

(*b*) The rate of growth of crystals in gels clearly was significantly less than for crystals growing in normal aqueous mother liquor.

(*c*) In the gel experiments, as illustrated in Fig. 1, there were significantly fewer nuclei formed, and this was particularly striking for the initial phases of crystallization. If the experiments are continued for several months the differences between gel and non-gel growth are less marked, but they are nonetheless apparent.

(*d*) Crystals grown in the silica gel, as seen in Fig. 2, generally grow to a larger size than those grown under equivalent conditions but without the gel matrix.

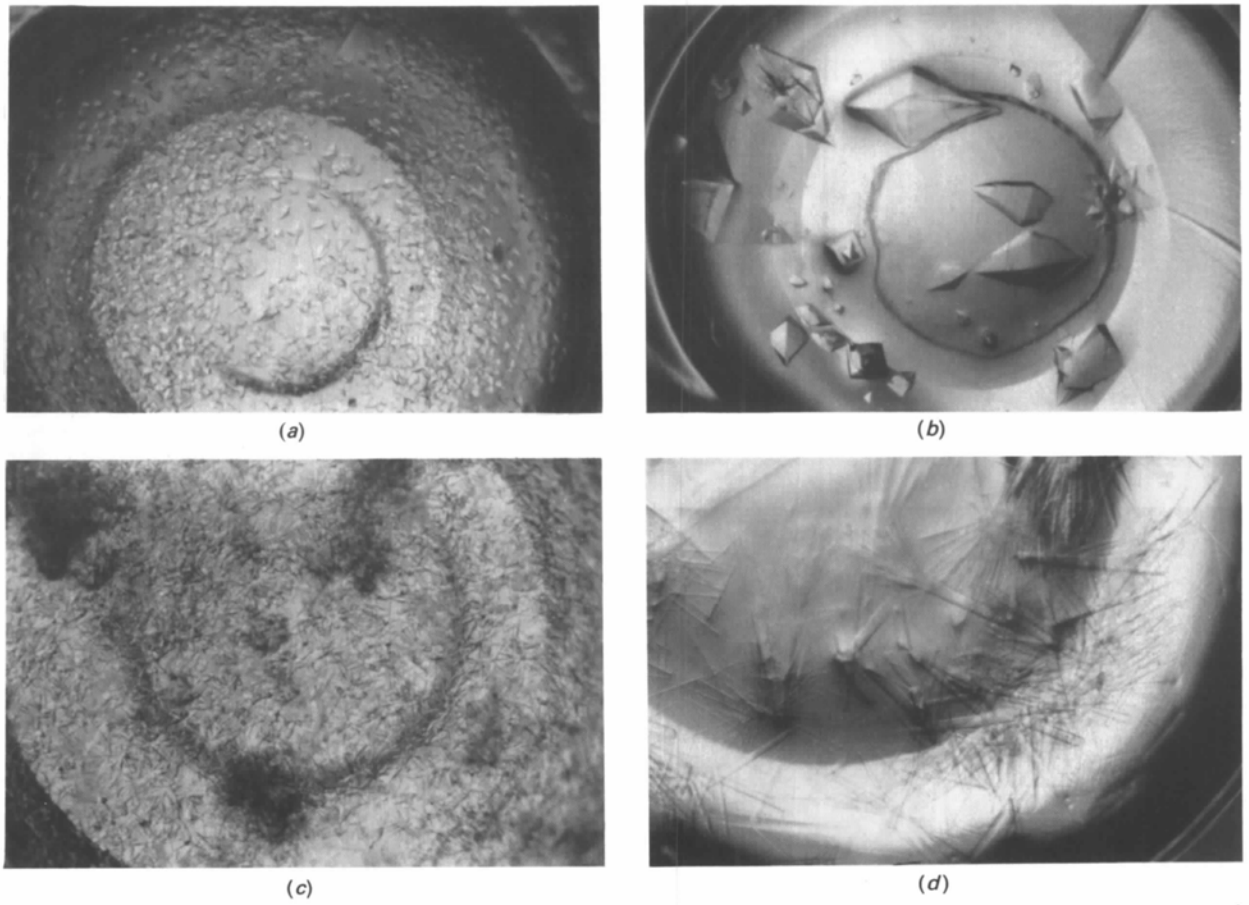

Fig. 1. (*a*), (*b*) Crystals of thaumatin grown in 0.8 *M* sodium potassium tartrate, 0.1 *M* ADA pH 6.5, protein concentration 40 mg ml^{-1}. In (*a*), crystals were grown in the absence of gel, whereas in (*b*), crystals were grown in the presence of gel. (*c*), (*d*) Crystals of pyruvate kinase grown in 2.0 *M* ammonium sulfate, 0.1 *M* Tris–HCl pH 8.5, protein concentration 20 mg ml^{-1}. In (*c*), crystals were grown in the absence of gel, whereas in (*d*), crystals were grown in the presence of gel.

(*e*) Crystals which grow in the gel matrix appear to be somewhat more stable. For example, canavalin and lysozyme, crystallized under certain conditions, may be stable for no more than a few days to a week after which time the crystals develop cracks. Crystals grown under otherwise identical conditions in gels, however, appear to be stable almost indefinitely.

(*f*) Some macromolecule crystals grow with altered habits in the gel matrix as compared with equivalent crystals grown in its absence. While the fundamental unit cell is probably not different, the ratio of cell edges and face development may be substantially altered.

(*g*) There appears to be less background precipitate in the gels as compared with normal aqueous mother liquor.

Discussion

The gel used here, silica gel, is quite innocuous and generally regarded as safe for use with most biological macromolecules and assemblies. The gel material is versatile and adaptable to other procedures and configurations than those described here. In addition, the gel matrix seems to tolerate a broad range of salts, organic precipitants, buffers and pH, and even the presence of detergents.

The first conclusion that may be drawn is that some positive advantages, in terms of ultimate size, quality and habit, may be obtained by using the silica-gel-based procedures described here. While this is not universal by any means *i.e.* crystals of some macromolecules were not improved upon at all, in many cases positive enhancements were observed.

The altered results observed for macromolecular crystal growth in silica gels are presumably a consequence of the limitation in the diffusion rate of macromolecules and a retardation in their presentation to a growing prenuclear aggregate (Malkin, Cheung & McPherson, 1993; Malkin & McPherson, 1993), a nucleus exceeding critical size, and a growing crystal surface. The kinetics of the overall process are clearly altered, as evidenced by the slower growth of crystals once formed and by alterations in the ultimate habits.

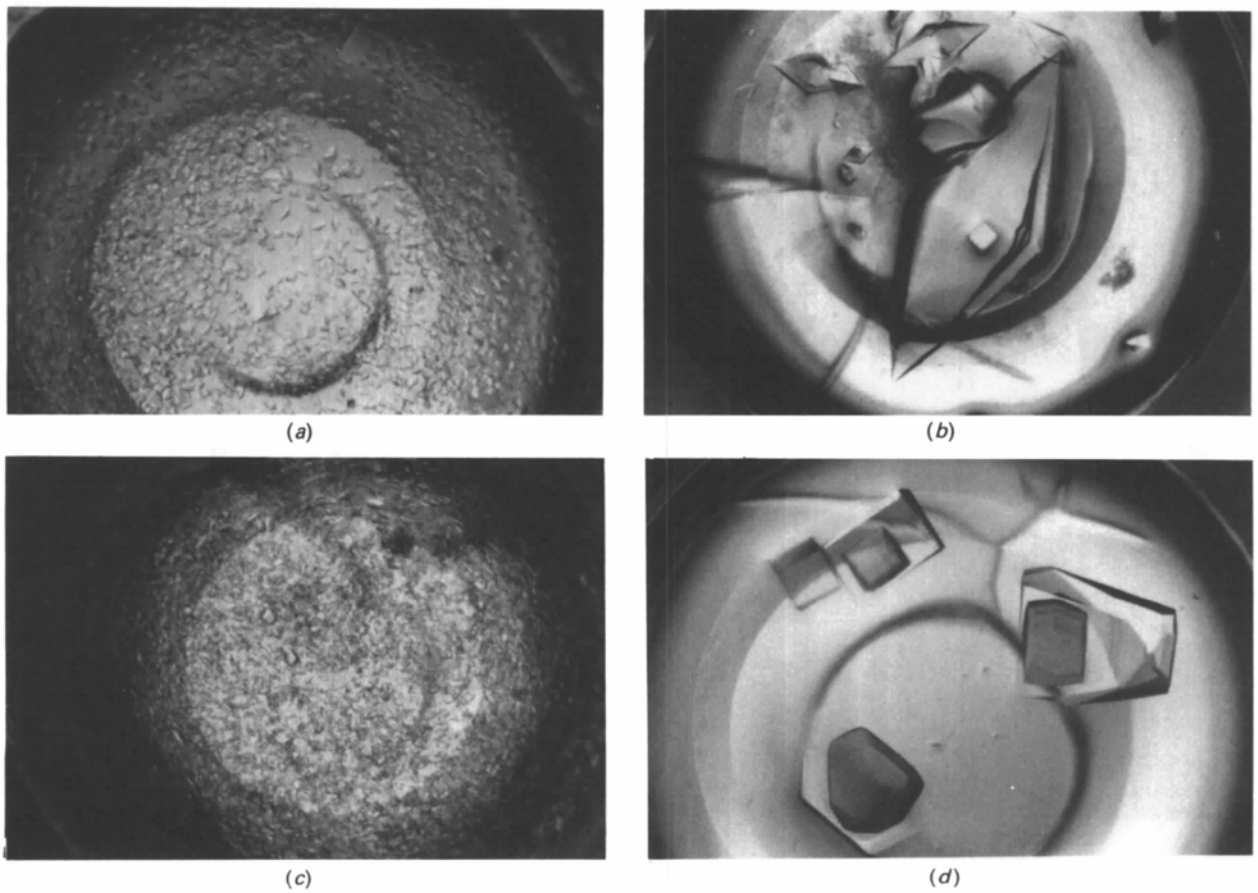

Fig. 2. Crystals of thaumatin [(*a*), (*b*)] and lysozyme [(*c*), (*d*)] grown in the absence [(*a*), (*c*)] and presence [(*b*), (*d*)] of gel illustrating reduced nucleation and larger terminal crystal size in the presence of the silica hydrogel.

Because of decreased diffusion of the macromolecules, and the virtual elimination of convection, stable depletion zones may form in the immediate environment of a growing nucleus. That may in turn impose more ordered and disciplined growth on developing crystals. That is, the crystallization process, which in the case of macromolecules occurs at very high levels of supersaturation, may become internally regulating and self controlled where convection is eliminated (McPherson, Greenwood & Day, 1991; McPherson, 1993).

In addition to altering, directly, the kinetics of the crystallization process, the gel may have other secondary kinetic effects. It almost certainly, for example, alters the rate of equilibration between macromolecules and precipitant, in turn a function of the particular technique or procedure employed. Thus, the presence of a gel matrix may both alter the kinetics of macromolecule addition to a growing crystal surface, as well as alter the rate and degree to which supersaturation is established.

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References

CUDNEY, R. & MCPHERSON, A. (1992). *J. Mol. Biol.* **229**, 253–254.

GARCIA-RUIZ, J. M. (1993). *Key Eng. Mater.* **58**, 87–106.

HENISCH, H. K. (1988). *Crystal Growth in Gels and Liesegang Rings*. Cambridge Univ. Press.

KO, T.-P., DAY, J., GREENWOOD, A. & MCPHERSON, A. (1994). *Acta Cryst.* D**50**. Submitted.

KO., T.-P., NG, J. D. & MCPHERSON, A. (1993). *Plant Physiol.* **101**, 729–744.

LARSON, S. B., KOSZELAK, S., DAY, J., GREENWOOD, A., DODDS, J. A. & MCPHERSON, A. (1993). *J. Mol. Biol.* **231**, 375–391.

MCPHERSON, A. (1993). *Microgravity Sci. Technol.* **6**, 101–109.

MCPHERSON, A., GREENWOOD, A. & DAY, J. (1991). *Adv. Space Res.* **11**(7), 343–356.

MALKIN, A. J., CHEUNG, J. & MCPHERSON, A. (1993). *J. Cryst. Growth*, **126**, 544–554.

MALKIN, A. J. & MCPHERSON, A. (1993). *J. Cryst. Growth*, **126**, 555–564.

MILLER, T. V., HE, X. M. & CARTER, D. C. (1992). *J. Cryst. Growth*, **122**, 306–309.

NG, J. D., KO, T.-P. & MCPHERSON, A. (1993). *Plant Physiol.* **101**, 713–728.

ROBERT, M. C. & LEFAUCHEUX, F. (1988). *J. Cryst. Growth*, **90**, 358–367.

ROBERT, M. C., PROVOST, K. & LEFAUCHEUX, F. (1993). *Crystallization in Gels and Related Methods. Crystallization of Nucleic Acids and Proteins*, edited by A. DUCRUIX & R. GIEGÉ, pp. 127–143. Oxford Univ. Press.

SHARBAUGH, A. H. SR & SHARBAUGH, A. H. JR (1989). *J. Chem. Educ.* **7**, 589–594.