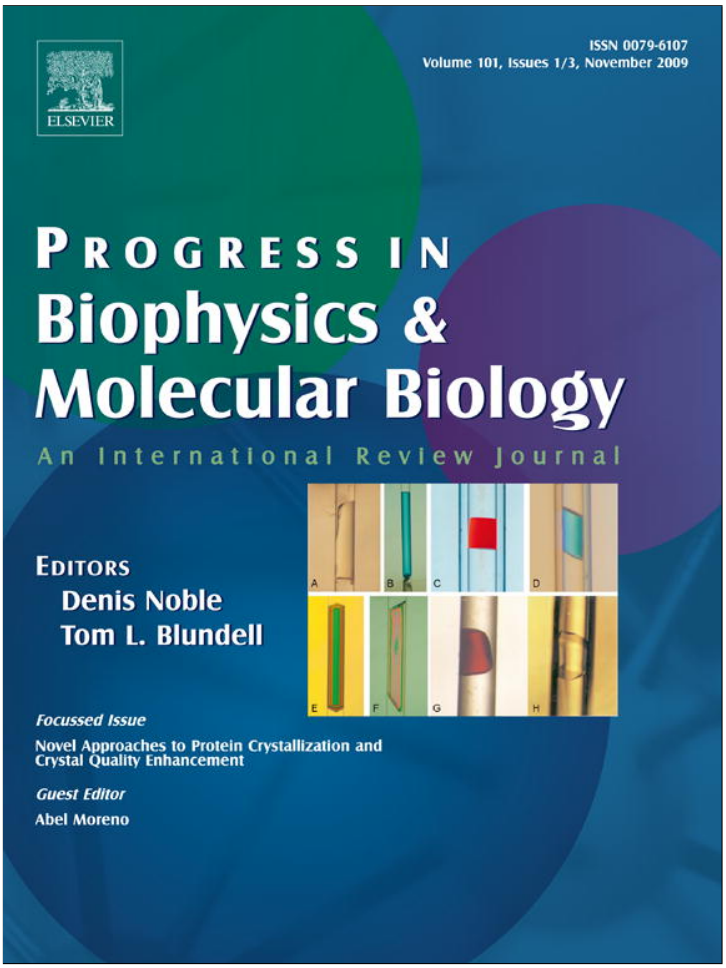

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Crystal growth of proteins, nucleic acids, and viruses in gels

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ABSTRACT

Medium-sized single crystals with perfect habits and no defect producing intense and well-resolved diffraction patterns are the dream of every protein crystallographer. Crystals of biological macromolecules possessing these characteristics can be prepared within a medium in which mass transport is restricted to diffusion. Chemical gels (like polysiloxane) and physical gels (such as agarose) provide such an environment and are therefore suitable for the crystallisation of biological macromolecules. Instructions for the preparation of each type of gel are given to urge crystal growers to apply diffusive media for enhancing crystallographic quality of their crystals. Examples of quality enhancement achieved with silica and agarose gels are given. Results obtained with other substances forming gel-like media (such as lipidic phases and cellulose derivatives) are presented. Finally, the use of gels in combination with capillary tubes for counter-diffusion experiments is discussed. Methods and techniques implemented with proteins can also be applied to nucleic acids and nucleoprotein assemblies such as viruses.

1. History of crystal growth in gels

For long chemists utilized silica gels to grow single faceted crystals of inorganic compounds reaching a macroscopic size and high optical perfection (for historical reviews see Henisch, 1973, 1988; Wilke, 1988). They added the gel in the crystallisation medium to control the nucleation rate (Henisch et al., 1965), suppress heterogeneous nucleation (e.g. Halberstadt and Henisch, 1968) or immobilise crystal nuclei in the mesh and favour their growth in three dimensions.

The very first trace of an attempt to crystallise a biological macromolecule in a gel dates back to the 1950s. It was at Harvard University where J. Lewin succeeded in growing well-developed single crystals of a barium derivative of albumin in a rigid gelatin gel (cited in Low and Richards, 1954). In the same Laboratory of Physical Chemistry related to Medicine and Public Health, B.W. Low and F.M. Richards (who invented the optical comparator box called after him) followed this example to prepare in two weeks large single crystals of pure albumin at 10% (m/v) in 5% (v/v) methanol and 1% (m/v) gelatin. They noted that Lewin's observation recalls the use of gels to slow down the mixing of components in the preparation of large single crystals of sparingly soluble compounds, a technique mentioned in Chamot and Mason's Handbook of Chemical Microscopy published in 1947. Their albumin crystals were morphologically and optically identical to those obtained by other methods but showed a marked tendency to twin on the (001) face. In the frame of their investigations they did not exploit these crystals for quantitative measurements of density, composition and

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unit cell dimensions because they were not sure about their gelatin content (Low and Richards, 1954). Then crystallisation in gelatin seems to have been abandoned either because of the ignorance of the original method or of its advantages. Twenty years later, the gel technique is not mentioned in the reference work on protein crystallography (Blundell and Johnson, 1976).

With the preparation of the first crystallisation experiments of proteins under microgravity in the mid 1980s the use of gels was reborn. In 1987, a short paper published in French by M.-C. Robert, a mineralogist and J. Berthou, a specialist of lysozyme, lays the foundations of the crystallisation method in gels applied to proteins. The convincing results of a simple experiment with lysozyme in tetramethoxysilane gel demonstrate that the approach is applicable. The also announce the real advantages of growing soft crystals in elastic gels which will actually be discovered and confirmed repeatedly over the next twenty years. These include the growth in suspension and in the absence of convection of crystals with well-developed facets which are superb by any standard and possess excellent diffraction properties. Moreover, despite of their fragility these crystals can be extracted without damage from the gel network (Robert and Berthou, 1987). In the absence of gel, similar crystal quality can only be obtained when the level of gravity is reduced. Thus, gels are ideal diffusive media to mimic on Earth crystal nucleation and growth under weightlessness. Agarose gels were introduced immediately after silica gels (Robert and Lefaucheux, 1988). The results obtained with lysozyme and trypsin triggered an avalanche of in-depth investigations of the process of protein crystal growth as well as of the direct and indirect effects of the gel on crystal quality (García-Ruiz, 1991 and references given below).

Unexpectedly, all this did not help popularise enough the gels since, all things considered, they were rarely used to crystallise soluble proteins. At the same time as the gel technique was totally overlooked, advances were made in the difficult field of membrane protein crystallisation. For these delicate macromolecules, crystallisation assays were exclusively limited to proteins which could be dissolved in an appropriate detergent solution. Consequently, many attempts to grow crystals were unsuccessful. After numerous years of effort, a cubic phase of monoolein revealed to be a key to the preparation of well-ordered 3D crystals of bacteriorhodopsin and of other trans-membrane proteins (Rummel et al., 1998). It was also rapidly discovered that the peculiar organization of lipids in gel-like cubic phases does not restrict the use of the latter to the crystallisation of hydrophobic macromolecules. Polar proteins crystallise very well in such media (Landau et al., 1997). Alike what happened to the gels, this convection-less crystallisation method has seriously never been applied to anything else than membrane proteins.

The fact that the last protein structure determined owing to crystals grown in a gel was published in 2006 means that crystallisation in gel has again fallen in disuse. For this reason, we present here in detail the silica and agarose gels employed most frequently to crystallise soluble proteins and icosahedral viruses. Since these media are essentially made of water, they are hydrogels. We explain why macromolecules should preferably be crystallised inside the network of gels which are diffusive media "par excellence", i.e. where molecules displace solely by diffusion. Further, we insist on the many advantages of crystallisation in gels can have over crystallisation in pure solution. Moreover, we emphasize the potential of gel-like media like lipidic phases for the crystallisation of soluble proteins. We also report results obtained with gels of other chemical compositions that may become useful in future. Further, we discuss the combined use of gels and capillary tubes. The range of applications of gels and of related methods is not limited. It goes from small proteins and nucleic acids to large macromolecular assemblies like nucleoprotein complexes or viruses.

2. Synthesis and structure of gels

2.1. Silica hydrogels

So far polysiloxane hydrogels have rarely been used. There are probably two reasons for this: generally they are not available in biology laboratories and their preparation requires some handling. The polycondensation of silicate ions leads to the formation of a dense network of silicon and oxygen atoms. Silica gels are chemical gel because all inter-atomic bonds are covalent (Fig. 1). So far the silicon-containing compounds (siloxanes) employed to prepare gels destined to protein crystallisation are sodium metasilicate (also called water glass), tetramethyl orthosilicate (TMOS) and tetraethyl orthosilicate (TEOS). These compounds possess a well defined chemical composition. Nevertheless, crystal growers should be aware that the hydrolysis of TMOS and TEOS preceding the condensation of the hydrated silica tetrahedral ions is accompanied by the production of significant amounts of methanol and ethanol, respectively. Hence, these gels must be cured to remove undesired alcohol molecules which may have deleterious effects to biological molecules. Chemical gels are irreversible, elastic and resist to deformation but swell in water.

In the scanning electron microscope (SEM), the flexible polymer network of the silica gel resulting from neutralization of a sodium metasilicate solution with acetic acid has a random pore size distribution from 50 to 150 nm (Moreno et al., 1999a). The distribution is more homogenous in gels prepared at alkaline pH. The pores of TMOS polysiloxane hydrogels have different sizes. Their size and the stiffness of the gel is concentration-dependent; the higher the concentration, the lower the pore size. Their diameter ranges from 150 to 250 nm in 10% (v/v) gels to 50–100 nm in 20% (v/v) gels. Small angle X-ray scattering (SAXS) analyses confirm that the pore size distribution of TEOS gels is also random. Further, condensation involves interactions between particles whose size and fractal dimension depend on the synthesis pathway and on their final location. Three stages of cluster generation have been

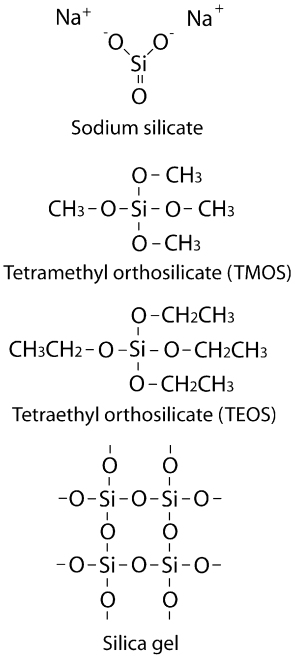

Fig. 1. Silicates and structure of silica gels. (Top) Sodium silicate; (Centre), Tetramethyl orthosilicate (TMOS) and tetraethyl orthosilicate (TEOS); (Bottom) Schematic representation of the silica gel network.

observed in silica gels using SAXS, atomic force microscopy (AFM) and SEM. The structure of these gels is discontinuous from their surface to their inner part (Vinogradova et al., 2003). Table 1 gives a protocol for the preparation of crystallisation assays with silica gel.

2.2. Agarose gels

Agarose is a gelling compound extracted from certain red sea weeds of the Rhodophyceae group (McHugh, 1987). Its long chains are repetitions of the non-charged and hydrophilic disaccharide unit agarobiose (Fig. 2). They are associated in double helices and the latter are grouped in bundles of several thousands. Interactions between chains rely on van der Waals forces or hydrogen bonds of the order of kT. Agarose is a physical gel because the association of its chains is reversible upon heating. The difference between its melting (T_m) and gelling (T_g) temperatures is called hysteresis (Guenet, 1992). At variance with chemical gels, physical gels do not swell but their network breaks apart when a deformation is applied on the rigid fibres.

Agarose was the first sieving medium used in electrophoresis. A cursory glance at manufacturer catalogues reveals a wide variety of commercially available products. Depending on the source, the natural biopolymer contains a variable proportion of β-D-galactose substituted with a methyl or a sulphate group in position 6. A low content of electronegative groups, transparency and absence of colour are indicators of purity. Agaroses distinguish also by the length of their chains and the strength of their gel (usually given for a concentration of 1 or 1.5% m/v). The process of gel formation is influenced by chain length, chemical composition, and degree of derivatisation (Griess et al., 1993). Agarose with shorter chains has a lower melting temperature. The presence of methyl groups increases the melting temperature. Low or ultra-low-gelling temperatures are useful for the crystallisation of biological macromolecules that do not withstand elevated temperatures. The first have a T_m close to 60 °C and a T_g of about 28 °C. For the others T_m is below 50 °C and T_g below 20 °C. Differences between one product and another and variations from one batch to the next make the comparison of the experimental data found in literature very difficult.

The reticular structure of the agarose mesh, its strength and its elasticity vary with concentration. Also, at a given concentration, the size of the pore varies with the nature and concentration of additives (as for example some precipitating agents). For this reason, the mesh of the gel is more reproducible when polymerization occurs in pure water or in the presence of a low concentration of buffering substance. In extreme cases, the polysaccharide chains may be segregated by the chemical additive and polymerization cannot occur (Gonzales-Ramirez et al., 2008). The mean pore diameter decreases with agarose concentration: at low ionic strength (i.e. 10 mM tris-borate-EDTA) it is 520, 500, 350, 300, 230 and 180 nm at 0.7, 1, 2, 3, 4 and 5% (m/v) respectively (Maaloum et al., 1998). It increases with the ionic strength: for a 1% (m/v) gel

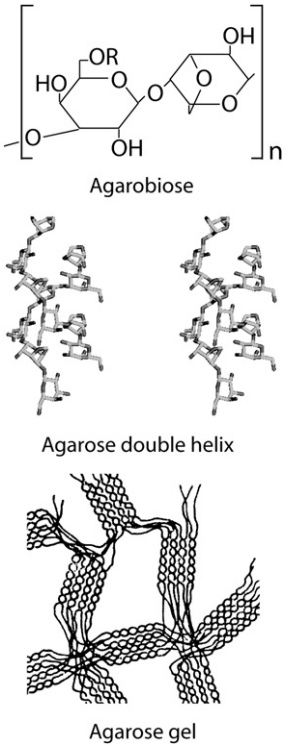

Fig. 2. Elementary unit and structure of agarose gel. (Top) Structure of agarobiose composed of β-D-galactose molecule linked to 3,6 anhydro α-L-galactose via an 1–4 bond. R is hydrogen in the absence of methyl or sulphate group. (Centre) Agarobiose units are linked together via 1–3 bonds. Their number ranges from a few dozens to several thousands. Resulting chains have a mean diameter of 8 nm and an average M_r estimated to 10^5 and adopt a double helical structure. Image created with coordinates from pdb file 1aga using *Pymol* (www.pymol.org). (Bottom) The gel network is an entanglement of chains associated reversibly and exclusively via van der Waals forces or hydrogen bonds (drawing adapted from Arnott et al., 1974). Upon heating agarose the chains separate and adopt a random coil conformation. When the heated solution cools they take the helical conformation and upon crossed linking they form an elastic and transparent gel. Further association of chains and additional crossed links consolidate the network; the gel becomes turbid and rigid. Finally, the gel expulses part of its liquid phase. This phase separation is called syneresis.

Table 1
Preparation of vapour-diffusion crystallisation assays containing silica gel.

Step

1. Dilute a commercial sodium silicate stock solution Na_2SiO_3 with a density of $\rho = 1.39$ g/ml (e.g. Aldrich Cat. No. 338443), with water to prepare a working solution with a density of 1.06 g/ml using the relationship $V_{SS} = (0.06\ V_a)/(1.39 - \rho^T)$, where V_{SS} is the volume of stock required to prepare a final volume V_a and ρ^T is the density of water at temperature T. This solution can be kept a few days.
2. Dispense reservoir solutions in the sample wells of crystallisation plates and cover temporarily.
3. Neutralise above silicate solution with a 1 M acetic acid solution to obtain a monosilicic acid (H_4SiO_4) solution. The final solution must be used immediately.
4. Prepare sample drops on glass coverslips, microbridges or depressions or microplates by mixing equal volumes of protein solution/reservoir solution/silica solution. The polymerization of the polysiloxane hydrogel occurs in less than 1 h.

Safety note: Siloxane solutions are corrosive. Skin and eyes protection is recommended for handling. Glassware must be thoroughly rinsed with ethanol prior to cleaning with water.

Comments: TMOS and TEOS are not chemically neutral and should never be in direct contact with proteins. In addition, these liquid compounds are not soluble in water. Their gelling process involves a hydrolysis followed by a polycondensation. Experimenters should be aware that the hydrolysis consumes water (vigorous stirring accelerates dissolution and clearing) and releases alcohol (methanol or ethanol, respectively). The molar ratio of water to TMOS is correlated with the size of the pores in the gel matrix (Brinker and Scherrer, 1990). For a TMOS gel of percentage p, the volume of water is $2.5 \times p$ ml and that of methanol $0.9 \times p$ ml. For other silica gel preparation protocols see refs Robert and Lefaucheux (1988), Cudney et al. (1994), and Vidal et al. (1998a,b). The component of the gel can be dispensed using a liquid dispensing apparatus for high-throughput applications (Chayen and Saridakis, 2002).

the mean values are 370 nm (1 mM), 509 nm (10 mM), 966 nm (100 mM) and 1777 nm (1 M). The pore size distribution is broadest at lowest agarose concentrations and at highest ionic strength (Maaloum et al., 1998). In 0.15–0.5% (m/v) gels prepared in 10 mM tris-borate-EDTA pores have diameters ranging from 300 nm to more than 1 μm (Pernodet et al., 1997). Independently of these fluctuations, pore diameters are always compatible with the diffusion of large particles like icosahedral viruses measuring 30–40 nm across. Once the gel is formed the pore size is invariable.

From the practical standpoint, agarose can easily be added to solutions destined to crystallisation. Table 2 gives a protocol for the preparation of the assays. Agarose is easy to handle manually or with an automated pipeting station. Fig. 3 depicts the material required to melt it and maintain a solution at the right temperature. An aluminium block designed to adapt underneath a transparent plastic plate keeps the agarose solution liquid while it is transferred in the crystallisation plate by a Mosquito® robot (Fig. 3). Equilibration kinetics derived from refractive index measurements performed at various time intervals, indicate that 0.2% (m/v) low-gelling agarose (T_g = 28 °C) does not alter the rate of water evaporation (Fig. 3).

3. Diffusive versus convective mass transfer

Any crystal growth process is accompanied by the formation of solute concentration gradients. Concentration differences generate density gradients. The latter lead to flow in crystallising solutions and the associated differences in refractive index are visible as shadow patterns on Schlieren photographs or as phase shifts on interferograms (see e.g. Chen et al., 1979). They were observed during the growth of macroscopic salt crystals and also around the 0.3–1.7 mm-long (110) faces of lysozyme crystals (Pusey et al., 1988). At the surface of the latter the velocity of convective plumes reaches 50 μm/s at 18 °C. Tiny crystals measuring <20 μm across see their growth rate decline when they are exposed to the direct flow of solution. Comparative studies of inorganic crystal growth revealed that the silica network restricts the transfer of mass and heat between nutrient solution and crystal surface to diffusion (e.g. Rubin, 1969; Gits-Léon et al., 1987). Further, the network of both silica and agarose gels suppresses the convectional flow triggered by density gradients in steady solutions as does weightlessness (Robert et al., 1988). Hence, depletions zones around growing crystals are stable in the gel alike under microgravity (Otalora et al., 2001). It may also keep impurities away from crystal surfaces (see e.g. Chernov, 2003).

The dimensionless Grashof number (Gr) can be used to understand transport processes in solution. It is defined as the quotient of buoyancy over viscosity forces and is represented by,

$$Gr = L^3\beta\Delta c g \nu^{-2}$$

where L (in cm) is the characteristic length of the system (for example the diameter of a spherical crystallisation drop or of a cylindrical capillary tube), β the solute expansivity (in cm^3/mg), Δc the concentration difference (in mg/cm^3), g the acceleration due to gravity (9.81×10^2 cm^2/s on Earth), and ν the kinematic viscosity (in cm^2/s). When Gr is less than 1, the contribution of convection is so weak that mass transfer occurs essentially by diffusion (see e.g. García-Ruiz et al., 2001a).

Above equation indicates that Gr is proportional to the level of gravity. It can be 10^{-4}–10^{-9}-fold smaller than on Earth's surface in the microgravity environments existing respectively aboard manned orbiters and satellites. On the other hand, it depends even more upon the characteristic length L of the container since this parameter appears as L^3. Thus, inside an agarose gel with a pore diameter of 1 μm, Gr is about 10^9-fold less than in a spherical drop of solution measuring 1 mm across. Consequently, silica and agarose are perfect media to prepare crystals of biological macromolecules. Crystal growth takes place in the solution trapped inside the mesh of the hydrogel. From above theoretical considerations, one may expect to be able to produce on Earth crystals having properties similar to those of crystals that grow under microgravity.

4. Protein crystal growth in gels

4.1. Results from silica gels

Silica gels were seldom used to grow biological crystals since their introduction in the field (Robert and Lefaucheux, 1988). This is surprising because a kit of ready-to-use sodium silicate and acetic acid solutions is commercially available since many years. On the basis of its chemical composition, the silica gel prepared from these chemicals is innocuous. In contrast, the alcohol produced during the polymerization of gels prepared with TEOS or TMOS should be removed before use. Repeated additions and withdrawal of water

Table 2
Preparation of crystallisation assays containing agarose gel and crystal recovery.

Step

1. Prepare a 2% (m/v) stock solution of low-gelling-point agarose (T_{gel} = 28 °C). In an Erlenmeyer flask, dissolve the appropriate amount of agarose powder in ultra-pure water and heat it in the microwave at 90–100 °C. Pass the warm solution over a 0.22 μm porosity filter membrane to remove dust and insoluble particles. Dispense the filtrate in 1 ml aliquots and store at 4 °C or 20 °C.
2. Heat a sample of 2% (m/v) agarose stock solution to 80–90 °C in a heating block. Once the solution is completely liquid, move it to a block heated at 30–35 °C. Care should be taken to heat agarose suspension long enough (at least 5 min) at a sufficiently high temperature to melt all chains. Otherwise the pore size may not be reproducible. It may be necessary to place the crystallisation plates for a short time at low temperature (e.g. at 5–10 °C) when working with an agarose having a gelling temperature below room temperature.
3. Dispense reservoir solutions in the wells of crystallisation plates, sample wells or depressions of microplates.
4. Prepare crystallisation drops on glass coverslips or polystyrene microbridges by mixing one protein solution with one volume of reservoir solution and add the adequate volume of 2% (m/v) agarose solution to have a final concentration of 0.1–0.4% (m/v). The time required for complete polymerization will depend on agarose concentration.
5. After crystallisation, the gel can be dissected away with microtools under a binocular microscope. A small incision opens the agarose network and liberates the crystal. The enzyme agarase can be used to hydrolyse the polysaccharide chains but it must be free of proteases and/or nucleases. Crystals in a capillary are recovered in a drop of mother liquor by applying a little air pressure on one end. They may also be analysed in situ at room or cryotemperature since they are immobilised in the gel.

Safety note: Skin and eye protection should be worn during handling of hot agarose solutions.
Comments: The gelling temperature of agarose is usually given for a 1, 1.5 or 2% (m/v) solution in water. At a lower concentration the gelling temperature is lower. A liquid agarose solution can be handled by an automated pipeting station (see Fig. 3). Agarose does not form a gel in the presence of some crystallising agents (Gonzales-Ramirez et al. 2008). In this case it can be added first to the macromolecular solution and, after polymerization, the crystallising agent can be diffused into the gel by contact. Proteins such as lectins which bind carbohydrates may interact with agarose chains.

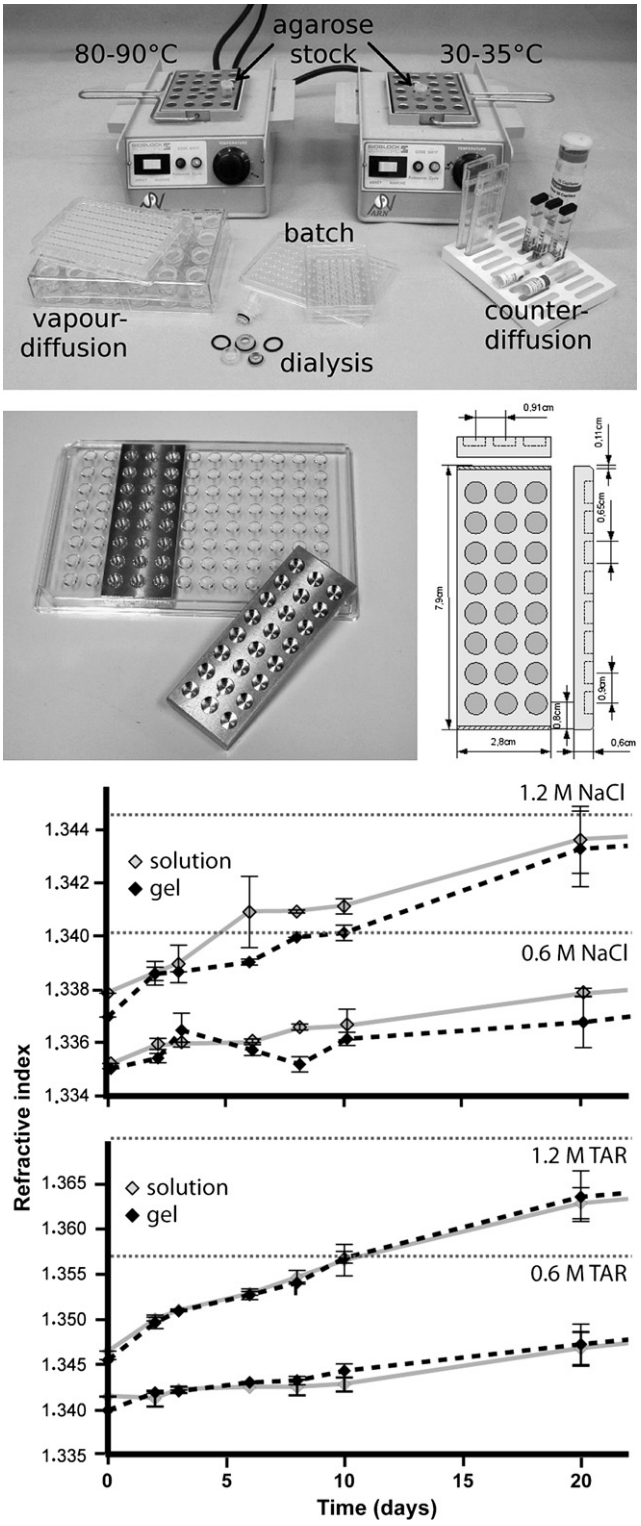

Fig. 3. Handling and properties of agarose gel. (Top) Heating blocks used for melting and maintaining agarose at the right temperature before addition to crystallisation assays. The gel is compatible with all crystallisation methods. (Centre) Aluminium plate fitting underneath a transparent Impact™ (from Greiner BioOne) plastic plate which can be warmed to maintain agarose liquid while it is transferred in crystallisation drops by a Mosquito® pipeting station (www.TTPLabtech.com). (Bottom) Equilibration kinetics for 0.6 and 1.2 M solutions of (upper panel) sodium chloride and (lower panel) sodium tartrate in the absence and in the presence of 0.2% (m/v) low-gelling temperature agarose. Buffers were 0.1 M ADA pH 6.8 and 0.1 M sodium acetate pH 4.5, respectively. Plots represent the variation of the refractive index of crystallisation drops (initial volume 30 μl) as a function of time. Data are means of four measurements. Horizontal dotted lines correspond to the equilibrium values reached by vapour diffusion in reservoir solutions.

or buffer solution are generally sufficient to reduce significantly the concentration of undesired contaminants. Convection currents are strongly attenuated by the silica gel network and above a critical concentration they are abolished. In the gel, crystals grow at the place were they nucleate (Fig. 4). Within a broad range of concentrations the mechanical properties (e.g. the elasticity) of silica gels are compatible with the growth of crystals with great dimensions (up to several mm on the edge).

The first crystallisations of hen lysozyme and porcine trypsin indicated that these macromolecules diffuse freely through the polysiloxane hydrogel matrix. The crystals are birefringent between crossed polarizers and have the same habit as controls prepared in pure solution but nucleation is less frequent (Robert and Lefaucheux, 1988). Interferential holography images revealed protein concentration gradients around growing crystals (Lefaucheux et al., 1984) (see Fig. 4). Cudney et al. (1994) experimenting with various proteins, one virus and one transfer ribonucleic acid, observed that the presence of the gel decreases the crystal growth rate and leads to fewer nuclei ending in crystals with larger sizes. Crystals were more stable but the habit of some of them was altered. On the other hand, the habit of crystals grown from mixtures of closely related lysozymes from hen and turkey egg-white was much less altered in the presence of TMOS gel (Provost and Robert, 1995).

At high TMOS concentrations surface energy anisotropy is so low that single lysozyme crystals become spherical (García-Ruiz et al., 1998a). The gel network is so strong that a ghost of silica is left after complete dissolution of the protein. In spite of the strength of the gel, the short-range order is maintained; these reinforced crystals diffract X-rays to 1.5 Å, are less sensitive to dehydration and can easily be handled at ambient conditions. The comparison of gel-free and gelled lysozyme solutions by small angle neutron scattering (SANS) over the time intervals of prenucleation and of crystal growth led to the conclusion that a proportion of the protein adsorbs on the gel. The concomitant decrease of the concentration of the soluble protein results in lower supersaturations and nucleation rates. Hence, the silica matrix can be considered as a nucleation inhibitor for small molecules and for lysozyme (Vidal et al., 1998b).

Silica-gel-grown lysozyme crystals produce diffraction patterns with much sharper reflections (extending to at least 1.6 Å resolution) than solution-grown ones. In X-ray topography these crystals distinguish by the presence of single Bragg peaks. The existence of a very good long-range order in the lattice was deduced from rocking curves with full-width at half-maximum (or mosaicity) of only 15 arc seconds and low level of defects visible on topographs (Vidal et al., 1999).

Altogether, these encouraging results should encourage crystal growers to test silica gels with their molecules. Fig. 5 displays photographs of crystals of icosahedral viruses grown in this medium. Their habit is identical to that of crystals prepared in the absence of gel. Crystallisations of concanavalin A, trypsin and C-phycocyanin in TMOS gel yielded fewer numbers of crystals with augmented volume (Chayen and Saridakis, 2002).

4.2. Clues from agarose gels

Few protein crystal growers have tried this easy-to-use polysaccharide available on the shelves of any molecular biology laboratory. Table 3 lists proteins and nucleic acids crystallized in agarose which had diffraction properties suitable for structure determination. The resolution of the final model is equal or better than 2 Å for 6 amongst the 15 proteins. It is noteworthy that (i) agarose is not only suitable for the crystallisation of proteins but also of nucleic acids, and that (ii) the diffraction limits can be better than 2 Å and even as high as 1.2 Å even when data collection is done at

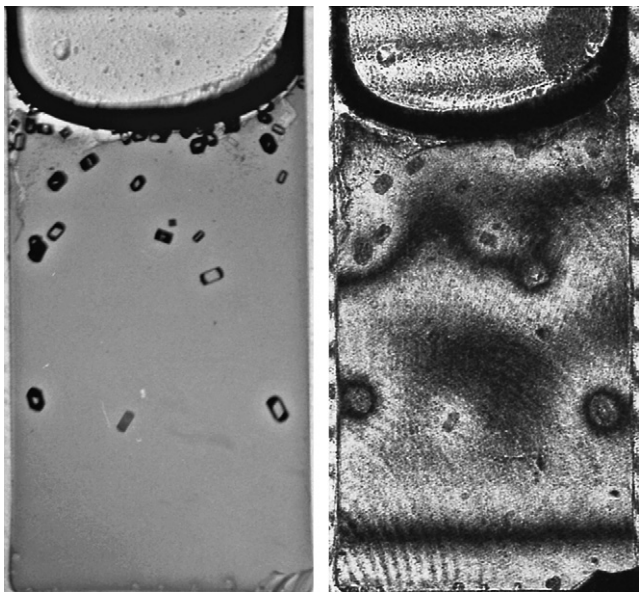

Fig. 4. Growth of hen egg-white lysozyme crystals in silica gel. The protein was at 30 mg/ml in a solution containing 0.4 M sodium chloride, 50 mM sodium acetate and 0.81% (w/v) tetramethyl-polysiloxane. (Left) Crystals visualized in transmitted white light. (Right) Salt gradient imaged by holographic interferometry. The width of the quartz cell is 10 mm. For experimental details, see Lefaucheux et al. (1984). The interference fringes are indicative of the mass transfer and reveal the lower concentration of soluble protein near the crystals.

a temperature between +15 and +20 °C (Table 3). This well illustrate that the presence of the polysaccharide is not deleterious to crystal quality. It is also a strong argument to analyse crystals at a temperature close to that at which they have grown and to which the protein is functional in vivo.

Numerous rigorous studies were designed to understand protein crystal growth in agarose. Protein concentration gradients around growing crystals like those seen in silica gel were revealed when optical phenomena were monitored (Lefaucheux et al., 1984). Refractive index variations during lysozyme nucleation in supersaturated solutions in a gel at a concentration of 0.1% (m/v) were interpreted as amorphous precipitate (appearing when protein and crystallising agent are mixed) that does not sediment alike in free solution. Hence, more protein is available for nucleation than in the absence of gel (Robert et al., 1994; Vidal et al., 1996). A study of the evolution of the fringe patterns in Michelson interferometry showed that the protein-depleted zone forms in three steps. First nucleation takes place, then purely diffusive mass transfer occurs, and finally the concentration of soluble protein decreases slowly until crystal growth stops (Vidal, 1997). The prenucleation clusters found by SANS led to the conclusion that the polysaccharide network promotes nucleation and to the hypothesis that lysozyme molecules first associated randomly before they rearrange as ordered nuclei (Vidal et al., 1998a). Agarose is also known to favour the nucleation of many small molecules. It is probably for technical reasons that SAXS analyses did not detect lysozyme aggregates in this gel (Finet et al., 1998).

Actually, the gel behaves as a non-Newtonian liquid when it is present at low concentration. At higher concentrations, it is viscoelastic (García-Ruiz et al., 2001a). Inside the loose network of a 0.15% m/v gel crystals grow as fast as in solution (Lorber and Giegé, 2001). As shown in Fig. 3 vapour diffusion is not slowed down by the gel. Ligands (such as natural substrates, analogs or inhibitors), compounds rich in electrons required for the preparation of heavy atom derivatives, and cryoprotectants like glycerol or PEG (Gavira et al., 2002) diffuse through the gel without any restriction. A recent kinetic analysis using a temperature-jump technique proposed that the network of the gel inhibits the nucleation of lysozyme and that in turn this results in bigger crystals (Wang and Liu, 2008).

An obvious advantage of the gel network is that crystal nuclei are trapped and cannot move in the solution, an invaluable advantage for photographic (e.g. as reported by Charron et al., 2002) or spectroscopic analyses (Bonneté et al., 1996; Finet et al., 1998; Vidal et al., 1998a,b). The addition of a low concentration of agarose (0.15% m/v) minimises the effects of gravity on fluid dynamics. This is a prerequisite for the implementation of counter-diffusion in glass capillaries (García-Ruiz et al., 1993, 1999; García-Ruiz and Moreno, 1997; Ng et al., 2003). In a similar investigation, 0.05% (m/v) gellan gum (a water-soluble bacterial polysaccharide) sufficed to depress convection and detect wider concentration gradients near the surface of growing lysozyme crystals and lower transport rates than in solution (Hou et al., 2001). On the opposite extreme of the concentration scale, semi-solid gels composed of up to 2% (m/v) agarose are also compatible with protein crystal growth (Sugiyama et al., 2009).

On many occasions agarose was employed to immobilise crystals in solution or inside capillary tubes (for some examples see Fig. 5 and Section 6.3 for details on crystallisation in capillaries). For instance, it was added in vapour diffusion (Provost and Robert, 1991; Bernard et al., 1994), in microdialysis (Thiessen, 1994) and counter-diffusion experiments (García-Ruiz et al., 2002) or in drops kept under oil (Moreno et al., 2002). In another instance it was used in the form of beads (Willaert et al., 2005) or to solidify the reservoir solution (Wong et al., 2009). Also, crystals grown under microgravity in 0.2% (m/v) gel stay at the place where they nucleate. They are less sensitive to mechanical shocks and to temperature fluctuation during their return from space to the laboratory. Their crystallographic quality is superior to that of controls prepared in parallel in the laboratory (Lorber et al., 1999b). The mesh of polysaccharide chains is strong enough to prevent crystal sedimentation (Normand et al., 2000) and its elasticity is no hindrance to the formation of the crystal lattice. Onboard an orbiter the gel attenuated crystal motions like drift and stirring provoked by acceleration peaks (Lorber et al., 2000). In weightlessness nucleation occurs mainly in the bulk of the sample, is more synchronous and 2 times faster than on Earth (Lorber and Giegé, 2001).

Agarose also facilitated the measure of rocking curves on proteins crystal grown in magnetic fields (Lübbert et al., 2004) as well as cryocrystallography analyses (Lopez-Jaramillo et al., 2001; Biertümpfel et al., 2005) and single-wavelength anomalous scattering diffraction (SAD) data collection inside X-ray capillaries (Gavira et al., 2002). It abolishes buoyancy-driven convection in samples subjected to high hydrostatic pressures (Kadri et al., 2002, 2003a,b, 2005) or magnetic fields (Gavira and Garcia-Ruiz, 2009).

The role of agarose in biological crystals has been investigated. SEM images of lysozyme crystals grown in this gel and subsequently frozen and fractured display randomly oriented gel fibers which run right through the crystalline surface (Gavira and Garcia-Ruiz, 2002). The soft material has an ordered structure neither in the core of the crystal nor near its surface and it does not seem to interfere with the diffraction quality of the lattice of biological molecules. On the contrary, crystals containing agarose are less fragile during irradiation either at room temperature (Sauter et al., 2002) or after cryocooling ex situ (Zhu et al., 2001; Biertümpfel et al., 2005) or in situ (Gavira et al., 2002).

Although some crystals grown in agarose gel have an unusual aspect and seem to be of inferior quality, they may diffract X-rays to

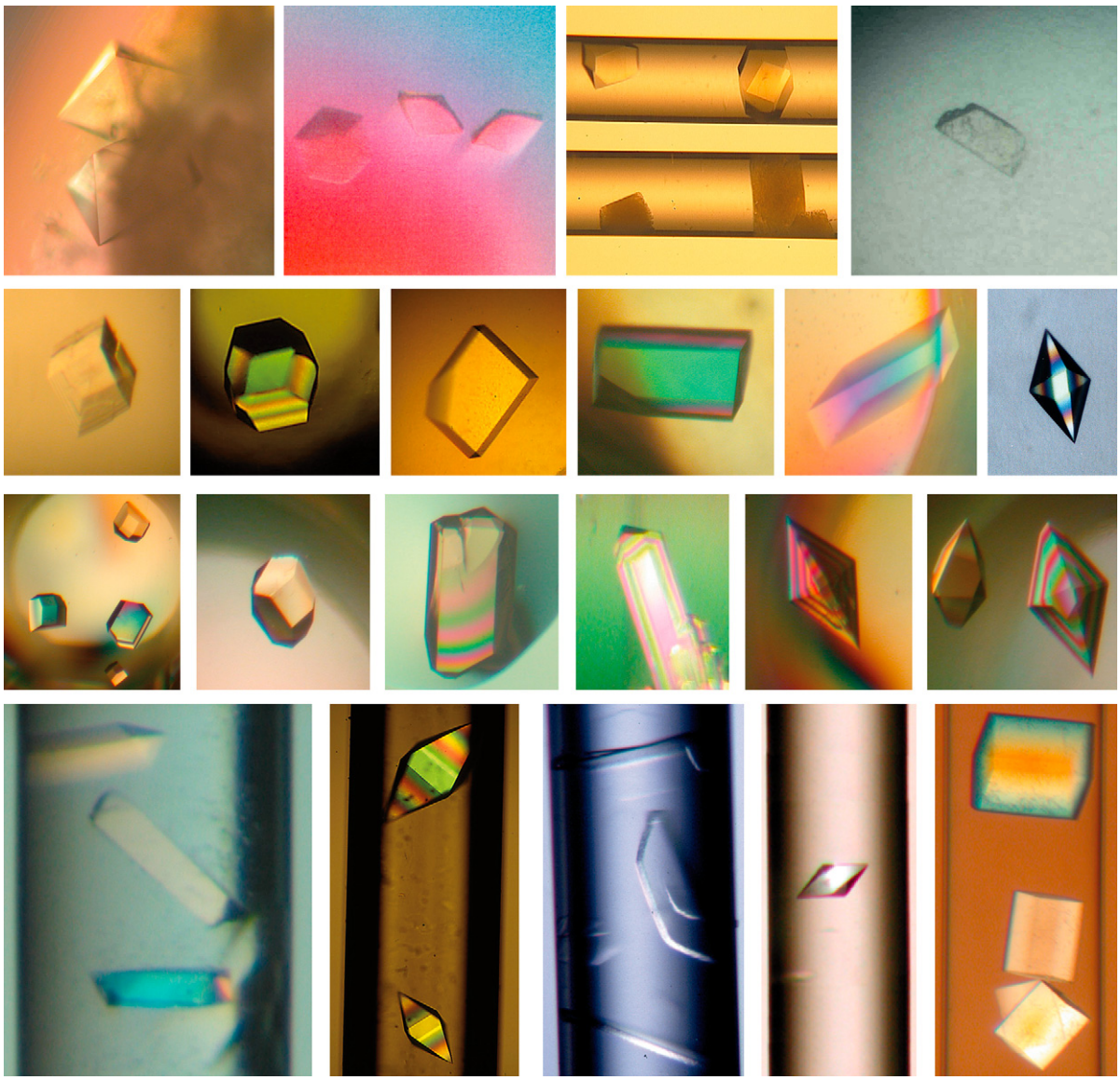

Fig. 5. Virus, protein and nucleoproteic complex crystals grown in gels. (From top to bottom, first row) Viruses in silicate or agarose gel. (From left to right) Brome mosaic virus at 55 mg/ml in 11% (m/v) PEG-8000 and 0.1 M sodium acetate pH 4.5; Turnip yellow mosaic virus at 8.5 mg/ml in 0.6 M ammonium phosphate with 0.1 M 2-(N-morpholino) ethanesulfonic acid pH 3.9 (controls without gel produce crystals with similar habits); Tomato bushy stunt virus at 25 mg/ml grown inside capillaries containing (top) solution without gel and (bottom) 0.2% (m/v) agarose with $T_g = 28\ ^\circ C$; Grapevine fanleaf virus at 3 mg/ml in 0.2% (m/v) agarose showing a granular structure. All crystals measure 150–300 microns across. (Second row) Proteins in agarose gel. (From left to right) Cubic concanavalin crystal prepared in batch; Tetragonal hen lysozyme crystal prepared by dialysis; Monoclinic plates (in polyethylene glycol) and orthorhombic prisms (in sodium formate) of *Thermus thermophilus* aspartyl-tRNA synthetase-1 (a dimer of M_r 130 000) prepared by vapour diffusion; Orthorhombic crystal of *Deinococcus radiodurans* glutaminyl-tRNA synthetase prepared by batch under paraffin oil in PEG-3350 solution; tetragonal dipyramide of sweet-tasting thaumatin grown by free-interface diffusion. Greatest crystals reach about 600 microns in length. (Third row) Protein crystallisation in novel organic gels. (From left to right) Tetragonal hen lysozyme in sodium chloride solution containing either 2% (m/v) methyl cellulose Metolose® 90SH30000 or 3% (m/v) hydroxyprolyl cellulose (M_r 10^5); Hexagonal turkey lysozyme prisms in a solution of the same crystallising agent containing either 2% (m/v) hydroxyethyl cellulose (M_r 1.3×10^6) or 2% (m/v) methyl cellulose Metolose® 90SH15000; Thaumatin dipyramides in sodium tartrate solution containing either 5% (m/v) hydroxyethyl cellulose (M_r 250 000) or 0.5% (m/v) hydropropylmethyl cellulose. All crystals were prepared in Cryschem® plates using 10 μl sitting drops equilibrated against 500 μl reservoirs. Hen lysozyme was at 30 mg/ml, turkey lysozyme at 15 mg/ml and thaumatin at 60 mg/ml. The crystals are up to 2 mm long. Metolose® is a registrated mark of Shin Etsu Chemical Co., Ltd. (Last row) Protein crystallisation in 0.2% (m/v) agarose gel inside glass capillaries. (From left to right) Prisms of *D. radiodurans* glutaminyl-tRNA synthetase grown in batch; Crystals of T4 endonuclease VII in complex with a DNA cruciform junction grown in the presence of agarose and PEG 6000 (Biertümpfel et al., 2002); Tetragonal thaumatin dipyramides grown by diffusing sodium tartrate through the gel containing the protein; Crystal of Sm1 protein from *Aeropyrum pernix* complexed with a 14-mer RNA in PEG 5000-MME and agarose; Orthorhombic crystals of *T. thermophilus* aspartyl-tRNA synthetase-1 grown by gel acupuncture method (for details see Moreno et al., 2005).

high resolution. Those of the collagen-like peptide (PPG_{10}) prepared on Earth or under microgravity exhibit a high degree of surface roughness (Berisio et al., 2002) and those of the tomato bushy stunt and grapevine fanleaf viruses have a granular structure (Fig. 5). The first diffract X-rays to 1.45 Å (Berisio et al., 2002) and the second (Lorber et al., 1999a) and third (Schellenberger et al., to be published) to at least 3 Å.

5. Crystal quality enhancement

Over the years it has been found that crystallisation in a gel-like agarose may have several benefits. Table 4 lists the advantages which can be expected from the usage of gels during the optimisation of crystallisation conditions. The suppression of convection and the immobilisation of nuclei are favourable to a growth in three

Table 3
Examples of protein and nucleic acid structures derived from crystals grown in agarose gel.

Macromolecule or complex and mass	Crystallising agent[a]	Agarose (% m/v)	Res. (Å)	T (K)	Reference
Protein					
Mitochondrial cytochrom b–c_1, 236 000	PEG-4000	0.1–0.4	4.5	n.g.	Yu et al., 1994
T-cell receptor β-chain – superantigen, 40 000	PEG-1000	0.025	3.5	278	Fields et al., 1996
tRNA-guanine transglycosylase, 43 000	PEG-8000	0.2	1.85	288	Romier et al., 1996
Cholera toxin receptor, 14 000	PEG-1000 (PSac)	0.2	2.3	295	Merritt et al., 1997
L-Asparaginase II, 38 000	MPD	1.0	2.3	295	Kozak and Jaskolski, 2000
Aspartyl-tRNA synthetase-1 (monoclinic), 130 000	PEG-8000	0.1	2.65	100	Zhu et al., 2001
Thaumatin, 22 400	Sodium tartrate	0.15	1.2	293	Sauter et al., 2002
Globular head of complement protein C1q, 50 000	PEG-4000 (NDSB)	0.2–0.4	1.9	n.g.	Gaboriaud et al., 2003
3-Dehydroquinate dehydratase, 16 000	Ammonium sulphate	0.1	1.7	100	Maes et al., 2004
Archeal Hjc resolvase, 15 000	PEG-1000	0.3	2.7	100	Biertümpfel et al., 2005
Bacterial metalloprotease FtsH, 51 000	PEG-400	0.1–0.2	2.8	110	Bieniossek et al., 2006
Clam hemoglobin II, 17 000	Ammonium sulphate	0.1	2.0	100	Gavira et al., 2006
Yeast ornithine acetyltransferase, 48 000	PEG-400	0.1	2.8	100	Maes et al., 2006
Sinorhizobium dihydropyrimidinase, 200 000	Sodium formate	0.1	1.85	100	Martinez-Rodriguez et al., 2006
RNA					
Bacterial ribonuclease P RNA, 130 00	MPD	0.05	3.3	100	Kazantsev et al., 2005

Protein structures are listed in chronological order.
Abbreviations: NDSB, non detergent sulfobetaine; n.g.: not given; PSac, pentasaccharide,; Res.: resolution; T: temperature.
[a] Peculiar additives are indicated between parentheses.

dimensions yielding crystals with fully developed faces and greater volumes (see Fig. 5 for some examples). The polymer chains filling randomly the solvent channels of the crystal make the protein lattice less sensitive to mechanical and osmotic shocks and also to thermal fluctuations or freezing. The quiescent gel medium is an optimal environment for defect-free nucleation and crystal growth. As in the case of small molecules, nucleation is better controlled and impurities have less deleterious effects.

Recent in situ observations performed by laser confocal interference contrast microscopy led to the conclusion that the polysaccharide has almost no effect when the protein solution is pure but it plays the role of a filter when the solution contains large impurities (van Driessche et al., 2008). This is well illustrated by the two following results. (i) In the presence of 0.3% m/v agarose, clear impurity depletion zones are visible around crystals of colourless apoferritin (M_r 450 000) crystallized in a solution contaminated with red-coloured holoferritin dimers (M_r 900 000) (Chernov et al., 2001). (ii) MALDI-TOF and electrospray analyses (performed according to established procedures, see Potier et al., 2000) detect no significant difference in composition between thaumatin I crystals grown in solution and those grown in 0.2% (m/v) agarose gel when thaumatin I (M_r 22 204) contains traces of thaumatin II (having 98% sequence identity and the same isoelectric point pI 8.4 but differing in M_r by only 84 or ~0.4%) (Lorber, Potier and Sanglier, unpublished results). There must be a minimum of structural differences between related macromolecules so that the discrimination can occur during the crystallisation process. In other words, the impurity filtering effect operates when structural dissimilarities are strong enough. The same conclusion was drawn from comparative analyses of thaumatin crystals grown in gel either under normal gravity or under microgravity for which chromatography, electrophoresis, N-terminal sequencing and mass spectrometry analyses were also unable to show a difference in macromolecular content (Lorber and Giegé, 2001). In the case of hen and turkey lysozymes which differ by 7 amino acids (M_r 14 313 and 14 209, respectively), by a net charge of 1, and consequently by solubility

Table 4
Some non-exclusive advantages of crystallisation in gel.

Effect of gel[a]	Example of macromolecule, M_r	Reference
3D growth in agarose gel results in crystals with greater volume	tRNA-G transglycosylase, 42 700	Romier et al., 1996
	Endonuclease-oligoDNA, 49 000	Biertümpfel et al., 2002
Reinforced crystal lattice in silica gel	Hen lysozyme, 14 500	Garcia-Ruíz et al., 1998a
Protection of crystals in agarose gel during:		
- Handling and irradiation	Thaumatin, 22 400	Sauter et al., 2002
- Osmotic stress when soaking with ligands	Aspartyl-tRNA synthetase-1, 130 000	Sauter et al., 2009
- Cryocooling	Aspartyl-tRNA synthetase-1, 130 000	Zhu et al., 2001
	Hjc resolvase, 15 400	Biertümpfel et al., 2005
	Grapevine fanleaf virus, 6×10^6	Schellenberger et al., to be published
Reproducible diffraction quality in agarose gel	Hen lysozyme, 14 500	Dong et al., 1999; Lorber et al., 1999a
Sharper and more intense reflections when crystals are grown in silica or agarose gels	Hen lysozyme, 14 500	Vidal et al., 1999; Lorber et al., 1999a
	Human serum albumin, 66 000	Miller et al., 1992
	Aspartyl-tRNA synthetase-1, 130 000.	Lorber et al., 1999a; Moreno et al., 2005
	Dehydroquinate dehydratase, 205 000	Maes et al., 2004
Nucleation favored or decreased	Hen lysozyme, 14 500	Robert et al., 1994
	Several proteins, virus & tRNA	Cudney et al., 1994
	Hen lysozyme, 14 500	Vidal et al., 1998a,b
	Turkey lysozyme, 14 500	Hirschler et al., 1995
Attenuation of impurities effects	Hen lysozyme, 14 500	Provost and Robert, 1995, Hirschler et al., 1995 Hirschler and Fontecilla-Camps, 1996.
Suppression of twinning	Alcohol dehydrogenase, 74 000	Sica et al., 1994
Improvement counter-diffusion setup	Insulin, 5740	Gavira and Garcia-Ruiz, 2002

[a] Agarose and polysiloxane concentrations were 0.1–0.5% (m/v) and 0.5–2% (m/v), respectively.

(Provost and Robert, 1995), positive effects were observed. In the case of iron-deprived apoferritin (M_r 476 000) contaminated by dimers of holoferritin containing ~4500 iron atoms per particle (M_r 1.44×10^6) (Chernov et al., 2001), the effect was amplified.

Most gel-grown crystals have superior diffraction properties with respect to crystals grown in the absence of gel (see Table 3). Their quality is more reproducible, their diffraction patterns exhibit more intense and sharper reflections. Plot of the average intensity-to-noice I/σ ratio as a function of the resolution for human serum albumin crystals grown in 1.5–0.3% (m/v) agarose gels showed for the first time the real improvement of crystal quality (Miller et al., 1992). Later this was confirmed with thaumatin (Lorber et al., 1999b). Further, the comparison of lysozyme crystals at a resolution of 1.8 Å indicated that laboratory gel-grown ones are close to microgravity-grown ones with regard to their superior diffraction properties (including more intense and sharper reflections) and the number of observable ordered water molecules which is greater than for crystals prepared in solution in the laboratory (Dong et al., 1999).

The enhanced diffraction properties of crystals of aspartyl-tRNA synthetase-1 support the observations made on small-M_r lysozyme, thaumatin and albumin (Moreno et al., 2005). The quality of the crystals of this large size and dimeric enzyme (M_r 130 000) with a multi-domain architecture also benefits from the presence of agarose gel (Fig. 6). Further, the diffraction limit of grapevine fanleaf virus crystals could be extended beyond 6 Å by adding 0.2% (m/v) low-gelling temperature agarose to the crystallisation droplets (Fig. 7).

6. Perspectives for crystallisation in gels

The choice of gels usable for crystallisation is not at all limited to silica and agarose. Two examples of other suitable gel-like media are given below. On the other hand, in addition to silica and agarose gels, convection is also minimal inside capillary tubes. As described below, gels can be used in combination with them.

6.1. Lipidic phases

Mixtures of water and certain lipids organise in complex phases under ambient conditions. These phases were extensively studied as models for the membranes of living organisms (see e.g. Luzzati and Tardieu, 1974). Crystallographic analyses revealed that they are continuous systems made of two compartments (e.g. Mariani et al., 1988; Lindblom and Rilfors, 1989; Luzzati et al., 1993). Later, it was found that cubic phases formed by the lipids 1-monooleyl-*rac*-glycerol (monoolein, Fig. 8) or 1-monopalmitoleyl-*rac*-glycerol are suitable as matrices for the 3D crystallisation of membrane proteins (Landau and Rosenbusch, 1996; Rummel et al., 1998; for a review of recent developments see Johansson et al., 2009). Indeed, the lipidic component of the mesophase interacts with the hydrophobic domains of membrane proteins and so crystallisation can take place

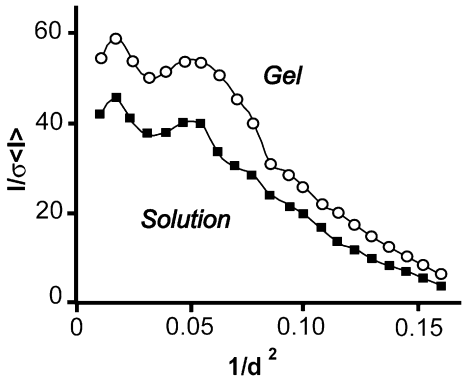

Fig. 6. I over sigma (I) plots for solution- and gel-grown *Thermus thermophilus* aspartyl-tRNA synthetase-1 crystals. The diffraction intensities of orthorhombic crystals with similar dimensions and volumes are compared. Data adapted from Moreno et al. (2005). A similar result had been reported for human serum albumin (Miller et al., 1992) and thaumatin (Lorber et al., 1999b).

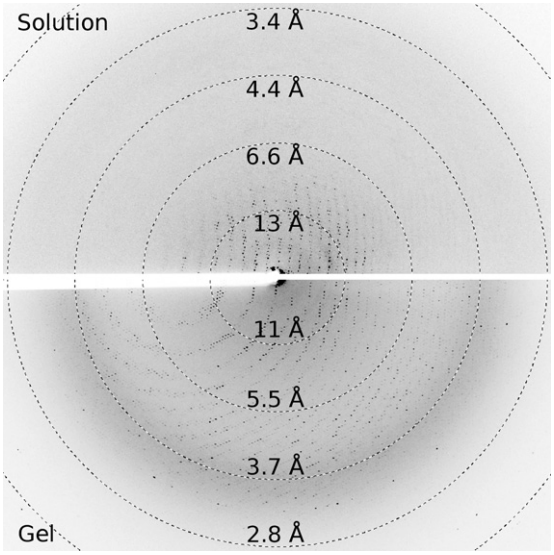

Fig. 7. Gel and cryocooling. Diffraction patterns after cryocooling of grapevine fanleaf virus crystals grown (top half) in the absence and (bottom half) in the presence of 0.2% (m/v) agarose. X-ray diffraction analyses were performed on crystals of comparable volumes. The wavelength was the same (λ = 0.98 Å) but the crystal-to-detector distance was 250 and 300 mm, respectively.

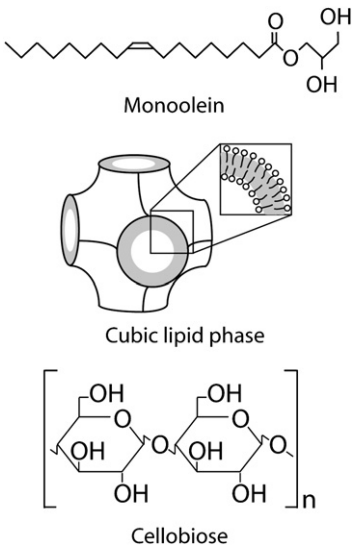

Fig. 8. (Top) Structure of 1-monooleyl-*rac*-glycerol (monoolein). (Centre) Schematic representation of the elementary building unit of a cubic mesophase with a close-up view of the lipid bilayer (Drawing adapted from Lindblom and Rilfors, 1989 and from Landau and Rosenbusch, 1996.) These units do not exist as such in solution. They form a periodical 3D network of nanochannels in which they are located at the nodes of a body-centered cubic lattice (i.e. 1 unit is placed at each of the 8 corners of a cube and one in its centre). The complete system is composed of a continuous hydrophobic phase (composed of the hydrocarbon chains of the lipid, shown in grey colour) and a continuous hydrophilic phase (composed of the polar heads of the lipid shown in white colour) running side-by-side. (Bottom) Cellobiose is made of two D-glucopyranose molecules linked by a β 1–4 bond. In cellulose compounds, the number of cellobiose units varies from a few hundred to several thousands. The hydrogen atoms of free hydroxyl groups can be substituted chemically by methyl, ethyl or propyl groups.

in the absence of detergent (Nollert et al., 1999). The preparation of lipidic mesophases was gradually standardized (see e.g. Caffrey, 2003; Cherezov and Caffrey, 2005). A detailed protocol has been published recently (Caffrey and Cherzov, 2009). Successful crystallisations are listed in the Membrane Protein Data Bank (Raman et al., 2006). Membrane protein crystals can be harvested with the help of microtools (see Caffrey, 2009). They may be recovered from the lipid matrix by enzymatic hydrolysis with *Candida rugosa* lipase (Nollert and Landau, 1998). Solubilization with a detergent like octyl-β-D-glucoside is an alternative.

Interestingly, the aqueous compartment of the cubic phase can accommodate water-soluble proteins. It can be employed as a gel to crystallise non-membrane proteins (Landau et al., 1997; Cherezov and Caffrey, 2003). In this case crystallisation is independent of the type of phase and the packing arrangement of the lipidic compartment. As with agarose the phases are not stable in the presence of all crystallising agents (for results of compatibility tests, see e.g. Landau et al., 1997; Cherezov et al., 2001). From the point of view of the Grashof number, the nanochannels composing the lipidic phases are expected to be even a better diffusive medium than the mesh of agarose gels since their dimensions are smaller (i.e. in the range of the size of protein or not more than 100 nm depending on the degree of swelling).

6.2. Cellulose derivatives

Besides polysiloxane and agarose, a number of chemicals and biochemicals form gels or gel-like media when they are dissolved in water. A priori all those which are not transparent (like carboxyvinyl polymers), not neutral (like polyacrylic and polygalacturonic acids) or free of ions (like alginates and carrageenans) are not suitable for protein crystallisation. This holds for substances of ill-defined composition (such as gums from higher plants or algae, pectins from fruit, chitosans extracted from crustacean carapace, or gelatines from animal skin and bones which may be contaminated by proteins like proteases or nucleases).

Amongst the chemically neutral and biochemically pure ones are cellulose derivatives. Their chains encompassing hundreds to thousands cellobiose units associate in bundles via hydrogen bonds (Fig. 8). An aqueous solution of these fibres (which are either microcrystalline or non-cristalline) behaves like a gel (see e.g. Fortin and Charlet, 1989; Kalinina et al., 2001; Saito et al., 2003; Kondo et al., 2004; Silva et al., 2008). Over a decade ago, carboxymethyl cellulose was already identified as a potential crystallising agent for proteins, nucleic acids and viruses (Patel et al., 1995).

We have used following cellulose derivatives to grow protein crystals at room temperature. For instance, methyl cellulose (at 1% m/v), hydroxyethyl cellulose (with M_r 250 000–1.3 × 10^6 at respectively 5% and 1% m/v), hydroxypropyl cellulose (M_r 10^5–10^6 at 3–4% and 10% m/v), and hydroxypropyl methyl cellulose (M_r 10^5 at 0.5–1% m/v) dissolved in water form gels which are moderately viscous, transparent and stable over a broad range of temperatures and crystallising agents. These compounds are fully compatible with the crystallisation of soluble model proteins. Tetragonal hen egg-white lysozyme and thaumatin crystals grown at 20 °C in the presence of methyl-, hydroxyl propyl-, hydroxyethyl- and hydroxypropylmethyl cellulose diffract X-ray at least to the same resolution and with the same apparent mosaicity as control crystals (Lorber, unpublished results). The same holds for 2 mm-long hexagonal prisms of turkey lysozyme prepared in hydroxyethyl cellulose and for thaumatin dipyramides grown in 5% m/v hydroxyethyl cellulose (M_r 250 000) or in 10% m/v hydroxypropyl cellulose (M_r 10^6) (Fig. 5). These examples demonstrate that the panel of diffusive media can be extended beyond silica and agarose gels. Further investigations will tell if these novel gels can replace them and have other hidden properties.

6.3. Gels and capillary tubes

Some crystal growers have used various gels as plugs or dialysis membranes (also sometimes called a physical buffer) to separate the macromolecular solution from the crystallising agent solution (Zeppezauer, 1971; Littke and Johns, 1984). Early devices consisting essentially of small-diameter (1 mm or less) glass tubes had the purpose to consume less sample volume. Delicate free-interface diffusion crystallisation experiments (Salemme, 1972) were performed inside very thin tubes. There, capillary forces counterbalance the buoyant forces and suppress any perturbation at the contact area between protein and crystallising agent solutions which would lead to irreversible mixing. Hence, the effects of gravity are minimized, convection is abolished, mass transport restricted to diffusion and concentration gradients around growing crystals are stabilised (for reviews, see García-Ruiz, 2003; Ng et al., 2003). This environment is close to the one attained under microgravity. Further advantages were then expected by combining gel and a capillary geometry.

Three crystallisation methods are represented in Fig. 9. The diameter of the capillary tubes should not exceed 0.3 mm in order to suppress crystal and precipitant sedimentation under gravity. This effect is not negligible when the diameter is superior (see e.g. Moreno et al., 1999b). The first and most simple is batch

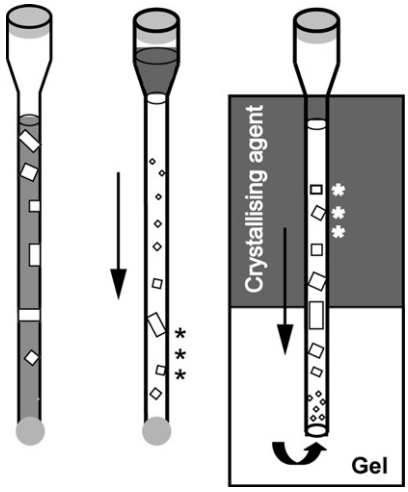

Fig. 9. Crystallisation in transparent capillary tubes. (Left) In batch crystallisation the macromolecule and the crystallising agent are mixed with the gel and transferred inside the capillary. When supersaturation is high enough, the initial mixture may become opaque. At places were crystals grow it will then gradually clear off and a halo will become visible around each crystal. (Centre) Macromolecule (protein or virus) is immobilised in the gel. When the crystallising agent is brought into contact at the top, it diffuses slowly inside the gel. Crystallisation occurs by counter-diffusion. The tomato bushy stunt virus crystals shown in Fig. 6 were prepared in this way in agarose gel inside capillaries. (Right) In the gel acupuncture method (GAME) an open capillary containing the macromolecule (in solution or in gel) is punched into a thick layer of silica or agarose gel. When crystallising agent solution is placed onto the gel, its molecules diffuse into the gel and after some time enter very slowly inside the capillary. As in the centre of the figure, a wave of higher supersaturation is then generated which propagates inside the capillary. This system is reminiscent of a micro-diffusion cell consisting of an X-ray capillary closed on one end by a plug of polyacrylamide gel (for a drawing, see Zeppezauer, 1971). To take advantage of capillarity effects, experiments with protein solution are performed in tubes with an inner diameter of 0.3 or less. Greater diameters (up to 1 or 2 mm) may be used with protein solution containing a low concentration of gel. Arrows indicate the direction of the diffusion of crystallising agent molecules. Stars mark the approximate areas were crystals of highest quality are expected to grow.

crystallisation. Capillary tubes were useful to study the effects of physical parameters such as electric (Mirkin et al., 2003) or magnetic fields (Moreno et al., 2007; Gavira and Garcia-Ruiz, 2009; Moreno, Sauter, Giegé and Lorber, unpublished results).

The second method is based on counter-diffusion: the macromolecule mixed with the gel is loaded in the capillary and the crystallising agent brought in contact with the gel diffuses through it to trigger nucleation and crystal growth (García-Ruiz, 2003). In the "gel acupuncture" method (GAME, García-Ruiz and Moreno, 1994, 1997; García-Ruiz et al., 1998b), crystallisation also proceeds very slowly by counter-diffusion. In both cases (Fig. 9), the diffusion of the crystallising agent across the macromolecular solution generates a wave of local supersaturation that displaces along the capillary (García-Ruiz et al., 2001b). The latter creates a self-optimization process (García-Ruiz et al., 2001b) which can be further improved by adding a layer of silicone oil. This fluid slows down the diffusion and thus enhances crystal growth (Moreno et al., 2002). When enough protein is available, crystals can fill completely the tube and take a cylindrical shape (García-Ruiz et al., 1995, Otalora et al., 1996; Moreno et al., 1996). The theory of counter-diffusion was established and the kinetics of the process was simulated (Otalora and García-Ruiz, 1997; García-Ruiz and Moreno, 1997; García-Ruiz et al., 1998b; Carotenuto et al., 2002).

Capillary tubes like X-ray glass capillaries or tubes made of synthetic polymers (Potter et al., 2004) are convenient sample container. A special box was designed to contain several experiments (García-Ruiz et al., 2002). When agarose is present in the protein solution, the crystals can be extracted by displacing it with either air or grease. (A more sophisticated method uses femtosecond laser ablation to detach crystals nucleated onto glass walls, see Kashii et al., 2006). The excess of gel is then removed as explained in Table 2. Another advantage of the method is that crystals can be soaked in situ with a cryoprotectant before freezing (Lopez-Jaramillo et al., 2001). Heavy atoms can be diffused into them for structure determination (Gavira et al., 2002) and other small molecules to act on crystal composition (Domínguez-Vera et al., 1996). Thus, crystal quality can be evaluated without manual handling (see e.g. Sugahara et al., 2009).

7. General conclusion

In retrospect the potential of gels has been so far largely underexploited by protein and nucleic acid crystal growers in spite of a body of experimental results which demonstrate that crystallisation in such diffusive media may be more advantageous than crystallisation in pure solution. It is no longer merely our claim that gels may be advantageous for the crystal growth of biological macromolecules. Indeed, the 3D structures of a wide variety of macromolecules derived from crystals prepared in gels are as many pieces of evidence that the technique is applicable not only to proteins but also to nucleic acids (RNA, DNA) as well as nucleoprotein complexes and viruses. Further, the crystals grown in gel may diffract X-rays more strongly and have a less mosaic structure, which in turn facilitates the crystallographic analysis. In addition, gel-grown crystals may be more stable against temperature fluctuations, mechanical shocks, or differences in osmotic pressure occurring upon the soaking with a ligand, inhibitor, cryoprotectant or heavy atom. Their reinforced lattice is more resistant to cryocooling or even to diffraction data collection at room temperature. A supplementary benefit is that all crystallisation methods can accommodate a gel without perturbing the equilibration pathway. For instance, a little amount of agarose can be added in batch, vapour diffusion, dialysis or free-interface diffusion experiments. Recently, agarose was even successfully used inside microfluidic channels (Dhouib et al., 2009). Besides, the combination of gel and counter-diffusion experiments in capillaries deserves more attention since it offers supplementary possibilities. Indeed, it enables the experimenter to optimize crystal growth under quasi-ideal conditions. We sincerely wish this review will help revive the crystallisation of biological macromolecules in gels and in capillaries and lead to an explosion of the number of well-resolved macromolecular structures.

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