Crystallization of biological macromolecules using agarose gel

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Gellified media prevent convection and crystal sedimentation, and provide an attractive growth environment for optimising biological crystals. Agarose gels are particularly easy to use and they are compatible with most of the common crystallization methods. They also offer new possibilities like counter-diffusion techniques. This paper gives a brief overview of their general properties and presents an application of a counter-diffusion setup combining agarose gel and capillaries to the crystallization of proteins and protein / nucleic acid complexes.

Keywords: agarose gel; crystallization; biological macromolecule; counter-diffusion.

1. Introduction

Gels have been known for long to promote the growth of high-quality crystals of small molecules (Henisch, 1988). During the last decade agarose and silica gels have been successfully tested on crystallization of biological macromolecules (reviewed in Robert *et al.*, 1999). Regardless of their favorable properties, they are still seldom used in bio-crystallization. Gellified media are characterized by the absence of convection and the prevention of crystal sedimentation and, in that respect, they can mimic microgravity (García-Ruiz *et al.*, 2001a). By reducing convection in the crystallization environment, gels provide a better control of mass transfer processes during crystal growth. Molecular transport is more homogenous and operates in a diffusive regime (García-Ruiz *et al.*, 2001b). In addition, crystals can no longer move or sedimentate, since nuclei are trapped in the pores of the gel matrix. They remain in suspension in the mother liquor and are able to grow in three dimensions (García-Ruiz *et al.*, 2001b; Lorber & Giegé, 2001; Sauter *et al.*, 2002). Such a more stable growth environment has been shown to prevent twinning of alcohol dehydrogenase crystals (Sica *et al.*, 1994) and to reduce the incorporation of impurities into apoferritin crystals (Chernov *et al.*, 2001). Furthermore, it may favor higher internal order and generate crystals with lower mosaicity and a more homogeneous lattice. This has actually been observed for lysozyme (Vidal *et al.*, 1999) but also for larger macromolecules crystallized in gel (Lorber *et al.*, 1999a). In the case of thaumatin, gel-grown crystals showed a lower mosaicity, produced a higher diffraction signal compared to solution-grown controls (Lorber *et al.*, 1999b), and led to the refinement of the structure at atomic resolution (Sauter *et al.*, 2002). In addition, lysozyme crystals grown in a diffusive regime, either in microgravity or in agarose gel, yielded a final model with a better-defined solvation shell (Dong *et al.*, 1999). Both examples demonstrate that the presence of gel does not interfere with crystallographic analysis or structure determination, but can even lead to improved three-dimensional information.

Considering the potential of gels for growing high quality-crystals, we recently started to use agarose in our attempts to crystallize several nucleic acid binding proteins. Gels can be applied to all classical crystallization methods (vapor diffusion, batch, dialysis) but also offer new possibilities like counter-diffusion techniques. Indeed, we obtained promising results using the method of counter-diffusion described by López-Jaramillo *et al.* (2001). Tests were performed with Endonuclease VII (EndoVII) from bacteriophage T4, an enzyme resolving DNA four-way junctions, and three RNA binding proteins, the Sm-related proteins Sm1 from *Aeropyrum pernix* (AP-Sm1) and *Archeoglobus fulgidus* (AF-Sm1), and Hfq from *Escherichia coli*. Practical aspects of crystallization in gel are discussed as well as examples illustrating the principle of the counter-diffusion method combining agarose gel and capillaries.

2. Materials and methods

2.1. Macromolecule samples

Bacteriophage T4 Endonuclease VII (EndoVII) was prepared as described previously (Raaijmakers *et al.*, 1999). Hfq protein from *E. coli*, AP-Sm1 protein from *A. pernix* and AF-Sm1 protein from *A. fulgidus* were cloned, overexpressed in *E. coli* host cells and purified to homogeneity (J. Basquin, personal communication). HPLC-purified DNA substrates for EndoVII and the RNA ligand for AF-Sm1 were purchased from Metabion GmbH, (Munich, Germany) and RNA-TEC NV (Leuven, Belgium), respectively. They were added to the proteins prior to performing crystallization assays. Hen egg white lysozyme was purchased form Sigma (cat. number L6876).

2.2. Gel preparation and crystallization setup

Low gelling temperature agarose (T_g = 28 °C) was purchased from Serva (cat. number 11408). The 2% (m/v) agarose stock solution was filtered using a Millipore membrane (0.22 µm) and stored at 4 °C. Before setting-up crystallization trials, the gel is melted at 95 °C and kept liquid at 35-45 °C. This agarose solution can be used to perform classical crystallization assays using vapor diffusion, dialysis or batch methods. To do so, it is either added to the crystallizing agent or to the macromolecule solution prior to setting up the drops. In counter-diffusion experiments, the agarose solution is directly added to the macromolecule stock solution to a final agarose concentration of 0.1-0.2% (m/v). This mixture is extemporaneously sucked into x-ray capillaries and their thin end is sealed with nail polish or bee wax. Filling a 0.3 mm diameter capillary requires ~6 µl of solution. When the macromolecule sample is gellified (after a few minutes), the concentrated crystallizing agent solution is poured on the top of it at the wide end of the capillary (see Figure 1). The latter is subsequently sealed using either nail polish or vacuum grease, as described in López-Jaramillo *et al.* (2001). Crystallizing solutions used in counter-diffusion are given in Table 1. All crystallization assays were performed at 20 °C in x-ray glass capillaries with 0.3 mm diameter (Hampton Research, CA). Preliminary crystallographic analyzes were carried out *in situ* using the in-house x-ray source (Nonius rotating anode) by fixing the capillaries directly on a goniometric head.

3. Why to choose agarose gel: practical aspects

Silica and agarose are the most frequently used gels in bio-crystallization and have similar properties. Although diluted silica gels (0.5-2% m/v) have first been used (Robert & Lefaucheux, 1988; Cudney *et al.*, 1994), agarose gels present several advantages: they are commonly available in molecular biology laboratories, easier to

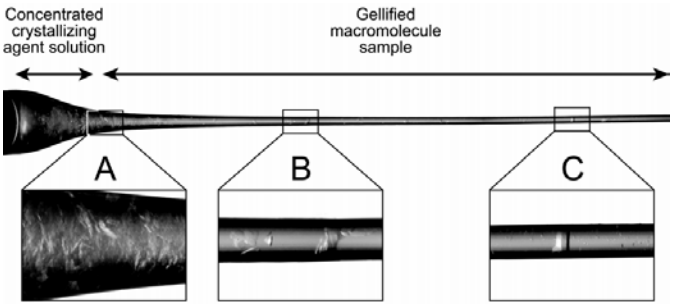

Figure 1 Typical counter-diffusion features of Hfq crystallization. When the crystallizing agent diffuses along the capillary containing a gellified macromolecule solution, it establishes a supersaturation gradient that generates microcrystalline precipitate at the beginning (A), then many small, intergrown crystals (B) and, finally, large monocrystals (C).

handle, do not interact with proteins (Vidal *et al.*, 1998) and providean effective gel at lower concentrations (0.12-0.15% m/v; García-Ruiz *et al.*, 2001a).

On a practical point of view, the gel matrix provides a mechanical protection of the crystals. The samples can be transported without the risk of undergoing severe damages. The soft gel matrix allows seeding and soaking experiments, and can easily be dissected for crystal mounting. It was shown that lysozyme crystals grown in 2% silica incorporate the gel matrix, thus, increasing their mechanical properties and stability against dehydration (García-Ruiz *et al.*, 1998). Similarly, lysozyme crystals trap the surrounding agarose fibers when they grow in agarose (Gavira & García-Ruiz, 2002) and, in the case of the large multi-domain enzyme aspartyl-tRNA synthetase, the presence of agarose gel was essential to ensure successful cryo-cooling of the crystals (Zhu *et al.*, 2001).

Finally, the addition of agarose gels is fully compatible with classical crystallization techniques. Various examples have been published showing the implementation in vapor diffusion (Bernard *et al.*, 1994; Sica *et al.*, 1994; Thiessen, 1994), microdialysis (Thiessen, 1994; Lorber *et al.*, 1999b) or microbatch (Moreno *et al.*, 2002). Furthermore gellified media can be used to perform counter-diffusion experiments either by gel-acupuncture (García-Ruiz and Moreno, 1994; Moreno *et al.*, 1999) or by using gel inside capillaries (López-Jaramillo *et al.*, 2001).

4. Crystallization by counter-diffusion in agarose gel

4.1. Principle

The counter-diffusion method has been developed by García-Ruiz and coworkers (García-Ruiz & Moreno, 1994; García-Ruiz *et al.*, 2001c). It allows the continuous screening of supersaturation states in a single experiment: the fast diffusion of the crystallizing agent into the macromolecule solution generates a concentration gradient throughout the whole crystallization chamber. This implies two important prerequisites which are the use of a long diffusion path (i.e. a capillary) and the absence of convection (in microgravity or in gel). During the experiment a supersaturation wave propagates along the crystallization chamber while the amplitude of the wave is gradually reduced (García-Ruiz *et al.*, 2001c). Such a procedure allows testing a broad range of nucleation and growth conditions yielding precipitate at the entry of the chamber and leading to monocrystals at the end (Figure 1). A simple setup has recently been designed using gellified macromolecule samples in capillaries (López-Jaramillo *et al.*, 2001).

4.2. Application to proteins and nucleic acid/protein complexes

We applied the counter-diffusion method to our current projects and it proved to be successful in producing individual well-shaped crystals in all cases. Our experiments covered proteins with different sizes and oligomeric states (Table 1). They include three RNA binding proteins: Hfq, AP-Sm1 and AF-Sm1. The latter was crystallized with a single strand RNA of 14 bases derived from a target RNA (Törö *et al.*, 2001). The crystallization of the resolvase EndoVII was tried either alone or in complex with two types of DNA substrates:a 36 bases cruciform junction and a 18 bp mismatched duplex. Lysozyme was used as a control reproducing the conditions described in the original work (López-Jaramillo *et al.*, 2001). The starting conditions for counter-diffusion can directly be adapted from those giving hits in sparse matrix screens using vapor diffusion: i) the concentration of the buffer and additives (e.g. salts) remains unchanged; ii) the concentration of crystallizing agent is increased (1.5-2 times the orginal one) to provoke precipitation at the entrance of the capillaries and to observe all counter-diffusion features (see Figure 1); iii) the buffer of the macromolecule sample can be replaced by that present in the crystallizing solution. Nevertheless, this step is not mandatory since the counter-diffusion process will provide an automatic buffer exchange. The macromolecule solution is gellified in a capillary using 0.1-0.2% (m/v) agarose and the crystallizing solution is poured on the top of it as described in section 2.2.

We obtained monocrystals in all cases in 1-2 weeks (Figure 2). The presence of both nucleic acids and proteins in complex crystals was confirmed by gel electrophoresis analysis and silver staining (result not shown). The results were especially interesting in the case of AP-Sm1 and Hfq proteins where we had severe problems of reproducibility using the vapor diffusion methods due to uncontroled nucleation. Instead of getting many small crystals, a few individual, well-shaped crystals were obtained at the end of the capillary (in low supersaturation conditions). For half of the cases, crystals were large enough to be characterized using the in-house x-ay source. For example, AF-Sm1 / RNA bipyramids (~0.1 mm - Figure 2C), thin EndoVII plates (~20 µm thick - Figure 2D) and lysozyme crystals (~0.3 mm – Figure 2F) diffracted up to 3.5 Å, 4 Å and below 2 Å, respectively.

Our results suggest that counter-diffusion can be applied to a wide range of molecules and complexes. Furthermore, it presents many practical advantages. First, it is suitable after a first screening procedure for discriminating amorphous from microcrystalline precipitates or improving microcrystals. Second, one should note that the crystals are protected by the gel in the capillary and can easily be observed under a microscope.

When they have reached a reasonable size, they can be directly analyzed at room temperature without taking them out of the capillary, since the gel matrix immobilizes them. After a first diffraction trial, the crystals can be removed from the capillary to be mounted in a cryo-loop. This procedure is easy because the gel is very soft. If the crystallization buffer already provides cryo-protection, the whole capillary can be flash-cooled in liquid nitrogen without any further crystal manipulation (López-Jaramillo *et al.*, 2001). Finally, the use of counter-diffusion is now facilitated by a specific crystallization device, the Granada Crystal Box (García-Ruiz *et al.*, 2002), which makes the method attractive for tasks ranging from lab-scale crystallization to high-throughput crystallography studies (Gavira *et al.*, 2002).

5. Conclusion

The crystallization in gels offers many advantages with respect to crystal growth and crystal handling. Moreover, it can substantially

Figure 2 Examples of crystals obtained by counter-diffusion from various proteins and complexes: A) Hfq, B) AP-Sm1, C) AF-Sm1 associated to a 14 bases RNA target RNA, D) EndoVII, E) EndoVII in complex with a DNA cruciform junction, F) EndoVII in complex with a mismatched DNA, G) lysozyme. The initial conditions in crystallizing solutions are indicated in Table 1. All crystals were grown in 0.3 mm diameter capillaries at 20°C.

Table 1 Macromolecules and crystallization solutions used in counter-diffusion experiments. All assays were performed at 20°C.

Macromolecules	Size (kDA)	Concentration	Crystallization solutions
Hfq	66 (hexamer)	10 mg/ml	30 % PEG 3.35 K, 0.1 M citric acid pH 5.4
AP-Sm1	59 (heptamer)	10 mg/ml	40 % PEG 8 K, 0.1 M Tris-HCl pH 8.0
AF-Sm1 / RNA	59 (heptamer) + 4	10 mg/ml	25 % PEG 5 K-MME, 0.1 M citric acid pH 4.9
Endo VII	36 (dimer)	18 mg/ml	20 % PEG 6 K, 1 M LiCl, 0.1 M Tris/HCl pH 8.0
EndoVII / DNA	36 + 13	10 mg/ml	15 % PEG 2 K-MME, 0.2 M Bis-Tris Propane pH 7.0
Lysozyme	14 (monomer)	20 mg/ml	1 M NaCl, 0.1 M acetate buffer pH 4.6

improve crystal quality and thereby facilitate the subsequent crystallographic analysis. Currently the use of gellified media is not well adapted for large-scale screening. This would require specific robotic implementation, which is not yet available but is being explored (Moreno *et al.*, 2002). Nevertheless, once a first hit has been obtained by standard screening, agarose gel should definitely be tried as an additive. Overall, crystallization in gel, particularly using the counter-diffusion method, appears to be a very effective optimization method that deserves to be used more routinely.

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