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Agarose gel as a medium for growing and tailoring protein crystals

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ABSTRACT

The nucleation inducing ability of agarose gels has been exploited to study the crystallization of proteins in diffusion-dominated environments. The crystal size was successfully tuned in a wide range of gel, protein and precipitant concentrations. The impact of the gel content on crystal size resulted to be independent of the specific protein, allowing the mathematical prediction of crystal size and pointing out the exclusivity of physical interactions between the gel and the protein. The versatility of the technique and the fine-tuning of the nucleation flux was demonstrated by crystallizing five different proteins and implementing batch and counter-diffusion crystallization. In addition, the potential of agarose gel to be used not only as a growth, but also as a delivery medium for serial crystallography applications, has been proven by preparing unidimensional micro-crystals slurries with 0.1% (w/v) gel.

KEYWORDS: protein crystallization, agarose-gel, nucleation, batch, counter-diffusion

1. INTRODUCTION

The growth of protein crystals in gelled media has proved to date to be the cheapest means to produce protein crystals of high quality similar to those obtained under microgravity conditions[1,2,3]. Gels create a stable environment for crystals to grow in convection-free conditions, avoiding sedimentation and formation of aggregates, increasing crystals uniformity, and avoiding the creation of protein depletion zones around the growing crystals. The use of agarose has allowed progress in crystal quality, size limitation, and even in obtaining protein crystals inaccessible by other techniques[4,5]. The incorporation of gel into the crystallization medium does not prevent the free diffusion of the protein macromolecules. At the same time, they certainly deal with a new surface whose chemical (stability, hydrophilicity, charge, etc.) and physical (porosity, elasticity, transparency, etc.) properties may affect crystallization[6]. In spite of these advantages, the use of agarose gel is not generalized mainly because its inclusion in a crystallization protocol is equivalent to the insertion of a new additive into the system.

It has been shown that agarose gels are strong promoters of nucleation, while silica gels act as nucleation inhibitors, without knowing the mechanism of action or the causes underlying these effects. In 1991 Provost and Robert proposed the use of agarose gels as an alternative means to silica gel[7]. The advantages of agarose are two-fold: it can be used at very low concentrations and polymerizes faster than silica gels, facilitating its application to any crystallization technique, i.e., hanging or sitting drop, batch, etc. In this work, agarose gels were found to strongly increase the nucleation density of lysozyme crystals, in contrast to the nucleation inhibition effect of silica gels. The total number of crystals increased linearly with the gel concentration in the range of 0-0.15% (w/v). However, if crystallization was carried out at 30 °C, this effect disappeared because, they argued, at this temperature the gel may not be completely set. The induction ability was explained

as an epitaxial effect of the gel walls. Robert and co-workers explained that agarose gel fibers impeded the sedimentation of the initial amorphous precipitate, which was typically observed in free solution and depleted the solution bulk, giving rise to the observed higher nucleation rate[8]. On the contrary, in the same period, Thiessen showed that for agarose concentrations greater than 0.4% (w/v) the induction of lysozyme nucleation was repressed independently of supersaturation[9]. Similar results have been recently published by Tanabe and co-workers[10]. They found a non-linear relationship between lysozyme nucleation rate and gel concentration; they observed induction of nucleation in the range of 0.2 - 0.4%, inhibition up to 0.8%, and then induction again up to 2%. They explained the general trends of induction over the whole range using the same justification found by Provost and Robert[11]. More specifically, nucleation induction may be related to the loss of water necessary to stabilize the double agarose helices. This phenomenon would have increased the effective supersaturation by increasing both salt and protein concentrations. Moreover, small angle neutron dispersion studies (SANS) ruled out the possibility of an increase in supersaturation during gelation[12]. It was not observed any type of interaction of the lysozyme molecules with agarose gel fibers or with any of the charged (sulfate) or hydrophobic (methyl) moieties present in the gel structure, and the authors went back to the previous hypothesis of clusters stabilization as the main reason for the observed nucleation induction effect.

Meanwhile hydrogels are back in the palestra of crystallization tools-box with many different purposes. Self-healing ferritin crystals with integrated polymer networks able to expand and contract have recently been reported[13]. These materials combined the structural order and periodicity of crystals to the adaptiveness and tunable mechanical properties of polymeric networks and to the chemical versatility of protein building blocks. Fmoc-CF dipeptide, incorporated within the lysozyme crystals, showed a clear protection of the most sensitive groups

(disulfide bonds and methionines) of the enzyme[14]. Moreover Fmoc-FF hydrogels have allowed the incorporation of a very highly hydrophilic material, i.e., single-walled carbon nanotubes, within lysozyme crystals transforming them from an isolator to a conductor material[15].

Still agarose is the most investigated hydrogel media, as demonstrates its incorporation as a simulating medium for biomineralization[16], as a selector of a particular crystallization polymorph[17], or even as a medium to observe the unusual phenomena named the Morse code effect[18], to cite just a few recent works. In protein crystallization, agarose has been proved to facilitate the soaking of different molecules (ligands, co-factors, cryo-protectants, heavy atoms)[19,20], even when exposed to organic solvents (DMSO)[21]. It is also an excellent medium to produce big size crystals for neutron diffraction studies[22] and can be used as a delivery medium of nano/micro-metre size crystals for X-Ray Free-Electron Laser (XFEL) experiments[23].

The aim of the present study was to investigate and clarify the nature of the nucleation induction promoted by agarose, which is still not clear. To the best of our knowledge, this is the first study reporting that the tailoring of crystal size by agarose is independent of the specific protein, thus revealing the physical inducing action of the gel. Crystal size was also finely controlled, at constant agarose contents, by tuning protein or precipitant concentration. The use of agarose for the preparation of microcrystalline slurries for XFEL and Serial Milli-second Crystallography (SMX) is presented as a potential application. We also show a workaround strategy to set-up experiments when there is incompatibility between the precipitant and the agarose gel, i.e., when it is impossible to polymerize agarose, by using the capillary counter-diffusion crystallization (CDC) approach. In conclusion, agarose gels were employed as a medium to grow and tailor crystal size over a wide dimensional range, increasing not only the efficiency of protein crystallization but also the number of characterization techniques that can be used to gather the required structural information.

2. MATERIALS AND METHODS

2.1 Materials

OmniPur agarose (2120) was purchased from Calbiochem (San Diego, CA, USA). Lysozyme from chicken egg white (62971, HEWL, three times crystallized powder) and catalase from bovine liver (C30, microcrystalline aqueous suspension) were purchased from Sigma-Aldrich. Proteinase K (A3830, lyophilized) and human insulin (11376497001, lyophilized) were purchased as lyophilized powders from PanReac AppliChem (Barcelona, Spain) and Roche Diagnostics GmbH (Mannheim, Germany), respectively. All the proteins were directly dissolved in their own buffer and the concentration measured spectrophotometrically at 280 nm.

2.2 Crystallization of proteins

The crystallization of five commercially available proteins was studied in the presence of different amounts of agarose gel. All the solutions were filtered with 0.22 μm syringe filters to remove impurities. All the experiments were performed in triplicate. Different crystallization methods based on batch or CDC were selected depending on the constraints enforced by the precipitation cocktail on the crystallization protocol.

2.2.1 Batch crystallization

In a typical experiment, proteins were crystallized in batches of 80 μL each. Agarose was first dissolved in MilliQ water at 95 °C for 10 min. Once agarose has been fully dissolved, it was cooled down to 45 °C. Then, precipitant, protein, and agarose were mixed together inside PCR vials at 45 °C according to pre-determined ratios to achieve their target concentrations. The mixture was let to cool at room temperature to achieve agarose gelation. As an alternative for thermosensitive proteins, low melting (LM) point agaroses may be also used to carry out agarose preparation, mixing and gelation at room temperature. Nevertheless, since regular agarose is by far easier to

retrieve in the majority of labs, we propose its use as a first alternative for performing crystallization trials and leave LM agarose as a potential growth medium for proteins with scarce thermostability.

The crystallization conditions of three selected proteins are summarized in Table 1. Proteinase K was buffered in 50 mM HEPES (pH 7.0). Each batch involved 6.0 mg/ml of proteinase K, 25 mM sodium citrate and 0.5 M $NaNO_3$. Samples were prepared with gel concentration ranging from 0 to 1.9% (w/v). As regards insulin, each batch was made of 5.0 mg/mL of protein in 25 mM HCl, 35.2 mM Na citrate (pH 7.0), 5 mM $ZnCl_2$, and 5% (v/v) acetone. HEWL was buffered in 50 mM sodium acetate (pH 4.5). Samples were prepared with 27.6 mg/mL of protein and 0.75 M NaCl. For HEWL and insulin, the gel concentration ranged from 0 to 2.1% (w/v).

Once the crystallization had been completed, samples were analyzed via optical microscopy (AZ100 Nikon, Germany). For micrographs taken at high magnification (4X or more), a small amount of gel was withdrawn from the PCR vial and gently sandwiched between two glass coverslips. A characteristic length was selected for each protein: the distance between the two vertexes of proteinase K bipyramidal crystals, the height of insulin polyhedra, and the width of (110) face of HEWL crystals. The average size of crystals was evaluated considering 50 – 70 crystals per sample.

2.2.2 Counter-diffusion crystallization

When the crystallization of the target protein required the use of PEGs or ammonium sulfate, we proposed some variations of the batch method based on CDC techniques. For PEGs, counter-diffusion was carried out in PCR vials whereas, for ammonium sulfate, it involved the use of capillaries.

We selected two model proteins, namely catalase and trypsin, to be crystallized with PEGs having different molecular weights. Following the same protocol for the preliminary preparation of the agarose sol, the protein solution was then mixed with it at 45 °C. The solution was cooled down to trigger agarose gelation and achieve protein immobilization in the gel matrix. Then, the precipitant solution was added on top of the gel and let to diffuse inside its matrix to achieve crystallization conditions. As summarized in Table 1, catalase was dissolved in 50 mM K phosphate buffer. The final protein concentration was 3.0 mg/mL, and 0.1, 0.5 and 1.0% (w/v) gels were prepared. The total volume was 50 µL. The same volume of 50% (w/v) PEG4000 was added on top after agarose gelation. As regards trypsin, the mix between agarose and protein consisted in 0.1-1.5% (w/v) agarose and 67.2% (v/v) of trypsin at 30 mg/mL dissolved in 15 mM HEPES pH 7.5, 5 mM $CaCl_2$ and 5 mg/mL benzamidine. Precipitation solution consisted in 0.1 M HEPES pH 7.5, 30% PEG8000 and 0.2 M ammonium sulfate. The volumes of gel + protein and precipitation solution were 50 µL each.

For crystallization trials requiring ammonium sulfate, CDC was carried out in capillaries. Proteinase K was selected as case study. The agarose sol mixed with the protein solution was loaded into 0.7 mm (ID) capillaries (Triana Science & Technology, Spain). 0.1, 0.5, 1.0, and 1.5% (w/v) agarose concentrations were tested. The hot solution was gelled at room temperature. A 1.0% (w/v) agarose plug was added to the end of the capillary as a physical buffer, and the other end was sealed with plasticine. The capillary was then loaded inside a closed vial carrying 150 µL of 3 M ammonium sulfate.

Lastly, the gelation step was investigated in the absence of protein solutions. After preparing the agarose at 95 °C, the mixing between agarose and ammonium sulfate or PEGs was performed at 45 °C inside 1.5 mL Eppendorf tubes. Then, the solutions were left to cool down to room

temperature. Ammonium sulfate and PEGs solutions were all freshly prepared and filtered by a 0.22 μm pore size filter.

Table 1. Crystallization methods and final concentrations for the five model proteins of this study.

Protein	Crystallization method	Protein (mg/mL)	Buffer	Precipitant
Proteinase K	Batch	6.0	50 mM HEPES pH 7.0	0.5 M $NaNO_3$, 25 mM Na citrate pH 6.5
Insulin	Batch	5.0	25 mM HCl	5 mM $ZnCl_2$, 35.2 mM Na citrate pH 7.0, 5% (v/v) acetone
HEWL	Batch	27.6	50 mM Na acetate pH 4.5	0.75 M NaCl
Catalase	CDC in PCR vial	3.0	50 mM K phosphate pH 7.0	50% (w/v) PEG4000
Trypsin	CDC in PCR vial	20.2	15 mM HEPES pH 7.5, 5 mM $CaCl_2$, 5 mg/mL benzamidine	0.1 M HEPES pH 7.5, 30% PEG8000, 0.2 M $(NH_4)_2SO_4$
Proteinase K	CDC in capillary	25.0	50 mM HEPES pH 7.0	3 M $(NH_4)_2SO_4$

3. RESULTS AND DISCUSSION

3.1 The effect of agarose gel on crystal size in batch crystallization

Proteinase K was first selected to carry out crystallization in batches containing agarose gel. A gradient of gel content from 0 to 1.9% (w/v) was investigated. Gel-free experiments in batch led to few large protein crystals of approx. 300 μm and served as reference. When agarose was added to the system, the crystallization behavior was strongly affected, both in terms of nucleation

density and dimensional uniformity of crystals. Representative images showing crystals of proteinase K grown in gel-free and gelled batches are reported in Figure 1 (a). The introduction of a very small amount of gel in the system, i.e., 0.05% (w/v), was already highly effective in preventing crystal sedimentation and homogenizing the probability of finding crystals in different zones of the batch. The increase in the agarose gel content had a major impact on the final crystal size, leading to progressively smaller crystals as sketched in Figure 2 (a). The decrease in size was steep below 1% (w/v) gel and reached a plateau around 15 μm for higher gel contents. The presence of agarose gel in the crystallization environment allowed the establishment of a convection-free medium. The predominance of diffusion phenomena within increasingly concentrated gelled batches allowed the precise control of the dimensional distribution of crystals. In addition, it was beneficial for getting monodispersed crystal slurries. The population of crystals resulted to be increasingly homogeneous with the amount of gel added to the system, as proved by the dramatically smaller standard deviations of samples involving 0.5% (w/v) gel or more.

The crystallization of two other proteins in batches containing gradients of agarose gel displayed a similar trend to what had been observed for proteinase K. Optical microscopy images of samples involving insulin and HEWL are sketched in Figure 1 (b) and (c), respectively. The average size of insulin and HEWL crystals as a function of the agarose gel content is reported in Figure 2 (b) and (c). As regards insulin, we observed slightly bigger crystals, compared to those obtained with proteinase K. The crystal size was successfully tuned from 500 μm, in the absence of agarose, to 20 μm, for the highest gel content. Nevertheless, the introduction of 0.1% (w/v) agarose had a more important impact on crystal size, reducing it by more than 5 times, compared to gel-free samples. As regards HEWL, the average size of crystals in gel-free and gelled samples was higher compared to proteinase K and insulin. On average, HEWL crystals were one order of magnitude

bigger at plateau conditions. The ability of agarose gels in reducing crystal size was saturated for gel contents larger than 1% (w/v), resulting in characteristic lengths equal to 200 μm. Overall, the presence of agarose gel was also beneficial for the dimensional uniformity of the crystals, as highlighted by increasingly smaller standard deviations.

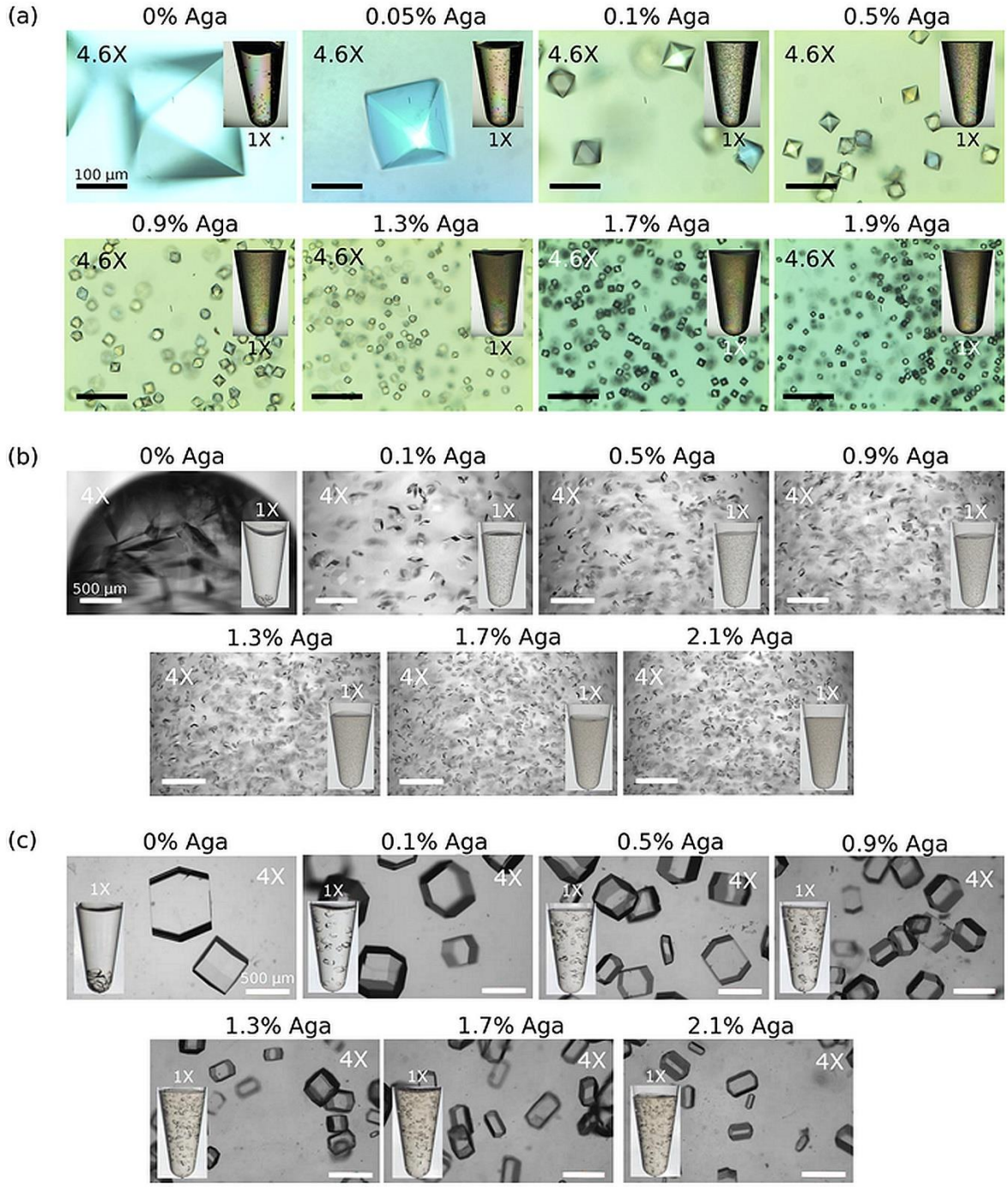

Figure 1. Overview of the proteins crystallized in batch with agarose gradients: (a) proteinase K, (b) insulin and (c) HEWL. Representative microscopy images of crystals grown in gel-free (0%) and in batches containing agarose gel (0.05 – 2.1%, depending on protein) are

illustrated. Embedded are the low-magnification micrographs of the crystallization batches. Scale bar is 100 μm for proteinase K and 500 μm for insulin and HEWL.

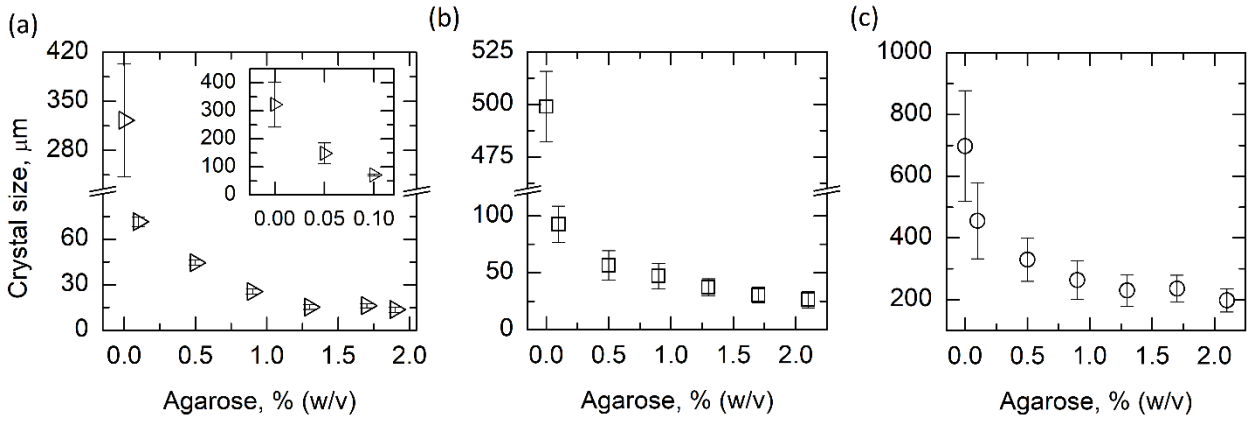

Figure 2. The crystal size as a function of the content of agarose gel in the system for (a) proteinase K, (b) insulin and (c) HEWL. Embedded in (a) is the enlargement of the 0 – 0.1% (w/v) agarose region. Error bars refer to standard deviations.

The analysis of the crystal size trends provided insight into the possible mechanism underlying protein-gel interactions. It has to be noticed that the protein-gel interactions firstly affect the nucleation flux, which is directly related to the number of crystals per unit volume. However, since the direct counting of crystals was not feasible in our experimental set-up, we focused on another physical variable which depends on the nucleation flux, i.e., the crystal size. Figure 3 displays the normalized crystal size ($\widehat{l_c}$) vs. agarose content (C_{aga}) trend for each protein. The normalized crystal size was defined as:

$$\widehat{l_c} = \frac{l_c}{l_{c,max}} \quad (1)$$

where l_c was the crystal size and $l_{c,max}$ was the maximum crystal size obtained for each protein. As a general trend, the experimental data were successfully fitted with a decay exponential law ($R^2 > 0.99$) in the form of Equation 2:

$$\widehat{l_c} = Ae^{-\frac{C_{aga}}{B}} \quad (2)$$

where A and B are two fitting parameters, whose values are listed in Table S1. Only data referring to agarose contents ≥ 0.1% had been considered. As a matter of fact, considering proteinase K as an example, if the data referring to 0.05% gel were included in the normalization process, the fitting of the size trend would require a double exponential decay law, as highlighted in Figure S1. We attributed such a deviation to the transition of agarose from sol to gel occurring in this region. As a general trend, the affection of crystallization by the gel resulted to be independent of the type of protein, proving that the nucleation induction ability of the gel was not related to specific protein features. A direct matching between electrostatic, i.e., charge, or dimensional, i.e., molecular weight, properties of the protein and the gel chemistry or pore size could thus be excluded. We concluded that the nucleation induction ability of the gel was due only to physical interaction, rather than chemical affinity. The entrapment of water inside the gel fibers would reduce the activity of water in the whole system, increasing the overall supersaturation. Such a finding was in agreement with a previous study carried out by small angle neutron scattering by Vidal *et al.,*[12]. Moreover, we found that the effect of the gel was not only unrelated to the protein nature but also to the average size of the crystals, as data referring to crystals whose size differed for an order of magnitude had been considered. For all the proteins, the nucleation induction ability of the gel was saturated for agarose contents above 1.3% (w/v). Such evidences highlighted that the use of agarose gel as a growth medium allows the *a priori* prediction of the size of the crystals, provided that experimental data for a single amount of agarose are known. Thanks to the mathematical modeling of the crystal size trends, the number of experiments to grow a target crystal size in gel can be drastically reduced, avoiding expensive and time-consuming trial-and-error experimentation. Moreover, we also concluded that the gel was able to control the crystal growth rate, in addition to the density of nucleation. Since the same behavior was identified for all the

proteins investigated, the control exerted by the gel prevailed over the diffusion and incorporation speeds of protein single molecules into crystals. Since the latter depends on the specific protein and growth mechanism, major deviations would have been observed if the gel did not override their typical timescale.

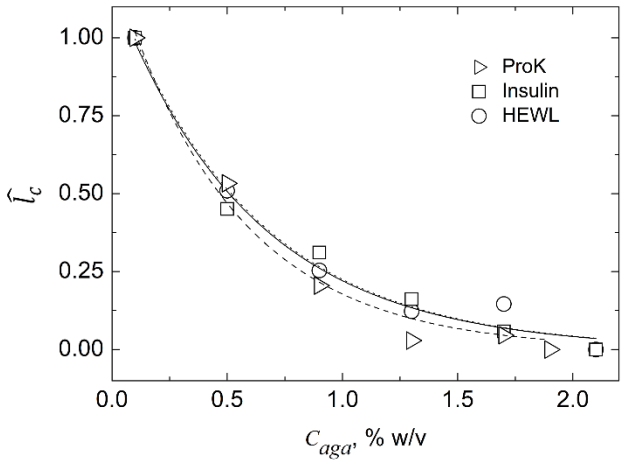

Figure 3. Normalized crystal size as a function of the agarose content. Fitting was carried out with an exponential decay law, considering points above 0.1% (w/v) agarose. Continuous, dashed, and dotted lines refer to insulin, proteinase K, and HEWL, respectively.

After proving the agarose gel suitability as a crystal growth medium, we tailored crystal size at fixed agarose contents. Proteinase K was selected as case-study. In particular, we investigated if our crystallization set-up could potentially be applied to the preparation of samples meeting the requirements of XFEL or SMX analyses. Two of the most compelling restrictions for crystal slurries suitable for XFEL and SMX regard the presence of micro-crystals and the sample flowability. Crystals size has to be below 25 µm and the suspension must be compatible to microfluidic devices, thus having low viscosity and being not prone to plugging. In this study, we propose the use of agarose gel not only for the preliminary growth of protein crystals, but also as a delivery medium for XFEL and SMX. The precipitant concentration has been tuned to

demonstrate the versatility of the gel-based technique in tuning crystal size even at constant gel content. In order to ensure good flowability of the crystalline suspension, the gel content was set at 0.1% (w/v). The selection was done considering that the transition from the non-Newtonian fluid to the viscoelastic gel regime appears at 0.12% (w/v) for agarose gels[24]. For this purpose, $NaNO_3$ concentrations equal to 0.1, 0.5, 1 and 2 M were studied, keeping all the other process variables constant. On the one hand, the smallest amount of salt led to the largest crystals, reaching sizes above 400 μm, as sketched in Figure 4 (a). On the other hand, when the salt concentration was increased, crystals populations centered at 25.3 (+/- 1.9) and 13.3 (+/- 1.3) μm could have been obtained with 1 M and 2 M $NaNO_3$, respectively. The combination of small and dimensionally uniform crystals together with the low amount of gel would be ideal for the application of such a crystal growth technique to femto- and milli-seconds serial crystallography experiments.

Further tailoring was carried out by increasing the protein concentration at fixed % of agarose gel. The concentrations of precipitants were the same of the agarose gradient study. In Figure 4 (b), the conditions leading to about 50% decrease in crystal size are highlighted for the three model proteins, each of them being crystallized at different gel contents. In every case, the increase in protein concentration was beneficial for the dimensional uniformity of the samples. Representative micrographs of proteinase K crystals grown with 0.5% (w/v) agarose are reported in Figure S2.

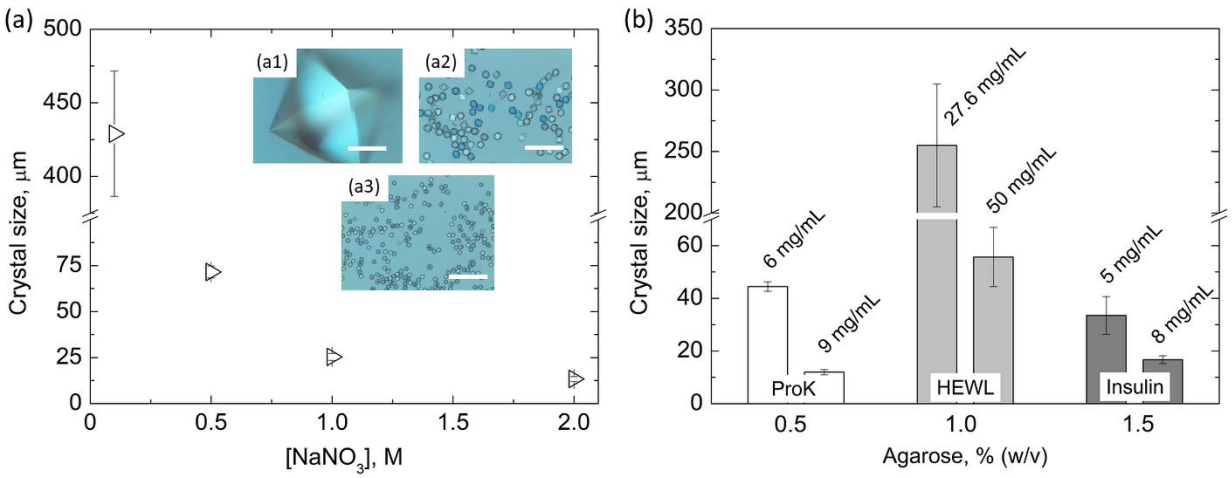

Figure 4. (a) The size of proteinase K crystals grown in 0.1% (w/v) agarose gel as a function of precipitant concentration. Embedded in the panel are the optical micrographs of crystals referring to (a1) 0.1, (a2) 1 and (a3) 2 M $NaNO_3$. Scale bar is 100 µm. (b) The decrease in crystal size with increased protein concentration at selected agarose contents: proteinase K at 0.5% (w/v), HEWL at 1.0% (w/v), and insulin at 1.5% (w/v).

As a final remark, the presence of agarose gel also favored crystallization at very low protein concentrations, which normally would not lead to crystals. Gel-free trials carried out with 3.0 and 3.5 mg/mL proteinase K did not crystallize in a time frame of 4 months. Conversely, experiments carried out in the presence of 0.5% (w/v) agarose produce crystals in just 4 days, as highlighted in Figure S3. Therefore, in addition to the ability to fine-tune the crystal size, agarose allows working at very low level of supersaturation extending the effective use of the protein sample.

3.2 The use of agarose gels in non-ideal conditions

The first part of the study was devoted to the proof of agarose gel suitability in inducing nucleation and tuning crystal size. However, its application to protein crystallization may be limited by the type of precipitant required by specific proteins. Small molecules like salts, i.e., ammonium sulfate, and polymers like PEGs may hinder the formation of double helices which build fibers

during the gelation process[25]. On the one hand, the action of salts on the stabilization of agarose double helices is linked to the lyotropic effect. Ammonium sulfate is believed to hinder proper agarose gelation because of salting-out, whereas other inorganic salts like NaCl stabilize the formation of agarose double helices thanks to salting-in effects[26, 27]. On the other hand, the competition of PEG for water molecules during the gelation step leads to the condensation of agarose into a thick precipitate, and heterogeneous gels are obtained[28, 29]. In this scenario, we propose a 2-step variation of the batch crystallization technique to extend the applicability of agarose as a growth and delivery medium of crystals. The preliminary confinement of the protein inside the gel phase enabled the physical separation between agarose and precipitants. Such a strategy ensured the undisturbed gelation of agarose in the first phase. Then, two methods based on CDC have been proposed for proteins requiring PEGs and ammonium sulfate in their precipitation cocktail.

3.2.1 Counter-diffusion with PEGs

After the immobilization of the protein inside the gel, the precipitant solution was added on top. In this way, a continuous gradient of supersaturation conditions was created along the gel phase thanks to the diffusion of PEG molecules through the network. First, the CDC of catalase was studied in systems containing 0.1, 0.5 and 1.0% (w/v) gel. Figure 5 (a) displays representative samples for each condition. A diffusion scheme was observed in the samples, going from high nucleation density near the interface with the precipitant solution to larger and fewer crystals in the bottom of the vial, see Figure S4. At constant gel and protein contents, the continuous screening of PEG4000 concentration was successfully carried out in each sample. The total absence of precipitate was observed for every gel content tested. More concentrated gels led to higher nucleation density on top of the gel layer, and smaller crystals. Interestingly, when the agarose gel

was 1.0% (w/v), nucleation was confined to the upper layers of the gel. The formation of the first crystals in the lower layers required more than 5 days, whereas it was generally accomplished in 24 hours. The slowing of nucleation kinetics was related to the denser gel network, hindering the diffusion of both precipitant and protein molecules.

Trypsin crystallization was also carried out in counter-diffusion with gel contents equal to 0.1, 0.5, 1.0 and 1.5% (w/v). A gradient of the size of the crystals grown in the gel was observed along the sample. The increase in gel content led to well defined gradients with smaller crystals as a function of the agarose concentration. Crystal sizes ranged from microcrystals on the top of the gel phase in all cases to huge macro crystals on the bottom for the lower and medium agarose concentrations.

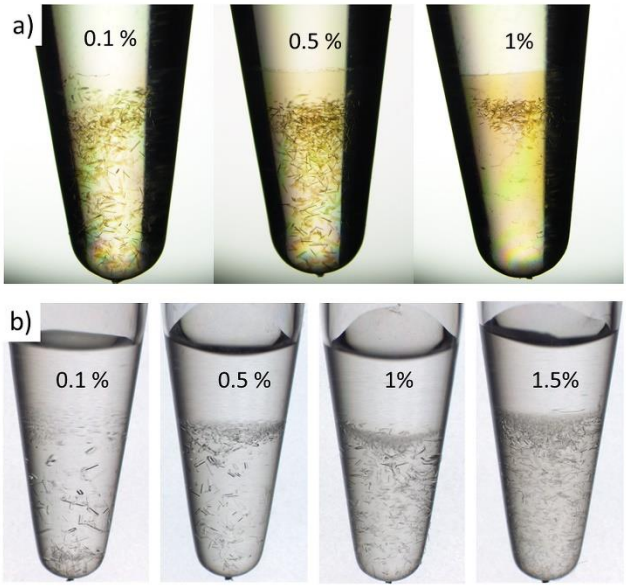

Figure 5. (a) Catalase and (b) trypsin crystals obtained by CDC with increasing amounts of agarose gel.

3.2.2 Counter-diffusion with ammonium sulfate

In order to carry out CDC with ammonium sulfate, proteinase K and a different implementation of the technique were selected. For this purpose, capillary tubes loaded with proteinase K immobilized in the gel phase were put in contact with 3 M ammonium sulfate solutions in a closed system, as sketched in Figure 6 (a). The presence of a 1% (w/v) agarose plug between the two phases acted as a physical buffer. On the one hand, it mitigated the initial supersaturation wave related to the diffusion of the salt along the capillary. On the other hand, it prevented the migration of the protein outside the gel matrix. In fact, preliminary tests carried out in PCR vials highlighted the escape tendency of proteinase K from the gel, leading to crystals in the precipitant compartment.

We observed a marked influence of the agarose gel on proteinase K nucleation in CDC too, as reported in Figure 6 (b) and (c). The average crystal size showed a quasi-linear decrease with the agarose gel content. Interestingly, we did not observe a gradient of nucleation density along the capillary, as it would be expected from a conventional CDC experiment. We hypothesized that this behavior was a consequence of the strong interaction between ammonium sulfate and agarose fibers. Crystallization in 6 cm long capillaries was achieved within 24 hours, indicating a very fast diffusion of the salt inside the gel, conversely to what had been observed for the diffusion of PEG4000 in the same matrix. Therefore, the technique did not allow for a real CDC experiment, but it rather explored simple diffusion conditions. A final remark could be made by comparing the size of proteinase K crystals obtained with the batch and the capillary techniques. Considering the same gel content, significantly bigger crystals were obtained in capillaries. Such an evidence pointed out that the gel pore size or content did not limit the growth of the crystalline entities within its matrix. A mechanical constraint exerted by the gel fibers on the crystals could thus be excluded.

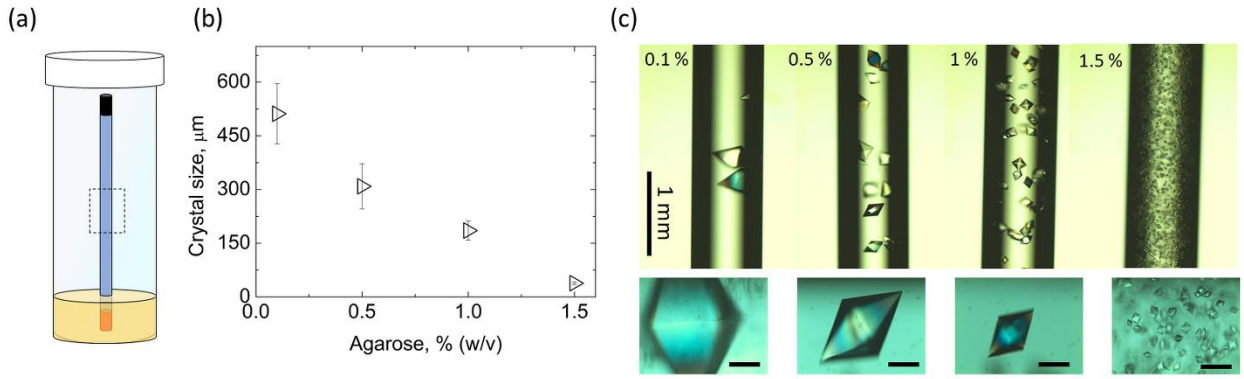

Figure 6. (a) Schematic representation of the set-up for crystallization in capillaries. The capillary filled with protein and agarose gel is immersed in the precipitant solution. A 1% (w/v) agarose plug has been placed at the bottom of the capillary as a physical buffer. (b) Crystal size as a function of the agarose gel content. Error bars refer to standard deviations. (c) Optical microscopy images of crystals grown in capillaries filled with increasing amounts of gel. Images refer to the capillary zone highlighted in (a). Below each capillary the corresponding zooms are reported. Scale bar is 100 µm.

Finally, in order to check which $(NH_4)_2SO_4$ and PEG concentrations could be compatible with agarose, both precipitants were separately mixed with the polysaccharide. The maximum amounts of $(NH_4)_2SO_4$ and PEGs tested were chosen for being the most popular concentrations used in standard crystallization screenings. Mixtures with $(NH_4)_2SO_4$ ranged from 0.1 to 2 M of salt and from 0.1 to 2.0% (w/v) of agarose. All mixtures with agarose containing $(NH_4)_2SO_4$ concentrations less than 0.5 M resulted in homogeneous and clear gels, regardless of the agarose concentration (Figure 7, first row, green subfigures). The appearance of non-homogeneous neither clear gels was observed with 1 M $(NH_4)_2SO_4$, except at 0.1% (w/v) of agarose. At 0.5% (w/v) of agarose the mixture resulted in an aggregation state of agarose fibers in suspension but still with a gel behavior (Figure 7, second row, orange subfigure). For the rest of agarose concentrations, all the mixtures

showed precipitation at the bottom of the tubes (Figure 7, second row, red subfigures). With 2 M $(NH_4)_2SO_4$ all the tubes showed a huge amount of agarose fibers precipitated at the bottom (Figure 7, third row). As regards PEGs, the mixtures between polymer and agarose were performed at 30% (v/v) for the former and from 0.1 to 1.0% (w/v) for the latter. The molecular weights of the tested PEGs ranged from 300 to 8000 Da. Results from mixtures with PEGs were all independent of the agarose concentration: the mixing between agarose and PEGs resulted in clear and homogeneous gels with PEGs below 400 Da, in a suspension of agarose fibers with PEGs between 600 and 1000 Da, and in strong precipitates at the bottom of the tubes for PEGs between 1500 and 8000 Da.

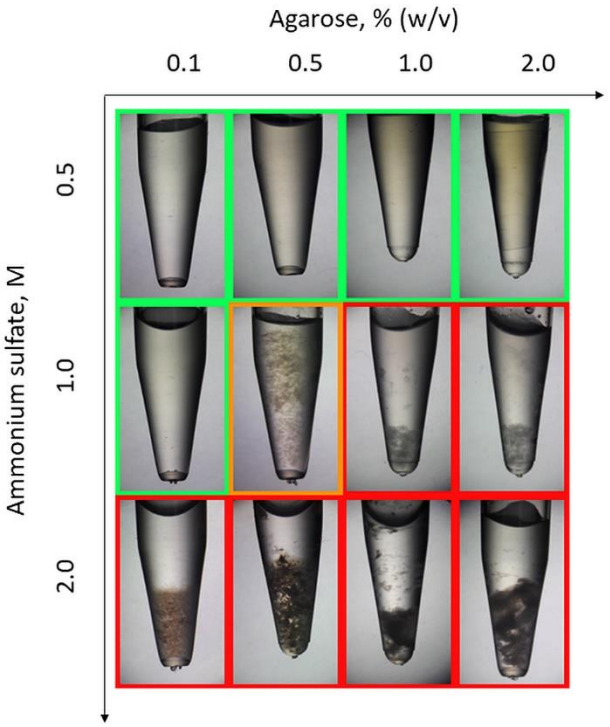

Figure 7. The effect of increasing ammonium sulfate contents on the formation of agarose gels: transparent gels (green), aggregated fibers (orange) and massive agarose precipitation (red) are illustrated.

4. CONCLUSION

The use of agarose gel for the controlled growth of protein crystals has been here investigated. As a proof of concept, insulin and lysozyme were crystallized in batch, whereas catalase and trypsin were crystallized with the aid of CDC techniques. Proteinase K was crystallized following the two techniques in order to prove the flexibility of our approach. The tuning of different parameters, such as gel content, precipitant and protein concentrations, served as a tool to elucidate their impact on nucleation. The ability of the gel to act as a nucleation promoter was demonstrated to be the consequence of physical interactions between the gel and the protein macromolecules. The tailoring of crystal size from several hundreds of μm to hundreds of nm makes agarose gels a powerful tool to grow protein crystals suitable for different applications, from neutron diffraction to femto-crystallography.

ASSOCIATED CONTENT

Supporting Information.

The following file is available free of charge, reporting additional details on the fitting of crystal size trends and on batch and CD crystallization of model proteins:

ESI_Artusio.pdf (PDF)

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

CDC, counter-diffusion crystallization; HEWL, hen egg white lysozyme; HEPES, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid; ID, internal diameter; LM, low melting; PCR, polymerase chain reaction; PEG, polyethylene glycol; SANS, small angle neutron scattering; SMX, serial millisecond crystallography; XFEL, X-ray free electron laser; CDC, counter-diffusion crystallization.

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For Table of Contents only

Agarose gel as a medium for growing and tailoring protein crystals

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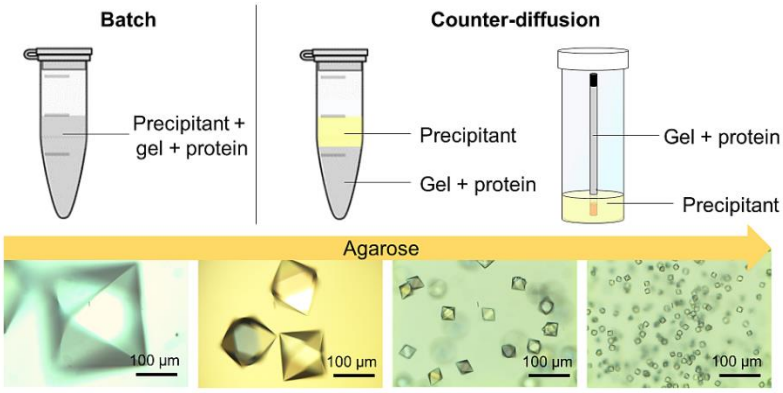
TOC GRAPHIC



SYNOPSIS

Protein crystal size was successfully tuned from few µm to several hundred µm by performing batch crystallization in gels prepared from variable amounts of agarose. Counter-diffusion crystallization in gel was proposed as an alternative for proteins requiring PEGs or ammonium sulfate as precipitants. The results pointed out the physical action of agarose gels in inducing protein nucleation.