Protein crystallisation in hydrogels

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Introduction

The determination of protein structures using X-ray diffraction techniques requires high quality protein crystals. The knowledge of the threedimensional structure of proteins at atomic resolution is necessary to obtain a detailed insight in the understanding of biomacromolecular interactions, such as enzyme-substrate or enzyme-inhibitor interactions, protein-carbohydrate interactions and rational drug design.

Mass transport in classical protein crystallisation methods – i.e. hanging and sitting drop (vapour diffusion), batch crystallisation, dialysis and liquid-liquid diffusion – is influenced by buoyant convective flows. Gravity causes either crystal sedimentation or density-driven and convective flows, generated around growing crystal surfaces (Carotenuto *et al*., 2002). Microgravity experiments have shown that crystallisation in a diffusive environment can result in high-quality crystals (e.g. García-Ruiz *et al*., 2001*a*; Snell *et al.*, 2005). For numerous examples, the extent of quality improvement has been reported (e.g. Declercq *et al.*, 1999; Vahedi-Faridi *et al.,* 2003).

Diffusion-limited growth of protein crystals can be easily obtained on earth by using hydrogel crystallisation (Henisch, 1988; Robert *et al*, 1988; Robert *et al*., 1999; García-Ruiz *et al*., 2001*b*). An agarose concentration as low as 0.04% (w/v) was able to overcome buoyancy and crystal sedimentation (García-Ruiz *et al.,* 2001*b*). Agarose, agar, silica and gellan gum have been successfully used as gel materials to obtain protein crystals. Especially, agarose has been frequently employed. Gel-grown crystals can lead to enhanced diffraction properties. Previously unachieved high-resolution X-ray diffraction data for thaumatin crystals, which were grown in 0.15% (w/v) agarose gel, have been obtained (Sauter *et al.*, 2002). Crystallisation in gelified media also prevents crystal sedimentation. This favours three-dimensional growth (García-Ruiz *et al.*, 2001*b*; Lorber *et al.*, 2001). Gels also provide an efficient protection of samples during handling (mounting, soaking, seeding, …) and transport without affecting their crystallographic analysis (Sauter *et al.*, 2002).

In this contribution, the technique of performing protein crystallisation in hydrogel beads is presented. A theoretical diffusion model is used to calculate the precipitant and protein concentration and relative supersaturation evolution in a gel bead at pre-nucleation conditions; and to characterise its behaviour. Due to its fast response time, supersaturation profiles in a gel bead can be well controlled by changing the external conditions. The usefulness of this technique is illustrated for the crystallisation of lysozyme in agarose and Ca-alginate hydrogel beads.

Materials and Methods

Mass transport calculations

The diffusion of salt into a gel bead containing protein is described by its mass balance:

$$\frac{\partial C_s'}{\partial t} = \frac{D_{e,s}}{R^2}\left(\frac{\partial^2 C_s'}{\partial r'^2} + \frac{2}{r'}\frac{\partial C_s'}{\partial r'}\right) \qquad (1)$$

with C'_s the dimensionless salt concentration which equals $C_s/C_{s,b}$ (C_s is the salt concentration in the gel bead and $C_{s,b}$ is the salt concentration in the bulk), R the bead radius, r' (= r/R) the dimensionless radial distance, t the time, and $D_{e,s}$ the salt effective diffusion coefficient in the gel. It is assumed that external mass transport limitation can be neglected. Boundary conditions are:

$$C_s'(0,r') = 0 \quad \text{for} \quad r' < 1 \quad \text{and} \quad C_s'(0,1) = 1 \tag{2}$$

$$C_s'(t,1) = 1 \quad \text{and} \quad \left(\frac{\partial C_s'}{\partial r'}\right)_{r'=0} = 0 \tag{3}$$

Likewise, the diffusion of protein in the gel bead towards the surrounding medium can be expressed in the same way. The supersaturation is computed as the relative supersaturation (Otálora *et al.*, 1997):

$$\sigma = \frac{C_p}{C_{p,s}} \tag{4}$$

with $C_{p,s}$ the protein solubility concentration. The solubility curve of lysozyme for sodium chloride can be expressed as the sum of two Green-type functions (Otálora *et al.*, 1997):

$$C_{p,s} = S_1 + S_2 \tag{5}$$

$$\ln(S_1) = \ln(0.97617) - 0.30376 C_s \tag{6}$$

$$S_2 = 65.69255 \ \exp(-1.71840 C_s) \tag{7}$$

where $C_{p,s}$ and C_s have dimensions % (w/v). The partial differential equations with the corresponding initial and boundary conditions, were integrated numerically by orthogonal collocation (Villadsen *et al.*, 1987), and 4th order Runge-Kutta-Gill method.

Experimental methods

Commercial hen egg white lysozyme was dissolved in sodium acetate buffer (pH 4.5) at room temperature. Agarose ("low gelling temperature", Gibco BRL) was dissolved in sodium acetate buffer (pH 4.5) at boiling temperature. The lysozyme and agarose solutions were mixed at 37°C. Beads were formed upon dripping into a cold (4°C) paraffin solution. The beads had an average diameter of 3.5 mm. Gel beads containing 0.5 and 1% (w/v) agarose have been produced. These beads contained a lysozyme concentration of 60 mg/ml and 40 mg/ml.

Ca-alginate beads were produced by dripping a sodium alginate (Fluka) solution into a well-stirred 0.27 M $CaCl_2$ solution. Gel beads containing 0.5, 1 and 1.5% (w/v) alginate were produced. The beads were cured during 1.5 h in the $CaCl_2$ solution and next washed in sodium acetate buffer (0.05 M, pH 4.5). The beads had an average diameter of 2.5 mm. Beads were filled with lysozyme by submerging them in a lysozyme solution (60 mg/ml, 0.05 M sodium acetate buffer, pH 4.5).

All crystallisation experiments were performed in a multi-well plate at 20°C. Lysozyme was crystallised at pH 4.5 (0.05 M sodium acetate) and NaCl was used as precipitating agent.

Results and Discussion

The evolution of NaCl and lysozyme concentration in a gel bead, which initially contained a uniformly distributed lysozyme concentration, upon immersion into the precipitant solution was calculated (Figure 1A). The calculated evolution of the relative supersaturation in the gel bead is shown in Figure 1B. Initially, a concentration peak is formed at the edge of the bead. This peak broadens and travels to the centre of the bead. Due to the diffusion of protein out of the gel bead, the supersaturation in the gel layer close to the bead edge is too low to give nucleation and no crystals will be produced in this part of the bead.

The growth of lysozyme crystals in agarose gel beads has been studied for varying NaCl bulk concentrations (1 ml of 4, 5, 6, 7 and 8% NaCl), agarose concentrations (0.5 and 1.0% w/v) and initial lysozyme concentrations in the gel beads (60 and 20 mg/ml) (Figure 2). As predicted by the simulation of the supersaturation profiles, there was no nucleation and growth of crystals in the outer layer of the gel beads. The density of crystals is the highest at the bead centre. The number of crystals increases with the NaCl, agarose and lysozyme concentration. The increase of agarose

concentration (0.5 to 1%) resulted in a higher number of crystals. This confirms the results of Vidal *et al.* (1998a) who indicated that agarose gel is a nucleation promotor. Other gel types can act as a nucleation inhibitor, like silica gel (Vidal *et al.*, 1998b).

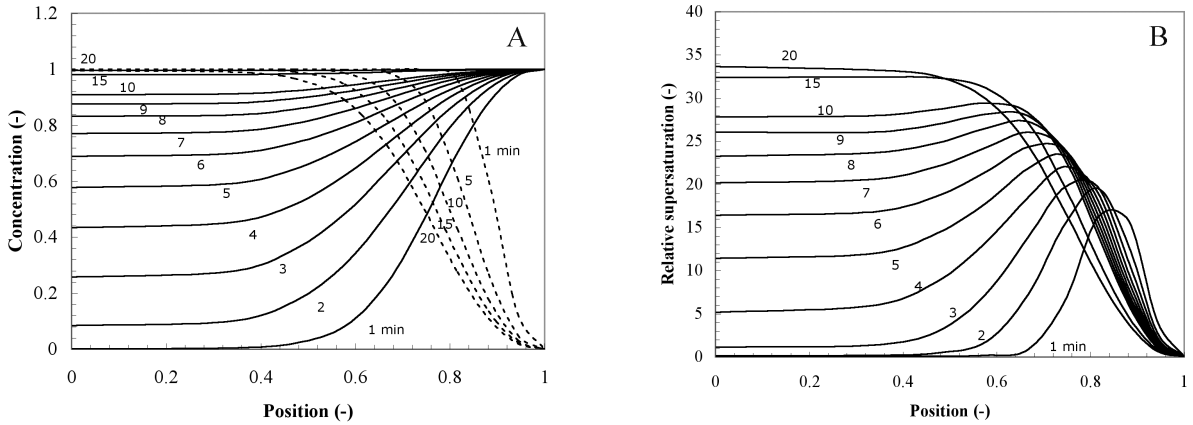

Figure 1: A. Evolution of the NaCl (—) and lysozyme (- - -) concentration in a 1% (m/v) agarose gel bead during the first 20 min (the gel bead edge is at position 1); bead diameter = 3.5 mm; $C_{p,i}$ = 40 mg/ml, $C_{s,b}$ = 7% (m/v) NaCl; **B.** Evolution of the relative supersaturation in a 1% (m/v) agarose gel bead during the first 20 min (the gel bead edge is at position 1); bead diameter = 3.5 mm; $C_{p,i}$ = 40 mg/ml, $C_{s,b}$ = 7% (m/v) NaCl.

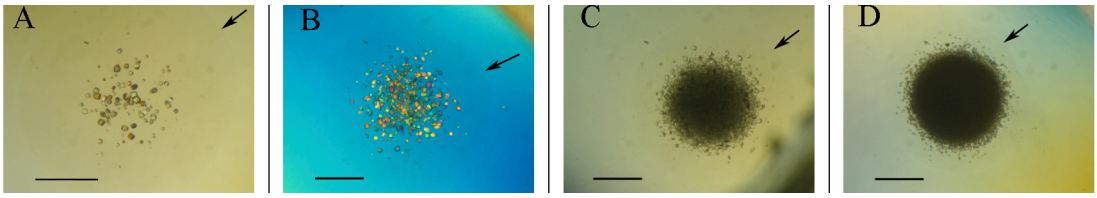

Figure 2: Crystallisation of lysozyme in agarose beads. Influence of the NaCl concentration (0.5 % agarose and 60 mg/ml lysozyme): A. 5%, B. 6%, C. 7%, and D. 8% w/v NaCl. The arrow indicates the edge of the gel bead; the scale bar length equals 1 mm.

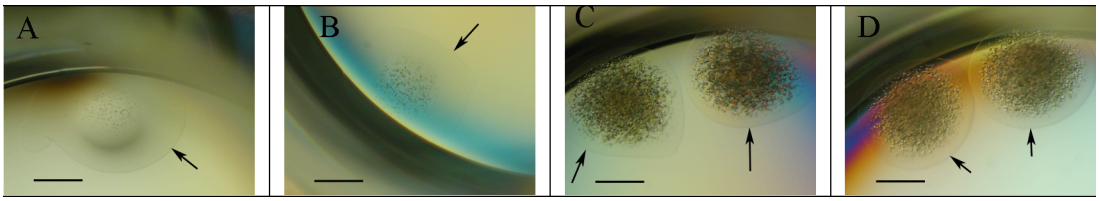

Figure 3: Crystallisation of lysozyme in Ca alginate beads. Influence of the NaCl concentration (1 % alginate and 60 mg/ml lysozyme): A. 5%, B. 6%, C. 7%, and D. 8% w/v NaCl. The arrow indicates the edge of the gel bead; the scale bar length equals 1 mm.

To our knowledge, protein crystallisation in alginate hydrogels have not yet been discussed in the scientific literature. Therefore, we tested Ca-alginate as a new protein crystallisation environment. The classical method of mixing equal volumes of a protein solution with the Na-alginate solution before gelation takes place by dropping the mixture in a stirred $CaCl_2$ solution could not be used since upon mixing the lysozyme solution with the Na-alginate solution a white precipitate was formed. Therefore, another method was developed. Firstly, Ca-alginate beads, which do not contain lysozyme, were produced. Next, the beads were submerged in a lysozyme solution, which allowed the diffusion of lysozyme into the beads and to fill them. This method can also be applied if other gel forming polymers, where the gelification process is performed under harsh conditions (like gelification at a high temperature, extreme pH values or/and in the presence of organic solvents) are used for protein crystallisation. An additional advantage of this method is that the protein solution is

purified upon diffusion into the gel bead (which can improve the quality of the obtained crystals in the beads). The influence of the alginate concentration (0.5, 1.0 and 1.5%) and NaCl concentration (4, 5, 6 and 7%) was investigated. As in the case of lysozyme crystallisation in agarose gel beads, the number of crystals increases with increasing NaCl (Figure 3) and gel concentration. Consequently, Ca-alginate gel is also a nucleation promotor.

Conclusions

We explored the use of hydrogel beads as a new protein crystallisation system. We showed that the protein crystallisation environment can be controlled in this system, i.e. nucleation rate and crystal growth can be influenced and optimised. A gel bead is a well-controlled system that can be used to direct the crystal growth; *e.g*. crystals growth at a particular supersaturation (and as a consequence at a specific growth rate). The supersaturation can not only be influenced by changing the precipitant concentration in the bulk solution but also by the surrounding protein concentration. This can easily be accomplished during the nucleation and/or the crystal growth phase of the crystallisation process.

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