Protein crystallisation in hydrogel beads and microcapsules

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Introduction

Protein crystallisation is a process that is used as an effective technique in bioseparations and protein structure determination by X-ray crystallography. Protein (enzyme) crystals can also be applied for the slow release of drugs and as enzymatic biocatalysts. The *in situ* crystallisation of proteins in hydrogel beads/microcapsules is a reaction-diffusion process (Willaert *et al*., 2005). The overall effectiveness of the process can be kinetically or diffusional controlled, which depends on the magnitude of the growth rate to the mass transport by diffusion or vice versa, respectively.
We studied the *in situ* crystallisation of hen egg white lysozyme (14.4 kDa) in Ca-alginate beads and alginate-poly-L-lysine (PLL) microcapsules. The dynamic behaviour of the internal precipitant and protein concentration, and relative supersaturation in a gel bead and microcapsule upon submerging in a precipitant solution is characterised theoretically using a transient diffusion model. The effect of the supersuturation and PLL reaction time on lysozyme crystallisation have been investigated experimentally.

Materials and Methods

Mass transport calculations
The transient diffusion equation of protein out and of salt into a gel bead and microcapsule is calculated in 2 spatial dimensions using a finite element technique (COMSOL Multiphysics):

$$\frac{\partial C_i}{\partial t} + \nabla\left(-D_i \nabla C_i\right) = 0 \qquad (1)$$

with C_i the protein or salt concentration and D_i the protein or salt effective diffusion coefficient, respectively. The supersaturation is calculated as the relative supersaturation σ (Otálora & García-Ruiz, 1997),

$$\sigma = \frac{C_p}{C_{p,s}} \qquad (2)$$

with C_p the protein concentration (mg/ml) and $C_{p,s}$ the protein solubility concentration. The solubility curve of lysozyme for sodium chloride can be expressed as (adapted expression, based on Otálora & García-Ruiz, 1997):

$$C_{p,s} = S_1 + S_2 \qquad (3)$$

$$\text{with } \ln(S_1) = \ln(0.97617) - 0.30376 C_s \quad \text{and} \quad S_2 = 65.69255 \exp(-1.71840 C_s) \qquad (4)$$

where $C_{p,s}$ and the salt concentration C_s have dimensions % (m/v).

Gel bead and microcapsule preparation
Sodium alginate solutions of 1.5% (w/v) were prepared. Ca-alginate beads were produced by blowing off the dripping Na-alginate solution from a syringe needle by compressed air into 4% (w/v) $CaCl_2$. Ca-alginate beads were "filled" with lysozyme by immersion the beads in a lysozyme solution (60 or 120 mg/ml in 0.05 M sodium acetate buffer, pH 4.5) overnight. Alginate-PLL microcapsules were prepared by shaking the alginate beads in a 0.1% (w/v) poly-L-lysine hydrochloride (Sigma) solution during 6 min (20°C), 10 min (8°C), 1 h (8°C) or 2 h (8°C).

Lysozyme crystallisation in beads/microcapsules
Crystallisation experiments with Ca-alginate beads were performed in a microbatch-well (72) plate at 20°C (1 well contained 1 - 5 beads and 5 µl precipitant solution; the plate was filled with paraffin oil). Crystallisation experiments with alginate-PLL microcapsules were performed in a multi-well

(24) plate at 20°C (1 well contained 5 - 10 microcapsules and 1 ml of the precipitation solution). Lysozyme was crystallised at pH 4.5 (0.05 M sodium acetate) using NaCl as the precipitating agent.

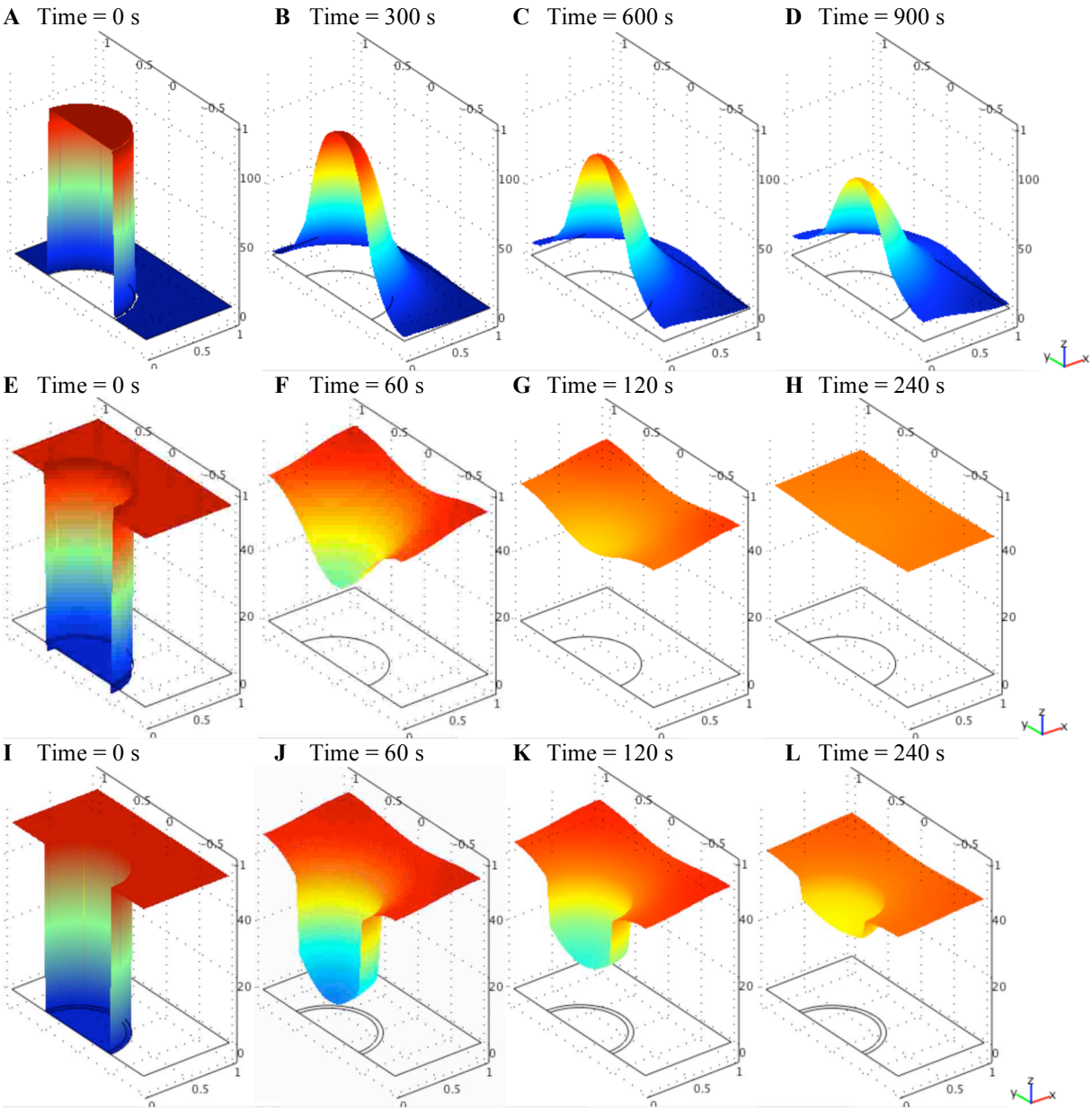

Figure 1: 2D calculations of the protein (A, B, C, D) and precipitant (E, F, G, H) concentration evolution in an alginate bead and bulk solution upon immersion in the precipitant solution. Evolution of the precipitant concentration in the microcapsule (I, J, K, L). X and Y axis represent the spatial coordinates (mm); the Z axis represents the protein or precipitant concentration (mg/ml).

Results and Discussion

Evolution of the NaCl, lysozyme and supersaturation profiles in alginate beads and alginate-PLL microcapsules

The evolution of the NaCl and lysozyme concentration in a gel bead, which initially contained a uniformly distributed lysozyme concentration, upon immersion into the precipitant solution is calculated (Fig. 1). The values of the used parameters were: diffusion coefficient of NaCl in the bulk solution $D_{s,bulk}$ = 1.6 10^{-9} m^2/s, in the gel bead $D_{s,gel}$ = 1.28 10^{-9} m^2/s, and PLL-membrane $D_{s,mem}$ = 1 10^{-10} m^2/s; diffusion coefficient of protein in the bulk solution $D_{p,bulk}$ = 1 10^{-10} m^2/s, in the gel bead $D_{p,gel}$ = 0.5 10^{-10} m^2/s, and PLL-membrane $D_{p,mem}$ = 1 10^{-13} m^2/s; initial lysozyme

concentration in the gel bead $C_{p,gel,i}$ = 120 mg/ml, and initial NaCl concentration in the bulk solution $C_{s,bulk,i}$ = 50 mg/ml; and a mean bead diameter of 1.0 mm. As can be seen in Figure 1, the response time of the diffusion of NaCl in the bead is much higher than the outward diffusion of lysozyme. The response time (τ) for diffusion of a molecule in or out a bead with radius R can be calculated as (Glueckauf, 1955): $\tau = R^2/(15D)$. The used "small" beads are characterised with a fast response time: the response time for NaCl is 0.217 min and for lysozyme 5.556 min.

The calculated evolution of the relative supersaturation in the gel bead and microcapsule are shown in Figure 2. Initially, a concentration peak, which is steeper in the case of a microcapsule since there is no outward diffusion of lysozyme, is formed at the edge of the bead. This peak broadens and travels to the centre of the bead. In gel beads, 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, due to the diffusion of protein out of the gel bead. This is confirmed in the experiments (see further). In microcapsules, the supersaturation reaches a maximum value which stays constant when steady-state condition is obtained, in contrast to the supersaturation evolution in the case of a gel bead where the steady-state value is strongly dependent on the total volume of the system.

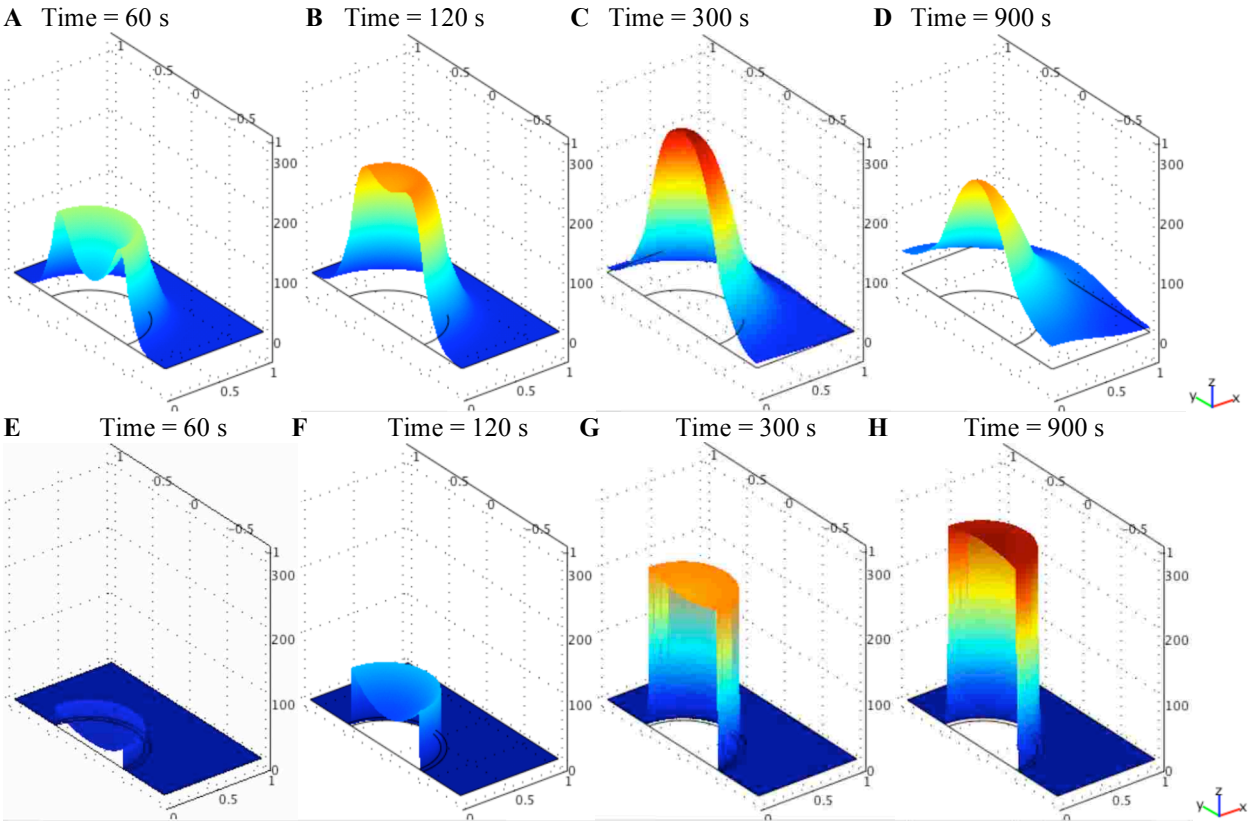

Figure 2: Evolution of the relative supersaturation profile in an alginate bead (A, B, C, D) and alginate-PLL microcapsule (E, F, G, H) upon immersion in the precipitant solution. X and Y axis represent the spatial coordinates (mm); the Z axis represents the relative supersaturation (dimensionless).

Growth of lysozyme in alginate beads and alginate-PLL microcapsules

Experiments in the microbatch system resulted in crystals in the gel beads and microcapsules for precipitant concentrations varying between 4 and 6% (w/v) (Fig. 3). A remarkable observation is that the morphology of the crystals that are embedded in the gel, is changed, i.e. crystals have a more globular, round shape. Due to the diffusion of lysozyme out of the gel beads and the microcapsules, ordinary lysozyme crystals can also be observed in the bulk solution (Fig. 3.C). This is an indication that the molecular weight cut-off of the PLL-membrane is too large to retain lysozyme in the microcapsule for a long time.

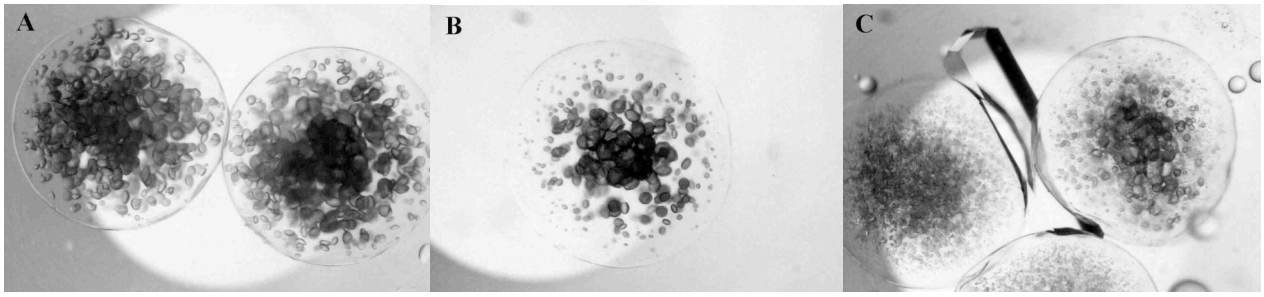

Figure 3: Crystallisation of lysozyme (60 mg/ml) in a microbatch system (400x magnification): **A.** alginate-PLL microcapsule, 6% (w/v) NaCl; **B.** alginate bead, 5% (w/v) NaCl; **C.** alginate-PLL microcapsule, 4% (w/v) NaCl.

In a second experiment, the reaction time of PLL with alginate was increased up to 2 h (at 8°C) to decrease the permeability of the PLL-membrane. The microcapsules were submerged in a relative large precipitant solution volume (1 ml). However, no crystals could be observed. This could be attributed to a still too large molecular weight cut-off of the PLL-membrane. To verify this hypothesis, empty alginate-PLL microcapsules were prepared and submerged overnight into a lysozyme solution (120 mg/ml) and next submerged in the precipitant solution (4%, 5%, and 6% NaCl). In this case, crystals could be observed (Fig. 4). A significant influence between 10 min reaction time and 1 or 2 h reaction time is observed.

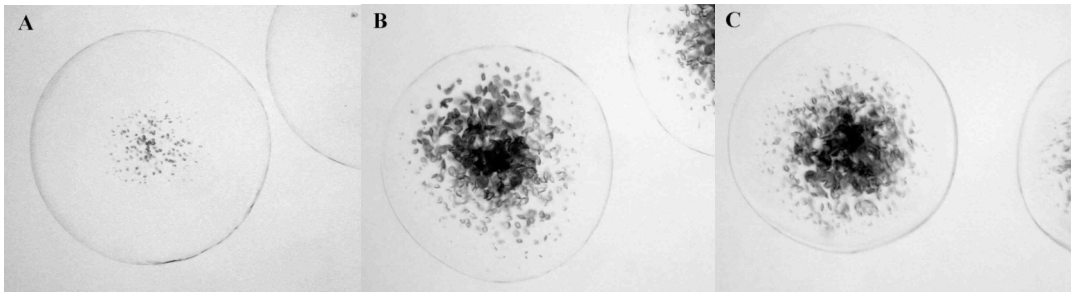

Figure 4: Lysozyme crystallisation in 6% (w/v) NaCl. Influence of the PLL reaction time at 8°C: **A.** 10 min, **B.** 1 h, and **B.** 2 h (400x magnification).

Conclusions

Lysozyme crystallisation in Ca-alginate beads and Ca-alginate-poly-L-lysine microcapsules has been demonstrated. The morphology of the crystals in the gel was significantly different from solution grown crystals. The molecular weight cut-off of the PLL-membrane was too large to retain lysozyme in the microcapsule for a long time. Further research will be focused on a reduction of the molecular weight cut-off of the microcapsule membrane.

References

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