Chapter 5

Marangoni convection and protein crystallisation

5.1 Introduction

Protein crystallisation is thought to yield better results when performed in a microgravity environment. It is assumed that the absence of gravity-driven buoyancy is one of the main factors responsible for an improvement of the quality of the grown crystals. However, while buoyancy is reduced significantly in microgravity, Marangoni convection might still be present. In co-operation with crystal growers and developers of protein crystallisation facilities a short-duration microgravity experiment was performed in order to investigate possible Marangoni effects in a hanging drop configuration. This experiment also served to test newly developed protein crystallisation units.

Firstly, an introduction to protein crystallisation (which is still considered more an art than a science [1]) and its relation to microgravity is provided. Then the Marangoni effect is discussed, as well as its possible occurrence in protein crystallisation systems. Convection is thought to be detrimental to protein crystal growth, and the arguments generally provided to support this hypothesis are presented subsequently. Finally, a description is given of the protein crystallisation hardware and the results of the microgravity experiment.

Some of the information in this chapter has already been provided in earlier chapters (specifically chapter 1). Since this chapter constitutes a separate part of this thesis, it was decided not to refer to these earlier chapters to improve readability.

5.2 Protein crystallisation

The knowledge of the three-dimensional structure of a protein is essential for the understanding and explanation of the biological activity of such a protein. For larger molecules (> 20000 Dalton), the only way to determine this structure is X-ray diffraction, which requires single crystals of the protein (see e.g. [2, 3, 4]). Failure to produce any crystals at all or crystals of high enough quality is currently the most important obstacle in protein X-ray crystallography.

Protein crystallisation is generally performed by dissolving the protein in a solution containing a precipitating agent, a pH-buffer and in some cases additional additives, such as anti-fungal agents. The physical properties of this solution are then altered in such a way that the solution becomes supersaturated. This can be done by changing the precipitant concentration, the temperature or the pH-value. Currently, most protein crystallisation methods are based on supersaturating the solution by changing the precipitant concentration and there are four such methods:

1. Vapour diffusion.
2. Liquid-liquid diffusion.
3. Dialysis.
4. Batch crystallisation.

Details concerning these methods can be found in various publications (for example [5, 6, 7, 8]). The vapour diffusion method, especially the hanging drop method, is the most widely used method for protein crystallisation. With this method, a hanging drop containing the protein solution is separated by an air space from a reservoir containing precipitant solution with a higher precipitant concentration than the protein solution. Usually, a buffered protein solution is mixed 1:1 with a buffered precipitant solution, forming the drop solution, while the pure precipitant solution is placed in the precipitant reservoir.

Three kind of precipitants are used. These are salts, such as. sodium chloride and ammonium sulphate, organic solvents, such as ethanol and acetone, and polyethylene glycol (PEG) of various molecular weights. In salt or PEG systems water is transferred from the protein to the precipitant solution, while in organic solvent systems the organic solvent is transferred to the protein solution from the precipitant solution. The use of organic solvents is limited, because organic solvents tend to induce protein denaturation [9].

The supersaturation is a function of pH, ionic strength, dielectric constant of the liquid, temperature and protein concentration. The influence of the various factors is described, for example, by Arakawa and Thimasheff [9]. The level of supersaturation should be higher for nucleation than for protein crystal growth. This explains the need for accurate control of supersaturation.

One phenomenon often seen in protein crystal growth is the “cessation of growth” phenomenon [10]. It is found frequently that protein crystals grow to a fixed terminal size, after which no further growth takes place. When these crystals are cleaved subsequently, both pieces start growing on their newly exposed faces till both have reached their terminal size. The phenomenon is probably due to surface contamination, which can be influenced by convection.

5.3 Microgravity

Under normal terrestrial conditions, gravity is generally assumed to disturb the crystal growth from solutions in two ways:

1. Due to mass transfer during the crystallisation process, concentration gradients develop around the growing crystal. The associated density gradients result in buoyancy forces and thereby initiate Rayleigh convection. The influence of convection on protein crystal quality has been the subject of many investigations. It is believed to be detrimental to crystal quality. There is no general agreement on the subject, however.
2. Crystals growing free in solution are subjected to sedimentation. This causes asymmetrical growth and intergrowth of crystals.

For these two reasons it is believed that the use of a microgravity environment can improve the quality of the growing crystal. In addition, under microgravity conditions it might be possible to grow crystals of proteins, which do not crystallise at all under terrestrial circumstances. Additional advantages of a microgravity environment might be found in containerless processing, as foreign surfaces affect nucleation and growth of protein crystals [11, 12].

Another aspect of the influence of gravity on protein crystals is outlined by Noever [13]. In his paper it is stated that gravitational strain can lead to a maximum stable size for protein aggregates and protein crystals, above which the aggregate becomes unstable or the crystal becomes susceptible to strain-induced flaws or cracks. The higher the gravity level is, the smaller the critical size becomes.

Protein crystallisation in microgravity has been the subject of many experiments. Experiments have been performed on board of longer duration flights such as Russian satellites (Photon and COSIMA-2) [14, 15, 16], the space station MIR [17], Chinese spacecrafts (COSIMA-1 [18], FSW-2 [19]), and on board of the US Space Shuttle [11, 12, 20, 21 ,22, 23, 24, 25, 26], but also on shorter duration sounding rocket flights (TEXUS [20, 21] and MASER [27]).

Some experiments thus far indicated that crystals grown in microgravity are on average larger and of better quality (with respect to X-ray diffraction properties). Usually, the improvement found is only with respect to experiments conducted in parallel on earth. In recent investigations, however, improvement in crystal quality (enhancement of X-ray diffraction resolution) with respect to the best crystals ever grown on earth has been found for various proteins [23, 24, 26]. Among the best results are those obtained by Day and McPherson [12] for the crystallisation of satellite tobacco mosaic virus by liquid-liquid diffusion. They found the X-ray resolution of microgravity crystals to be as good as 1.8 Å compared to 2.3 Å for the best crystals ever grown on earth.

Not only did the microgravity experiments sometimes produce better [12, 15, 17, 18, 19, 22, 23, 24, 25, 26, 27] and larger [11, 12, 14, 17, 18, 19, 20, 21, 22, 24, 25, 27] crystals (than in the reference experiments), in some cases a different morphology (space group and unit cell dimensions) [11, 12] and a faster growth was observed. Occasionally, a different (better) crystal habit was found [11, 12, 14, 15, 16, 17, 19, 22], sometimes as a result of the absence of sedimentation. Uniform quality is the major difference between space- and lab-grown crystals according to Day and McPherson [12, 23]. They also observed an absence of secondary nucleation and crystal aggregation. In some other experiments no crystals were found on earth in contrast to the microgravity experiments [14, 21], from which it was concluded that microgravity may favour the nucleation step. Other authors, however, have concluded from the fact that multiple crystals were found on earth in contrast to only a small number of crystals in space, that nucleation may be suppressed in microgravity [11, 15, 24]. McPherson and Day argue that, in the case of vapour diffusion, nucleation is increased in microgravity, while nucleation is reduced in microgravity when liquid-liquid diffusion is used [12]. They also found a faster equilibration of the protein droplet and the precipitant reservoir (vapour diffusion) in

microgravity, contrary to the (preliminary) findings of Sibille and Baird [28]. Strong et al. found that in microgravity a higher lysozyme concentration was necessary to produce crystals than on earth [17]. During the STS-29 flight showers of small crystals were produced in each crystallisation chamber for each of 15 proteins. No explanation was given [22]. Littke and John used Schlieren photography to demonstrate that no convection plumes arise around growing crystals in microgravity in a liquid-liquid diffusion set-up [21]. Hilgenfeld et al. [16] were the only investigators finding a reproducibly worse crystal size and quality in microgravity. They crystallised lysozyme (from Streptomyces coelicolor) and thermolysin on the COSIMA-1 and 2 missions.

All these findings are somehow contradictory and no sufficient explanation has yet been given for all results obtained in microgravity. A summary of observations regarding the influence of microgravity has been given by McPherson [29].

5.4 Marangoni convection in protein crystallisation systems

Although microgravity might prevent the density-driven Rayleigh convection from developing, it does not rule out another type of convection; the surface-tension-driven Marangoni convection. In many protein crystallisation systems, solvent evaporates from the solution in order to create and maintain the supersaturation conditions necessary for crystal growth. Due to differences in surface tension along these evaporating interfaces, the Marangoni effect may introduce unwanted flows, even under microgravity conditions. It should be noted that Marangoni effects are only possible in the vapour diffusion and the liquid-liquid diffusion crystallisation methods, as a phase boundary is only present when these methods are used.

Relatively little is know about the possibility of Marangoni convection occurring during protein crystallisation. In one paper, some qualitative remarks have been made about the subject [30]. Another paper describes Marangoni convection as a result of temperature gradient in solidifying lysozyme solution drops, but this paper is not pertinent to protein crystallisation [31]. In two more recent papers [32, 33], Monti and Savino try to model vapour diffusion systems from a fluid dynamic point of view. They included both Marangoni and Rayleigh convection in their model. These papers are commented on at the end of this section. Firstly, the possibility of a Marangoni effect occurring in protein crystallisation is discussed qualitatively. Then, some aspects of interfacial behaviour of protein solutions are reviewed. Finally, some aspects relating to Marangoni convection in protein crystallisation systems are assessed quantitatively.

5.4.1 Qualitative description of the phenomenon

Marangoni convection can be the result of concentration and temperature gradients along an interface. Increasing, or sometimes decreasing, temperature as well as increasing the concentration of a surface-tension-lowering solute may locally decrease the surface tension, and liquid is drawn from places with low surface tension to high surface tension areas. It has been demonstrated in the past [e.g. 34, 35] that Marangoni convection can occur in two different forms:

1. Macro-convection, where convection originates from concentration or temperature differences due to an asymmetry in the system.
2. Micro-convection, where the convection is initiated by small (random) temperature or concentration disturbances that grow in time.

Micro-convection can only develop when the system under consideration is stationary unstable with respect to the Sternling-Scriven criteria [36, 37], i.e. roll-cell instabilities can appear if mass transfer takes place out of the phase of higher kinematic viscosity and lower molecular diffusivity if the gradient of static surface tension with concentration is negative, or into this phase if the gradient of static surface tension with concentration is positive. When analysing protein crystallisation systems, distinction should be made between the various precipitant systems. The analysis is complicated by the fact that a protein crystallisation system is a multi-component system. When a salt is used as a precipitant, water is transferred from the protein solution to the air. Both salt and protein concentration then increase. Salt increases the surface tension while proteins decrease it (see section 5.4.2). Protein is likely to have a larger influence on surface tension. Therefore, according to Sternling and Scriven, the system is oscillatory unstable, rather than stationary unstable. When an organic solvent is used as a precipitant, the organic solvent is transferred to the protein solution from the air, thereby lowering the surface tension. Also in this case, the protein crystallisation system is oscillatory unstable with respect to the Sternling-Scriven criteria. In either case, Marangoni convection is theoretically possible, but micro-convection is very unlikely.

Macro-convection in a hanging drop configuration could occur as a result of an asymmetry with respect to the concentration field. Asymmetry is present in a protein crystallisation system, for example, due to the fact that the distance between precipitant reservoir and drop is not the same everywhere, and that generally the drop is shaped asymmetrically. Macro-convection is more intense in a stationary unstable system, and is therefore less intense in a protein/salt system.

Even when no mass transfer occurs between the liquid and the gas phase, concentration gradients might develop along the liquid-gas interface in a crystallisation system. An example is given for a protein/salt system. According to various authors [10, 29, 38] a protein depletion zone might develop around a growing crystal. This depletion zone can have dimensions of the order of 10^{-4}-10^{-3} m [10, 39], about an order of magnitude smaller than crystallisation drop size. It is disturbed by Rayleigh convection in normal gravity, which is believed to lead to inferior crystal growth (see section 5.5). However, Marangoni effects might also disturb the depletion zone profile. Suppose the distance of a crystal from the interface is comparable to the thickness of the depletion zone. The protein concentration (c_p) at the interface nearest to the crystal is then lower than in the surrounding areas. Liquid with higher protein concentration is

drawn to this spot, increasing the protein concentration and increasing the growth speed of the crystal face facing the liquid-gas interface (figure 1). In this way the Marangoni effect may serve to augment the crystallisation growth rate or cause the crystal habit to change.

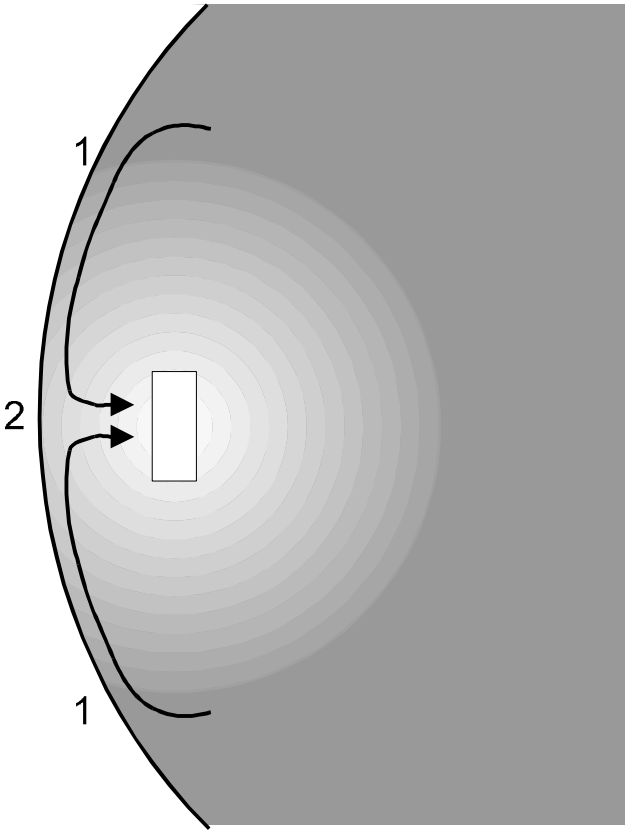

*figure **Fout! Bladwijzer niet gedefinieerd.*** *Crystal growing in a drop, surrounded by a protein depletion halo. When the crystal is growing close to the interface, the Marangoni effect increases the mass transfer rate of protein toward the crystal face which is closest to the interface. The protein concentration at 1 is larger than at 2.*

A Marangoni effect caused by temperature gradients, also called thermocapillarity, can occur in a protein crystallisation system whenever a macroscopic temperature gradient is applied, or whenever releasing or absorbing heat of solution causes temperature gradients. When surface tension decreases with temperature, as is usually the case, then a system that releases heat during mass transfer is stable, e.g. an organic solvent crystallisation system, while a system that needs heat during mass transfer, is always unstable, like a protein/salt system [40].

A macroscopic gradient can occur when the supersaturation is brought about by slowly changing the temperature (however, in this case no gas-liquid interface is usually present) or by placing heat sources such as lamps in the vicinity of the drop. This should be avoided in any case.

One additional comment should be made. It is the requirement of some protein crystallographers to control the supersaturation levels in a protein solution [41, 42, 43]. For example, it could be advantageous to decrease supersaturation levels after nucleation has taken place. In that case, mass and heat transfer to the drop are exactly the other way as described above. The mass transfer systems described above are then stationary unstable with respect to the Sternling-Scriven criteria and convection is more likely.

The intensity of Marangoni convection due to concentration gradients can be expressed by the dimensionless Marangoni number (analogous to e.g. [44]).

$$Ma = \frac{\left(-\frac{\partial\gamma}{\partial c}\right)H}{\mu D}\Delta c \qquad (1)$$

In this equation, γ is the static surface tension, c the concentration of the component influencing the surface tension, H a characteristic dimension of the system (film thickness, drop diameter), μ the dynamic viscosity and D the diffusivity of the component causing the surface tension gradients. When the Marangoni convection is a result of temperature gradients, the Marangoni number reads:

$$Ma = \frac{\left(-\frac{\partial\gamma}{\partial T}\right)H}{\mu\, a}\Delta T \qquad (2)$$

In this equation, a is the thermal diffusivity. Marangoni convection is enhanced by a strong dependence of surface tension on either concentration or temperature, low viscosity, small (thermal) diffusivity and large gradients in concentration or temperature. Large concentration and temperature gradients parallel to the interface are favoured by large driving forces for mass and heat transfer, and by approximately equal mass (or heat) transfer resistances in the two phases.

Given an unstable system with respect to the Sternling-Scriven criteria, micro-convection only develops when the Marangoni number is larger than a critical Marangoni number. This critical number depends on the geometry and the boundary conditions of the system [45]. Furthermore, these critical numbers depend on various factors concerning the condition of the liquid-gas interface, such as Gibbs absorption [46], the presence of insoluble surfactants, and the surface viscosity of the liquid [47]. These factors are discussed in more detail in section 5.4.3.

5.4.2 Surface tension of protein solutions.

The size of surface tension gradients is a function of, among other things, the concentration dependence of the surface tension. For aqueous solutions of organic solvents, this surface tension dependence on concentration is often well known, as for example with

acetone [48], or it can be calculated from surface tension values of the pure components [49]. The surface tension of solutions of various salts in water is also known [48]. For protein solutions less is known about surface tension and its dependence on concentration.

Greenley reports on the surface tension of various proteins in aqueous solutions as a function of concentration [50]. Surface tensions are measured in salt-free and 3 M NaCl solutions. All proteins show quite a large influence on surface tension. An increase of the protein concentration always results in a decrease of the surface tension. This is also found for various plasma proteins by Katona et al. [51]. However, the magnitude of this dependence can differ a factor 15 from one protein to another. Some proteins show surfactant-like behaviour. When the concentration is increased beyond a critical micelle concentration, no decrease of static surface tension is found when the protein concentration is further increased [52]. Adding a salt to a protein solution sometimes alters the surface tension of the solution. In general, the surface tension of enzymatic protein solutions is more sensitive to salt than that of non-enzymatic proteins. The salt usually increases the surface tension [50]. Changing the temperature and pH can also affect the surface tension [50,51]. The higher the temperature is, the lower the surface tension. Some authors state that all these changes in surface tension (pH, temperature, effect of a salt) reflect conformational changes of the protein in solution [51, 53].

The surface tension of a protein solution also depends on the time after which a new gas-liquid interface is created [52, 54]. During times varying between somewhat more than 60 minutes and 150 hours [51,52,54] the surface tension of a protein solution decreases (at first exponentially) to a fixed value. The rate of decrease depends on the protein (and determines the foamability of the protein solution). Graham and Phillips studied the kinetics of the absorption and subsequent denaturation of three different proteins at the air-water interface [55]. A more flexible protein such as β-casein is absorbed and changes its conformation at the same time, in contrast with lysozyme, which has a more static structure.

Greenley also reported on the dependence of surface tension on concentration of PEG-4000. For very small concentrations the dependence is quite large and negative. However, in the concentration ranges often encountered in protein crystallisation, the surface tension becomes independent of the PEG-concentration [50].

In our laboratory, a preliminary investigation was performed into the dependence of surface tension on concentration of lysozyme under typical protein crystallisation conditions. Results of these experiments indicate that the value of $(d\gamma/dc)$ directly after forming the gas-liquid interface has the same order of magnitude as the value for the water/acetone system around 5 % w/w, which is $-1.6\ 10^{-4}\ m^3/\ s^2$. It is important to note that the results are only applicable for freshly formed interfaces. Details of these experiments are presented elsewhere [56].

All these observations show that the surface tension of a protein solution is a complicated function of protein-concentration, salt or organic solvent concentration, temperature, pH, concentration of impurities and time.

5.4.3 Quantitative analysis of Marangoni effects.

As a protein crystallisation system is a very difficult system to model from first principles, this section presents an assessment of the magnitude of the Marangoni effect based on comparison.

In the first part of this section, an estimate is presented on the importance of thermal Marangoni convection relative to solutal Marangoni convection, both originating from mass transfer during crystallisation. Subsequently, with the help of a simple model, an attempt is made to describe the mass transfer of water in a protein/salt crystallisation system and the mass transfer of organic solvent in a protein/organic solvent crystallisation system. From this model, the rates of mass transfer are found, as well as the magnitudes of the mass transfer resistance in liquid and gas phase. This leads to a comparison between the two different crystallisation systems with respect to the magnitude of gradients in concentration of the surface tension determining solute parallel to the interface. From this a ratio is estimated, indicating the size of a possible solutal Marangoni effect in one protein crystallisation system compared to the other. Finally, some remarks are made on the interfacial properties of a protein solution.

Solutal versus thermal Marangoni convection.

In order to compare the effect of temperature gradients caused by evaporation with the effect of concentration gradients caused by the same evaporation, the equations describing the problem need to be studied. When Newtonian behaviour of the liquid is assumed and interfacial properties other than the surface tension are neglected (which is very crude as the last part of this section indicates), and the influence of salt concentration on surface tension is ignored, the following dimensionless boundary condition to the Navier-Stokes equations remains (time (t), velocity (**v**) and length are non-dimensionalised using the kinematic viscosity (ν) and the drop diameter (d_d); protein concentration (c) and temperature are non-dimensionalised using the initial concentration (c_0) and temperature(T_0)).

$$\left(\nabla_{\perp}\cdot\mathbf{v}_{\Gamma}\right)=\frac{\mathrm{Ma_c}}{\mathrm{Sc}}\nabla_{\Gamma}\,\mathrm{c}+\frac{\mathrm{Ma_T}}{\mathrm{Pr}}\nabla_{\Gamma}\,\mathrm{T} \tag{3}$$

$$\mathrm{Ma_c}=\frac{\left(-\frac{\partial\gamma}{\partial\mathrm{c}}\right)\mathrm{d_d}\;\mathrm{c_0}}{\mu\,\mathrm{D}} \tag{4}$$

$$\mathrm{Ma_T}=\frac{\left(-\frac{\partial\gamma}{\partial\mathrm{T}}\right)\mathrm{d_d}\;\mathrm{T_0}}{\mu\,\mathrm{a}} \tag{5}$$

In these equations, the Schmidt number (Sc = ν/D) and the Prandtl number (Pr = ν/a) have been introduced. The symbol $\nabla_{\perp}$ indicates the nabla operator normal to the gas-liquid interface. The symbol ∇_{Γ} indicates the nabla operator parallel to the interface. To get an impression of which term on the right hand side of equation (3) is more important, it is also necessary to involve the convection-diffusion equation and the heat balance. These equations determine the size of the gradients in protein concentration and temperature parallel to the interface present in equation (3).

$$\frac{\partial}{\partial\mathrm{t}}\mathrm{c}=-\left(\mathbf{v}\cdot\nabla\mathrm{c}\right)+\frac{1}{\mathrm{Sc}}\nabla^{2}\mathrm{c} \tag{6}$$

$$\frac{\partial}{\partial\mathrm{t}}\mathrm{T}=-\left(\mathbf{v}\cdot\nabla\mathrm{T}\right)+\frac{1}{\mathrm{Pr}}\nabla^{2}\mathrm{T} \tag{7}$$

The approximated boundary conditions to these two equations are (all heat is assumed to be dissipated in the liquid phase):

$$\nabla_{\perp}\mathrm{c}=-\frac{\dot{m}\mathrm{d_d}}{\mathrm{Dc_0}}\mathrm{w_p} \tag{8}$$

$$\nabla_{\perp}\mathrm{T}=\frac{\dot{m}\mathrm{d_d}}{\lambda\mathrm{T_0}}=\frac{\dot{m}\mathrm{d_d}}{\mathrm{aT_0}}\frac{\Delta\mathrm{H_v}}{\rho\,\mathrm{c_p}} \tag{9}$$

In these equations, λ is the thermal conductivity, ΔH_v is the heat of vaporisation, ρ and c_p are the density and the heat capacity of the liquid, $\dot{q}$ is the local heat flux and $\dot{m}$ is the local mass flux, which depends amongst other things on local interface concentration, Biot number and gas phase concentration. Furthermore, w_p is the mass fraction of protein. The term $-w_p$ in equation (8) accounts for the fact that water evaporates while this equation relates to the protein concentration.

To get an idea of the ratio of $\nabla_\Gamma c$ and $\nabla_\Gamma T$, a relationship needs to be obtained for the influence of diffusivity and boundary conditions on the interfacial concentration. Consider a one-dimensional problem in which a flux of mass or heat is dissipated by diffusion (a pre-convective state). The larger the coefficient of diffusion is, the larger the interface concentration or temperature is. The problem is the problem of a semi-infinite space ($y \geq 0$) in which at $t = 0$ and $y = 0$ a flux is applied to the boundary. The quantity of interest is called X:

$$\frac{\partial^2 X(t,y)}{\partial y^2} = k\frac{\partial X(t,y)}{\partial t} \qquad (10)$$

boundary conditions $\quad t = 0 \quad X(t,y) = 1$

$$t > 0 \qquad \kappa\left(\frac{\partial X}{\partial y}\right)_{y=0} = Q$$

A solution for the interfacial quantity (at $y = 0$) can be derived [57].

$$X(t,0) = 1 - \frac{2Q}{\kappa}\sqrt{\frac{t}{k\pi}} \qquad (11)$$

Suppose the flux differs from one place to another by a factor (1+f). The difference in the value of the interfacial quantity between these two places can be expressed as:

$$\Delta X = \frac{2Qf}{\kappa}\sqrt{\frac{t}{k\pi}} \qquad (12)$$

When one wants to compare the ratio of differences in interfacial concentration and interfacial temperature caused by evaporation, the following substitutions should be made (see equations (6) - (9)) for the quantities X, Q, k and κ.

concentration: $\quad X = c \qquad k = \frac{\nu}{D} \qquad \kappa = D \qquad Q = -\frac{\dot{m}d_d}{c_0}w_p$

temperature: $\quad X = T \qquad k = \frac{\nu}{a} \qquad \kappa = \lambda \qquad Q = \frac{\dot{m}d_d}{T_0}\Delta H_v$

These substitutions lead to:

$$\frac{\Delta X(\text{concentration})}{\Delta X(\text{temperature})} = \frac{\nabla_\Gamma c}{\nabla_\Gamma T} = -w_p\frac{T_0}{c_0}\frac{\rho c_p}{\Delta H_v}\sqrt{\frac{a}{D}} \qquad (13)$$

Defining the ratio R as the ratio between the solutal part and the thermal part of the boundary condition (3) helps in analysing the relative importance of thermal and solutal Marangoni convection.

$$R \equiv \frac{\frac{Ma_c}{Sc}}{\frac{Ma_T}{Pr}} \frac{\nabla_\Gamma c}{\nabla_\Gamma T} = -\frac{\left(-\frac{\partial\gamma}{\partial c}\right) c_0}{\left(-\frac{\partial\gamma}{\partial T}\right) T_0} w_p \frac{T_0}{c_0} \frac{\rho c_p}{\Delta H_v} \sqrt{\frac{a}{D}} \qquad (14)$$

$$R = -\frac{\left(-\frac{\partial\gamma}{\partial c}\right)}{\left(-\frac{\partial\gamma}{\partial T}\right)} w_p \frac{\rho c_p}{\Delta H_v} \sqrt{Le} \qquad (15)$$

If the absolute value of R is much larger than one, solutal Marangoni convection dominates. On the other hand, for values of R much smaller than one, thermal Marangoni convection prevails. If all physical properties are taken equal to those of water, except for the diffusion coefficient (equal to that of lysozyme in water), the following expression for this ratio is found (all physical constants used for the calculations are tabulated in table 1):

$$R \approx -65\, w_p \frac{\left(\frac{\partial\gamma}{\partial c}\right)}{\left(\frac{\partial\gamma}{\partial T}\right)} \qquad (16)$$

The value of (dγ/dT) depends on the kind of protein. Examples are presented by Katona et al. [51]. The maximum average value in the range 20-80 °C found in that paper is -2.2 10^{-4} N/mK (the value for pure water is -1.53 10^{-4} N/mK). The value of (dγ/dc) depends very much on the kind and the concentration of protein. Taken from the work of Greenley [50], values range from -1.6 10^{-4} m^3/s^2 (glucose oxydase) to -2.4 10^{-3} m^3/s^2 (thyroglobulin) (at 3 M NaCl and isoelectrical points). These are values at low protein concentrations. A value of about -2 10^{-4} m^3/s^2 at lysozyme concentrations between 5 and 25 mg/ml was found in our laboratory for short times after creating the interface [56]. Surface tension gradients at low concentrations are generally larger. When a value of -2 10^{-4} N/mK is taken for (dγ/dT), R takes a value in the order -10^{-1} to -10^{1}, depending on the type and the concentration of protein. Therefore, in protein/salt systems, solutal and thermal Marangoni convection are expected to be roughly of the same order of magnitude (but competing). Typically, for lysozyme at a concentration of 10 mg/ml and at a short time after creating the interface, R takes a value of -0.6. It should be noted that solutal convection is absent when the protein concentration no longer influences the surface tension, which is the case for high protein concentrations and long times after creating the gas-liquid interface.

The same analysis can of course be given for protein/organic solvent systems. However, diffusion coefficient, vaporisation enthalpy and concentrations are those of the organic solvent. Furthermore, $-w_p$ is dropped from formula (14) as the organic solvent is the evaporating as

well as the surface-tension-determining component. When acetone is chosen as an example, R takes the value of 84 ((dγ/dc) of this system is taken to be equal to that of 5 % w/w acetone-water without protein). R is generally in the order 10^1 to 10^2, indicating that in organic solvent-protein systems solutal Marangoni convection is dominant.

table 1 Values used for the calculations in this chapter

quantity	value	dimension	reference
a (water)	$1.43\ 10^{-7}$	$m^2\ s^{-1}$	[58]
c_p (water)	$4.185\ 10^{6}$	$J\ kg^{-1}\ K^{-1}$	[58]
$(d\gamma/dc)_o$ (acetone in water, 25 °C)	$-1.6\ 10^{-4}$	$m^3\ s^{-1}$	[59]
$(d\gamma/dc)_o$ (ethanol in water, 25 °C)	$-1.9\ 10^{-4}$	$m^3\ s^{-1}$	[59]
$(d\gamma/dc)_p$ (lysozyme)	$-2\ 10^{-4}$	$m^3\ s^{-1}$	[56]
$(d\gamma/dT)$ (water, close to 20 °C)	$-1.53\ 10^{-4}$	$kg\ s^{-2}\ K^{-1}$	[58]
$(d\gamma/dT)$ (protein in water, maximal)	$-2.2\ 10^{-4}$	$kg\ s^{-2}\ K^{-1}$	[52]
D_{oa} (acetone in air, 21 °C)	$1.04\ 10^{-5}$	$m^2\ s^{-1}$	[60]
D_{oa} (ethanol in air, 25 °C)	$1.19\ 10^{-5}$	$m^2\ s^{-1}$	[58]
D_{wa} (water in air, 25 °C)	$2.56\ 10^{-5}$	$m^2\ s^{-1}$	[58]
D_{wo} (acetone in water, 21 °C)	$1.27\ 10^{-9}$	$m^2\ s^{-1}$	[60]
D_{wo} (ethanol in water, 25 °C)	$1.27\ 10^{-9}$	$m^2\ s^{-1}$	[61]
D_{wp} (lysozyme in water, 20 °C)	$1.04\ 10^{-10}$	$m^2\ s^{-1}$	[62]
D_{ws} (NaCl in water, 25 °C)	$1.5\ 10^{-9}$	$m^2\ s^{-1}$	[48]
m (acetone in air/water, 25 °C)	$1.6\ 10^{-3}$	-	[63]
m (ethanol in air/water, 25 °C)	$2.3\ 10^{-4}$	-	[64]
p_0 (water, 20 °C)	2337	Pa	[58]
W (NaCl)	2	-	[65]
ΔH_v (acetone)	$5.5\ 10^{5}$	$J\ kg^{-1}$	[48]
ΔH_v (water, 25 °C)	$2.4\ 10^{6}$	$J\ kg^{-1}$	[48]
ρ (water, 20 °C)	998	$kg\ m^{-3}$	[58]

Solutal Marangoni convection in organic solvent- and salt-protein systems.

In this subsection, the magnitude of the solutal Marangoni effect caused by mass transfer in a protein/salt system is compared to the magnitude in a protein/organic solvent system. To

do this, an estimate is required for the size of the concentration gradients parallel to the interface existing in both systems as a result of geometrical effects. In the first part of this section it was established that these concentration gradients parallel to the interface are dependent on the flux at the interface and the diffusion coefficient of the surface tension determining component. Therefore, the mass transfer rate in protein crystallisation systems is first calculated with the help of a simple one-dimensional, stationary model.

In this model it is assumed that there is no convection yet and all mass transfer is by diffusion (pre-convective state). To calculate the flux of water, the interfacial concentration of protein and the interfacial concentration of water in a protein/salt system, equations are needed for transfer of water in the drop, transfer of protein in the drop, transfer of water in the vapour phase and an equilibrium relation describing the vapour pressure of water as a function of the salt concentration. For the transport equations the simplified Maxwell-Stefan equations [66] are used. It is presumed that no mass transfer limitation is present between the vapour phase and the precipitant reservoir and that there is no mass transfer limitation at the vapour-liquid interface itself. The latter assumption can in some cases be an oversimplification [67], as surface active agents can form a mass transfer barrier. Further, the water and protein concentration in the centre of the drop is considered to be constant for this analysis. The mole fraction of protein is neglected compared to that of water and salt. This leads to (see figure 2.a):

$$x_{w,i} - x_{w,b} = -\delta_l \frac{2 - x_{w,i} - x_{w,b}}{2 D_{ws} C_{tot}} N_w - \delta_l \frac{x_{p,i} + x_{p,b}}{2 D_{wp} C_{tot}} N_w \qquad (17)$$

$$C_{w,g,L} - C_{w,g,i} = -\delta_g \frac{N_w}{D_{wa}} \qquad (18)$$

$$x_{p,i} - x_{p,b} = \delta_l \frac{x_{p,i} + x_{p,b}}{2 D_{wp} C_{tot}} N_w \qquad (19)$$

$$C_{w,g,i} = \frac{p_0}{R T} e^{-W \frac{1 - x_{w,i}}{x_{w,i}}} \qquad (20)$$

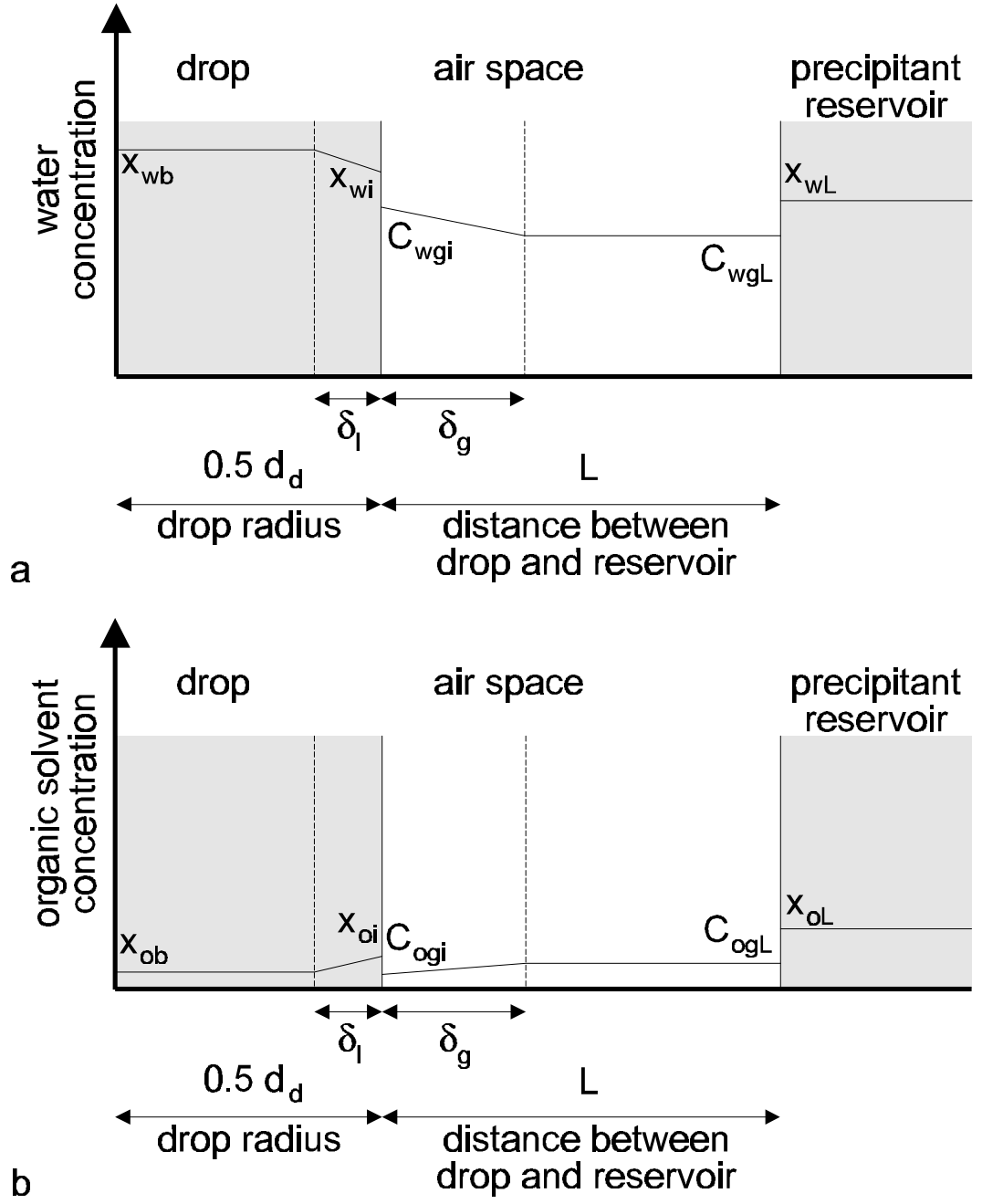

figure 2 *Sketch of the system used for the calculations in this subsection. The film thicknesses (d_l and d_g) used are either calculated using the penetration theory or equal to drop radius and distance between drop and reservoir respectively.*
a. A protein/salt system. The concentration in the graph is the mole fraction of water. Water is evaporating from the drop to the precipitant reservoir.
b. A protein/organic solvent system. The concentration in this graph is the mole fraction of organic solvent. Organic solvent is evaporating from the precipitant reservoir to the drop.

In these equations, C is a concentration in mole/m^3, x is a mole fraction and N is a mole flux. The subscripts p, s, w, g, i, a, tot, and b refer to protein, salt, water, gas phase, air, total, and bulk of the drop, respectively. R and p_0 are the universal gas constant and the vapour pressure of pure water. The D's in the equations are Maxwell-Stefan diffusion coefficients, and the two subscripts refer to the components. For example, D_{wp} is the diffusion coefficient resulting from the friction between protein and water. The last equation is taken from a paper by Fowlis et al. [68]. The δ's in the equations are film thicknesses (from film theory). The magnitude of this film thickness is estimated from penetration theory for short times t after starting the evaporation process (using the diffusion coefficient of salt in water and of water in air respectively):

$$\delta = \sqrt{\pi D t} \tag{21}$$

Whenever these film thicknesses (δ_l and δ_g) are calculated to be larger than drop radius and distance between drop and precipitant reservoir, respectively, these last parameters are used as film thicknesses. The water concentration in the air adjacent to the precipitant reservoir ($C_{w,g,L}$) can be calculated from equation (20) by substituting $x_{w,i}$ with the water fraction in the precipitant reservoir ($x_{w,L}$, taken to be constant). These equations can now be solved for N_w, $c_{w,g,i}$, $x_{w,i}$ and $x_{p,i}$.

To calculate the flux of organic solvent and the interfacial concentration of organic solvent in a protein/organic solvent system, equations for the transfer of organic solvent in the drop and in the air and an equilibrium relation are needed. For this system, the contribution of protein to the transfer equations and to a possible Marangoni effect are neglected. The equations are (see figure 2.b):

$$x_{o,i} - x_{o,b} = -\delta_l \frac{2 - x_{o,i} - x_{o,b}}{D_{ow} C_{tot}} N_o \tag{22}$$

$$C_{o,g,L} - C_{o,g,i} = -\delta_g \frac{N_o}{D_{oa}} \tag{23}$$

$$C_{o,g,i} = x_{o,i} C_{tot} m \tag{24}$$

In these equations, m is a distribution coefficient, defined as the ratio between the concentration of organic solvent in the gas phase and the liquid phase at the interface ($m = C_{g,i} / C_{l,i}$). Film thicknesses are calculated from penetration theory as well. The organic solvent concentration in the air can be calculated using equation (24) by substituting $x_{o,i}$ with the organic solvent fraction in the precipitant reservoir, $x_{o,L}$.

Some calculations have been performed with typical values listed in tables 1 and 2. From these calculations an indication of the magnitude of the ratio of mass transfer resistances can be obtained by evaluating the Biot number:

$$Bi = \frac{x_b - x_i}{x_i - x_L} \tag{25}$$

If Bi is much larger than one, the resistance to mass transfer is located in the liquid phase and if it is much smaller than one, the resistance is located in the gas phase. Calculations for the lysozyme-NaCl system indicate that Biot numbers are in the order 10^{-3}-10^{-5} for a typical crystallisation system (parameters are indicated in table 2) depending on time. For very short times, Biot numbers are smaller but insensitive to the dimensions of the system. For longer times, Biot numbers and fluxes depend on the distance between drop and reservoir. These calculations indicate that mass transfer resistance in protein/salt systems, even during microgravity, is almost completely located in the gas phase. When convection is present, the Biot number is even smaller. This is contradictory to the hypothesis of Fowlis et al [68]. They suspect that during microgravity the evaporation rate of water might be determined by the transfer of water in the drop. This result is in agreement with the experimental results of Sibille et al. [69] for evaporation rates in Plaas-Link capillaries, and the more recent experimental work of DeTitta and Luft [70].

table 2 System constants used for calculating fluxes, Biot numbers and R_{sol}

geometric constants		
	distance between drop and reservoir (m)	0.01
	drop radius (m)	$2\ 10^{-3}$
lysozyme/NaCl		
	bulk protein concentration (kg/m^3)	50
	droplet salt concentration (w/w %)	5
	reservoir salt concentration (w/w %)	10
organic solvent		
	bulk organic solvent concentration (v/v %)	2.5
	reservoir organic solvent concentration (v/v %)	5

The model equation (17) shows that, although mass transfer for water is limited by the gas phase resistance, protein crystallisation gradients are nevertheless present. The gradients in protein concentration are determined both by the magnitude of the water flux, which is limited by the gas phase resistance, and the film thickness for the protein transport.

Biot numbers for organic solvent systems have been calculated for ethanol and acetone, and are in the order of 10^{-1}-10^{1} for large times and 10^{-2}-10^{-1} for small times. The different Biot numbers in the two systems are due to the difference in distribution coefficient, which is 10 times as high for acetone as for ethanol, leading to ten times higher Biot numbers. As the Biot number in an organic solvent system is much closer to one than in a protein/salt system, a Marangoni effect is anticipated to be stronger for the former system.

To obtain an indication of the magnitude of concentration gradients of the surface-tension-determining component parallel to the interface, a second interface concentration is calculated by disturbing the flux by a factor. A new perturbed interface concentration is calculated by either keeping the bulk liquid concentration of the transferring component constant when Bi < 1 or the precipitant concentration of the transferring component when Bi > 1. The final step in the calculation is the computation of the ratio R_{sol}, indicating the relative magnitude of a solutal Marangoni effect in a protein/salt and a protein/organic solvent system.

$$R_{sol} = \frac{\Delta c_{ps}}{\Delta c_{po}} \frac{\left(\frac{\partial \gamma}{\partial c}\right)_p}{\left(\frac{\partial \gamma}{\partial c}\right)_o} \qquad (26)$$

In these calculations, a thermal Marangoni effect is neglected as well as differences in rheological and other interfacial properties (see next subsection) in the two systems. Values of R_{sol} have been calculated to compare lysozyme-NaCl to either acetone or ethanol systems. For the typical conditions listed in table 2, R_{sol} takes a value in the order of 10^{-2}-10^{-1}, the values for acetone being smaller than for ethanol, and the values for small times being smaller than for large times. The value of R_{sol} proves to be approximately independent of the factor used to disturb the flux. From these calculations it can be concluded that solutal Marangoni effects as a result of mass transfer between drop and precipitant reservoir are of the order 10^{1}-10^{2} times as small in protein/salt as in protein/organic solvent systems. These numbers should be read keeping the assumptions made in mind.

Interfacial properties of protein-solutions.

The gas-liquid interface in a protein crystallisation system rarely behaves as a clean Newtonian interface with low surface shear and dilatational properties. Apart from proteins, crystallisation systems contain, more often than not, (traces of) other substances, which can affect the behaviour of the gas-liquid interface seriously. Detergents, trace amount of fats, fatty acids and other proteins can all function in a surfactant-like manner, i.e. they can lower the surface tension of a solution when present in only small concentrations. Apart from lowering the surface tension, gas-liquid interfaces can be influenced in other ways by these substances and the various influences are discussed below.

- Proteins or impurities that form an insoluble monolayer on the interface inhibit the Marangoni effect by their surface elasticity stabilisation (Plateau-Marangoni-Gibbs effect). When an interface is disturbed in such a way that it is partly cleared and absorbed surfactant is swept outward, the concentration of surfactant is lowered on the cleared patch and increased in its immediate surroundings. The movement is therefore counteracted by a surface tension gradient caused by the surfactant surface concentration

gradient. This effect is able to make an interface completely stable towards micro-convection. The effect is quantified by the elasticity number N_{el}.

$$N_{el} = -\frac{\Gamma_0 H}{D_s}\left(\frac{\partial\gamma}{\partial\Gamma_0}\right) \qquad (27)$$

In this equation, Γ_0 is the surface concentration in kg/m^2. Berg and Acrivos [47] showed that even a layer of an insoluble contamination of relatively low surface-tension-lowering capability can dramatically increase the critical Marangoni number for the onset of thermocapillary (micro-)convection. As quite a number of proteins change their conformation in the interface relatively slowly and irreversibly (denaturation) to a conformation which resembles an insoluble surfactant, this effect may render Marangoni convection non-existent in many protein crystallisation systems after a certain amount of time. When data by Graham and Phillips [71] is used, the elasticity number of lysozyme, bovine serum albumin (BSA) and β-casein is calculated to be in the order 2 10^5, in the region where surface tension decreases linearly with surface concentration. The critical Marangoni number for the onset of convection is thereby increased thousand-fold [47]. However, this elasticity number is not big enough to inhibit convection completely. For higher bulk concentrations, which are more common in protein crystal growth, equilibrium surface tension is independent of surface and bulk concentration, making the elasticity number and the Plateau-Marangoni-Gibbs effect vanish.

- Brian and Ross [46] studied the theoretical instability limits of a system in which mass transfer induces solutal Marangoni convection. They demonstrated that the more surface active the transferring component is, the larger the critical Marangoni number for the onset of convection is. They quantified the Gibbs absorption effect using the absorption number N_a and found that increasing the absorption number beyond 0.5 renders the system completely stable.

$$N_a = \frac{\Gamma_0}{\delta(c_b - c_i)} \qquad (28)$$

However, Brian and Ross' analysis is not applicable to the protein/salt crystallisation system as the protein is the surface tension determining component, but not the transferring component. A more quantitative analysis, using their results, is therefore not possible.

- As mentioned in section 5.4.2, some protein solutions do not change their static surface tension when the concentration is increased beyond a specific concentration. This does not mean that in such a case any kind of surface tension gradient is impossible, since the dynamic surface tension can still vary and also gradients caused by temperature fluctuations are possible. Nevertheless, it limits the possibility of Marangoni effects occurring in protein/salt systems. Graham and Phillips investigated the equilibrium surface tension of solutions of lysozyme, BSA and β-casein as a function of protein

concentration and found it to be a constant in the concentration ranges used in protein crystallisation. Moreover, they found that protein was irreversibly absorbed to the interface and that decreasing the protein concentration beneath the surface layer did not change the surface tension for any of the three proteins investigated [71]. This indicates that solutal Marangoni effects are impossible once the equilibrium surface tension is attained, i.e. once the protein has formed a skin of denatured protein on the gas-liquid interface.

- For a planar (x-y plane), non-deformable Newtonian interface the stress boundary condition in one direction becomes (cf. [72]):

$$\frac{\partial \gamma}{\partial x} = \mu \frac{\partial v_x}{\partial z} - \mu^s \frac{\partial^2 v_x}{\partial y^2} - \left(\kappa^s + \mu^s\right) \frac{\partial^2 v_x}{\partial x^2} \qquad (29)$$

In this equation, z is the co-ordinate normal to the interface. This equation was obtained by ignoring the velocity components in y-direction. Apart from the bulk viscosity two (Newtonian) surface viscosity coefficients appear in this equation, the interfacial shear (μ^s) and the interfacial dilatational viscosity (κ^s). For most systems the extra transport of momentum constituted by these coefficients is neglected. Usually the surface viscosity is too small. The relative importance of interfacial shear and dilatational viscosity is expressed by the Boussinesq number Bo_μ or Bo_κ:

$$Bo_\mu = \frac{\mu^s}{H\mu} \qquad Bo_\kappa = \frac{\kappa^s}{H\mu} \qquad (30)$$

When either Bo is larger than 1, bulk flow is strongly influenced by the interfacial shear properties [73]. Normally, the interfacial shear viscosity is in the order of 10^{-5} to 10^{-7} kg/s [74]. Therefore, the interfacial shear viscosity does not play a significant role unless the characteristic dimension H (e.g. drop diameter) is smaller than respectively 10^{-2} to 10^{-4} m. However, in protein crystallisation systems, gel-like films of protein can form at the liquid-gas interface. These films consist of partly denatured protein [71, 75] and can be thicker than 100 Å [71]. The surface shear viscosity of absorbed proteins has been measured by Graham and Phillips for lysozyme, BSA and β-casein and has maximum values of 10 kg/s, 4 10^{-1} kg/s and smaller than 1 10^{-3} kg/s respectively [76, see also 77]. In a hanging drop configuration (typical dimension is 1 mm), this leads to values of Bo_μ of the order of 10^7, 10^5 and smaller than 10^3, respectively. These data indicate that velocities due to Marangoni convection are strongly reduced by the presence of protein films.

Apart from reducing flows, a large interfacial viscosity also increases the critical Marangoni number for the onset of micro-convection, as discussed by Berg and Acrivos [47]. This effect is also quantified by the Boussinesq number Bo_μ. The influence of the surface viscosity is expressed in the sum (α^2 Bo_μ + N_{el}). In this sum α is the dimensionless wave number, which typically has a value of 2. Since in their examples the elasticity number was always much larger than the Boussinesq number, Berg and Acrivos

concluded the effect of interfacial viscosity to be negligible. However, in protein crystallisation systems the Boussinesq number can be of the same order of magnitude as the elasticity number, or even larger. Therefore, the large interfacial shear viscosity that can occur in protein crystallisation systems has a stabilising influence. A flexible protein, such as β-casein, generally forms interfacial films of lower viscosity than a globular protein, such as lysozyme [76].

Generally, surface rheology of protein solutions is not Newtonian. The interfacial stress can depend on the history of the interface, resulting in viscoelastic behaviour [73, 78]. For some interfaces a viscoplastic behaviour (Bingham surface fluid) has been suggested. A Bingham surface only moves when the applied stress is larger than a specific yield stress. Such a surface is completely rigid when the applied stress is smaller. However, no such models have yet been proposed for protein solutions [73].

In the papers by Monti and Savino [32, 33], Marangoni convection is modelled numerically in vapour diffusion systems. From their calculations they conclude that Marangoni effects may play a role in microgravity for non-symmetrical drops, and around a growing crystal. However, in their calculations none of the interfacial properties of protein solutions discussed in this section were taken into account. For their calculations, a value for the dependence of surface tension on concentration has been taken from our previous paper [56], which is only valid for short times after creating the interface. Ground experiments were used to validate their numerical code, and this validation was based on the observation of nucleation zones and not on the observation of any convection. Not surprisingly, crystals nucleated close to the interface in hanging drop configurations (sitting drops were not tested), from which it was concluded that the numerical code was qualitatively all right. At best, this reflects the buoyancy part of their model, but this part should be checked with sitting drop experiments as well. With respect to the Marangoni part of their calculations, their findings give at best an upper bound to the effect of Marangoni convection on the distortion of the concentration field.

5.5 Protein crystallisation in relation to convection

In this section a summary is presented with theory and proof for the influence of convection in protein crystallisation.

Evidence for the existence of convection during protein crystallisation and its detrimental effect on protein crystal quality.

Various studies have demonstrated the existence of Rayleigh convection during protein crystallisation. Using Schlieren optics, some authors found plumes of low density fluid rising from crystals growing in solution [79, 80]. Convection was observed during protein crystallisation in two microscope studies, in which precipitated protein acted as tracer particles [81, 82].

Direct proof for the detrimental effect of convection on protein crystal growth has been given in several papers [79, 83, 84]. Pusey et al. observed that the growth of one of the faces of a growing lysozyme crystal was progressively and irreversibly inhibited when it was exposed to an increasing solution flow [79, 83]. Nyce and Rosenberger found that tetragonal lysozyme crystals did not grow when suspended in a thermosyphon, but similar crystals started growing when placed in a stagnant solution. They speculated that this might be due to the shear forces exerted by the solution flow on the crystals, but it could also be a result of the varying temperatures used in their experiment. The influence of the solution flow was not found for orthorhombic crystals [84]. Miller et al. compared gel-grown crystals of human serum albumin with crystals obtained with a normal vapour diffusion method, both in microgravity and on earth. From this comparison it was concluded that crystal quality is probably deteriorated by solutal convection as the best crystal quality was obtained with gel-grown crystals and crystals grown in microgravity [85]. Broom et al. demonstrated that orientation of a crystal with respect to solutal flow can have a pronounced influence on crystal morphology [86].

Pusey et al. demonstrated by means of a simple model combined with experiments that Rayleigh convection plays an important role (relative to diffusion) for crystals larger than 10 microns and that even in microgravity convection starts to be significant for crystals approaching sizes of 1 cm [87, 88].

Indirect proof for the detrimental effect of convection on crystallisation is found in the observation that crystals grown in gels can be of better quality than crystals grown in ordinary solutions [85]. Convection is suppressed by the gel network, which is believed to be one of the advantages of gels. Nevertheless, other factors may also be of influence, such as suppression of heterogeneous and secondary nucleation and the reduction of sedimentation rates [89, 90, 91]. Besides positive effects, gels can also have negative effects on protein crystal growth, such as rupture of mechanically weak protein crystals [21] or increasing the number of primary crystallisation nuclei [19].

Theories explaining the influence of convection on protein crystal growth.

Theories explaining the effect of convection on protein crystal growth can be divided in two: they are either fluid-dynamic in nature or they are related to the influence of convection on mass transfer to and from a growing crystal. Hypotheses with a fluid-dynamic nature are partly summarised by McPherson [29]:

1. As proteins are held in the crystal by relatively weak bonds, shear flow along the crystal might break these crystal bonds and remove protein molecules from the crystal (suggested in various papers, for example [11, 22, 23]).
2. As protein molecules can be oriented in the flow, they are not always susceptible to incorporation in the crystal lattice.
3. Protein molecules are deformed by the flow, which might reduce their attachment probability, but which might also cause deformed molecules to get incorporated in the lattice, resulting in crystal defects.

4. The attachment probability of protein molecules is extremely low and this might even be further reduced by a convective flow which sweeps the molecules past the interface. Furthermore, the incorporation of impurities which have a higher attachment probability than the protein might be enhanced by convection.
5. Whenever shear flow is present, fluctuations in liquid temperature, concentration, and velocity can occur, which could result in local growth irregularities. This is a recent result from low velocity turbulence theory.

However, by means of order-of-magnitude analyses, Grant and Saville [92] established that the effect of shear flow on protein deformation and orientation is negligible, and that the forces exerted by shear flow are not large enough to break the bonds in the protein crystal.

Convection around a growing crystal can influence the mass transfer of protein from bulk solution to the crystal interface. This influence is also thought to be important in explaining the detrimental effect of convection on protein crystal growth. The crystal growth rate is determined by the rate of transport of protein from bulk solution to the crystal interface and the rate of incorporation in the crystal of protein present at the solid-liquid interface. If the incorporation rate is much faster than the transport rate in the concentration boundary layer surrounding the crystal, the protein concentration at the crystal face is minimal, i.e. it is equal to the saturation concentration. If, to the contrary, the incorporation rate is much slower than the transport rate, then the concentration close to the crystal face is maximal, i.e. it is equal to the bulk protein concentration. The rate of transport of protein in the concentration boundary layer is determined by convection and diffusion, and since convection increases this transport rate, protein concentration close to the crystal interface generally increases due to the presence of convection. It is the hypothesis of McPherson [29] that low protein concentration favours controlled growth kinetics. High protein concentrations in the boundary layer close to the interface might lead to secondary nucleation (cf. [93]), which leads to crystal showers and intergrowing crystals. If, to the contrary, the concentration near the crystal interface is lower than in bulk solution, concentrations could be in the so-called metastable region of the solubility diagram. In the metastable region, the so-called Ostwald region, crystal growth is sustained, but crystal nucleation is impossible. Furthermore, low concentrations lead to lower growth rates, reducing the chance for lattice defects (cf. [10]) and can also lead to different growth kinetics [94]. Another way in which lower concentrations at the interface can influence crystal growth, is the way concentration determines protein cluster size close to the interface. Tay et al. [93] explain that this influence can work two ways. A higher supersaturation can either increase cluster size or diminish it. As protein crystallisation might take place by addition of monomer clusters of a specific size (see also [95, 96, 97]), the way convection influences cluster size affects growth rate and crystal habit.

The hypothesis of McPherson is supported by the fact that protein diffusion is a very slow process (diffusion coefficients are of the order of 10^{-10} m^2/s or even smaller). It is therefore very likely that growth rates in microgravity are limited by diffusion, resulting in very low concentrations close to the crystal interface. An observation supporting this hypothesis has been made by Malkin and McPherson [96]. Using a light-scattering technique, they found that

growth rates of satellite tobacco mosaic virus (STMV) crystals were limited by transport of the virus from the bulk to the growth interface (diffusion-limited aggregation). Feher and Kam [10] found quite large concentration gradients around a growing lysozyme crystal of 100 to 200 microns (up to 50 % of bulk concentration) using UV-absorption. They used a set-up in which convection was probably hindered seriously. Contrary to these findings, experimental and modelling results of Pusey, Grant, and co-authors on the crystallisation of lysozyme indicate that attachment kinetics is the limiting step up to crystal dimensions of 100 microns [87, 88, 92]. For lysozyme the hypothesis stated above is therefore not sufficient to explain the detrimental effect of convection. Nevertheless, it might still explain why crystals grown in microgravity can grow substantially larger. In a later paper [83], Pusey found experimentally that growth rates temporarily increased when small (~20 μm) lysozyme crystals were subjected to solution flows, indicating that mass transfer is more important, when compared to attachment kinetics, than his previous modelling results had indicated. Using interferometry, Miyashita and co-workers found only slight concentration gradients (2.5 % of a bulk concentration of 2 w/v %) across the boundary layer around a lysozyme crystal of approximately 500 microns [38]. Summarising, no experimental and theoretical agreement on the validity of the hypothesis stated by McPherson has been found. The validity of the hypothesis might very well vary from protein to protein.

Apart from influencing protein transport to the crystal, convection can also influence transport of impurities to and precipitant from the crystal [30]. Grant and Saville [92] suggested that this might explain the effect of convection on crystal growth for particular crystals and particular impurities (convection can change the ratio of protein and impurity incorporation, i.e. segregation). Pusey [83] rejected this hypothesis since it cannot explain why solutal flow does not seem to hinder orthorhombic lysozyme crystal growth, while it does hinder tetragonal lysozyme crystal growth. As these two crystal forms are very much alike it does not seem logical that the same impurity has so great an influence on crystal growth of one form and so little on the other.

The influence of convection on the transport of rejected precipitant is a beneficial one, when the same line of reasoning is applied as is used to explain the detrimental effect of convection on the protein transport. Convection decreases the concentration gradient of precipitant across the boundary layer, which leads to a reduction of precipitant concentration at the interface and subsequently a lower supersaturation. Also in other cases, convection can be beneficial to crystal quality. If segregation of an impurity takes place at a crystal interface and this impurity is preferentially rejected, then convection can increase segregation with respect to the bulk concentrations of protein and impurity [30]. As mentioned before, crystal growth kinetics depend on supersaturation and growth may either be favoured by large or small values of supersaturation. Convection usually increases the value of supersaturation at the crystal interface and this might for some proteins and for some crystal habits be a preferable situation. Furthermore, studies of protein crystals growing in a flow cell showed that forced convection can provide stable, uniform growth conditions [94].

Considering all the evidence in literature, our opinion is that the influence of convection on protein crystallisation should mainly be sought in the influence convection has on mass

transfer rates from and to the protein crystal. Mass transfer rates influence concentration and thereby supersaturation levels. The exact value of the supersaturation close to the crystal interface determines attachment rates, local cluster size of the protein and incorporation rate of impurities. However, it is our view that the influence of convection on protein crystallisation can not be generalised too much. That is, the situation changes from protein to protein, crystallisation condition to crystallisation condition, and it even changes from crystal habit to crystal habit (cf. orthorhombic and tetragonal lysozyme).

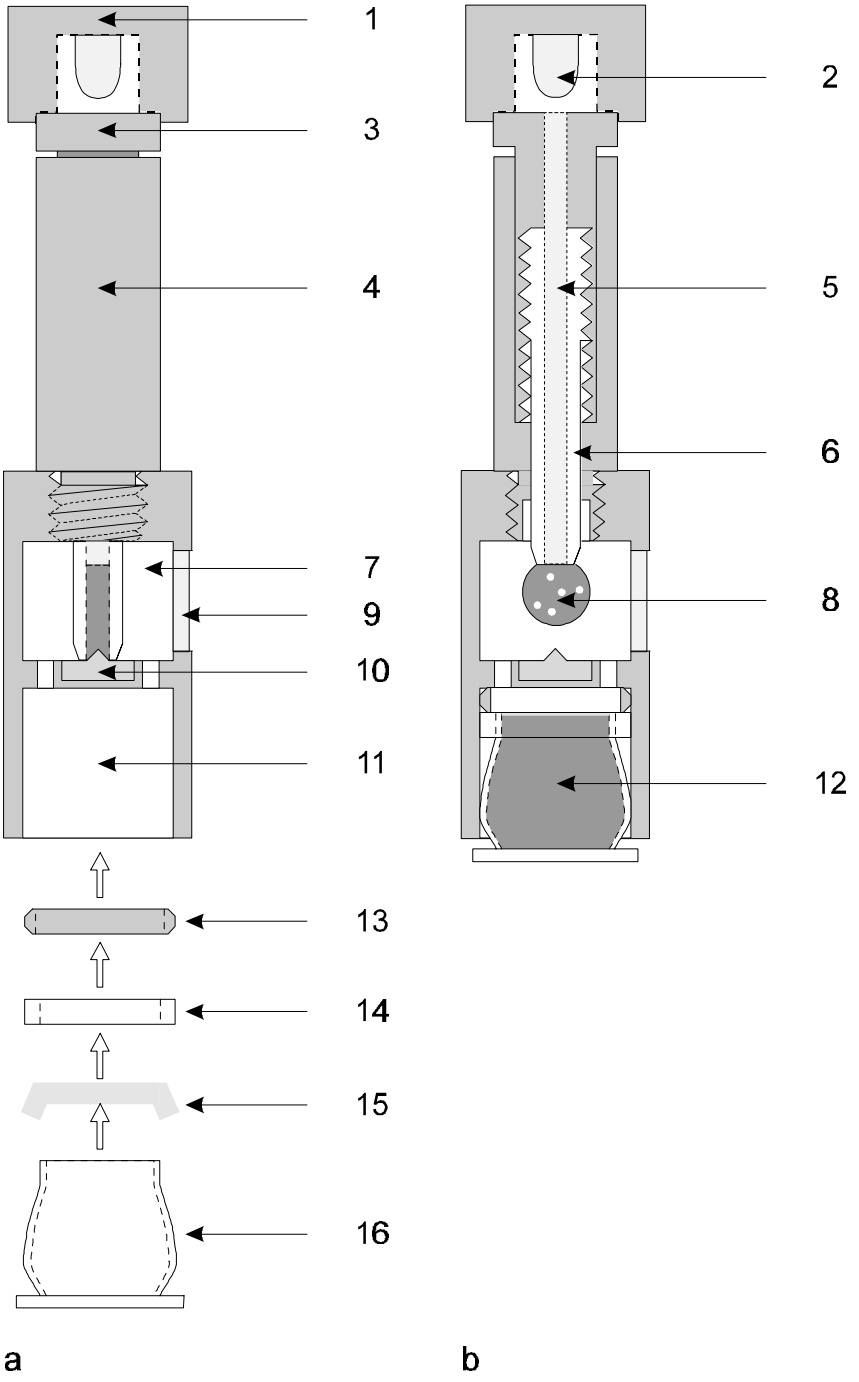

figure 3 *Sketch of a protein crystallisation unit. Figure 3a shows the launch position of the crystallisation cell. Figure 3b shows the microgravity configuration.*

1. Driving head. 2. Lamp. 3. Turning knob. 4. Syringe (crystallisation cell). 5. Glass plunger. 6. Teflon tube (containing protein solution). 7. Crystallisation chamber. 8. Protein solution. 9. Observation window. 10. Teflonised sealing plate. 11. Precipitant chamber. 12. Precipitant solution. 13. Metal ring. 14. Teflon ring. 15. Teflon ultrafiltration membrane. 16. Precipitant container.

5.6 A microgravity experiment

A microgravity experiment was performed to investigate the possibility of Marangoni convection occurring during protein crystallisation. The experiment consisted of observing hanging drops during microgravity. The experiment also served to evaluate crystallisation units, which are to be used in a Dutch Protein Crystallisation Facility (DPCF) [98]. The evaluation of the crystallisation units is described elsewhere [56].

The experimental hardware

The design of the crystallisation units used in the experiment is sketched in figure 3. The crystallisation unit consisted of a crystallisation cell (a kind of syringe), a precipitant container and the body of the crystallisation unit. A space was drilled out of the stainless steel body, in which the cell could be screwed and the precipitant container could be clasped.

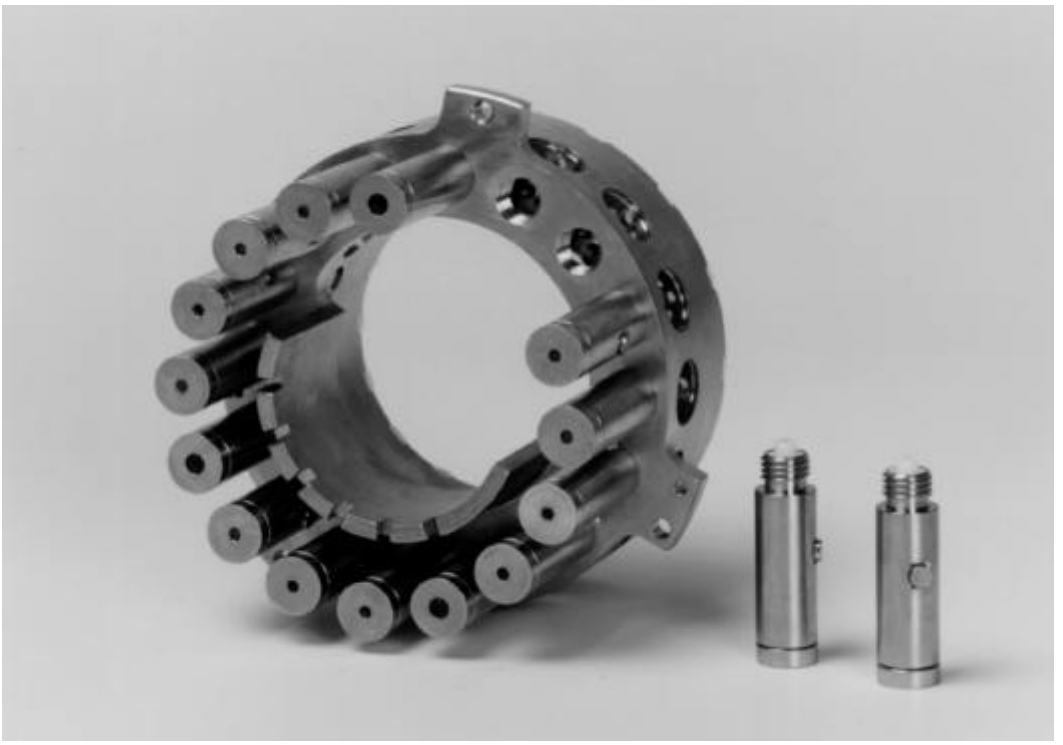

figure 4 *Photograph of the experiment ring with the experiment cells. The windows visible on the outer face of the ring were sealed with a metal insert before the experiment. The drops were observed through the windows on the inner face of the ring.*

*figure **Fout! Bladwijzer niet gedefinieerd.** Photograph of the experiment ring integrated in the mechanism, which rotates the mirror and opens and closes the experiment cells. The windows visible on the outer face of the ring were sealed with a metal insert before the experiment. The cylinders protruding from the equipment are the motors driving the mechanism.*

The crystallisation cell (figure 4) contained a glass plunger, a Teflon cylinder and a turning knob to move the cylinder. The glass plunger was glued into the turning knob. When the knob was turned, the glass plunger turned around, but stayed in the same axial position. The Teflon cylinder, however, moved in axial direction. In this way, a droplet could be

extruded from the cylinder. The droplet keeps hanging from the tip of the plunger. Figure 3a shows the cylinder position during launch and re-entry, while figure 3b depicts the cylinder in microgravity position. In launch position, the cylinder was pressed on a Teflonised metal plate. A conical bulge was present on the metal plate. This bulge exactly fitted into the cylinder and served to facilitate the detachment of the drop from the plate. The Teflon tubes used during the experiment had inner diameters of 1.0, 1.5 and 1.8 mm, corresponding to drop volumes of 6, 13 and 19 μl.

The precipitation solution was contained in a precipitant container, manufactured from Teflon. This container was sealed with an ultrafiltration membrane (the diameter of the pores is 0.2 μm). A Teflon membrane on a supporting polyester layer was used (Schleicher & Schuell, TE35). No liquid could pass this membrane. However, vapour transport was not restricted by the membrane.

The drops could be observed via a window with which each crystallisation chamber is provided. The droplet was illuminated by a LED, positioned at the other end of the glass plunger (by which the light is transported). Tracer particles were added to the droplets in order to visualise possible flows. No measurable heat is transported from the LED to the drop. The hardware for the microgravity experiment consisted of one ring with 16 crystallisation units. All 16 windows were facing towards the centre of the ring (figure 4). The system was equipped with one CCD-camera with which 4 drops could be observed at the same time by means of a pyramid-shaped mirror in the centre of the ring.

Apart from the experiment ring with the cells, the experimental equipment consisted of a mechanism, which could rotate the mirror and the knobs on the cells (figure 5), a CCD-camera, a video-8 recorder, an electronic control unit, a temperature logger and a battery package. All these elements were accommodated in a vacuum tight late access container.

Overview of the experiments

Solutions were prepared in the laboratory in Groningen and transported in a cooling box to the launch site Esrange near Kiruna (North-Sweden). In the laboratory and at Esrange the solutions were kept at 2-4 °C. Precipitant chambers and experiment cells were filled 2.5 days before launch (the launch was postponed for two days due to weather conditions). Experiment cells 2 to 4 were refilled one day before launch (lysozyme started to crystallise in these cells). The late access container with the experiment cells was transferred from the refrigerator to the rocket 4 hours before the start of each countdown, where the experiment units were given time to equilibrate to the temperature in the launch tower (18-19 °C). After the first two countdowns the container was replaced in the refrigerator.

Maser 5 was launched on April 9, 1992. Microgravity levels during flight were of exceptional quality and continuously less than 10^{-6} g [99]. The late access container was recovered from the rocket within two hours after impact. The container was then transported in thermal insulation with a helicopter back to the laboratory at the launch range. After

demounting, the experiment ring was transported back to the University of Groningen where it was opened for inspection 15 days after the experiment. The ring was kept at a temperature of 2-4 °C.

Two months after the microgravity experiment a reference experiment was performed at the University of Groningen using exactly the same equipment.

Description of the 16 experiments.

The contents of the various experiment cells and of the various precipitant containers are listed in table 3.

The various lysozyme (Boehringer, crystallised from hen egg white) and NaCl- solutions were prepared in a 0.2 M HAc/Ac^- buffer (pH 4.70). Microcentrifuge cups were filled with lysozyme and filled up with buffer solution till solutions of 50 mg/ml were obtained. The cups were centrifuged for 10 minutes at 10000 rpm and supernatant solution was transferred in clean cups. NaCl solutions were prepared by dissolving NaCl (analytical grade) in buffer solution (the pH of the resulting salt solutions was 4.43).

The human serum albumin (HSA) and PEG-400 solutions were prepared with 500 mM potassium phosphate buffer (pH 6.72). Microcentrifuge cups were filled with HSA (Sigma, essentially fatty acid free, fraction V) and filled up with buffer solution and water till 100 mg/ml HSA in 50 mM K-phosphate solutions were obtained. The cups were centrifuged for 10 minutes at 10000 rpm and the supernatant solution was transferred in clean cups. PEG-400 solution was prepared by dissolving PEG-400 (Merck) in buffer solution and water till a 25 % (v/v) PEG in 50 mM K-phosphate solution was obtained.

The phospholipase A_2 and accompanying organic solvent solutions were prepared in a 10 mM TRIS (2-amino-2(hydroxymethyl)-1,3-propanediol)-HCl /5 mM $CaCl_2$ buffer. The TRIS (Boehringer, crystallised) buffer had pH 7.01. One microcentrifuge cup was filled with 10 mg/ml porcine phospholipase A_2, centrifuged for 10 minutes at 10000 rpm and clean cups were filled with supernatant solution. The 10 % methanol, 20 % acetone and 20 % ethanol solutions were prepared by mixing the respective organic solvents (Merck, analytical grade) with the appropriate amount of buffer solution.

The 5 % (w/w) acetone-solutions were prepared in water. De-ionised water was used for all solutions. To all buffer solutions NaN_3 was added in 1 mM concentration.

Table 3. Description of the cell contents

cell number	cell contents	contents precipitant container	cell diameter
1	water	8 % (w/v) NaCl	1.8 mm

2	lysozyme / 2 % (w/v) NaCl	8 % (w/v) NaCl	1.8 mm
3	lysozyme / 2 % (w/v) NaCl	solid NaCl	1.8 mm
4	lysozyme / 4 % (w/v) NaCl	8 % (w/v) NaCl	1.8 mm
5	human serum albumin / 12.5 % (v/v) PEG 400	25 % PEG 400	1.8 mm
6	phospholipase A_2 / 5 % (v/v) methanol	10 % v/v methanol	1.8 mm
7	phospholipase A_2 / 10 % (v/v) acetone	20 % v/v acetone	1.8 mm
8	phospholipase A_2 / 10 % (v/v) ethanol	20 % v/v ethanol	1.8 mm
9	lysozyme / 2 % (w/v) NaCl / 5 % (w/v) acetone	active charcoal	1.8 mm
10	5 % (w/w) acetone / water	8 % w/v NaCl	1.8 mm
11	lysozyme / 2 % (w/v) NaCl / 5 % (w/v) acetone	8 % w/v NaCl	1.8 mm
12	lysozyme / 4 % (w/v) NaCl / 5 % (w/v) acetone	solid NaCl	1.8 mm
13	water	active charcoal	1.8 mm
14	5 % (w/w) acetone / water	active charcoal	1.8 mm
15	5 % (w/w) acetone / water	active charcoal	1.0 mm
16	5 % (w/w) acetone / water	active charcoal	1.5 mm

Tracer particles consisted of ceramic microballoons (Grace), coated (by precipitation reaction) with silver to improve their visibility and to increase their density. The particles were classified according to size (diameter between 100 and 200 μm) and density (approximately the same as water).

The final protein solutions, which were placed in the experiment cells, were prepared at the launch base by mixing the protein solution with the precipitant solution (and in some cases

buffer solution and/or acetone) in appropriate volumetric amounts. Tracer particles were first inserted in the experiment cells. Special equipment, devised to select the right amount of tracers, was used for this task. In some cases problems arose, because particles moved due to static electricity. In these cases the procedure was repeated. The crystallisation cells were subsequently filled with the appropriate solutions using syringes equipped with small needles. Slow filling speeds were required, due to the foamability of the protein solutions. Finally, the cells were screwed into the experiment ring.

The precipitant containers were filled with the appropriate precipitant solutions, with solid NaCl or small cylinders of active carbon (Ceca, AC35, 1.8 mm).

All crystallisation cells, precipitant containers and syringes as well as the crystallisation ring were ultrasonically cleaned with water, acetone (p.a.) and a mixture of those liquids. They were subsequently flushed with these liquids and dried with compressed nitrogen (absolutely oil free).

Three experiments were chosen to investigate a protein/salt crystallisation system (2-4, table 3). Lysozyme was chosen, because this protein has been the subject of many crystallisation studies. Different salt concentrations were used to vary driving forces for evaporation. Acetone was added in two cases (11-12) to investigate whether the protein might inhibit Marangoni effects which are usually observed in a pure acetone-water system. Only one experiment was chosen to investigate a protein/PEG crystallisation system (5). Human serum albumin was selected, because it is characteristically crystallised with PEG as a precipitant. Three experiments were chosen to examine a protein/organic solvent system (6-9). Phospholipase A_2 was selected as a protein as it is easily crystallised with methanol, acetone and ethanol using the conditions employed in this experiment [100]. Three experiments with an acetone-water system were performed (14-16) to check the occurrence of Marangoni effects in this protein crystallisation set-up. With this stationary unstable system, violent Marangoni effects are usually observed. Three different experiments were done to investigate whether drop volume might have any influence. Finally, three reference experiments were included (1, 10 and 13).

Timeline

As described before, four different cells can be observed at the same time. The cells can also be opened four at a time. The first eight drops were observed twice and only these experiment cells were closed towards the end of the experiment. The other eight drops were lost during re-entry. A precise time schedule is given in table 4.

The experiment sequence was activated by the microgravity signal received from the Service Module of the sounding rocket.

Table 4 Timeline of microgravity experiment

time (seconds)	action
0-36	cells 1-4 opened and observed
41-82	cells 5-8 opened and observed
87-207	cells 9-12 opened and observed
212-332	cells 13-16 opened and observed
337-378	cells 1-4 observed and closed
382-420	cells 5-8 observed and closed

Results

The contents of cells 1-8 were checked for crystals when they were opened at the laboratory. This was done by transferring the solution to a vapour diffusion set-up as they are commonly used in the lab. This set-up consists of plastic depression trays in which the precipitant solution is brought prior to placing the protein drop on a siliconised glass cover slip. This cover slip is then placed upside down above the precipitant solution. The slip is sealed with vacuum grease. The drops were checked under a normal light microscope.

All lysozyme drops contained many imperfect crystals. However, these crystals probably grew during the entire time the liquid was contained in the cell, because the solutions were already supersaturated. The HSA drop contained many, very small, needles, which were not birefringent, however. Movement of these needles in the liquid was observed under the microscope, indicating some kind of convection. When the phospholipase A_2/methanol and /ethanol drops were unscrewed, tracers started circulating very violently, due to evaporation of the alcohol. After the drops were placed in the microscope set-up no further tracer movements were observable with the naked eye. However, microscope observations showed that convection was occurring in the ethanol drop. Very small particles (probably precipitated protein) were circulating during quite a long time in the drops to and from the place where the liquid-gas interface met the glass wall of the objective plate. The convection was probably surface tension gradient induced, as the particles seemed to accelerate at the interface. The convection was of considerable intensity (larger than in the HSA drop). The convection could have been influenced by temperature gradients caused by the lamp of the microscope, heating the drop. Nevertheless, it can be concluded from this that the tracers used in the experiments were too large to show microscopic convection in the drops. It should be remarked that other microscope studies in literature also demonstrated the existence of various convection patterns during protein crystallisation by observation of precipitated protein [81, 82].

Hard copies were made of pictures of drops 1-4 and 5-8 at the beginning and at the end of the experiment and tracer positions were compared. Results of this and other video tape observations are summarised in table 5.

The observations of drops 1 and 7 give reason to believe that the contents of these cells have been interchanged accidentally. It is not very likely that a pure water drop shows convection as is also demonstrated by the reference experiment (which only showed sedimentation of tracer particles). In cell 7 no convection was observed, in contrast to cells 6 and 8. Moreover, cells 1 and 7 appeared to be empty when unscrewed in the lab. Therefore, these experiments were not taken into account.

The water-acetone drops (10, 14, 15, 16) showed Marangoni convection in all cases. It appeared that neither drop volume nor the kind of precipitant used was of considerable influence on convection intensity.

No Marangoni convection was observed for the lysozyme-salt systems. In drops 6 and 8 (phospholipase A_2 / organic solvent) Marangoni convection was observed for a short time, despite these systems not being stationary unstable with respect to mass transfer. Extruding the drops might cause some unexpected effects as no movements were observed after ten seconds. However, comparing the drops at the end of the first observation period with the start of the second observation period showed that some tracer movements have occurred in between. One other reason for decreasing Marangoni activity might be that phospholipase inhibits interfacial movements. Violent tracer movements which occurred when the drop was transferred to the microscope equipment showed nevertheless that the protein does not completely stop interfacial movements (especially not in a stationary unstable system) as could also be concluded from the microscope observations.

Comparing 9, 11, 12 and 14 leads to the conclusion that lysozyme tends to make the interface more rigid. No explanation can yet be given for differences between 9,11 and 12.

Table 5 Observations during microgravity experiment.

cell	observation
1	movement of tracers first minute; air bubble present; no movement of tracers after 6 minutes
2	no movement of tracers
3	no movement of tracers
4	no movement of tracers
5	drop not observable

6	tracers move during first ~10 seconds after which they come to a stop; tracers seem to have moved slightly after 7 minutes
7	low tracer content; no movements observed after unscrewing; tracers are still at the same place after 7 minutes
8	tracers move during first ~10 seconds after which they come to a stop; tracers seem to have moved slightly after 7 minutes
9	only part of small drop visible; no movement of tracers
10	tracers move during the whole observation time
11	tracers move during first ~35 seconds after unscrewing the drop; movements are at most minimal afterwards
12	tracers move during first ~10 seconds after unscrewing the drop; movements are at most minimal afterwards
13	only a small drop is observable; no tracer movements observed
14	tracer movements during the whole observation time
15	tracer movements during the whole observation time
16	tracer movements during the whole observation time; no discernible differences in tracer velocities are observed between drops 10, 14, 15 and 16; initial movements in drop 1, 6, 11 and 12 are somewhat less than in 10, 14, 15 and 16

One reference experiment showed anomalous behaviour compared to the microgravity experiment (11), as no convection was observed on earth and tracer movements were observed in microgravity. Some other reference experiment observations were affected by the presence of air bubbles (5-8, 12). As the microgravity equipment and the tracers used for this experiment impose some extra limitations on the observation of convection, it was decided not to repeat this reference experiment. However, some other reference experiments were performed with hanging drop equipment as it is commonly used in the laboratory (as described above). The drops have been seeded with cross-linked (DVB) polystyrene spheres (chemically inert) of 1-85 μm diameter (Duke Scientific) and observed under the microscope, using an infra-red mirror to avoid thermal Marangoni effects. Sedimentation was observed in every experiment. In some of the protein experiments a layer of protein was formed at the interface. Convection was observed for experiments 7, 10 (very minimal) and 14 (violently) and furthermore for experiments similar to 9, in which b-casein was used instead of lysozyme (very minimal as well). Even the smaller particles were too large to observe every movement in the drop, as was demonstrated for experiment number 8, in which no movement was found of the polystyrene spheres, whereas an experiment with precipitated phospholipase A_2 showed convection on a very small scale. The movement in the drop was definitely not entirely due to

sedimentation, as precipitated protein moved upwards as well as downwards. The intensity of convection was less than found after the microgravity experiment, indicating that convection induced by temperature gradients had been reduced by the infra-red mirror.

5.7 Conclusions and discussion

The major drawbacks to the microgravity experiment were the small number of cells and the magnification of the drop observation. Convection magnitudes due to buoyancy have been measured for lysozyme and are of the order of 10^{-5} m/s for a crystal of 1 micron [79]. These velocities are very small, but supposedly still sufficient to be detrimental to crystal growth. In order to establish whether Marangoni convection might have any influence on protein crystal growth it is necessary to check for the occurrence of very small flows, not seen with normal magnifications. This requires larger magnifications and smaller tracer particles than were used in the experiment described in this study.

Despite this, the experiments revealed that Marangoni effects can occur in protein crystallisation systems, at least for a short period, but possibly on a smaller scale for longer periods. The experiment leads to the conclusion that Marangoni effects are probably larger in organic solvent systems than in salt or PEG systems. This is partly explained in terms of concentration gradients in section 5.4.3. Another explanation could be that protein inhibits interfacial movements in an organic solvent system less than it would do in a protein/salt system. Protein that is not denatured by the organic solvent is probably also less prone to denaturation in the gas-liquid interface.

Both a protein/salt system and a protein/organic solvent system are oscillatory unstable with respect to solutal Marangoni convection. Marangoni convection probably has less influence on crystal quality than buoyancy has, as it is primarily relevant in the drop regions close to the gas-liquid interface. Furthermore, Marangoni convection is more probable in protein/organic solvent systems than in protein/salt systems, is more likely induced by thermal effects in protein/salt systems and by solutal effects in protein/organic solvent systems and is more likely to occur shortly after creating the interface and with proteins with a flexible or random conformation. The considerations in the last subsection of section 5.4.3 give reason to believe that Marangoni convection does not occur in protein/salt crystallisation systems, at least not a long time after creation of the interface and when proteins are used that can form an insoluble layer on the air-liquid interface. Nevertheless, as long as no more definite answers can be given, it should be taken into account. Marangoni convection can be minimised by:

- Avoiding a gas-liquid interface (when dialysis is used, or when temperature is used to control supersaturation, no gas-liquid interface is present; temperature control might have additional advantages [101, 102])
- Minimising mass transfer (equilibration) rates
- Adding surfactants, which do not alter crystal quality (detergents [103])
- Containerless processing. No macroscopic gradients due to evaporation develop in a spherical drop.

- Increase of viscosity (as with gel crystallisation [89, 90, 91] or the use of high PEG concentrations)

None of these measures is in conflict with other protein crystallisation objectives, except, in some cases, the first and the last [21].

With the simple multi-component model presented in section 5.4.3, it can be concluded that in the hanging drop configuration for protein/salt systems mass transfer resistance is located exclusively in the gas phase. The model does not need the assumptions of Fowlis et al. [68] to demonstrate this. Protein gradients in the liquid exist nevertheless. For protein/organic solvent systems, mass transfer resistance is usually located in the gas phase as well, but under certain conditions, the resistance to mass transfer may also be mainly located in the liquid phase. The model indicates that when the Biot number is close to one, Marangoni effects are largest.

List of symbols

a	thermal diffusivity	$[m^2\ s^{-1}]$
Bi	Biot number	[-]
Bo	Boussinesq number	[-]
c	concentration	$[kg\ m^{-3}]$ or [-]
C	concentration	$[mole\ m^{-3}]$
d_d	drop diameter	[m]
D	diffusivity	$[m^2\ s^{-1}]$
D_s	surface diffusivity	$[m^2\ s^{-1}]$
f	factor used in section 5.4.3	[-]
$\dot{q}$	heat flux	$[J\ m^2\ s^{-1}]$
H	characteristic liquid dimension (equation (1))	[m]
k	constant in equation (10)	$[s\ m^{-2}]$
Le	Lewis number	[-]
m	distribution coefficient ($C_{g,i}/C_{l,i}$)	[-]
$\dot{m}$	mass flux	$[kg\ m^{-2}\ s^{-1}]$
Ma	Marangoni number	[-]
N	mole flux	$[mole\ m^{-2}\ s^{-1}]$
N_a	absorption number	[-]
N_{el}	elasticity number	[-]
p_0	vapour pressure of pure water	[Pa]
Q	flux at interface in equation (10)	$[m^2/s]$ or $[W\ m^{-1}\ K^{-1}]$
Pr	Prandtl number	[-]
R	ratio as defined in equation (14)	[-]
R	gas constant	$[J\ mole^{-1}\ K^{-1}]$
R_{sol}	ratio as defined in equation (26)	[-]

Sc	Schmidt number	[-]
T	temperature	[K] or [-]
v	velocity	[m s^{-1}]
w	mass fraction	[-]
W	coefficient in equation (20)	[-]
x	mole fraction in liquid	[-]
X	dimensionless quantity in equation (10)	[-]
y	co-ordinate normal to the interface	[-]
γ	surface tension	[N m^{-1}]
Γ_0	surface concentration	[kg m^{-2}]
δ	mass transfer film thickness	[m]
Δc	concentration difference	[kg m^{-3}]
ΔH_v	heat of vaporisation	[J kg^{-1}]
ΔT	temperature gradient	[K]
κ	constant in equation (10)	[m^2 s^{-1}] or [W m^{-1} K^{-1}]
κ^s	surface dilatational viscosity	[kg s^{-1}]
λ	thermal conductivity	[W m^{-1} K^{-1}]
ρ	density	[kg m^{-3}]
μ	dynamic viscosity	[Pa s]
μ^s	surface shear viscosity	[kg s^{-1}]

subscripts

a	air
b	bulk (of droplet)
g	gas phase
i	interfacial
l	liquid phase
L	precipitant reservoir
o	organic solvent
p	protein
s	salt
w	water
0	initial

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