Nucleation rate determination by a concentration pulse technique: application on ferritin crystals to show the effect of surface treatment of a substrate

Daniela Tsekova*, Snejina Popova and Christo Nanev

Institute of Physical Chemistry, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria. E-mail: dtsekova@ipchp.ipc.bas.bg

The nucleation of horse spleen ferritin (HSF) crystals on substrates was investigated using a new modification of the double pulse technique. The influence of three different structureless[1] substrates (glass, glass covered by methyl groups and poly-L-lysin template) on the nucleation was studied. The boundaries in the phase-diagram, which separate zones of crystal nucleation and growth were obtained by keeping pH = 5.0, and using $CdSO_4$ as crystallizing agent. The steady-state nucleation rates were determined. The energy required for critical nuclei formation was evaluated (10^{-13} erg) and the sizes of critical nuclei were found (5 and 2 molecules).

Keywords: protein crystallization, ferritin, concentration pulse technique, nucleation, structureless substrates

1. Introduction

It is well known that the solubility of proteins depends on many factors, e.g. buffers with certain pH, electrolyte concentration, type and concentration of salts and any other additives (Ries-Kautt & Ducruix, 1999). So when trying to crystallize macromolecules one has to consider the influence of many factors. It has been claimed that there are pronounced similarities between mechanisms and kinetics of protein and small molecule crystal growth (Durbin & Feher, 1990; Durbin *et al.*,1993; Vekilov & Rosenberger, 1998). Less data concerning the mechanisms and kinetics of the processes of macromolecule crystal nucleation exist. Indeed, there are data for the light scattering investigations of protein solutions and corresponding speculations for the processes of protein aggregation and crystal formation (Georgalis *et al.*, 1993; Heinrichs *et al.*, 1993; Bishop *et al.*, 1992; Malkin & McPherson, 1993; Rosenberger *et al.*, 1996). But as far as the interpretation of these experimental data depends on the model of the solution interactions, ambiguity cannot be avoided. Temperature regimes applied for direct measurement of the rate of nucleation and kinetics studies of protein crystal nucleation were discussed recently (Tsekova *et al.*, 1999; Nanev & Tsekova, 2000; Galkin & Vekilov, 1999, 2000). In each of these papers lysozyme was used as a model protein, its solubility has very strong dependence on the temperature variation (Sazaki *et al.*, 1996). There are also proteins having very weak or no solubility dependence on the temperature. In these cases it is clear that the temperature control cannot be used. It was found that ferritin is a protein without noticeable solubility dependence with temperature (Petsev *et al.*, 2001). The purpose of this work is the direct determination of the nucleation rates with a model protein, selected to be horse spleen ferritin (HSF) and the elucidation of the effects of some structureless substrates on the crystal nucleation. In part of our experiments we used glass cells treated by organic reagents for making thin template film on the substrate, which itself has a specific influence on the processes of nucleation (Tsekova *et al.*, 1999; Nanev & Tsekova, 2000; Pechkova & Nikolini, 2001; Fermani *et al*, 2001). Here we offer to control the supersaturation by changes in the solute concentration. Dilution of microbatch and vapour diffusion drops was also applied to yield improved crystals by Saridakis (Saridakis & Chayen, 2000).

[1] Structureless substrate means substrate without crystallographic structure.

2. Theoretical background

The nucleation rate is defined as $I = \frac{dn}{dt}$, where n is the number of nuclei formed on 1 cm^2 and t is the time for their formation. Direct information for the nucleation rate can be obtained from experimental *"number of nuclei versus time"*, n(t) relationship. For separation the nucleation from subsequent growth we used pulse technique that cause fast supersaturation changes (Tammann, 1922; Kaischev *et al.*, 1953). First "pulse" enables a nucleation process, the second changes the supersaturation to a level where no nucleation occurs, but existing crystals grow to detectable dimensions. Such double-pulse technique has successful implementation and development mainly in the case of electrocrystallization, where changes in the electrode overpotential result in changes in supersaturation (Kaischev *et al.*, 1953; Mutaftshiev & Kaischev, 1955; Kaischev & Mutaftshiev, 1965; Milchev, 1991 and ref. therein). As far as the electrode overpotential cannot be applied to a protein solution, successful trials were made with temperature changes in case of temperature-dependent solubility (Tsekova *et al.*, 1999, Nanev & Tsekova, 2000; Galkin & Vekilov, 1999, 2000). Because of the temperature-independent solubility of ferritin we changed here the level of the supersaturation through changes in protein and salt concentrations.

The Volmer's expression for nucleation rate I [nuclei cm^{-2} s^{-1}] can be written as:

$$I = B\exp\left(-\frac{A_k}{kT}\right) = B\exp\left(-\frac{K_2}{(\ln\sigma)^2}\right) \qquad (1)$$

where B and K_2 are constants, k is Boltzmann's constant, T is the temperature [K], A_k is the energy required for the nucleus formation,

$$\ln\sigma = \ln\left(\frac{C}{C_e}\right)$$

is the supersaturation, where C is the actual protein concentration and C_e is the equilibrium one. It is clear from the above expression (1) that the slope of the plot lnI vs. $\ln\sigma^{-2}$ yields K_2, allowing calculation of A_k for every value of supersaturation $\ln\sigma$. So from the experimentally determined nucleation rates, using this way of transformation, the energy A_k can be evaluated.

There is a dependence between the rate of nucleation I and the size of the nucleus N_k at the corresponding supersaturation $\ln\sigma$ (Stoyanov, 1973; Milchev *et al.*, 1974a, 1974b; Milchev & Stoyanov, 1976; Oxtoby & Kashchiev, 1994; Kashchiev, 1981), given by the following equation:

$$N_k = \frac{d(\ln I)}{d(\ln\sigma)}, \qquad (2)$$

where N_k is the number of molecules constituting the nucleus. The size of the nucleus can be found from the slope of the linear fit of the plot lnI vs. $\ln\sigma$ - equation (2).

3. Experimental set-up

3.1. Materials

Horse spleen ferritin (Sigma Chemical Co.) was used after additional purification by FPLC (fast protein liquid chromatography), as described for apoferritin (Thomas *et al.*, 1998). The purified ferritin monomer fraction was stored at 5°C as a solution of 22 mg/ml in 0.2 M sodium acetate buffer at pH = 5. In all experiments ferritin crystals were grown applying the batch method. Capillaries with inner diameter from about 400 μm and up to 1000 μm and quasi-two dimensional glass cells with 500 μm distance between two inner surfaces were used.

Experiments on heterogeneous nucleation of ferritin were performed on the following substrates: 1) bare glass surface, which carries unstable negative charges in contact with water; 2) hydrophobic surface, covered by methyl groups (CH_3), which was obtained by treatment of the glass with hexamethyl-disilazane (HMDS); 3) glass substrate covered by poly-L-Lysine (PLL) molecules (in aqueous solution with pH < 9, PLL is positively charged).

For the treatment of the glass surface, we used HMDS purchased from Aldrich Chemical Company, Inc. and poly-L-Lysine from Sigma Chemical Co.

All the temperature control experiments described here were carried out in the 20 to 25°C range, as allwed by the insensitivity of ferritin crystallization to temperature variations (Petsev *et al.*, 2001).

3.2. Phase diagram investigation

In order to impose sharp changes in the supersaturation of a solution, a very important condition is to know the phase diagram and in particular the nucleation and metastable zones. Sets of screening experiments were carried out in order to find the boundaries between these two zones for the three different surfaces. For this purpose we preferably used capillaries (because of their smaller volume) and for conformation some of the experiments were repeated in quasi-two dimensional cells The phase-diagram of the ferritin/ $CdSO_4$ system was obtained by keeping pH = 5.0 (0.2 M sodium acetate buffer) and using $CdSO_4$ as crystallizing agent. As seen in fig. 1, the obtained boundaries of the metastable zones for the three surfaces are different. We found that the increasing of the $CdSO_4$ concentration over 1.4 % is connected with very weak decreasing of ferritin concentration (fig. 1) and the boundary line comes nearly horizontal. We suppose that the solubility line in fig. 1 (dotted) is also nearly horizontal between 1.5 % and 2.5 % $CdSO_4$ concentrations, i.e. that the ferriitin concentration changes very slight. The only value found in literature for the solubility of ferritin (apoferritin) is 23 μg/ml at 2.5% (w/v) concentration of $CdSO_4$ (Yau *et al.*, 2000b) and the same as our conditions: 0.2 M sodium acetate buffer, pH = 5, 22 °C. We used this value of C_e (0.023 mg/ml) to draw schematically the solubility line between 1.5 ang 3.5 % $CdSO_4$, because of the lack of any other data.

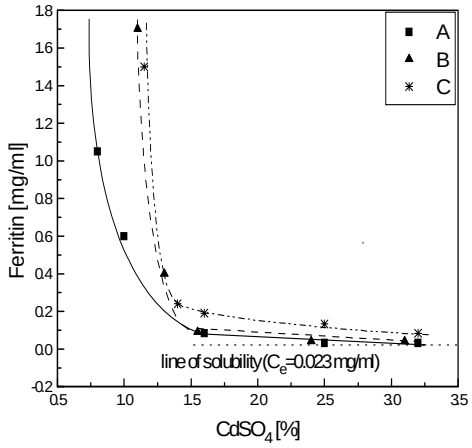

Figure 1
Phase-diagram of the ferritin/ $CdSO_4$ system in 0.2 M sodium acetate buffer, pH = 5.0. Curves A, B and C present the boundaries between the nucleation zone (where crystals can nucleate) and the metastable zone (where crystals can only grow, but cannot nucleate). Curve A is for the surface covered by CH_3 groups, curve B - for poly-L-lysine template and curve C is for glass substrate.

3.3. Concentration pulse technique

As it was mentioned above, the aim of the technique is to separate nucleation from ensuing growth. The time for nucleation - *t*, can be controlled in this way. Since the level of the supersaturation in the investigated system was changed through changes in the concentration, we call this procedure concentration pulse technique (Tsekova, 2002). Note that the slow growth rate of protein crystals allows adequate changes in the supersaturation even in case of relatively slow concentration equalisation of the diluted protein solution. The experiments have been done in the following way. At

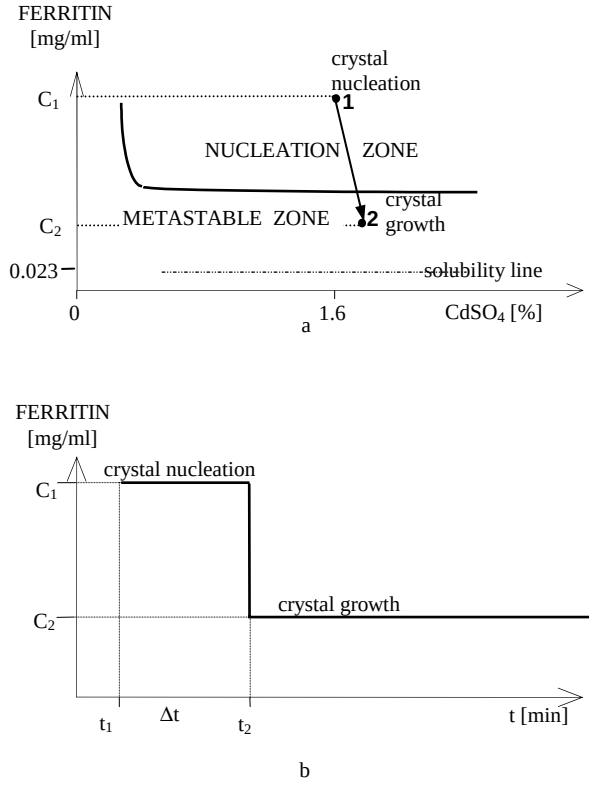

Figure 2
The rapid changes in the protein concentration ($C_1 \rightarrow C_2$) at a given moment (t_2) after the beginning of the nucleation (t_1), $CdSO_4$ concentration being also slightly changed.
a) in coordinates: ferritin concentration / $CdSO_4$ concentration;
b) in coordinates: ferritin concentration / time t [min].

the very beginning a relatively high supersaturation was applied necessary for creating crystal nuclei (see point 1 in figure 2 a). For all of our experiments point 1 had coordinates (1.6 % $CdSO_4$; C_1). To achieve the initial supersaturation equal amounts (1:1) 3.2 % $CdSO_4$ in 0.2 M sodium acetate buffer and $2\times C_1$ ferritin in the same buffer were mixed. For C_1 were used concentrations from 0.4 mg/ml to 1.2 mg/ml ferritin. After a time interval, $\Delta t = t_2 - t_1$ (fig. 2 b), which is the period of nucleation, by adding solvent (buffer + $CdSO_4$) the supersaturation was decreased to a value corresponding to the metastable zone. So, the final concentration of the solution can be described by point 2 on fig. 2 a. The final concentration corresponds to a supersaturation value, that is enough for the existing nuclei to grow but insufficient for nucleation of new crystals. Besides the position of point 2. depends on the surface. In case of glass surface we used 0.13 mg/ml ferritin and 2.5 % $CdSO_4$, while on both organic templates point 2. had coordinates: 0.08 mg/ml ferritin and 2.5 % $CdSO_4$. When growing crystals became visible in the optical microscope they were counted. $n(t)$ dependencies were plotted, n[number of nuclei on cm^2] *vs* t[minutes]. This procedure was applied at several initial concentrations (C_1) for each substrate.

4. Results and discussion

Ferritin molecules have quasi-spherical shape and crystallize in octahedral crystals (fig. 3 a) with {111} face in the crystal habit (Yau *et al.*, 2000a). In case of crystallization on a substrate we observed ferritin crystals with three general types of orientation (fig. 3 b): with {111} plane parallel to the substrate (triangle form, 1),

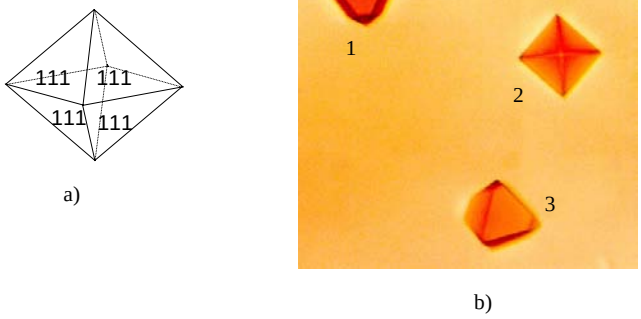

Figure 3
Ferritin crystals
a) cubic octahedral habitus of ferritin crystals;
b) a microphotograph of three general types of orientation of the ferritin crystals grown on a substrate.

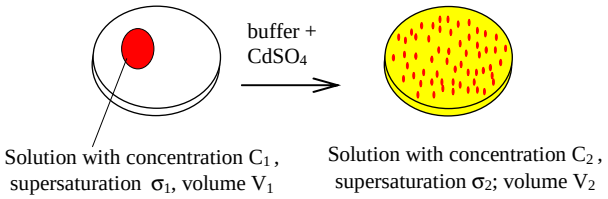

Figure 4
Scheme of the dilution of the concentrated solution and distribution of crystals, when they are grown to visible sizes by microscope.

with {100} plane parallel to the substrate (like a pyramid, 2) and when {110} is parallel or near to parallel position in respect to the substrate, 3.

We found that changes in the $CdSO_4$ concentration resulted in different ratios between the three orientations (especially on a hydrophobic surface). At the lowest salt concentration (from 0.8 to 1.1 %) orientations 2 and 3 are dominant, while it is more than 1.6%, the dominant is orientation 1. A similar effect of PLL coated substrate on the orientations of lysozyme crystals depending on supersaturation was reported in our previous work (Tsekova *et al.*, 1999) and by other authors too (Rong *et al.*, 2002).

For applying a concentration pulse a given quantity (2 to 10 times more than primer protein solution) of solvent (buffer + $CdSO_4$) was added to the initial solution in the quasi-two-dimensional cell (fig. 4). That means that the contact area between the solution with supersaturation σ_1 and the substrates was several times smaller than the final contact area. At the end of the experiment, the protein crystals had a uniform distribution on the whole final area of the substrates. At first sight it may be interpreted as a contradiction with our assertion for heterogeneous formation of crystals. However we have found that the parameters (I, A_k, N_k) of the nucleation processes on the three different substrates differ (table 1). Our working hypothesis is that the formation of the subcritical crystalline clusters starts in the bulk. We consider that such clusters with sub-critical dimensions have affinity to the substrates and after adhering to the substrate become critical (or super critical) particles.

The crystals grown by means of the "pulse" technique were counted and $n(t)$ dependencies were plotted for different supersaturations

$$\ln\sigma = \ln\left(\frac{C}{C_e}\right).$$

For our calculations at 1.6 % $CdSO_4$ we used an equilibrium solubility C_e = 0.023 mg/ml although this value was found for 2.5 % $CdSO_4$ (Yau *et al.*, 2000b). As already mentioned above, we suppose that this leads to a small enough error. On the other hand if we consider that the equilibrium concentration C'_e at 1.6 % $CdSO_4$ is $C'_e = C_e \times \chi$ where χ is a constant and the real supersaturations can be expressed as:

$$\ln\sigma' = \ln\left(\frac{C}{C'_e}\right) = \ln\left(\frac{C}{C_e \times \chi}\right) = \ln\sigma - \ln\chi$$

It is obvious that this difference ($\ln\chi$) between $\ln\sigma$ and $\ln\sigma'$ does not affect the slope of the line $\ln I/\ln\sigma$, i.e. the crystal nucleus size defining.

Fig. 5 shows a set of n(t) dependencies in case of PLL-templates as a substrate. We obtained such dependencies for the other two substrates too. From the slope of the linear fits in all cases the steady-state nucleation rates were measured and the results are shown in the table 1. The data given in the table show that, in comparison to the bare glass substrate, both templates activate the nucleation of ferritin. The highest nucleation rate was measured on the hydrophobic coverage, CH_3 groups. It decreased for the PLL-coverage, the lowest one being measured on bare glass. We suppose that the hydrophobic interactions between some parts of the protein molecule and the CH_3 groups are the strongest. This conclusion is supported by the fact that most of the ferritin crystals were nucleated and grown on the boundary air / solution (air is also hydrophobic) (fig. 6), while on the substrate only few crystals have been observed. Douillard (Douillard, 1997), reports for the preferred adsorption of lysozyme molecules on the air-solution interfaces, as well.

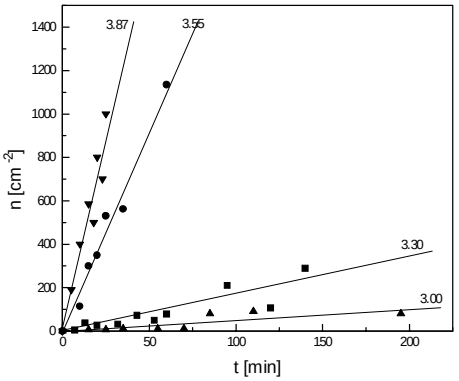

Figure 5
n *vs* t dependencies. The supersaturation {ln σ = ln (C/C$_e$)} is marked on the figure. These data are for crystallization of ferritin on poly-L-lysine surface.

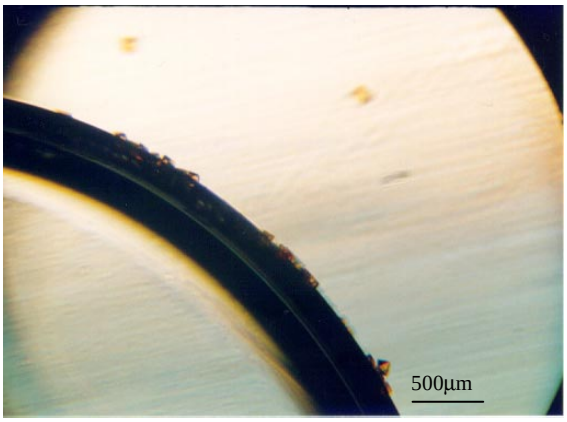

Figure 6
Plenty of ferritin crystals nucleated on the surface of the bubble situated in the protein solution (only few ones are nucleated on the substrate) in the glass quasi-two dimensional cell.

Besides, some electrostatic interaction between the charged substrate and the protein molecules has to be taken into account. At pH = 5.0 the ferritin molecule is negatively charged. The glass surface carries some unstable negative charges, while the PLL surface is positively charged. In our opinion electrostatic repulsion between ferritin molecule (or cluster) and bare glass substrate is the main reason for the lowest rate of nucleation measured on this surface.

Using the classical nucleation theory on the basis of the data for the nucleation rates, the energies required for critical nucleus formation were evaluated (eq. (1). In all cases the energies are of the order of 10^{-13} erg. It is the same order of magnitude that was found for lysozime crystals (Tsekova *et al.*, 1999) and for for small molecule crystal formation as well.

Applying the so called nucleation theorem (eq. (2)) the sizes of the critical nuclei were estimated, as well. We found 5 molecules for both glass and surface covered by PLL and 2 molecules for substrate covered by CH_3 groups. These clusters (5 and 2 molecules), which are critical nuclei, differ from ferritin dimers and oligomers, as it is shown by Petsev (Petsev *et al.* 2001). The binding sites within the dimer differ from the Cd^{2+} mediated bonds in the crystal lattice and the arrangement of the monomers in the dimer is different from that of a pair of monomers in the crystal.

Table 1 Heterogeneous nucleation of cubic ferritin crystals

(a) on the surface covered by CH_3 groups

ln σ	2.28	2.97	3.26	
I_{st}[nuclei.cm^{-2}.sec^{-1}]	0.122	0.372	0.820	
A_k[erg]		1.1×10^{-13}		
N_k		2		

(b) on the surface, treated by poly-L-lysin

ln σ	3.00	3.30	3.55	3.87
I_{st}[nuclei.cm^{-2}.sec^{-1}]	0.008	0.027	0.300	0.670
A_k[erg]		3.5×10^{-13}		
N_k		5		

(c) on the bare glass surface

ln σ	3.26	3.48	3.65	3.77	3.96
I_{st}[nuclei.cm^{-2}.sec^{-1}]	0.010	0.010	0.065	0.142	0.142
A_k[erg]			3.4×10^{-13}		
N_k			5		

Nevertheless the glass plates were polished to optically flatness, some roughness exist on the surface, due to the manufacturing process that makes the process of nucleation easier according to the nucleation theory (Kashchiev, 2000). We suppose that it is the reason that the critical nucleus size on glass (although the electrostatic repulsion) is equal to those obtained on PLL.

Using AFM technique Yau and Vekilov (Yau & Vekilov, 2000) investigated the processes of nucleation of ferritin crystals in the bulk solution. They claim that before nuclei are formed the ferritin molecules are arranged in rods of about 7-20 molecules in a rod. Our work is at higher supersaturations and nucleation occurred on substrates, so the smaller size of the nuclei is expected.

6. Conclusions

We suggest the use of a technique for separation of the two stages of nucleation and growth of protein crystals. It can be used for kinetics studies of crystal nucleation of all kind of proteins. The fact that at the same supersaturation of the protein solution the nucleation threshold depends on the substrate, shows that we can use templates with specific characteristics (e.g. hydrophobic surface) to initiate nucleation of ferritin crystals under lower supersaturations. In other words, the influence exerted by the different coverages can be used in order to rule the nucleation rate.

Acknowledgements

The authors express their gratitude to Dr. P. Vekilov for supplying them with the purified protein.

References

Bishop, J. B., Fredericks, W. J., Haward, S. B. & Sawada, T.(1992). *J. Cryst. Growth*, **122**, 41-49.
Douillard, R. (1997). *Thin Solid Films*, **292**, 169-172.
Durbin, S. D. & Feher, G. (1990). *J. Mol. Biol.* **212**, 763-774.
Durbin, S. D., Carlson, W. E. & Saros, M. T. (1993). *J. Phys. D:Appl. Phys.* **26**, B128-B132.
Fermani, S., Falini, G., Minnucci, M. & Ripamonti, A. (2001). *J. Cryst. Growth*, **224**, 327-334.
Galkin, O. & Vekilov, P. G. (1999). *J. Phys. Chem. B*, **103**, 10965-10971.
Galkin, O. & Vekilov, P. G. (2000). *J. Am. Chem. Soc.* **122**, 156-163.
Georgalis, Y., Zouni, A., Eberstain, W. & Saenger, W. (1993). *J. Cryst. Growth*, **126**, 245-260.
Heinrichs, W., Georgalis, Y., Schonert, H. & Saenger W. (1993). *J. Cryst. Growth*, **133**, 196-200.

Kaischev, R, Scheludko, A. & Bliznakov, G. (1953). *Compt. Rend.l'Acad. Bulg. Sci*. **6**, 1-4.
Kaischev, R. & Mutaftshiev, B. (1965). *Electrochimica Acta*, **10**, 643-650.
Kashchiev, D. (1981). *J. Chem Phys.* **76**, 5098-5102.
Kashchiev, D. (2000). *Nucleation Basic Theory with Applications*, Butterworth-Heinemann, Oxford.
Malkin, A. J. & McPherson, A. (1993). *J. Cryst. Growth*, **128**, 1232-1235.
Milchev, A. (1991). *Contemp. Phys.* **32**, 321-332.
Milchev, A. & Stoyanov, S. (1976). *J. Electroanal. Chem.* **72**, 33-43.
Milchev, A., Stoyanov, S. & Kaischew R. (1974a). *Thin Solid Films*, **22**, 255-265.
Milchev, A., Stoyanov, S. & Kaischew, R (1974b). *Thin Solid Films*, **22**, 267-274.
Mutaftshiev, B. & Kaischev, R. (1955). *Bulg. Chem. Commun.* **5**, 77-85.
Nanev, C. & Tsekova, D. (2000). *Cryst. Res. Technol.* **35**, 189-195.
Oxtoby, D. & Kashchiev, D. (1994). *J. Chem. Phys.* **100**, 7665-7671.
Pechkova, E. & Nikolini, C. (2001). *J. Cryst. Growth*, **231**, 599-602.
Petsev, D., Thomas, B., Yau, S.-T., Tsekova, D., Nanev, C., Wilson, W. & Vekilov, P. (2001). *J. Cryst. Growth*, **232**, 21-29.
Ries-Kautt, M. & Ducruix, A. (1992). *Crystallization of nucleicacids and proteins: A practical approach*, edited by Ducruix, A. & Giege, R., ch. 10, pp. 269-312. Oxford: IRL Press.
Rong, L., Komatsu, S. & Yoda, S. (2002). *J. Cryst. Growth*, **235**, 489-493.
Rosenberger, F., Vekilov, P. G., Muschol, M. & Thomas,B. R. (1996). *J. Cryst. Growth*, **167**, 1-27.
Saridakis, E. & Chayen, N. E., (2000). *Protein Sci.* **9**, 755-757.
Sazaki, G., Kurihara, K., Nakada, T., Miyashita, S. & Komatsu, H. (1996). *J. Cryst. Growth*, **169**, 355-360.
Stoyanov, S. (1973). *Thin Solid Films*, **18**, 91- 98.
Tammann, G. (1922). *Die Aggregatzustaende*, 2nd ed. Voss, Leipzig.
Thomas, B. R., Carter, D. & Rosenberger, F. (1998). *J. Cryst. Growth*, **187**, 499-510.
Tsekova, D., Dimitrova, S. & Nanev, C. (1999). *J. Cryst. Growth*, **196**, 226-233.
Tsekova, D. (2002). PhD Thesis IPC - BAS, Sofia, Bulgaria.
Vekilov, P. G. & Rosenberger, F., (1998). *J. Cryst. Growth*, **186**, 251-261.
Yau, S.-T. & Vekilov, P. (2000). *Nature*, **406**, 494-497.
Yau, S.-T., Thomas, B. R. & Vekilov, P. G. (2000a). *Phys. Rev. Lett.* **85**, 353-356.
Yau, S.-T., Petsev, D. N., Thomas, B. R. & Vekilov, P. G. (2000b). *J. Mol. Biol.* **303**, 667-678.