

Protein Nanocrystallization

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1.1 Introduction

There is no theory that allows us to predict when or where proteins will crystallize. However, for several reasons the problem is a very pertinent one, especially when we consider crystallization of proteins that are physically confined within a very small volume.

There is also a practical reason for studying protein crystallization in small, confined volumes: crystals are required for determining three-dimensional protein structures by X-ray crystallography. As crystallization conditions can only be found through trial and error, current practice requires simultaneous testing of many different conditions. The obvious idea that minimizing the volume of single tests maximizes the number of different conditions that can be screened with a given quantity of protein prompted the development of high-throughput nanocrystallization systems (Stevens 2000; Rupp 2003a, b; Bard et al. 2004).

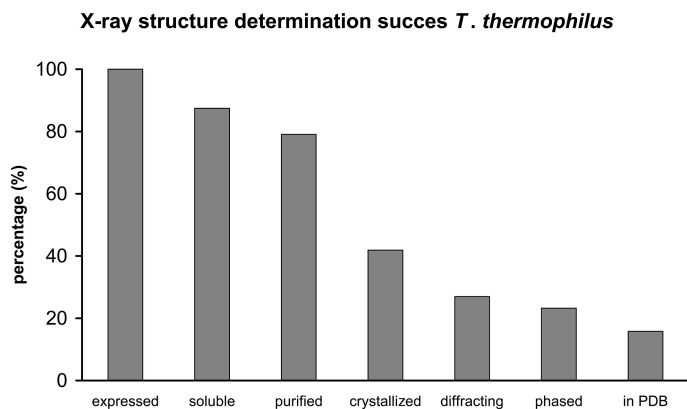


Fig. 1.1. The success rate of high-throughput crystallization. The overall success of the different stages in the high-throughput approach used by the RIKEN consortium is shown. The numerical data were presented at the ICCBM10 conference in Beijing by S. Yokoyama and represent the throughput obtained using expression in *Thermus thermophilus*. The high overall success rate in this example is not typical and expression in higher organisms shows a lower success rate

Although nanocrystallization is quickly becoming a mainstream method, the crystallization step remains the major bottleneck in the structure production process (Blundell and Patel 2004). This is illustrated by recent data from a large structural genomics initiative, indicating that the least successful step in going from sequence to structure is the one from purified protein to crystal. Note that the overall trend illustrated in Fig. 1.1 is not very different from a report predating the widespread use of nanocrystallization (Chayen and Saridakis 2002; Chayen 2004). Probably micro-heterogeneity of the proteins is the prime cause of this bottleneck.

Constructing genetic variants and developing more advanced means of protein production and purification might increase the success rate. Nevertheless, advances in nanocrystallization should also accompany this, as nanocrystallization favors throughput whilst substantially reducing demands on large-scale production and purification platforms.

Here we focus on miniaturization aimed at increasing the probability of finding crystallization conditions when the amount of protein available is limited. First we will review current understanding of nucleation and crystallization of proteins, and focus mainly on those aspects affected by the volume of the mother liquor. Subsequently we will review in detail the major practical obstacles typical of protein nanocrystallization. Problems typically associated with nanovolumes (500 nL or less) concern their dispensing, evaporation and mixing¹. We also discuss the limits imposed by the design of substrates suitable for storing liquid arrays, the robotic accuracy of dispensing strategies, and strategies for scoring nanocrystallization trials.

1.2

Nucleation and Crystallization in Nanovolumes

Naively, one might think that the protein concentration determines the level of supersaturation regardless of the volume. However, this may not be the case, considering that in tiny droplets the surface tension forces become relevant and below a certain volume even predominant. Inside a small nanodroplet the pressure can be substantially higher than the ambient pressure and can be calculated using the Young–Laplace equation (for a review see de Gennes 1985; Blokhuis 2004). However, these effects are less likely to influence protein crystallization in the microliter range. The pressure difference between the inside of a water droplet of 100- μm radius and the gas phase for a surface tension of 72 mN/m is only equal to 1.44 kPa (kN/m^2). Giegé and coworkers studied the influence of external hydrostatic pressure on the nucleation and growth of lysozyme crystals and reported that increasing the pressure from 0.1 MPa (atmospheric pressure) to 250 MPa leads to reduction of the size and number of lysozyme crystals. Moreover a transition to urchinlike particles made of crystalline needles progressively occurs (Lorber et al. 1996; Kadri et al. 2003).

These considerations are obviously irrelevant when the protein is confined within a lipid membrane and thus do not apply for proteins dissolved in the cytoplasm

¹ Classical numerical rounding separates the nanoliter from the microliter range: less than 0.5 is rounded to zero, if one wants to define the nanoliter regime its upper boundary is 500 nL.

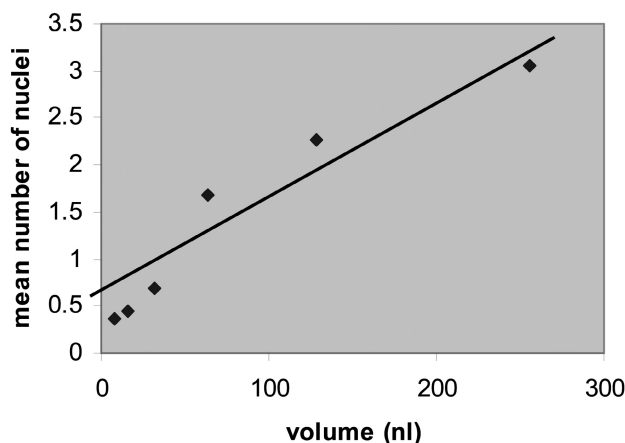


Fig. 1.2. Heterogeneous nucleation in submicroliter volumes. The average number of tetragonal crystals per droplet detected 24 h after mixing as a function of the volume of the droplet. Each data point is the count obtained from 16 droplets. In the smaller droplets needlelike crystals showed a higher relative abundance. (From Bodenstaff et al. 2002)

of living cells. The pressure inside a living cell is well regulated and partially determined by the presence of surrounding tissue. In plant cells the turgor or intracellular pressure can reach several atmospheres at most (Tomos and Leigh 1999).

For practical purposes it is more important that the homologous nucleation rate in protein crystallization is theoretically determined by the level of supersaturation, and it is independent of the volume of the mother liquor. If at a certain level of supersaturation it takes on average a full day to form a stable nucleus that grows into a macroscopic protein crystal in say 1 μL , then it would take 50 days on average for a similar event to occur in a volume of 20 nL. If the nucleation rate per unit volume is constant, reduction of the crystallization volume therefore results in a reduced chance of finding crystals. In other words, one has to increase the level of supersaturation in nanoliter crystallization trials in order to observe rare nucleation events. The relation between the crystallization volume in submicroliter volumes and the observed number of crystals is shown in Fig. 1.2 and indicates that there is a dependence on the droplet volume (Bodenstaff et al. 2002). The relation appears to be linear, but does not go through the origin, indicating that a basic assumption of the homogeneous nucleation theory is not satisfied. This suggests that heterogeneous nucleation plays an important role in low volumes. Vekilov et al. report that despite precautions, heterogeneous nucleation is always observed in their experiments and led to a nonzero intercept of the linear dependence of N (mean number of observed crystals) as a function of the induction time, Δt , in a volume of 700 nL (Galkin and Vekilov 1999; Chernov 2003; Vekilov and Galkin 2003).

Note that although the probability of finding a crystal is very low, a nucleus can always be formed owing to a spontaneous (homogeneous) nucleation event because of density fluctuations (ten Wolde and Frenkel 1997). At this point two types of heterogeneous nucleation should be distinguished: heterogeneous nucleation that de-

depends on nuclei that float in the bulk volume and heterogeneous nucleation that is somehow related to the surface of the mother liquor. In the first case, homogeneous and heterogeneous nucleation cannot be distinguished by changing the crystallization volume. In the latter case reduction of the crystallization volume would increase the relative contribution of heterogeneous nucleation. On the basis of the experimental results it can be argued that there may exist a certain (very low) volume below which heterogeneous nucleation will be the dominant nucleation mechanism (Galkin and Vekilov 1999; Bodestaff et al. 2002). The early stages of crystallization have been probed using fluorescence energy transfer (Pusey and Nadarajah 2002), but the mechanism of nucleation (homogeneous or heterogeneous) remains poorly understood. Most of the atomic force microscopy work has focused on crystal growth (McPherson et al. 2001, 2003) with the notable exception of work from the Vekilov group (Yau and Vekilov 2001). To induce nucleation or to reduce the induction time of crystallization, different engineered and natural seeding materials have been tested, but they turned to be successful only for certain proteins. This indicates that, probably there is no “universal nucleating surface,” so finding a suitable substrate is another process of trial and error in the quest for crystals (Chayen et al. 2001; Pechkova and Nicolini 2001; Sanjoh et al. 2001; Bergfors 2003; d’Arcy et al. 2003).

The critical radius, r^* , of the crystal nucleus is the same for homogeneous and heterogeneous nucleation. Following the notation and arguments given by Veesler and Boistelle, we can express the critical radius as (Veesler and Boistelle 1999)

$$r^* = \frac{2\gamma_1 V_m}{kT \ln \beta} \quad (1.1)$$

The supersaturation is given by the ratio of the actual concentration, C , and the equilibrium saturation concentration, C_s , k is Boltzmann’s constant, T the absolute temperature and γ_1 is the interfacial free energy of the nucleus with respect to the solution. The volume of one molecule in the nucleus is V_m . At the critical size r^* the nucleus is in a very labile equilibrium. If it gains one molecule such that $r > r^*$, it will continue to grow. But if it loses one molecule such that $r < r^*$, it will spontaneously dissolve. If a cap-shaped nucleus with radius r is formed on a surface it contains fewer molecules than a sphere with the same radius in bulk solution. In heterogeneous nucleation three surface free energies play a role: γ_1 between the nucleus and the solution, γ_a between the nucleus and the substrate and γ_o between the substrate and the solution. Depending on the values of these energies the probability of a nucleation event may increase. The substrate can induce nucleation at even lower supersaturation as less energy is required to form the nucleus on the surface (Veesler and Boistelle 1999). As below a certain volume homogeneous nucleation becomes highly improbable, introducing heterogeneous nucleation sites could be an attractive approach to induce crystallization in a controlled manner in very small volumes. Although the chance of finding crystals decreases with decreasing volume, protein nanocrystallization has been shown to be a viable approach. A relatively small increase in supersaturation can easily compensate for the decreasing chance of finding crystals in the screening phase. The important optimization of the crystal growth phase can only be started after the identification of suitable nucleation conditions. In this respect the use of heterogeneous surfaces may help us to develop even small

assays to find these nucleation conditions. Carefully designed growth strategies are subsequently needed to provide us with X-ray diffraction quality crystals needed for successful structural biology.

1.3

Creating and Dispensing Small Liquid Volumes

The controlled dispensing of very small liquid volumes was first demonstrated by Elmqvist (in the context of printing) in the Siemens–Elema Minograf recording mechanism (US patent 2,566,443, issued September 1951). Important factors in the dispensing of small liquids volumes are:

- Dynamic range of the dispensed volume
- Dispensing frequency (determines throughput)
- Precision and accuracy
- Linearity
- Reliability
- Ease of operation and maintenance
- General compatibility of surfaces and liquids (compatible with labile compounds)

The preferred size range for (protein) droplets is between 20 pL and 20 nL, as the total trial volume should be low to realize significant advantage over classical methods. The manual, classical, dispensing of small volumes is by pipetting, but below a volume of roughly 200 nL pipetting becomes notably inaccurate and unreliable. Although manual dispensing can be used for small volumes, convenience and accuracy rules out their use in high-throughput experimentation. Low-volume manual dispensing in protein crystallization was reported by Yeh for drops above 100 nL using a handheld nanoject pipettor with an error of the order 5–9%. For drops smaller than 100-nL volume the error rises rapidly (Yeh 2003). For most applications a standard error of 5% is considered the upper limit (Rose 1999). As manual dispensing is neither accurate nor convenient at volumes below 100 nL, especially when variation in droplet composition is essential for the experiment, different methods are clearly needed. Three established methods used in the field that can dispense in the nanoliter and picoliter ranges are the inkjet, electrospray and pin-transfer methods.

1.3.1

Inkjet Technology

Several dispensing systems in protein nanocrystallization have been described in the literature (Stevens 2000; Bodestaff et al. 2002; Howard and Cachau 2002; Krupka et al. 2002; Kuil et al. 2002; Santesson et al. 2003; Blundell and Patel 2004). Inkjet nanodispensing involves application of a force – electrical, thermal or acoustic – that generates a pressure wave through the fluid. The liquid stream created is allowed to escape through a small orifice. When the liquid passes through the ori-

Advanced Techniques in Biophysics

Arrondo, J.L.R.; Alonso, A. (Eds.)

2006, XIV, 280 p., Hardcover

ISBN: 978-3-540-30700-6