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José Luis R. Arrondo Alicia Alonso (Eds.)

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Professor José Luis R. Arrondo

Professor Alicia Alonso

Unidad de Biofísica (Centro Mixto CSIC-UPV)
Departamento de Bioquímica y Biología Molecular
P.O. Box 644
48080 Bilbao
Spain

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Preface

The present book of the Springer Series in Biophysics deals with some techniques that are being implemented nowadays. One of the motors that have driven the biosciences, like daily life, has been the technological boost produced by the advancement of microprocessor technology. A whole array of possibilities have been opened to develop the classical techniques that were used some years ago.

Abrahams and coworkers contribute with a chapter on protein nanocrystallography which deals with obtaining protein crystals in small, confined volumes, trying to overcome one of the setbacks in crystallography, the amount of material needed to obtain good samples for diffraction. This chapter is followed by one by Ibarra-Molero and Sanchez-Ruiz reviewing the recent advances of differential scanning calorimetry in the field of protein energetics and also in the energetic analysis of other biological systems. The following two chapters look at recent advances of IR spectroscopy. IR reflection--absorption spectroscopy (IRRAS) looks at the air--water interface of membranes and in the chapter by Mendelsohn and coworkers the general basis as well as the application to lipids and peptides or proteins are reviewed. Arrondo and coworkers address the analysis of IR spectra by a new approach called two-dimensional generalized spectroscopy, where information on protein changes after a perturbation is analysed by synchronous or asynchronous maps. This approach, essentially different from that of 2D-NMR spectroscopy, uses correlation analysis of the dynamic fluctuations caused by an external perturbation to enhance spectral resolution.

Three chapters are devoted to different technical developments of NMR. Szypersky deals with the principles of ultrafast NMR spectroscopy through the use of **G**-matrix Fourier transform (GFT) NMR as a technique for rapid sampling of multidimensional NMR data. Freeman and Kup e approach the problem of fast multidimensional NMR by outlining two radical new approaches, one using spatially encoded single-scan multidimensional NMR and the other using projection--reconstruction of multidimensional spectra. Size is one of the problems that NMR has to face in the study of proteins, Fernández and Wider analyse the use of transverse relaxation-optimized spectroscopy (TROSY) in combination with isotope-labelling techniques to extend applications of NMR spectroscopy in solution to much larger molecules, such as integral membrane proteins in detergent micelles, large proteins in monomeric form and in macromolecular complexes, and intermolecular interactions in large complexes.

Carrión-Vázquez and coworkers have addressed protein nanomechanics, a new multidisciplinary area of research to directly measure mechanical forces in single molecules, by applying atomic force microscopy (AFM). Large unilamellar

vesicles are the subject of the chapter by Bagatolli, who reviews the use of two-photon fluorescence microscopy in studying the lateral structure of compositionally simple vesicles and more complicated membranes. San Martín and Valle look at the three-dimensional organization and structural features of macromolecular assemblies, knowledge of which is indispensable for understanding their functions, by using three-dimensional electron microscopy.

This book constitutes a privileged observatory for reviewing novel applications of biophysical techniques that can help the reader utilize the efforts of the scientists contributing to the volume to enter an area where the technology is progressing quickly and where a comprehensive explanation is not always to be found.

Bilbao, June 2006

José Luis R. Arrondo
Alicia Alonso

Contents

CHAPTER 6
Emerging Techniques in Fast Multidimensional NMR
Ray Freeman, Eriks Kupče

CHAPTER 7
Principles and Application of Projected Multidimensional NMR Spectroscopy – G-matrix Fourier Transform NMR
Thomas Szyperski

CHAPTER 8
Protein Nanomechanics – as Studied by AFM Single-Molecule Force Spectroscopy
Mariano Carrión-Vázquez, Andrés F. Oberhauser, Héctor Díez, Rubén Hervás, Javier Oroz, Jesús Fernández, David Martínez-Martín

List of Contributors

Abrahams, Jan Pieter
Section Biophysical Structure Chemistry, Leiden Institute of Chemistry, Leiden University, PO Box 9502, 2300 RA Leiden, The Netherlands

Arrondo, José Luis R.
Unidad de Biofísica (Centro Mixto CSIC-UPV/EHU) and Departamento de Bioquímica, Universidad del País Vasco, Apdo. 644, 48080 Bilbao, Spain

Bagatolli, Luis A.
MEMPHYS – Center for Biomembrane Physics, Department of Biochemistry and Molecular Biology, University of Southern Denmark. Campusvej 55, 5230 Odense M, Denmark

Cai, Peng
Department of Chemistry, Newark College of Arts and Science, Rutgers University, Newark, NJ 07102, USA

Carrión-Vázquez, Mariano
Instituto Cajal/CSIC, Avda. Doctor Arce 37, 28002 Madrid, Spain

Díez, Héctor
Instituto Cajal/CSIC, Avda. Doctor Arce 37, 28002 Madrid, Spain

Fernández, César
Novartis Institutes for Biomedical Research, 4002 Basel, Switzerland

Fernández, Jesús
Instituto Cajal/CSIC, Avda. Doctor Arce 37, 28002 Madrid, Spain

Flach, Carol R.
Department of Chemistry, Newark College of Arts and Science, Rutgers University, Newark, NJ 07102, USA

Freeman, Ray
Jesus College, University of Cambridge, Cambridge CB5 8BL, UK

Garcia-Pacios Marcos
Unidad de Biofísica (Centro Mixto CSIC-UPV/EHU) and Departamento de Bioquímica, Universidad del País Vasco, Apdo. 644, 48080 Bilbao, Spain

Georgieva, Dilyana
Section Biophysical Structure Chemistry, Leiden Institute of Chemistry, Leiden University, PO Box 9502, 2300 RA Leiden, The Netherlands

Goñi, Félix M.
Unidad de Biofísica (Centro Mixto CSIC-UPV/EHU) and Departamento de Bioquímica, Universidad del País Vasco, Apdo. 644, 48080 Bilbao, Spain

Hervás, Rubén
Instituto Cajal/CSIC, Avda. Doctor Arce 37, 28002 Madrid, Spain

Ibarra-Molero, Beatriz
Facultad de Ciencias, Departamento de Quimica Fisica, Universidad de Granada, Fuentenueva s/n, 18071 Granada, Spain

Iloro, Ibon
Unidad de Biofísica (Centro Mixto CSIC-UPV/EHU) and Departamento de Bioquímica, Universidad del País Vasco, Apdo. 644, 48080 Bilbao, Spain

Kuil, Maxim E.
Section Biophysical Structure Chemistry, Leiden Institute of Chemistry, Leiden University, PO Box 9502, 2300 RA Leiden, The Netherlands

Kupče, Eriks
Varian Ltd., 6 Mead Road,, Oxford Industrial Park, Yarnton, Oxford OX5 1QU, UK

Martínez-Martín, David
Instituto Cajal/CSIC, Avda. Doctor Arce 37, 28002 Madrid, Spain

Mendelsohn, Richard
Department of Chemistry, Newark College of Arts and Science, Rutgers University, Newark, NJ 07102, USA

Oberhauser, Andrés F.
Department of Neuroscience and Cell Biology, University of Texas Medical Branch, Galveston, TX 77555, USA

Oroz, Javier
Instituto Cajal/CSIC, Avda. Doctor Arce 37, 28002 Madrid, Spain

Sanchez-Ruiz, Jose M.
Facultad de Ciencias, Departamento de Quimica Fisica, Universidad de Granada, Fuentenueva s/n, 18071 Granada, Spain

San Martín, Carmen
Biocomputing Unit, Centro Nacional de Biotecnología (CNB-CSIC), Darwin 3, 28049 Madrid, Spain

Szyperski, Thomas
Departments of Chemistry and Structural Biology, State University of New York at Buffalo, Buffalo, NY 14260, USA

Valle, Mikel K.
Biocomputing Unit, Centro Nacional de Biotecnología (CNB-CSIC), Darwin 3, 28049 Madrid, Spain

Wider, Gerhard
Institut für Molekularbiologie und Biophysik, ETH Zürich, 8093 Zurich, Switzerland

Protein Nanocrystallization

Dilyana Georgieva, Jan Pieter Abrahams, Maxim E. Kuil

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).

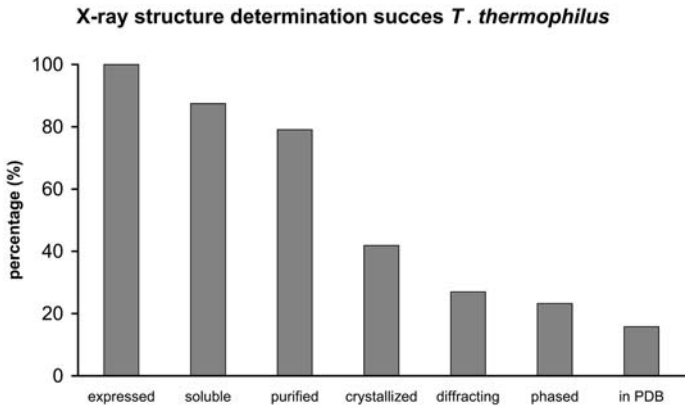

Fig. 1.1. The success rate of high-throughput crystallization. The overall success of the different stages in the high-throughput approach used by the RIKEN consortium is shown. The numerical data were presented at the ICCBM10 conference in Beijing by S. Yokoyama and represent the throughput obtained 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

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Advanced Techniques in Biophysics

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 microheterogeneity 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 mixing[1]. 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.

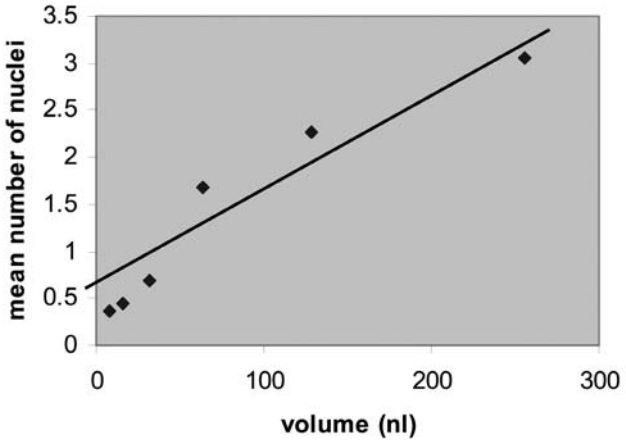

Fig. 1.2. Heterogeneous nucleation in submicroliter volumes. The average number of tetragonal crystals per droplet detected 24 h after mixing as a function of the volume of the droplet. Each data point is the count obtained from 16 droplets. In the smaller droplets needlelike crystals showed a higher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

pends 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; Bodenstaff 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)

$$r^* = \frac{2\gamma_1 V_m}{kT \ln\beta} \,. \tag{1.1}$$

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 looses 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 a 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; Bodenstaff 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-

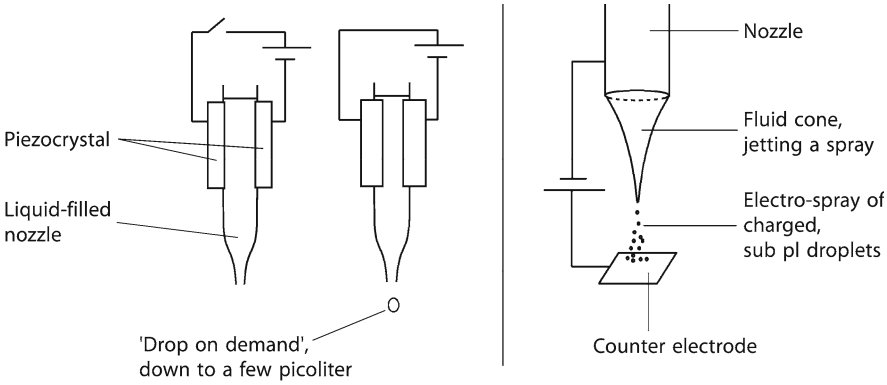

Fig. 1.3. Piezoelectric dispensing versus electrospray dispensing. In the piezoelectric dispensing method (*left*) a pressure wave is generated that allows detachment and propelling of the droplet from the fluid body. Electrospray dispensing uses a different principle: an electric potential difference pulls droplets out of a fluid body. (From Bodenstaff et al. 2002)

fice the pressure difference allows the stream to overcome the surface tension forces and to be ejected as a drop. In a continuous inkjet the liquid supply is pressurized sufficiently to create a jet. The breakup of the jet can be synchronized by applying a periodic modulation of the velocity of the fluid exiting the nozzle. One of the most successful applications of the continuous inkjet technology is the Hertz continuous mist inkjet. In this method charged drops with volume of about 3 pL are produced at very high drop repetition frequency. Although this method is very fast and allows for high throughput, it has not been applied for protein crystallization. In drop-on-demand inkjet methods the forces to overcome surface tension forces and emit a drop or a cluster of drops are generated in response to a signal. The liquid supply is not sufficiently pressurized to form a (continuous) jet. The liquid is held in a nozzle, forming a meniscus, and remains in place until some force overcomes the inherent surface tension that keeps the liquid together. The commonest approach is to suddenly raise the pressure on the liquid, propelling it from the nozzle to the surface. It is also possible to pull the liquid out of a nozzle by an attractive force overcoming the surface tension (Pond 2000). For protein nanocrystallization the precise positioning and timing of the droplet deposition is of great importance; therefore, the drop-on-demand method of dispensing appears more suitable than the Hertz technologies. In view of the required drop volume, the precise positioning and the nature of the dispensed liquid, the relevant technologies are piezoelectric, electrostatic and acoustic drop-on-demand dispensing. To dispense solutions containing proteins that are possibly heat-sensitive, the bubble jet technology is considered less suitable. In piezoelectric dispensing a piezocrystal changes its shape in response to an electrical pulse. This results in "squeezing" a glass capillary and thus creates a pressure difference, either by opening a valve leading out of the pressurized container or by pushing against the fluid. As a result a drop is created "on demand." This type of piezoelectric dispensing is known as "squeeze mode." Various other methods such as the "bend", "shear" and "push" modes have been developed and differ in aspects

less relevant for our purpose (Pond 2000). On-demand piezoelectric dispensers can typically create single drops in the picoliter size range and have been successfully used to dispense liquids and solutions with various properties in volumes as small as 0.3 pL (Howard and Cachau 2002). With present-day technology it is possible to very reliably produce droplets with a volume of 25 pL or more. These can be produced at a rate of at least 1,000 droplets per second. The range of viscosities that can be dispensed with piezoelectric inkjet technology ranges from 0.4 to 100 mPa s, e.g., 100 times the viscosity of water at 20°C. This somewhat limited viscosity range is extended by the use of electrospray dispensing, where an electric potential difference is used to pull droplets out of a fluid body (for a review see Rohner et al. 2004). The viscosity of the fluids and the presence of detergents are less relevant for electrospray dispensing compared with piezoelectric dispensing. However, there are currently no commercial dispensing stations that make use of drop-on-demand electrospray methods although electrospray dispensing has significant advantages for the dispensing of very viscous liquids. The use of pulsed electrospray is relatively new (Wei et al. 2002), and recently we constructed several prototypes that can successfully dispense viscous liquids on flat substrates. A schematic comparison between the setup for piezoelectric dispensing and electrospray dispensing is given in Fig. 1.3.

A major problem in the reliable operation of any dispensing system is the problem of clogging, e.g., accumulation of solid material, given the small size of the orifice and the nature of the components to be dispensed. Clogging of the orifice starts and often results in drop misdirection with a disastrous effect on the overall reliability. Careful design of the chamber and orifice, including the proper choice of materials (and their wetting properties), results in reliable operation. For prolonged reliable operation good cleaning procedures and the purity and stability of the liquids to be dispensed are crucial.

1.3.2 Acoustic Dispensing

A novel technology that avoids the clogging problem is acoustic drop-on-demand dispensing where the free surface of a liquid is disrupted by a strong acoustic field. If the acoustic energy is well focused into a small volume of liquid near the surface, a drop of variable size can be ejected. The droplet size depends on the acoustic energy field, the acoustic frequency in combination with the liquid properties. In acoustic droplet ejection (ADE), nanoliter or picoliter droplets are ejected from a conventional microplate by means of the acoustic energy generated by a piezoelectric transducer. The energy is focused via acoustic lenses on the surface of the liquid, causing a droplet of precise volume to be ejected without any physical contact between the acoustic device and the liquid being dispensed. Drops are collected on another surface (e.g., a microplate) positioned in the path of the droplet. The "nozzleless" ADE method avoids the reliability issues associated with the small orifice such as clogging followed by drop misdirection. As acoustic ejection is a noncontact method, cross-contamination between samples caused by the transfer device – a common problem in liquid handling – is largely avoided. A smallest drop volume of 2 pL is possible and the volume can continuously be adjusted up to 40 pL. The acoustic drop-on-de-

mand dispensing technology can generate variable drop volumes at megahertz rates. ADE can dispense a wide variety of solvents, DNA, proteins and even live mammalian cells without detectable loss of activity or viability (Ellson 2003). Although acoustic dispensing has not been applied so far in protein nanocrystallization, the high speed and contactless mode of operation are substantial advantages. The major advantage of ADE is the fast and powerful transfer of liquids. It is less clear how suitable the method is when the total amount of material is very limited and has to be dispensed in nanoliter volumes as in protein crystallography.

1.3.3 Fast Solenoid Valve Technology

The fast solenoid valve microdispensing method couples the accuracy of a stepper-motor-driven syringe pump with the high-speed actuation of a microsolenoid valve. The syringe creates a steady hydraulic pressure within the system fluid, essential to obtain consistent and accurate droplet sizes. For a given pressure the desired

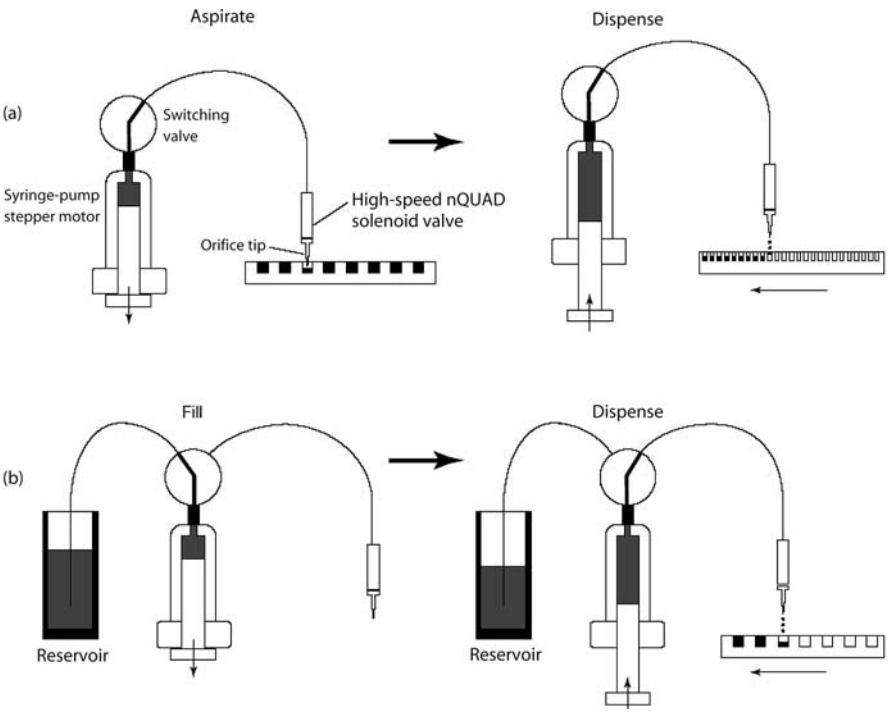

Fig. 1.4. Fast solenoid–syringe method. **a** In the aspirate dispensing mode liquid is collected from microvials and redistributed into nanovials. This mode is used for small volume rearrangements. **b** In the bulk dispensing mode a larger volume is aspirated and distributed into the microvials. This step is used to rearrange moderate volumes. As shown in Fig. 1.7 the Cartesian instrument shows good linearity in both dispensing modes. (From Rose 1999)

drop volume is obtained by choosing an appropriate valve opening time. The technology can be used in two different ways, aspirate dispensing and bulk dispensing. Using the syringe pump, either the sample can be aspirated from a source and then deposited into a destination, or the sample can be introduced through the entire fluidic path and dispensed in a continuous fashion. A schematic view of the bulk and aspirate fast solenoid dispensing mechanisms is given in Fig. 1.4 (Rose 1999). The system is robust and adequate for setting up crystallization in the nanoliter range. In this method the potential of cross-contamination of the dispensed liquids makes a washing step between dispensing different liquids essential. The use of parallel dispensing nozzles each dedicated to a single fluid reduces such demands. As in most dispensing technologies, calibration of the correct parameters in the dispensing process is needed for each solution.

1.3.4 Pin-Transfer Technology

Pin-transfer technology transfers liquid from a source to a location using a solid pin. The process requires dipping a pin into a sample and taking it out. A small volume of liquid remains on the tip of the pin and by placing the pin on a solid surface the liquid is dispensed. The major advantage of this technology is that the pins are simple and relatively inexpensive, while volumes in the low-nanoliter range can be transferred. The pin method has limited flexibility as the pin dispenses a fixed small volume that depends on the tip properties and geometry in combination with the liquid properties. This limits the use in applications requiring variable volumes. Another disadvantage of this technology is that clogging can occur, especially when suspended particles are relatively large with respect to the tip or the gap in the pin. If throughput and cost are more important parameters than high precision and accuracy, pin-transfer technology is a possible alternative to noncontact dispensing. When a screen using fixed volumes of liquids optimized for protein crystallization has been identified, a very fast system with disposable pins could be mass-produced.

1.3.5 Comparison of Liquid Dispensing Methods

Important aspects of the dispensing techniques related to protein crystallization are summarized in Table 1.1. Some highly relevant factors for protein crystallization cannot be compared easily for all techniques and all strategies. As an example, the dead volume of a dispensing system is not a problem for most liquids but for the component of most interest, the protein to be studied, it is highly relevant. No matter how accurate and reliable a dispensing system is, if it is necessary to inject some hundred microliters of precious protein the system it will be of little to no value. Evaluation and reduction of the dead volume is an issue that is not widely addressed in the field of nanodispensing, which mostly focuses on throughput and accuracy. If one would like to compare the amount of protein needed per trial between methods, the dead volume should be included. Equally relevant for all dispensing techniques

Table 1.1. Comparison of nanodispensing techniques. Some important aspects of different dispensing techniques related to protein crystallization are compared. Electrospray has a great potential in protein crystallization, but it is not yet commercially available

Property	Pin method	Piezoelectric inkjet	Thermal inkjet	Acoustic inkjet	Electrospray	Fast solenoid valve column
Driving force	Mechanical	Pressure	Heat	Sound wave pressure	Electric	Pressure (release)
Biocompatible	Yes	Yes	No	Yes	Yes	Yes
Viscous solutions	No	No	Yes	No	Yes	No
Accurate, reproducible	No	Yes	Yes	Yes	Yes	Yes
Small volumes (nanoliter and subnanoliter)	Yes	Yes	Yes	Yes	Yes	No
Low energy transfer to sample	Yes	Yes	No	Yes	Yes	Yes

is the (cross-) contamination of the dispensing system that ultimately results in unreliable operation and failure. The problem of tip contamination has been addressed in the Mosquito dispensing system where an automated system simply discards the tips after use for a single compound. An elegant and rapid way of cleaning and drying for nondisposable tips and equipment is still to be found. In this respect the ADE method seems a viable method as it eliminates the need for disposing and/or washing steps altogether.

1.4 Droplet Evaporation

Langmuir (1918) reached the amazing conclusion that the evaporation rate of a droplet is proportional to the droplet radius. As the droplet volume is proportional to the radius cubed the study of small droplets will eventually be hampered by evaporation. Although droplet evaporation has been studied extensively it is not yet completely understood. Three different situations, most relevant for protein crystallization, can be ranked in complexity:

1. Free droplet in a supporting gas (liquid–gas interface)
2. Sessile or hanging droplets on a supporting solid (liquid–gas–solid interface)
3. Droplet inside a small container (liquid–gas–solid and geometry of container)

The general evaporation problem is complex and many aspects are of importance; especially the evaporation of sessile droplets with dissolved particles differs fundamentally from pure solvent evaporation. The factors that influence the evaporation of a fluid are:

- The droplet volume and shape
- The temperature of the fluid and surrounding vapor
- The composition of the fluid
- The external pressure and the partial vapor pressure

In addition, for a droplet in contact with a solid surface, the surface roughness, composition, temperature and wetting properties as well as the geometry are of importance.

As examples, ingenious experiments have been performed on the evaporation of flying droplets of pure solvents trapped in electric or acoustic fields. In these experiments the presence and influence of a support is eliminated. Beauchamp and coworkers studied the evaporation of charged droplets in the so-called ping-pong configuration (Grimm and Beauchamp 2002; Smith et al. 2002). They used droplets generated by electrospray dispensing and controlled the motion of the charged droplets by reversing the electric field direction when the droplets moved outside the observation volume. In the thesis of Eberhardt (1999), the evaporation of levitated droplets trapped in an acoustic field was studied. Both studies confirmed Langmuir's findings and show that the droplet diameter d_p as a function of time is given by $d_p^2(t) = d_p^2(0) + Ct$, where $d_p(t)$ represents the radius at time t and $d_p(0)$ the initial radius of a pure liquid droplet, with C (a negative constant) representing the evaporation.

1.4.1 Evaporation of a Binary Mixture of Pure Liquids

Eberhardt (1999) and Sefiane et al. (2003) have studied the evaporation of binary mixtures from free and sessile droplets respectively. They both observed the evaporation of the most volatile component first, as expected, but in the presence of a supporting surface there appears an intermediate phase related to the wetting properties of the surface. The addition of a less volatile component to the droplets will slow down the overall evaporation. This observation may allow the reduction of the evaporation of very small droplets of mother liquor too.

1.4.2 Evaporation of a Solvent with a Solute

The evaporation of a solvent with a (nonevaporating) solute proceeds significantly differently from the case discussed before. The most general case is the evaporation of a solvent with a solute without the presence of a substrate as used in the crystallization in acoustically and/or electrostatically levitated droplets (Chung and Trinh 1998; Santesson et al. 2003; Knezic et al. 2004). In the presence of a surface due to the pinning of the contact line, e.g., the rim of the droplet on the surface, there is a significant flow inside the droplet to supply solvent to the surface of the droplet where the evaporation takes place (as in levitated droplets). The flow of solvent causes the relative accumulation of solute close to the contact line. The effect is illustrated by looking at the ring left by a spilled coffee droplet (Deegan et al. 1997). The solute flow in levitated droplets has been visualized but is difficult to control to improve the crystallization (Chung and Trinh 1998).

1.4.3 Evaporation from Microcompartments

In a series of publications the liquid evaporation from silicon microwells showed solute accumulation as a result of contact pinning in very small wells (Hjelt et al. 2000; Young et al. 2003; Rieger et al. 2003). The accumulation of solute particles and their dynamics has been studied by fluorescence spectroscopy; some characteristic phases in the evaporation process are shown in Fig. 1.5. The white lines represent the trajectory of the solute during the evaporation. These results are relevant for protein crystallogenesis, as they suggest that a concentration gradient builds up during the initial stages of the crystallization experiment.

1.4.4 Practical Approaches to Reduce Evaporation

The aforementioned considerations suggest the following precautions to reduce evaporation. The most important factor (if not interfering with the crystallization process) is the reduction of the temperature of the droplet (and container). Covering

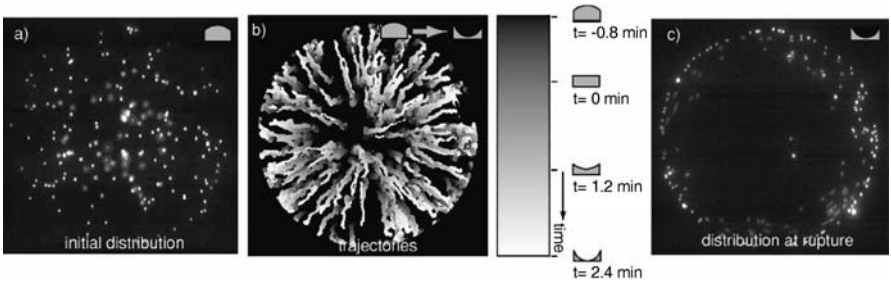

Fig. 1.5. Contact line pinning in nanovials. In this experiment the trajectories of small fluorescent spheres dissolved in a water–glycerol mixture contained in a nanovial are recorded. The *inserts* in grey reflect the shape of the meniscus of the evaporating liquid. It is clear that nonevaporation solutes such as the fluorescent beads travel to the liquid boundary and accumulate owing to contact line pinning in nanovials with a radius of 100 µm. (From Rieger et al. 2003)

the droplet with an immiscible liquid with a high boiling point can further reduce the evaporation. This approach has been successfully applied in batch crystallization where the droplets are covered with oil. By choosing the appropriate oil–solvent combination, the evaporation can be controlled to a certain extent. Evaporation can be prevented also by controlling the surrounding vapor pressure. During the filling of microarrays the evaporation can hardly be avoided. In our own experiments we use a contact-cooled microarray to prevent evaporation. After dispensing, the array can be covered with oil and sealed with adhesive transparent tape. Cooling the microarray has the additional advantage of reversing the convective flow and thereby reducing the solvent transport form the array (Bodenstaff et al. 2002). For most applications in microbatch crystallization it is also possible to fill the (nano) wells first with oil that has lower density than the volatile component(s) (Chayen et al. 1992; Kuil et al. 2002). Subsequently added components sink to the bottom of the well and are covered with a layer of oil. However, sinking droplets do not always merge and mix if this approach is used. Mayer and Köhler (1997) studied the effect of droplet evaporation in microarrays and reported a linear dependence of the evaporation rate on the opening surface of the container. However, more recently a linear dependence of the evaporation rate on the radius of cylindrical microcompartments was reported and it was concluded that the evaporation is diffusion-limited (Rieger et al. 2003).

1.5 Liquid Mixing in Small Volumes and Microfluidics

Two consecutive processes dominate the mixing of liquids: fusion of the liquid boundaries and diffusion of the components. Although both processes are similar in bulk fluid and microfluidics, their outcome is significantly different; unexpected mixing results in microfluidics were reported. Two liquid streams can flow alongside

in a tube a few micrometers wide over a period of time without mixing, almost as if they were separated by glass (Knight 2002). The fusion step is not only the first step, but is also the rate-limiting step in the mixing process. Macroscopically stirring can speed up fusion, as turbulence increases the interfacial area between the liquids, but in small channels it is almost impossible to produce such a turbulent flow. Mechanical forces as in shaking or thermal forces inducing convective flows are less effective and more difficult to apply in very small volumes. A recent approach to accelerate mixing of small volumes is electrosmosis, where components are displaced by electric fields. For the aim of protein nanocrystallization mixing of protein droplets on a small scale can best be achieved during the dispensing phase. For example, in the Microdrop robot system droplets are shot from the nanodispenser with a linear velocity of 3–5 m/s and they dive in the bulk solution without splashing as there is not enough energy for splashes to be formed – droplets with a diameter less than 100 μm have more surface energy than kinetic energy at the speeds generated (see http://www.microdrop.de).

When two droplets meet on a solid surface they usually fuse if the liquids involved are miscible. Little is known about what happens next: the subsequent diffusion of dissolved components (e.g., proteins) in high concentrations within the fused nanovolumes. Techniques like dynamic light scattering and fluorescence correlation spectroscopy probe diffusion in (very) small volumes but are always conducted in a total volume of a few microliters, far away from interfaces (Nijman et al. 2001; Schmauder et al. 2002). Although usually homogeneous mixing is aimed for, the lack of homogeneous mixing can sometimes also be an advantage as demonstrated in free-interface diffusion methods used in protein crystallization without evaporation (Hansen et al. 2002; Zheng et al. 2004).

1.6 Design and Making of Nanostructures

1.6.1 Nanoarrays

Making small droplets of mother liquor is not enough: they should also be stored for prolonged periods without evaporation. For this purpose, nanoarrays are used. In robotic dispensing systems the positional accuracy is mostly better than 5 μm and repositioning within 10–20 μm is possible. The latter value limits the minimal size of the wells at present to about 40 μm (and subsequently the volumes to roughly 40 μm × 40 μm × 40 μm). Apart from size considerations a practical geometry for the containers, which minimizes the evaporation, should be chosen. Irrespective of the method of dispensing, the evaporation is reduced when the opening surface is minimal, implying the height should be as large as possible. This suggests that a tall, straight, cylindrical structure is optimal. However, because the wells also have to be monitored for crystal growth, in practice other geometries are chosen. For example, we designed our wells as truncated inverted pyramids with a bottom and top width of 300 and 800 μm, respectively. For manufacturing nanoarrays, the choice of the material is important and compatibility with proteins is vital in this application; sur-

faces that denature proteins should be avoided. Other essential issues are transparency in the visible part of the spectrum, noncrystalline structure and high uniformity at the length scales studied. If birefringence is used as a detection or recognition technique, the material should in addition also be nonbirefringent. In an early approach we used the elastomer poly(dimethylsiloxane), PDMS, to produce the microwells (Sia and Whitesides 2003). It meets all the requirements mentioned earlier; moreover, the time to produce a prototype is short and the material is not prohibitively expensive. A brass master was micro-machined using a CNC cutting machine to produce deep wells of the desired size. Alternatively, multilayered photolithography can be used to manufacture a master with higher accuracy. Currently, we use polyurethane to make a rigid and more-solvent-resistant array, as shown in Fig. 1.6. The outside dimensions are 29 mm × 78 mm and the array contains 720 wells. There are numerous high-density microplates on the market that can be used for protein nanocrystallization. Hanging and sitting drop vapor diffusion methods are possible with some plates. Other plates can be exposed to X-rays in order to check the quality of the crystals in the droplet directly. The plates having a low birefringence are suitable if polarization techniques are used for crystal recognition. As the current trend in the high-throughput-screening market is to reduce costs, this reduction is accomplished not only with low-volume but also with high-density plates such as the 6,144-well format made in PDMS used by King at Harvard Medical School and even higher densities such as the 24,576 array available from BioTrove.

1.6.2 Microfluidic Systems

Hansen and Quake introduced a completely different approach to protein nanocrystallization. They designed nanofluidic networks with incorporated valves that could be used to bring protein and precipitant solution in contact in nanoliter volumes. The solutions are introduced using excess pressure forcing the liquids into the fluidic network. The elastomer used is permeable to gas and the channels can be completely filled with liquid. The permeability is also a disadvantage, as crystalliza-

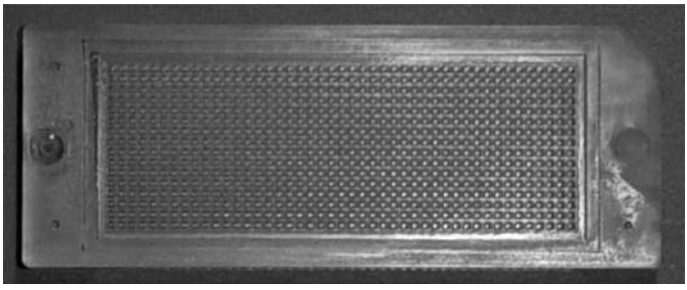

Fig. 1.6. Polurethane array. The array consists of 720 wells (45 × 16) each with a square opening of 800 μm and a square bottom 300-μm wide. The well depth is 2 mm. The array is made by polymerizing polyurethane in a micromachined metal mould

tion trials eventually can dry out or equilibrate with neighboring trials. By applying a pressure to a second network that controls the valves, the protein and precipitant solutions can be pumped around, mixing and/or sealing them (Hansen et al. 2002). The network and channels are made of a very transparent elastomer, allowing a good visualization of the crystallization process. A particular advantage is the use of rectangular chambers for crystallization, allowing for an even illumination of the entire volume when inspecting crystallization trials. The total use of protein is 150 μL for 6,048 crystallization conditions; the time needed to grow crystals was reported to be shorter than by vapor diffusion. Moreover, diffraction-quality crystals can be recovered from the system, indicating that the method is not only suited for screening. The equipment for fluid handling and the nanofluidic chips are commercially available as the Topaz Crystallization System from the Fluidigm Company. The LabChip system from Caliper Life Sciences is another microfluidic system that is used for protein-crystallization experiments.

1.7 Robotics

There are four important aspects in the robotics of protein nanocrystallization:

1. Motion during the dispensing
2. Motion involved in filling/refilling and cleaning
3. Dispensing control
4. Motion involved in inspection of plates

The last aspect, inspection of plates, is not discussed at length as most of the critical steps are found in the first three aspects. Several commercial systems storing, retrieving and moving the plates with reduced vibration, accurate alignment and good temperature control are available (e.g., the Bruker Nonius Crystal Farm).

Robotics required for dispensing should be fast in order to reduce evaporation and increase throughput. Massive parallel-dispensing systems, such as 1,536-nozzle inkjet systems, have almost eliminated the speed problem as all compounds can be dispensed in one step (Gast and Fiehn 2003). Cocktails can be freshly prepared in microliter to milliliter quantities using existing technology and can be "uploaded" to a "multinozzle" pipette and multiple 1,536 arrays can be processed with a very high throughput. The overall speed is determined by the dispensing speed of the protein – the most precious component. As the amount of protein is often limited, the amount of protein needed for dispensing should be minimal. To reduce dead-volume losses, the optimal dispensing strategy is to dispense the protein sequentially from a highly reliable single nozzle system, which will then take most of the processing time. This time can only be reduced by accurate in-flight dispensing. For example, to optimize the protein stability, a chilled protein holder has been developed. One microliter can be dispensed to all wells of a 96-well plate in approximately 15 s (Cartesian synQUAD technology). Most pipetting stations use standard size microtitre plates and the spacing and sizes of future-generation plates can easily be extrapolated. The plate height has not been considered too much in the early design considerations. When the standard footprint size is kept constant we will likely be