

Springer Series  
in Biophysics 10

José Luis R. Arrondo  
Alicia Alonso (Eds.)

# Advanced Techniques in Biophysics

José Luis R. Arrondo   Alicia Alonso (Eds.)

# **Advanced Techniques in Biophysics**

With 93 Figures, 17 in Color and 2 Tables

 Springer

**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

**ISSN 0932-2353**

**ISBN 10 3-540-30700-1 Springer Berlin Heidelberg NewYork**

**ISBN 13 978-3-540-30700-6 Springer Berlin Heidelberg NewYork**

Library of Congress Control Number: 2005938927

This work is subject to copyright. All rights are reserved, whether the whole or part of the material is concerned, specifically the rights of translation, reprinting, reuse of illustrations, recitation, broadcasting, reproduction on microfilms or in any other way, and storage in data banks. Duplication of this publication or parts thereof is permitted only under the provisions of the German Copyright Law of September 9, 1965, in its current version, and permission for use must always be obtained from Springer. Violations are liable for prosecution under the German Copyright Law.

**Springer is a part of Springer Science+Business Media**

springeronline.com

© Springer-Verlag Berlin Heidelberg 2006

Printed in Germany

The use of general descriptive names, registered names, trademarks, etc. in this publication does not imply, even in the absence of a specific statement, that such names are exempt from the relevant protective laws and regulations and therefore free for general use.

Editor: Dr. Sabine Schreck, Heidelberg

Desk Editor: Anette Lindqvist, Heidelberg

Production: LE-TeX Jelonek, Schmidt & Vöckler GbR, Leipzig

Typsetting: Satz-Druck-Service, Leimen

Cover Design: Design & Production, Heidelberg

Printed on acid-free paper

39/3100/YL

5 4 3 2 1 0

Springer Series in Biophysics 10

## 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. Szyperksy 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

## 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 Química Física, 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 Química Física, 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



# Contents

## CHAPTER 1

### Protein Nanocrystallization

DILYANA GEORGIEVA, JAN PIETER ABRAHAMS, MAXIM E. KUIL

1.1	Introduction . . . . .	1
1.2	Nucleation and Crystallization in Nanovolumes . . . . .	2
1.3	Creating and Dispensing Small Liquid Volumes . . . . .	5
1.3.1	Inkjet Technology. . . . .	5
1.3.2	Acoustic Dispensing . . . . .	7
1.3.3	Fast Solenoid Valve Technology. . . . .	8
1.3.4	Pin-Transfer Technology. . . . .	9
1.3.5	Comparison of Liquid Dispensing Methods. . . . .	9
1.4	Droplet Evaporation . . . . .	11
1.4.1	Evaporation of a Binary Mixture of Pure Liquids . . . . .	12
1.4.2	Evaporation of a Solvent with a Solute . . . . .	12
1.4.3	Evaporation from Microcompartments . . . . .	12
1.4.4	Practical Approaches to Reduce Evaporation . . . . .	12
1.5	Liquid Mixing in Small Volumes and Microfluidics . . . . .	13
1.6	Design and Making of Nanostructures. . . . .	14
1.6.1	Nanoarrays. . . . .	14
1.6.2	Microfluidic Systems . . . . .	15
1.7	Robotics . . . . .	16
1.8	Crystal Recognition. . . . .	18
1.9	Outlook. . . . .	21
	References . . . . .	22

## CHAPTER 2

### Differential Scanning Calorimetry of Proteins: an Overview and Some Recent Developments

BEATRIZ IBARRA-MOLERO, JOSE M. SANCHEZ-RUIZ

2.1	Introduction . . . . .	27
2.2	What Is DSC? . . . . .	28
2.3	What Is Heat Capacity?. . . . .	29
2.4	Equilibrium Thermodynamics Analysis Versus Kinetic Analysis . . . . .	30
2.5	The Calorimetric Criterion for Two-State Behaviour . . . . .	34
2.6	Two-State Versus Continuous (Barrierless, Downhill) Transitions. . . . .	36
2.7	Characterization of Ligand-Binding Effects . . . . .	39

2.8	Absolute Heat Capacities and the Residual Structure in the Unfolded State . . . . .	42
2.9	Calorimetrically Determined Denaturant $m$ Values . . . . .	44
	References . . . . .	45

### CHAPTER 3

#### **IR Reflectance–Absorbance Studies of Peptide Structure, Orientation, and Conformational Flexibility in Langmuir Films: Relevance for Models of Pulmonary Surfactant Action**

CAROL R. FLACH, PENG CAI, RICHARD MENDELSON

3.1	Introduction . . . . .	49
3.2	Instrumentation . . . . .	50
3.3	Information from IRRAS Measurements . . . . .	50
3.4	Pulmonary Surfactant . . . . .	57
3.4.1	Biochemistry and Models for Function . . . . .	57
3.4.2	Application of IRRAS to the Pulmonary Surfactant . . . . .	60
3.4.2.1	Squeeze-Out . . . . .	60
3.4.2.2	Structure, Orientation, and Possible Mechanism of Action of Therapeutic Agents for RDS . . . . .	61
3.5	Future Possibilities for IRRAS . . . . .	67
	References . . . . .	69

### CHAPTER 4

#### **Two-Dimensional Infrared Correlation Spectroscopy**

JOSÉ LUIS R. ARRONDO, IBON ILORO, MARCOS GARCIA-PACIOS,  
FÉLIX M. GOÑI

4.1	Introduction . . . . .	73
4.2	Description of the Technique . . . . .	74
4.3	Spectral Simulations . . . . .	76
4.3.1	Intensity Changes . . . . .	76
4.3.2	Band Shifting . . . . .	77
4.3.3	Bandwidth . . . . .	79
4.4	D-IR Studies of Proteins . . . . .	80
4.4.1	2D Maps of Homopolypeptides . . . . .	80
4.4.2	Protein Denaturation . . . . .	81
4.4.3	Protein Unfolding and Aggregation . . . . .	84
4.5	Summary . . . . .	86
	References . . . . .	87

### CHAPTER 5

#### **NMR Spectroscopy of Large Biological Macromolecules in Solution**

CÉSAR FERNÁNDEZ, GERHARD WIDER

5.1	Introduction . . . . .	89
5.2	Technical Background . . . . .	90

5.2.1	The NMR Signal. . . . .	90
5.2.2	NMR and Molecular Size . . . . .	92
5.2.3	Isotope Labeling. . . . .	93
5.2.4	Transverse Relaxation-Optimized Spectroscopy (TROSY) . . . . .	93
5.2.4.1	The Foundations of TROSY . . . . .	93
5.2.4.2	Field-Strength Dependence of TROSY for $^{15}\text{N}$ - $^1\text{H}$ Groups . . . . .	95
5.2.4.3	Implementation of TROSY: 2D [ $^{15}\text{N}$ , $^1\text{H}$ ]-TROSY . . . . .	95
5.2.4.4	[ $^{13}\text{C}$ , $^1\text{H}$ ]-TROSY . . . . .	97
5.2.5	Cross-Correlated Relaxation-Induced Polarization Transfer for Studies of Very Large Structures . . . . .	98
5.2.6	The Water Resonance. . . . .	98
5.3	Isotope-Labeling Techniques . . . . .	99
5.3.1	Uniform Isotope Labeling . . . . .	99
5.3.2	Selective Isotope-Labeling Strategies . . . . .	100
5.4	TROSY for NMR Studies of Large Biological Macromolecules . . . . .	102
5.4.1	2D [ $^{15}\text{N}$ , $^1\text{H}$ ]-TROSY. . . . .	102
5.4.2	[ $^{13}\text{C}$ , $^1\text{H}$ ] Correlation Experiments. . . . .	104
5.4.3	TROSY for Resonance Assignments in Large Molecules. . . . .	105
5.4.3.1	[ $^{15}\text{N}$ , $^1\text{H}$ ]-TROSY for Sequential Assignment of Protein Backbone Resonances . . . . .	105
5.4.3.2	[ $^{15}\text{N}$ , $^1\text{H}$ ]-TROSY for Assignment of Protein Side-Chain Resonances . . . . .	106
5.4.4	TROSY for Studies of Intermolecular Interactions and Drug Design . . . . .	108
5.4.5	TROSY for Observation of Scalar Couplings Across Hydrogen Bonds . . . . .	109
5.4.6	TROSY for Measurements of RDCs . . . . .	109
5.4.7	TROSY for Studies of Dynamic Processes . . . . .	111
5.4.8	TROSY in NOESY . . . . .	111
5.4.9	Applications to Nucleic Acids . . . . .	112
5.4.10	TROSY, CRIPT, and CRINEPT for Studies of Very Large Structures . . . . .	112
5.5	Solution NMR Studies of Membrane Proteins. . . . .	114
5.5.1	Resonance Assignments and Collection of Structural Constraints for Membrane Proteins . . . . .	114
5.5.2	D Structure Determination . . . . .	115
5.5.3	Studies of Intermolecular Interactions Between a Membrane Protein and Detergent Molecules in Micelles . . . . .	116
5.5.4	Dynamic Properties of Membrane Proteins . . . . .	118
5.6	Conclusion and Outlook . . . . .	119
	References . . . . .	120

## CHAPTER 6

**Emerging Techniques in Fast Multidimensional NMR**

RAY FREEMAN, ERIKS KUPČE

6.1	Introduction . . . . .	129
6.2	Spatially Encoded Single-Scan Multidimensional NMR . . . . .	130
6.2.1	Evolution . . . . .	130
6.2.2	Detection . . . . .	132
6.2.3	Resolution . . . . .	133
6.2.4	The $F_2$ Dimension . . . . .	134
6.2.5	Signal-to-Noise Ratio . . . . .	134
6.2.6	Application of Single-Scan Two-Dimensional Spectroscopy . . . . .	136
6.3	Projection–Reconstruction of Multidimensional Spectra . . . . .	136
6.3.1	Projection of NMR Spectra . . . . .	138
6.3.2	Reconstruction from Projections . . . . .	140
6.3.3	Application of Projection–Reconstruction NMR . . . . .	142
6.3.4	Related Methods . . . . .	142
6.4	Conclusions . . . . .	144
	References . . . . .	145

## CHAPTER 7

**Principles and Application of Projected Multidimensional NMR Spectroscopy – G-matrix Fourier Transform NMR**

THOMAS SZYPSKI

7.1	Introduction . . . . .	147
7.2	Background . . . . .	147
7.2.1	RD and Accordion NMR Spectroscopy . . . . .	148
7.2.2	RD NMR Spectroscopy . . . . .	148
7.2.2.1	Joint Sampling of Two Shift Evolution Periods . . . . .	148
7.2.2.2	Time-Proportional Phase Incrementation in RD NMR . . . . .	150
7.2.2.3	Editing Peak Pairs in RD NMR . . . . .	151
7.2.2.4	Peak Pattern in RD NMR Spectra . . . . .	151
7.2.2.5	Double RD NMR . . . . .	151
7.2.2.6	Use of Heteronuclear Magnetization . . . . .	151
7.2.2.7	Application of RD NMR in Structural Genomics . . . . .	152
7.3	GFT NMR . . . . .	152
7.3.1	Theory of GFT NMR . . . . .	152
7.3.2	Sensitivity of GFT NMR . . . . .	156
7.3.3	Precision of Measurements in GFT NMR . . . . .	156
7.3.4	Survey: GFT NMR and Comparison with FT NMR . . . . .	158
7.3.5	Application of GFT NMR . . . . .	159
7.3.5.1	Protein Resonance Assignment . . . . .	159
7.3.5.2	G-matrix Transformation for Measurement of Residual Dipolar Couplings . . . . .	159
7.3.6	GFT projection-reconstruction . . . . .	160
	References . . . . .	160

## 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

8.1	Introduction . . . . .	163
8.2	Biological Machines, Single-Molecule Manipulation and Protein Mechanics . . . . .	164
8.2.1	Protein Machines . . . . .	164
8.2.2	Range of Relevant Forces in Biology . . . . .	166
8.2.3	Mechanical Proteins and Mechanical Nanomachines . . . . .	167
8.2.4	Single-Molecule Techniques . . . . .	170
8.2.5	Single-Molecule Manipulation Techniques . . . . .	171
8.3	SMFS of Proteins: Physical Principles and Methodology . . . . .	172
8.3.1	Mechanical Force Transduction by AFM . . . . .	174
8.3.1.1	Cantilever and Tip Choices . . . . .	174
8.3.1.2	Calibration of the AFM . . . . .	175
8.3.1.3	Obtaining the Final Force–Distance Curve . . . . .	177
8.3.2	Establishing a “Mechanical Circuit”: Protein Attachment and Functionalization . . . . .	178
8.3.3	Mechanical Unfolding of Proteins . . . . .	178
8.3.3.1	Length-Clamp (Standard) SMFS . . . . .	179
8.3.3.2	Force-Clamp SMFS . . . . .	206
8.3.3.3	Computer Simulations of Mechanical Unfolding . . . . .	206
8.3.4	Mechanical Refolding of Proteins . . . . .	207
8.4	Intramolecular Interactions . . . . .	208
8.4.1	Model System: I27 Module, an Immunoglobulin Domain from Titin . . . . .	211
8.4.2	Other Protein Folds: from “Mechanical” and “Nonmechanical” Proteins . . . . .	214
8.4.3	Supramolecular Mechanical Properties of Protein Complexes . . . . .	216
8.4.4	Achievements . . . . .	217
8.4.4.1	Single-Molecule Mechanics vs. Bulk Biochemical Assays . . . . .	217
8.4.4.2	Detection of Rare Misfolding Events . . . . .	218
8.4.4.3	Directional Aspects of the Force: Shear vs. Zipper Configurations and “Achilles Heels” . . . . .	219
8.4.4.4	Molecular Determinants of the Mechanical Stability of Proteins . . . . .	220
8.4.4.5	Muscle Elasticity, a Macroscopic Biological Property, Reduced to the Single-Molecule Level . . . . .	222
8.5	Intermolecular Interactions: Findings and Limitations . . . . .	223
8.5.1	Model System: Biotin–(Strept)avidin . . . . .	224
8.5.2	Other Protein–Biomolecule Pairs . . . . .	225
8.6	Limitations and Perspectives . . . . .	226
8.6.1	Technical Limitations . . . . .	226
8.6.2	Methodological Limitations . . . . .	231

8.6.3	SMFS and Protein Mechanics in Vivo . . . . .	232
8.7	Conclusions . . . . .	233
	References . . . . .	235

## CHAPTER 9

### **Multiphoton-Excitation Fluorescence Microscopy and Membranes**

LUIS A. BAGATOLLI

9.1	Introduction . . . . .	247
9.2	Model Systems. . . . .	248
9.3	Fluorescence Microscopy and Membrane Domains in GUVs. . . . .	249
9.4	Fluorescent Probes . . . . .	250
9.5	Two-Photon-Excitation Microscopy . . . . .	251
9.6	LAURDAN Probe: the Tips . . . . .	252
9.7	Membrane Lateral Structure in Artificial Lipid Mixtures and Natural Lipid Extracts as Seen by LAURDAN . . . . .	255
9.7.1	The Importance of Visual Information to Ascertain Lateral Structure in Compositionally Complex Mixtures . . . . .	257
9.7.2	LAURDAN in Cell Membranes and Tissues. . . . .	258
9.8	Concluding Remarks . . . . .	260
9.9	Summary. . . . .	261
	References . . . . .	262

## CHAPTER 10

### **Three-Dimensional Electron Microscopy. The Coming of Age of a Versatile Structural Biology Technique**

CARMEN SAN MARTÍN, MIKEL K. VALLE

10.1	Introduction . . . . .	267
10.2	Basis of 3D-EM . . . . .	268
10.3	Recent Evolution of the 3D-EM Field. . . . .	270
10.4	The Cutting Edge . . . . .	270
10.5	Dynamic Structures and the Fourth Dimension in Cryo-EM. . . . .	272
10.6	Cryo-electron Tomography . . . . .	273
10.7	Concluding Remarks . . . . .	276
	References . . . . .	276

<b>Subject Index</b> . . . . .	279
--------------------------------	-----

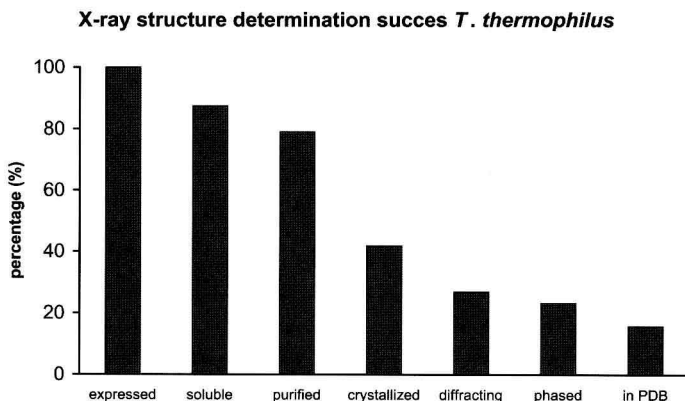
# Protein Nanocrystallization

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

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 mixing<sup>1</sup>. 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

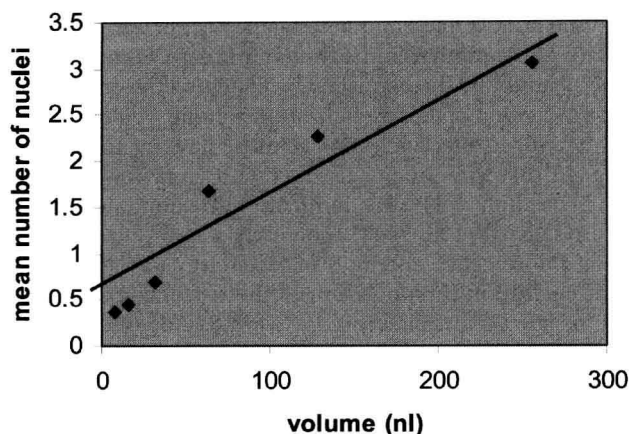
Naively, one might think that the protein concentration determines the level of supersaturation regardless of the volume. However, this may not be the case, considering that in tiny droplets the surface tension forces become relevant and below a certain volume even predominant. Inside a small nanodroplet 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- $\mu\text{m}$  radius and the gas phase for a surface tension of 72 mN/m is only equal to 1.44 kPa ( $\text{kN/m}^2$ ). 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

---

<sup>1</sup> 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  $\mu\text{L}$ , 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,  $\Delta t$ , 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-