

Contents

Part I Directed Evolution 1

| | | |
|----------|---|-----------|
| 1 | Continuous Evolution of Proteins <i>In Vivo</i> | 3 |
| | <i>Alon Wellner, Arjun Ravikumar, and Chang C. Liu</i> | |
| 1.1 | Introduction | 3 |
| 1.2 | Challenges in Achieving <i>In Vivo</i> Continuous Evolution | 5 |
| 1.3 | Phage-Assisted Continuous Evolution (PACE) | 10 |
| 1.4 | Systems That Allow <i>In Vivo</i> Continuous Directed Evolution | 13 |
| 1.4.1 | Targeted Mutagenesis in <i>E. coli</i> with Error-Prone DNA Polymerase I | 13 |
| 1.4.2 | Yeast Systems That Do Not Use Engineered DNA Polymerases for Mutagenesis | 16 |
| 1.4.3 | Somatic Hypermutation as a Means for Targeted Mutagenesis of GOIs | 18 |
| 1.4.4 | Orthogonal DNA Replication (OrthoRep) | 20 |
| 1.5 | Conclusion | 22 |
| | References | 22 |
| 2 | <i>In Vivo</i> Biosensors for Directed Protein Evolution | 29 |
| | <i>Song Buck Tay and Ee Lui Ang</i> | |
| 2.1 | Introduction | 29 |
| 2.2 | Nucleic Acid-Based <i>In Vivo</i> Biosensors for Directed Protein Evolution | 32 |
| 2.2.1 | RNA-Type Biosensors | 32 |
| 2.2.2 | DNA-Type Biosensors | 35 |
| 2.3 | Protein-Based <i>In Vivo</i> Biosensors for Directed Protein Evolution | 37 |
| 2.3.1 | Transcription Factor-Type Biosensors | 37 |
| 2.3.2 | Enzyme-Type Biosensors | 41 |
| 2.4 | Characteristics of Biosensors for <i>In Vivo</i> Directed Protein Evolution | 44 |
| 2.5 | Conclusions and Future Perspectives | 45 |
| | Acknowledgments | 46 |
| | References | 46 |

| | | |
|----------|--|------------|
| 3 | High-Throughput Mass Spectrometry Complements Protein Engineering | 57 |
| | <i>Tong Si, Pu Xue, Kisurb Choe, Huimin Zhao, and Jonathan V. Sweedler</i> | |
| 3.1 | Introduction | 57 |
| 3.2 | Procedures and Instrumentation for MS-Based Protein Assays | 59 |
| 3.3 | Technology Advances Focusing on Throughput Improvement | 62 |
| 3.4 | Applications of MS-Based Protein Assays: Summary | 63 |
| 3.4.1 | Applications of MS-Based Assays: Protein Analysis | 64 |
| 3.4.2 | Applications of MS-Based Assays: Protein Engineering | 66 |
| 3.5 | Conclusions and Perspectives | 68 |
| | Acknowledgments | 68 |
| | References | 69 |
| | | |
| 4 | Recent Advances in Cell Surface Display Technologies for Directed Protein Evolution | 81 |
| | <i>Maryam Raeeszadeh-Sarmazdeh and Wilfred Chen</i> | |
| 4.1 | Cell Display Methods | 81 |
| 4.1.1 | Phage Display | 81 |
| 4.1.2 | Bacterial Display Systems | 83 |
| 4.1.3 | Yeast Surface Display | 84 |
| 4.1.4 | Mammalian Display | 85 |
| 4.2 | Selection Methods and Strategies | 86 |
| 4.2.1 | High-Throughput Cell Screening | 86 |
| 4.2.1.1 | Panning | 86 |
| 4.2.1.2 | FACS | 86 |
| 4.2.1.3 | MACS | 87 |
| 4.2.2 | Selection Strategies | 88 |
| 4.2.2.1 | Competitive Selection (Counter Selection) | 88 |
| 4.2.2.2 | Negative/Positive Selection | 89 |
| 4.3 | Modifications of Cell Surface Display Systems | 89 |
| 4.3.1 | Modification of YSD for Enzyme Engineering | 89 |
| 4.3.2 | Yeast Co-display System | 91 |
| 4.3.3 | Surface Display of Multiple Proteins | 91 |
| 4.4 | Recent Advances to Expand Cell-Display Directed Evolution Techniques | 93 |
| 4.4.1 | μSCALE (Microcapillary Single-Cell Analysis and Laser Extraction) | 93 |
| 4.4.2 | Combining Cell Surface Display and Next-Generation Sequencing | 94 |
| 4.4.3 | PACE (Phage-Assisted Continuous Evolution) | 94 |
| 4.5 | Conclusion and Outlook | 96 |
| | References | 97 |
| | | |
| 5 | Iterative Saturation Mutagenesis for Semi-rational Enzyme Design | 105 |
| | <i>Ge Qu, Zhoutong Sun, and Manfred T. Reetz</i> | |
| 5.1 | Introduction | 105 |
| 5.2 | Recent Methodology Developments in ISM-Based Directed Evolution | 108 |

- 5.2.1 Choosing Reduced Amino Acid Alphabets Properly 109
- 5.2.1.1 Limonene Epoxide Hydrolase as the Catalyst in Hydrolytic Desymmetrization 109
- 5.2.1.2 Alcohol Dehydrogenase TbSADH as the Catalyst in Asymmetric Transformation of Difficult-to-Reduce Ketones 110
- 5.2.1.3 P450-BM3 as the Chemo- and Stereoselective Catalyst in a Whole-Cell Cascade Sequence 112
- 5.2.1.4 Multi-parameter Evolution Aided by Mutability Landscaping 115
- 5.2.2 Further Methodology Developments of CAST/ISM 117
- 5.2.2.1 Advances Based on Novel Molecular Biological Techniques and Computational Methods 117
- 5.2.2.2 Advances Based on Solid-Phase Chemical Synthesis of SM Libraries 118
- 5.3 B-FIT as an ISM Method for Enhancing Protein Thermostability 120
- 5.4 Learning from CAST/ISM-Based Directed Evolution 121
- 5.5 Conclusions and Perspectives 121
- Acknowledgment 124
- References 124

Part II Rational and Semi-Rational Design 133

- 6 Data-driven Protein Engineering 135**
Jonathan Greenhalgh, Apoorv Saraogee, and Philip A. Romero
- 6.1 Introduction 135
- 6.2 The Data Revolution in Biology 136
- 6.3 Statistical Representations of Protein Sequence, Structure, and Function 138
- 6.3.1 Representing Protein Sequences 138
- 6.3.2 Representing Protein Structures 140
- 6.4 Learning the Sequence-Function Mapping from Data 141
- 6.4.1 Supervised Learning (Regression/Classification) 141
- 6.4.2 Unsupervised/Semisupervised Learning 144
- 6.5 Applying Statistical Models to Engineer Proteins 145
- 6.6 Conclusions and Future Outlook 147
- References 148
- 7 Protein Engineering by Efficient Sequence Space Exploration Through Combination of Directed Evolution and Computational Design Methodologies 153**
Subrata Pramanik, Francisca Contreras, Mehdi D. Davari, and Ulrich Schwaneberg
- 7.1 Introduction 153
- 7.2 Protein Engineering Strategies 154
- 7.2.1 Computer-Aided Rational Design 155
- 7.2.1.1 FRESCO 155
- 7.2.1.2 FoldX 157

- 7.2.1.3 CNA 158
- 7.2.1.4 PROSS 159
- 7.2.1.5 ProSAR 160
- 7.2.2 Knowledge Based Directed Evolution 161
- 7.2.2.1 Iterative Saturation Mutagenesis (ISM) 161
- 7.2.2.2 Mutagenic Organized Recombination Process by Homologous *In Vivo* Grouping (MORPHING) 161
- 7.2.2.3 Knowledge Gaining Directed Evolution (KnowVolution) 162
- 7.3 Conclusions and Future Perspectives 171
- Acknowledgments 171
- References 171

8 Engineering Artificial Metalloenzymes 177

Kevin A. Harnden, Yajie Wang, Lam Vo, Huimin Zhao, and Yi Lu

- 8.1 Introduction 177
- 8.2 Rational Design 177
- 8.2.1 Rational Design of Metalloenzymes Using *De Novo* Designed Scaffolds 177
- 8.2.2 Rational Design of Metalloenzymes Using Native Scaffolds 179
- 8.2.2.1 Redesign of Native Proteins 179
- 8.2.2.2 Cofactor Replacement in Native Proteins 181
- 8.2.2.3 Covalent Anchoring in Native Protein 184
- 8.2.2.4 Supramolecular Anchoring in Native Protein 187
- 8.3 Engineering Artificial Metalloenzyme by Directed Evolution in Combination with Rational Design 188
- 8.3.1 Directed Evolution of Metalloenzymes Using *De Novo* Designed Scaffolds 188
- 8.3.2 Directed Evolution of Metalloenzymes Using Native Scaffolds 189
- 8.3.2.1 Cofactor Replacement in Native Proteins 189
- 8.3.2.2 Covalent Anchoring in Native Protein 192
- 8.3.2.3 Non-covalent Anchoring in Native Proteins 194
- 8.4 Summary and Outlook 200
- Acknowledgment 201
- References 201

9 Engineered Cytochromes P450 for Biocatalysis 207

Hanan Alwaseem and Rudi Fasan

- 9.1 Cytochrome P450 Monooxygenases 207
- 9.2 Engineered Bacterial P450s for Biocatalytic Applications 210
- 9.2.1 Oxyfunctionalization of Small Organic Substrates 211
- 9.2.2 Late-Stage Functionalization of Natural Products 220
- 9.2.3 Synthesis of Drug Metabolites 224
- 9.3 High-throughput Methods for Screening Engineered P450s 227
- 9.4 Engineering of Hybrid P450 Systems 229
- 9.5 Engineered P450s with Improved Thermostability and Solubility 230

- 9.6 Conclusions 231
- Acknowledgments 232
- References 232

Part III Applications in Industrial Biotechnology 243

10 Protein Engineering Using Unnatural Amino Acids 245

Yang Yu, Xiaohong Liu, and Jiangyun Wang

- 10.1 Introduction 245
- 10.2 Methods for Unnatural Amino Acid Incorporation 246
- 10.3 Applications of Unnatural Amino Acids in Protein Engineering 247
 - 10.3.1 Enhancing Stability 248
 - 10.3.2 Mechanistic Study Using Spectroscopic Methods 248
 - 10.3.3 Tuning Catalytic Activity 250
 - 10.3.4 Tuning Selectivity 252
 - 10.3.5 Enzyme Design 252
 - 10.3.6 Protein Engineering Toward a Synthetic Life 255
- 10.4 Outlook 256
- 10.5 Conclusions 258
- References 258

11 Application of Engineered Biocatalysts for the Synthesis of Active Pharmaceutical Ingredients (APIs) 265

Juan Mangas-Sanchez, Sebastian C. Cosgrove, and Nicholas J. Turner

- 11.1 Introduction 265
 - 11.1.1 Transferases 266
 - 11.1.1.1 Transaminases 266
 - 11.1.1.2 Oxidoreductases 267
 - 11.1.1.2.1 Ketoreductases 267
 - 11.1.1.2.2 Amino Acid Dehydrogenases 271
 - 11.1.1.2.3 Cytochrome P450 Monooxygenases 272
 - 11.1.1.2.4 Baeyer–Villiger Monooxygenases 273
 - 11.1.1.2.5 Amine Oxidases 274
 - 11.1.1.2.6 Hydroxylases 276
 - 11.1.1.2.7 Imine Reductases 276
 - 11.1.1.3 Lyases 278
 - 11.1.1.3.1 Ammonia Lyases 278
 - 11.1.1.4 Isomerases 278
 - 11.1.1.5 Hydrolases 279
 - 11.1.1.5.1 Esterases 279
 - 11.1.1.5.2 Haloalkane Dehalogenase 279
 - 11.1.1.6 Multi-enzyme Cascade 281
 - 11.2 Conclusions 282
 - References 287

| | | |
|-----------|--|------------|
| 12 | Directing Evolution of the Fungal Ligninolytic Secretome | 295 |
| | <i>Javier Viña-Gonzalez and Miguel Alcalde</i> | |
| 12.1 | The Fungal Ligninolytic Secretome | 295 |
| 12.2 | Functional Expression in Yeast | 297 |
| 12.2.1 | The Evolution of Signal Peptides | 297 |
| 12.2.2 | Secretion Mutations in Mature Protein | 300 |
| 12.2.3 | The Importance of Codon Usage | 301 |
| 12.3 | Yeast as a Tool-Box in the Generation of DNA Diversity | 302 |
| 12.4 | Bringing Together Evolutionary Strategies and Computational Tools | 305 |
| 12.5 | High-Throughput Screening (HTS) Assays for Ligninase Evolution | 306 |
| 12.6 | Conclusions and Outlook | 309 |
| | Acknowledgments | 309 |
| | References | 310 |
| 13 | Engineering Antibody-Based Therapeutics: Progress and Opportunities | 317 |
| | <i>Annalee W. Nguyen and Jennifer A. Maynard</i> | |
| 13.1 | Introduction | 317 |
| 13.2 | Antibody Formats | 318 |
| 13.2.1 | Human IgG1 Structure | 318 |
| 13.2.2 | Antibody-Drug Conjugates | 319 |
| 13.2.3 | Bispecific Antibodies | 320 |
| 13.2.4 | Single Domain Antibodies | 321 |
| 13.2.5 | Chimeric Antigen Receptors | 321 |
| 13.3 | Antibody Discovery | 322 |
| 13.3.1 | Antibody Target Identification | 322 |
| 13.3.1.1 | Cancer and Autoimmune Disease Targets | 323 |
| 13.3.1.2 | Infectious Disease Targets | 323 |
| 13.3.2 | Screening for Target-Binding Antibodies | 324 |
| 13.3.2.1 | Synthetic Library Derived Antibodies | 324 |
| 13.3.2.2 | Host-Derived Antibodies | 325 |
| 13.3.2.3 | Immunization | 325 |
| 13.3.2.4 | Pairing the Light and Heavy Variable Regions | 326 |
| 13.3.2.5 | Humanization | 327 |
| 13.3.2.6 | Hybrid Approaches to Antibody Discovery | 328 |
| 13.4 | Therapeutic Optimization of Antibodies | 328 |
| 13.4.1 | Serum Half-Life | 328 |
| 13.4.1.1 | Antibody Half-Life Extension | 329 |
| 13.4.1.2 | Antibody Half-Life Reduction | 331 |
| 13.4.1.3 | Effect of Half-Life Modification on Effector Functions | 331 |

| | | |
|----------|---|-----|
| 13.4.2 | Effector Functions | 331 |
| 13.4.2.1 | Effector Function Considerations for Cancer Therapeutics | 332 |
| 13.4.2.2 | Effector Function Considerations for Infectious Disease Prophylaxis and Therapy | 333 |
| 13.4.2.3 | Effector Function Considerations for Treating Autoimmune Disease | 334 |
| 13.4.2.4 | Approaches to Engineering the Effector Functions of the IgG1 Fc | 334 |
| 13.4.3 | Tissue Localization | 335 |
| 13.4.4 | Immunogenicity | 335 |
| 13.4.4.1 | Reducing T-Cell Recognition | 336 |
| 13.4.4.2 | Reducing Aggregation | 336 |
| 13.5 | Manufacturability of Antibodies | 336 |
| 13.5.1 | Increasing Antibody Yield | 337 |
| 13.5.1.1 | Codon Usage | 337 |
| 13.5.1.2 | Signal Peptide Optimization | 337 |
| 13.5.1.3 | Expression Optimization | 338 |
| 13.5.2 | Alternative Production Methods | 338 |
| 13.6 | Conclusions | 339 |
| | Acknowledgments | 339 |
| | References | 339 |

14 Programming Novel Cancer Therapeutics: Design Principles for Chimeric Antigen Receptors 353

Andrew J. Hou and Yvonne Y. Chen

| | | |
|--------|--|-----|
| 14.1 | Introduction | 353 |
| 14.2 | Metrics to Evaluate CAR-T Cell Function | 354 |
| 14.3 | Antigen-Recognition Domain | 356 |
| 14.3.1 | Tuning the Antigen-Recognition Domain to Manage Toxicity | 356 |
| 14.3.2 | Incorporation of Multiple Antigen-Recognition Domains to Engineer “Smarter” CARs | 356 |
| 14.3.3 | Novel Antigen-Recognition Domains to Enhance CAR Modularity | 359 |
| 14.3.4 | Engineering CARs that Target Soluble Factors | 360 |
| 14.4 | Extracellular Spacer | 360 |
| 14.5 | Transmembrane Domain | 362 |
| 14.6 | Signaling Domain | 362 |
| 14.6.1 | First- and Second-Generation CARs | 362 |
| 14.6.2 | Combinatorial Co-stimulation | 363 |
| 14.6.3 | Other Co-stimulatory Domains: ICOS, OX40, TLR2 | 364 |
| 14.6.4 | Additional Considerations for CAR Signaling Domains | 364 |
| 14.7 | High-Throughput CAR Engineering | 366 |
| 14.8 | Novel Receptor Modalities | 367 |
| | References | 369 |

Part IV Applications in Medical Biotechnology 377

| | |
|-----------|--|
| 15 | Development of Novel Cellular Imaging Tools Using Protein Engineering 379 |
| | <i>Praopim Limsakul, Chi-Wei Man, Qin Peng, Shaoying Lu, and Yingxiao Wang</i> |
| 15.1 | Introduction 379 |
| 15.2 | Cellular Imaging Tools Developed by Protein Engineering 380 |
| 15.2.1 | Fluorescent Proteins 380 |
| 15.2.1.1 | The FP Color Palette 380 |
| 15.2.1.2 | Photocontrollable Fluorescent Proteins 381 |
| 15.2.1.3 | Other Engineered Fluorescent Proteins 383 |
| 15.2.2 | Antibodies and Protein Scaffolds 383 |
| 15.2.2.1 | Antibodies 383 |
| 15.2.2.2 | Antibody-Like Protein Scaffolds 384 |
| 15.2.2.3 | Directed Evolution 384 |
| 15.2.3 | Genetically Encoded Non-fluorescent Protein Tags 385 |
| 15.3 | Application in Cellular Imaging 386 |
| 15.3.1 | Cell Biology Applications 386 |
| 15.3.1.1 | Localization 386 |
| 15.3.1.2 | Cell Signaling 387 |
| 15.3.2 | Application in Diagnostics and Medicine 390 |
| 15.3.2.1 | Detection 390 |
| 15.3.2.2 | Screening for Drugs 392 |
| 15.4 | Conclusion and Perspectives 393 |
| | References 394 |
| | Index 403 |