METHODS IN MOLECULAR BIOLOGY

Series Editor
John M. Walker
School of Life and Medical Sciences
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Peptide Libraries

Methods and Protocols

Edited by

Ratmir Derda

Department of Chemistry, University of Alberta, Edmonton, AB, Canada

Humana Press

Editor
Ratmir Derda
Department of Chemistry
University of Alberta
Edmonton, AB, Canada

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Dedication

I would like to dedicate this book to the memory of the colleague, mentor, and friend Carlos Barbas the IIIrd.

Preface

Peptides are a unique class of drugs that combine the advantages of "small-molecule" and "biological" classes of drugs. Due to their small size, peptide derivatives can have tissue permeability akin to that of small molecules, while genetic selection allows for rapid discovery and optimization of these molecules. The number of peptide-based drugs recently approved by the FDA constituted a significant fraction of the approved small-molecule drugs. Furthermore, in the past 50 years, natural peptide ligands have been used as inspiration for discovery of small-molecule-based drugs. Development of ACE inhibitors is one of the classical examples of such peptide-inspired drug discovery [1].

The utility of peptides extends beyond lead structures in drug pipelines and ranges from identification of probes that can be used for drug delivery or cell imaging (e.g., *see* Chapters 15 and 16) to molecules for noncovalent capture of heavy metals to yield medical imaging agents and peptide-based fluorogenic tags for microscopy and life science applications (*see* Chapter 14). Short peptide sequences that elicit potent molecular recognition towards a specific target fuel the discovery of biomaterials and even functional inorganic materials. The source for discovery of novel bioactive and functional peptides, in many cases, is a "library of peptides."

The purpose of this Preface is to revisit the origin of the word "peptide library," a term widely used by contemporary chemists and biologists, and indicate how historical differences in its origin and definitions lead to the rise of the modern libraries at the chemistry-biology interface. Where possible, we emphasize the role of the enabling technologies in developing modern peptide libraries highlighted in this book. The purpose of the subsequent book chapters is to provide an overview of modern and emerging methods for production, analysis, and utility of peptide libraries that "stand on the shoulders" of the rich history of this field. The topics include (1) synthesis, genetic expression, or hybrid synthesis-expression routes to library production; (2) examples of modern utility of these libraries with emphasis on complex screens that utilize cell-based assays, de novo discovery of reactions, or hybrid organic-inorganic materials; and (3) emerging tools for the analysis of these libraries by method of genetic selection and next-generation sequencing.

Chemical Peptide Libraries

The 1990s is often considered the decade of the birth and rise of the concept of "chemical libraries" in the academic and industrial discovery process [2]. One of the first reports demonstrating the power of combinatorial organic synthesis could be considered John Ellman's report in *PNAS* describing synthesis of a library of 192 structurally diverse benzodiazepine derivatives [3]. The 1990s was also the decade of the rise of classes of compounds that resided between Ellman's "nonoligomer libraries" and classical "oligomer libraries" (DNA, peptides). Examples could be either nonpeptide oligomers or the chemically synthesized hybrid libraries that combined features from peptides and unnatural moieties. This book presents two great modern examples of hybrid motifs. Chapter 6 describes

peptides that include an unnatural beta-turn element to create a stable hairpin motif in a short peptide-derived structure. Chapter 5 describes an amphoteric aziridine-aldehyde linchpin that can be incorporated into linear peptides and promote their rapid and enantio selective cyclization.

While many Chapters (1–3, 13, 15, 16) use elements of peptide library synthesis, no chapters focus solely on "how to synthesize a peptide library." The reason is that the field has matured 10 years before the rise of the modern "chemical library synthesis." A decade prior to Ellman's pioneering report, Mario Geysen and coworkers reported synthesis of a "library of peptides" [4] on an array of 4 × 40 mm polyethylene rods. In the 3 years between 1984 and 1987, Geysen and his coworkers synthesized more than 200,000 peptides and optimized the procedure to allow for synthesis of 4,000 hexapeptides per 10 days and testing of 2,000 per day [5]. This scale, driven simply by clever engineering, is not so different from that of modern chemical libraries empowered by robotics and information technology. The pin synthesis introduced by the Geysen group was the first of many technological advances that accelerated assembly of medium-scale libraries of peptides. To gain an understating of the state of the art of "peptide libraries" and technologies of the early 1990s, we recommend an excellent 1992 review by Jung and Beck-Sickinger [6].

Notable additions to techniques for peptide libraries appearing in the 1980s and 1990s were SPOT synthesis, tea-bag synthesis, automated synthesis, light-directed synthesis on the surface, and one-bead one-compound (OBOC) synthesis. All additions were rapidly adapted in the pharmaceutical industry and led to incorporation of technology-oriented companies. This book has several chapters that overview the modern utility of SPOT and OBOC methods for discovery of functional epitopes in cell-based assays. The book starts from Chapter 1 by the pioneer of OBOC technology Kit Lam and coworkers, who describe an example of one-bead two-compound synthesis. They screen their library to identify compounds that elicit a dual effect—cell capture and cell killing. In Chapter 3, Pei and coworker apply OBOC strategy for synthesis of therapeutically valuable macrocyclic peptides. Imperiali and Martin in Chapter 14 describe the use of OBOC libraries for the selection of lanthanide-binding peptides. Conveniently, the beads with the best peptide chelators are also "the brightest" beads in their screen, allowing for simple and elegant identification of improved La-binding tags. Chapter 15 describes application of OBOC for discovery of binding epitopes for receptors located on the surface of live cells. A notable addition by Len Luyt and coworkers is the utility of a large particle sorter to delineate hit from nonhit peptides and avoid traditional "manual picking" of beads using microscopy assays.

In the areas of SPOT synthesis, Kaur and coworkers in Chapter 16 describe implementation of one of the contemporary models of automated SPOT synthesizers to generate peptide arrays for discovery of cell-binding peptide epitopes. In Chapter 13, Eldridge and Weiss use SPOT synthesis technology as one of the final validation tools in their discovery of novel hydrazine-reactive peptides.

One fundamental discovery at the heart of library-oriented OBOC, SPOT, and pin and array technologies is a 1969 report of solid phase synthesis by Bruce Merrifield. Another, perhaps less known advance, was the 1977 report by Smith, Hurrel, and Lynch describing testing of the peptide directly on the solid support. Reports prior to 1977 utilized synthesis on, cleavage from, and subsequent testing of the peptides off the solid support [7]. The report by Smith et al. aimed to "remove the protecting groups without cleavage of the peptides from the support" and "to estimate the antigenicity of each immobilized peptide [by their ability to bind] radioactivity-labelled antibodies" [7]. Interaction of biomolecules with surface-immobilized molecules was already known for many years, owing to the 1968

report of affinity chromatography [8] and 1975 report of Southern blot technology, [9] which is often quoted as an inspiration for modern DNA/protein/peptide-array technology [10]. Unfortunately, the material of the support used for chromatography or blots was not satisfactory for chemical synthesis. The report of Smith et al. can be considered as an "enabling technology report," showing that it is possible to find a solid support material that (1) tolerates harsh conditions required for the organic synthesis of peptides and (2) permits diffusion of proteins and minimizes nonspecific binding. An echo of this technology can be seen in Chapters 1, 13, 15, and 16 that relies on heterogeneous assays with immobilized synthetic peptides.

Biological Peptide Libraries

The history of peptide libraries can be conceptually pushed to the mid-twentieth century if one equates biologically synthesized protein libraries to chemically synthesized peptide libraries (note that they are the same chemical structures that differ in origin). Testing of random variations in protein sequences was the basis of molecular biology, and technologies for the production of large protein libraries, such as phage lambda libraries, have been used in the 1960s and 1970s to study the function of proteins. A major technological breakthrough in molecular biology was high-throughput genome sequencing. This technology promised to revolutionize the discovery of peptide-based ligands and materials from peptide libraries. In Chapter 17 we describe application of one of the next-generation gene sequencing technologies (Ion Torrent) to analyze genetically encoded peptide libraries displayed on phage.

Another, biological breakthrough in peptide libraries, which is at the heart of technologies in Chapters 9–12, was made possible in the late 1980s by Smith and coworkers. They started from development of a cloning vector, fd-tet, derived from filamentous bacteriophage [11] and, in 1985, identified a variant of fd-tet that permitted cloning of antigens as a fusion with the coat of M13 bacteriophage [12]. While Smith created low-diversity libraries in 1988 [13], it is the later report by Smith and Scott in 1990 [14] that is often cited as the birthplace of phage display technology. Inspection of the 1988 report highlights the importance of another "enabling technology" behind modern phage display technology. In 1988, Smith reported that: "Libraries with more than $l0^6$ clones are difficult to achieve with any vector that must be introduced into host cells by transfection because of the limited capacity of competent cells" [13]. A mere 2 years later, the discovery of high-efficiency electroporation by William Dower and coworkers [15] put these problems to rest. New transfection techniques allowed Smith to create libraries with unprecedented diversity of 10^9 or 10^{10} peptides and cause "viral" spread of this technology across many research groups in Europe, Canada, and the USA in 1990–1992. An example of the use of electroporation technology for peptide library production can be seen in Chapter 11.

Today, phage display is one of the established technologies for lead discovery of polypeptide-based drugs in the pharmaceutical industry. Brian Kay and coworkers describe one of the examples of the utility of phage display for this purpose via alanine-scanning mutagenesis in Chapter 12. Heinis and coworkers in Chapter 8 describe another example of phage display discovery of pharmaceutically valuable bicyclic peptides. Phage display was an inspiration to a wide range of other display technologies. The optimization of display from a relatively simple phage unit made of 1 DNA and 5 proteins followed two trends: (1) simpler and (2) more complex display unit. The example of "simpler" display is mRNA

display, which has benefits such as immense diversity and resistance to chemical treatment that can kill phage. Hartman and coworkers capitalize on both features and describe mRNA display of chemically modified peptides in Chapter 9. An example of more complex display is that on the surface of cells. Patrick Daugherty and coworkers in Chapter 10 use bacteria display to screen libraries of highly folded, cysteine-rich cyclic peptides. One advantage of such display is the ability to use sorting techniques to select hit peptides with desired potency and even binding kinetics. Another benefit is temporally controlled expression, which allows "hiding" of the displayed peptide during reamplification to avoid the undesired bias in peptide diversity that often plagues phage display [16].

Peptide Libraries on the Chemistry-Biology Interface

Libraries of polypeptides displayed on phage are a validated source of new chemical entities and FDA-approved drugs. In the 2000s, it was recognized that these libraries could also be used as starting material for organic synthesis. If the chemical reaction does not destroy the phage, the synthesis can proceed for any number of steps and yield a product that remains genetically encoded. Peptides displayed on RNA can be used analogously. These peptides with chemical posttranslational modifications (cPTM) can be selected, identified by their DNA tag, or amplified to regenerate the starting materials. This approach is identical to Nature's approach to diversification of proteins by posttranslational modification. Our book describes one of the first diverse collections of protocols from this unique chemistry-biology interface.

Modern examples of cPTM-peptide libraries are often empowered by unnatural amino acid mutagenesis or novel chemical ligation strategies, which permit incorporation of residues with functionalities not encountered in natural proteins. Chapter 2, for example, is a great demonstration that combines both UAA and intein-mediated protein ligation to generate macrocyclic peptides and, potentially, peptide libraries. Development of new bioorthogonal strategies for protein modifications, thus, is the major driver for development of cPTM-peptide libraries. As an example of new modification strategies, Chapter 4 by Raines and coworkers describes a powerful technology used for protein ligation to install side chain modifications that mimic PTM observed in ubiquitin-mediated protein degradation. Chapter 13 by Eldrich and Weiss describe an elegant de novo discovery of chemical reaction for site-specific modification of peptide sequences by hydrazine probes. With the advent of novel techniques for site-specific protein modification, it will be possible to create a wider variety of genetically encoded cPTM-peptide libraries.

On the other hand, not all modern examples of cPTM libraries used modern chemistry or cutting-edge UAA mutagenesis. Chapters 8, 11, and 15 in this book describe examples of chemical modification of libraries made only of 20 natural amino acids. Chapter 8 by Hartman and coworkers describes synthesis of macrocyclic libraries made via classical alkylation of cysteine by bromomethyl benzene linkers. Chapter 11 from our group describes the synthesis of glycosylated libraries using 1970s chemistry that converts N-terminal serine to an N-terminal aldehyde. Chapter 15 by Murray and Santoso describes another method for modification of phage-displayed peptide libraries by alkylation of natural cysteines with maleimide-containing reactive partner. An interesting example of noncovalent PTM library is described by Ghosh and coworkers in Chapter 7. Instead of site-specific covalent modifications, the authors use classical Jun-Fos coiled-coil interaction to tether staurosporine noncovalently to a library of translationally encoded cyclic peptides.

The libraries from Chapters 11 and 18 exhibit all benefits of encoded cPTM libraries, but they are mere logical hybrids of two technologies already in place in the early 1990s. The first half, phage display of peptides, was in place in the early 1990s. The other half, site-specific chemical modification of the *natural* proteins, reached the peak of its development in the late 1980s as well (Robin Offord, personal communication). From this perspective, Chapters 11 and 18 that describe cPTM of natural peptide libraries could be considered as an example of "postmature discovery." This term, coined by Joshua Lederberg and Harriet Zuckerman, describes "discovery [...] deterred by a hindrance [...] prior to publication, most of the other ingredients being in place." Although cPTM-peptide libraries might be considered ~15 years overdue, the development of this concept is rapidly marching forward. Enabling technology from new bio-orthogonal chemistry, new sequencing tools (Chapter 17), and new library construction tools (Chapter 12) fuel this area. The number of publications describing cPTM libraries is rising each year. cPTM technology is already used by a number of startup companies and "big pharma" (Chapter 18), and it holds the promise to become a major pipeline for the discovery of peptide-derived drug candidates.

One of the outstanding contemporary chemists who could have provided a valuable contribution to this book is Carlos Barbas the IIIrd, whom I have invited to contribute to this book in 2013 but who, unfortunately, passed away in 2014 [17]. At the age of 49, Carlos left behind a legacy in many areas that parallel the areas described in this book. Owing to his vast contributions to phage display, site-specific protein modification, genetically encoded discovery of reactions of peptides, and peptide-catalyzed reactions, I would like to dedicate this book to the memory of the colleague, mentor, and friend Carlos Barbas the IIIrd.

Edmonton, AB, Canada *Ratmir Derda*

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Contents

Contributors

Sara Ahadi • *Department of Biochemistry & Molecular Medicine, University of California Davis, Sacramento, CA, USA*

Sahar Ahmed • *Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, AB, Canada; Medicinal Chemistry Department, Faculty of Pharmacy, Assiut University, Assiut, Egypt*

Mohammed Almohaini • *Department of Chemistry and Massey Cancer Center, Virginia Commonwealth University, Richmond, VA, USA*

Aruna Anbazhagan • *Department of Chemistry and Massey Cancer Center, Virginia Commonwealth University, Richmond, VA, USA*

Kristen A. Andersen • *Department of Biochemistry, University of Wisconsin-Madison, Madison, WI, USA*

Lara Anwar • *Department of Biochemistry & Molecular Medicine, University of California Davis, Sacramento, CA, USA*

Sarfuddin Azmi • *Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, AB, Canada*

Fernanda C. Bononi • *Department of Chemistry, The University of Western Ontario, London, ON, Canada; Cancer Research Laboratory Program, London Regional Cancer Program, London Health Sciences Centre, London, ON, Canada*

Carine B. Bourguet • *Département de Chimie de l', Université de Montréal, Montréal, QC, Canada*

Karla Camacho-Soto • *Department of Chemistry and Biochemistry, University of Arizona, Tucson, AZ, USA*

Shiyu Chen • *Ecole Polytechnique Fédérale de Lausanne (EPFL), Institute of Chemical Sciences and Engineering, Lausanne, Switzerland*

Patrick S. Daugherty • *Department of Chemical Engineering, University of California, Santa Barbara, Santa Barbara, CA, USA*

Xiaojun Deng • *Department of Biochemistry & Molecular Medicine, University of California Davis, Sacramento, CA, USA*

Ratmir Derdra • *Department of Chemistry, Alberta Glycomics Centre, University of Alberta, Edmonton, AB, Canada*

Glenn M. Eldridge • *Science and Math Division, West Valley College, Saratoga, CA, USA*

Rudi Fasan • *Department of Chemistry, University of Rochester, Rochester, NY, USA*

Indraneel Ghosh • *Department of Chemistry and Biochemistry, University of Arizona, Tucson, AZ, USA*

David E. Hacker • *Department of Chemistry and Massey Cancer Center, Virginia Commonwealth University, Richmond, VA, USA*

Matthew C.T. Hartman • *Department of Chemistry and Massey Cancer Center, Virginia Commonwealth University, Richmond, VA, USA*

Christian Heinis • *Ecole Polytechnique Fédérale de Lausanne (EPFL), Institute of Chemical Sciences and Engineering, Lausanne, Switzerland*

Jennifer L. Hickey • *Davenport Research Laboratories, Department of Chemistry, University of Toronto, Toronto, ON, Canada*
Barbara Imperiali • *Department of Biology, Massachusetts Institute of Technology, Cambridge, MA, USA; Department of Chemistry, Massachusetts Institute of Technology, Cambridge, MA, USA*
Kamaljit Kaur • *Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, AB, Canada; Chapman University School of Pharmacy, Harry and Diane Rinker Health Science Campus, Chapman University, Irvine, CA, USA*
Brian K. Kay • *Department of Biological Sciences, University of Illinois at Chicago, Chicago, IL, USA*
Malgorzata E. Kokoszka • *Department of Biological Sciences, University of Illinois at Chicago, Chicago, IL, USA*
Pappanaicken Kumaresan • *Department of Pediatrics, University of Texas MD, Anderson Cancer Center, Houston, TX, USA*
Kit S. Lam • *Department of Biochemistry & Molecular Medicine, University of California Davis, Sacramento, CA, USA*
Ruiwu Liu • *Department of Biochemistry & Molecular Medicine, University of California Davis, Sacramento, CA, USA*
William D. Lubell • *Département de Chimie de l', Université de Montréal, Montréal, QC, Canada*
Leonard G. Luyt • *Departments of Oncology, Medical Imaging, Chemistry, The University of Western Ontario, London, ON, Canada; The University of Western Ontario, London Regional Cancer Program, London Health Sciences Centre, London, ON, Canada*
Zhong Ma • *Department of Chemistry and Massey Cancer Center, Virginia Commonwealth University, Richmond, VA, USA*
Langdon J. Martin • *Department of Chemistry and Physics, Warren Wilson College, Asheville, NC, USA*
Wadim L. Matochko • *Department of Chemistry, Alberta Glycomics Centre, University of Alberta, Edmonton, AB, Canada*
Brion W. Murray • *Pfizer Worldwide Research and Development, San Diego, CA, USA*
Simon Ng • *Department of Chemistry, Alberta Glycomics Centre, University of Alberta, Edmonton, AB, Canada*
Christopher J. Noren • *New England Biolabs, Ipswich, MA, USA*
Beth M. Paschal • *New England Biolabs, Ipswich, MA, USA*
Dehua Pei • *Department of Chemistry and Biochemistry, The Ohio State University, Columbus, OH, USA*
Ziqing Qian • *Department of Chemistry and Biochemistry, The Ohio State University, Columbus, OH, USA*
Ronald T. Raines • *Department of Biochemistry, University of Wisconsin-Madison, Madison, WI, USA*
Elizabeth Restituyo • *Department of Chemistry and Biochemistry, University of Arizona, Tucson, AZ, USA*
Buyung Santoso • *Pfizer Worldwide Research and Development, San Diego, CA, USA; Ferring Research Institute, San Diego, CA, USA*
Tsung-Chieh Shih • *Department of Biochemistry & Molecular Medicine, University of California Davis, Sacramento, CA, USA*
Amol V. Shivange • *Department of Chemical Engineering, University of California, Santa Barbara, Santa Barbara, CA, USA*

Jessica M. Smith • *Department of Chemistry, University of Rochester, Rochester, NY, USA*
Rania Soudy • *Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, AB, Canada*
Joanne Tan • *Davenport Research Laboratories, Department of Chemistry, University of Toronto, Toronto, ON, Canada*
Katrina F. Tjhung • *Department of Chemistry, Alberta Glycomics Centre, University of Alberta, Edmonton, AB, Canada*
Punit Upadhyaya • *Department of Chemistry and Biochemistry, The Ohio State University, Columbus, OH, USA*
Gregory A. Weiss • *Department of Chemistry, University of California, Irvine, CA, USA*
Andrei K. Yudin • *Davenport Research Laboratories, Department of Chemistry, University of Toronto, Toronto, ON, Canada*
Serge Zaretsky • *Davenport Research Laboratories, Department of Chemistry, University of Toronto, Toronto, ON, Canada*