Part III
Amino Acids in Combinatorial Synthesis

12
Combinatorial/Library Peptide Synthesis

Michal Lebl

12.1
Introduction

Combinatorial chemistry is a phenomenon that appeared swiftly in the 1990s and in retrospective it is surprising that it took so long to bring it to the arsenal of modern chemists [1] as it seems to be self-evident that compounds can be synthesized in a parallel fashion so much faster and cheaper than at one-at-a-time pace. First came the parallel synthesis of peptides, pioneered by Geysen *et al.* [2] and Houghten [3], enabled by the concept of solid-phase chemistry invented by Merrifield [4–6] and Letsinger and Kornet [7]. Peptides were an obvious choice for the application of parallel synthesis, since their chemistry was well developed, and did not need long reaction times, inert atmosphere, or increased temperature and pressure. Even though solid-phase chemistry was also proposed to be applicable for the synthesis of other types of organic molecules, these early attempts were not taken seriously and were considered as more of a curiosity than mainstream technological progress [8–10]. Leznoff was one of the first to recognize the potential of solid-phase organic reactions and predicted that such reactions would be easy to automate [11]. Today, there are many examples of syntheses that could be achieved only by use of a solid support or in which a solid support acts as convenient "pseudo-diluent" allowing reactions like cyclizations being performed in manageable volumes. The fact that most chemical transformations can be performed in solid phase, sometimes actually more effectively than in solution [12], is now generally accepted and thus it seems quite strange that solid-phase synthesis met such resistance at the very beginning [13–16]. Even in the peptide arena, a number of established laboratories resisted the transition to solid-phase technology for decades. Only the successes of numerous applications in both research laboratories and industrial processes, at scales ranging from milligrams to kilograms and more (tons of the pharmaceutically relevant peptide Fuzeon® were produced using solid-phase synthesis by Roche [17]), convinced the skeptics that solid phase is the technology of the future.

Solid-phase chemistry has been extensively reviewed and only some of most relevant articles can be mentioned here [18]. Solid-phase peptide synthesis was

covered in detail in the most authoritative book – *Houben–Weyl Methods of Organic Chemistry* [19]. More personal stories of the development of various types of solid-phase materials are available in the comprehensive assembly of recollections of scientists developing these supports [20, 21].

The term combinatorial, or library, synthesis was coined only after two seminal papers were published in 1991. The fact that the time was right for the arrival of a new technique was evidenced by the appearance of these two papers in the same issue of *Nature*. Houghten *et al.*'s paper [22] described the generation of peptide mixtures, which after biological testing could be deconvoluted to identify the active component of the mixture. Lam *et al.*'s paper [23] presented the generation of millions of beads each containing only one type of peptide. This mixture of beads could be tested for binding to the biomolecule (receptor, antibody, enzyme), and beads with ligands showing binding could be isolated and the structure of the peptide determined by sequencing. Both of these papers used the technique called "split and mix," or "divide, couple, and recombine," which was actually published earlier by Furka *et al.* [24, 25]. Pioneers of combinatorial chemistry were asked later to write their recollections of the history of discovery of their techniques, and their memories were collected [1] in the inaugural article of the new journal dedicated to combinatorial chemistry, appropriately named the *Journal of Combinatorial Chemistry*. Combinatorial synthesis developed far beyond peptides and it is now very difficult to find a class of molecules that is not subjected to a certain degree of combinatorial technology. This chapter is dedicated to chemically synthesized peptide libraries (biological libraries are covered in another chapter), but most of the principles discussed are applicable to any chemical entities. Earlier reviews and discussions of synthetic concepts can be found elsewhere [13–16, 26–56].

12.2
High-Throughput Synthesis of Peptides

12.2.1
Parallel Peptide Synthesis

The idea that the effectiveness of peptide synthesis could be enhanced by automation [57, 58] or by synthesizing more than one peptide at a time was around since the introduction of solid-phase synthesis, but it required prepared minds to make a quantum leap from just several parallel reactions to several hundred reactions. Three papers can be traced to the beginning of massively parallel synthetic approaches – Frank's synthesis on paper circles [59], Houghten's technique of tea-bag synthesis [3], and Geysen's pin synthesis [60].

Ronald Frank used marked pieces of cellulose paper as a substrate for DNA synthesis [59] (later extended into synthesis of peptides [61]), and showed that separation of segments requiring the coupling of the same building block into the same pool (reagent vessel) and resorting the segments before the next step of the synthesis increases the throughput of the synthesis substantially.

Houghten's approach [3] is based on the same concept of compartmentalizing the synthetic substrate (polystyrene resin beads). Beads are contained in polypropylene mesh bags, which could be individually labeled and would survive the synthetic sequence without losing the encapsulated solid carrier. This so-called tea-bag synthetic technology [62, 63] was the basis of formation of a company Multiple Peptide Synthesis and was used for the synthesis of peptides in scales reaching from milligrams to multigrams. Automation of the tea-bag technology proved difficult in the version requiring multiple resorting of hundreds of polypropylene containers. In the case that only a limited number of tea-bags are used, an automated synthesizer utilizing centrifugation for liquid removal was built [64, 65].

Geysen's pin synthesis utilizes plastic pins covered with a functionalized poly-styrene layer arrayed in a grid following the pattern of 96-well microtiter plates. Simply dipping these pins into the wells with appropriate activated building blocks (amino acids) allows for simultaneous parallel synthesis. The material, shape, and functionalization of the pins went through multiple generations [66–81]. The optimized version of the multipin technology uses SynPhase™ "lanterns" or "crowns" – polymeric barrels in a shape of miniature Chinese lanterns or crowns [82, 83] (Figure 12.1). The lanterns are constructed from inert polymer core material in a barrel shape, onto which the functionalized "synthetic" layer is grafted. This geometry allows for the attachment of individual lanterns (one or more per pin) to the pin holders for individualized synthetic steps or combining all lanterns for common synthetic steps. Lanterns can be color-coded or labeled by attachment of radiofrequency transmitters. Supplies for multipin synthesis are available from the company Mimotopes (www.mimotopes.com). For a compilation of papers using SynPhase products, see http://www.mimotopes.com/files/editor_upload/File/CombinatorialChemistry/SynPhase_Publications.pdf.

A convenient and general method of peptide cleavage from the solid support is the use of gaseous ammonia. Since the ammonia treatment leaves the polymer support dry upon cleavage, further extraction of the peptide is necessary. This allows for the cleavage to be performed in parallel/batch fashion and to sort the polymer (lanterns,
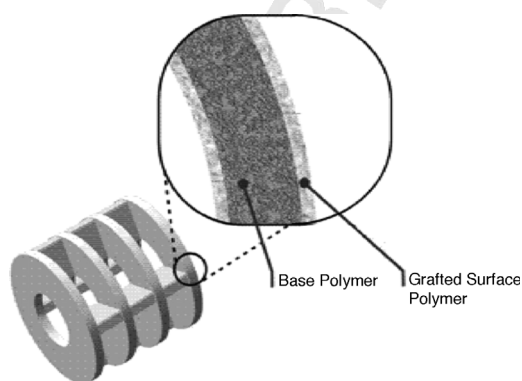

Base Polymer   Grafted Surface Polymer

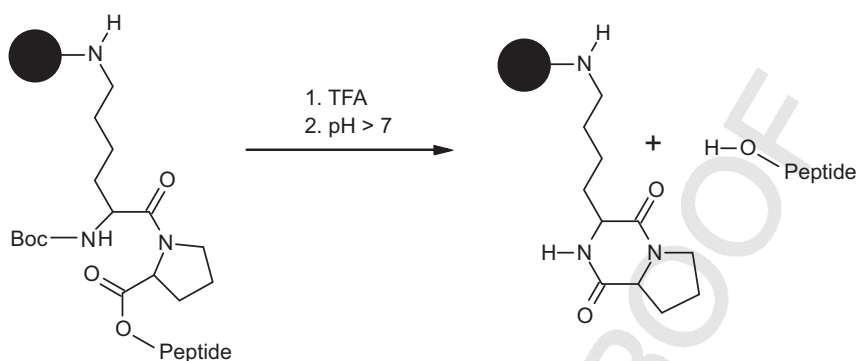**Figure 12.1**   Structure of the SynPhase lantern.

Figure 12.2 Scheme of the diketopiperazine linker release [84].

sheets, beads) by identity of the peptide later in the dry state followed by the peptide's release into a plate/vial by addition of a solvent. This ammonia release and dry-sorting technique was originally utilized in pin synthesis using the diketopiperazine linker [84] (Figure 12.2), but works well even with a simple benzyl ester linkage.

A number of papers were dedicated to the parallel synthesis in specialized synthetic blocks, where activated amino acids are delivered into individual locations by pipetting (manual or automated), and common reagents are delivered by multichannel liquid distributors and removed by filtration. Reactors in these blocks are equipped with a frit at the bottom, and utilize various techniques for simultaneous closing and opening of the exit ports (overpressure, individual plugs, membranes, squeezed tubing, etc.). These techniques and available instruments were extensively reviewed by us in 2005 [51], and therefore only new instruments and some nonmainstream techniques will be discussed here. The list of available instrumentation is kept updated at http://www.peptideresource.com/synthesizer.html.

Thuramed (a division of Creosalus; thuramed.com) introduced an elegant solution to the multiple peptide synthesizer. Their machine, Tetras™, is capable of 106 parallel syntheses, where each reactor can be operating at a different scale and using a different synthetic protocol, thus making the claim of being the only asynchronous multiple synthesizer true. Reactor cartridges are placed on the perimeter of the rotor allowing positioning of each reactor under a dedicated nozzle delivering reagents or washing solvents. This design eliminates the danger of cross-contamination and wastage of reagents needed for flushing the common lines. Reagent nozzles can be supplied from a local vessel of very small volume, minimizing the need for using larger than necessary volumes of expensive components, or from the tanks of solvents placed in a dedicated off-machine location.

The availability of solvent-resistant 96-well or even 384-well microtiter filter plates allowed for techniques in which the reactor (filter plate) was disposable. However, for institutions and companies interested in the synthesis of tens of thousands of individual peptides, even the cost of these filter plates was carefully evaluated and more economical techniques using standard deep-well microtiter plates were conceived. One of these techniques uses "surface suction" for removal of the reagent

solutions [85, 86]. To be able to use this technique, the solid support suspended in the solution must sediment when the stirring (shaking, magnetic or mechanical stirring, bubbling) is stopped. When the array of wide-mouth suction tubes is slowly lowered towards the surface of the liquid, only the surface layer is taken away by suction and the tube can be lowered close to the level of sedimented solid support without disturbing it. A simple method for the uniform distribution of solid support into the wells of microtiter plates was described [87].

Tilted centrifugation is another technique for liquid removal using classical microtiter plates and sedimenting solid support [88, 89]. Again, the solid support must sediment at the end of the reaction prior to the solution removal. Synthetic plates are placed on the perimeter of the centrifuge and tilted slightly towards the center of rotation. Rotation creates a "pocket" of the given volume, depending on the distance from the center of rotation, speed of rotation, and the plate tilt, from which the solid support cannot escape. This technique can process multitudes of plates at the same time extremely fast and was a basis of several automated solid-phase synthesizers [51, 90, 91]. This technology was tested in the synthesis of tens of thousands peptides for finding the optimal coupling reagent in multiple synthesis (surprisingly, good old N,N'-diisopropylcarbodiimide (DIC) was found to be the best due to its stability in solution and performance in situations where the speed of condensation is not the most desired feature [90]) and replacement for regulated piperidine by 4-methylpiperidine [92]. A simple synthesizer based on the same principle was built to process 24 peptides in a plastic disposable rotor [93] (Figure 12.3).
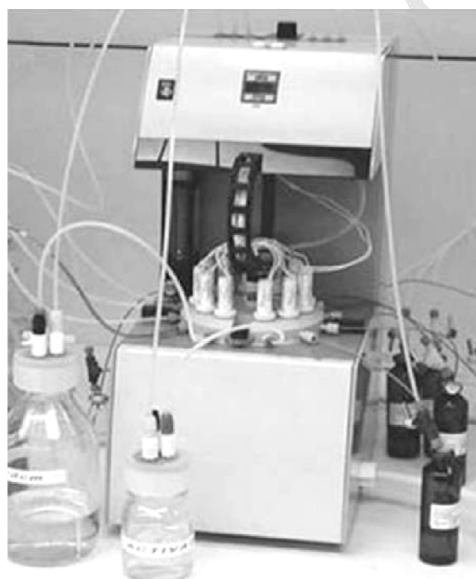

Figure 12.3 Simple centrifugal DNA and peptide synthesizer [93].

Any technique that removes liquids in parallel, but delivers solutions in a series is bound to be less efficient than a technique with balanced solution addition and removal. An ideal process of synthesis performs all steps of the synthesis at the same time at the different sections of the synthetic substrate (machine), as shown, for example, in the synthesis utilizing a cotton strip as the solid support [94]. A high-throughput synthesizer was realized for parallel synthesis of 12 000 DNA sequences by a San Diego company, but can be easily imagined to be built for peptide synthesis. This synthesizer has 36 384-well filter plates arranged in a circular fashion, moving in 10-s increments under stations dedicated to perform one step of the synthesis (addition of building blocks, activation, washing, deprotection) so that each plate is in a different stage of the individual cycle of the synthesis at the same time. After one round of passage around the circle, one building block is attached to the growing polymer in all wells of the plate and the process continues with another cycle. Reagents are delivered from pressurized containers by actuation of an array of solenoid valves as they pass above the wells of the microtiter plate [95].

The parallel deprotection and cleavage of peptides from the solid support is as important as the parallel synthesis. Gaseous HF was found to be a convenient reagent for processing multiple reactors [96] or even microtiter plates [97, 98] simultaneously. The ultimate convenience of processing the solid supported synthetic product is treatment of the silica gel support with HF. Silica gel dissolves into SiF_4 and evaporates, leaving behind only the deprotected peptide [99, 100].

12.2.2
Directed Sorting

The previously mentioned tea-bag technology or paper disk synthesis are examples of synthetic methodologies utilizing directed sorting – reorganization of the synthetic compartments in such a way that in each step of the synthesis the compartments requiring the attachment of the same building block (amino acid) can be combined and the reaction can be performed in the same reaction vessel. This requires either some means of labeling the individual container, or keeping the containers in an order, so that at any moment the identity of the individual container can be established.

Labeling was achieved by simply writing the code on the tea-bag [3], including a radiofrequency tag [101, 102], or a one- or two-dimensional barcode. Radiofrequency tags or barcodes allow for automating the sorting process [103, 104]. Exciting technologies can be designed using barcodes miniaturized to a level that each particle of the solid support has its own "barcode" [105–109]; unfortunately, at this level of miniaturization, the cost of the individual particles and the need for specialized equipment used for their reading may be prohibitive for widespread application.

Two-dimensional barcode labels (MiniKan™, MicroKan™, or NanoKan™) became commercially available from the company IRORI (later Discovery Partners International) and, together with the sorter system capable of sorting tens of thousands of cans in a reasonable timeframe, can make synthesis of multimilligram quantities of

tens of thousands of peptides quite feasible [110, 111]. However, alternative (and less-expensive) technologies are available for peptide synthesis and NanoKans are used in a majority of cases for parallel synthesis of small molecules. A very impressive automation was built around these solid-phase carriers [112].

Krchňák *et al.* used plastic syringes equipped with a frit for resin compartmentalization [113–116]. This manual technique was later automated and a robot handling plastic syringes was built [117]. Manual handling of multiple syringes can be also simplified by using so called Domino Blocks [118], available commercially (www.torviq.com).

A promising way of compartmentalization of the solid support is the sintering of its particles in the mixture with an inert polymer into so called "plugs" [119]. These plugs were commercially available from Polymer Laboratories (now part of Agilent Technologies).

Compartmentalization similar to tea-bags was also achieved in porous wafers made from a Teflon® ring covered on both sides by a porous Teflon membrane to form a cylinder-shaped permeable container [120]. Wafers became the basis for building a solid-phase DNA synthesizer and due to their proprietary character never made it to mainstream peptide synthesis, even though they seem to be an ideal tool for multiple synthesis. Krchňák *et al.* later designed "resin capsules" constructed from a polypropylene ring sealed with a polyetheretherketone membrane [121]. These capsules can be easily made using common tools in the chemical laboratory and sized appropriately for the synthetic vessels (plastic syringes), thus optimizing the use of reagents (one disadvantage of tea-bags is the excessive use of reagent solutions).

Convenient containers/particles for directed sorting are SynPhase lanterns mentioned earlier. They can be equipped with radiofrequency tags, or they can be arranged on pins or strings, putting them in ordered arrays. If lanterns are transferred according to a defined algorithm from one array to another without any errors, they thus make assemblies ("necklaces") that could then be used for coupling the next amino acid. The identity of each particle (lantern) can be established at the end of the synthesis by the position on a particular necklace (pin). Techniques utilizing these concepts were pursued by Krchňák *et al.* [122, 123] and later also by Furka *et al.* [124–126]. The ENCORE system [122, 127], originally developed for organic combinatorial synthesis, but easily applicable for multiple peptide synthesis, utilizes lanterns attached to pins and dedicated tools to arrange them efficiently into particular reactors.

Solid carriers capable of convenient compartmentalization and therefore parallel multiple synthesis include functionalized polymeric sheets and disks [128, 129], membranes [130], paper, cellulose [61, 131], cotton [132, 133], or functionalized acrylate-grafted polypropylene fabric [134]. Some of them will be mentioned later since they act as the support of choice for array syntheses. The case close to the author's heart is the case of multiple synthesis on cotton. Cotton is the most inexpensive solid support that, due to its polysaccharide structure, allows efficient synthesis of difficult sequences [132, 135], can be easily compartmentalized, and automated multiple [64, 65] or continuous [94] synthesizers can be designed for its use. Solid-phase synthesis on planar supports was reviewed earlier [136].

On the borderline between random library synthesis and synthesis using directed sorting is the strategy using dividable material (fabric, paper, sheets), where the material is divided after each coupling step utilizing different building blocks. This technique does not end up with a coded individual piece of the solid support and positively responding pieces must still be decoded, but it guarantees that all components of the library are synthesized and none of them is synthesized in more than one copy [137, 138].

12.3
Synthesis of Peptide Arrays

For a multitude of applications the availability of individual peptides for testing in solutions is not necessary. For example, the evaluation of binding to a biologically relevant target can be performed with the peptide still immobilized on the support. Realization of high-throughput screening can be then easily implemented on "chips" containing hundreds to hundreds of thousands individual peptides on their surface [139–143].

The first arrays of peptides were prepared on planar cellulose paper sheets by Ronald Frank [144]. The activated paper was spotted (the reason for calling this technique SPOT synthesis) with solutions of Fmoc-protected activated amino acids. After attachment of the first building block, the remaining free functional groups on the surface were acetylated (capped), thus creating an array of available synthetic locations. The Fmoc group was removed by immersing the paper sheets in a piperidine solution and after washing the free amino group-containing spots were revealed by application of bromophenol blue solution [145, 146]. The synthesis was continued by repeated cycles of delivering activated amino acids to appropriate locations, incubation, and deprotection by the immersion into the deprotecting solution. The building block delivery process, originally manual pipetting, was automated to eliminate the potential for operator error and became the flagship technology for the company Jerini Peptide Technologies (www.jpt.com). SPOT synthesis and its applications were reviewed in detail elsewhere [147–159].

The pioneering approach to the synthesis of peptides on chips was published by Fodor [160–167] and became the basis for the formation of Affymax (www.affymax.com). Later the company (in its Affymetrix incarnation; www.affymetrix.com) concentrated on production of DNA chips for genotyping and gene profiling [168, 169].

Fodor's technology [160], based on photolithography, is illustrated in Figure 12.4. The surface of the synthetic substrate protected by a photocleavable protecting group is deprotected in defined regions by light irradiation through the mask. The chip is then flooded with the activated amino acid, which couples on the deprotected regions of the surface. After the completed coupling, the activated amino acid is washed away, the next set of regions is deprotected by light, and the next amino acid is coupled to the deprotected areas. This process is repeated until all amino acids needed for the first coupling are applied. Then the amino acids for the second cycle of peptide synthesis
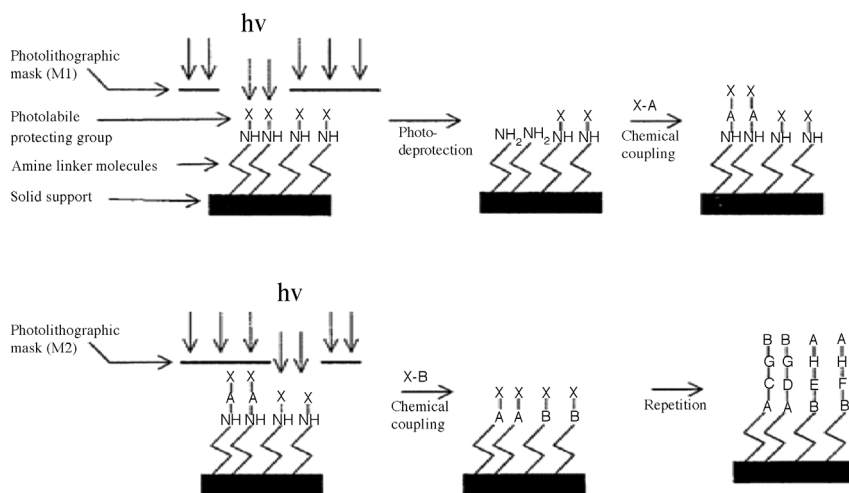
Figure 12.4 Scheme of photolithographic peptide synthesis. Irradiation of the surface covered with the growing peptides protected on the N-terminus by photocleavable protecting groups through the mask creates a patterned surface with deprotected locations onto which the next amino acid can be coupled. A mask is then repositioned to allow deprotection of the next set of locations.

are attached in the same way and the process is repeated until the peptides of desired sequence are built on all chip locations. The obvious disadvantage of this process is the multitude of repetitions necessary for each step of the synthesis and the necessity to apply activated solutions across the whole surface, even though only a fraction of it is actually engaged in the reaction extending the peptide chain. Each step of the process also requires a dedicated photo mask. The mask issue was later removed by the application of the actuated micromirror chip for selective irradiation of the synthetic chip surface [170]. This technology led to the formation of yet another company, Nimblegen (www.nimblegen.com), which is, however, totally dedicated to the synthesis of DNA arrays.

Their Maskless Array Synthesizer (MAS) technology instrument is comprised of a maskless light projector, a reaction chamber, and a fluidic unit. The Digital Micromirror Device (DMD), a solid-state array of miniature aluminum mirrors, is able to pattern 786 000 to 4.2 million individual pixels of light. These "virtual masks" reflect the desired pattern of UV light onto the microscope slide in the reaction chamber, which is coupled to the synthesizer. The UV light selectively cleaves a UV-labile protecting group at the precise location where the next building block will be coupled. A maskless photolithography peptide synthesizer was also reported recently by Korean scientists [171].

An interesting alternative to the approach described above is based on photolytic generation of an acid at a specific location of the chip. This *in situ* generated acid then deprotects the Boc-protected peptide fragment to allow the next coupling step [172–175]. Therefore, the site of the reaction is also addressed by light, but in

contrast to photosensitive protecting groups, conventional Boc chemistry building blocks can be used, making this approach cheaper. Since the Boc synthetic strategy is used, generation of a relatively strong acid is required. It is critical to ensure that the generated acid will not diffuse into the neighboring location and deprotect unwanted loci. This was achieved by using the polymeric coating which slowed the diffusion rate of the acid on a silicon-based microfluidics chip manufactured by Applied MEMS that contains either 4000 or 8000 chambers on an area of little more than $1\,cm^2$. Each chamber serves as an independent reaction vial to synthesize a specific type of oligonucleotide or peptide. In addition to linear peptides, photolithography was used, for example, for the synthesis of arrays of peptoids and cyclic peptides [176, 177].

A similar in concept is strategy employed by Maurer *et al.* [178], who generated deprotecting acid (for removal of the Boc group in peptide synthesis) by application of an electric current on an array of electrodes analogously to Egeland and Southern [179], who developed this technique for DNA array synthesis. An acid was generated by applying $+3\,V$ onto an array of platinum electrodes in a solution of diphenylhydrazine. Diffusion of the acid was eliminated by the fact that diphenylhydrazine is a weak base, which neutralized the acid everywhere besides the closest proximity to the active electrodes. Even though this technology was developed for peptide synthesis, it became the basis for DNA chip synthesis in the company Combimatrix [180].

All these technologies require repetition of each coupling step n times, where n is the number of amino acids needed for coupling in that particular step, and result in a very slow process.

A revolutionary approach to peptide arrays is being developed by the company PepPerPrint in Heidelberg, Germany [181]. They have developed glass slides modified with a multifunctional poly(ethylene glycol) (PEG)-based polymer. PEG films starting from self-assembled alkyl silane monolayers via monolayer peroxidation and subsequent graft polymerization of PEG methacrylate (PEGMA) were optimized for substitution and film thickness. The novel support material allows a versatile modification of the amino group surface density up to $40\,nmol/cm^2$. The polymer coating is stable to a wide range of chemical and thermal conditions, and prevents the glass surface from unspecific protein adsorption [182, 183].

Their first method of synthesis on microelectronic chips utilizes solid amino acid microparticles charged by friction and transferred to defined pixel electrodes onto the chip's surface, where they couple to a functional polymer coating simply upon melting.

The building blocks are distributed to a location on the glass substrate in the form of a specially formulated toner by electrostatic deposition analogously to printing by a "Xerox®" machine. This "printer," however, has 30 or more ink cartridges. Toner is created by encapsulating Fmoc-protected pentafluorophenylesters in diphenylformamide. The diphenylformamide is solid at room temperature and the micron-size beads of activated amino acids can be stored for months without losing activity. After distribution of amino acids to the locations on the slide, the "dust" particles
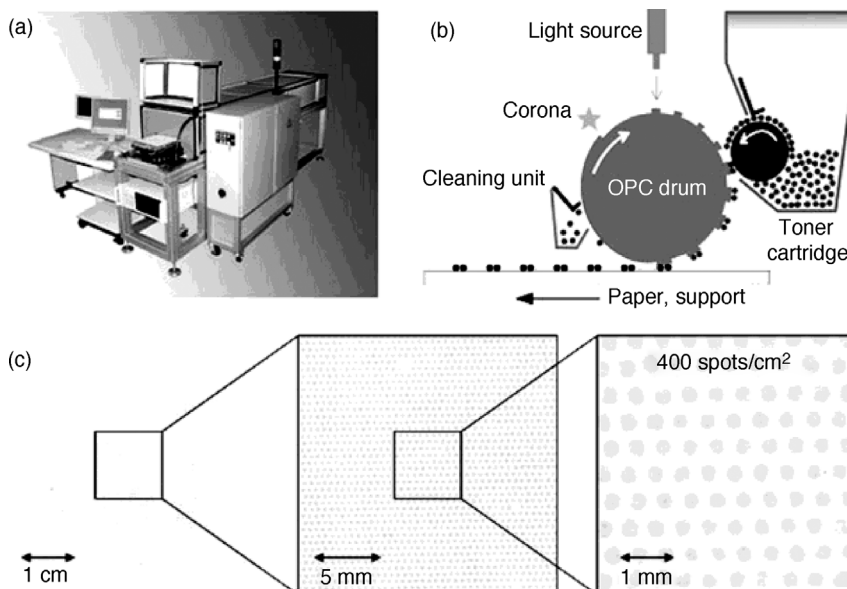
Figure 12.5 Laser printing-based peptide synthesizer. (a) Peptide laser printer with 20 different printing units aligned; the mounting for the support is visible at the front of the printer. (b) A light source (LED row) illuminates and thereby neutralizes selected areas of an organic photoconductor drum, which is first uniformly charged by a corona. Triboelectrically charged toner particles are transferred to these neutralized areas and from there by a strong electric field to a solid support. (c) Amino acid toner was printed by the peptide laser printer onto a glass slide derivatized with free amino groups. The active pentafluorophenyl esters embedded in the particles were released by heat, the residual material was washed away with DMF, and the remaining free amino groups were blocked with 10% acetic anhydride in DMF. Finally, the Fmoc protecting groups were removed with 20% piperidine in DMF and the newly introduced free amino groups were stained with 0.1% bromophenol blue in methanol. (Modified from [184].)

are melted at elevated temperature and the coupling of pentafluorophenyl ester proceeds. After coupling completion, the glass slide substrate is transferred to the compartment in which washing and deprotection is performed. By applying standard Fmoc chemistry, peptide array densities of up to 40 000 spots/cm^2 were prepared [185]. The scheme of the synthesizer is illustrated in Figure 12.5.

This method solves the problem of technologies using photo- or electro-deprotection (lithography) due to the monomer-by-monomer repeated consecutive coupling of 20 different amino acids, which adds up to an excessive number of coupling cycles. Synthesis based on electrically charged solid amino acid particles is more efficient. A laser printer or a chip addresses the different charged particles consecutively to a solid support, where, when completed, the whole layer of solid amino acid particles is melted at once. This allows coupling of all 20 different amino acids to the support in one single coupling reaction [181, 186–188].

12.4
Peptide Libraries

12.4.1
Synthesis of Peptide Mixtures

Furka realized that by the repetitive process of coupling, mixing, and recombination of the solid support resin one can create almost equimolar mixtures of peptides [24, 25, 189–191]. The same conclusion was reached independently by Houghten, and his group has shown that keeping portions of the resin support separated after the last two couplings generates mixtures differing by only two amino acids in their sequence, and allows one to test these mixtures in any biological test and define whether the defined positions play a significant part in the biological activity of those peptides [22, 27, 192–205]. In the next step, the second generation of peptide mixtures is generated, this time defining the next position of the sequence. Repetition of this process defines, by using simpler and simpler mixtures, the most active sequence from original mixtures. This technique was used for identification of antibody epitopes and epitope mapping, enzyme substrate and inhibitor discovery, receptor ligand elucidation, or identification of substances responsible for physiological effects in *in vivo* tests [206–208].

Instead of the split-and-mix technique for the generation of equimolar mixtures of peptides by coupling in "mixed positions," equikinetic mixtures of amino acids can be introduced [209] making the synthesis faster and more effective. In this method, relative reactivities of building blocks are determined first and then amino acids are mixed in such a ratio that speed of all possible extension reactions is the same (normalized). Example of ratios of amino acids used in coupling with DIC [210] and pentafluorophenyl esters [211] can be found in Table 12.1. This ratio has to be established empirically not just for different amino acids, but for different side-chain protecting groups as well. However, it was shown in a detailed study that since the equikinetic mixture cannot take into account the character of the amino acid to be extended, a significant portion of the sequence space can be under-represented in the synthesized library [212].

An alternative procedure to incorporate all amino acids in an equimolar ratio is based on coupling of a slightly subequivalent amount of a mixture containing the same amount of all amino acids, followed by the second coupling with an excess of the same mixture [213, 214]. The first coupling step guarantees that no amino acid is incorporated preferentially and the second step ensures completeness of the coupling.

Positional scanning libraries were later invented by Houghten's laboratory as an alternative synthetic and screening technique [215–222]. In this version of mixture screening, the libraries in which all positions are defined are synthesized and screened, and ligands deduced from the most active mixtures are synthesized and their activities tested. An illustrative (imaginary) example of deduction of the active sequence by this technique is given in Figure 12.6. Eighty tetrapeptide mixtures of 8000 peptides (composed of all 20 natural amino acids) were prepared with all 20

Table 12.1 Composition of amino acids mixture for coupling in equimolar quantities (isokinetic ratio).

Amino acid	Molar ratio	Amino acid	Molar ratio
Boc-Ala	1.18	Fmoc-Ala-OPfp	1.20
Boc-Arg(Tos)	2.26		
Boc-Asn	1.86	Fmoc-Asn-OPfp	5.05
Boc-Asp(OBzl)	1.22	Fmoc-Asp(OBut)-OPfp	1.00
Boc-Gln	1.85	Fmoc-Gln(Trt)-OPfp	1.66
Boc-Glu(OBzl)	1.26	Fmoc-Glu(OBut)-OPfp	1.35
Boc-Gly	1.00	Fmoc-Gly-OPfp	1.11
Boc-His(Dnp)	1.24	Fmoc-His(Trt)-OPfp	2.49
Boc-Ile	6.02	Fmoc-Ile-OPfp	13.01
Boc-Lys(2-Cl-Z)	2.16	Fmoc-Lys(Boc)-OPfp	1.84
Boc-Leu	1.72	Fmoc-Leu-OPfp	1.39
Boc-Met(O)	0.80		
Boc-Phe	0.88	Fmoc-Phe-OPfp	1.15
Boc-Pro	1.50	Fmoc-Pro-OPfp	2.00
Boc-Ser(Bzl)	0.97		
Boc-Thr(Bzl)	1.66		
Boc-Trp(For)	1.32	Fmoc-Trp(Boc)-OPfp	1.74
Boc-Tyr(2-Br-Z)	1.44	Fmoc-Tyr(But)-OPfp	1.22
Boc-Val	3.91	Fmoc-Val-OPfp	9.62

The ratio depends strongly on the protecting groups and the activation procedure used (left column, DIC activation [210]; right column, coupling of Pfp esters [211]).

amino acids defined in each position of the peptide chain. In this case these 80 mixtures represented 32 000 individual peptides. The most active mixture for the defined position 1 was the one containing methionine as the defined amino acid. The most active mixtures with the defined position 2 were those ones with isoleucine and alanine, position 3 has shown significantly enhanced binding activity for asparagine and arginine, and position 4 has shown a strong preference for aspartic acid and serine. To retest these sequences in solution and identify the most active one, it would be necessary to resynthesize only those eight peptides representing the combination of all selected possibilities. If the same exercise would be performed with an iterative technique, it would be necessary to synthesize new libraries after each consecutive screening step (seven libraries; 140 mixtures in total) and these iterative libraries would be probably useless in screening other targets. Positional screening libraries are generic and can be used against a plethora of targets. The disadvantage of iterative scanning is that the amino acid residues defined at the beginning do not have to be the optimal and all subsequently synthesized libraries are biased towards these earlier defined parts of the sequence. The positional scanning library technique was successfully used to define the consensus peptide structures in a multitude of studies with libraries representing up to 10^{12} peptides, the screening of which would be impossible by the application of other techniques.

Iterative

Positional scanning

OXXX=AXXX, DXXX, EXXX, FXXX, GXXX, ..., ..., YXXX (O = defined position, X = mixed position, other letter = individual amino acid)
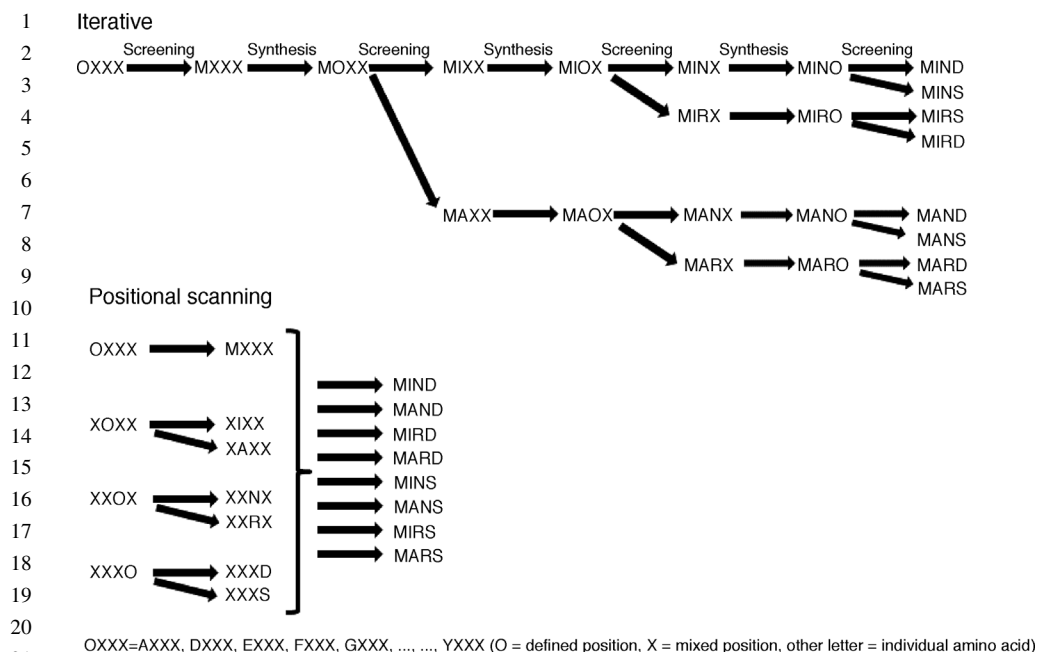
Figure 12.6 Comparison of iterative and positional scanning library techniques. (Modified from [49].)

The structure of positional scanning libraries is not limited to linear peptide sequences. Cyclic as well as scaffolded libraries with randomized "arms" attached to a semirigid scaffold based on a bicyclic peptide template were successfully synthesized and screened [197, 223, 224].

Deconvolution of so-called "omission libraries" (libraries with defined amino acid missing instead of fixed in a given position) was much less successful and did not find wide application [225].

In so-called "orthogonal" libraries used by Deprez et al. [226] each library member is present in two (or three) different mixtures and any two (or three) mixtures of the library have one, and only one, peptide in common. Consequently, the testing of these libraries should show activity of the mixtures containing the active compound in each sublibrary. If only one compound in the library is active, the identification of this compound by "mental deconvolution" is simple. However, if activities are observed in a number of sublibraries, the identification of the active components may be excessively complicated.

The concept of creating the orthogonal mixture can be explained on mixing the compounds synthesized in 100, 96-well microtiter plates. If aliquots are taken from all wells in a plate, 100 "plate mixes" of 96 compounds each can be created. Then aliquots are taken from all A1 wells of all plates and this create 96 "well mixes" of 100 compounds each. If the activity is found in only one plate and one well mix, then the active compound can be determined by testing 196 mixtures instead of testing 9600

individual peptides. "Column, row, diagonal, ..." mixes can also be created to confirm the finding. "Orthogonal" libraries by Deprez et al. are, of course, synthesized as mixtures and not created by mixing the individual compounds [226].

Mixture-based peptide libraries were also used as a basis for transformation into nonpeptidic structures by global modifications – the most notorious example being the creation of "libraries from libraries" in which the peptides were reduced into polyamines or polyalkylated [227–230].

Results achieved in Houghten's laboratories utilizing libraries of peptide mixtures can be found at http://www.tpims.org/scientists_richard-houghten.asp. An application of mixture libraries was comprehensively reviewed by Eichler [49].

Even though most of the libraries were synthesized on the solid phase, the technique using a soluble carrier can be used as well [231].

Other types of soluble peptide libraries were prepared by individual coupling of peptides with DNA [232, 233] or peptide nucleic acid [234] tags. After biotransformation of the peptide library in the solution assay (cleavage by protease, phosphorylation by kinase), the modified substrate was captured on a complementary DNA array and the structure of the substrate peptide was defined.

12.4.2
Synthesis of Peptides on a Mixture of Particles

Furka et al. [24] synthesized libraries in 1988, but he did not recognize the fact that each individual bead resulting from the split-and-mix synthesis contains just one peptide sequence. A fraction of the resin in every step is exposed only to one amino acid and therefore only one peptide sequence is being built on each bead (Figure 12.7). After mixing all beads together the identity of the beads is lost, but after another separation into individual reactors, again, only one amino acid can be attached to the growing chain. This fact did not escape the prepared mind of Kit Lam [23], who came up with the split-and-mix synthetic scheme to create mixtures of beads containing multiple copies of the individual sequences (one-bead–one-compound (OBOC) technology). The synthesized peptides, still attached to the beads, can be then submitted to binding studies and after isolation of beads expressing binding (observed by color, fluorescence, radioactivity, or detailed observation of cells sticking to the bead surface), the structure of active peptides can be elucidated by Edman degradation, mass spectrometry (MS), or reading the code built in during the library construction. Even though the OBOC dedicated solid-phase library synthesizer was constructed [235, 236], owing to the fact that the library design varies significantly in a number of randomized positions and used building blocks, these libraries are usually prepared by manual synthesis.

Since the application of these OBOC libraries was reviewed [23, 236–251] on a number of occasions and in a multitude of journals and monographs, this chapter will not go into the individual results achieved with OBOC libraries (as listed, for example, in [252]), but rather concentrate on the various techniques applicable for their production and testing. Results achieved by Lam's laboratory, which remains
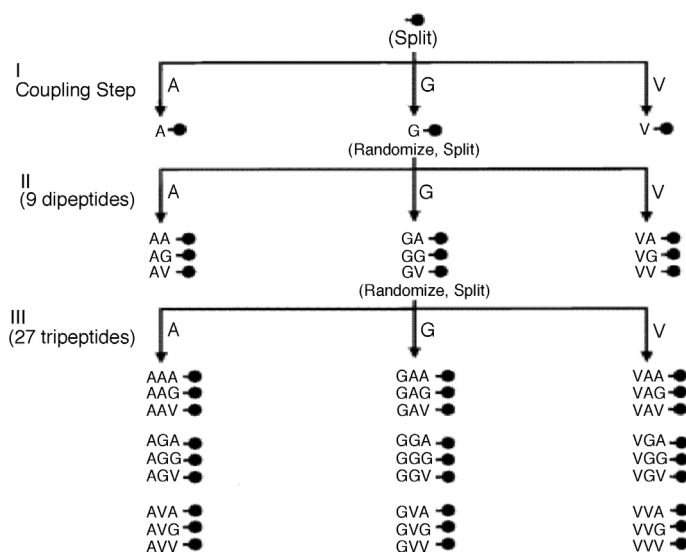
Figure 12.7 Principle of split-and-mix combinatorial synthesis [23]. A batch of beads (e.g., 1 000 000 beads) is separated into three aliquots and each aliquot is coupled with a different amino acid. Beads are mixed together and the process is repeated twice. At the end of this tripeptide synthesis, there are 27 populations of approximately 37 000 beads each with a unique sequence. Obviously, if we would try to synthesize a hexapeptide using all 20 amino acid in each coupling, we would prepare only 1.5% of possible structures with just 1 000 000 beads (64 000 000 possible sequences).

very active in developing OBOC technology and applying it especially in the area of cancer research, are available at http://oboc.ucdavis.edu/html/publications.htm.

In almost 20 years since the invention of one-bead–one-peptide technology [23], a great effort by numerous laboratories has been dedicated to eliminating its drawbacks, and making it more efficient and reliable. The greatest problem is the fact that screening is performed on the surface of the polymeric bead and not in solution (this issue will be discussed later), and that the generated signal representing the binding of the target to the bead does not correlate with the affinity of the ligand on the bead surface. First, the unspecific binding due to interaction with components of the detection system, or any unspecific protein, must be excluded, such as by using the dual-color screening system [253], or by scanning immobilized beads treated by two different labeled proteins or protein mixtures consecutively. By subtracting the two optical images it is possible to define beads binding uniquely to only one protein mixture and not to proteins common to both mixtures or to chemical and protein components of the assay itself [254].

Indeed, a ligand on a surface may have different binding properties. The resins typically used for library synthesis have a high loading capacity (e.g., 90-μm TentaGel™ resin with a loading capacity of 0.3 mmol/g has a ligand density of around 100 mM). This high loading is, however, only necessary for subsequent hit identification and not for actual biomolecule binding. High ligand density may

contribute to undesired binding of a target molecule to low-affinity ligands (due to high local ligand concentrations) and may also result in unintended multidentate interactions with the target leading to false-positives. As a result of this known fact, it is common to resynthesize and test the initial hits individually in solution. Since screening of a large library can produce hundreds of hits or more, it is not uncommon (due to the expense of sequencing and resynthesis) to define the structure and resynthesize only a fraction of "hits," potentially missing the best compound.

An alternative to the sole bead binding evaluation is the technique of "bead blot." The library of beads is incubated with the protein or protein mixture and protein-loaded beads are immobilized on a porous surface. Proteins bound to beads are then directionally eluted onto the surface and captured on a membrane superimposed on the beads. The location of the target protein is then defined by specific immunostaining and the bead responsible for its binding can be recovered for structure evaluation. This method allows evaluation of binding to a multitude of proteins contained in the original incubating mixture for which a selective labeled antibody is available [255, 256].

Meldal *et al.* [257–263] prepared a library of internally quenched fluorescent substrates on a support susceptible to penetration of the proteolytic enzymes into its structure (PEG acrylate). Appearance of fluorescence on the beads after incubation then pointed to the bead carrying the good substrate for the particular enzyme. This technique was modified into a "one-bead–two-compounds" assay [264, 265], in which the resin bead in addition to a combinatorial library member contains a reporter compound that can act as a beacon to monitor the activity of the library member. In the screen for enzyme inhibitors, the bead contains a quenched substrate and a library of potentially inhibiting peptides. Upon incubation of the beads with enzyme, the beads lacking fluorescent signal are likely to carry inhibitor, and can be selected and sequenced. This concept can be generally applied in all fields of combinatorial chemistry, including drug, catalyst, and material development.

Lam *et al.* speculated that lowering the substitution on the surface of the bead allowed an increase in the stringency of the assay [266]. They achieved it by segregating the internal volume of the beads from the surface layer. The internal content of the bead contains enough material for the sequencing and can actually be different from the bead surface. This allows for encoding nonsequencable parts of the peptide or nonpeptide structure on the bead surface, or for increasing the efficiency of Edman degradation by not including conserved parts of the peptide structure (if any) in the bead interior. Debenham *et al.* [267] used decreased substitution of the surface for the screening of high-affinity lectin ligands in an attempt to mimic the solution assay and avoid bivalent interaction. They found that actually 99.9% of the ligands had to be eliminated from the surface to prevent bidentate binding. It was later shown and explained by Chen *et al.* [268] that the decrease of the surface substitution is the best way to reduce the nonspecific binding of the macromolecular targets. High surface loading with the test compound was probably the reason for the earlier reported unsuccessful results from screening OBOC libraries.

The first attempts to segregate the surface and internal volume of the bead used enzymes to cleave the substrate-linker only on the bead surface, leaving the enzyme inaccessible core intact – to "shave the bead" [269]. The "shaved" bead can then be

functionalized with a different protecting group to segregate the two compartments – the surface and interior. This technique was later replaced by simple acylation of the amino groups of the bead matrix in a biphasic solvent environment. The water-soaked TentaGel bead surface is exposed to an organic solvent that contains the derivatizing reagent (e.g., Fmoc-OSu or Alloc-OSu), while the interior of the bead remains in water without any derivatizing reagent. In this way only the outer layer of the bead is derivatized. By varying the ratio of diethyl ether and dichloromethane (DCM) and adjusting the amount of Fmoc-OSu used, the thickness of the outer layer can be controlled [251, 270].

Astle *et al.* [271] simplified the process of hit isolation through magnetic bead sorting. The target protein was labeled with a magnetic nanoparticle. The binding of the target protein to a positive bead made the bead magnetic and allowed it to be separated from the rest of the library by placing a magnet on the side of the tube (Figure 12.8). The positive (magnetic) beads were separated into individual wells of a microtiter plate and the compounds were released by treatment with CNBr (methionine was used as part of the linker). The resulting samples (about 50%) were spotted onto a glass slide to generate a peptide microarray, which was then incubated with varying concentrations of the fluorescently labeled target protein to confirm and
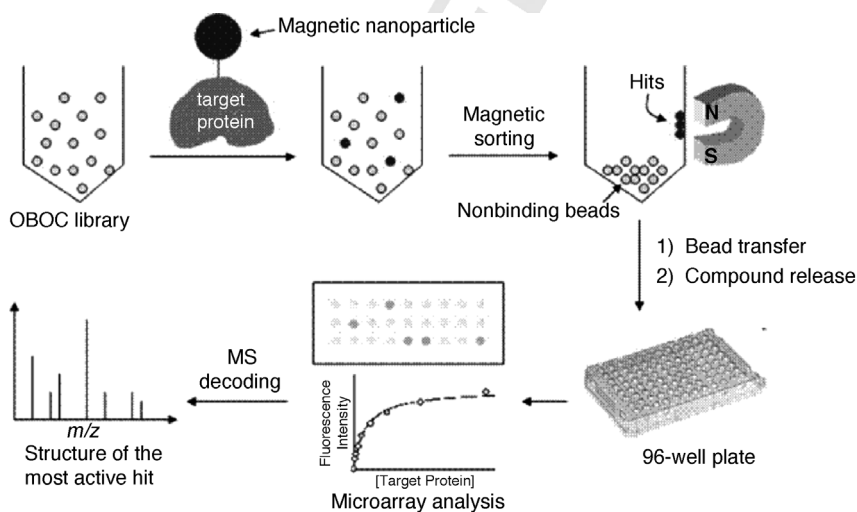

Figure 12.8 Scheme of the integrated magnetic screening and testing of hits on microarrays. TentaGel beads (75 μm) from a OBOC library are incubated with target protein, washed, and then incubated with antitarget protein antibodies linked covalently to iron oxide-containing particles (Dynabeads). Beads that bind the target protein are now magnetically labeled and retained on the side of the tube using a magnet, while nonbinding beads are removed. Each of the selected magnetic beads is separated into the well of a microtiter plate and the compounds are removed from the beads by cleavage of a linker. The compounds are then spotted onto a glass slide and formed microarrays are probed with different concentrations of the target protein to determine the intrinsic affinity of each compound for the target. The structure of selected hit is then deduced by tandem MS. In this way, no resynthesis of the hits is necessary until the best binders are confirmed [271]. (Illustration adapted from [272].)

reinvestigate its binding to these hits. A plot of the fluorescence intensity against the protein concentration gave the dissociation constant for each protein–ligand pair. This technique was tested on a 64-million mixed peptide/peptoid library and screen for binding to an anti-FLAG antibody. An initial on-bead screening produced 63 hits. A microarray analysis of the 63 hits revealed 27 low-nanomolar ligands against the antibody. The identity of the most active hits was then defined by matrix-assisted laser desorption ionization (MALDI)-MS.

The original paper [23] teaches the isolation of "positive" beads under a microscope. This technique is obviously a bottleneck in the application utilizing large libraries as it is negatively influenced by the subjective interpretation of bead color intensities. Affymetrix scientists successfully applied an automated fluorescence-activated cell sorter for selecting active beads from the peptide library coded by DNA tags tested against labeled antibodies [273]. Selectide Corporation (now part of Aventis), a company based on Kit Lam's technology, tested early on automated separation of fluorescently tagged beads using cell sorter instruments with surprisingly very limited success [236]. Later, a dedicated particle sorter COPAS™ (Union Biometrica) became a commercially available bead sorting instrument for the OBOC technology. It was successfully used, for example, for the screening of fluorescent internally quenched substrate libraries [264]. The success of this technique can be attributed to the use of highly porous solid support with minimal autofluorescence. Alternatively, the library can be first presorted to eliminate beads with high auto-fluorescence [274–276] or a different fluorescent labeling agent (such as quantum dots) with a different fluorescence emission wavelength can be used [277]. Validation of the selected (resynthesized) structures has to be performed in any case (as discussed, for example, by Kodadek and Bachhawat-Sikder [275]) to eliminate hits resulting from artifactual interactions.

The collaboration of scientists from the University of Edinburgh, Novartis, and Evotec Technologies (now PerkinElmer) resulted in the development of an automated system for selection and bead picking of fluorescently tagged positive beads utilizing confocal microscopy [278, 279] (Figure 12.9). Fluorescence-based detection of positive beads is complicated by the autofluorescence of the bead material or the
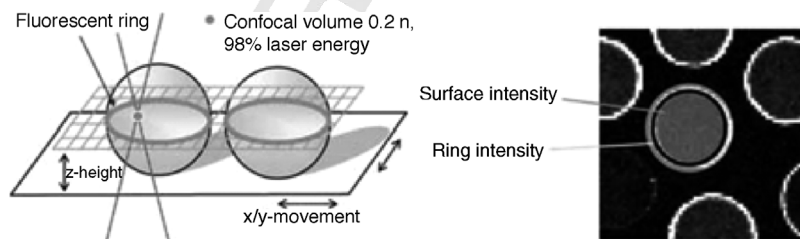
Figure 12.9 Principle of automated confocal microscopy screening of OBOC libraries. Beads in the monolayer are moved in the *xy* plane where the confocal plane is offset by about half of the bead size from the bead support. Observation of increased "ring" intensity represents the binding to macromolecular fluorescently labeled target. Fluorescence in the middle of the bead represents autofluorescence of the bead material or synthesized compound and is irrelevant to the biological interaction with the target [280].
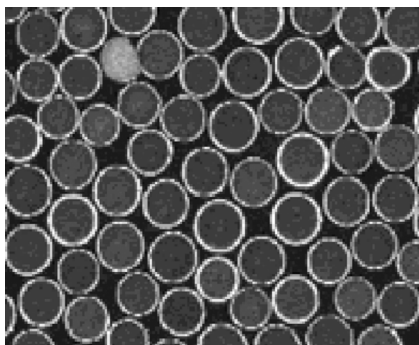
Figure 12.10 Resynthesized and retested (by confocal microscopy) beads with identical ligand show clearly the typical ring signifying the real binding effect [281]. At the same time large differences in ring intensity exemplifies the nonhomogeneity of the bead surface.

synthesized compounds, which can be higher than the fluorescence of the bound target macromolecule [276, 277]. However, the fact that the macromolecular fluorescently labeled target does not penetrate into the interior of the polymeric bead can be used for distinguishing between autofluorescence and fluorescence from the molecules bound to the surface of the bead. Confocal microscopy focuses only at a couple of microns "slice" of the bead and specific surface binding of the fluorophor-labeled target is clearly visible as the ring around the perimeter of the bead – unlike autofluorescence, which is distributed throughout the bead material. The bead library after incubation with the fluorescently labeled target is distributed as a monolayer of beads in the wells of a 96-well glass-bottom microtiter plate. Each well contains approximately 2000 beads. The plate is placed in an xy positioner above an inverted confocal microscope capable of autofocusing slightly under the center of the polymeric bead. The individual wells are then scanned in approximately 4 min each (for the 96-well plate, 00 000 beads can be scanned in 7 h), and a table of locations and internal and ring fluorescence intensities is built. After completion of the scan, the hits are ranked by their intensities, and the best are picked by an actuated capillary and placed into individual containers for structural evaluation. The bead picking process is slower and can retrieve about 1 bead/min [280–282]. Resynthesis and retesting of the active compounds has shown that surface inhomogeneity prevents precise ranking of the ligand quality based on the solid support fluorescence reading (Figure 12.10).

To validate the results obtained with the automated bead picking system, Hintersteiner et al. [281] designed a system utilizing postsynthesis/postscreening labeling for verification of the binding of ligand on the positive bead in solution assay. For the screening, the tested compound (peptide) is attached to the bead via a complex linker (Figure 12.11) composed of the segment allowing eventual detachment from the bead, followed by an amino acid with a terminal alkyne in the side-chain (labeling segment), a hydrophilic spacer to separate the library segment from the labeling segment, and the library (combinatorial) part. The identified and picked beads are then labeled via "click" reaction [283, 284] of the alkyne moiety with an
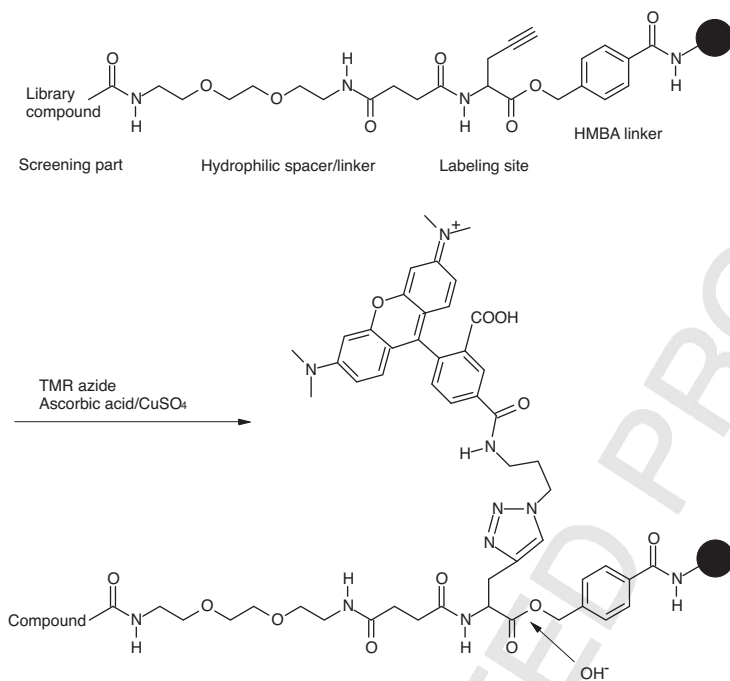
Figure 12.11 Structure of the attachment to the bead used in postsynthesis/postscreening labeling method. After on-bead screening with confocal microscopy and bead picking, individual hit beads are treated with an azide-modified dye (e.g., TMR-azide) to convert all compounds into fluorescent ligands for further affinity testing via fluorescence spectrometry. Compound is released to solution from the HMBA linker via hydrolysis [281].

azide-containing fluorophore. The ligands, now fluorescently labeled, are then cleaved from the beads and their concentration is quantified by fluorescence reading. Subsequently, binding to the unlabeled target is evaluated in a solution by two-dimensional fluorescence intensity distribution analysis (2D-FIDA). Only the structures of compounds with high and validated binding are then analyzed by MALDI-MS. The process was illustrated on an identification of ligands to the S_H2 domain of the adaptor protein Grb2. This automated technology is obviously not available for average laboratories due to the required dedicated instrumentation, but it is probably the most important addition to OBOC technology.

The fusion of OBOC technology and libraries utilizing a deconvolution of mixtures is the so-called "library of libraries" [285] (often confused with libraries from libraries, where one type of library is globally transformed into a different type of library by chemical reaction). A library of libraries is an assembly of individual beads, where each bead contains a sublibrary characterized by several defined positions in a sequence. For example, the hexapeptide library of libraries with three defined positions would be composed of 160 000 different types of beads, each of which would contain 8000 different peptides with three defined positions in their sequence.
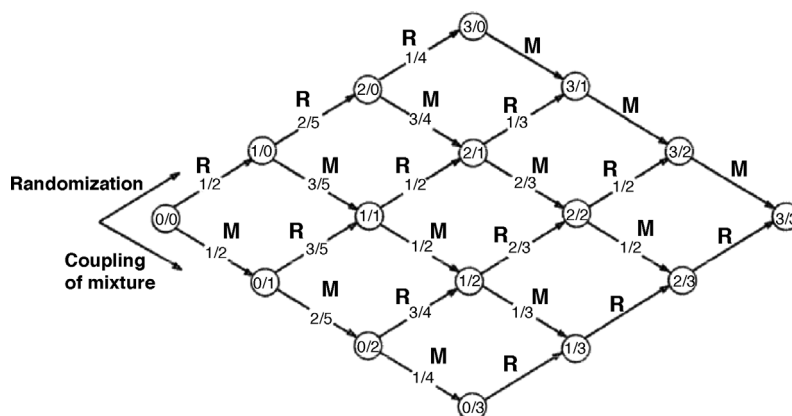
Figure 12.12 Scheme of the synthesis of a hexapeptide library of libraries with a three-amino-acid motif. Arrows carrying the symbol R represent performed randomization and arrows carrying the symbol M mean that the mixture of amino acids was coupled. Numbers inside of a circle show the number of randomized positions versus fixed position in the particular portion of the library at that particular point – the completed library (3/3) has three fixed and three mixed positions. The numbers in the arrows specify the ratio in which the portion of the library from the previous step was split for performing randomization or coupling of mixed amino acids. (Adapted from [285].)

This represents an enormous saving in comparison with an OBOC library, where we would need 64 000 000 beads to cover the same diversity. However, due to the slightly complicated synthetic scheme (see Figure 12.12), the potential of this type of library for quick definition of a peptide pharmacophore has, up to now, been underutilized. The concept of a library of libraries is clearly applicable to libraries built on planar substrates or labeled macroscopic substrates.

12.4.2.1 Determination of the Structure of a Peptide on an Individual Bead

For natural peptides the Edman degradation is still used for the sequence determination of "active" beads retrieved from a library. However, this technique is slow, cannot distinguish between L and D configurations of the building blocks, and unsequencable blocks (anything but α-amino acids) cannot be incorporated in the library structure. Therefore, alternative techniques were evaluated for structure determination.

Youngquist *et al.* [286, 287] developed a method in which during the generation of the library a small portion of the growing peptide chain is capped, so that at the end each bead contains "the history" of its synthesis. This history is then easily deconvoluted into the peptide sequence by MS.

An increase in the throughput of decoding the peptidic structures was achieved by the application of a partial Edman degradation (PED) followed by MALDI-MS [288–290]. In this method, beads carrying unique peptoid (or peptide–peptoid) sequences were subjected to multiple cycles of treatment with a 1 : 3 (mol/mol) mixture of phenyl isothiocyanate (PITC) and Fmoc-Cl to generate a series of
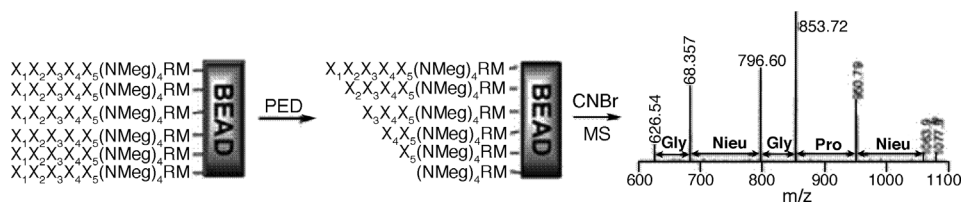
Figure 12.13 Example of rapid sequence determination by PED followed by MALDI-MS [288].

N-terminal truncation products for each resin-bound peptoid. At the end the Fmoc group was removed from the N-terminus and any reacted side-chains via piperidine treatment. The resulting mixture of the full-length sequence and its truncation products was analyzed by MALDI-MS. From the typical spectrum shown in Figure 12.13, the sequence could be easily deduced [288].

The peptide can also be tagged by sulfobenzoic acid on the N-terminus, which promotes an efficient charge site-initiated fragmentation of the backbone amide bonds to selectively enhance the detection of a single fragment ion series that contains the C-terminus of the molecule (y-ions) [291]. Another method uses a cleavable linker that is hydrophilic to help reduce nonspecific binding to biological samples and allows for the attachment of a halogen tag, which greatly facilitates postscreening sequencing by tandem MS (MS/MS). The linker is based on a tartaric acid unit, which, upon cleavage from resin, generates a C-terminal aldehyde. This aldehyde can then be derivatized with a bromine-containing amino-oxy compound that serves as an isotope tag for subsequent MS/MS analysis of y-ion fragments [292].

As it became obvious that library techniques are applicable not only to peptides, but also to small organic molecules, various techniques for coding/decoding the structure of a binding compound (complicated and available in limited quantity) on the bead surface by some entity hidden in the core of the bead (simply detectable and relatively abundant) were sought. The coding became especially relevant with the possibility to effectively differentiate the two compartments [266, 270, 293–295].

Coding for structure elucidation of nonpeptide molecules was covered in many reviews [37, 244, 296–299], often using very interesting and creative concepts, but in the context of peptide libraries is of only limited relevance.

As the MS techniques [300, 301] are becoming more sensitive and more readily available, more reports based on direct structure determination by MS/MS experiments are being published [302]. A single bead can even be physically cut into several pieces and still provide adequate quantities of peptides for this type of analysis [302, 303]. The hydrogen–deuterium exchange can simplify interpretation of spectra obtained from electrospray MS [304]. Hits from cysteine-rich libraries were alkylated to transform cysteine residues into acetamidomethyl derivatives, cleaved from the beads, and the structure determined by an MS/MS experiment [305]. Another example of the direct application of MS/MS for structure determination of peptides from noncoded library of peptides was shown by Brown *et al.* [306]. The library was synthesized on a hydroxymethyl benzoic acid (HMBA) linker. The peptides were cleaved from the beads by an application of gaseous ammonia. It

was shown that even a short (5 min) exposure to high-pressure (117 psi) ammonia gas detaches the majority of the peptide from the linker. Individual beads were then placed on a glass surface. A nanomanipulator then placed a 1-μm nanoelectrospray tip next to the bead and delivered the extraction solvent to the bead. After 30 s, the solvent was backfilled into the nanoelectrospray tip and the tip was introduced into the mass spectrometer. MS/MS analysis delivered high-quality data in 100% of tested cases.

12.4.3
Solution-Based Screening of OBOC Libraries

The disadvantage of OBOC libraries with their limitation to binding assays was quickly recognized by Lam *et al.* and this technique was modified by the introduction of selectively cleavable linkers to allow assaying of libraries in solution. Since the identity of the tested peptide is not known until the sequence is read from the "active" bead, the test must guarantee that the observed activity of the solution would be traceable back to the bead from which the active compound was cleaved. Moreover, there must be enough of peptide still available on that source bead to allow the structure determination. There are two general approaches to screen a OBOC library with solution-phase assays: (i) the 96-well two-stage release assays and (ii) the *in situ*-releasable solution-phase assay with immobilized beads. In both approaches, ligands are attached to the solid support via cleavable linker(s). The ligands are then released from each bead into solution phase where the biological assays take place. The bead of origin of the positive releasate can subsequently be identified and isolated for structure determination. The spatial relationship between the active bead and the solution activity can be realized by embedding the bead in a semisolid medium (e.g., gel), to which the peptide is slowly diffusing from the entrapped bead. Beads can be also immobilized into microwells, in which the activity will be tested.

In the 96-well two-stage releasable assay [307], the double orthogonally cleavable linker [308–310] (e.g., the linker of the structure in Figure 12.14) is incorporated into the preparation of the library. Approximately 100–500 beads are added into each well of a 96-well filtration plate (e.g., Millipore). Upon neutralization, the first aliquot of the library is released with the formation of a diketopiperazine structure on the bead. After incubation overnight, suction is applied so that the filtrates (each with 100–500 compounds) are collected in a 96-well plate placed beneath the filtration plate. The filtrates are then assayed for biological activity in solution. Beads from the positive wells are then redistributed into filtration plates, now with one bead per well. With alkali treatment (e.g., gaseous ammonia), the second aliquot of the library is released and the filtrates from each well are then tested for biological activity. The beads that correspond to the wells with active compounds are then identified and isolated for structure determination. This two-stage release assay is needed if a high number of beads (e.g., more than 5000) are assayed. If the number of beads to be assayed is limited, a single release assay with one bead per well may be sufficient, particularly if the transfer of individual beads into each well (one bead per well) can be automated. For a 100-μm bead, approximately 100 pmol of compound can, in principle, be
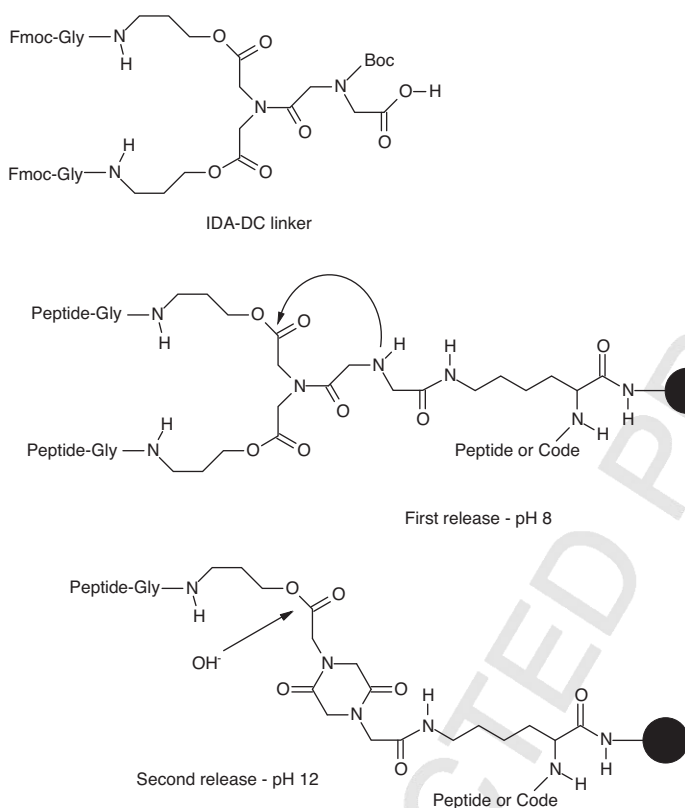
Figure 12.14 Scheme of the sequential releases from doubly cleavable linker [308]. If the linker is attached through trifunctional amino acid (Lys), a copy of nonreleasable peptide or a code is still attached to the bead after both releases.

recovered giving a final concentration of 1 μM (if the final assay volume is 100 μl). In order to increase the concentration of the recovered compound, one may (i) miniaturize the assay volume (e.g., using the 384-well plate), (ii) use bigger beads, or (iii) use beads with a higher substitution level.

For *in situ* solution-phase releasable assay, Salmon *et al.* proposed the use of soft agar to immobilize beads [311]. After the linker has been cleaved the compounds were released and diffused into the surrounding agar, where the solution-phase assay takes place. Jayawickreme *et al.* [312–314] reported a related method to identify ligands that interact with the α-melanocyte-stimulating hormone (MSH) receptor. They first immobilized the bead library on a thin film of polyethylene and exposed the beads to gaseous trifluoroacetic acid (TFA) for 10 h at room temperature. After neutralization with gaseous ammonia, the beads were layered on the surface of a dish of melanocytes growing in soft agar. As a result of pigment dispersion, the cells located underneath and around the positive beads with MSH agonist activity turned dark within 15 min. This elegant assay system has also been adapted to other G-protein-coupled receptors by transfecting those receptors into a cell line with
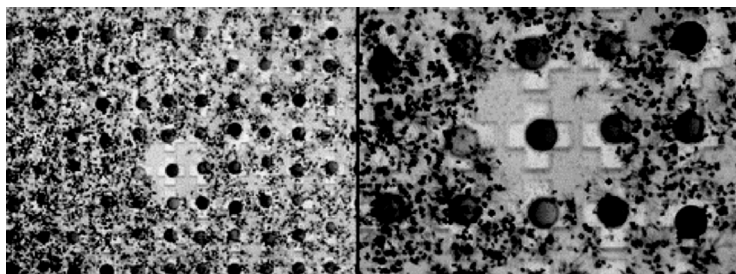
Figure 12.15   Cell viability assay on a dedicated microfabricated microtiter plate. Plate wells can accept only one bead (120 μm size). Clearing in the cell lawn clearly defines location of the source bead of the cytotoxic peptide [252].

melanocyte background. Salmon *et al.* [311] have applied a related *in situ* solution-phase releasable assay to anticancer agent discovery. The *in situ*-releasable assay is highly efficient and, in principle, only a single cleavable linker is needed since the beads are already spatially separated; the two-stage release assay as described in the 96-well releasable method is not needed. The *in situ* assay has an added advantage in that the concentration of the released compound could be rather high (e.g., above 10 μM) in close proximity to the bead and the potency of the compound could be estimated on the basis of the size of the activity ring surrounding each positive bead (Figure 12.15).

A recently published technique [252] uses a dedicated photolithographically microfabricated plate in which each well is sized to accommodate only one 120-μm TentaGel library bead. This plate is loaded with 10 000 beads and beads are covered with a suspension of cancer cells in Matrigel™ to create the three-dimensional cell culture. After the cell colonies reach a desired confluency, the library compound connected by a disulfide-containing linker is released by application of a buffer containing dithiothreitol. After an additional incubation the regions in which cytotoxic peptide was released are clearly observable by a decreased cell viability associated with particular beads. Those beads were retrieved and the structure was determined by Edman degradation.

In some instances, it may be advantageous to combine solution-phase assays with on-bead assays to screen a specific target. Positive beads isolated by this approach are more likely to be true-positives. For example, the compound-beads are partitioned into 1000 beads per well and a portion of the compound on each bead is released into the solution for biological testing. The 1000 beads from a positive well can then be recycled and an on-bead binding assay performed to identify the single positive bead. Using this approach, Salmon *et al.* successfully isolated ligands that bind to an anti-β-endorphin monoclonal antibody. This strategy may have higher throughput than the earlier discussed system for automated picking of beads from binding assays followed by solution evaluation of binding constants; however, the future is clearly favoring automated systems. It remains to be seen whether large pharmaceutical companies will be able to revitalize their development pipelines by the application of the new (highly automated and expensive) technologies, or whether simple semi-

automated or completely manual techniques applied in small biotech companies or academic laboratories will still provide a major resource in supplying new drug candidates.

12.5
Future of Peptide Libraries

As can be seen in this chapter, there is a multitude of concepts for generating diversity of peptide or peptide-like structures. All of them have a clear goal of discovering a new functional molecule that can be applied in a practical application (as a pharmaceutical, diagnostic, catalyst, or anything that can be imagined). It is still to be determined which technology will be dominant in the future, but at this moment it can be predicted that the winning technique will be highly automatable, simple, with a low demand on the amount of necessary target and reagents, and, most importantly, provide the most reliable and validated results. The author's personal prediction is that it would be the automated OBOC technology applying the library of libraries strategy.

12.6
Synthetic Protocols

12.6.1
Pin Synthesis [2, 60, 76, 315]

The polyethylene rods (diameter, 4 mm; length, 40 mm) immersed in a 6% (v/v) aqueous solution of acrylic acid were irradiated at a dose of 1 000 000 rads (1 rad = 0.01 Gy). The rods so prepared were assembled into a polyethylene holder with the format and spacing of a microtiter plate. Subsequent reactions at the tips of the rods were carried out in a Teflon tray with a matrix of wells to match the rod spacing. Conventional methods of solid-phase peptide chemistry were used to couple Boc-L-lysine methyl ester to the polyethylene/polyacrylic acid via the amino group of the side-chain. Carboxy substitution of the support was determined by treating NH_2-lysine(OMe)-polyethylene/polyacrylic acid with ^{14}C-labeled butyric acid and was found to be 0.15–0.2 nmol/mm^2. Removal of the Boc group was followed by the coupling of Boc-L-alanine to complete a peptide-like spacer. Successive amino acids were added as dictated by the sequence to be synthesized. At the completion of the final coupling reaction, and after removal of the Boc protecting group, the terminal amino group was acetylated with acetic anhydride in dimethylformamide (DMF)/triethylamine. All N,N-dicyclohexylcarbodiimide (DCC)-mediated coupling reactions were carried out in DMF in the presence of 1-hydroxybenzotriazole (HOBt). The following side-chain protecting groups were used: O-benzyl for threonine, serine, aspartic acid, glutamic acid, and tyrosine; carbobenzoxy for lysine; tosyl for arginine; 4-methylbenzyl for cysteine; and 1-benzyloxycarbonylamido-2,2,2-trifluoroethyl for histidine. Side-chain-protecting groups were removed by treatment with

borontris(trifluoroacetate) in TFA for 90 min at room temperature. After hydrolysis with HCl/propionic acid, sequences included in the synthesis as controls were analyzed to confirm that, although coupling at each stage had occurred, it was incomplete for several of the amino acids, notably arginine. Before testing by enzyme-linked immunosorbent assay (ELISA), support-coupled peptides were washed several times with phosphate-buffered saline (P_i/NaCl).

12.6.2
SPOT Synthesis [144, 153]

A sheet of chromatographic paper Whatman Chrl (Maidstone, UK) was marked with a pencil (spot positions) and the sheet was dried under vacuum overnight. A solution of 0.2 M Fmoc-amino acid (Pro or β-Ala), 0.24 M DIC, and N-methylimidazole in DMF was soaked into the paper sheet, and the reaction was run for 3 h in a closed container. The paper sheet was washed 3 times with DMF and treated with piperidine/DMF (1 : 4, v/v) for 20 min. After three washes with DMF and two washes with ethanol, the sheet was dried in a desiccator. Solutions of Fmoc-amino acid HOBt esters (0.5 ml, 0.3 M) were then spotted onto pencil-marked spots and reactions proceed for 20 min on a plastic tray covered with a glass plate. The paper sheet was then washed twice with acetic anhydride/DMF (1 : 49, v/v) and treated with the same solution in the presence of 1% diisopropylethylamine (DIPEA) for 30 min. After washing with DMF (4 times), deprotection by piperidine/DMF (1 : 4, v/v, 5 min), and washing with DMF (4 times), the sheet was washed in bromophenol blue solution (0.01%) to reveal blue spots with available amino groups. The sheet was washed with ethanol (2 times) and dried by cold air from a hair dryer between two layers of Whatman 3MM paper. Blue spots were used as a target for spotting activated solutions of amino acids (0.3 M, 0.5–1 ml). The paper sheet was optionally respotted after 15 min (if disappearance of blue color was slow). Final deprotection was performed by immersing the dry sheet into a solution of TFA/DCM/diisobutylsilane/water (50 : 45 : 3 : 2, v/v/v/v) for 2 h. After washes with DCM (4 times), DMF (3 times), and ethanol (2 times), the sheet was dried and ready for binding assay or storage (−20 °C in a sealed plastic bag). In the case of peptide synthesis on a linker cleavable by intramolecular diketopiperazine formation, the sheet has to be washed exclusively by acidic solutions so as to not lose the synthesized peptides prematurely. The spots can be cut or punched out of the dried sheet into polypropylene tubes or into wells of a microtiter plate and after addition of neutral buffer the peptides are released into solution.

12.6.3
Synthesis in Tea-Bags [3]

Q4 Standard Boc-amino acid resin (50–100 mg; 0.2–0.8 meq/gm) was contained in polypropylene 74-μm mesh packets having approximate dimensions of 15 × 20 mm. After a number was placed at the top of the unsealed bag with a black marker pen, the packet was closed and the number was permanently sealed into the polypropylene to

give an easily readable label for each bag. These resin packets can be used for simultaneous multiple peptide syntheses (i.e., syntheses in which many different peptides are produced concurrently) or for multiple analog peptide syntheses (i.e., syntheses in which many analogs of a particular peptide are produced concurrently). The method is not limited to the Boc strategy, which is described in this example. The standard deprotecting, neutralization, coupling, and wash protocols, and basically any type of the resin support, can be used for the synthesis.

Synthesis of analogs having single-amino-acid variations can be achieved by using various peptide synthesizers or completely manual methods. Between 40 and 80 individual packets containing the desired starting resin were carried through their common Boc removal, washing, and neutralization steps. After their methylene chloride washes to remove excess base, the packets containing the neutralized peptide resins were removed from the reaction vessel and added to solutions containing preformed symmetrical anhydrides of the next protected amino acid. The individual coupling steps were carried out for 60 min with stirring or shaking at room temperature. After completion of the coupling steps, the resin packets were returned to the reaction vessel, and the synthesis process was continued through additional cycles of common wash, deprotection, neutralization, and coupling steps until the syntheses were completed. Specific variations of analogous peptides, such as single-residue replacement or omission analogs or chain-lengthened or -shortened analogs, were easily accomplished by removing the individual coded packets at the point of variation during the synthesis, carrying out the desired variation separately, and, if appropriate, returning the packet to the common reaction vessel for completion. After the preparation of a series of protected peptide resins, the resin-filled packets were washed thoroughly, dried, and weighed to give an initial indication of coupling completion. The protected peptide resins, still contained within their packets, were then cleaved by using conventional HF/anisole procedures in a vessel modified to allow cleavage of 20 peptide resins at once. After extraction of the residual anisole with ether or ethyl acetate, the peptides were extracted from the resin packets by 5% acetic acid and either lyophilized directly or put through a Sephadex G-10 desalting column prior to lyophilization. The crude peptides were characterized by high-performance liquid chromatography and found to have an average purity of 84% (70–94%).

12.6.3.1 Synthesis on Cotton [132]

12.6.3.1.1 Modification of the Cotton Carrier

DMAP-Catalyzed Carbodiimide/HOBt Acylation

i) The cotton sheet (10 cm^2) was shaken successively in 25% TFA/DCM (3 ml) for 20 min, DCM (2 × 3 ml) for 3 min each, 10% DIPEA/DCM (2 × 3 ml) for 3 min each and DCM (2 × 3 ml) for 3 min each and dried between filter papers. Subsequently, the carrier was shaken overnight in 0.1 M Fmoc-amino acid/DCC/HOBt/0.03 M 4-dimethylaminopyridine (DMAP) in DMF (1 ml). The carrier was washed successively with DMF (2 × 3 ml) for 3 min each, ethanol

(2×3 ml) for 3 min each, and DCM (2×3 ml) for 3 min each, and dried between filter papers. A sample of the carrier was used for the determination of substitution. After shaking in 20% piperidine in DMF (5 ml) and washing by the same solution, the absorption of the solution was read at 301 nm. The substitution was calculated according to the formula:

Substitution (mmol/cm^2) $= (A \times V$ (ml) $\times 1000)/(8100 \times F$ (cm^2))

where A is the absorption, V is the volume, and F is the area of the carrier. The absorption coefficient (8100) was determined by a calibration curve using solutions of Fmoc-Ala in 20% piperidine/DMF.

ii) The reaction was performed in the same way, with the exception that the solution was placed 4 times for 15 min in an ultrasonic bath. In this way, larger quantities of cotton were modified (3×300 cm).

NMI-Catalyzed Carbodiimide/HOBt Acylation The cotton or paper carrier (10 cm^2) was pretreated with TFA/DCM, as described above, and shaken overnight in 0.1 M Fmoc-amino acid/DIC/HOBt/0.2 M *N*-methylimidazole (NMI) in DMF (1 ml) or soaked with 0.5 M Fmoc-amino acid/DIC/HOBt/1 M NMI (0.2 ml) and left overnight. After that the carrier was shaken successively in DMF (2×3 ml) for 3 min each and DCM (2×3 ml) for 3 min each, and dried between filter papers.

Attachment of the Acid-Labile Handle and the Starting Amino Acid The handle HO-CH$_2$-C$_6$H$_4$-O-(CH$_2$)$_2$-COOTcp (HPP-OTcp) was synthesized according to the original procedure described by Albericio and Barany [316]. The amino acid carrier (10 cm^2) was shaken in 0.1 M HPP-OTcp/HOBt in DMF (1 ml) after the addition of a 0.01 M bromophenol blue/DMA solution (20 µl) until the disappearance of the blue color from the carrier (30–60 min) or the carrier was soaked with 0.3 M HPP-OTcp/HOBt in DMF (0.2 ml) and left after the addition of bromophenol blue until decolorization. After that, the carrier was shaken successively in DMF (2×3 ml) for 3 min each and DCM (2×3 ml) for 3 min each, and dried between filter papers. The HPP-amino acid-carrier formed by this reaction was then acylated with the starting amino acid as described above.

Peptide synthesis using Fmoc-protected amino acids using standard protocol (DIC/HOBt activation, monitored with bromophenol blue, 20% piperidine in DMF for deprotection) is recommended for assembly of peptides. Boc-based peptide synthesis gives much inferior products.

12.6.3.2 Split-and-Mix Synthesis of OBOC Noncleavable Libraries [237]

The library is synthesized on 130-µm TentaGel (Rapp Polymere, Tubingen, Germany) resin beads. Alternatively, ArgoGel™ (Argonaut Technologies, Foster City, CA) polydimethylacrylamide beads or Pepsyn Gel Resin (Cambridge Research Biochemicals, Northwitch, UK) can be used. In general, any resin that is compatible with organic solvents, as well as aqueous media, is adequate. Spacers, such as aminocaproic acid, aminobutyric acid, and/or β-Ala, may be attached to the resin prior to assembling the library. The resin beads are divided into 19 aliquots contained in 19 polypropylene vials or plastic syringes equipped with a plastic frit at the bottom

(CSPS, San Diego, CA). (The number of amino acids used in randomization, equal to the number of reaction vessels, is limited only by the patience of the chemist.) Nineteen Fmoc-amino acids (all proteinogenic amino acids except Cys) are added separately into each of the resin aliquots using a minimal amount of DMF. The amino acids are added in 3-fold excess, and coupling is initiated by adding a 3-fold excess of benzotriazol-l-yl-oxytris-(dimethylamino) phosphonium hexafluorophosphate (BOP) and DIPEA (or DIC) and HOBt. A trace amount of bromophenol blue is added to the reaction mixture. The vials are tightly sealed (syringes are capped) and rocked gently for approximately 30 min at room temperature or until all beads turn from blue to colorless. Completion of the coupling is confirmed by the ninhydrin test. When the coupling is incomplete, the beads are allowed to settle and the supernatant is gently removed; alternatively, in the case of synthesis in a syringe, it is expelled from the syringe. A fresh activated Fmoc-amino acid is added and the reaction proceeds for an additional 1 h. The resin is washed by DMF and the resin pools are mixed in a siliconized cylindrical glass vessel fitted at the bottom with a frit. Dry nitrogen is bubbled through to mix the resin. After washing 8 times with DMF, piperidine/DMF (1 : 4, v/v) is added. After 10 min of bubbling with nitrogen, the piperidine is removed and the resin is washed 10 times with DMF. The amount of released dibenzofulvene–piperidine adduct is determined by measuring the absorbance at 302 nm. A stable level of substitution determined in this manner throughout the library synthesis serves as one of the quality control criteria. The resin is again divided into 19 aliquots for the coupling of the next 19 amino acids. After the coupling steps are completed, the Fmoc group is removed with piperidine/DMF (1 : 4, v/v), and the resin is washed with DMF and DCM. The side-chain protecting groups are removed by treatment with reagent K (TFA/phenol/water/phenolethanedithiol, 82.5 : 5 : 5 : 5 : 2.5; v/w/v/w/v) for 5 + 120 min. This treatment is performed in the common container (glass bubbler) or, in cases that separate pools of resin are required, in individual syringes. The resin is washed thoroughly with TFA, DCM, DMF, DMF/water (1 : 1, v/v), and 0.01% HCl in water, and stored in DMF at 4 °C. Again, small individual pools can be conveniently stored in the plastic syringes in which the whole synthesis is performed. Larger library batches (up to 80 g) are stored with protected side-chains in 0.2% HOBt/DMF at 4°C. To verify the quality of the library, several randomly chosen beads are sequenced, and the average amount of peptide per bead is determined. This value is confirmed by quantitative amino acid analysis of a random sample from the library (1 mg). Whereas amino acid analysis is used to determine the overall amino acid composition of the library, sequence analysis confirms the random distribution of amino acids at each position

12.6.3.3 Preparation of Dual-Layer Beads [251, 270]

Preparation of topologically segregated bifunctional TentaGel resin beads with 60% Boc outside and 40% Fmoc-linker inside (outside-Boc/inside-Fmoc-linker bifunctional resin). TentaGel S NH$_2$ resin beads (1.0 g, 0.26 mmol) were swollen in water for 48 h. The water was drained and a solution of Alloc-OSu (31.1 mg, 0.156 mmol) in a DCM/diethyl ether mixture (50 ml, v/v, 55 : 45) was added to the resin, followed by

addition of DIPEA (55 μl, 0.312 mmol). The resulting mixture was shaken vigorously for 1 h. The resin was washed 3 times with DCM and 6 times with DMF. Fmoc-linker was then built in the inner region of the resin. The resin was washed 3 times with DCM. In the presence of argon, a solution of $PhSiH_3$ (770 μl, 6.24 mmol) in DCM (4 ml) was added to the resin followed by a solution of $Pd(PPh_3)_4$ (75.1 mg, 0.065 mmol) in DCM (12 ml). The mixture was shaken in an argon atmosphere for 30 min. This process was repeated. The resin was washed with DCM, DMF, and DCM 3 times each. A solution of di-*tert*-butyl dicarbonate (1.19 ml, 5.2 mmol) in DCM (10 ml) was added to the resin, followed by the addition of DIPEA (226.4 μl, 1.3 mmol). The mixture was shaken until the ninhydrin test was negative. The obtained outside-Boc/inside-Fmoc-linker bifunctional resin was washed with DCM, DMF, DCM, and MeOH 3 times each, and then dried *in vacuo*. The percentage of inner region was determined to be 39% using quantitative UV absorption analysis of the dibenzofulvene–piperidine adduct released by treatment with piperidine.

12.6.3.4 Preparation of Library of Libraries [285]

Libraries of peptides were constructed on TentaGel Resin S Amino-NH_2 (Rapp Polymere, Tubingen, Germany). Standard solid-phase peptide synthesis chemistry (Fmoc chemistry) was used. In every step the resin was divided into a number of pools in ratios according to the scheme in Figure 12.12. The mixture of protected amino acids with molar ratios adjusted according to the results from the pilot experiment was used in steps in which the amino acids in the mixture were coupled. The randomization steps were performed according to split-synthesis methodology. Finally, the Fmoc groups were removed with 20% (v/v) piperidine in DMF and the side-chain protecting groups were removed with a mixture of TFA/phenol/anisole/ethanedithiol, 94 : 2: 2: 2; v/w/v/v or with reagent K (TFA/phenol/water/thiophenol/ethanedithiol, 82.5 : 5: 5: 5: 2.5 (v/w/v/w/v)). The resin was then washed thoroughly with DMF, neutralized with 10% (v/v) DIPEA in DMF, thoroughly washed again, hydrated, and stored in 0.01% HCl at 4 °C.

12.6.4
Preparation of OBOC Libraries for Testing in Solution [308, 317]

12.6.4.1 Synthesis of Multicleavable Linker

Boc-iminodiacetic acid (Boc-Ida)   A solution of iminodiacetic acid (30.0 g, 225 mmol) in 1 M NaOH (225 ml) and dioxane (200 ml) was stirred and cooled in an ice-water bath. Di-*tert*-butyl pyrocarbonate (53.9 g, 247 mmol) was added in several portions and stirring was continued at room temperature for 1 h. Dioxane was evaporated *in vacuo*, and the residue was covered with a layer of ethyl acetate (100 ml) and acidified with a saturated solution of $KHSO_4$ to pH 2–3. The aqueous phase was extracted with ethyl acetate (3 × 150 ml). Combined ethyl acetate extracts were washed with water (100 ml), dried over anhydrous $MgSO_4$, and evaporated *in vacuo*. The product was crystallized from a solvent mixture of ethyl acetate and petroleum ether. Yield 47.0 g (90%).

HN(CH$_2$-CO-O-(CH$_2$)$_3$-NH<-Gly<-Fmoc)$_2$·HCl
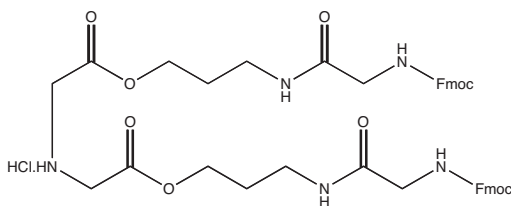
A solution of Fmoc-Gly-NH-(CH$_2$)$_3$-OH (7.08 g, 20 mmol) in DMF (30 ml) was added to the solution of Boc-Ida (2.33 g, 10 mmol), HOBt (2.7 g, 20 mmol), and DIC (3.14 ml, 20 mmol) in DMF (30 ml). DMAP (0.48 g, 4 mmol) was added and the reaction mixture was stirred at room temperature for 5 h. DMF was evaporated under reduced pressure, and the oily residue was dissolved in AcOEt (100 ml), filtered, and extracted 3 times with water, 5% aqueous HCl, water, a saturated solution of NaHCO$_3$, water, and a saturated solution of NaCl in water. The organic layer was dried by anhydrous MgSO$_4$, and the AcOEt was evaporated under reduced pressure. Yield 7.2 g (80%) of a crispy foam. The product was dissolved in DCM (30 ml), TFA (30 ml) was added, and the reaction mixture was stirred for 30 min. DCM and TFA were evaporated under reduced pressure, and the oily residue was dissolved in AcOEt and the solution was washed 3 times with water and a saturated solution of NaHCO$_3$. After addition of a solution of Na$_2$SO$_4$, three layers were formed. The bottom layer containing the product was separated, acidified by shaking with 5% HCl, dissolved in chloroform, dried by anhydrous MgSO$_4$, concentrated to a small volume, and poured into a large excess of ether. The resulting precipitate was collected, washed with ether, and dried. Yield 5.4 g (64%) of a crispy foam, single spot on preparative layer chromatography, R_f 0.28 in CHCl$_3$/MeOH/AcOH (90 : 9 : 1).

Boc-N(CH$_2$-COOH)-CH$_2$-CON(CH$_2$-CO-O-(CH$_2$)$_3$-NH<- Gly<-Fmoc)$_2$ (IDA-DC)
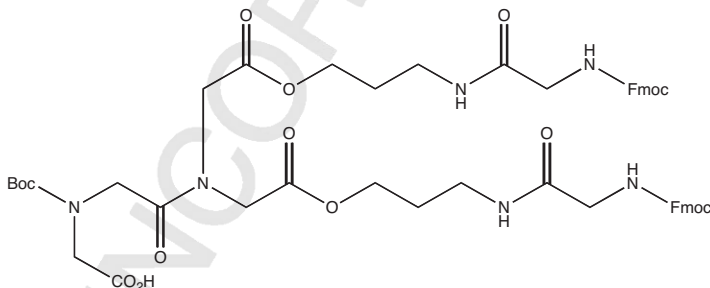
Boc-Ida (2.33 g, 10 mmol) was dissolved in DCM/DMF (10 : 1, 50 ml), DIC (1.57 ml, 10 mmol) was added, and the reaction mixture was stirred for 30 min.

Then the solution of $HN(CH_2\text{-}CO\text{-}O\text{-}(CH_2)_3\text{-}NH<\text{-}Gly<\text{-}Fmoc)_2 \cdot HCl$ (8.4 g, 10 mmol) in DMF (50 ml) was added, the pH was brought to about 8 by addition of DIPEA, and the reaction mixture was stirred for 1 h. DMF and DCM were evaporated under reduced pressure, and the oily residue was dissolved in AcOEt (100 ml) and washed 3 times with water, 5% aqueous HCl, water, and finally it was extracted with a saturated solution of $NaHCO_3$. The $NaHCO_3$ extracts were combined and acidified with aqueous HCl, and the solution was extracted 3 times with AcOEt and the organic phase was dried with anhydrous $MgSO_4$, and then the AcOEt was evaporated under reduced pressure. The oily residue was triturated with ether and the product crystallized. Yield 6.8 g (67%). Linker (300 mg) was dissolved in acetonitrite (ACN) (6 ml) and diluted with water (6 ml). A solution of linker (3 ml) was applied to a RP column equilibrated with 40% ACN in water (100 ml). The column was washed with 40% cerium ammonium nitrate (CAN) (100 ml), 45% CAN (100 ml), 50% CAN (200 ml), and 70% CAN (200 ml). Fractions (10 ml) were collected and their purity was checked by thin-layer chromatography in the system chloroform/toluene/MeOH: water (10:10:10:1). Fractions containing homogeneous linker were combined, the ACN was evaporated under reduced pressure, and the residual solution was lyophilized. Yield 188 mg, single spot on thin-layer chromatography, R_f 0.28 in $CHCl_3$/MeOH/AcOH (90:9:1). ^1H nuclear magnetic resonance (300 MHz, dimethylsulfoxide, 27 °C) δ: 1.36 (9H, Boc), 1.75 (2H, CβH$_2$), 3.16 (2H, CαH$_2$), 3.60 (2H, Gly CH$_2$), 3.76–4.23 (8H, Ida CH$_2$), 4.11 (2H, CγH$_2$), 4.19–4.34 (3H, Fmoc CH$_2$ and CH), 7.49 (1H, Gly NH), 7.34, 7.42, 7.72, and 7.89 (8H, Fmoc aromatic H).

12.6.4.2 Synthesis of the Library

TentaGel (5 g, 0.23 mmol/g, 130 μm average particle size) was swollen in DMF (swollen resin volume 25 ml) and Fmoc-Lys(Boc)/DIC/HOBt (3 equiv. each) in DMF was coupled. After 2 h the resin was washed 5 times with DMF and once with DCM and the Boc group was removed with TFA/DCM (1:1, v/v, 1 + 20 min). After washing with DCM (5 times) and DMF (4 times), the resin was neutralized with DIPEA/DMF (1:49, v/v), washed with DMF (3 times), and the linker IDA-DC (Figure 12.14, 3 equiv.) was activated by DIC and HOBt (3 equiv. each) in DMF and coupled overnight. The resin was washed with DMF (5 times) and the Fmoc group was removed with piperidine/DMF (1:4, v:v, 20 min). After washing with DMF (3 times) and distribution of the resin into m reaction vessels (plastic vials or fritted syringes), individual Fmoc-protected amino acids were coupled to each part of the resin using DIC and HOBt (3 equiv. each). The reaction was monitored using bromophenol blue. When complete coupling was observed in all reaction vessels (all resin particles were decolorized), completeness of the coupling was verified using the ninhydrin test. All resin portions were combined, washed with DMF (5 times), and the Fmoc group was removed as described above. This procedure (separate couplings and deprotection after combining the resin) was repeated $n-1$ times (n = number of library positions). The side-chain protecting groups were cleaved by reagent K for 2 h, and washed with TFA (3 times), DCM (5 times), DMF containing 0.1% HC1 (4 times), and

0.1% HC1 in water (5 times). The library has to be stored in an acidic solution in order to prevent premature loss of peptides.

12.6.4.3 Quality Control of the Doubly Releasable Library

Dried resin (5–10 mg) was shaken overnight in 2–5 ml of 0.1 M HEPES buffer (pH 8.5) in a polypropylene syringe equipped at the bottom with a polypropylene or Teflon frit and a polypropylene plunger. The absorbance of the solution (diluted, if necessary) at 280 nm was measured and the amount of released peptide was calculated according to the following formula:

$$\text{Release (mmol/g)} = (\text{absorbance} \times \text{volume} \\ \times \text{dilution})/(1197 \times n/x + 5559 \times m/y) \times \text{mass},$$

where mass is the quantity of library beads in grams, x is the number of amino acids in positions where Tyr is used, y is the number of amino acids in positions where Trp is used, n is the number of positions in the library where Tyr is used, and m is the number of positions in the library where Trp is used. If other amino acids with absorbance at 280 nm were used in library construction, the above formula must be modified. A solution of 0.2% NaOH was drawn into the syringe containing the library sample and the syringe was shaken for 4 h. The solution was expelled from the syringe and the absorbance measured at 280 nm. The same calculation was performed using the formula shown above using coefficients 1507 and 5377 instead of 1197 and 5559, respectively. The amount of released peptide in each step should not differ by more than 10% from the theoretical value, which was calculated according to

$$\text{Theoretical release (mmol/g)} = \text{Subst.}/(1 + \text{Subst.}(3\text{MW} + 686)/1000), s$$

where Subst. is the original substitution of the resin (in mmol/g), MW is the average molecular weight of the library peptides, and 686 is the molecular weight of the linker (without Fmoc groups), plus one Lys residue, minus one molecule of water. The average molecular weight of a natural amino acid is 119.7 (19 amino acids, Cys excluded). Therefore, the average molecular weight of a pentapeptide library made from these 19 amino acids is 598.5. Starting with a resin substitution of 0.2 mmol/g, 0.134 mmol of pentapeptide should be released at each step using 1 g of dried library resin.

12.6.4.4 Two-Stage Release Assay in 96-Well Microassay Plates

Library beads were transferred into pH 4.5 buffer containing 1.0% carboxymethyl-cellulose (to slow sedimentation), shaken, and rapidly pipetted into the upper chambers of a vacuum-controlled 96-well filtration manifold (Model 09 601; Millipore, South San Francisco, CA). Approximately 500 beads were placed in each filtration well, so that each plate contains approximately 48 500 unique peptides. The filtration plates serve as "master" plates for retaining subsets of peptides in unique locations. The transfer buffer was removed by vacuum filtration, and the first stage release of peptides was accomplished by dispensing the appropriate buffer or tissue culture medium (neutral pH) to each well and incubating overnight. The released

peptides were vacuum filtered into 96-well microassay test plates where the biological activity was determined. In some experiments the released peptides were distributed into several replicate plates for multiple simultaneous assays against different molecular targets. Wells identified as "positive" were marked and the beads of origin were recovered from the corresponding well(s) of the filtration master plate with the aid of a low-power stereomicroscope. The recovered beads were transferred one by one (one bead per well) into individual microwells of 96-well filtration plates. Cleavage of the ester (second) linker was then accomplished by overnight incubation in ammonia vapors in a desiccator or dedicated pressurized chamber. After drying, the appropriate buffer was added and the plates were gently shaken for several hours. Thereafter, the peptide-containing buffer was filtered into the test plates for bioassay. The individual peptide beads corresponding to each positive well in the second-stage assay were recovered and submitted for microsequencing.

12.6.4.5 Synthesis of the Positional Scanning Library [210]

The positional scanning hexapeptide library was composed of six sublibraries (O1XXXXX-NH$_2$, XO2XXXX-NH$_2$, XXO3XXX-NH$_2$, XXXO4XX-NH$_2$, XXXXO5X-NH$_2$, and XXXXXO6-NH$_2$), where one position (O) is individually defined with one of 19 amino acids and the remaining five positions (X) are mixtures of 19 amino acids. Thus, the entire library is made up of 114 (19 × 6) distinct peptide mixtures. Amino acids are mixed for coupling in a molar ratio that ensures equimolar incorporation [210] of amino acids into peptides (Table 12.1). One hundred fourteen polypropylene mesh bags were labeled and loaded with 400 mg 4-methylbenzhydrylamine (MBHA) resin each. Nineteen Boc-protected amino acids were activated by DIC and coupled to bags 96–114, whereas the mixture of 19 amino acids was coupled to bags 1–95. Resins bags 96–114 have a defined amino acid at position 6. The other bags have a mixture of amino acids at that site. After Boc removal, 19 individual amino acids were coupled to bags 77–95 and the amino acid mixtures to the remaining bags. Resin bags 77–95 have a defined amino acid at position 5. This procedure was repeated through to the sixth coupling. The peptides were cleaved, extracted, and lyophilized. Peptide mixtures were dissolved in water at 10–20 mg/ml and stored for 1–2 weeks at 4 °C or were frozen for prolonged storage. The higher final concentration of peptide mixtures in this library compared to the dual defined peptide library compensates for the presence of 19 times more peptides when compared to the latter peptide mixtures (five versus four mixture positions).

12.6.4.6 Synthesis of the Dual Defined Iterative Hexapeptide Library [210]

Nineteen (or any number corresponding to the number of used building blocks, depending on the capability of the chemist) individually labeled porous polypropylene mesh packets were charged with 20 g of MBHA resin each. Each of 19 of the 20 genetically coded Boc-amino acids (Cys excluded) was activated by DIC and coupled to one of the 19 resin packets. The coupling reaction was monitored for completion by using bromophenol blue or the ninhydrin test. The resin packets were washed with DCM and dried; the resins of all packets were recombined and mixed thoroughly.

This one-position resin was referred to as X-resin. The X-resin was divided into 19 equal portions and placed into new polypropylene mesh packets. The Boc group was removed with TFA/DCM (11 : 9, v/v), and the resin was washed with DCM and 2-propanol, neutralized with DIPEA/DCM (1 : 19, v/v), and washed with DCM. The 19 amino acids were activated by DIC and coupled to the resin packets to generate 361 (19^2) dipeptides. These dipeptides were termed OX-resins. Mixing of all OX-resins affords XX-resin. The coupling steps were repeated twice more to generate a 130 321 tetrapeptide mixture resin (XXXX-resin). The XXXX-resin was divided into 400 equal aliquots and placed in labeled polypropylene mesh packets. The Boc group was removed and the resin neutralized. Two amino acids were coupled to each of the packets using standard coupling procedures. The result was a hexapeptide mixture resin (O1O2XXX-resin) with two defined (O) and four mixture (X) positions. The 400 separate peptide mixtures were deprotected and cleaved using the high/low HF method in a multivessel apparatus. The peptides were extracted from the resins with water or a mixture of acetic acid and water, the solution was lyophilized twice, and peptide mixtures were dissolved in water at 1–5 mg/ml. The peptide library was stored for 1–2 weeks at 4 °C or frozen for prolonged storage. Sonication facilitates the solubilization of peptide mixtures with hydrophobic amino acids at the defined positions.

12.6.4.7 Acylation Monitoring by Bromophenol Blue [145, 146]

Couplings performed in neutral solution (DIC/HOBt, preformed anhydrides, active esters) can be monitored by addition of trace amounts of bromophenol blue. The sensitivity of the method can be significantly diminished by application of a large amount of bromophenol blue and therefore no more indicator than the amount equal to 1% of available amino groups should be applied. Usually several drops of 0.1% solution of bromophenol blue in DMF or N-methylpyrrolidone (if the dissolution provides blue solution, it can be decolorized by the addition of HOBt) are added into the last wash before coupling or directly to the solution of activated acid. Blue-colored beads turn green, greenish yellow, and eventually yellow. The speed of some couplings can be puzzling. Most are complete within 2–5 min; in library synthesis, however, care should be taken about the slowest couplings, which may require much longer exposure. Bromophenol blue monitoring allows the evaluation of coupling at the level of individual beads. In this case a sample of the reaction slurry is placed on the Petri dish and inspected under a microscope. It is easy to detect one incompletely coupled bead in the middle of tens of thousands of beads. Successful application of bromophenol blue monitoring requires the absence of quaternary ammonium salts on the resin, as resin containing these residual functionalities never becomes bromophenol blue-negative. Before using a new batch of solid support, the resin should be peracetylated (if it is not fully protected) and treated with bromophenol blue solution. If blue coloration is observable, bromophenol blue cannot be used for monitoring. In the presence of sulfonium salts (modified side-chains of Met), the resin would be greenish even without the presence of a free amino group.

References

1 Lebl, M. (1999) Parallel personal comments on "classical" papers in combinatorial chemistry. *Journal of Combinatorial Chemistry*, **1**, 3–24.

2 Geysen, H.M., Meloen, R.H., and Barteling, S.J. (1984) Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid. *Proceedings of the National Academy of Sciences of the United States of America*, **81**, 3998–4002.

3 Houghten, R.A. (1985) General method for the rapid solid-phase synthesis of large numbers of peptides: specificity of antigen–antibody interaction at the level of individual amino acids. *Proceedings of the National Academy of Sciences of the United States of America*, **82**, 5131–5135.

4 Merrifield, R.B. (1963) Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. *Journal of the American Chemical Society*, **85**, 2149–2154.

5 Merrifield, R.B. (1985) Solid phase synthesis (Nobel Lecture). *Angewandte Chemie (International Edition in English)*, **24**, 799–810.

6 Merrifield, R.B. (1993) *Life During a Golden Age of Peptide Chemistry: The Concept and Development of Solid-Phase Peptide Synthesis*, American Chemical Society, Washington, DC.

7 Letsinger, R.L. and Kornet, M.J. (1963) Popcorn polymer as a support in multistep syntheses. *Journal of the American Chemical Society*, **85**, 3045–3046.

8 Leznoff, C.C. (2000) 1999 Alfred Bader Award Lecture. From early developments in multi-step organic synthesis on solid phases to multi-nuclear phthalocyanines. *Canadian Journal of Chemistry*, **78**, 167–183.

9 Leznoff, C.C. (1978) The use of insoluble polymer supports in general organic synthesis. *Accounts of Chemical Research*, **11**, 327–333.

10 Leznoff, C.C. and Wong, J.Y. (1972) The use of polymer supports in organic synthesis. The synthesis of monotrityl ethers of symmetrical diols. *Canadian Journal of Chemistry*, **50**, 2892–2893.

11 Leznoff, C.C. and Fyles, T.M. (1976) Synthesis of insect sex attractants on solid phases. *Journal of the Chemical Society, Chemical Communications*, 251–252.

12 Burkett, B.A. (2008) Solid-phase synthesis: from revolution to evolution. *COSMOS*, **4**, 17–37.

13 Seneci, P. (2001) *Solid-Phase Synthesis and Combinatorial Technologies*, John Wiley & Sons, Inc., New York.

14 Sucholeiki, I. (2001) *High-Throughput Synthesis*, Dekker, New York.

15 Bannwarth, W. and Felder, E. (2000) *Combinatorial Chemistry: A Practical Approach*, Wiley-VCH Verlag GmbH, Weinheim.

16 Burgess, K. (2000) *Solid-Phase Organic Synthesis*, John Wiley & Sons, Inc., New York.

17 Roche Laboratories Inc. and I. Trimeris (2004) About Fuzeon. Manufacturing 2004.

18 Seneci, P. (2007) CHAPTER TITLE???, in *Combinatorial Chemistry. Comprehensive Medicinal Chemistry II* (eds B.T. John and J.T. David), Elsevier, Oxford, pp. 697–760.

19 Giralt, E., Albericio, F., Rapp, W., Stewart, J.M., Loffet, A., Guibe, F., Holmes, C.P., Rolland, M., Amblard, M., Fehrentz, J.A., Martinez, J., Rivier, J.E., Miranda, M.T.M., Otteson, K.M., Kates, S.A., Eichler, J., Houghten, R.A., and Antonenko, V. (2004) Synthesis of peptides on solid supports, in *Synthesis of Peptides and Peptidomimetics*, vol. **E22a** (eds M. Goodman, A. Felix, L. Moroder, and C. Toniolo), Thieme, Stuttgart, pp. 665–877.

20 Hudson, D. (1999) Matrix assisted synthetic transformations: a mosaic of diverse contributions. II. The pattern is completed. *Journal of Combinatorial Chemistry*, **1**, 403–457.

21 Hudson, D. (1999) Matrix assisted synthetic transformations: a mosaic of diverse contributions. I. The pattern emerges. *Journal of Combinatorial Chemistry*, **1**, 333–360.

22 Houghten, R.A., Pinilla, C., Blondelle, S.E., Appel, J.R., Dooley, C.T., and Cuervo, J.H. (1991) Generation and use

Q9

Q10

of synthetic peptide combinatorial libraries for basic research and drug discovery. *Nature*, **354**, 84–86.

23 Lam, K.S., Salmon, S.E., Hersh, E.M., Hruby, V.J., Kazmierski, W.M., and Knapp, R.J. (1991) A new type of synthetic peptide library for identifying ligand-binding activity. *Nature*, **354**, 82–84.

24 Furka, A., Sebestyen, F., Asgedom, M., and Dibo, G. (1988) Cornucopia of peptides by synthesis, in *Highlights of Modern Biochemistry, Proceedings of the 14th International Congress of Biochemistry* (ed. A. Kotyk), VSP, Utrecht, p. 47.

25 Furka, A., Sebestyen, F., Asgedom, M., and Dibo, G. (1991) General method for rapid synthesis of multicomponent peptide mixtures. *International Journal of Peptide and Protein Research*, **37**, 487–493.

26 Geysen, H.M., Armstrong, R.W., and Kay, B.K. (1995) *Chemical Diversity: The Synthetic Techniques of Combinatorial Chemistry*, American Chemical Society, Washington, DC.

27 Houghten, R.A. (1995) *Combinatorial Libraries: The Theory and Practice of Combinatorial Chemistry*, ESCOM, Leiden.

28 Abelson, J.N. (1996) *Combinatorial Chemistry*, Academic Press, San Diego, CA.

29 Cortese, R. (1996) *Combinatorial Libraries: Synthesis, Screening and Application Potential*, de Gruyter, New York.

30 Jung, G. (1996) *Combinatorial Peptide and Nonpeptide Libraries: A Handbook*, VCH, New York.

31 Krchňák, V. and Lebl, M. (1996) Synthetic library techniques: Subjective (biased and generic) thoughts and views. *Molecular Diversity*, **1**, 193–216.

32 Rinnova, M. and Lebl, M. (1996) Molecular diversity and libraries of structures: synthesis and screening. *Collection of Czechoslovak Chemical Communications*, **61**, 171–231.

33 Cabilly, S. (1997) *Combinatorial Peptide Library Protocols*, Humana Press, Totowa, NJ.

34 Czarnik, A.W. and DeWitt, S.H. (1997) *A Practical Guide to Combinatorial Chemistry*, American Chemical Society, Washington, DC.

35 Wilson, S.R. and Czarnik, A.W. (1997) *Combinatorial Chemistry. Synthesis and Applications*, John Wiley & Sons, Inc., New York.

36 Cargill, J.F. and Lebl, M. (1997) New methods in combinatorial chemistry: robotics and parallel synthesis. *Current Opinion in Chemical Biology*, **1**, 67–71.

37 Lebl, M. and Krchňák, V. (1997) Synthetic peptide libraries. *Methods in Enzymology*, **289**, 336–392.

38 Bunin, B.A. (1998) *The Combinatorial Index*, Academic Press, San Diego, CA.

39 Gordon, E.M. and Kerwin, J.F.J. (1998) *Combinatorial Chemistry and Molecular Diversity in Drug Discovery*, John Wiley & Sons, Inc., New York.

40 Terrett, N.K. (1998) *Combinatorial Chemistry*, Oxford University Press, New York.

41 Jung, G. (1999) *Combinatorial Chemistry: Synthesis, Analysis, Screening*, Wiley-VCH Verlag GmbH, Weinheim.

42 Miertus, S. and Fassina, G. (1999) *Combinatorial Chemistry and Technology. Principles, Methods, and Applications*, Dekker, New York.

43 Lebl, M. (1999) Solid-phase synthesis of combinatorial libraries. *Current Opinion in Drug Discovery and Development*, **2**, 385–395.

44 Schreiber, S.L. (2000) Target-oriented and diversity-oriented organic synthesis in drug discovery. *Science*, **287**, 1964–1969.

45 Dorwald, F.Z. (2000) *Organic Synthesis on Solid Phase: Supports, Linkers, Reactions*, Wiley-VCH Verlag GmbH, Weinheim.

46 Fenniri, H. (2000) *Combinatorial Chemistry: A Practical Approach*, Oxford University Press, Oxford.

47 Koch, J. and Mahler, M. (2002) *Peptide Arrays on Membrane Supports*, Springer, Berlin.

48 Eichler, J. and Houghten, R.A. (2004) Combinatorial synthesis, in *Synthesis of Peptides and Peptidomimetics*, vol. **E22a** (eds. M. Goodman, A. Felix, L. Moroder, and C. Toniolo), Thieme, Stuttgart, pp. 844–866.

49 Eichler, J. (2005) Synthetic peptide arrays and peptide combinatorial libraries for the exploration of protein–ligand interactions and the

design of protein inhibitors. *Combinatorial Chemistry & High Throughput Screening*, **8**, 135–143.

50 Shin, D.S., Kim, D.H., Chung, W.J., and Lee, Y.S. (2005) Combinatorial solid phase peptide synthesis and bioassays. *Journal of Biochemistry and Molecular Biology*, **38**, 517–525.

51 Lebl, M. and Hachmann, J.P. (2005) CHAPTER TITLE???, in *High-Throughput Peptide Synthesis* (ed. J. Howl), Humana Press, Totowa, NJ, pp. 167–194.

52 Marasco, D., Perretta, G., Sabatella, M., and Ruvo, M. (2008) Past and future perspectives of synthetic peptide libraries. *Current Protein and Peptide Science*, **9**, 447–467.

53 Villar, H.O. and Hansen, M.R. (2009) Design of chemical libraries for screening. *Expert Opinion on Drug Discovery*, **4**, 1215–1220.

54 Boschetti, E. and Righetti, P.G. (2009) The art of observing rare protein species in proteomes with peptide ligand libraries. *Proteomics*, **9**, 1492–1510.

55 Boschetti, E., Bindschedler, L.V., Tang, C., Fasoli, E., and Righetti, P.G. (2009) Combinatorial peptide ligand libraries and plant proteomics: a winning strategy at a price. *Journal of Chromatography A*, **1216**, 1215–1222.

56 Righetti, P.G., Boschetti, E., Zanella, A., Fasoli, E., and Citterio, A. (2010) Plucking, pillaging and plundering proteomes with combinatorial peptide ligand libraries. *Journal of Chromatography A*, **1217**, 893–900.

57 Merrifield, R.B. and Stewart, J.M. (1965) Automated peptide synthesis. *Nature*, **207**, 522–523.

58 Merrifield, R.B., Stewart, J.M., and Jernberg, N. (1966) Instrument for automated synthesis of peptides. *Analytical Chemistry*, **38**, 1905–1914.

59 Frank, R., Heikens, W., Heisterberg-Moutsis, G., and Blocker, H. (1983) A new general approach for the simultaneous chemical synthesis of large numbers of oligonucleotides: segmental solid supports. *Nucleic Acids Research*, **11**, 4365–4377.

60 Geysen, H.M., Rodda, S.J., and Mason, T.J. (1986) A *priori* delineation of a peptide which mimics a discontinuous antigenic

determinant. *Molecular Immunology*, **23**, 709–715.

61 Frank, R. and Doring, R. (1988) Simultaneous multiple peptide synthesis under continuous flow conditions on cellulose paper discs as segmental solid supports. *Tetrahedron*, **44**, 6031–6040.

62 Houghten, R.A. (1987) Is automated peptide synthesis slowing the advance of biochemical research? *Trends in Biotechnology*, **5**, 322–324.

63 Houghten, R.A., DeGraw, S.T., Bray, M.K., Hoffmann, S.R., and Frizzell, N.D. (1986) Simultaneous multiple peptide synthesis: the rapid preparation of large numbers of discrete peptides for biological, immunological, and methodological studies. *BioTechniques*, **4**, 522–528.

64 Lebl, M., Stierandova, A., Eichler, J., Pátek, M., Pokorny, V., Jehnicka, J., Mudra, P., Zenisek, K., and Kalousek, J. (1992) An automated multiple solid phase peptide synthesizer utilizing cotton as a carrier, in *Innovation and Perspectives in Solid Phase Peptide Synthesis* (ed. R. Epton), Intercept, Andover, pp. 251–257.

65 Pokorny, V., Mudra, P., Jehnicka, J., Zenisek, K., Pavlik, M., Voburka, Z., Rinnova, M., Stierandova, A., Lucka, A.W., Eichler, J., Houghten, R.A., and Lebl, M. (1994) Compas 242. New type of multiple peptide synthesizer utilizing cotton and tea bag technology, in *Innovation and Perspectives in Solid Phase Synthesis* (ed. R. Epton), Mayflower, Birmingham, pp. 643–648.

66 Bray, A.M., Maeji, N.J., and Geysen, H.M. (1990) The simultaneous multiple production of solution phase peptides; assessment of the Geysen method of simultaneous peptide synthesis. *Tetrahedron Letters*, **31**, 5811–5814.

67 Geysen, H.M. (1990) Molecular technology: peptide epitope mapping and the pin technology. *Southeast Asian Journal of Tropical Medicine and Public Health*, **21**, 523–533.

68 Maeji, N.J., Bray, A.M., and Geysen, H.M. (1990) Multi-pin peptide synthesis strategy for T cell determinant analysis. *Journal of Immunological Methods*, **134**, 23–33.

69 Bray, A.M., Maeji, N.J., Valerio, R.M., Campbell, R.A., and Geysen, H.M. (1991) Direct cleavage of peptides from a solid support into aqueous buffer. Application in simultaneous multiple peptide synthesis. *Journal of Organic Chemistry*, **56**, 6659–6666.

70 Bray, A.M., Maeji, N.J., Jhingran, A.G., and Valerio, R.M. (1991) Gas phase cleavage of peptides from a solid support with ammonia vapour. Application in simultaneous multiple peptide synthesis. *Tetrahedron Letters*, **32**, 6163–6166.

71 Valerio, R.M., Benstead, M., Bray, A.M., Campbell, R.A., and Maeji, N.J. (1991) Synthesis of peptide analogues using the multipin peptide synthesis method. *Analytical Biochemistry*, **197**, 168–177.

72 Rodda, S.J., Mason, T.J., and Maeji, N.J. (1993) Multipin technology in the preparation and screening of peptide libraries. *Australasian Biotechnology*, **3**, 346–347.

73 Valerio, R.M., Bray, A.M., Campbell, R.A., Dipasquale, A.J., Margellis, C., Rodda, S.J., Geysen, H.M., and Maeji, N.J. (1993) Multipin peptide synthesis at the micromole scale using 2-hydroxyethyl methacrylate grafted polyethylene supports. *International Journal of Peptide and Protein Research*, **42**, 1–9.

74 Bray, A.M., Jhingran, A.G., Valerio, R.M., and Maeji, N.J. (1994) Simultaneous multiple synthesis of peptide amides by the multipin method. Application of vapor-phase ammonolysis. *Journal of Organic Chemistry*, **59**, 2197–2203.

75 Bray, A.M., Lagniton, L.M., Valerio, R.M., and Maeji, N.J. (1994) Sonication-assisted cleavage of hydrophobic peptides. Application in multipin peptide synthesis. *Tetrahedron Letters*, **35**, 9079–9082.

76 Maeji, N.J., Valerio, R.M., Bray, A.M., Campbell, R.A., and Geysen, H.M. (1994) Grafted supports used with the multipin method of peptide synthesis. *Reactive Polymers*, **22**, 203–212.

77 Valerio, R.M., Bray, A.M., and Maeji, N.J. (1994) Multiple peptide synthesis on acid-labile handle derivatized polyethylene supports. *International Journal of Peptide and Protein Research*, **44**, 158–165.

78 Bray, A.M., Valerio, R.M., Dipasquale, A.J., Greig, J., and Maeji, N.J. (1995) Multiple synthesis by the multipin method as a methodological tool. *Journal of Peptide Science*, **1**, 80–87.

79 Dapremont, L., Valerio, R.M., Bray, A.M., Stewart, K.M., Mason, T.J., Wang, A.W., and Maeji, N.J. (1995) Multiple synthesis using the multipin method. *Physiological Chemistry and Physics and Medical NMR*, **27**, 339–343.

80 Maeji, N.J., Bray, A.M., Valerio, R.M., and Wang, W. (1995) Larger scale multipin peptide synthesis. *Peptide Research*, **8**, 33–38.

81 Rasoul, F., Ercole, F., Pham, Y., Bui, C.T., Wu, Z., James, S.N., Trainor, R.W., Wickham, G., and Maeji, N.J. (2000) Grafted supports in solid-phase synthesis. *Biopolymers (Peptide Science)*, **55**, 207–216.

82 Parsons, J.G., Sheehan, C.S., Wu, Z., James, I.W., and Bray, A.M. (2003) A review of solid-phase organic synthesis on SynPhase™ lanterns and SynPhase™ crowns, in *Methods in Enzymology: Combinatorial Chemistry, Part B* (eds G.A. Morales and B.A. Bunin), Academic Press, New York, pp. 39–74.

83 Ede, N.J. (2002) Multiple parallel synthesis of peptides on SynPhase™ grafted supports. *Journal of Immunological Methods*, **267**, 3–11.

84 Bray, A.M., Valerio, R.M., and Maeji, N.J. (1993) Cleavage of resin-bound peptide esters with ammonia vapour. Simultaneous multiple synthesis of peptide amides. *Tetrahedron Letters*, **34**, 4411–4414.

85 Lebl, M., Krchňák, V., Ibrahim, G., Pires, J., Burger, C., Ni, Y., Chen, Y., Podue, D., Mudra, P., Pokorny, V., Poncar, P., and Zenisek, K. (1999) Solid-phase synthesis of large tetrahydroisoquinolinone arrays by two different approaches. *Synthesis*, 1971–1978.

86 Krchňák, V., Weichsel, A.S., Lebl, M., and Felder, S. (1997) Automated solid-phase organic synthesis in micro-plate wells. Synthesis of N-(alkoxy-acyl)amino alcohols. *Bioorganic and Medicinal Chemistry Letters*, **7**, 1013–1016.

87 Lebl, M., Pokorny, V., and Krchňák, V. (2005) Simple tools for resin distribution.

Journal of Combinatorial Chemistry, **7**, 42–45.

88 Lebl, M. (1999) New technique for high-throughput synthesis. *Bioorganic and Medicinal Chemistry Letters*, **9**, 1305–1310.

89 Lebl, M. (1998) A new approach to automated solid phase synthesis based on centrifugation of tilted plates. *Journal of the Association for Laboratory Automation*, **3**, 59–61.

90 Hachmann, J.P. and Lebl, M. (2006) Search for optimal coupling reagent in multiple peptide synthesizer. *Biopolymers (Peptide Science)*, **84**, 340–347.

91 Lebl, M. (2003) Centrifugation based automated synthesis technologies. *Journal of the Association for Laboratory Automation*, **8**, 30–36.

92 Hachmann, J.P. and Lebl, M. (2006) Alternative to piperidine in Fmoc solid phase synthesis. *Journal of Combinatorial Chemistry*, **8**, 149.

93 Lebl, M., Pistek, C., Hachmann, J.P., Mudra, P., Pesek, V., Pokorny, V., Poncar, P., and Zenisek, K. (2007) Economical parallel oligonucleotide and peptide synthesizer – Pet Oligator. *International Journal of Peptide Research and Therapeutics*, **13**, 367–375.

94 Lebl, M. and Eichler, J. (1989) Simulation of continuous solid phase synthesis: synthesis of methionine enkephalin and its analogs. *Peptide Research*, **2**, 297–300.

95 Lebl, M. and Heiner, D. (2008) US Patent 7,390,459.

96 Houghten, R.A., Bray, M.K., DeGraw, S.T., and Kirby, C.J. (1986) Simplified procedure for carrying out simultaneous hydrogen fluoride cleavages of protected peptide resins. *International Journal of Peptide and Protein Research*, **27**, 673–678.

97 Kerschen, A., Kanizsai, A., Botros, I., and Krchňák, V. (1999) Apparatus and method for cleavage of compounds from solid support by gaseous reagents. *Journal of Combinatorial Chemistry*, **1**, 480–484.

98 Lebl, M., Pires, J., Poncar, P., and Pokorny, V. (1999) Evaluation of gaseous hydrogen fluoride as a convenient reagent for parallel cleavage from the solid support. *Journal of Combinatorial Chemistry*, **1**, 474–479.

99 Li, Y., Yu, Y., Giulianotti, M., and Houghten, R.A. (2008) High throughput synthesis of peptide alpha-thioesters through the use of "volatilizable" support. *Journal of Combinatorial Chemistry*, **10**, 613–616.

100 Houghten, R.A. and Yu, Y. (2005) "Volatilizable" supports for high-throughput organic synthesis. *Journal of the American Chemical Society*, **127**, 8582–8583.

101 Moran, E.J., Sarshar, S., Cargill, J.F., Shahbaz, M.M., Lio, A., Mjalli, A.M.M., and Armstrong, R.W. (1995) Radio frequency tag encoded combinatorial library method for the discovery of tripeptide-substituted cinnamic acid inhibitors of the protein tyrosine phosphatase PTP1B. *Journal of the American Chemical Society*, **117**, 10787–10788.

102 Nicolaou, K.C., Xiao, X.Y., Parandoosh, Z., Senyei, A., and Nova, M.P. (1995) Radiofrequency encoded combinatorial chemistry. *Angewandte Chemie (International Edition in English)*, **34**, 2289–2291.

103 Xiao, X., Zhao, C., Potash, H., and Nova, M.P. (1997) Combinatorial chemistry with laser optical encoding. *Angewandte Chemie (International Edition in English)*, **36**, 780–782.

104 Xiao, X.Y. and Nova, M.P. (1997) Radiofrequency encoding and additional techniques for the structure elucidation of synthetic combinatorial libraries, in *Combinatorial Chemistry: Synthesis and Application* (eds S.R. Wilson and A.W. Czarnik), John Wiley & Sons, Inc., New York, pp. 135–152.

105 Pregibon, D.C., Toner, M., and Doyle, P.S. (2007) Multifunctional encoded particles for high-throughput biomolecule analysis. *Science*, **315**, 1393–1396.

106 Nicewarner-Pena, S.R., Freeman, R.G., Reiss, B.D., He, L., Pena, D.J., Walton, I.D., Cromer, R., Keating, C.D., and Natan, M.J. (2001) Submicrometer metallic barcodes. *Science*, **294**, 137–141.

107 Sha, M.Y., Walton, I.D., Norton, S.M., Taylor, M., Yamanaka, M., Natan, M.J., Xu, C., Drmanac, S., Huang, S., Borcherding, A., Drmanac, R., and Penn, S.G. (2006) Multiplexed SNP genotyping

using nanobarcode particle technology. *Analytical and Bioanalytical Chemistry*, **384**, 658–666.

108 Evans, M., Sewter, C., and Hill, E. (2003) An encoded particle array tool for multiplex bioassays. *Assay and Drug Development Technologies*, **1**, 199–207.

109 Zhi, Z.L., Morita, Y., Hasan, Q., and Tamiya, E. (2003) Micromachining microcarrier-based biomolecular encoding for miniaturized and multiplexed immunoassay. *Analytical Chemistry*, **75**, 4125–4131.

110 Nicolaou, K.C., Pfefferkorn, J.A., Mitchell, H.J., Roecker, A.J., Barluenga, S., Cao, G.Q., Affleck, R.L., and Lillig, J.E. (2000) Natural product-like combinatorial libraries based on privileged structures. 2. Construction of a 10 000-membered benzopyran library by directed split-and-pool chemistry using NanoKans and optical encoding. *Journal of the American Chemical Society*, **122**, 9954–9967.

111 Xiao, X.Y., Li, R.S., Zhuang, H., Ewing, B., Karunaratne, K., Lillig, J., Brown, R., and Nicolaou, K.C. (2000) Solid-phase combinatorial synthesis using MicroKan reactors, Rf tagging, and directed sorting. *Biotechnology and Bioengineering*, **71**, 44–50.

112 Pátek, M., Šafář, P., Smrcina, M., Wegrzyniak, E., Bjergarde, K., Weichsel, A., and Strop, P. (2004) 2D and 3D spatially addressed arrays for high-throughput automated synthesis of combinatorial libraries. *Journal of Combinatorial Chemistry*, **6**, 43–49.

113 Krchňák, V., Vágner, J., and Hirsch, I. (1988) Simultaneous synthesis of sequence unrelated peptides derived from proteins of human papilomaviruses. *Collection of Czechoslovak Chemical Communications*, **53**, 2645.

114 Krchňák, V., Vágner, J., and Mach, O. (1989) Multiple continuous-flow solid-phase peptide synthesis. Synthesis of an HIV antigenic peptide and its omission analogues. *International Journal of Peptide and Protein Research*, **33**, 209–213.

115 Krchňák, V. and Vágner, J. (1990) Color-monitored solid-phase multiple peptide synthesis under low-pressure continuous flow conditions. *Peptide Research*, **3**, 182–193.

116 Vágner, J., Krchňák, V., Picha, J., Pichova, D., and Fusek, M. (1993) Color-monitored solid-phase multiple peptide synthesis under low-pressure continuous-flow conditions. Synthesis of medium-size peptides: the propart of human procathepsin D and the growth-hormone releasing factor. *Collection of Czechoslovak Chemical Communications*, **58**, 435–444.

117 Krchňák, V., Cabel, D., and Lebl, M. (1996) MARS: multiple automated robotic synthesizer for continuous flow of peptides. *Peptide Research*, **9**, 45–49.

118 Krchňák, V. and Padera, V. (1998) The domino blocks: a simple solution for parallel solid-phase organic synthesis. *Bioorganic & Medicinal Chemistry Letters*, **8**, 3261–3264.

119 Atrash, B., Bradley, M., Kobylecki, R., Cowell, D., and Reader, J. (2001) Revolutionizing resin handling for combinatorial synthesis. *Angewandte Chemie International Edition*, **40**, 938–941.

120 Beattie, K.L. and Fowler, R.F. (1991) Solid-phase gene assembly. *Nature*, **352**, 548–549.

121 Bouillon, I., Soural, M., and Krchňák, V. (2008) Resin capsules: permeable containers for parallel/combinatorial solid-phase organic synthesis. *Journal of Combinatorial Chemistry*, **10**, 714–720.

122 Krchňák, V., Smith, J., and Vágner, J. (2001) Solid-phase traceless synthesis of selected nitrogen-containing heterocyclic compounds. The encore technique for directed sorting of modular solid support. *Collection of Czechoslovak Chemical Communications*, **66**, 1078–1106.

123 Smith, J.M., Gard, J., Cummings, W., Kanizsai, A., and Krchňák, V. (1999) Necklace-coded polymer-supported combinatorial synthesis of 2-arylaminobenzimidazoles. *Journal of Combinatorial Chemistry*, **1**, 368–370.

124 Furka, A., Dibo, G., and Gombosuren, N. (2005) Preparation of cherry-picked combinatorial libraries by string synthesis. *Current Drug Discovery Technologies*, **2**, 23–27.

125 Furka, A., Christensen, J.W., and Healy, E. (2003) Split–mix synthesis using macroscopic solid support units, in

Methods in Enzymology: Combinatorial Chemistry, Part B (eds G.A. Morales and B.A. Bunin), Academic Press, New York, pp. 99–112.

126 Furka, A., Christensen, J.W., Healy, E., Tanner, H.R., and Saneii, H.H. (2000) String synthesis. A spatially addressable split procedure. *Journal of Combinatorial Chemistry*, **2**, 220–223.

127 Krchňák, V. and Padera, V. (2003) The Encore technique: a novel approach to directed split-and-pool combinatorial synthesis, in *Methods in Enzymology: Combinatorial Chemistry, Part B* (eds G.A. Morales and B.A. Bunin), Academic Press, New York, pp. 112–124.

128 Berg, R.H., Almdal, K., Pedersen, W.B., Holm, A., Tam, J.P., and Merrifield, R.B. (1989) Long-chain polystyrene-grafted polyethylene film matrix: a new support for solid-phase peptide synthesis. *Journal of the American Chemical Society*, **111**, 8024–8026.

129 Hird, N., Hughes, I., Hunter, D., Morrison, M.G.J.T., Sherrington, D.C., and Stevenson, L. (1999) Polymer discs – an alternative support format for solid phase synthesis. *Tetrahedron*, **55**, 9575–9584.

130 Daniels, S.B., Bernatowicz, M.S., Coull, J.M., and Koster, H. (1989) Membranes as solid supports for peptide synthesis. *Tetrahedron Letters*, **30**, 4345–4348.

131 Eichler, J., Beyermann, M., and Bienert, M. (1989) Application of cellulose paper as support material in simultaneous solid-phase peptide synthesis. *Collection of Czechoslovak Chemical Communications*, **54**, 1746–1752.

132 Eichler, J., Bienert, M., Stierandova, A., and Lebl, M. (1991) Evaluation of cotton as a carrier for solid phase peptide synthesis. *Peptide Research*, **4**, 296–307.

133 Schmidt, M., Eichler, J., Odarjuk, J., Krause, E., Beyermann, M., and Bienert, M. (1993) Multiple peptide synthesis using cellulose-based carriers: synthesis of substance P – diastereoisomers and their histamine-releasing activity. *Bioorganic & Medicinal Chemistry Letters*, **3**, 441–446.

134 Xiang, B., Lam, K.S., and Sun, G. (2009) Functional fibrous polypropylene solid support and its application in solid phase peptide synthesis and cell specific binding. *Reactive and Functional Polymers*, **69**, 905–914.

135 Rinnova, M., Jezek, J., Malon, P., and Lebl, M. (1993) Comparative multiple synthesis of fifty linear peptides: evaluation of cotton carrier vs T bag-benzhydrylamine resin. *Peptide Research*, **6**, 88–94.

136 Lebl, M. (1998) Solid-phase synthesis on planar supports. *Biopolymers (Peptide Science)*, **47**, 397–404.

137 Staňková, M., Wade, S., Lam, K.S., and Lebl, M. (1994) Synthesis of combinatorial libraries with only one representation of each structure. *Peptide Research*, **7**, 292–298.

138 Dittrich, F., Tegge, W., and Frank, R. (1998) "Cut and combine": an easy membrane-supported combinatorial synthesis technique. *Bioorganic and Medicinal Chemistry Letters*, **8**, 2351–2356.

139 Houseman, B.T., Huh, J.H., Kron, S.J., and Mrksich, M. (2002) Peptide chips for the quantitative evaluation of protein kinase activity. *Nat Biotechnol*, **20**, 270–274.

140 Uttamchandani, M., Chan, E.W., Chen, G.Y., and Yao, S.Q. (2003) Combinatorial peptide microarrays for the rapid determination of kinase specificity. *Bioorganic and Medicinal Chemistry Letters*, **13**, 2997–3000.

141 Uttamchandani, M. and Yao, S.Q. (2008) Peptide microarrays: next generation biochips for detection, diagnostics and high-throughput screening. *Current Pharmaceutical Design*, **14**, 2428–2438.

142 Min, D.H. and Mrksich, M. (2004) Peptide arrays: towards routine implementation. *Current Opinion in Chemical Biology*, **8**, 554–558.

143 Breitling, F., Nesterov, A., Stadler, V., Felgenhauer, T., and Bischoff, F.R. (2009) High-density peptide arrays. *Molecular BioSystems*, **5**, 224–234.

144 Frank, R. (1992) SPOT synthesis: an easy technique for the positionally addressable, parallel chemical synthesis on a membrane support. *Tetrahedron*, **48**, 9217–9232.

145 Krchňák, V., Vágner, J., Šafář, P., and Lebl, M. (1988) Noninvasive continuous monitoring of solid phase peptide

synthesis by acid–base indicator. *Collection of Czechoslovak Chemical Communications*, **53**, 2542–2548.

146 Krchňák, V., Vágner, J., and Lebl, M. (1988) Noninvasive continuous monitoring of solid phase peptide synthesis by acid–base indicator (preliminary communication). *International Journal of Peptide and Protein Research*, **32**, 415–416.

147 Adler, S., Frank, R., Lanzavecchia, A., and Weiss, S. (1994) T cell epitope analysis with peptides simultaneously synthesized on cellulose membranes: fine mapping of two DQ dependent epitopes. *FEBS Letters*, **352**, 167–170.

148 Frank, R. (1995) Simultaneous and combinatorial chemical synthesis techniques for the generation and screening of molecular diversity. *Journal of Biotechnology*, **41**, 259–272.

149 Martens, W., Greiser-Wilke, I., Harder, T.C., Dittmar, K., Frank, R., Orvell, C., Moennig, V., and Liess, B. (1995) Spot synthesis of overlapping peptides on paper membrane supports enables the identification of linear monoclonal antibody binding determinants on morbillivirus phosphoproteins. *Veterinary Microbiology*, **44**, 289–298.

150 Frank, R. and Overwin, H. (1996) SPOT synthesis. Epitope analysis with arrays of synthetic peptides prepared on cellulose membranes. *Methods in Molecular Biology*, **66**, 149–169.

151 Frank, R., Hoffmann, S., Kiess, M., Lahmann, H., Tegge, W., Behn, C., and Gausepohl, H. (1996) Combinatorial synthesis on membrane supports by the SPOT technique: imaging peptide sequence and shape space, in *Combinatorial Peptide and Nonpeptide Libraries: A Handbook* (ed. G. Jung), VCH, New York, pp. 363–386.

152 Frank, R. (1997) Multiple peptide synthesis with SPOT technique, in *Immunology Methods Manual* (ed. I. Lefkovits), Academic Press, San Diego, CA, pp. 783–795.

153 Frank, R. (2002) The SPOT-synthesis technique. Synthetic peptide arrays on membrane supports – principles and applications. *Journal of Immunological Methods*, **267**, 13–26.

154 Reineke, U., Volkmer-Engert, R., and Schneider-Mergener, J. (2001) Application of peptide arrays prepared by the SPOT-technology. *Current Opinion in Biotechnology*, **12**, 59–64.

155 Heine, N., Ast, T., Schneider-Mergener, J., Reineke, U., Germeroth, L., and Wenschuh, H. (2003) Synthesis and screening of peptoid arrays on cellulose membranes. *Tetrahedron*, **59**, 9919–9930.

156 Reimer, U., Reineke, U., and Schneider-Mergener, J. (2002) Peptide arrays: from macro to micro. *Current Opinion in Biotechnology*, **13**, 315–320.

157 Kramer, A., Reineke, U., Dong, L., Hoffmann, B., Hoffmüller, U., Winkler, D., Volkmer-Engert, R., and Schneider-Mergener, J. (1999) Spot synthesis: observations and optimizations. *Journal of Peptide Research*, **54**, 319–327.

158 Schneider-Mergener, J., Kramer, A., and Reineke, U. (1996) Peptide libraries bound to continuous cellulose membranes: tools to study molecular recognition, in *Combinatorial Libraries: Synthesis, Screening and Application Potential* (ed. R. Cortese), de Gruyter, Berlin, pp. 53–68.

159 Wenschuh, F.H., Gausepohl, H., Germeroth, L., Ulbricht, M., Matuschewski, H., Kramer, A., Volkmer-Engert, R., Heine, N., Ast, T., Scharn, D., and Schneider-Mergener, J. (2000) Positionally addressable parallel synthesis on continuous membranes, in *Combinatorial Chemistry: A Practical Approach* (ed. H. Fenniri), Oxford University Press, Oxford, pp. 95–116.

160 Fodor, S.P.A., Read, J.L., Pirrung, M.C., Stryer, L., Lu, A.T., and Solas, D. (1991) Light-directed, spatially addressable parallel chemical synthesis. *Science*, **251**, 767–773.

161 Holmes, C.P., Adams, C.L., Fodor, S.P.A., and Yu-Yang, P. (1992) New techniques in random screening: the use of light-directed spatially addressable chemical synthesis, in *Perspectives in Medicinal Chemistry* (eds B. Testa, E. Kyburz, W. Fuher, and R. Giger), VHCA, Basel, pp. 489–500.

162 Rozsnyai, L.F., Benson, D.R., Fodor, S.P.A., and Schultz, P.G. (1992) Photolitographic immobilization of

biopolymers on solid supports. *Angewandte Chemie (International Edition in English)*, **31**, 759–761.

163 Cho, C.Y., Moran, E.J., Cherry, S.R., Stephans, J.C., Fodor, S.P.A., Adams, C.L., Sundaram, A., Jacobs, J.W., and Schultz, P.G. (1993) An unnatural biopolymer. *Science*, **261**, 1303–1305.

164 Fodor, S.P.A., Rava, R.P., Huang, X.C., Pease, A.C., Holmes, C.P., and Adams, C.L. (1993) Multiplexed biochemical assays with biological chips. *Nature*, **364**, 555–556.

165 Gallop, M.A., Barrett, R.W., Dower, W.J., Fodor, S.P.A., and Gordon, E.M. (1994) Applications of combinatorial technologies to drug discovery. 1. Background and peptide combinatorial libraries. *Journal of Medicinal Chemistry*, **37**, 1233–1251.

166 Gordon, E.M., Barrett, R.W., Dower, W.J., Fodor, S.P.A., and Gallop, M.A. (1994) Applications of combinatorial technologies to drug discovery. 2. Combinatorial organic synthesis, library screening strategies, and future directions. *Journal of Medicinal Chemistry*, **37**, 1385–1401.

167 Jacobs, J.W. and Fodor, S.P.A. (1994) Combinatorial chemistry: applications of light-directed chemical synthesis. *Trends in Biotechnology*, **12**, 19–26.

168 Pease, A.C., Solas, D., Sullivan, E.J., Cronin, M.T., Holmes, C.P., and Fodor, S.P.A. (1994) Light-generated oligonucleotide arrays for rapid DNA sequence analysis. *Proceedings of the National Academy of Sciences of the United States of America*, **91**, 5022–5026.

169 Fodor, S.P.A. (1997) Massively parallel biochemical assays. *Laboratory Automation News*, **2**, 50–51.

170 Singh-Gasson, S., Green, R.D., Yue, Y., Nelson, C., Blattner, F., Sussman, M.R., and Cerrina, F. (1999) Maskless fabrication of light-directed oligonucleotide microarrays using a digital micromirror array. *Nature Biotechnology*, **17**, 974–978.

171 Shin, D.S., Lee, K.N., Yoo, B.W., Kim, J., Kim, M., Kim, Y.K., and Lee, Y.S. (2010) Automated maskless photolithography system for peptide microarray synthesis

on a chip. *Journal of Combinatorial Chemistry*, **12**, 463–471.

172 Gao, X., Yu, P., LeProust, E., Sonigo, L., Pellois, J.P., and Zhang, H. (1998) Oligonucleotide synthesis using solution photogenerated acids. *Journal of the American Chemical Society*, **120**, 12698–12699.

173 Pellois, J.P., Wang, W., and Gao, X. (2000) Peptide synthesis based on t-Boc chemistry and solution photogenerated acids. *Journal of Combinatorial Chemistry*, **2**, 355–360.

174 Leproust, E., Pellois, J.P., Yu, P., Zhang, H., Gao, X., Srivannavit, O., Gulari, E., and Zhou, X. (2000) Digital light-directed synthesis. A microarray platform that permits rapid reaction optimization on a combinatorial basis. *Journal of Combinatorial Chemistry*, **2**, 349–354.

175 Gao, X., LeProust, E., Zhang, H., Srivannavit, O., Gulari, E., Yu, P., Nishiguchi, C., Xiang, Q., and Zhou, X. (2001) A flexible light-directed DNA chip synthesis gated by deprotection using solution photogenerated acids. *Nucleic Acids Research*, **29**, 4744–4750.

176 Li, S., Marthandan, N., Bowerman, D., Garner, H.R., and Kodadek, T. (2005) Photolithographic synthesis of cyclic peptide arrays using a differential deprotection strategy. *Chemical Communications*, 581–583.

177 Li, S., Bowerman, D., Marthandan, N., Klyza, S., Luebke, K.J., Garner, H.R., and Kodadek, T. (2004) Photolithographic synthesis of peptoids. *Journal of the American Chemical Society*, **126**, 4088–4089.

178 Maurer, K., McShea, A., Strathmann, M., and Dill, K. (2005) The removal of the t-BOC group by electrochemically generated acid and use of an addressable electrode array for peptide synthesis. *Journal of Combinatorial Chemistry*, **7**, 637–640.

179 Egeland, R.D. and Southern, E.M. (2005) Electrochemically directed synthesis of oligonucleotides for DNA microarray fabrication. *Nucleic Acids Research*, **33**, e125.

180 Maurer, K., Cooper, J., Caraballo, M., Crye, J., Suciu, D., Ghindilis, A., Leonetti, J.A., Wang, W., Rossi, F.M., Stover, A.G.,

Larson, C., Gao, H., Dill, K., and McShea, A. (2006) Electrochemically generated acid and its containment to 100 micron reaction areas for the production of DNA microarrays. *PLoS ONE*, **1**, e34.

181 Beyer, M., Nesterov, A., Block, I., Konig, K., Felgenhauer, T., Fernandez, S., Leibe, K., Torralba, G., Hausmann, M., Trunk, U., Lindenstruth, V., Bischoff, F.R., Stadler, V., and Breitling, F. (2007) Combinatorial synthesis of peptide arrays onto a microchip. *Science*, **318**, 1888.

182 Beyer, M., Felgenhauer, T., Bischoff, F.R., Breitling, F., and Stadler, V. (2006) A novel glass slide-based peptide array support with high functionality resisting non-specific protein adsorption. *Biomaterials*, **27**, 3505–3514.

183 Stadler, V., Beyer, M., Konig, K., Nesterov, A., Torralba, G., Lindenstruth, V., Hausmann, M., Bischoff, F.R., and Breitling, F. (2007) Multifunctional CMOS microchip coatings for protein and peptide arrays. *Journal of Proteome Research*, **6**, 3197–3202.

184 Stadler, V., Felgenhauer, T., Beyer, M., Fernandez, S., Leibe, K., Guttler, S., Groning, M., Konig, K., Torralba, G., Hausmann, M., Lindenstruth, V., Nesterov, A., Block, I., Pipkorn, R., Poustka, A., Bischoff, F.R., and Breitling, F. (2008) Combinatorial synthesis of peptide arrays with a laser printer. *Angewandte Chemie International Edition*, **47**, 7132–7135.

185 Beyer, M., Block, I., Konig, K., Nesterov, A., Fernandez, S., Felgenhauer, T., Schirwitz, C., Leibe, K., Bischoff, R.F., Breitling, F., and Stadler, V. (2009) A novel combinatorial approach to high-density peptide arrays. *Methods in Molecular Biology*, **570**, 309–316.

186 Breitling, F., Felgenhauer, T., Nesterov, A., Lindenstruth, V., Stadler, V., and Bischoff, F.R. (2009) Particle-based synthesis of peptide arrays. *ChemBioChem*, **10**, 803–808.

187 Felgenhauer, T., Stadler, V., Bischoff, R., and Breitling, F. (2008) Peptide chip: Highly complex peptide arrays from microchips. *BIOspektrum*, **14**, 151–153.

188 Schirwitz, C., Block, I., Konig, K., Nesterov, A., Fernandez, S., Felgenhauer, T., Leibe, K., Torralba, G., Hausmann, M.,

Lindenstruth, V., Stadler, V., Breitling, F., and Bischoff, F.R. (2009) Combinatorial peptide synthesis on a microchip. *Current Protocols in Protein Science*, **18**, 2.13–2.13.

189 Sebestyen, F., Dibo, G., Kovacs, A., and Furka, A. (1993) Chemical synthesis of peptide libraries. *Bioorganic and Medicinal Chemistry Letters*, **3**, 413–418.

190 Furka, A. and Bennett, W.D. (1999) Combinatorial libraries by portioning and mixing. *Combinatorial Chemistry and High Throughput Screening*, **2**, 105–122.

191 Furka, A., Hamaker, L.K., and Peterson, M.L. (2000) Synthesis of combinatorial libraries using the portioning–mixing procedure, in *Combinatorial Chemistry: A Practical Approach* (ed. H. Fenniri), Oxford University Press, Oxford, pp. 1–32.

192 Houghten, R.A., Appel, J.R., Blondelle, S.E., Cuervo, J.H., Dooley, C.T., and Pinilla, C. (1992) The use of synthetic peptide combinatorial libraries for the identification of bioactive peptides. *BioTechniques*, **13**, 412–421.

193 Houghten, R.A. (1993) The broad utility of soluble peptide libraries for drug discovery. *Gene*, **137**, 7–11.

194 Houghten, R.A., Dinh, K.T., Burcin, D.E., and Blondelle, S.E. (1993) The systematic development of peptides having potent antimicrobial activity against E. coli through the use of synthetic peptide combinatorial libraries, in *Techniques in Protein Chemistry IV* (ed. R.H. Angeletti), Academic Press, San Diego, CA, pp. 249–256.

195 Houghten, R.A. (1993) Peptide libraries: criteria and trends. *Trends in Genetics*, **9**, 235–239.

196 Dooley, C.T., Chung, N.N., Wilkes, B.C., Schiller, P.W., Bidlack, J.M., Pasternak, G.W., and Houghten, R.A. (1994) An all D-amino acid opioid peptide with central analgesic activity from a combinatorial library. *Science*, **266**, 2019–2022.

197 Eichler, J., Lucka, A.W., and Houghten, R.A. (1994) Cyclic peptide template combinatorial libraries: synthesis and identification of chymotrypsin inhibitors. *Peptide Research*, **7**, 300–307.

198 Houghten, R.A. (1994) Combinatorial libraries: finding the needle in the haystack. *Current Biology*, **4**, 564–567.

199 Houghten, R.A. (1994) Soluble combinatorial libraries: extending the range and repetoire of chemical diversity. *Methods: A Companion to Methods in Enzymology*, **6**, 354–360.

200 Houghten, R.A. (1995) The theory and practice of combinatorial chemistry. *Perspectives in Drug Discovery and Design*, **2**, 249–250.

201 Houghten, R.A., Pinilla, C., Appel, J.R., Blondelle, S.E., Dooley, C.T., Eichler, J., Nefzi, A., and Ostresh, J.M. (1999) Mixture-based synthetic combinatorial libraries. *Journal of Medicinal Chemistry*, **42**, 3743–3778.

202 Houghten, R.A., Wilson, D.B., and Pinilla, C. (2000) Drug discovery and vaccine development using mixture-based synthetic combinatorial libraries. *Drug Discovery Today*, **5**, 276–285.

203 Houghten, R.A. (2000) Parallel array and mixture-based synthetic combinatorial chemistry: tools for the next millennium. *Annual Review of Pharmacology & Toxicology*, **40**, 273–282.

204 Hoesl, C.E., Nefzi, A., Ostresh, J.M., Yu, Y., and Houghten, R.A. (2003) Mixture-based combinatorial libraries: from peptides and peptidomimetics to small molecule acyclic and heterocyclic compounds, in *Methods in Enzymology: Combinatorial Chemistry, Part B* (eds G.A. Morales and B.A. Bunin), Academic Press, New York, pp. 496–517.

205 Pinilla, C., Appel, J.R., Borras, E., and Houghten, R.A. (2003) Advances in the use of synthetic combinatorial chemistry: mixture-based libraries. *Nature Medicine*, **9**, 118–122.

206 Carroll, F.I. and Houghten, R.A. (2009) From rapid *in vitro* screening to rapid *in vivo* screening in the drug discovery process. *Neuropsychopharmacology*, **34**, 251–252.

207 Houghten, R.A., Pinilla, C., Giulianotti, M.A., Appela, J.R., Dooley, C.T., Nefzi, A., Ostresh, J.M., Yu, Y., Maggiora, G.M., Medina-Franco, J.L., Brunner, D., and Schneider, J. (2008) Strategies for the use of mixture-based synthetic combinatorial libraries: scaffold ranking, direct testing *in vivo*, and enhanced deconvolution by computational methods. *Journal of Combinatorial Chemistry*, **10**, 3–19.

208 Houghten, R.A., Dooley, C.T., and Appel, J.R. (2006) In *vitro* and direct *in vivo* testing of mixture-based combinatorial libraries for the identification of highly active and specific opiate ligands. *AAPS Journal*, **8**, E371–E382.

209 Ostresh, J.M., Winkle, J.M., Hamashin, V.T., and Houghten, R.A. (1994) Peptide libraries: determination of relative reaction rates of amino acids in competitive couplings. *Biopolymers*, **34**, 1681–1689.

210 Pinilla, C., Appel, J.R., and Houghten, R.A. (1994) Identification of antigenic determinants using synthetic peptide combinatorial libraries, in *Current Protocols in Immunology* (eds J.E. Coligan, A.M. Kruisbeek, D.H. Margulies, E.M. Shevach, and W. Strober), John Wiley & Sons, Inc., New York, pp. 9.8.1–9.8.15.

211 Ljungdahl, T., Veide-Vilg, J., Wallner, F., Tamas, M.J., and Grotli, M. (2010) Positional scanning peptide libraries for kinase substrate specificity determinations: straightforward and reproducible synthesis using pentafluorophenyl esters. *Journal of Combinatorial Chemistry*, **12**, 733–742.

212 Boutin, J.A., Gesson, I., Henlin, J.M., Bertin, S., Lambert, P.H., Volland, J.P., and Fauchere, J.L. (1997) Limitations of the coupling of amino acid mixtures for the preparation of equimolar peptide libraries. *Molecular Diversity*, **3**, 43–60.

213 Udaka, K., Wiesmuller, K.H., Kienle, S., Jung, G., and Walden, P. (1995) Decrypting the structure of major histocompatibility complex class I: restricted cytotoxic T lymphocyte epitopes with complex peptide libraries. *Journal of Experimental Medicine*, **181**, 2097–2108.

214 Kramer, A., Volkmer-Engert, R., Malin, R., Reineke, U., and Schneider-Mergener, J. (1993) Simultaneous synthesis of peptide libraries on single resin and continuous cellulose membrane supports: examples for the identification of protein, metal and DNA binding peptide mixtures. *Peptide Research*, **6**, 314–319.

215 Houghten, R.A., Dooley, C.T., and Appel, J.R. (2004) *De novo* identification of highly active fluorescent kappa opioid ligands from a rhodamine labeled tetrapeptide

positional scanning library. *Bioorganic and Medicinal Chemistry Letters*, **14**, 1947–1951.

216 Pinilla, C., Appel, J.R., Blondelle, S.E., Dooley, C.T., Eichler, J., Nefzi, A., Ostresh, J.M., Martin, R., Wilson, D.B., and Houghten, R.A. (2000) Synthesis and screening of positional scanning synthetic combinatorial libraries, in *Combinatorial Chemistry: A Practical Approach* (ed. H. Fenniri), Oxford University Press, Oxford, pp. 51–74.

217 Pinilla, C., Appel, J.R., and Houghten, R.A. (1999) Positional scanning combinatorial libraries: how and why complex mixtures work, in *Innovation and Perspectives in Solid Phase Synthesis and Combinatorial Libraries* (ed. R. Epton), Mayflower, Birmingham, pp. 159–162.

218 Rothman, R.B., Baumann, M.H., Dersch, C.M., Appel, J., and Houghten, R.A. (1999) Discovery of novel peptidic dopamine transporter ligands by screening a positional scanning combinatorial hexapeptide library. *Synapse*, **33**, 239–246.

219 Dooley, C.T. and Houghten, R.A. (1998) Synthesis and screening of positional scanning combinatorial libraries. *Methods in Molecular Biology*, **87**, 13–24.

220 Pinilla, C., Appel, J.R., and Houghten, R.A. (1996) Tea bag synthesis of positional scanning synthetic combinatorial libraries and their use for mapping antigenic determinants. *Methods in Molecular Biology*, **66**, 171–179.

221 Pinilla, C., Appel, J.R., Blondelle, S.E., Dooley, C.T., Eichler, J., Ostresh, J.M., and Houghten, R.A. (1994) Versatility of positional scanning synthetic combinatorial libraries for the identification of individual compounds. *Drug Development Research*, **33**, 133–145.

222 Dooley, C.T. and Houghten, R.A. (1993) The use of positional scanning synthetic peptide combinatorial libraries for the rapid determination of opioid receptor ligands. *Life Sciences*, **52**, 1509–1517.

223 Eichler, J. and Houghten, R.A. (1999) Scaffolded peptide combinatorial libraries, in *Innovation and Perspectives in Solid Phase Synthesis and Combinatorial Libraries* (ed. R. Epton), Mayflower, Birmingham, pp. 155–158.

224 Eichler, F. (2004) Rational and random strategies for the mimicry of discontinuous protein binding sites. *Protein and Peptide Letters*, **11**, 281–290.

225 Campian, E., Peterson, M.L., Saneii, H.H., and Furka, A. (1998) Deconvolution by omission libraries. *Bioorganic and Medicinal Chemistry Letters*, **8**, 2357–2362.

226 Deprez, B., Williard, X., Bourel, L., Coste, H., Hyafil, F., and Tartar, A. (1995) Orthogonal combinatorial chemical libraries. *Journal of the American Chemical Society*, **117**, 5405–5406.

227 Ostresh, J.M., Dörner, B., and Houghten, R.A. (1998) Peralkylation. "Libraries from libraries": chemical transformation of synthetic combinatorial libraries. *Methods in Molecular Biology*, **87**, 41–49.

228 Dörner, B., Ostresh, J.M., Husar, G.M., and Houghten, R.A. (1996) Generation of peralkylated peptidomimetic combinatorial libraries. *Methods in Molecular and Cellular Biology*, **6**, 35–40.

229 Houghten, R.A., Blondelle, S.E., Dooley, C.T., Dörner, B., Eichler, J., and Ostresh, J.M. (1996) Libraries from libraries: generation and comparison of screening profiles. *Molecular Diversity*, **2**, 41–45.

230 Ostresh, J.M., Husar, G.M., Blondelle, S.E., Dörner, B., Weber, P.A., and Houghten, R.A. (1994) "Libraries from libraries": chemical transformation of combinatorial libraries to extend the range and repertoire of chemical diversity. *Proceedings of the National Academy of Sciences of the United States of America*, **91**, 11138–11142.

231 Han, H., Wolfe, M.M., Brenner, S., and Janda, K.D. (1995) Liquid-phase combinatorial synthesis. *Proceedings of the National Academy of Sciences of the United States of America*, **92**, 6419–6423.

232 Kozlov, I.A., Melnyk, P.C., Hachmann, J.P., Srinivasan, A., Shults, M.D., Zhao, C., Musmacker, J., Nelson, N., Barker, D.L., and Lebl, M. (2008) A high-complexity, multiplexed solution-phase assay for profiling protease activity on microarrays. *Combinatorial Chemistry and High Throughput Screening*, **11**, 24–35.

233 Shults, M.D., Kozlov, I.A., Nelson, N., Kermani, B.G., Melnyk, P.C., Shevchenko, V., Srinivasan, A.,

Musmacker, J., Hachmann, J.P., Barker, D.L., Lebl, M., and Zhao, C. (2007) A multiplexed protein kinase assay. *ChemBioChem*, **8**, 933–942.

234 Diaz-Mochon, J.J., Bialy, L., and Bradley, M. (2006) Dual colour, microarray-based, analysis of 10 000 protease substrates. *Chemical Communications*, 3984–3986.

235 Bartak, Z., Bolf, J., Kalousek, J., Mudra, P., Pavlik, M., Pokorny, V., Rinnova, M., Voburka, Z., Zenisek, K., Krchňák, V., Lebl, M., Salmon, S.E., and Lam, K.S. (1994) Design and construction of the automatic peptide library synthesizer. *Methods: A Companion to Methods in Enzymology*, **6**, 432–437.

236 Lebl, M., Krchňák, V., Sepetov, N.F., Seligmann, B., Strop, P., Felder, S., and Lam, K.S. (1995) One-bead–one-structure combinatorial libraries. *Biopolymers (Peptide Science)*, **37**, 177–198.

237 Lam, K.S. and Lebl, M. (1994) Selectide technology: bead-binding screening. *Methods: A Companion to Methods in Enzymology*, **6**, 372–380.

238 Lam, K.S. and Wu, J. (1994) Selectide technology: substrate motif identification for post-translational modifications. *Methods: A Companion to Methods in Enzymology*, **6**, 401–403.

239 Lebl, M., Krchňák, V., Šafář, P., Stierandova, A., Sepetov, N.F., Kocis, P., and Lam, K.S. (1994) Construction and screening of libraries of peptide and non-peptide structures, in *Techniques in Protein Chemistry V* (ed. J.W. Crabb), Academic Press, San Diego, CA, pp. 541–548.

240 Lam, K.S. and Lebl, M. (1995) Synthesis and screening of a "one-bead–one-compound" combinatorial peptide library. *Methods in Molecular and Cellular Biology*, **6**, 15–25.

241 Lebl, M., Krchňák, V., Sepetov, N.F., Nikolaev, V., Staňková, M., Kocis, P., Pátek, M., Flegelova, Z., Ferguson, R., and Lam, K.S. (1995) Synthetic combinatorial libraries: a new tool for drug design. Methods for identifying the composition of compounds from peptide and/or nonpeptide libraries, in *Methods in Protein Structure Analysis* (eds M.Z. Atassi and E. Appella), Plenum Press, New York, pp. 335–342.

242 Chen, C.L., Strop, P., Lebl, M., and Lam, K.S. (1996) One bead–one compound combinatorial peptide library: different types of screening. *Methods in Enzymology*, **267**, 211–219.

243 Lam, K.S. and Lebl, M. (1996) Combinatorial library based on the one-bead–one-compound concept, in *Combinatorial Peptide and Nonpeptide Libraries: A Handbook* (ed. G. Jung), VCH, New York, pp. 173–201.

244 Lam, K.S., Lebl, M., and Krchňák, V. (1997) The "one-bead–one-compound" combinatorial library method. *Chemical Reviews*, **97**, 411–448.

245 Zhao, Z.G. and Lam, K.S. (1997) Synthetic peptide libraries, in *Annual Reports in Combinatorial Chemistry and Molecular Diversity*, vol. **1** (eds W.H. Moos, M.R. Pavia, B.K. Kay, and A.D. Ellington), ESCOM, Leiden, pp. 192–209.

246 Lam, K.S. and Lebl, M. (1998) Synthesis of a one-bead one-compound combinatorial peptide library. *Methods in Molecular Biology*, **87**, 1–6.

247 Seligmann, B., Lebl, M., and Lam, K.S. (1998) Solid-phase peptide synthesis, lead generation, and optimization, in *Combinatorial Chemistry and Molecular Diversity in Drug Discovery* (eds E.M. Gordon and J.F.J. Kerwin), John Wiley & Sons, Inc., New York, pp. 39–109.

248 Liu, G. and Lam, K.S. (2000) One-bead one-compound combinatorial library method, in *Combinatorial Chemistry: A Practical Approach* (ed. H. Fenniri), Oxford University Press, Oxford, pp. 33–50.

249 Lam, K.S. and Renil, M. (2002) From combinatorial chemistry to chemical microarray. *Current Opinion in Chemical Biology*, **6**, 353–358.

250 Lam, K.S., Lehman, A.L., Song, A., Doan, N., Enstrom, A.M., Maxwell, J., and Liu, R. (2003) Synthesis and screening of "one-bead one-compound" combinatorial peptide libraries, in *Methods in Enzymology: Combinatorial Chemistry, Part B* (eds G.A. Morales and B.A. Bunin), Academic Press, New York, pp. 298–322.

251 Liu, R., Marik, J., and Lam, K.S. (2003) Design, synthesis, screening, and decoding of encoded one-bead one-compound peptidomimetic and small

252 Townsend, J.B., Farzana, S., Liu, R., and Lam, K.S. (2010) Jeffamine derivatized TentaGel beads and poly (dimethylsiloxane) microbead cassettes for ultrahigh throughput *in situ* releasable solution-phase cell-based screening of one-bead–one-compound combinatorial small molecule libraries. *Journal of Combinatorial Chemistry*, **12**, 700–712.

253 Lam, K.S., Wade, S., Abdul-Latif, F., and Lebl, M. (1995) Application of a dual color detection scheme in the screening of a random combinatorial peptide library. *Journal of Immunological Methods*, **180**, 219–223.

254 Lehman, A., Gholami, S., Hahn, M., and Lam, K.S. (2006) Image subtraction approach to screening one-bead–one-compound combinatorial libraries with complex protein mixtures. *Journal of Combinatorial Chemistry*, **8**, 562–570.

255 Lathrop, J.T. and Hammond, D. (2007) The bead blot: a method for selecting small molecule ligands for protein capture and purification. *Nature Protocols*, **2**, 3102–3110.

256 Lathrop, J.T., Fijalkowska, I., and Hammond, D. (2007) The bead blot: a method for identifying ligand–protein and protein–protein interactions using combinatorial libraries of peptide ligands. *Analytical Biochemistry*, **361**, 65–76.

257 Meldal, M., Svendsen, I., Breddam, K., and Auzanneau, F.I. (1994) Portion-mixing peptide libraries of quenched fluorogenic substrates for complete subsite mapping of endoprotease specificity. *Proceedings of the National Academy of Sciences of the United States of America*, **91**, 3314–3318.

258 Meldal, M. and Svendsen, I. (1995) Direct visualization of enzyme inhibitors using a portion mixing inhibitor library containing a quenched fluorogenic peptide substrate. Part 1. Inhibitors for subtilisin Carlsberg. *Journal of the Chemical Society, Perkin Transactions*, **1**, 1591–1596.

259 Meldal, M. (1998) The solid-phase enzyme inhibitor library assay. *Methods in Molecular Biology*, **87**, 75–82.

260 Renil, M., Ferreras, M., Delaisse, J.M., Foged, N.T., and Meldal, M. (1998) PEGA supports for combinatorial peptide synthesis and solid-phase enzymatic library assays. *Journal of Peptide Science*, **4**, 195–210.

261 Spetzler, J.C., Westphal, V., Winther, J.B., and Meldal, M. (1998) Preparation of fluorescence quenched libraries containing interchain disulphide bonds for studies of protein disulphide isomerases. *Journal of Peptide Science*, **4**, 128–137.

262 Buchardt, J., Schiodt, C.B., Krog-Jensen, C., Delaisse, J.M., Foged, N.T., and Meldal, M. (2000) Solid phase combinatorial library of phosphinic peptides for discovery of matrix metalloproteinase inhibitors. *Journal of Combinatorial Chemistry*, **2**, 624–638.

263 Stennicke, H.R., Renatus, M., Meldal, M., and Salvesen, G.S. (2000) Internally quenched fluorescent peptide substrates disclose the subsite preferences of human caspases 1, 3, 6, 7 and 8. *Biochemical Journal*, **350**, 563–568.

264 Meldal, M. (2002) The one-bead two-compound assay for solid phase screening of combinatorial libraries. *Biopolymers*, **66**, 93–100.

265 Meldal, M. (2004) "One bead two compound libraries" for detecting chemical and biochemical conversions. *Current Opinion in Chemical Biology*, **8**, 238–244.

266 Wang, X., Peng, L., Liu, R., Xu, B., and Lam, K.S. (2005) Applications of topologically segregated bilayer beads in one-bead one-compound combinatorial libraries. *Journal of Peptide Research*, **65**, 130–138.

267 Debenham, S.D., Snyder, P.W., and Toone, E.J. (2003) Solid-phase synthesis for the identification of high-affinity bivalent lectin ligands. *Journal of Organic Chemistry*, **68**, 5805–5811.

268 Chen, X., Tan, P.H., Zhang, Y., and Pei, D. (2009) On-bead screening of combinatorial libraries: reduction of nonspecific binding by decreasing surface ligand density. *Journal of Combinatorial Chemistry*, **11**, 604–611.

269 Vágner, J., Barany, G., Lam, K.S., Krchňák, V., Sepetov, N.F., Ostrem, J.A.,

molecule combinatorial libraries. *Methods in Enzymology*, **369**, 271–287.

Strop, P., and Lebl, M. (1996) Enzyme-mediated spatial segregation on individual polymeric support beads: application to generation and screening of encoded combinatorial libraries. *Proceedings of the National Academy of Sciences of the United States of America*, **93**, 8194–8199.

270 Liu, R., Marik, J., and Lam, K.S. (2002) A novel peptide-based encoding system for "one-bead one-compound" peptidomimetic and small molecule combinatorial libraries. *Journal of the American Chemical Society*, **124**, 7678–7680.

271 Astle, J.M., Simpson, L.S., Huang, Y., Reddy, M.M., Wilson, R., Connell, S., Wilson, J., and Kodadek, T. (2010) Seamless bead to microarray screening: rapid identification of the highest affinity protein ligands from large combinatorial libraries. *Chemistry and Biology*, **17**, 38–45.

272 Pei, D. (2010) On-bead library screening made easier. *Chemistry and Biology*, **17**, 3–4.

273 Needels, M.C., Jones, D.G., Tate, E.H., Heinkel, G.L., Kochersperger, L.M., Dower, W.J., Barrett, R.W., and Gallop, M.A. (1993) Generation and screening of an oligonucleotide-encoded synthetic peptide library. *Proceedings of the National Academy of Sciences of the United States of America*, **90**, 10700–10704.

274 Reddy, M.M., Bachhawat-Sikder, K., and Kodadek, T. (2004) Transformation of low-affinity lead compounds into high-affinity protein capture agents. *Chemistry and Biology*, **11**, 1127–1137.

275 Kodadek, T. and Bachhawat-Sikder, K. (2006) Optimized protocols for the isolation of specific protein-binding peptides or peptoids from combinatorial libraries displayed on beads. *Molecular BioSystems*, **2**, 25–35.

276 Marani, M.M., Ceron, M.C.M., Giudicessi, S.L., De Oliveira, E., Cote, S., Erra-Balsells, R., Albericio, F., Cascone, O., and Camperi, S.A. (2009) Screening of one-bead–one-peptide combinatorial library using red fluorescent dyes. Presence of positive and false positive beads. *Journal of Combinatorial Chemistry*, **11**, 146–150.

277 Olivos, H.J., Bachhawat-Sikder, K., and Kodadek, T. (2003) Quantum dots as a visual aid for screening bead-bound combinatorial libraries. *ChemBioChem*, **4**, 1242–1245.

278 Uhl, V., Gstach, H., Seifert, J.M., Jung, T., Falchetto, R., Fetsch, A., Stange, R., Schmied, M., Graf, C., Adam, A., Amberger, F., Monteux, D., Prechelmacher, S., Pruckner, A., Dautz, W., Graf, P., Hermes, E., Van Hooft, P., Berces, A., Muller, J., and Auer, M. (2002) CONA-HTS: confocal nanoscanning–bead picking–AIDA technology. A methodology for drug discovery with orphan molecular targets and combinatorial chemistry on the solid surface. *Single Molecules*, **3**, 174–175.

279 Hintersteiner, M. and Auer, M. (2008) Single-bead, single-molecule, single-cell fluorescence: technologies for drug screening and target validation. *Annals of the New York Academy of Sciences*, **1130**, 1–11.

280 Hintersteiner, M., Buehler, C., Uhl, V., Schmied, M., Muller, J., Kottig, K., and Auer, M. (2009) Confocal nanoscanning, bead picking (CONA): pickoscreen microscopes for automated and quantitative screening of one-bead one-compound libraries. *Journal of Combinatorial Chemistry*, **11**, 886–894.

281 Hintersteiner, M., Kimmerlin, T., Kalthoff, F., Stoeckli, M., Garavel, G., Seifert, J.M., Meisner, N.C., Uhl, V., Buehler, C., Weidemann, T., and Auer, M. (2009) Single bead labeling method for combining confocal fluorescence on-bead screening and solution validation of tagged one-bead one-compound libraries. *Chemistry and Biology*, **16**, 724–735.

282 Meisner, N.C., Hintersteiner, M., Seifert, J.M., Bauer, R., Benoit, R.M., Widmer, A., Schindler, T., Uhl, V., Lang, M., Gstach, H., and Auer, M. (2009) Terminal adenosyl transferase activity of posttranscriptional regulator hur revealed by confocal on-bead screening. *Journal of Molecular Biology*, **386**, 435–450.

283 Rostovtsev, V.V., Green, L.G., Fokin, V.V., and Sharpless, K.B. (2002) A stepwise Huisgen cycloaddition process: copper (I)-catalyzed regioselective "ligation" of azides and terminal alkynes. *Angewandte*

Chemie International Edition, **41**, 2596–2599.

284 Tornoe, C.W., Christensen, C., and Meldal, M. (2002) Peptidotriazoles on solid phase: [1,2,3]-triazoles by regiospecific copper(I)-catalyzed 1,3-dipolar cycloadditions of terminal alkynes to azides. *Journal of Organic Chemistry*, **67**, 3057–3064.

285 Sepetov, N.F., Krchňák, V., Staňková, M., Wade, S., Lam, K.S., and Lebl, M. (1995) Library of libraries: approach to synthetic combinatorial library design and screening of "pharmacophore" motifs. *Proceedings of the National Academy of Sciences of the United States of America*, **92**, 5426–5430.

286 Youngquist, R.S., Fuentes, G.R., Lacey, M.P., and Keough, T. (1995) Generation and screening of combinatorial peptide libraries designed for rapid sequencing by mass spectrometry. *Journal of the American Chemical Society*, **117**, 3900–3906.

287 Youngquist, R.S., Fuentes, G.R., Lacey, M.P., Keough, T., and Baillie, T.A. (1994) Matrix-assisted laser desorption ionization for rapid determination of the sequences of biologically active peptides isolated from support-bound combinatorial peptide libraries. *Rapid Communications in Mass Spectrometry*, **8**, 77–81.

288 Thakkar, A., Cohen, A.S., Connolly, M.D., Zuckermann, R.N., and Pei, D. (2009) High-throughput sequencing of peptoids and peptide–peptoid hybrids by partial Edman degradation and mass spectrometry. *Journal of Combinatorial Chemistry*, **11**, 294–302.

289 Sweeney, M.C. and Pei, D. (2003) An improved method for rapid sequencing of support-bound peptides by partial Edman degradation and mass spectrometry. *Journal of Combinatorial Chemistry*, **5**, 218–222.

290 Wang, P., Arabaci, G., and Pei, D. (2001) Rapid sequencing of library-derived peptides by partial Edman degradation and mass spectrometry. *Journal of Combinatorial Chemistry*, **3**, 251–254.

291 Keough, T., Youngquist, R.S., and Lacey, M.P. (1999) A method for high-sensitivity peptide sequencing using postsource decay matrix-assisted laser desorption ionization mass spectrometry. *Proceedings of the National Academy of Sciences of the United States of America*, **96**, 7131–7136.

292 Paulick, M.G., Hart, K.M., Brinner, K.M., Tjandra, M., Charych, D.H., and Zuckermann, R.N. (2006) Cleavable hydrophilic linker for one-bead–one-compound sequencing of oligomer libraries by tandem mass spectrometry. *Journal of Combinatorial Chemistry*, **8**, 417–426.

293 Wang, X., Zhang, J., Song, A., Lebrilla, C.B., and Lam, K.S. (2004) Encoding method for OBOC small molecule libraries using a biphasic approach for ladder-synthesis of coding tags. *Journal of the American Chemical Society*, **126**, 5740–5749.

294 Franz, A.H., Liu, R., Song, A., Lam, K.S., and Lebrilla, C.B. (2003) High-throughput one-bead–one-compound approach to peptide-encoded combinatorial libraries: MALDI-MS analysis of single TentaGel beads. *Journal of Combinatorial Chemistry*, **5**, 125–137.

295 Song, A., Zhang, J., Lebrilla, C.B., and Lam, K.S. (2003) A novel and rapid encoding method based on mass spectrometry for "one-bead–one-compound" small molecule combinatorial libraries. *Journal of the American Chemical Society*, **125**, 6180–6188.

296 Czarnik, A.W. (1997) Encoding strategies in combinatorial chemistry. *Proceedings of the National Academy of Sciences of the United States of America*, **94**, 12738–12739.

297 Czarnik, A.W. (1997) Encoding methods for combinatorial chemistry. *Current Opinion in Chemical Biology*, **1**, 60–66.

298 Barnes, C. and Balasubramanian, S. (2000) Recent developments in the encoding and deconvolution of combinatorial libraries. *Current Opinion in Chemical Biology*, **4**, 346–350.

299 Uffman, E.W. and Gilbertson, S.R. (2002) A novel peptide-based encoding system for "one-bead one-compound" peptidomimetic and small molecule combinatorial libraries. *Chemtracts*, **15**, 606–610.

300 Swali, V. and Bradley, M. (1997) Analytical techniques for single bead analysis in combinatorial chemistry. *Analytical Communications*, **34**, 15H–18H.

301 Swali, V., Langley, G.J., and Bradley, M. (1999) Mass spectrometric analysis in combinatorial chemistry. *Current Opinion in Chemical Biology*, **3**, 337–341.

302 Marani, M.M., Oliveira, E., Cote, S., Camperi, S.A., Albericio, F., and Cascone, O. (2007) Identification of protein-binding peptides by direct matrix-assisted laser desorption ionization time-of-flight mass spectrometry analysis of peptide beads selected from the screening of one bead–one peptide combinatorial libraries. *Analytical Biochemistry*, **370**, 215–222.

303 Marani, M.M., Paradis-Bas, M., Tulla-Puche, J., Cote, S., Camperi, S.A., Cascone, O., and Albericio, F. (2007) From the one-bead–one-compound concept to one-bead–one-reactor. *Journal of Combinatorial Chemistry*, **9**, 395–398.

304 Sepetov, N.F., Issakova, O.L., Lebl, M., Swiderek, K., Stahl, D.C., and Lee, T.D. (1993) The use of hydrogen–deuterium exchange to facilitate peptide sequencing by electrospray tandem mass spectrometry. *Rapid Communications in Mass Spectrometry*, **7**, 58–62.

305 Juskowiak, G.L., McGee, C.J., Greaves, J., and Van Vranken, D.L. (2008) Synthesis, screening, and sequencing of cysteine-rich one-bead one-compound peptide libraries. *Journal of Combinatorial Chemistry*, **10**, 726–731.

306 Brown, J.M., Hoffmann, W.D., Alvey, C.M., Wood, A.R., Verbeck, G.F., and Petros, R.A. (2010) One-bead, one-compound peptide library sequencing via high-pressure ammonia cleavage coupled to nanomanipulation/nanoelectrospray ionization mass spectrometry. *Analytical Biochemistry*, **398**, 7–14.

307 Salmon, S.E., Lam, K.S., Lebl, M., Kandola, A., Khattri, P.S., Wade, S., Pátek, M., Kocis, P., Krchňák, V., Thorpe, D., and Felder, S. (1993) Discovery of biologically active peptides in random libraries: solution-phase testing after staged orthogonal release from resin beads. *Proceedings of the National Academy of Sciences of the United States of America*, **90**, 11708–11712.

308 Kocis, P., Krchňák, V., and Lebl, M. (1993) Symmetrical structure allowing the selective multiple release of a defined quantity of peptide from a single bead of polymeric support. *Tetrahedron Letters*, **34**, 7251–7252.

309 Lebl, M., Pátek, M., Kocis, P., Krchňák, V., Hruby, V.J., Salmon, S.E., and Lam, K.S. (1993) Multiple release of equimolar amounts of peptides from a polymeric carrier using orthogonal linkage–cleavage chemistry. *International Journal of Peptide and Protein Research*, **41**, 201–203.

310 Pátek, M. and Lebl, M. (1998) Safety-catch and multiply cleavable linkers in solid-phase synthesis. *Biopolymers (Peptide Science)*, **47**, 353–363.

311 Salmon, S.E., Liu-Stevens, R.H., Zhao, Y., Lebl, M., Krchňák, V., Wertman, K., Sepetov, N., and Lam, K.S. (1996) High-volume cellular screening for anticancer agents with combinatorial chemical libraries: a new methodology. *Molecular Diversity*, **2**, 57–63.

312 Jayawickreme, C.K., Quillan, J.M., Graminski, G.F., and Lerner, M.R. (1994) Discovery and structure–function analysis of α-melanocyte-stimulating hormone antagonists. *Journal of Biological Chemistry*, **269**, 29846–29854.

313 Jayawickreme, C.K., Graminski, G.F., Quillan, J.M., and Lerner, M.R. (1994) Creation and functional screening of a multi-use peptide library. *Proceedings of the National Academy of Sciences of the United States of America*, **91**, 1614–1618.

314 Jayawickreme, C.K., Sauls, H., Bolio, N., Ruan, J., Moyer, M., Burkhart, W., Marron, B., Rimele, T., and Shaffer, J. (1999) Use of a cell-based, lawn format assay to rapidly screen a 442,368 bead-based peptide library. *Journal of Pharmacological and Toxicological Methods*, **42**, 189–197.

315 Rodda, S., Tribbick, G., and Geysen, M. (1996) Epitope mapping with the use of peptide libraries, in *Combinatorial Peptide and Nonpeptide Libraries: A Handbook* (ed G. Jung), VCH, New York, pp. 303–326.

316 Albericio, F. and Barany, G. (1985) Improved approach for anchoring N^α-9-fluorenylmethyloxycarbonylamino acids as *p*-alkoxybenzyl esters in solid-phase peptide synthesis. *International Journal of Peptide and Protein Research*, **26**, 92–97.

317 Lebl, M., Krchňák, V., Salmon, S.E., and Lam, K.S. (1994) Screening of completely random one-bead one-peptide libraries for activities in solution. *Methods: A Companion to Methods in Enzymology*, **6**, 381–387.

Abstract

Techniques of parallel and combinatorial synthesis of peptides are reviewed together with methods of their testing and screening. Methods for structure elucidation of compounds identified in combinatorial mixtures are discussed. The chapter also contains experimental details for selected techniques.

Author Query

1. Please check the order/numbering of ALL section headings.

2. Editor: Which chapter? ('another chapter'?)

3. Which San Diego company?

4. Check units: meq/gm or meq/g?

5. The symbol $<$ means that amino acid Gly is bound in a reverse orientation (NH2 on right COOH on the left).

6. The symbol $<$ means that amino acid Gly is bound in a reverse orientation (NH2 on right COOH on the left).

7. Check definitions of ACN and CAN

8. Check layout of all display equations

9. Supply full published publication details for [17] (or delete from reference list)

10. Supply chapter title for [18]

11. Supply chapter title for [51]

12. Check definition of OPC?