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The "One-Bead-One-Compound" Combinatorial Library Method

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I. Introduction

During the last six years, combinatorial chemistry has attracted enormous attention from the pharmaceutical industry because it can greatly facilitate the drug discovery process.[1] At the present time, virtually every major pharmaceutical company has a form of combinatorial chemistry program included in their drug discovery effort. Combinatorial chemistry is regarded as one of the most important recent advances in medicinal chemistry. In addition, it is also an extremely powerful tool in basic research.

Combinatorial library methods were first applied to peptides[2−5] and oligonucleotides.[6−12] Since then, the field has been expanded to include proteins,[13] synthetic oligomers,[14,15] small molecules,[16] and oligosaccharides.[17−19] Figure 1 summarizes the various types of combinatorial libraries. The method of library preparation is dependent on the type of library desired. For instance, peptide libraries can be synthesized either chemically[4,5] or biologically[3] using, for example, a phage display approach. Protein libraries on the other hand are prepared primarily by biological methods. Small molecules, chemical oligomers, and oligosaccharide libraries are generally prepared by synthetic approaches. In general, the synthetic combinatorial libraries are prepared on a solid phase, a technique first described by Merrifield for synthesis of peptides.[20−22] However, with the advances in enzyme-catalyzed chemical reactions, some of these libraries can, in principle, be prepared by a biosynthetic approach.[23] Polynucleotide libraries are usually prepared chemically but amplification by PCR is necessary for further enrichment.

Many reviews of combinatorial chemistry are available. Only the most recent ones are quoted here.[16,24−32] The comprehensive coverage of the literature pub-

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Kit S. Lam was born in Hong Kong in 1954. He received his B.A. degree in microbiology from the University of Texas at Austin in 1975, his Ph.D. degree in oncology from the University of Wisconsin, Madison, in 1980, where he worked on phosphorylation of intracellular membranes, and structure and function of nuclear envelope. He then attended Stanford University School of Medicine where he developed and patented a heteromyeloma cell line fusion partner for human monoclonal antibody production. He received his M.D. degree from Stanford in 1984. He then did his residency training in internal medicine as well as medical oncology fellowship training at the University of Arizona, where he is currently a faculty member. He is an associate professor of medicine, microbiology, and immunology; a member of the Arizona Cancer Center; and a practicing medical oncologist. He invented the "one-bead-one-compound" library method. He is one of the founding scientists of Selectide Corporation. His laboratory is engaged in the development and application of the "one-bead-one-compound" combinatorial library method.

Viktor Krchňák was born in Brno, Moravia, in 1947. He studied chemistry at the Faculty of Natural Sciences of the Masaryk University in Brno. He received his Ph.D. in organic chemistry from the Institute of Organic Chemistry and Biochemistry of the Czechoslovak Academy of Sciences in Prague, under the supervision of Professor Zdenek Arnold. He then continued working at this Institute in the laboratory of peptides, his main focus being solid-phase peptide synthesis. In 1983 he joined the peptide research department in Leciva Pharmaceuticals, Prague, where he initiated the research program of synthetic antigens, focusing on diagnostic kits using peptides for antibody detection. In 1992 he joined Selectide Corporation, Tucson, AZ, turning his attention to the emerging new field of combinatorial chemistry. Currently he is at Houghten Pharmaceuticals, San Diego, developing new approaches to automation of combinatorial solid-phase synthesis. His research interest covers combinatorial chemistry in general and solid-phase chemistry in particular. He admires gothic architecture, renaissance devices, baroque music, impressionist painting, and modern science.

Michal Lebl was born in Prague, Czechoslovakia, in 1951. He obtained M.S. degree in organic chemistry in 1974 from Technical University of Chemical Technology, Prague. In 1974 he joined Institute of Organic Chemistry and Biochemistry of the Czechoslovak Academy of Sciences and under the supervision of Dr. Jost he obtained his Ph.D. degree in 1978. He continued working in the same institute for another 13 years. During this time he worked (as postdoctoral associate, visiting professor, and consultant) in the laboratory of Professor Hruby in the University of Arizona (1983, 1986, 1989). In 1992 he defended the title D.Sc. His achievements in the peptide chemistry were rewarded by the Leonidas Zervas award of the European Peptide Society in 1990. In 1991 he became director of chemistry in small startup company Selectide Corporation. His task was to build the laboratories, hire the people, validate the library technology invented by Dr. Lam, and continue the development of the technology. Due to a very strict budget, he hired many scientists from former eastern Europe, built the laboratories from kitchen furniture, and equipped it with used instrumentation. However, Selectide was able to show that the technology is successfully working in the real target situation, develop the techniques for screening in solution, and transform the peptide technology to nonpeptide one. Selectide was one of the first of the combinatorial chemistry-based companies to identify lead, which is now being considered as a good clinical candidate. The company was sold to Marion Merrell Dow in 1995. Michal Lebl and Viktor Krchňák left the company in 1996 to join Houghten Pharmaceuticals to develop automation of combinatorial solid-phase synthesis. Michal Lebl has published over 200 scientific papers and coauthored more than 30 patents. He is a member of the board of *Collection of Czechoslovak Chemical Communication*, *International Journal of Peptide and Protein Research*, *Peptide Research*, and *Molecular Diversity* and he is responsible for the Internet version of the last journal. He is a member of Czechoslovak Chemical Society, European Peptide Society, American Peptide Society, American Chemical Society, American Go Association, and Societas Amicorum Bamae. He enjoys photography, playing Go, and the works of Jara da Cimrman.

lished in the field of molecular diversity can be found in the dynamic database available on the Internet.[33] One review discussing aspects of library techniques in a form of a debate of two imaginary persons appeared recently in the new journal dedicated to molecular diversity.[34]

All combinatorial library methods involve three main steps: (i) preparation of the library, (ii) screening of the library components, and (iii) determination of the chemical structures of active compounds. There are five distinct combinatorial library methods: (i) biological library methods such as phage displayed,[3,35,36] plasmid,[37] or polysomes;[38] (ii) spatially addressable library approaches such as the multipin system,[39] multiple synthetic techniques using segmentable carriers,[40,41] SPOT synthesis on cellulose paper or polymeric membrane,[42,43] light-directed synthesis on chips,[44] or diversomer technology;[45] (iii) combinatorial library methods that require deconvolution—the iterative approach,[5,46] positional scanning,[47] recursive deconvolution,[48] and orthogonal partition;[49] (iv) the one-bead-one-compound combinatorial library method;[4,50] and (v) synthetic solution library methods—affinity chromatography selection,[51,52] affinity capillary electrophoresis,[53] or affinity-directed mass spectroscopic techniques.[54] The biological library approach has thus been applied only to peptides and proteins, and in general includes only

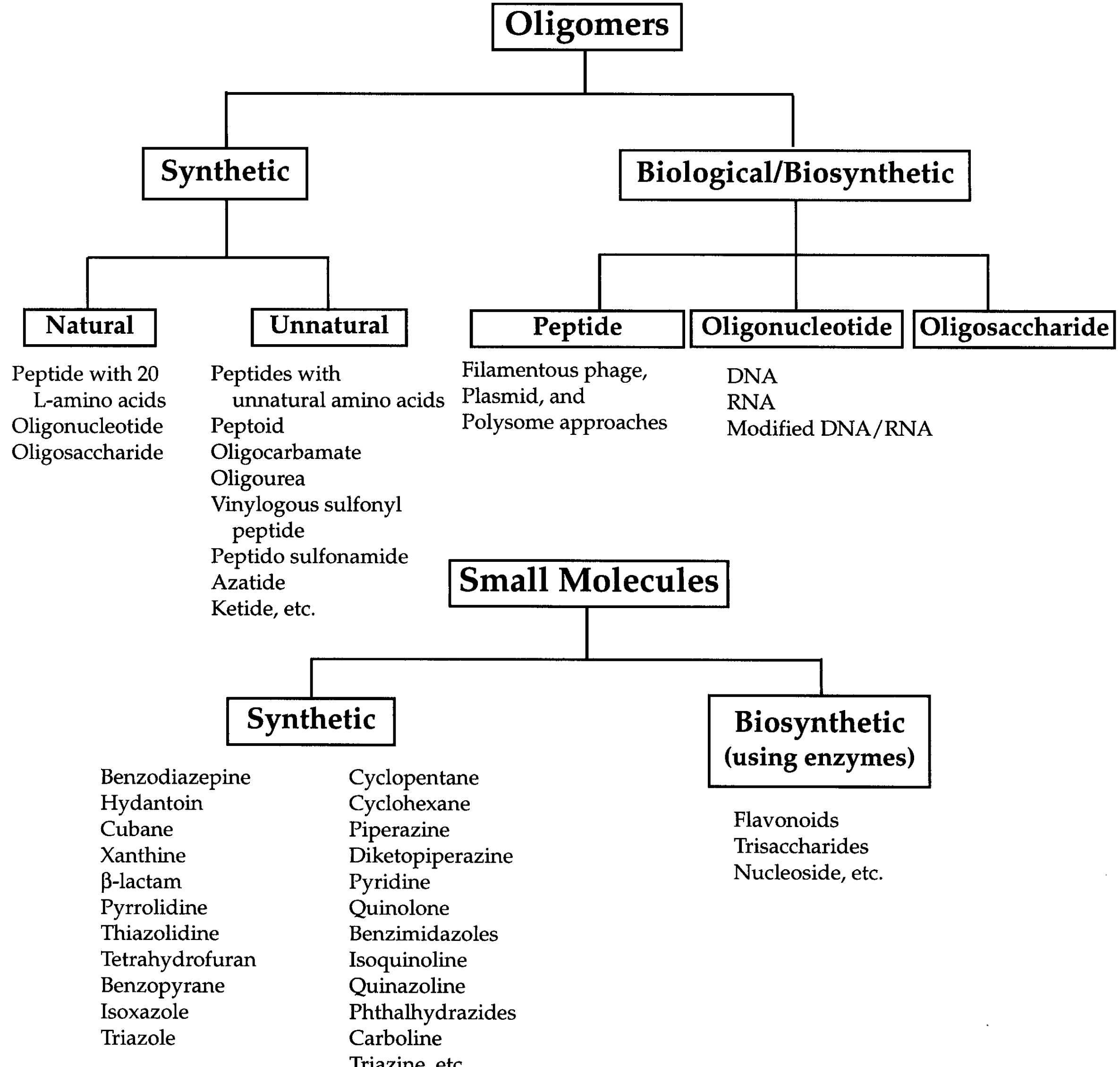

Figure 1. Various library types.

the 20 eukaryotic amino acids. In principle, unnatural amino acids could be incorporated into peptide libraries using the polysome approach; however, this remains to be proven. The remaining four methods involve synthetic or biosynthetic chemistry and may incorporate peptides with unnatural amino acids, chemical oligomers, oligosaccharides, oligonucleotides, and small molecules into the libraries. There are advantages and disadvantages of each of the above combinatorial library approaches. The method chosen depends largely on the nature of the target, the assay system, and the resources and the expertise available in a specific laboratory. In some instances, a combination of approaches may be appropriate.

The unique features of the one-bead-one-compound combinatorial library approach are (i) the synthesis of a large library is rapid using the "split synthesis" approach, (ii) the compounds are spatially separable and therefore all the compounds can be tested concurrently but independently, (iii) once identified, the chemical structure of the compound on a positive bead may be determined directly or by an encoding strategy, and (iv) both on-bead binding or functional assays and solution-phase assays can be used by this approach. The one-bead-one-compound library method has been recently reviewed.[55,56] In this article, we will give a brief discussion of the one-bead-one-compound concept. Following this will be a detailed account of the chemistry used for library synthesis (applicable, but not always actually applied to one-bead-one-compound libraries), various screening methods available, and methods for structure determination of identified hits. Lastly, successful applications of the one-bead-one-compound library method on various biological and chemical systems will be given.

II. The One-Bead-One-Compound Concept

The one-bead-one-compound concept first recognized by Lam et al.[4] is based on the fact that combinatorial bead libraries, prepared via a "split synthesis" approach (Figure 2), contain single beads displaying only one type of compound although there may be up to 10^{13} copies of the same compound on a single 100 μm diameter bead. The "split synthesis" method was first described by Furka et al.[57,58] in two abstracts in 1988. A mixture of 27 tetrapeptides was prepared, and the peptides were cleaved from the resin and analyzed by paper electrophoresis and Edman degradation. In 1991, Furka et al.[59] described the use of "portioning mixing" or "split

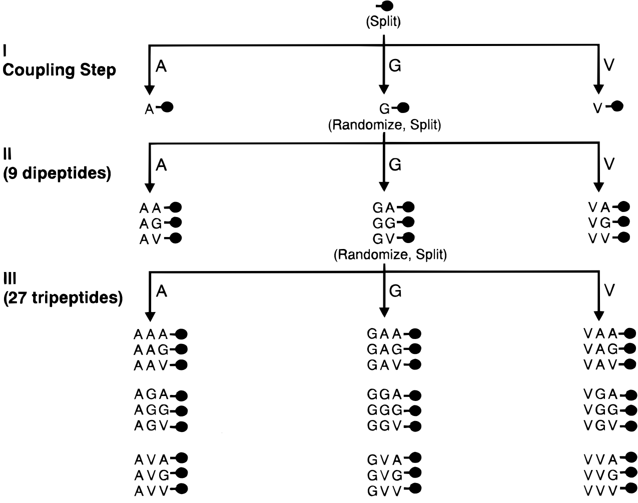

Figure 2. Scheme of the "split synthesis" method. (Adapted with permission from *Nature* **1991**, *354*, 82. Copyright 1991 MacMillan Magazines Limited.)

synthesis" in the synthesis of 27 tetrapeptides and 180 pentapeptides, along with their analysis using paper electrophoresis and HPLC. The authors proposed to use this approach to synthesize several peptides concurrently followed by HPLC purification as a rapid method of synthesizing multiple peptides. They also proposed to prepare simple mixtures of peptides for biological screening. Additionally, they intended to use peptide mixtures to elucidate the factors influencing the retention times in HPLC separation. However, they did not recognize the one-bead-one-compound concept and its application to synthesis and screening of millions of different compounds while still attached to the beads.

Lam et al.[4] and Houghten et al.[5] also developed the "split synthesis" method. Lam[4] further recognized that because each bead encountered only one amino acid at each coupling cycle and that the reaction was driven to completion, only one type of peptide was displayed on and within each bead. With an appropriate detection method such as those commonly used in immunological research, one could detect and therefore isolate a peptide bead that interacts specifically with a protein target such as an antibody. This one-bead-one-compound library method was first successfully applied on over a million peptide beads to isolate peptide ligands that interact with anti-β-endorphin monoclonal antibody and streptavidin using an enzyme-linked colorimetric detection method.[4] Even though the on-bead binding assay is highly efficient, there are some targets that require a solution-phase assay. Therefore, orthogonal cleavable linkers were developed[60,61] such that the compound from each bead could be released from the solid support and tested in solution while the beads remained spatially separated; in this way, beads that caused a positive response could be identified and isolated for structure determination.[50,62−66]

In the last six years, the one-bead-one-compound library approach has been successfully applied to many different targets including antibodies, avidin, streptavidin, proteases, protein kinases, and other enzymes, adhesion molecules, G-protein coupled receptors, artificial receptors, small organic dyes, bacteria, and even whole cells. This library approach has been applied to peptides, chemical oligomers, and small molecule libraries. Both "on-bead" binding or functional assays as well as solution-phase assays have been used to screen these libraries. Although almost all published work on the one-bead-one-compound library method deals with biological systems, this library approach can in principle be applied to physical problems. In fact, because of the spatially separable nature of this type of library, it is particularly suited for the discovery of new compounds with novel physical characteristics such as optical, electromagnetic, electrochemical, or photochemical properties. Depending on the application, the library does not necessarily need to be in the form of a bead. In some cases, a disk or thin film may be desirable. Regardless of the chemical component, the shape or nature of the solid support, or the biological or physical property of interest, the advantage of the one-bead-one-compound concept can be retained.

III. Synthesis of One-Bead-One-Compound Libraries

A. Choice of Solid Support

Synthetic approaches to one-bead-one-compound libraries are often determined by the selected polymeric carrier. A beaded polymer has to fulfill certain criteria depending on the synthetic and screening strategies. For one-bead-one-compound libraries, the size and substitution homogeneity is of the utmost importance (the quantitative evaluation of the biological signal created by a peptide released from a single bead depends on the uniformity of the amount of compound released from each bead), as well as the resin resistance to the formation of clusters (formation of clusters may prevent the statistical distribution of resin beads and may also substantially lower the number of structures created). The ability of the resin to swell in both organic and aqueous media is especially important when on-bead binding assays are used for screening. Polydimethylacrylamide beads fulfill some of the above-mentioned criteria and this resin was originally used for evaluation of technology feasibility.[4] Later, TentaGel, polyoxyethylene-grafted polystyrene,[67] became the resin of choice for solid-phase library (both peptide and organic) synthesis due to its uniformity in size as well as its nonstickiness. PEG-PS resin,[68] developed by Barany, has a similar composition and properties as TentaGel, the major difference being the placement of the chemically reactive groups (amino groups), in relation to the polystyrene matrix. TentaGel has the functionalizable group at the end of polyoxyethylene chains, therefore far from the hydrophobic polystyrene chain (this feature is especially important for compound display on the bead surface for bead-binding assay). In contrast, PEG-PS has the functional group next to the polystyrene chain, and the polyoxyethylene chain in this case does not serve as the "linker" connecting the synthetic compound with the polymer, but rather as a "modifier" of polymer properties. A new support introduced by Argonaut Technologies (ArgoGel)[69] differs from the TentaGel by branching of the polyoxyethylene chain at the attachment point to the polystyrene core—this modification effectively doubles the resulting substitution level (0.4 mmol/g). A new polymeric carrier described

by Meldal et al.,[70-72] based on copolymerization of acrylamide and polyoxyethylene, seems to be very promising, as does a recently described highly cross-linked hydrophilic polymer.[73]

Although classical resin beads are more suited for the one-bead-one-compound library approach, alternative carriers such as grafted polypropylene and cellulose membranes can also be used in a one-particle-one-compound library format. The application of some of these carriers to the technique of one-bead-one-compound is not obvious—we may have to redefine the technique as one-location-one-compound, but let us mention some of these carriers for completeness. Polyacrylamide-grafted polypropylene "pins" were used for the synthesis of the first library.[74] This type of support was shown to be very useful for multiple peptide synthesis[75] and was adapted to a larger scale by the application of "crowns" attached to the pins;[76] it was later shown to be applicable for nonpeptide syntheses.[77] Cellulose paper was shown by Frank to be a good support for the multiple ("SPOT") synthesis of thousands of peptides[43] or for the synthesis of libraries.[43,78] Synthesis on paper is performed by "spotting" the solution of protected amino acids onto the functionalized cellulose paper in the presence of an activating reagent. Here, the reaction vessel is the carrier itself and liquid manipulation during the synthesis (usually shaking in the case of solid-phase synthesis) is eliminated; the reaction is driven by diffusion of the liquid in the carrier. This principle of internal volume synthesis was tested using polymeric carriers on a multiple synthesizer utilizing centrifugation for liquid elimination,[79] and was found comparable, if not better than the classical arrangement of solid-phase peptide synthesis.[80] The purest form of cellulose, cotton, was found to be a convenient solid-phase support, especially for multiple synthesis,[81] or library generation.[82] A specific advantage of membrane, sheet, or thread-like carriers is ease of divisibility. This feature can be used for the synthesis of libraries with a nonstatistical distribution of library members.[83] The synthesis of this library starts with n pieces of the carrier which are coupled with n different building blocks (first randomization). Each of the n pieces is then divided into m parts and these smaller parts are distributed into m reaction vessels in which m reactions are performed (second randomization). The process can be repeated as many times as required within the physical limits of handling polymer particles. This process produces a library of $n \times m \times ... = X$ compounds on X polymeric particles, where no compound is missing and none is represented more than once. This is the classical one-particle-one-compound technique. If the individual pieces are large enough to be individually labeled, the structure determination step can be omitted.

B. Peptide Libraries

Solid-phase peptide synthesis lent itself to the combinatorial approach since the available collection of amino acids represented a manageably large collection of subunits, and solid-phase amide bond-forming chemistry was well developed. There are, however, significant differences between synthesizing a single peptide and synthesizing a million peptides incorporating both readily reacted amino acids and difficult combinations of amino acids, amines, and carboxylic acids and other commercially available or internally available building blocks used to extend the diversity incorporated into a library.

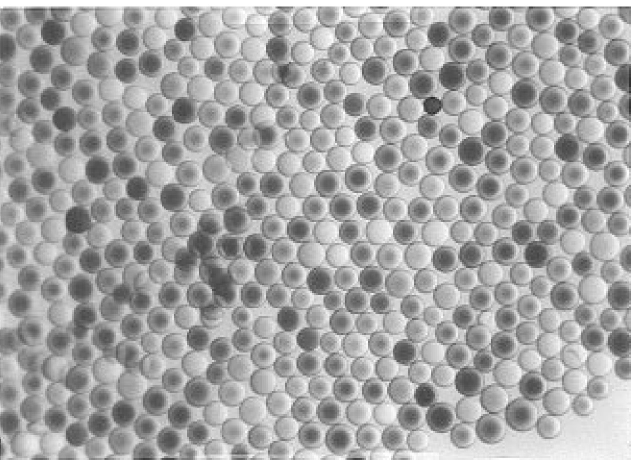

Figure 3. Monitoring of coupling reaction at the level of individual beads. A library of tetrapeptides with free amino terminal amino groups was treated with a solution of bromophenol blue[90]—all beads were uniformly colored. A sample of the library (~20 000 beads) was placed onto a petri dish under a microscope and a solution of Fmoc-Val-OH, HOBt, and DIC was added. Progress of the reaction can be observed by the color change of individual beads. The figure shown here was taken after 5 min of reaction. The different kinetics of coupling are clearly visible, as well as spatial inequality of reaction (sites located inside the beads react much slower then those on the bead surface).

Coupling in the peptide library synthesis is performed in the usual way (within a battery of bubblers, in closed plastic vials, in polypropylene syringes,[84] or in tea bags[85]), and standard coupling reagents are used.[21,86,87] (Detailed descriptions of library synthetic procedures are available, e.g. refs 64 and 88.) Difficult couplings can be forced to completion by the application of more energetic coupling reagents such as tetramethylfluoroformamidinium hexafluorophosphate (TFFA).[89] However, due to the easy handling and simple deprotection performed in multiple vessels in parallel, the Fmoc protecting group is favored over the Boc group, which has to be deprotected by trifluoroacetic acid solution, and in the application of which, the final deprotection has to be performed by strong acids (TFMSA in TFA or HF). It is important to stress that coupling monitoring requires modified techniques. In the synthesis utilizing the "split synthesis" method,[4,5,59] every bead in the mixture may contain a different peptide sequence, and therefore coupling kinetics of each bead may be different. Since we are not interested in knowing whether the coupling reaction is complete in average, but rather whether all beads in the sample were coupled, one should follow the reaction at the level of individual beads. This can be achieved by the application of nondestructive methods such as bromophenol blue monitoring.[90] An example of such monitoring is given in Figure 3. Obviously the majority of beads are already fully coupled (clear beads); however, coupling on several beads is not complete (colored beads). The classical ninhydrin test[91] does not reveal any problems at this coupling stage.

Synthesis of libraries can be performed manually or by the application of automated instruments.[92−94] The distribution of the resin is achieved either by volume distribution of a homogeneous (nonsedimenting) suspension (suspension of beads in an isopycnic solution)[93] or a suspension continuously stirred during the distribution.[92] Another design utilizes a symmetrical distribution vessel for the distribution by sedimentation of the suspension.[94]

Equimolarity of the incorporation of the amino acid mixture into the growing peptide can be achieved in several ways. The most reliable is the split synthesis,[4,5,59] which was actually designed for the purpose of generating equimolar peptide mixtures.[59] Equimolarity, however, can be achieved only in the cases when the number of particles used for the synthesis is substantially larger then number of synthesized peptides (1 000 000 compounds cannot be synthesized on 100 000 beads). Split synthesis results in the collection of polymeric particles containing individual sequences—the basis of the one-bead-one-compound library technique.[4] The split synthesis method is, however, very inconvenient in the case of iterative libraries with a fixed position inside of the peptide sequence. In this case the synthesis of a library containing 20 amino acids in the fixed position and 20 amino acids in positions requiring equimolar incorporation of amino acids would require the use of 400 reaction vessels. Alternatively one can use a mixture of amino acids in which the ratio is adjusted according to the reactivity of the particular protected building block,[74,95−97] or double or triple coupling of subequimolar (0.8 molar) amounts of equimolar mixtures of amino acids.[98−100] Equimolarity of incorporation is not an issue in real one-bead-one-compound libraries, where equimolarity is a priori achieved by the synthesis of only one compound on each bead; however, it becomes important in techniques synthesizing multiplicity of peptides (motifs) on each particular bead (see below).

Challenging syntheses are frequently encountered in combinatorial peptide chemistry. For example, some biological targets require a free carboxy terminus (or both termini free) displayed on the polymeric bead; this demands a modified synthetic strategy. Synthesis of peptides from the amino to carboxy terminus is not feasible due to the significant problems with racemization in every step. This problem was solved by three groups[101−103] by synthesizing cyclic peptides with cleavable linkers in their cyclic structures and cleavage of the intramolecular linker exposing the free terminus of the molecules (see Figure 4).

Cyclization has been used to decrease conformational flexibility of peptides. This conformational constraint may sometimes result in more potent ligands for biological receptors. This was shown to be true in the case of disulfide cyclic libraries containing ligands for the gp IIbIIIa receptor, which, due to low concentration of library components, could not be found in a linear library, but were selected from a heterodetic cyclic library.[63] The fact that cyclization clearly changes the preference of a biological receptor toward a particular target was shown in the study of streptavidin binders from cyclic libraries of various ring sizes.[104] Cyclic peptides composed of lysines and glutamic acid were used as a template onto which various carboxylic acids were attached.[105] Cyclic peptide libraries were intensely studied by Spatola et al.,[106−108] and an optimal strategy for their syntheses was devised. Ellman et al.[109] synthesized a β-turn mimetic library with the same goal in mind (see Figure 5).

(i) DCC, HOBt, DMF, overnight
(ii) peptide synthesis, standard Fmoc protocol
(iii) 50% piperidine in DMF
(iv) 3% TFA in DCM
(v) DIC, HOBt
(vi) 50% TFA in DCM
(vii) 0.5% NaOH, 30 min

Figure 4. Scheme of synthesis of peptide libraries with exposed carboxy terminus.

Conformational fixation was also achieved by constructing libraries with a high bias toward α-helical conformation. A library was synthesized by randomizing four positions in the sequence of an amphipathic helix (YKLLKKLLKKLKKLLKKL) on either the hydrophobic or hydrophilic sites of the molecule. Peptides with increased antimicrobial activity were identified in this way.[110]

Several libraries combining peptidic structure with nonpeptidic elements have recently been prepared; some examples are discussed below. Most potent and specific zinc endopeptidase inhibitors were identified in a library of peptides with the amino terminus modified by the Z-Phe(PO_2CH_2) peptidomimetic group.[111] A similar approach was used in Pfizer's study of the endothelin antagonist developed by Fujisawa.[112] N-Terminal substitution was kept intact, and all amino acids were randomized by an array of natural and unnatural α- and non-α-amino acids. Promising leads were generated from library constructed by acylation of the peptide chain by an array of three building blocks containing amino and

(i) α-bromoacetic acid, DIC
(ii) 2-aminoethanethiol t-butyl disulfide or 3-aminopropanethiol t-butyl disulfide
(iii) Fmoc-amino acid, HATU
(iv) 20% piperidine/DMF
(v) symetric anhydride of α-bromocarboxylic acid
(vi) PBu_3, H_2O
(vii) tetramethylguanidine, PrOH, DMF, H_2O

Figure 5. Scheme of synthesis of a β turn mimetic.

(i) levulinic acid, cat H_2SO_4, rt, overnight
(ii) EtOAc, Pd/C, H_2
(iii) Fmoc-OSu, $NaHCO_3$, H_2O/dioxan
(iv) DCC
(v) automated parallel synthesis

(i) Fmoc-OSu, pyridine, DCM, overnight
(ii) allyl 10-(vinyloxy)decanoate, pyridinium p-toluenesulfonate, DCM, 3h
(iii) dimedone, $Pd(PPh_3)_4$, THF, overnight
(iv) p-methylbenzhydrylamine resin, DCC

Figure 6. Scheme of synthesis of HIV protease inhibitor library.

carboxyl functionality and capped by a set of carboxylic acids.[113] The peptide chain in this case was used as the "biasing element" targeting the binding pocket of the Src SH_3 domain. Vinylogous sulfonyl peptide synthesis was developed,[114] and libraries were used for studies of synthetic receptors.[115] Boc-protected vinylogous sulfonyl chlorides were coupled in dichloromethane and catalyzed by (dimethylamino)pyridine, and the excess of base (DBU) was monitored with bromophenol blue. Monitoring of the reaction was found to be critical—excess base destroyed the sulfonyl chloride, and even an excess of sulfonyl chloride did not result in complete amino group modification. Libraries of synthetic receptors were generated by the combinatorial synthesis of peptides on "scaffold" molecules such as macrocyclic tetramine–cyclen[116] or steroid[117] molecules.

Palladium-mediated macrocyclization was used in the synthesis of cyclic libraries. The carboxy-terminal lysine side chain was acylated with acrylic acid and the terminal amino group of the linear peptide was acylated by iodobenzoic acid. Pd(0)-mediated cyclization provided clean products in high yield.[118] The synthesis of C2 symmetric inhibitors of HIV protease[119] (Figure 6) serves as an example of solid-phase synthesis utilizing the specific feature of the target molecule (in this case the diol structure) as the opportunity to simultaneously protect a functional group critical to the function of the constructed molecule and to use this protection as the attachment to the solid support.

The one-bead-one-compound technique can be modified and results in the one-bead-one-motif technique. This technique combines one-bead-one-compound library with the multiple defined positional scanning library. The power of this approach was demonstrated on screening with several model systems.[120] This technique addresses the problem of generation and screening of incomplete libraries. A library of hexapeptides composed from 20 amino acids would contain 64 000 000 compounds. However, it is very probable that not all amino acids in the peptide with significant affinity toward the biological target are essential for the binding. Some of the amino acids in the sequence can easily be replaced without significant loss of activity, whereas several "critical" amino acids cannot be replaced by any substituent.

Figure 7. Scheme of synthesis of variable length "library of libraries". For description of the process see the text: M, coupling of amino acids mixture; R, randomization (separation of the resin into n portions and coupling n amino acids individually).

The task is therefore to find the critical amino acids, or motif, required for the binding. If the motif is defined as an arrangement of three amino acids, we can create 20 different arrangements within the hexapeptide framework. Each of these arrangements are represented by 8000 individual motifs. Therefore, the complete library of tripeptide motifs in the hexapeptide framework or "library of libraries", is composed of 160 000 species. This is a substantialy lower number than 20^6 or 64 000 000 individual compounds. The synthesis of library of libraries requires splitting of the resin into several aliquots (in the above example of libraries of hexapeptides into up to six aliquots—in three of them a mixture of amino acids is coupled and three aliquots are randomized by coupling 20 amino acids simultaneously); in total 252 couplings are performed in the synthesis of this library (for the scheme see ref 120). The synthesis of an alternative library with variable length is shown in Figure 7. At the beginning of synthesis, and after each randomization step, one-quarter of the resin is separated, and the mixture of amino acids is coupled to the remaining part. After this coupling, one-third of the resin is separated, and the remainder undergoes coupling with the mixture of amino acids. The next coupling is performed with half of the resin from the previous coupling. All portions of the resin are then combined, and a randomization is performed. Synthesis of a library of libraries with a three amino acid motif, by this method, consists of three randomization steps and four stages of multiple couplings of amino acid mixtures. As a result, each solid-phase particle of the library went through three mandatory randomization steps and as many as 12 acylations with the mixture of amino acids. This library, containing peptides of lengths from three to 15 residues, consists of 256 positional motif sublibraries. Among sublibraries of up to hexapeptides, all positional motifs are represented. However, because this synthetic scheme does not allow more then three successive acylations with the amino acid mixture, motifs in which "pharmacophore" positions are separated by more than three adjacent "structural unit" positions are not represented.

The impossibility of covering all peptides from longer libraries with a limited number of beads was addressed by Hornik and Hadas,[121] who coupled mixtures of amino acids of varying complexity in individual positions of the library. The amino-terminal position was least complex and the carboxy-terminal position was the most complex. Each bead thus contained up to millions of peptides. However, the authors speculated that concentrations of individual peptides on the bead would still be recognizable by the receptor. Sequencing of positive beads semiidentified only N-terminal residues and secondary libraries had to be synthesized to elucidate the structure of the binder. The density of the peptide display can also be an important factor in the success of the screening assay. Wallace et al. were able to detect activity only after the peptide was displayed as an octameric complex.[122]

To optimize the primary hit from an incomplete peptidic library Selectide scientists designed a so called "homolog library". In every step of construction of this library, certain percentage (40%) of the solid support is not divided into n reaction vessels, but the amino acid found in the sequencing of the primary hit is coupled to this part of the carrier instead. The remaining carrier is divided into $n - 1$ parts and the remaining amino acids are coupled separately. After completion of coupling, the carrier is recombined and the next unequal division is performed. The percentage of resin to which the original amino acid was coupled depends on the length of peptide and number of amino acids used in each randomization step and is calculated in such a way that the sample of library that is intended to be screened contains a reasonable number of original sequences (e.g. 10 in one million). The probability of finding the bead with original sequence is thus increased as well as the probability of finding sequences with one or more substitutions. Positive hits identified in the screening are sequenced and distribution of amino acids in particular positions is evaluated. Positions in which the frequency of given amino acid is at the level in which the same amino acid was introduced during the synthesis (40%) can be considered unimportant for the studied binding. Positions in which the frequency is significantly higher can be considered critical. Most interestingly, the position where the frequency of the original amino acid is lower than 40% did not have optimal residue in the original hit and the hits from the secondary homolog library contain optimized residue(s). An example of this approach is given in Figure 8. A primary hit from 12-mer peptidic library was used as the basis for the synthesis of homolog library with 40% enrichment in every position. Positive beads from this library were sequenced, and the frequency of the original amino acid found in each position was plotted. A clearly identifiable motif of

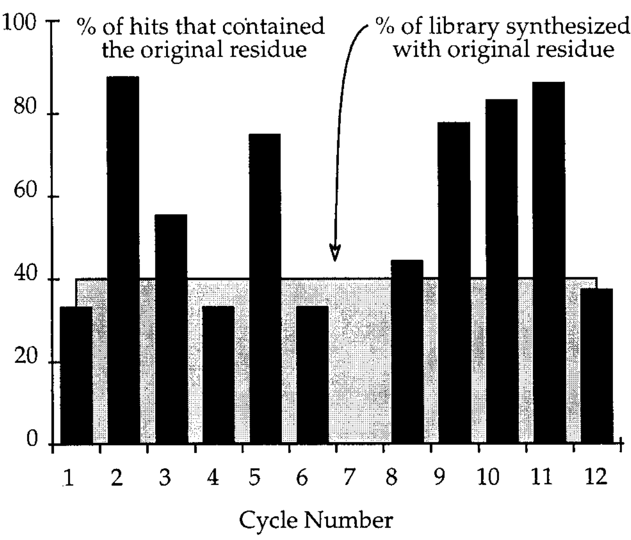

Figure 8. Results (cumulation) of sequencing of positive beads found in "homolog library" with 40% bias toward originally found sequence.

(i) DIEA, DCM
(ii) $R_1R_1'NH$, HBTU, DIEA, DMF
(iii) piperidine/DMF, followed by $(Fmoc\text{-}Ida)_2O$, DIEA and (ii)

Figure 9. Synthesis of library based on iminodiacetic acid.

amino acids originally presented in positions 2, 5, 9, 10, and 11 was detected. Positions 1, 4, 6, 8, and 12 can be replaced by many alternative amino acids—the percentage of amino acid from the original hit equals the ratio expected from the synthetic algorithm. An additional important residue was found for position 7, in which the original hit contained the nonoptimal amino acid. In the illustrated case the binding of peptide synthesized based on this structural information improved from IC_{50} 4 to 0.03 μM.[55]

Significant increases in the diversity of peptide libraries can be achieved by incorporating not only α but also other amino acids in the construction of the peptidic chain (see e.g. ref 123). Peptide backbones can serve as a scaffold onto which a variety of building blocks can be attached via coupling to the three-functional amino acids (aminoglycine, diaminopropionic acid, diaminobutyric acid, ornithine, lysine, iminodiacetic acid, aspartic acid, glutamic acid, serine, hydroxyproline, cysteine, etc.).[101,124-126] Iminodiacetic acid served as a convenient structural unit allowing construction of peptide-like libraries[127] (see Figure 9). Attachment of carboxylic acids onto free amino groups was the basis for the construction of a so

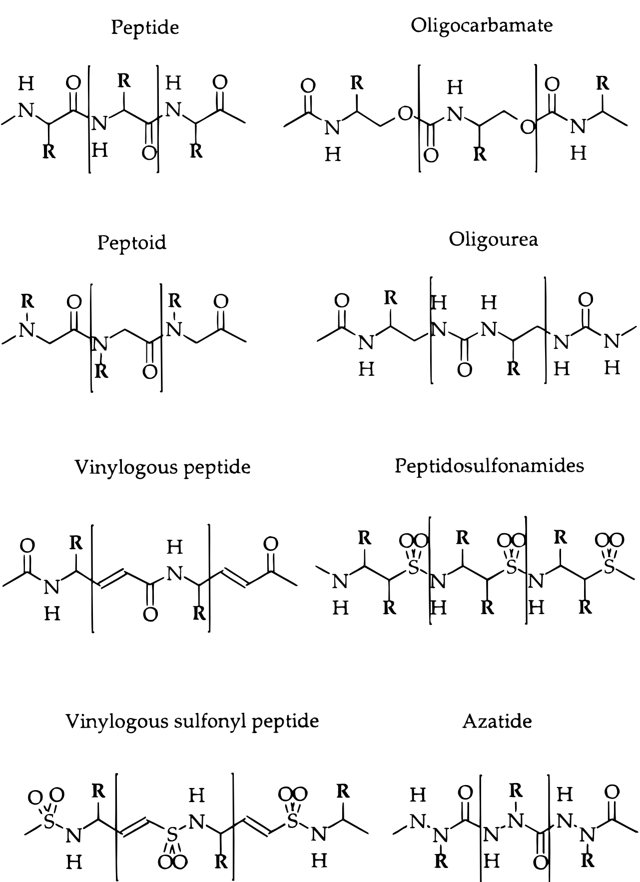

Figure 10. Biopolymer mimetics.

called α, β, γ library in which both the backbone and side chain arrangements were randomized.[128]

C. Libraries of Nonpeptide Oligomeric Compounds

Peptides synthesized from bifunctional building blocks, amino acids, are not the only examples of oligomeric compound libraries. A variety of other biopolymer mimetics have been designed and synthesized; their structures are shown in Figure 10. Biopolymer mimetic libraries are characterized by a repeating motif (monotonous backbone) throughout the backbone, synthesized by repetition of a limited number of chemical reactions and usually only one type of building block. The linear arrangement of building blocks in a peptide-like fashion was the basis for several library designs (for reviews see e.g. refs 16, 24, and 129–131). Since it is sometimes very difficult to guess whether the described library was synthesized for the screening with the use of one-bead-one-compound method, we have decided to cover in this review both chemistries which were developed with the vision of applying it to this technique and chemistries which would be readily applicable for synthesis of one-bead-one-compound library.

1. Peptoids

Peptoids, oligomeric compounds composed of N-alkylated glycines, first described by Simon and coworkers,[14,132-135] are the best known examples of oligomeric nonpeptide compounds. The only difference between peptides and peptoids is the position of the variable side chain. Whereas in the case of peptides the side chain is always connected to the

α-carbon of the amino acid, the characteristic feature of peptoids is that the side chain is linked to the amide nitrogen of the backbone. Thus, from the chemical point of view, peptoids are oligomeric N-substituted glycines. This change introduces two important features. Firstly, peptoids are resistant to proteolytic degradation. Secondly, the diversity of peptoids can be greater, as a wider range of building blocks are available for their synthesis. Peptoids are easily synthesized from bromoacetic acid and amines; the structural variety of commercially available amines is much larger than the limited variety of available amino acids. Nevertheless, peptoids can also be prepared from preformed building blocks, *N*-alkyl amino acids. Specific ligands binding to α1-adrenergic receptor and μ-specific opioid receptor were found from peptoid libraries.[134]

2. Oligocarbamates

Oligocarbamates, composed of chiral ethylene units connected to relatively rigid carbamate groups, are the next class of oligomeric compounds that were exploited in combinatorial fashion by Schultz et al.[15,136] N-Protected amino alcohols derived from amino acids (prepared by reduction with borane) were converted to *p*-nitrophenyl carbonates (reaction with *p*-nitrophenyl chloroformate) and reacted with polymer-supported amino groups. Then the amino protecting group (Fmoc) was removed, and the synthetic cycle was repeated.

The oligocarbamates library was synthesized by photolithographical methods,[15] originally developed by Fodor and co-workers for peptide synthesis.[44] The amino groups of amino alcohols were protected by the nitroveratryl group, removable by irradiation at 365 nm. The library of 256 oligocarbamates was tested for binders against a monoclonal antibody raised against synthetic oligocarbamate. Five specific ligands were found with IC_{50} values in the 60−180 nM range. Later, the solid-phase synthesis of *N*-alkylcarbamate oligomers was described by the same group.[137] N-Alkylation increased the density of side chains, removed the backbone hydrogen-bond donor, and decreased flexibility of the backbone. The synthesis was done on Rink resin and involved acylation of polymer-supported amino groups (with bromophenol blue (BB) monitoring[90]), followed by amide reduction via borane and coupling of *p*-nitrophenyl carbonate of Fmoc protected amino acid.

3. Oligoureas

Solid-phase synthesis of oligomers composed of repeating urea units was described by Burgess et al.[138] N-Boc-protected amino acids were reduced to amino alcohols and reacted under Mitsunobu conditions with phthalimide. Resulting mono protected diamines were converted to isocyanates by treatment with triphosgene. These intermediates were used to assemble the urea oligomers.

4. Vinylogous Sulfonyl Peptides

Since α-amino sulfonamides are known to be unstable, Genari and co-workers developed the synthesis of chiral vinylogous amino sulfonic acids and used them for the synthesis of vinylogous sulfonyl peptides[114,139] (see above). The sulfonamide group can mimic the tetrahedral geometry of amide bond hydrolysis by proteolytic enzymes and sulfonyl peptides may be pharmacophores for protease inhibition. Molecular receptors based on vinylogous sulfonyl peptides have been screened with a tripeptide library and specific binders have been found.[115]

5. Peptidosulfonamides

Replacement of α-amino acids by β-amino sulfonic acids in peptide backbone yields peptidosulfonamides, whose solid-phase synthesis was recently reported.[140] N-β-Boc sulfonyl chlorides were coupled to polymer-supported amino groups followed by Boc group removal. However, the chemistry is not straightforward and requires careful optimization and selection of an appropriate solid support.

6. Azatides

Peptide mimetics composed of α-aza amino acids were termed azatides and their solid-phase synthesis has been developed.[141] A set of Boc-protected alkyl hydrazines were prepared, either by reduction of protected hydrazones or by alkylation of hydrazine with an alkyl halide followed by Boc protection. These hydrazine derivatives were converted to activated species by bis(pentafluorophenyl) carbonate and used in a stepwise manner to build azatides.

7. Ketides

Polyketides represent a class of compounds exhibiting a number of interesting pharmacological properties, including antibiotics, anticancer agents, immunosupressants, etc. Asymmetric aldolization of polymer-supported aldehydes has been suggested as one possible way to prepare polyketides in a combinatorial fashion.[142] Combinatorial biosynthesis of polyketides was recently reported.[143]

D. Small Molecule Libraries

The first synthetic combinatorial libraries were composed of peptides for obvious and pragmatic reasons. The chemistry used for synthesis on solid phase[20] was well described (for review see e.g. ref 21 and references cited therein), as were methods for structure determination with small amounts of material. However, currently, pharmaceutical companies do not consider peptides as fashionable, high-priority lead compounds. This attitude may alter especially if drugs that originated from peptide leads are launched. Combinatorial chemistry is a most suitable tool to help peptides find their correct place in the toolbox of modern drug discovery.

Currently, the major effort in combinatorial chemistry is focused on combinatorial libraries of nonpeptide-like small molecules, reviewed e.g. in refs 16, 24, 25, 34, 131, 144, and 145. The rational for this activity is 2-fold. First, the structural diversity of peptides is limited by the character of the peptide backbone. Secondly, other classes of compounds have been shown to be structurally and chemically diverse and have characteristics not present in peptides that are important for drug candidates such as oral bioavailability and resistance to protease degradation.

Since the number of synthetic combinatorial libraries is growing fast, one has a tendency to categorize these libraries. There are several criteria that can be applied to classify synthetic combinatorial libraries: (i) design of the library, (ii) novelty of structures,

(iii) type of chemistry used for synthesis, (iv) structural features of library members, (v) chemical strategy, etc.

(i) The vast majority of combinatorial libraries have been designed to accelerate the drug discovery process. Two kinds of libraries were developed and synthesized in this respect. Generic (unbiased) libraries are used to discover a novel structural motif or a feature that possesses desired biological activity, either to replace an already existing drug, or to search for lead compounds for new targets, where no small molecule organic compounds with the desired properties are known. Dedicated, secondary, or biased libraries serve the purpose of optimizing the properties of existing lead compounds.

(ii) The search for pharmacologically interesting compounds can be carried out among structures that have already provided successful drugs. Benzodiazepines serve as the best example known so far.[146–149] Alternatively, the search can focus on design and synthesis of generic libraries of diverse and previously unexplored structures to discover pharmacophores of completely novel structure, e.g. cubanes,[150] or Kemp's acid derivatives.[151]

(iii) The most prominent chemical reaction practiced on solid phase, the amide bond formation, rewarded its discoverer, Bruce Merrifield, with the Nobel prize. Several books have been dedicated to its formation. However, combinatorial chemistry, for obvious reasons, started to favor a variety of chemistries, particularly those forming C−C bonds and heterocyclic compounds. Valuable compilations of solid-phase organic reactions have been published by Hermkens et al.,[145] or in Chiron's booklet "Solid Phase Chemistry Publications",[152] or can be found within a dynamic database on the Internet.[33]

(iv) The classification of generic synthetic combinatorial libraries can be based on structural characteristics of individual library members. Libraries with a common central unit, a scaffold, that is present in all library members are termed structurally homogeneous.[153,154] This term stresses the uniformity of all library members as far as the presence of one central scaffold unit is concerned. Libraries of another type are composed of structurally unrelated bifunctional building blocks. Joints connecting these building units are the only common feature of library members. We refer to these libraries as structurally heterogeneous. These libraries are composed of compounds having higher diversity when compared to homogeneous libraries and therefore they should be more suitable for discovery of novel lead compounds, i.e., for design of generic libraries. Both types of libraries are depicted schematically in Figure 11.

(v) The central structural unit (scaffold) of structurally homogeneous libraries can be introduced in two different ways. The first method we may call the "glucose approach" according to Hirschmann,[155] who derivatized a β-D-glucose scaffold for the synthesis of a selective substance P antagonist. This approach is based on the solution-phase preparation of suitably protected scaffold molecules. These precursors are then used for the combinatorial synthesis of libraries (e.g. cyclopentane scaffold[156] or steroid scaffold[157]). Alternatively, the scaffold can be synthesized during library synthesis. This approach may be termed the "benzodiazepine approach" according to Bunin and Ellman,[146] who first described combinatorial synthesis of small heterocyclic compounds from simple building blocks.

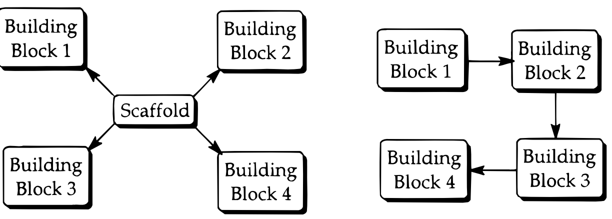

Figure 11. Structurally homogeneous and heterogeneous libraries.

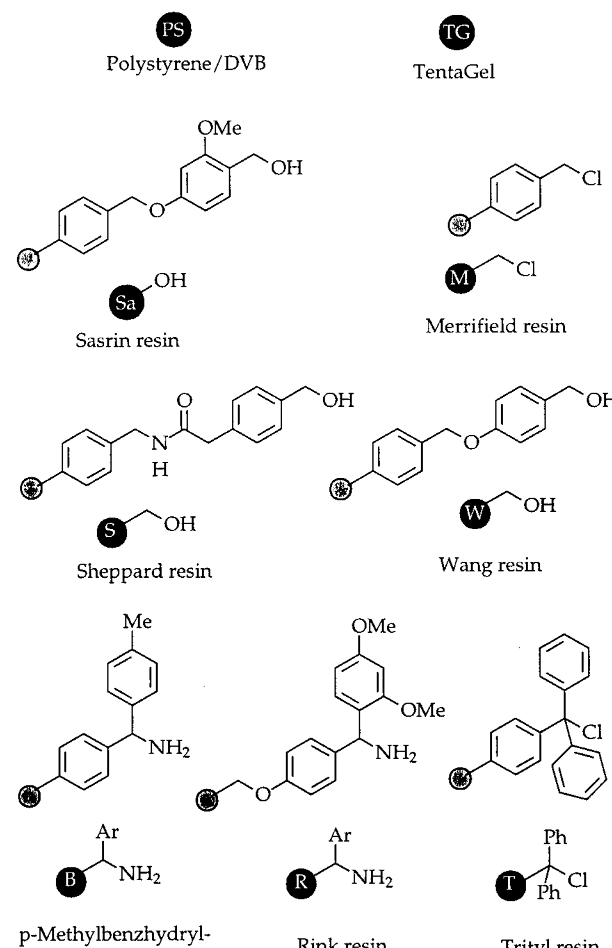

Figure 12. Structures of commonly used linkers. The symbols introduced here are used in the rest of the text.

In this review article, the small molecule libraries are divided into four sections: acyclic libraries assembled in linear fashion, libraries built using a preformed scaffold, libraries that include a heterocyclization step, and structurally heterogeneous libraries. A number of different linkers have been used to attach synthesized compounds to the insoluble support. The structures of the most commonly used linker−resin constructs are summarized in Figure 12. For the sake of simplicity, each one is coded by a letter that is used in synthetic schemes later on.

(i) Piperidine in DMF
(ii) Fmoc amino acids
(iii) R_3-SO_2Cl, R_3-NCO, R_3-COOH, or R_3-COCl

Figure 13. Library of peptide-like structures.

(i) MeOH/DCM (1:1), 24 h, rt

Figure 14. Ugi four-component condensation reaction.

1. Acyclic Libraries

The first attempt to depart from the structure of peptides, although still using mostly amide bond chemistry, is documented by the synthesis of a library of 8000 *N*-alkyl-*N*-acyl amino acids.[158,159] Polymer-supported amino acids were alkylated under the conditions of reductive amination with a variety of aldehydes, resulting in secondary amines which were acylated with carboxylic acids. The library was screened to determine specific binders to a model target, streptavidin, and the structure of positively reacting compounds was determined using mass spectrometry. Positive compounds, together with various structural analogs were synthesized and their binding confirmed. Structures containing both an imidazole moiety and a substituted aromatic residue demonstrated specific binding.[159]

Baldwin and co-workers described the synthesis of a 6727 member one-bead-one-compound tagged library of peptide-like structures[160,161] (Figure 13). In the first combinatorial step seven building blocks were used (four amino alcohols and three amino acids, both Fmoc protected), the second reaction included 31 Fmoc-protected amino acids and the last step included 31 sulfonyl chlorides, isocyanates, carboxylic acids, and chloroformates.

Linear, structurally homogeneous libraries were prepared also using multicomponent condensation reactions, e.g. by the Ugi four-component condensation (4CC) reaction[27,162−165] (Figure 14). A 96 member library of *N*-acyl amino alkyl amides was synthesized on Rink resin from four components: 12 carboxylic acids, one amine, eight aldehydes, and one isocyanide.[165] The library was synthesized in a 96-well microtiter plate.

(i) Piperidine in DMF
(ii) R_1COCl, DIEA in DCM
(iii) R_1COOH, HBTU, HOAt, DIEA in NMP/DMF, 12h
(iv) R_2X, DBU in DMSO
(v) R_2SO_2Cl, DIEA in DCM

Figure 15. Scheme of scaffold-based library synthesis.

(i) TEA in DCM, 12 , rt
(ii) 20% piperidine in DCM, 12 h
(iii) preformed betaine in DCM, alcohol in toluene, 3 days
(iv) HOAc, TBAF, 12 h

Figure 16. Scheme of biphenyl library synthesis.

A library of β-amino alcohols was proposed by Kobayashi et al.[166] based on the reaction of polymer-supported silyl enol ethers with imines in the presence of catalytic amount of scandium triflate yielding β-amino thioesters later reduced to amino alcohols.

2. Libraries on Preformed Scaffolds

A 2001 member small-molecule library using 3-amino-5-hydroxybenzoic acid as a core structure was reported by Dankwardt et al.[167] (Figure 15). Fmoc-protected 3-amino-5-hydroxybenzoic acid was

(i) xylene, reflux under Ar, 19h
(ii) Boc-ethylenediamine, DMF, 6h, rt
(iii) DCC, DCM
(iv) Fmoc-ethylenediamine.TFA, DIEA, DMF
(v) AA-β-Ala-Gly-β-Ala-Gly-O-TG, BOP, DMF
(vi) 20% piperidine in DMF, 10 min
(vii) $R_{2(3)}$-COOH, DCC (2:1), 30 min
(viii) 50% TFA in DCM (1:1), 1h, followed by DIEA

Figure 17. Synthesis of a library based on Kemp's triacid.

attached to Rink resin, the Fmoc group was cleaved by piperidine/DMF and the liberated amino group was acylated by a set of 69 carboxylic acids. In the next synthetic step the polymer-supported hydroxyl groups were alkylated with 28 alkyl halides.

A library of tetrasubstituted biphenyls as a representative of a novel scaffold system of the universal library concept was communicated by Pavia et al.[168] The aim of this approach is to generate functionalized small molecule scaffolds with a wide variety of spatial configurations. The first library described was assembled from a biphenyl unit attached to resin via a benzyl ester linkage at position 3 of the first ring and derivatized with suitably protected hydroxyl groups at position 4 of the first ring and 3 and 5 on the second ring (Figure 16).

Kočiš et al.[151] built a one-bead-one-compound library around a cyclohexane scaffold, making use of Kemp's triacid (Figure 17). Two carboxylic groups on a cyclohexane ring were extended by differentially protected ethylenediamine, the third carboxyl group was activated and used to attach the scaffold to resin-bound amino groups of amino acids. Three points of diversity allowed preparation of a 75 000 compounds library that was screened for enzyme inhibitors. Surprisingly, a specific thrombin inhibitor was found. Its structure was determined from MS experiments and the activity ($K_i = 4\ \mu M$) was confirmed in solution after resynthesis.

(i) Carbonates prepared in solution, then coupled to TentaGel
(ii) piperidine in DMF
(iii) Fmoc amino acids
(iv) R_3-SO_2Cl, R_3-NCO, R_3-COOH, or R_3-COCl

Figure 18. Acylpiperidine library synthesis.

A one-bead-one-compound library of 6727 acylpiperidines was reported by Pharmacopeia's combinatorial chemists[161,169] (Figure 18). Five piperidines were attached to a photolabile resin-supported linker and the amino group was acylated by 31 amino acids. In the last step, 31 reagents were used to derivatize the amino group to carboxamides, carbamates, sulfonamides, and ureas. The library was screened against a model target, carbonic anhydrase, and a 4 nM active compound, [*N*-(4- sulfamoylbenzoyl)-L-leucyl]piperidine-3-carboxylic acid, was identified.

A library of 12 000 triazines was described by Stanková and Lebl[170] (Figure 19). An excess of cyanuric chloride was reacted with immobilized amines producing single substituted dichlorotriazine. Under mild conditions the second chlorine atom was replaced with amines. The third, less reactive chlorine, was substituted at elevated temperature with another set of amines. The synthesis of this library utilized unique features of solid-phase chemistry and cannot be duplicated in solution.

3. Heterocyclic Libraries

The current chemical literature is almost flooded with description of polymer-supported chemistries that provide a variety of heterocyclic compounds, amenable for the combinatorial synthesis of libraries. Those include β-lactam,[171,172] tetrahydrofuran,[173,174] benzofuran,[175] pyrroles,[163,172,176-179] isoxazole,[180] thi-

(i) DIEA in DCM, 1h, rt
(ii) R_2-NH_2 in DCM, 2h, rt
(iii) R_3-NH_2 in dioxane, 2h, 90 oC
(iv) thioanisol, ethanedithiol, H_2O in TFA, 2.5h, rt
(v) BrCN, HCl in H_2O, 12h, rt

Figure 19. Synthesis of library based on gradual substitution of trichlorotriazine.

(i) Piperidine/NMP, 45 min
(ii) 0.8 M R_2-CHO in DCM/$CH(OMe)_3$, 3 h
(iii) 0.8 M R_3-CH_2-COCl, 1.1 M TEA in DCM, 0 oC, 16 h

Figure 20. β-Lactam library synthesis.

azolidine,[172,181−183] imidazole,[184−186] benzimidazole,[187] hydantoins,[45,148,188−191] benzopyran,[169] pyridine,[192−197] quinoline,[198,199] isoquinoline,[200−204] piperazine,[205,206] diketopiperazine,[112,127,154,207−209] phthal hydrazide,[210] quinazoline,[211−213] benzodiazepines,[146,147,149,214−218] and carboline.[219,220] However, most of them describe reaction conditions for only a limited number of building blocks without being used for real library synthesis but merely for parallel synthesis of single compounds. The overview of polymer-based chemistries leading to different heterocyclic compounds that were utilized for combinatorial synthesis of real libraries is summarized below.

β-Lactams. Scientists from Affymax described the solid-phase synthesis of β-lactams via the [2+2] cycloaddition reaction of ketenes with resin bound imines[171] (Figure 20). Five amino acids immobilized to the Sasrin resin were reacted with five aldehydes yielding resin-bound imines. The cycloaddition reaction was performed using three acid chlorides and

(i) Piperidine/DMF
(ii) 1 M Ar-CHO in $CH(OMe)_3$, 4 h
(iii) Ac_2O, DIEA, 15 min
(iv) 1 M olefin, 1 M $AgNO_3$, 1 M TEA in MeCN, 8 h
(v) mercaptoacyl chlorides

Figure 21. Cycloaddition-based library.

the products (25 compounds) were cleaved by 3% TFA in DCM for 45 min. The same reaction sequence has also been tested on TentaGel resin using a photocleavable linker. The amino group of the linker was used to form the imine; therefore, N-unsubstituted β-lactams were obtained. The synthesis is applicable for tagged one-bead-one-compound libraries and was shown to be compatible with coding using secondary amine tags.[172]

Pyrroles. A polymer-supported 1,3-dipolar cycloaddition reaction was used to prepare 1,2,4- trisubstituted pyrrolidines[176] (Figure 21). TentaGel AC or Sasrin resin were esterified with four amino acids and, after removal of the amino protecting group, the imine was formed with four aldehydes in neat orthoformate. Silver-catalyzed ($AgNO_3$) cycloaddition of five electron-poor olefins provided proline derivatives, whose amino function was further acylated. A library from four amino acids, four aldehydes, five olefins, and three mercaptoacyl chlorides was prepared by the split synthesis method and potent angiotensin converting enzyme inhibitors were identified. This synthetic route was also applied for coding using secondary amine tags.[172]

The Ugi four component condensation reaction was utilized for the synthesis of α-acyl amino esters and pyrroles.[163] Both Rink and Wang resins were reacted with succinic anhydride; polymer-supported carboxylate together with amine, aldehyde, and 1-isocyanocyclohexene produced α-acylamino acid amides. Treatment with dimethyl acetylenedicarboxylate gave polymer-supported pyrroles.

Thiazolidine. Three 540 member 4-thiazolidinone libraries were reported by Look et al.,[181] using acids, aldehydes, and mercapto acids as building blocks. Five Fmoc-protected amino acids were immobilized to TentaGel S AC or Tentagel RAM and the Fmoc groups were removed with piperidine in DMF. The polymer-supported imines were formed from five aromatic aldehydes and cyclized to thiazolidinones with five mercapto acids. The libraries were screened for cyclooxygenase-1 inhibitors. Selective ligands were found, and their structures deconvoluted.

A one pot, three component solid-phase synthesis of 4-thiazolidinones and 4-metathiazanones on acid-

(i) 0.25 M aldehyde, THF, mol sieves, 70 °C, 2 h
(ii) 0.5 M α–mercapto acid, THF, 70 °C, 2 h
(iii) 0.5 M β–mercapto acid, THF, 70 °C, 2 h

Figure 22. Thiazolidinone and metathiazanone library synthesis.

(i) KHMDS
(ii) Bu_3SnH, $Pd(PPh_3)_4$
(iii) TentaGel S NH_2, DIC, HOBt
(iv) R_1-NH_2
(v) $NaBH_4$-$Cu(acac)_2$
(vi) *n*-BuOH/DMF, 90 °C, 24 h

Figure 23. Benzimidazole library synthesis.

cleavable and photocleavable linkers was described by Holmes et al.[182] (Figure 22). Five polymer-supported Fmoc-protected amino acids were deprotected and condensed with five aldehydes and three mercapto acids in THF at 70 °C. α-Mercapto and *β*-mercapto carboxylic acids lead to the formation of five and six member rings, respectively. Alternatively, the reaction can be performed stepwise; resin-bound amino groups were condensed with aldehydes producing resin bound imines which were washed and treated with mercapto acids. Heterocyclic compounds were cleaved from the Wang resin by a TFA/DCM mixture (1:1). Coding using secondary amine tags[172] was shown to be compatible with this synthetic scheme.

(i) amino acid, BSA, DMAP in DMF, 48 h, rt
(ii) DCC, HOBt, R_4-NH_2 in DMF, 24 h, rt
(iii) TEA in MeOH, 55-90 °C, 48 h

Figure 24. Hydantoin library synthesis.

(i) TFMSA anhydride, lutidine in DCM, -78 - 0 °C
(ii) O-benzylhydroxylamine, 0 °C
(iii) Ar-NCO, DCE, reflux 24 h
(iv) t-BuOK, R_2-OH, rt

Figure 25. Alkoxyhydantoin library synthesis.

Benzimidazoles. A small library of benzimidazoles attached to amino TG via a TFA labile ether linkage was described by Phillips and Wei[187] (Figure 23). The synthesis started with the preparation of the fluoronitroaryl linker construct in solution followed by its immobilization via carboxylate to amino TentaGel. Polymer-supported fluoronitroaromatic compounds were treated with amines. Reduction of the nitro group followed by cyclization with aryl imidates provided 1,2-substituted benzimidazoles.

Hydantoins. An 800 compound library of hydantoins attached to carbamate linker was described by Dressman and co-workers[188] (Figure 24). Hydroxymethyl polystyrene resin was converted to activated carbonate using *p*-nitrophenyl chloroformate in the presence of *N*-methylmorpholine. Amino acids, dissolved in DMF and *N*,*O*-bis(trimethylsilyl)acetamide, were then reacted with immobilized activated carbonate under DMAP catalysis yielding polymer-supported carboxy carbamate. Carboxyl groups were activated with DCC and reacted with amines in the next step. These intermediates yielded the desired hydantoins upon heating in the presence of a base (triethylamine).

A library of 50 5-alkoxyhydantoins was described by Hanessian and Yang[189] (Figure 25). Merrifield

(i) PPh_3, DEAD in THF, rt
(ii) N-Boc aminoketone
(iii) TFA in DCM
(iv) R_3-SO_2Cl, R_3-NCO, R_3-COOH, or R_3-COCl

Figure 26. Dihydrobenzopyran library synthesis.

resin was esterified with α-hydroxy acid via its cesium salt and the resulting resin-bound ester was transformed to *N*-(benzyloxy)amino ester. The resulting polymer-supported amino acid derivatives were condensed with aryl isocyanates to yield urea derivatives. Cyclization and release from the resin was achieved by treatment with potassium *tert*-butoxide.

Benzopyrans. A tagged one-bead-one-compound library of 1143 dihydrobenzopyrans has been described by Burbaum et al.[169] (Figure 26). Three dihydroxyacetophenones were attached to a photolabile linker under Mitsunobu reaction conditions. The whole construct was immobilized on polystyrene resin and cyclized with a set of seven ketones, four of which contained protected amino groups. These were modified in the next synthetic step with 31 building blocks. The ketones were reduced with $NaBH_4$, converted to dithiolanes with ethanedithiol, or left unchanged to yield a library of 1143 dihydrobenzopyrans.

One active compound, 2-[*N*-(4-sulfamoylbenzoyl)-4′-aminocyclohexanespiro]-4-oxo-7-hydroxy-2,3-dihydrobenzopyran, was identified, having a 15 nM dissociation constant with carbonic anhydrase.

Isoquinoline. A library of 43 000 tetrahydroisoquinolines was reported by Griffith et al.[204] (Figure 27). Benzhydrylamine was acylated with α-amino acids and the resin bound imine was formed from the polymer-supported amino group with a set of aldehydes. The cyclization was performed with homophthalic anhydride. Free carboxyl groups of polymer-supported tetrahydroisoquinolines were activated in the next step and reacted with a set of amines.

(i) $HC(OMe)_3$ in DMF, 3h, rt
(ii) DIEA in DMF, 18h, rt
(iii) HATU in DMF, 20 min, rt followed by R_3-NH_2, rt, overnight

Figure 27. Tetrahydroisoquinoline library synthesis.

(i) Piperidine/DMF
(ii) Ar-CHO, $NaBH(OAc)_3$ in DCM
(iii) Boc-amino acid, PyBrop
(iv) TFA
(v) Toluene, reflux 5h

Figure 28. Diketopiperazine library synthesis.

Library compounds were cleaved by HF in pools of 836 compounds each.

Diketopiperazine. Diketopiperazines (DKP) were described by several groups.[112,127,154,207] Gordon and Steele[207] (Figure 28) prepared a combinatorial library of 1000 compounds starting from 10 amino acids immobilized to Wang resin. After Fmoc removal, the resin-bound amino groups were treated with aldehydes forming imines that were reduced by NaBH-$(OAc)_3$. The secondary amino group was acylated by a set of Boc-protected amino acids, activated using PyBrop. TFA-mediated Boc cleavage was followed by 5 h reflux in toluene to achieve the DKP cyclization. The initial work was extended, a library of 30 752 DKP was prepared and screening results for endothelin antagonists were reported.[112]

Šafár and co-workers reported the use of iminodiacetic acid (Ida) for DKP formation[127] (Figure 29). Fmoc-protected Ida mono methyl ester was coupled to the polymer-supported amino group, the Fmoc group was removed and an Fmoc protected N-α amino acid was coupled. The DKP ring was closed during subsequent Fmoc deprotection. Ida was later replaced with Asp, immobilized via its β-carboxyl group, while the α-carboxyl was employed in the cyclization.[154]

(i) DIC, HOBt in DMF
(ii) piperidine in DMF
(iii) Fmoc amino acid, DIC, HOBt in DMF

Figure 29. Synthesis of diketopiperazine library based on spontaneous iminodiacetic acid monomethyl ester intramolecular cyclization.

(i) R_2-NH_2, DMSO, 50 °C
(ii) PyBrop, DIEA, THF, 50 °C
(iii) R_4-NH_2, DMSO, 70 °C
(iv) TFA

Figure 30. Diketopiperazine and diketomorpholine library synthesis.

Scott et al.[209] reported the solid-phase synthesis of libraries containing 1,3,4,6-tetrasubstituted diketopiperazines and 3,4,6-trisubstituted diketomorpholines (Figure 30). Wang resin was esterified with α-bromo carboxylic acid and the resin-bound bromine was displaced with a primary amine. The resulting secondary amine was acylated with α-bromo carboxylic acid. Resin-bound bromoacyl amino acid afforded substituted morpholines upon cyclization in TFA. Alternatively, the resin-bound bromoacyl amino acids were treated with amines and cyclized to diketopiperazines. The morpholine library consisted of seven pools of nominally 980 compounds, and the library of diketopiperazines was composed of 23 pools of 980 compounds.

Benzodiazepines. The first example of combinatorial solid-phase synthesis of heterocyclic compounds, 1,4-benzodiazepines, was described by Bunin and Ellman[146] (Figure 31). Two 2-aminobenzophenones

(i) Piperidine in DMF
(ii) Fmoc-amino acid fluoride
(iii) 5% AcOH in DMF
(iv) lithiated 5-(phenylmethyl)-2-oxazolidinone in THF followed by alkylating agent in DMF

Figure 31. Benzodiazepine library synthesis.

were immobilized via a hydroxy or carboxy functionality to polystyrene resin via the acid labile linker, [4-(hydroxymethyl)phenoxy]acetic acid.[221] After removing the amino protecting group, six Fmoc-protected amino acid fluorides were coupled to the aniline type of amino groups. Fmoc groups were cleaved and the product exposed to 5% acetic acid in DMF to promote the diazepine cyclization. The last synthetic step was alkylation of the anilide with four alkyl halides. Ten benzodiazepines were cleaved from the support by TFA/H_2O/DMS. Combinatorial solid-phase synthesis of benzodiazepines was recently reviewed.[218]

4. Structurally Heterogeneous Libraries

The concept of structurally heterogeneous libraries is based on a combination of various types of structurally unrelated building blocks connected using different chemical reactions.[153,154] This approach allows one to combine polymer-supported chemistries in a true combinatorial manner, the only condition being compatibility of chemical reactions and building blocks used for the particular library.

The use of structurally unrelated bifunctional building blocks, that is, blocks with a full variety of structures between the two functional groups, results in a library for which there is no common scaffold or backbone feature. The diversity of compounds within this kind of library increases substantially. The dissimilarity of compounds within one such library is documented with an example of two compounds

Figure 32. Two compounds from structurally heterogeneous library.

Figure 33. Examples of structurally heterogeneous libraries.

composed of structurally unrelated aromatic hydroxy acids connected to amino alcohols (Figure 32). Note that compounds composed of structurally related bifunctional building blocks only, e.g. N-α-amino acids in the case of peptides, accordingly share a common backbone, a feature which contributes to the relatively low diversity of structural space that such compounds can probe.

Five types of functional groups on bifunctional building blocks were used in the first set of diverse libraries of this type that covered amino, carboxy, carbonyl, hydroxy, and aromatic hydroxy groups.[153,154] Various bifunctional building blocks were employed in the design and synthesis of libraries, including amino acids, amino alcohols, aromatic hydroxy acids, aromatic hydroxy aldehydes, and diamines. Reactions between functional groups employed in these libraries included acylation, esterification, reductive amination, and etherification. Six examples of libraries were reported: *N*-(alkoxyacyl) amino acids, *N*,*N'*-bis(alkoxyacyl) diamino acids, *N*-acyl amino ethers, *N*-(alkoxyacyl) amino alcohols, *N*-alkylamino ethers, and *N*-(alkoxyaryl) diamines (Figure 33). Specific streptavidin ligands were reported from the library of 4200 *N*-(alkoxyacyl) amino acids.[222]

Numerous organic reactions useful for solid-phase combinatorial chemistry have been reported in the literature. These include reductive alkylation;[77,158,207,223] Mitsunobu etherification[224−226] and esterification;[227] carbon−carbon bond-forming reactions such as Horner/Wittig,[228,229] Grignard,[179] Heck,[118,230] Stille,[149,231−233] and Suzuki reactions;[180,234−236] Michael addition;[223] and amide reduction.[137,179] One should be able to use these reactions in the design of heterogeneous libraries.

E. Cleavable Linkers

In many cases, libraries are screened after cleavage from a solid support. Under these circumstances, the synthesis of combinatorial libraries requires immobilization of the first building block to the resin bead via a linker, and cleavage of the compound once the library synthesis is complete. There are several requirements for a linker to fulfill. (i) The linker has to be stable to all chemical reactions performed during the library synthesis. (ii) The end products must be readily cleaved from the resin beads (ideally quantitatively). (iii) The cleavage conditions should not degrade the library compounds. (iv) The cleavage conditions should be (if possible) user friendly and preferably provide the released compounds ready for screening. (v) It would be optimal if the linker does not leave any residual functional groups on the library compounds. This last condition has not been met in most linkers described; however, it does not seem to be a critical disadvantage. Any compound of interest will normally possess functional group(s) usable as the source of attachment to the insoluble support during library synthesis.

Two strategies have been applied to provide the first building block attached to the resin via a suitable linker. Typically the linker−resin construct can be derivatized with building blocks. Alternatively, the hybrid of first building block plus linker is synthesized in solution and then attached to functionalized resin (see e.g. refs 187, 212, and 237).

Library compounds can be cleaved from resin beads in one single step, or gradually in several steps. Particularly for one-bead-one-compound libraries a two-step release (double releasable linker) allows instant library screening pinpointing any active compounds without further deconvolution (see section IV.B).

1. Single Cleavable Linkers

Many of the single cleavable linkers used in combinatorial synthesis were not specially designed for library synthesis, but were known and used for many years in solid-phase synthesis. This review does not contain an exhaustive list of linkers instead it will summarize the structures of linkers and resins that are most often used in the solid-phase combinatorial synthesis of small molecules (Figure 12).

The most popular linkers are acid labile, and the majority of them can be viewed as a variation on the benzyl-type linkage. The general formulae are shown in Figure 34. Benzyl esters of type **1** provide carboxylates upon cleavage from the resin, benzyl amides **2** are cleaved to carboxamides, benzyl ethers **3** provide alcohols, and benzylamines **4** yield amines. Chloromethylated copoly(styrene-divinylbenzene) resin, referred to as Merrifield resin,[20] is used to immobilize carboxylic acids via an unsubstituted benzyl ester. However, the unsubstituted benzyl-type linkers where Y1 and Y2 are hydrogens require harsh deprotection conditions, usually liquid HF, even in the case of the most labile type of compounds, the esters. Therefore the acid lability was increased by two kinds of substitution. Replacing the Y1

Figure 34. Linkers based on benzyl-type attachement.

Figure 35. Linkers developed for solid-phase synthesis.

hydrogen with a phenyl forms a benzhydryl linker[238] or *p*-methylbenzhydryl linker,[239] widely used to prepare carboxamides, however still requiring HF cleavage. The trityl linker[240,241] replaces both Y1 and Y2 with a phenyl ring and represents the most acid labile linker of this type. It has been used to immobilize acids, alcohols, thiols and amines and compound release can be achieved with either gaseous HCl or TFA vapors.[154]

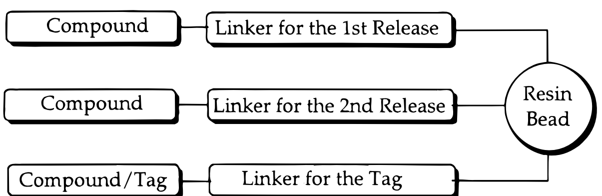

Figure 36. Scheme of multiply releasable library.

The ease of linker cleavage can be further fine-tuned by substitutions on the aromatic ring. Linker **5** uses a carboxamide group to attach the linker to the resin and increases the acid stability. PAM linker can serve as an example. Conversely, electron-donating anchoring alkoxy groups in the linker **6** increase acid lability and were used in Wang[242] and Sheppard[221] linkers. Additional methoxy groups further increase acid lability, as documented on Sasrin,[243] PAL,[244] or Rink[245] linkers.

A linker can also contain electron-withdrawing groups that increase acid stability. Prior to release from the linker these groups are chemically modified to electron-donating groups, making the linker more acid labile. For example, the safety-catch benzhydrylamine-type linker (SCAL linker) was described by Pátek and Lebl[246,247] wherein electron-withdrawing methyl sulfoxides were reduced to methylthio groups after the synthesis is complete and prior to release of compounds. A similar approach was used by Kiso et al.[248] and applied to a benzyl ester-type linker.

Ester bond-based linkers can also be cleaved by alkali or nucleophiles (both inter- and intramolecular).[198,203,249] The ester linkage can also be reversed, i.e. the carboxyl group is immobilized and library compounds are attached via their hydroxyl groups.[153,154,163]

A variety of new linkers that were developed for combinatorial chemistry (Figure 35) include linkers **7**−**10** containing cleavable silicon−carbon or oxygen bonds,[175,234,237,250] carbamate linkers,[188,211,213] sulfonamide-based linkers **11**−**13**,[251−253] new safety-catch linkers **14**[254] and **15**,[255] a linker **16** for immobilization of alcohols,[256] and traceless linkers **17** using Hofmann elimination[257] or desulfurization.[258] A methionine containing linker was based on the known lability of methionine−amino acid bond toward cyanogen bromide.[170,259,260]

2. Multiply Cleavable Linkers

The first multiply releasable linkers were designed for peptide libraries, and the residual functional groups from the linker remaining on a released peptide were not considered to be a critical disadvantage at that time. Releasable libraries were constructed according to the scheme depicted in Figure 36. Functional groups (amino groups) on the resin were branched to produce three independent arms, two of which were used for attaching the test compounds. The third branch linked the compound used for identification, which can be the same compound or a tag encoding the chemical history of a test compound (coding is described in section VI.B). Test compounds

Figure 37. Double releasable linkers based on iminodiacetic acid.

Figure 38. Double release from Ida-based linker.

were attached to two releasable arms via an ester bond; however, the ester bond was cleaved by two unique mechanisms entropically favored cyclization resulting in diketopiperazine (DKP) formation and alkaline hydrolysis.[60] The single release of a peptide based on DKP formation was described by Bray et al.;[261] however, the DKP moiety was cleaved from the resin and stayed with the released compound. To release compounds with the same terminus in both stages and without the pendant DKP, a new "reverse" DKP linker was designed, in which the DKP remains on the resin. Compounds are attached to the linker via an ester bond of Fmoc-Gly-NH-$(CH_2)_3$-OH (Fmoc-Gly-HOPA) and when released to the aqueous solution they contain an identical carboxy terminus, the hydroxypropylamide of glycine (Gly-HOPA).

Figure 39. Double release of compound without Glu-HOPA tag.

The first generation of new linkers was based on the Glu-Pro motif (Glu provides a side-chain function and Pro enhances the tendency to cyclize). (Actually five independent releases are possible utilizing selective deprotection of the Glu-Pro motif combined with photolytic cleavage and alkaline hydrolysis.[60]) The next generation of novel double cleavable linkers were based on the use of iminodiacetic acid (Ida) as a key component.[61] Iminodiacetic acid was found suitable for several reasons: (i) The imino group is in the α position relative to the carboxyl groups; (ii) both carboxyl groups are chemically equivalent; (iii) as an N-substituted amino acid it is prone to cyclization via DKP formation with practically any other α-amino acid; (iv) it is not chiral; (v) it is inexpensive. In general, there are three variations of the Ida-based linker. They can be schematically depicted as dipeptides containing Aaa-Ida, Ida-Aaa, or Ida-Ida, where Aaa is any α-amino acid, preferably one that is prone to cyclization via DKP formation. We found that the position of Ida in such a dipeptide was not important.

Figure 40. Double releasable linker for alcohols.

Figure 41. Photolytically cleavable linkers.

This is not true for the combination of Glu and Pro. While the dipeptide Glu-Pro provides satisfactory kinetics of DKP formation, the Pro-Glu cyclization takes more than 24 h.

The dipeptide motif Ida-Ida was found particularly suitable for designing double cleavable linkers.[61] The Ida-Ida dipeptide is prone to DKP formation; it provides three carboxyl groups, one on the amino terminal Ida and two on the carboxy terminus. To construct the double cleavable linker, two carboxyl groups are needed for derivatization and subsequent synthesis of test compounds, one for attaching the linker to the resin beads. Two Fmoc-Gly-HOPAs are either coupled to both carboxyls of the carboxy terminal Ida (Figure 37, linker **18**), or each Ida bears one Fmoc-Gly-HOPA (linker **19**). In either case there is one free carboxyl group that serves for connecting the linker to the solid support, e.g. via Lys which provides one extra amino group for a third, nonreleasable copy of the compound or the code. The chemistry of both releases is shown on Figure 38. By using peptide libraries built on a double cleavable linker, ligands for the anti-β-endorphin antibody and the glycoprotein IIb/IIIa receptor have been identified.[63]

The released compounds from both double releasable linkers **18** and **19** contained Gly-HOPA. Since it may be desirable to release a compound without the Gly-HOPA, but having a free carboxyl group instead, we designed a modified linker that incorporates an additional ester linkage[144] (Figure 39). The appended ester bond is introduced into the linker by attaching a hydroxy acid (e.g. 3-hydroxypropylamide of glutaric acid) to both arms of the linker. During the first release at pH 8 the DKP is formed and a compound with Gly-HOPA is released into solution. The beads are then separated from the solution by filtration, the pH is raised to ca. 13 using NaOH, and after ester hydrolysis (typically ca. 30 min) the pH is adjusted for biological screening. The second release is performed using NaOH as previously described and yields the desired compound with a free carboxyl group.

The practical advantage of these linkers is the aqueous buffer used for releasing the library compounds. The solutions of released compounds can be directly used in a biological assay. The chemical disadvantage lies in the limited chemistry that can be used during library synthesis, since the ester bond is labile toward nucleophiles.

The use of these linkers for small molecule organic libraries is also handicapped by the bulky group that is attached to the released library compounds. This disadvantage was eliminated by using amino alcohols as the first building blocks in a library synthesis, as depicted on Figure 40.[144] The chemistry of double release is exactly the same; however, the only common functional group in library compounds of this type is the hydroxyl group.

The release of compounds from resin beads in two steps using *o*-nitrobenzyl photolabile linker was described by Baldwin et al.;[160] the linker has recently been modified to produce less reactive ketone derivative upon cleavage.[262,263] Two new facile syntheses of *o*-nitrobenzyl alcohol for this linker were published recently.[264] An alternative design of photocleavable linker was used for solid-phase synthesis of fully

(i) 1% TFA in DCM
(ii) 95% TFA in DCM

Figure 42. System of gradually releasable linkers.

protected and unprotected peptides.[265] Different photolabile linkers are shown in Figure 41.

Another form of staged release takes advantage of reaction kinetics. Thus, gradual partial release of products from benzhydrylamine linker was achieved by Jayawickreme et al.[65] using TFA vapors. The synthesis of a multiply releasable system based on a combination of benzyl ester type acid labile linkers with different sensitivities toward acid has been described[266] and is shown in Figure 42. The most acid labile linker releases the compound in 1% TFA, while the second linker needs 95% TFA. The third copy of the same compound serves the purpose of analysis or on-bead binding assays; however, it can be cleaved by HF if needed.

Various approaches to the stepwise release of the compounds from polymeric carriers are discussed in the review article in ref 123.

IV. Screening Methods

A reliable high-throughput assay is essential to successfully screen a combinatorial library. As indicated earlier, both solid-phase and solution-phase assays have been developed for the one-bead-one-compound combinatorial library method. In the solid-phase assays, the ligands are still covalently attached to the solid support and the assays involve either (i) direct binding of molecular target to the bead-bound ligand[88] or (ii) detection of functional properties of the bead-bound ligand such as identifying phosphorylation or proteolytic substrates.[267,268] Solution-phase assays require cleavable linkers so that the ligands can be released while the beads are still spatially separated and the positive beads from which the positive ligands are released can be identified. Two solution-phase assays have been developed: the 96-well two-stage approach,[63,64] and the in situ-releasable solution-phase assay with immobilized beads.[50,62,65,66,269] Figure 43 illustrates the various methods used in the author's laboratory to screen one-bead-one-compound libraries.

A. On-Bead Screening

In this assay system the ligands are still covalently attached to the solid support. Most biological assays require aqueous media. Thus the solid support and its linkers must be water compatible. Two common solid supports fit this criterion: the polyethylene glycol grafted polystyrene bead (e.g. TentaGel) initially described by Rapp et al.,[270−272] and the polydimethylacrylamide bead (e.g. PepSyn gel) initially described by Sheppard et al.[273−275] Ideally, the beads should be uniform in both size and substitution. Unfortunately, although the size distribution for TentaGel is relatively uniform (Figure 43A and B), its functional substitution is not. In contrast, the substitution of the polydimethylacrylamide bead is fairly uniform (Lam et al., unpublished data) but the size is not (Figure 43C). Furthermore the polydimethylacrylamide beads are more sticky and have a tendency to clump together. There is a need for nonsticky beads with both uniform size and substitution that are fully compatible with aqueous media. The two approaches to screening bead-bound ligand libraries involve the detection of (i) target binding to the ligand or (ii) other functional properties of the ligand.

1. Binding Assay

The binding of target to bead-bound ligand can be detected either by direct visualization (e.g. a color target such as a dye,[276,277] or a larger target such as a cell[278]), or indirectly by using a reporter group such as an enzyme, a radionuclide, a fluorescent probe, or a color dye covalently attached to a target. The enzyme-linked colorimetric assay first described in 1991[4] is simple and highly efficient (Figure 43A). The screening of a library of 10^7 bead bound ligands can easily be accomplished by one person in one day.

Nonspecific binding or specific binding to an undesirable site of the receptor could be a significant problem in screening a library of millions of beads. Nonspecific binding can usually be eliminated by using a high ionic strength buffer (e.g. 0.3−0.4 M NaCl) with nonionic detergent (e.g. 0.1% Triton X-100 or Tween 20) and blocking proteins (e.g. bovine serum albumin or gelatin). The chromogenic substrates used in the initial screening system with alkaline phosphatase coupled target[4] were a combination of nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). Alkaline phosphatase hydrolyzes the phosphate and BCIP is then oxidized into a turquoise color with indigo dye. In turn the NBT is reduced to formazan, a dark purple insoluble precipitate deposited on the surface of the positive bead. The addition of NBT to the BCIP substrate greatly amplifies the sensitivity of the assay. However, NBT can also be reduced by any trace amount of reducing agent (e.g. ethanedithiol) in the beads left over from the library synthesis.[121] Additionally, certain peptide sequences, such as Asn-Asn-Asn were able to reduce NBT without addition of alkaline phosphatase.[50]

Many other enzyme/substrate systems such as horseradish peroxidase/tetramethyl benzidine have been tested in the author's laboratory. The horseradish peroxidase detection system has a tendency to produce more false positive beads, perhaps in part due to the nonspecific binding of the enzyme to the ligands. When using TentaGel beads, BCIP substrate alone at pH 8.5−9.0, although less sensitive than BCIP plus NBT, is superior and creates less

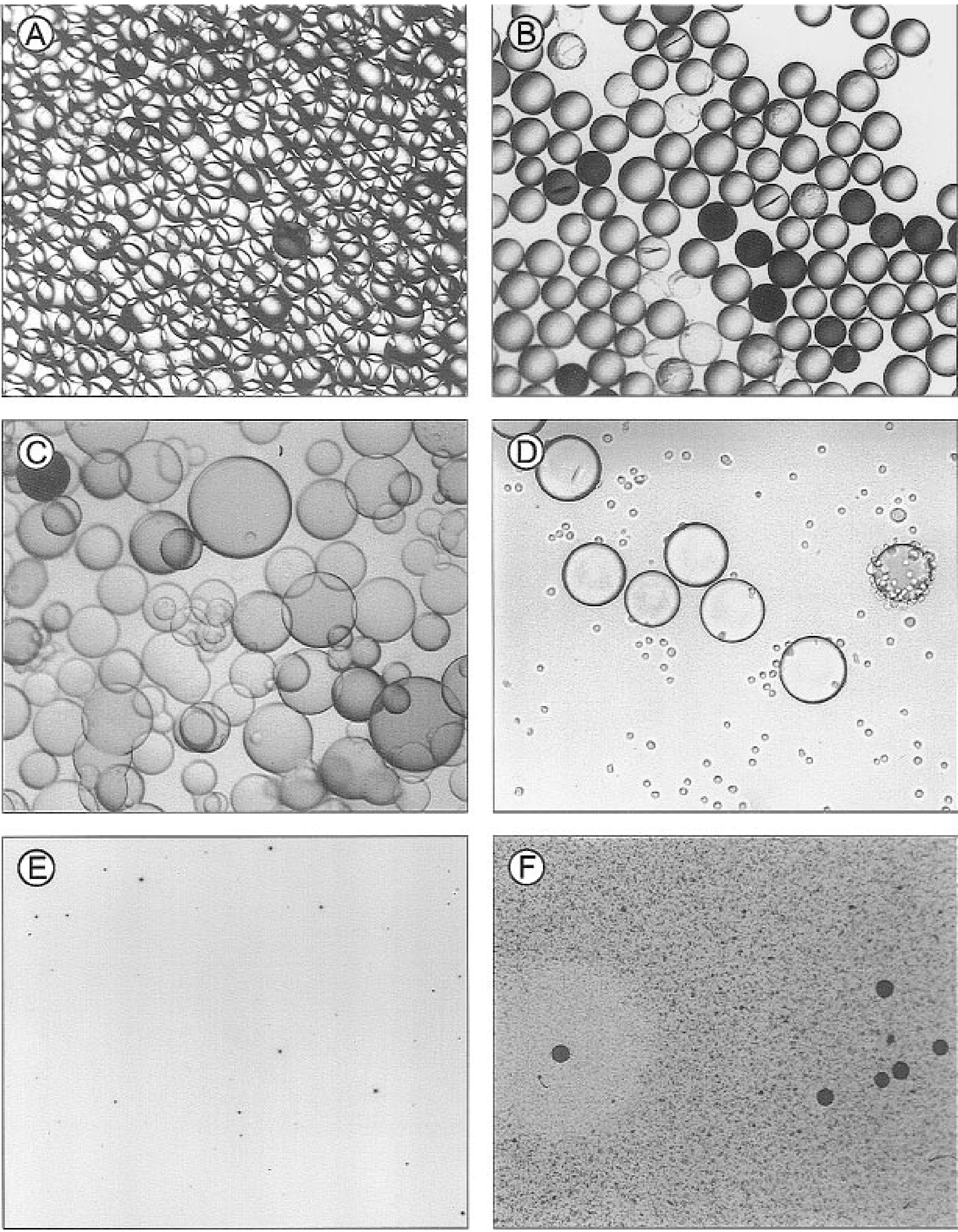

Figure 43. Examples of various screening methods for the one-bead-one-compound combinatorial libraries: (A) a positive bead in an enzyme-linked colorimetric assay using 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as the substrate; (B) a collection of positive beads with various color intensity; darker beads correspond to higher affinity; (C) a positive bead in an enzyme-linked colorimetric assay using a combination of nitroblue tetrazolium (NBT) and BCIP as substrates (please note that the size of PepSyn gel beads in this panel, unlike the TentaGel beads in the remaining panels, are not uniform); (D) a whole cell on-bead binding assay with a monolayer of intact myeloma cells attached to the surface of a positive bead; (E) an autoradiogram showing positive [^{32}P]-labeled peptide beads in an on-bead functional assay to discover peptide substrates for protein kinase; (F) an in situ solution phase releasable assay to discover anticancer agents (a clear zone of inhibition surrounds a positive bead). Please refer to the text and references for details of each method.

background.[88] In order to minimize the number of false positive beads, a dual-color detection scheme has been developed.[279] Alternatively, the positive beads can be decolorized with dimethylformamide and reprobed either in the presence of a competing ligand, or with a different secondary antibody.[55,56] In this way, many of the initially positive beads can be eliminated prior to microsequencing, and the chances of obtaining a true positive bead will be greatly enhanced.

Ohlmeyer et al.[280] used a dye-labeled target to screen bead-bound peptide libraries. A dual-color screening method using two different color dyes to detect specific binding has also been designed.[117] The main problem with dye-labeled targets is that the dye tends to bind to many of the library ligands complicating the screening process. Chen et al.[281] used a fluorescently labeled SH_3 domain to screen a peptide–bead library and isolated the positive beads with a fluorescent microscope. Alternatively, the bead library can be rapidly analyzed and the positive beads isolated with a fluorescence-activated cell sorter (FACS).[55,282] However, care must be taken that background autofluorescence of many commercially available beads does not interfere with the selection process.

Radionuclide-labeled targets have been used to screen bead libraries. Kassarjian et al.[283] used a [^{125}I]-labeled anti-β-endorphin to probe a random peptide library and used the autoradiographic detection method to localize the positive beads. This detection method appears more tedious when compared to the enzyme-linked colorimetric approach, particularly when macromolecular targets were used. However, in certain instances, particularly for small molecule targets, tagging with a radionuclide probe (e.g. ^{3}H or ^{14}C) rather than with a large enzyme may be sterically preferable. Nestler et al.[284] recently reported the development of a microautoradiographic screening method using a [^{14}C]-radiolabeled analog of a macrobicyclic receptor. The bead library was first immobilized on a glass slide with a thin layer of photographic emulsion. After two days of exposure, the slides were then developed and the positive beads were identified as a black halo and isolated under a microscope.

For molecular targets that are intrinsically colored or fluorescent, a bead library can be screened directly. Lam et al.[276] and others[277] have used color dyes to directly screen combinatorial peptide libraries and have isolated peptides that interact with the dye molecule. Besides screening for small molecule or macromolecular targets such as antibodies, enzymes, and structure proteins, one can also use intact cells to probe a bead library. Since intact cells are 6–10 μm in diameter, their binding to ligand beads (100 μm beads) can be directly observed, without a reporting group, under a dissecting microscope (Figure 43D).[285] With the use of this method, peptides that bind to the surface integrin of a prostate cancer cell line were identified.[278] In principle, the one-bead-one-compound library method can also be applied to intact viral particles, bacteria, or yeast. Since these organisms are so small, a reporting group of some sort may be needed for identification of positive beads.

2. Functional Assay

Inasmuch as most functional assays have been traditionally performed in solution phase (e.g. using a 96-well plate format), specific functional assays have been developed for detecting ligands that are covalently attached to beads. Lam and Wu[286] reported the use of a one-bead-one-compound library method to identify peptide substrate motifs for posttranslational modifications such as protein phosphorylation. In this assay, a random peptide–bead library is incubated with [γ-^{32}P]ATP and protein kinase. After incubation, the beads are washed thoroughly and heated to 100 °C for 5 min with 0.1 M HCl in order to hydrolyze the [γ-^{32}P]ATP thus eliminating any nonspecific binding of [γ-^{32}P]ATP to the positively charged peptide beads.[287] The beads are then immobilized on a glass plate with 0.5% molten low-gelling temperature agarose. After drying, the immobilized beads are exposed to an X-ray film. Autoradiography is then used for localization of the positive beads (Figure 43E). Because of the limited resolution of autoradiography, precise localization of the positive beads in the primary screen is impossible. The beads collected at or around the dark spot are excised, heated to 100 °C, diluted with additional molten agarose, and immobilized on a second glass plate, and then the autoradiography repeated. With this secondary screen, individual positive beads can be precisely located and isolated for structure determination. This approach can be applied to many other posttranslational modifications such as prenylation, methylation, glycosylation, ubiquitination, adenylation, sulfation, and hydroxylation.

Meldal et al.[268] devised a novel fluorogenic quenching assay for the identification of a proteolytic substrate motif from a bead library. In this assay system, a fluorescent molecule is attached to the carboxyl terminus of a random peptide and a fluorescent quenching molecule is attached to the amino terminus. After incubating with a specific protease, peptides on the bead with the appropriate substrate sequence will be cleaved, and the quencher will be released resulting in an increase in fluorescence of the positive bead. More recently, Meldal and Svendsen[288] reported the incorporation of a well-defined efficient substrate for trypsin Carlsberg into all the beads in a random library. As with previous experiments, a fluorescent group and a fluorescent quenching group were also incorporated into this peptide substrate, at the carboxyl and amino terminus respectively. As a result, each ligand of the random library is in close proximity with the fluorescent-tagged substrate. In the presence of the protease, the fluorescent quenching group will be removed and most of the beads will fluoresce. However, the beads carrying potent inhibitory peptides were able to block the proteolytic activity and the positive beads remained dark.

In principle, many other on-bead functional assays could be envisaged. For example, peptide beads with specific catalytic activity will change color in the presence of a colorimetric substrate; particles with photogenic material will emit light in the presence of an electric current.

B. Solution-Phase Screening

Solution-phase assays, usually in the 96-well plate format, have been used in mass screening for most drug discovery programs. Synthetic compounds, or natural products derived from fermentation broth, plants, or marine invertebrates, are usually added in a soluble form into each individual well for biological testing. There are many solution-phase assays available, e.g. competitive receptor binding

assays with radiolabeled ligands, various enzymatic assays, cell-based signal transduction assays, antibacterial assays, antiviral assays, and anticancer assays. All these solution-phase assays, in principle, can be adapted to combinatorial chemistry. Because the number of compounds or mixtures of compounds generated by combinatorial methods are enormous, the current trend is to miniaturize and automate these solution-phase assays.

There are two general approaches to screen a one-bead-one-compound library with solution-phase assays: (i) the 96-well two-stage release assays,[63] and (ii) the in situ-releasable solution-phase assay with immobilized beads.[50,62,65,66,269] In both approaches, ligands are attached to the solid support via cleavable linker(s).[60,61] 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.

1. The 96-Well Two-Stage Releasable Assays

In this assay, double orthogonally cleavable linkers are incorporated into the preparation of the library.[60,61,63,64] Approximately 100-500 beads are added into each well of a 96-well filtration plate (e.g. Millipore). Upon neutralization, the first linker is cleaved with the formation of a diketopiperazine molecule 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 tested for biological activity. Beads from the positive wells are then redistributed into filtration plates with one bead per well. With alkali treatment (e.g. gaseous ammonia), the second linker (ester linker) is cleaved, and the filtrates from each well are then tested for biological activity. The beads that correspond to the positive wells 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 bead into each well (one bead per well) can be automated. For a 100 μm bead, approximately 100 pmol of compound can, in principle, be 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 higher substitution. However, if the bead is too big, the efficiency of compound extraction from a bead may greatly diminish.

2. In Situ Solution-Phase Releasable Assay

Lam and Salmon[62] proposed the use of soft agar to immobilize beads. After the linker has been cleaved the compounds will be released and diffuse into the surrounding agar, where the solution-phase assay takes place. In 1994, Jayawickreme et al. reported a related method to identify ligands that interact with the MSH receptor. They first immobilized the bead library on a thin film of polyethylene and exposed the library to gaseous trifluoroacetic acid 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 melanocyte background.[65,269] Salmon et al.[66] have recently applied a related in situ solution phase releasable assay to discover anticancer agents.

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. >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 43F). Currently, not all solution-phase assays can be adapted to this assay format. However, undoubtedly in the near future, additional new assay systems will be developed for this in situ approach.

3. Combination of On-Bead and Solution-Phase Screening Assay

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.[63] successfully isolated ligands that bind to an anti-β-endorphin monoclonal antibody.

Alternatively, an on-bead binding assay can first be performed. The positive beads can then be collected for a releasable functional solution-phase assay to identify the true positive beads. For example, the beads that bind to a protein kinase can first be identified and isolated by an enzyme-linked colorimetric assay. Compounds from each positive bead can then be released and tested for protein kinase inhibitory activity.

C. Statistical Considerations

A few papers have been published on the statistical aspects of the "split synthesis" method used in the construction of the one-bead-one-compound combinatorial library.[289−292] Burgess et al.[289] used computer simulations to estimate the number of beads necessary in the library to cover a certain percentage of structures at a confidence level of 99%. Because of the enormous amount of computer time necessary for simulating any library longer than a pentapep-

tide, they derived an algebraic equation to approximate the library statistics. The equation only works well for large libraries (e.g. pentapeptide libraries). Using either the computer simulation or calculated methods, they estimated that for a library to cover 95% of all possible permutations at a 99% confidence level, the total number of beads in the library has to be approximately three times the number of all possible permutations. For example, a pentapeptide library (containing 20 amino acids) with 20^5 (or 3.2×10^6) possible permutations will require 9.6×10^6 beads to ensure that 95% of all possible permutations are included at the completion of library synthesis.

The frequently asked question is "is it necessary to have full representation of every possible permutation in a bead library?" From our experience, it is probably not necessary in most cases. The practical limit of a library that one can synthesize and screen is probably 10^8 beads (80 μm beads). By using an on-bead assay, 10^7 beads can routinely be screened by one person in one day. For a library with $<3 \times 10^6$ permutations (e.g. a pentapeptide library with 20 amino acids), one can easily cover 95% of all possible permutations by screening 10^7 beads. However, for a heptapeptide library (20^7 or 1.28×10^9 permutations), only a fraction of all possible permutations can be screened. This should not be a problem for many biological targets as usually only three to five of the seven amino acids are contact residues necessary for specific interaction. The remaining residues can be considered as spacers. This is certainly true for many antibodies.[293] In fact, this is the scientific basis behind the development of the library of libraries method (section III.B). The number of critical (irreplaceble) residues in the peptide ligand can be assessed from the number of positively reacting beads selected from the given library. The formula describing this relationship was developed and proven on several model targets.[55]

V. Library Analysis and Structure Determination of Identified Hits

There are two areas in which a novel approach to analytical chemistry (and a new way of thinking by analytical chemists) has been required. The first is the characterization of the synthesized library—making sure that all components are present and that they are present in equimolar amounts. The second is the determination of the structure present in very small amounts on an individual solid-phase particle.

A. Analytical Evaluation of Synthesized Libraries

Numerous analytical techniques have been applied for quality evaluation of prepared libraries. Taking into account that a one-bead-one-compound library is composed of individual beads containing unique chemical entities, which should be (in theory) pure, but as a whole, the library is a heterogeneous mixture, two approaches can be taken toward evaluation of library quality. The first approach is to analyze individual beads. This is limited by the question of a "statistically significant" sample size (i.e. number of beads which can be evaluated individually and which are representative of the whole library), the sensitivity of the analytical method (only picomolar quantities of compounds are available on the bead), and throughput of the method.

The method of choice for peptide libraries is microsequencing and amino acid analysis. Sequencing can identify incomplete couplings ("preview" of amino acids from the following cycle) or incomplete deprotection of side chain functional groups (obviously only protecting groups not removed during the sequencing cycle can be detected). Sensitivity of modern automatic microsequencers (in high femtomolar range) allows detection of these synthetic problems at the level of 1 to 2%. Sequencing based on Edman degradation can be combined with mass spectroscopic evaluation of mixtures generated by degradation using mixture of phenylisothiocyanate (PITC) and phenylisocyanate (PTC). The use of the second reagent does not result in cleavage of the amino terminal amino acid—this amino acid is capped and the resulting phenylcarbamoyl peptide resists further degradation. Repetition of this procedure provides a mixture of peptide fragments differring by one amino acid, which can be then analyzed by mass spectrometry. This method, originally designed for protein sequencing,[294] was recently applied for library analysis.[295]

One very sensitive analytical tool is mass spectrometry (for the review of the latest developments see e.g. ref 296). Its drawbacks, however, are (i) poor quantitation (signal intensity depends on ionizability of each component of the mixture, which can be dramatically different) and (ii) the fact that the compound has to be detached from the bead prior to the analysis. The most convenient method for compound detachment is exposure of the bead to gaseous reagents (ammonia, hydrogen fluoride, cyanogen bromide, trifluoroacetic acid) or photolytical cleavage of the compound. Here the beads do not have to be treated in separate vessels since the detached compounds remain physically attached inside of the bead matrix and can be extracted just prior to introduction, or even inside the mass spectrometer.[260,297−300] Mass spectrometry can identify all types of impurities arising from the synthesis or cleavage from the solid support, and it can therefore aid in the optimization of reaction conditions and selection of building blocks for the particular library (see e.g. ref 259). Mass spectrometric analysis of several hundred samples can be performed automatically (see e.g. refs 301−303).

Infrared spectroscopy is a valuable technique for evaluating the solid-phase transformation of functional groups (see e.g. ref 304). Especially promising seems to be those techniques addressing individual beads.[305−307] Spectra from a single bead are basically equivalent to the spectra obtained from a KBr pellet made of several milligrams (several thousands) of beads. Bead can be "flattened" between NaCl windows to a thickness of 10−15 μm (calculated value based on the volume and observed diameter of a flattened bead). Spectra obtained in this way show greater detail and are influenced less by moisture and carbon dioxide than spectra obtained from physically unmodified beads.[306] The sensitivity and selectivity

of infrared spectroscopy can be increased by application of carbon−deuterium bond-containing blocks, the quantitation of which is very reliable.[308]

Although NMR spectroscopy of solid-phase samples is widely used for the evaluation of solid-phase chemistries (see e.g. refs 309−312), its use in single bead analysis lies only with extremely large beads (∼600 μm) which can accomodate more than 10 nmol of compound.[313]

The second approach to the library analysis is to evaluate a sample of the library for its "statistical" features, where the library sample must have predictable properties. For example amino acid analysis of five beads from a tetrapeptide library has essentially the same probability of containing any particular amino acid only once as the probability of not containing this amino acid at all. On the other hand, 5000 beads from the same library (about 5 mg of resin, assuming an average bead size 130 μm) should provide equimolar representation of all amino acids. This analysis addresses very efficiently any gross synthetic problems such as omission of certain amino acids, or loss of the product from the carrier during synthesis or deprotection. Amino acid analysis is quantitative and is equally applicable to peptides with unnatural building blocks, for quantitation of organic molecule libraries built around amino acid scaffolds or utilizing amino acids as building blocks. Similar conclusions as from amino acid analysis, addressing individual positions in the library, can be drawn from multiple sequencing, which was applied for library characterization by Metzger et al.[314] The significance of this method can be increased by sampling the library after every condensation step. Sequencing by Edman degradation is, however, limited to peptide libraries with free amino termini and containing only α-amino acids.

Use of mass spectrometry for mixture analysis is based on the straightforward prediction of mass distribution of the library. Computer-generated distribution profiles (see e.g. ref 315) can be compared with the actual profile obtained from the library sample.[100,314] Depending on the ionization technique, the theoretical profile may need to be modified with a correction factor. For example peptide libraries evaluated by FAB mass spectrometry should take into consideration high proton affinity of arginine after multiplication of abundances of arginine-containing peptides by the factor of 10 the correspondence of theoretical and experimental spectrum can be achieved.[316] Evaluation of mass distribution detects synthetic problems based on incomplete coupling (shift toward lower molecular weights), incomplete deprotection or unwanted library modification, such as oxidation, acylation, or alkylation (shift toward higher molecular weights). Very useful information may be obtained from tandem mass spectrometry about classes of ions fragmenting into a common daughter ion or about compound classes losing the same neutral mass. This technique is helpful in determining the completeness of removal of specific protecting groups or about formation of side products during the synthesis.[317]

HPLC and capillary electrophoresis are often used for analysis of relatively small compound mixtures. The power of these methods can be enhanced by coupling with mass spectroscopic evaluation of the column eluate. In this arrangement mixtures of several hundred compounds can be analyzed. Using a microcolumn with a long gradient (up to 240 min), combined with MS/MS analysis, Nugent et al.[318] were able to identify all 272 components of a peptoid mixture. It is advisable to use a desalting trap cartridge to remove Na^+ and K^+ adducts which can complicate analysis of complex samples. Electrospray mass spectrometry has been used for characterization of numerous libraries.[303,314,317,319−324] Boutin et al.[316] compared mass spectrometry (FAB), capillary electrophoresis, and NMR for library characterization and concluded that existing analytical techniques can provide large amounts of information about prepared libraries.

B. Structure Determination of Identified Hits—Direct Sequencing

Peptides are especially good "substrates" for one-bead-one-compound (or one-bead-one-motif) libraries due to the fact that peptide structure can be ascertained from picomolar amounts on individual beads. Only minimal modification of standard sequencing protocols of automatic sequencers is required for most of the solid carriers. The only serious difficulties were encountered in sequencing beads from libraries synthesized on Sepharose.[325] To avoid unacceptably high carryover it was necessary to mechanically desintegrate the beads and/or apply the isothiocyanate solution in several cycles. The biggest obstacle in bead sequencing is the uniqueness of the individual bead and the potential loss of very valuable information due to "unforeseeable" problems such as malfunctioning instruments or power failure. For a unique bead (i.e. where only one bead was found positive and verified to be specific) it is possible (even advisable) to cut the bead in half and subject only half of the bead to sequencing. The techniques of single bead manipulation are impressive in the simplicity of the instrumentation needed for addressing individual beads. The critical component is a good dissecting microscope. The rest of the equipment consists, quite simply of a petri dish and an injection needle. The needle is sharp enough to cut the beads and its point can be used for lifting the bead and transferring it to the sequencing support. It is a good practice to use the needle to "pack" the bead into the fibers of the support filter to prevent its "jumping out". The micromanipulator described in the original publication[4] was quickly abandoned in favor of the micropipet and needle techniques.

Sequencing provides the sequence of linear peptides composed of α-amino acids. Non-α-amino acid containing peptides can be sequenced to the point of the first occurence of a nonsequenceable building block. Sequencing beyond this point (not addressing the identity of this particular building block—in cases where only one or a limited number of unnatural building blocks are used in defined positions of a library) can be achieved by coupling nonequimolar amounts of nonsequenable building blocks, creating thus the omission (but sequenceable) peptide on the same bead.[50,62] Alternatively a sequenceable (usually glycine) amino acid can be cocoupled with the unnatural one, thus creating a mixture of two peptides

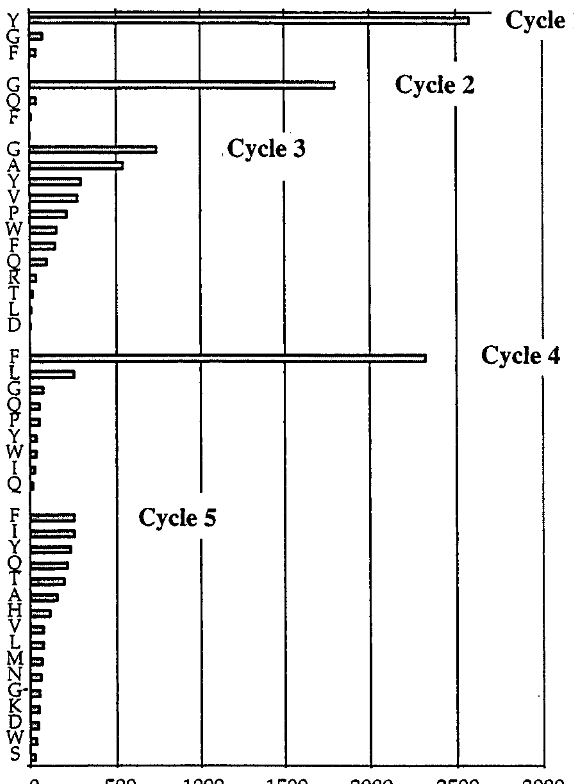

Figure 44. Results of multiple sequencing. Ninety-nine beads selected in the test evaluating binding to anti-β-endorphin antibody were sequenced in three batches of 33 beads and all data were cumulated. The first cycle provided almost exclusively Tyr, the second cycle revealed Gly, and the fourth cycle Phe. Several amino acids were found in the third and fifth cycle of the sequencing. Consensus structure Tyr-Gly-Xxx-Phe-Xxx derived from this experiment corresponds closely with results found by analysis of individual beads.

on one bead. This approach closely resembles that of structural coding, discussed below. Cocoupling of the second amino acid was also used for "tagging" D-amino acids in a sequence, although chiral sequencing can be applied in this case.[326]

Optimization of a chromatographic gradient can achieve separation of more than just the 20 natural amino acids. An example of good separation of unnatural and side chain-modified trifunctional amino acids was published.[55] Sequencing of cyclic peptides is usually straightforward; the bridging amino acid is not revealed in the cycle addressing this amino acid (confirming the cyclic structure); however, the structure containing both bridging amino acids is eluted in the cycle addressing the second amino acid. More problematic is the sequencing of "reversed" peptides—peptides attached to the resin, but having the carboxy terminus exposed.[101–103] Instead of applying C-terminal sequencing, we attached (or rather reattached) the peptide to the resin via an amino acid side chain, leaving the α-amino group free and therefore available for sequencing (see Figure 4). Sequencing in this case has to be performed on support modified for peptide sequencing since after the sequencing cycle cleaving the point of attachment (blank cycle on HPLC trace—this amino acid remains attached to the resin), the peptide is released from the bead and has to be "caught" by the support.

For one-bead-one-compound libraries, it is not always necessary to sequence individual beads. In cases where the structural consensus can be expected (small libraries or libraries with fixed structural elements), it is sometimes much faster to sequence a multiplicity of beads. Evaluation of signals in individual cycles can clearly show the requirement of the given position for the particular amino acid. In the cycles with high specificity requirement only one amino acid can be found; in positions not essential for the binding (activity) numerous amino acids can be found. The first conclusion about structure–activity relationships can be drawn from these sequencing data. This approach has been successfully applied to the anti-β-endorphin antibody ligands[327] and streptavidin ligands[55] (see Figure 44).

Peptide sequencing can reveal whether the mixture or individual amino acids were coupled in a particular position of structure found in library of libraries[120] representing motifs and not individual peptides (see above). "Tagging" by addition of an unnatural amino acid to the mixture of amino acids was used to identify a mixture composed of L- or D-amino acids. Hornik and Hadas[121] mixed unnatural (nonsequenceable) amino acids with sequenceable amino acids in the synthesis of a peptide library. The sequencing result was interpreted as the presence of either the α-amino acid or the nonsequenceable one. In their approach a multiplicity of peptides were synthesized on each bead and a high number of potentially active sequences had to be resynthesized.

C. Coding and Decoding

The inclusion of non-α-amino acids and novel building blocks required new methods for compound identification, since direct sequencing was no longer feasible. The idea of coding was suggested by Brenner and Lerner,[328] who contemplated the use of polynucleotides for coding peptide libraries. The advantage lies in the sensitivity and possibility of sequencing tens or hundreds of samples of nucleic acid codes in parallel. Nucleic acid coding has been applied in several cases;[282,329,330] however, its application for coding organic libraries is rather limited due to the incompatibility of nucleic acid chemistry with other organic reactions. Additional coding strategies were developed for nonsequencable one-bead-one-compound libraries.[280,331] Here, coding subunits, different from the test compound subunits, are attached to the solid support in a separate reaction from the addition of the test compound subunit. Coding subunits are those which can be easily identified by well-established analytical microtechniques: amino acids (Edman degradation and HPLC),[331,332] electrophoric tags (halocarbon molecules determined after silylation by gas chromatography),[280,333] or amines.[172] This last technique uses very simple chemistry of polyamide formation from protected iminodiacetic acid and secondary amines. (The same chemistry was also used for library formation.[127,334]) Amines are recovered after total hydrolysis of the active bead, dansylated and analyzed by HPLC. The repertoire of coding subunits can be extended by the application of the so-called digital coding principle, in which more than one subunit is used for coding individual building blocks.[55,280] To prevent the undesirable interaction between the coding structure and the biological target, the technique of sequester-

Table 1. Structures Determined from Individual Bead by Mass Spectrometry

bead material	size (μm)	technique	structure	model/library	reference
polystyrene	40	TOF-SI	Val-Tyr-Val	model	Brummel et al.[297]
MBHA	50 (?)	MALDI		model	Zambias et al.[298]
polystyrene	?	MALDI	Peptides	model	Egner et al.[260]
polystyrene	?	MALDI	Heptapeptides	model	Egner et al.[300]
polystyrene	40	TOF-SI, MALDI, ES		model	Brummel et al.[345]
TentaGel	130	ES		library	Stanková et al.[159]
TentaGel	130	ES		library	Kočiš et al.[151]
TentaGel	130	ES		library	Krchňák et al.[222]

ing the two structures in the internal volume of the bead (coding structure), and on the surface (screening structure), was developed.[335,336] This technique is based on the limited accessibility of the interior of the polymeric bead for the macromolecular reagents (enzymes), allowing the generation of orthogonally (Fmoc/Boc) protected volumes of the polymer. An alternative tagging method incorporating a radio frequency transmitting chip to record each coupling reaction[337,338] can be applied to a bead library only after the radiofrequency transmitting chips are appropriately miniaturized.

D. Mass Spectrometry

The only alternative technique for structure determination of a compound synthesized on one solid phase particle is mass spectrometry. Mass spectrometry can be used to complement amino acid sequencing (e.g. ref 339), or to determine nonsequenceable components of peptides composed from unnatural amino acids. Sequencing of peptides by mass spectrometry was developed in several laboratories (for review see refs 340 and 341). Sequencing can be substantially simplified by using deuterium exchange[342] since determination of exchangeable protons can decrease the number of possible sequences by an order of magnitude (for a simulation of this see ref 315). Another approach to peptide sequence determination was described by Youngquist et al.[259] It is based on partial (~10%) capping of a growing peptide chain in each step of the synthesis. Each bead thus contains all partial sequences, and the synthetic history can easily be interpreted from mass differences between these capped peptides. Residues with the same molecular weight (Ala and β-Ala, or enantiomeric amino acids) can be distinguished by using mixtures of different capping reagents. If the capping reagent contains an isotope with a characteristic isotopic "signature", e.g. bromine, the individual shorter sequences can easily be identified, even at very low levels (close to the noise level of an experiment).[343]

The most useful application of mass spectrometry is to determine the chemical structure of hits from small organic molecule libraries. The compound must be chemically detached from the polymeric

matrix, but does not necessarily have to be physically separated from the bead.[260,297−300,344] Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) or time-of-flight secondary-ion mass spectrometry (TOF-SIMS) techniques are used in this instance. The bead, which has been exposed to cleavage conditions (e.g. in reagent vapors), is placed on the sample plate, matrix solutions plus internal standards are added (in MALDI), and the plate is introduced into the spectrometer. Molecules available on the bead surface provide a sufficiently strong signal which can be used for molecule characterization or even for bead imaging.[297,345] Electrospray mass spectrometry (ESMS) requires cleavage and extraction of the compound from the bead. Brummel et al.[345] evaluated the above-mentioned techniques on an angiotensin II antagonist-based library synthesized on 40 μm polystyrene beads, and concluded that any of these techniques can be used for direct bead analysis of combinatorial libraries. All techniques provided sufficiently high precision, accuracy (<0.01 Da), and sensitivity. TOF-SIMS can address individual beads in the bead matrix thus eliminating the need for physical separation of beads. ESMS can, on the other hand, easily be automated for processing of multitudes of beads. Improvement in the exact mass determination can be achieved with the employment of Fourier transform ion cyclotron resonance mass spectrometry.[346] Examples of structure determination from individual bead are given in Table 1.

VI. Applications of One-Bead-One-Compound Libraries

Since the initial publication of the one-bead-one-compound combinatorial library method about five years ago,[4] there have been numerous reports on the application of this library approach to various biological targets (Table 2). Most of the published work employed the solid-phase screening approach. There are only a few articles published on the use of solution-phase assay methods.

A. Monoclonal Antibodies

Using an enzyme-linked colorimetric assay, Lam et al.[4] screened a random pentapeptide library with anti-β-endorphin monoclonal antibody (clone 3-E7) and identified six peptides (with K_i ranging between 17 and 9000 nM) that bind specifically to this monoclonal antibody (mAb). This mAb recognizes a continuous epitope YGGFL. When pentapeptide libraries containing D-amino acids were screened, additional peptides related to this motif were identified.[347,348] The binding affinity of peptides that differ from the YGGF motif were at least 100-fold lower.

When an antiinsulin monoclonal antibody (clone AE906) that recognizes a discontinuous epitope was used to screen a number of different peptide libraries, several distinct motifs were identified.[293] The libraries screened include linear L-amino acids containing tetra-, penta-, hexa-, hepta-, nona-, 12-, 15-mer peptide libraries; cyclic hepta-, octa-, nonapeptide libraries; “turned library” with a D-proline in the middle; and all D-amino acid containing hexa- and octapeptide libraries. A sequential screening approach with secondary and tertiary libraries enabled them to optimize the initial lead and identify ligands with significantly higher affinity.[349] Lam et al.[293] recently reviewed their experience of using the one-bead-one-compound library method for B-cell epitope mapping.

Table 2. Applications of One-Bead-One-Compound Combinatorial Library Methods

target	ref(s)
on-bead binding assay	
monoclonal antibodies	4, 293, 347−352
surface idiotype of lymphoma cells	285
streptavidin/avidin	4, 104, 353, 354
factor Xa	55, 355
thrombin	55, 355, 356
SH_3 domain	113, 281
MHC-class I molecule	357
dopamine D_2 receptor	358
glycosomal phosphoglycerate kinase	359
synthetic receptor	29, 117, 360
small organic dye	276, 277
prostate cancer surface integrin	278
on-bead functional assay	
cAMP-dependent protein kinase	267, 286
p60c-src protein tyrosine kinase	287, 361
c-abl protein tyrosine kinase	Wu and Lam, manuscript in preparation
protease substrate	268
protease inhibitor	288
solution-phase assay	
anti-β-endorphin MoAb	63
gpIIb/IIIa integrin	63
MSH	65, 269, 362
G-protein coupled receptor	362
anticancer agent	66
antibacterial agent	unpublished
carbonic anhydrase	169

Mattioli et al.[350] screened a random hexapeptide library with an antihepatitis A virus (HAV) mAb and identified a peptide that reacts specifically with monoclonal and polyclonal anti-HAV antibodies. This peptide, not only could induce a specific humoral response in mice, but could also be used in an ELISA to detect a primary IgM immune response in sera of acutely infected human patients. Interestingly, no sequence homology was found between the peptide and the HAV capsid proteins VP1 and VP3.

Steward et al.[351] screened an octapeptide bead library with antimeasles virus F protein monoclonal antibody and identified several peptide mimotopes. When these peptide mimotopes were used to immunize BALB/c mice, only one of the mimotopes was able to induce antibodies that cross react with Rn and inhibit measles virus plaque formation. Coimmunization of mice with this mimotope and a T-helper epitope induced a humoral response that protected the animal against fatal encephalitis following challenge with measles virus and with a structurally related canine distemper virus.

On the basis of the data obtained from a phage displayed peptide library, de Koster et al.[352] synthesized two dedicated 12-mer bead libraries and screened them with a mAb against glycoprotein D of Herpes simplex virus type 1. They were able to identify a peptide with an affinity 50 times higher than that of the natural epitope.

B. Surface Idiotype of Lymphoma Cell Lines

Lam[363] proposed the use of idiotype-specific peptides for targeted therapy of B-cell lymphoma. Idiotypes are surface monoclonal antibodies of B-cell lymphoma. These surface antibodies are specific for lymphoma cells, as well as specific for each individual patient. Using a murine lymphoma model with WEHI-231 and WEHI-279 cell lines (both with surface IgM), Lam et al.[285] identified a series of all L-amino acid hepta- nona-, and 11-mer peptides and all D-amino acid octapeptides that bind specifically to the lymphoma line. These peptides were able to bind specifically to the lymphoma line. When presented to the cell in a tetrameric form, they triggered signal transduction resulting in an elevated level of protein tyrosine phosphorylation.

C. Streptavidin and Avidin

Lam et al.[4] initially identified HPQ and HPM as binding motifs for streptavidin. The result resembles that of the streptavidin screen using a filamentous phage library.[36] Lam and Lebl[353] screened a library devoid of histidine and identified additional non-HPQ motifs with an apparent lower affinity. Interestingly, when the libraries were cyclized by disulfide bridges, HPQ and HPM motifs were also identified but the position of HPQ within the ring was greatly dependent on the size of the ring.[104] When D-amino acid containing peptide libraries were screened, the following motifs specific for streptavidin were identified: W(y/f)₋(y/e/f)a, ₋WpH, Y₋fP, and ₋w(F/Y)pH. In addition to streptavidin, avidin was also screened as a target.[353] Despite the fact that avidin and streptavidin both bind strongly to biotin, the peptide motifs identified from both avidin and streptavidin were different. Using a similar approach, Ostergaard et al.[354] screened several linear L and D libraries and identified additional motifs for these proteins.

D. Protease

Several groups have used the bead library approach to screen for protease substrates or inhibitors. Using an enzyme-linked colorimetric assay, scientists at Selectide Corporation identified novel peptides that bind to and inhibit the proteolytic activity of thrombin or Factor Xa. On the basis of the peptide motifs, peptide and peptidomimetic inhibitors for factor Xa with IC_{50} values in the low nanomolar range were identified.[55,355,356,364,365]

As indicated earlier, Meldal et al.[268,366] employed a novel fluorogenic quenching screening method to identify proteolytic substrate motifs from a bead library. In this assay system, a fluorescent molecule and a quenching molecule are built into the amino and carboxyl terminus, respectively, of a random peptide library. Peptides cleaved by a protease will fluoresce. In order to directly detect protease inhibitors, Meldal et al.[288,367] modified their assay system by constructing a fluorescent and quenching molecule into a selected peptide substrate which were then incorporated into a bead library with random peptides. The beads displaying potent inhibitory peptides were able to block the proteolytic activity and the positive beads remained dark. With this approach, inhibitors with IC_{50} values ranging between 3−90 μM were identified for sustilisin Carlsberg.

E. MHC-Class I Molecule

Although number of groups have applied combinatorial peptide library methods to map anchor motifs for MHC molecules,[261,368−371] Smith et al.[357] were the first to describe the use of the bead library approach to determine the anchor residues for MHC-class I molecules. The α-chains of HLA-A2.1 or -B7 were first purified, dissociated with 5 M KSCN, and mixed with a random nonapeptide library. The α-chain then reassociated with β_2 microglobulin (in excess) on the appropriate peptide bead when the KSCN was slowly removed by dialysis. The reassociated HLA complex was then detected with an enzyme-linked colorimetric assay using anti-MHC Class I molecule monoclonal antibody−enzyme conjugate. A series of peptides with specific anchor residues, which corroborate with those in the published literature, were isolated.

F. Protein Kinase

Lam et al.[267,286] developed a solid-phase phosphorylation assay to screen random peptide libraries for substrate motifs of protein kinase. Peptide-beads are first mixed with [γ-^{32}P]ATP and a specific protein kinase. After thorough washing, the beads are immobilized and autoradiography is used to localize the [^{32}P]-labeled bead for structure determination. (Please refer to the section on functional assays for detail.) With this approach, Wu et al.[267] identified RR₋S as the preferred substrate motif for cAMP-dependent protein kinase.

When p60^{c-src} protein tyrosine kinase (PTK) was screened, YIYGSFK was identified as an efficient and specific substrate for p60^{c-src} PTK.[361] Optimization with a secondary library, XIYXXXX, enabled them to identify GIYWHHY as a better substrate.[287] On the basis of this peptide sequence, pseudosubstrate inhibitors with IC_{50} values at low micromolar levels were developed. Recently, c-abl PTK was used to screen a XIYXXXX peptide library and four distinct peptide substrate motifs were identified (Wu and Lam, manuscript in preparation).

G. Enzymes of *T. brucei*

Samson et al.[359] screened an L-amino acid−bead library with either biotinylated or fluorescein-labeled glycosomal phosphoglycerate kinase (gPGK) of *T. brucei*. The pentapeptide NWMMF identified was able to selectively inhibit gPGK with an IC_{50} of approximately 80 μM. The same group used a similar approach to screen a D-amino acid pentapeptide library with biotinylated fructose-1,6-biphosphate aldolase of *T. brucei*. However, none of the identified peptides inhibited fructose-1,6-biphosphate significantly.[359]

H. Carbonic Anhydrase

Burbaum et al.[169] synthesized small molecule−bead libraries that had been encoded with a chemical tag and screened against carbonic anhydrase. The beads were first distributed into a 96-well plate with one

bead per well. After UV irradiation (350 nm), the compounds from each bead were released, extracted, and tested for carbonic anhydrase inhibitory activity. With this approach, two compounds, 2-[*N*-(4-sulfamoylbenzoyl)-4′-aminocyclohexanespiro]-4-oxo-7-hydroxy-2,3-dihydrobenzopyran and [*N*-(4-sulfamoylbenzoyl)-L-leucyl]piperidine-3-carboxylic acid, with nanomolar dissociation constants (15 and 4 nM respectively) were isolated.

I. SH_3 Domains

Chen et al.[281] labeled the SH_3 domain of phosphatidylinositol 3-kinase (PI3 kinase) with a fluorescent tag and used it to screen bead libraries for binding peptides. No definitive positive leads were identified when a random hexapeptide and cyclic heptapeptide libraries were screened. However, when a biased library of XXXPPXPXX was screened, several positive beads with two classes of consensus sequences were identified: R_LPP(R)P_ _ and _ _ _PPLP_R. The dissociation constant (K_d) of the better ligands range between 8 and 30 μM. Similar experiments were performed in the SH_3 domain of $p60^{c-src}$ PTK. Some of the isolated ligands exhibit selectivity toward one over the other SH_3 domain. For example, RKLPPRPSK, the PI3 kinase SH_3 domain ligand, binds to the PI3 kinase and Src SH_3 domains with K_D of 9.1 μM and 53 μM respectively. More recently, Combs et al.[113] from the same group screened a X′Y′Z′ -PLPPLP library (wherein X′Y′, and Z′ are nonpeptide components) with fluorescently labeled Src SH_3 domains and identified several peptide ligands with nonpeptidic elements.

J. Integrin

Using a 96-well solution phase-releasable assay, Salmon et al.[63] screened a cyclic peptide library for peptides that bind to platelet derived gp IIb/IIIa integrin, identifying cyclic CRGDC as an active ligand. Using a totally different approach with a whole-cell binding assay, Pennington et al.[278] screened two linear nona- and 11-mer all L-amino acid peptide libraries with intact prostate cancer cell lines (DU145) and used antiintegrin antibody as a blocking agent to select for integrin-specific binding peptides. They isolated two peptides that have some sequence homology with AG-73 and GD-2 peptides that were derived from laminin. They further demonstrated by flow cytometry that the isolated peptide, LNIVSVNGRH, was able to bind to the intact prostate cell line. Additionally, when the biotinylated peptide was added to streptavidin-coated plastic, the prostate cancer line was able to bind to the plastic.

K. G-Protein-Coupled Receptor

As mentioned earlier, Jayawickreme et al.[65] developed a “multi-use peptide library” of which a functional screen can be used to detect agonists to G-protein-coupled receptors. This is essentially an in situ-releasable assay in which the bead library was first immobilized on polyethylene sheets and then exposed to TFA vapor for 6 h followed by neutralization with NH_3 vapor. Melanophores that had been electroporated with bombesin receptor plasmid DNA (pJG3.6BR) and transiently expressing bombesin receptors were used as target cells. The immobilized beads on the polyethylene film were then placed face down on the agar bed of melanophores. After 5-10 min, pigment dispersion induced by the released peptides were monitored by video image subtraction using TLC-Image software (Biological Detection Systems, Pittsburgh). Beads corresponding to the positive area were recovered and thinned out, and the assay was repeated. A tertiary screen was also performed by placing individual beads 0.5 cm from each other and the assay repeated. Individual beads binding with bombesin receptor could be identified and microsequenced. In principle, ligands for any other G-protein-coupled receptor can also be discovered with this method. Using this approach, Jayawickreme et al.[269] identified several novel α-MSH receptor antagonists, of which the most potent, MPfRwFKPV, had an IC_{50} value of 11 $\pm$ 7 nM.

L. Dopamine D_2 Receptors

Sasaki et al.[358] synthesized the extracellular nonapeptide sequence of CNS dopamine D_2 receptors (residues 7−15: SWYDDDLER) and coupled it to magnetic beads via the amino terminus. These magnetic beads were then used as a probe to screen random pentapeptide−bead libraries. The positive peptide−bead rosetted by the magnetic beads were separated from the remaining bead library with an external magnet. Six peptides were isolated and their binding affinity to the dopamine D_2 receptor nonapeptide were determined. The K_d values of the best ligands identified were estimated to be 0.1 μM.

M. Synthetic Receptors

Still[29] recently reviewed their experience on the discovery of sequence-selective peptide binding by synthetic receptors using an encoded one-bead-one-compound combinatorial library. The synthetic receptor was first linked to a dye and used as a probe to screen the bead library. The colored beads were then isolated for structure determination.[360,372] In another experiment, Boyce et al.[117] synthesized a bead library peptidosteroidal receptor with two random tripeptide appendages. Dye-labeled enkephalin peptides were used as probes to screen the bead library. In the same paper, they also reported a two-color assay system in which two closely related peptide probes were labeled with different color dyes (e.g. red and blue). The peptidosteroidal receptors that were least specific turned reddish-purple, whereas beads that stained either blue or red were more specific.

N. Small Organic Dyes

Lam et al.[276] reported the use of a small organic dye, indigo carmine, to screen an L-amino acid heptapeptide library and two D-amino acid hexa- and octapeptide libraries. Peptide−beads that bound to the dye turned bluish turquoise color. Several peptides (whether from an L library or a D library) with the motif X+OOO+X were isolated (X = no preference, + = Lys or Arg, O = hydrophobic residues).

Using a similar approach, Wennemers and Still[277] screened acylated peptide bead libraries with a

number of organic dyes and identified several tripeptides interacting with these dyes.

O. Anticancer Agent Development

Salmon et al.[66] recently reported the use of an in situ solution phase-releasable assay to discover anticancer agents. Peptidic or peptidomimetic bead libraries were synthesized with cleavable Ida linkers (see section III.E). The bead library was then mixed with a cancer cell line and plated in soft agarose (0.5%) containing tissue culture medium. After 48 h, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added. The live cells metabolize MTT into a purple colored formazan product but the dead cells do not. As a result, a clear zone of inhibition is readily identifiable, even with the naked eye, surrounding a positive bead from which an anticancer compound was released. The positive bead was then isolated for structure determination. With this approach, peptidic and nonpeptidic anticancer agents were identified. This in situ solution-phase soft agar approach has also been used by scientists at Selectide Corporation for the discovery of antimicrobial agents (unpublished), and certainly has the potential to discover new antibiotics for bacterial, fungal, or viral pathogens.

VII. Perspectives

Combinatorial chemistry is now considered one of the most important recent developments in medicinal chemistry. The field has advanced rapidly over the past five years. The molecular biology revolution of the last two decades enables researchers to routinely clone and express biological receptors, enzymes, and proteins of pharmacologic interest. Many new drug targets for various diseases have now been identified. Relying on traditional drug discovery methodology to screen this many targets would almost be an impossible task. The development of combinatorial chemistry is timely and undoubtedly will contribute to the discovery of new drugs that can benefit mankind. Combinatorial chemistry is especially useful when applied in conjunction with modern computational chemistry and molecular modeling techniques since many leads can often be generated from a single combinatorial library screen. In addition to the discovery of initial leads, combinatorial chemistry can also be applied to the optimization of drug leads.

The one-bead-one-compound library method is one of five general approaches to combinatorial chemistry (see section I). As indicated earlier, there is no single best library method. Each of these methods has advantages and disadvantages. The choice of the method largely depends on the nature of the drug target or the basic science question one is asking, and the expertise and resources available. Sometimes it is advantageous to combine some of these approaches to solve a specific problem. One of the advantages of the one-bead-one-compound combinatorial library method is the spatially separable nature of the library. This enables one to test all the compounds concurrently but independently. As a result, often several completely different motifs can be identified from one single screen (e.g. see ref 293). This differs greatly from the library methods requiring deconvolution, where mixtures of compounds are generated and tested, and the chemical structure of the active compound is deduced by the assay results as well as the synthetic history of the mixtures. Often, this method enables one to determine only one predominant motif. If there were multiple equally important but completely different motifs for a specific target, the results may be difficult to interpret. The main disadvantage of the one-bead-one-compound library method is that after the positive bead has been identified, one still needs to determine the chemical structure either directly by Edman degradation and mass spectrometry or indirectly by decoding. In the last few years, there have been significant advances in this area. The radiofrequency encoding strategy has great potential but miniaturization of the chip to less than 100 μm is needed to make it effective in a large library format. Magnetic encoding may be feasible but requires further development. Currently, chemical encoding is the only practical method available. Ideally, the solid-phase support for the one-bead-one-compound library method should be (i) uniform in size and substitution; (ii) nonsticky under synthetic and assay conditions; (iii) compatible with both aqueous and organic conditions; and (iv) available in different sizes, shapes, and porosity. None of the currently commercially available solid supports fulfill all these criteria, and further development in this area is definitely needed.

There have been only a few reports on solution-phase assays to screen the one-bead-one-compound libraries, indicating much room for development in this area. New cleavable linkers that are compatible with a variety of organic syntheses and assay systems are needed. Miniaturization and automation of liquid and bead handling of individual assay compartments such as development of an automatic machine to rapidly manipulate, extract, and separate releasates from each bead into tiny assay compartments may greatly facilitate this process. In situ-releaseable assays with immobilized beads work well in a few biological systems (see section IV.B.2) but adaptation of this principle to a variety of other assay systems is needed.

Development of new coupling chemistries and new building blocks has been the focus of many combinatorial chemists in the last few years. We are confident that in the next several years, solid-phase organic synthesis with high coupling efficiency will be routine and numerous building blocks specially designed for combinatorial chemistry will be commercially available. This will parallel the current developments in solid-phase peptide synthesis.

Currently, most of the drugs used in the clinic are small molecules with molecular weight ranging between 300 and 600. Therefore, the pharmaceutical industry has been primarily interested in small-molecule combinatorial chemistry. While the small-molecule combinatorial library approach is the method of choice for developing drugs for intracellular targets, one should not forget that the field of synthetic combinatorial chemistry began with peptide libraries.[2−5] We believe that peptide library methods still play an important role in identifying drug leads and for the development of drug candidates for extracellular targets. Since peptide−protein, protein−

protein, and protein–nucleic acid interactions represent the majority of molecular interactions and signaling within a living cell or a living organism, combinatorial peptide library methods, using either biological or synthetic approaches, will remain extremely powerful tools for basic research in the area of molecular recognition.

Combinatorial chemistry has been applied almost exclusively to biological systems and was only recently applied to the physical sciences. For example, Schulz and co-workers recently used a spatially addressable library approach to develop a class of cobalt oxide magnetoresistant,[373] or superconducting, materials.[374] Various metal surfaces were assembled by combinatorial deposition of metals from solutions.[375] However, because these methods are spatially addressable, only a limited number of compounds can be generated. Since individual compounds are spatially separable in the one-bead-one-compound library method, this method is particularly well suited for the development of new compounds with a desirable physical property such as optical, electromagnetic, electrochemical, or photochemical properties. The one-bead-one-compound concept holds whether the solid support is a bead, a disk or a thin film; whether it is a biological problem, a chemical problem, or a physical problem. Only one's imagination in designing an appropriate synthetic scheme or finding a clever detection method restricts our ability in solving a scientific problem that might otherwise prove difficult.

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IX. List of Abbreviations

AA, amino acid; Ac, acetyl; acac, acetylacetone; All, allyl; Alloc, allyloxycarbonyl; Ar, aryl; Boc, *tert*-butyloxycarbonyl; BOP, (benzotriazol-1-yloxy)tris-(dimethylamino)phosphonium hexafluorophosphate; BSA, *N*,*O*-bis(trimethylsilyl)acetamide; Bu, butyl; Bzl, benzyl; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; Cbz, benzyloxycarbonyl; DCC, *N*,*N*-dicyclohexylcarbodiimide; DCE, 1,2-dichloroethane; DCM, dichloromethane; Ddz, α,α-dimethyl-3,5-dimethyoxybenzyloxycarbonyl; DEAD, diethyl azadicarboxylate; DIBAL-H, diisobutylaluminum hydride; DIC, *N*,*N*-diisopropylcarbodiimide; DIEA, diisopropylethylamine; DMAP, 4-(dimethylamino)pyridine; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; DVB, divinylbenzene; Et, ethyl; Fmoc, fluorenylmethyloxycarbonyl; HATU, 2-(1*H*-9-azabenzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate; HOAt, 2-hydroxy-9-azabenzotriazole; HOBt, *N*-hydroxybenzotriazole; Ida, iminodiacetic acid; KHMDS, potasium hexamethyldisilazane; LDA, lithium diisopropylamide; Me, methyl; NMP, *N*-methylpyrrolidone; HOSu, *N*-hydroxysuccinimide; PBu_3, tributylphosphine; Pd_2dba_3, tris-(dibenzylideneacetone)dipalladium; Ph, phenyl; Pr, propyl; PyBrop, bromotrispyrrolidinophosphonium hexafluorophosphate; TBAF, tetrabutylammonium fluoride; TFMSA, trifluoromethanesulfonic acid; THF, tetrahydrofuran; TEA, triethylamine; TFA, trifluoroacetic acid; Trt, trityl.

X. References

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