Combinatorial Peptide and Nonpeptide Libraries

A Handbook

Edited by Günther Jung

VCH Weinheim · New York · Basel · Cambridge · Tokyo

6 Combinatorial Library Based on the One-Bead–One-Compound Concept

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6.1 Introduction

Combinatorial peptide library methods not only offer great potential for facilitating the drug discovery process but also provide a powerful tool for basic research of various disciplines. Currently, there are four general approaches for preparing and screening huge random combinatorial peptide libraries: (i) the biologic peptide library method using filamentous phage [1–4], plasmids [5], or polysomes [6], (ii) the combinatorial library method with iterative processes on polyethylene pins [7], in solution [8–11], and on paper ("spot synthesis") [12, 13], or libraries utilizing the "positional scanning" principle [14, 15], (iii) the combinatorial library method based on the "one-bead–one-compound" concept, or the Selectide process [16–30], and (iv) the combinatorial library method using affinity chromatography selection techniques [31, 32].

In this chapter, we shall focus on the development and application of the Selectide process. This method uses the solid phase "split synthesis" method [8, 16, 33], to generate random peptide libraries such that each individual bead displays only one peptide entity [16] (see Fig. 6-1). The random library of millions of beads is then screened in parallel for a specific acceptor molecule (receptor, antibody, enzyme, virus, etc.). The beads that interact with the acceptor molecule are then isolated, and the structure of the peptide determined by microsequencing. Much of our earlier efforts concerned the development of peptide libraries using the "on-bead binding assay" screening method and the use of automatic Edman degradation by microsequencer as the sole method for structure determination. Over the last three to four years, several important developments have been made in the Selectide process. We are now able to synthesize chemical libraries with releasable linkers such that ligands are released into solution for screening with any existing solution-phase assay [34]. Unnatural amino acids have been incorporated into our peptide libraries and specific ligands were isolated [35, 36].

In addition to linear and constrained peptide libraries, combinatiorial nonpeptide libraries with small organic molecules as subunits have been synthesized and screened for the identification of nonpeptide ligands [37–43]. A peptide coding scheme has been developed by us [44], as well as by others [26, 27, 45–48] for the nonpeptide libraries. Peptide and nonpeptide scaffoldings have been designed and

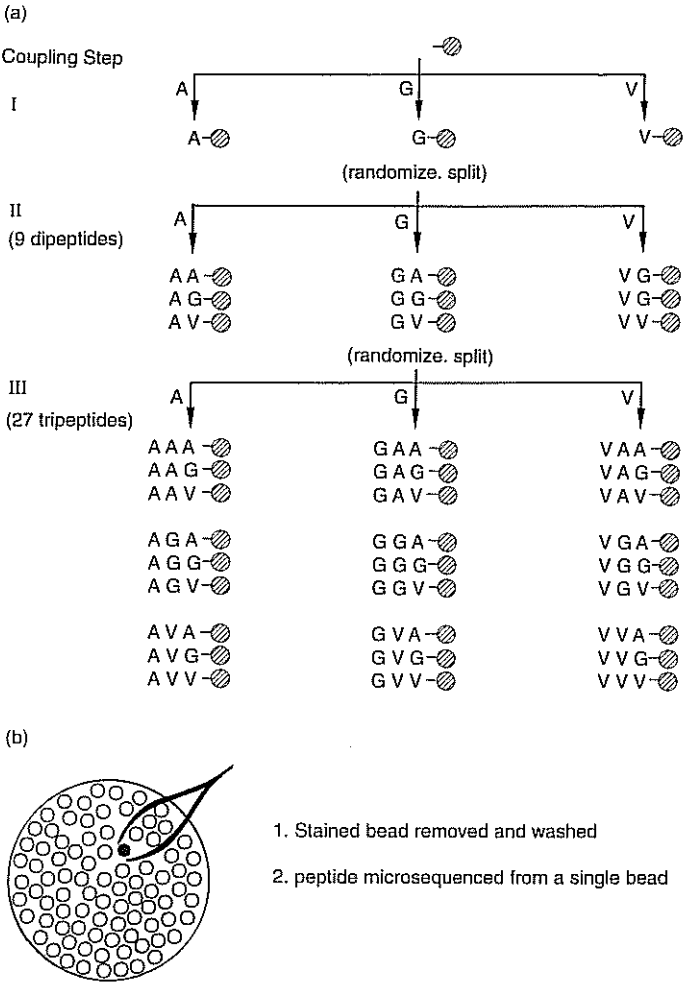

Figure 6-1. Scheme of split synthesis illustrated by an example of tripeptide library in which three amino acids were randomized in each step (from [16]).

built for the synthesis and screening of libraries [37–39, 41, 49]. Strategies for the synthesis of resin beads with differential protecting groups on the surface and inside of the beads have also been developed [50]. Structure determination of small nonpeptide ligands by mass spectroscopy [40] and digital coding have been evaluated. The concept of "library of libraries" has been applied and proven on model targets [51, 52]. More recently, using a novel screening method where the peptide library was radiolabeled with [γ^{32}P]ATP and protein kinase, substrate motif for these important enzymes were determined [53, 54]. We have been successful in identifying short linear peptides (both D- and L-amino acids) that interacted significantly

with a small organic dye, indigo carmine [55]. A fully automated random peptide library synthesizer has been built and used routinely in the synthesis of our standard peptide libraries [56]. We have also applied a dual color screening method to eliminate false positive beads resulting in the more efficient use of the microsequencer [57]. In this chapter, the concept, the basic method, as well as some of these newer developments will be explored. In addition, examples of how to apply Selectide process to various disciplines will be given. Technical details concerning the Selectide process have been recently reviewed [58, 59].

6.2 The Basic Concept of "One-Bead-One-Compound"

Furka *et al.* [33, 60, 61] first described the "split synthesis method" to overcome the differential coupling rates of various activated amino acids in the synthesis of peptide mixtures. The resin was first divided into several aliquots, and different activated amino acids were added into each resin aliquot. After coupling, the resin was mixed, washed, deprotected, and split into several aliquots again. The same process was repeated a few more times. The side chain protecting groups were then removed, the linker cleaved, and an equimolar mixture of solution peptides was ready for testing.

The "split synthesis method" was conceived independently later by us and others [8, 16]. We recognized that this synthetic method not only produced equimolar mixture of peptides but more importantly, since each resin bead encountered only one single activated amino acid at each coupling cycle and the coupling was driven to completion, each bead would have expressed only one peptide-entity [16, 17]. By using a noncleavable linker, the deprotected peptide remained bead bound. We also noted that each 100 μm bead contained approximately 100 pmole of peptide, which was plenty of material for microsequencing. Using an enzyme-linked colorimetric assay similar to Western blot, we were able to identify beads that interacted with an anti-β-endorphin antibody and streptavidin. The color beads were then physically isolated for microsequencing [16]. Since synthetic chemistry is used in the synthesis of our libraries, in addition to eukaryotic amino acids, we may also use unnatural amino acids [35, 36] as well as small organic subunits resulting in a nonpeptide library [37, 40–43].

This "one-bead-one-compound" concept offers numerous potential applications. Besides identifying binding ligands for acceptor molecules, or compounds that elicit a biological response, we can potentially screen for compounds with specific physical, chemical, photochemical, or electromagnetic properties. In addition, we may also screen for compounds with catalytic activity.

6.3 Synthesis of Random Peptide Library

Details on the synthesis of a random peptide library have been described elsewhere [59]. Standard Fmoc chemistry and split synthesis method [8, 16, 33] were used. The resin has to be compatible with solid-phase peptide synthetic methods (i.e., stable to base, trifluoroacetic acid, and organic solvents) used during the synthesis. In addition, it has to be compatible with the aqueous condition used in the screening process. The commercially available TentaGel S (polystyrene grafted with polyoxyethylene, Rapp Polymere, Germany) [62] or polydimethylacrylamide resin (Pepsyn Gel) first described by Sheppard's group [63] have been found to be satisfactory. The resin (0.2–0.4 meq/g) is first divided into several aliquots, and a fourfold excess of specific Fmoc–amino acids are added into each aliquot of resin. The coupling reaction is initiated with the addition of either diisopropylcarbodiimide (DIC) or benzotriazol-1-yl-oxy-tris-(dimethylamino)-phosphonium–hexafluorophosphate (BOP) and *N*-hydroxybenzotriazole (HOBt) with gentle mixing. In the case of DIC coupling, coupling reaction can be monitored by incorporating a trace of bromophenol blue into the reaction mixture [64], and the completion of the reaction is confirmed by the ninhydrin test [65]. On rare occasions, double or even triple coupling is needed to ensure completion of each coupling cycle. If couplings are not complete, deletion sequences will appear. After each coupling cycle, all the resin aliquots are mixed, washed, N^{α}-Fmoc group deprotected, the resin is washed, and divided into several aliquots for the next cycle of synthesis. The same process is repeated several times until the desired length of the library is achieved. The N^{α}-Fmoc group is deprotected by 20% piperidine in dimethylformamide (DMF) (v/v) and side chain protecting groups are removed by a mixture of trifluoroacetic acid/p-cresole/water/thioanisole/ethanedithiol (82.5 : 5 : 5 : 5 : 2.5) [66].

6.4 Screening with an "On-Bead Binding Assay"

The on-bead binding assay allows one to rapidly identify peptide beads that interact with an acceptor molecule. The acceptor needs to be tagged directly either with an enzyme [16, 67], a fluorescent probe [26], a radionuclide [24], or a chromophore [19]. Alternatively, a secondary reagent with the above tags can be used. For example, the primary acceptor molecule may be biotinylated, and the secondary reagent may then be streptavidin–alkaline phosphatase conjugate.

The fully deprotected bead library is first washed extensively with double distilled water, then twice with phosphate buffer saline (PBS, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, and 2.7 mM KCl, pH 7.2) containing 0.1% gelatin (w/v) and 0.1% Tween 20 (v/v). Other blockings agents such as bovine serum albumin or other nonionic detergents may also be used. The 0.28 M salt in the 2 × PBS prevents

some of the nonspecific ionic interaction. The library is then incubated in the same buffer with, for example, the biotinylated acceptor molecule. After at least one hour, the library is washed and streptavidin–alkaline phosphatase is added. After incubating for one hour, the library is washed extensively, mixed with 5-bromo-4-chloro-3-indolyl phosphate (BCIP), and plated in a dozen of polystyrene petri dishes (100 × 20 mm). Usually within one hour, the positive beads change to turquoise in color. The turquoise beads are then isolated for microsequencing. To eliminate false positive beads, the positive beads are decolorized with DMF and restainded in the presence of specific ligand. Alternatively, a dual color screening system may also be used [17, 57].

6.5 Screening with a "Releasable Assay"

The "on-bead binding assay" is extremely rapid, and up to 10^8 beads can be screened readily. However, with this assay, one is not able to screen for biological activities where the soluble receptor is unavailable or when the assay requires that the ligand be in solution in order to elicit the biological response. This is true for many standard drug screening assays available in the pharmaceutical industry. To develop a solution phase screen and maintain the "one-bead–one-compound concept", we modified the library and applied a dual releasable linker technology in a two-stage release and screening process [33, 58, 68, 69]. Screening in solution requires equimolar amounts of test compound be released at each step. We selected two release methods which are independent and can be performed in distinct steps, intramolecular cyclization and hydrolysis, for construction of releasable libraries. Linkers utilizing intramolecular cyclization [70–72] have the disadvantage of leaving the diketopiperazine structure attached to the released peptide molecule. We have overcome this limitation by redesigning the cleavable linker [68], and later we developed a more convenient and substantially less expensive two-stage releasable linker [69] composed of the dipeptide motif Ida–Ida (Ida = iminodiacetic acid), which is particularly suitable for the construction of double cleavable linkers. The structure of Ida–Ida dipeptide-based linkers and the mechanisms of the staged release are shown in Fig. 6-2.

For the screening, approximately 500 beads are first pipetted into each well of the 96-well filtration plate (Millipore). The double cleavable linker is first cleaved with the addition of neutral buffer into each well. After at least 4 hours, the peptide supernatants are suctioned into the 96-well receiving plate below. A solution-phase biologic assay is then performed, and the positive wells are identified. The amount of ligands released in each stage depends on the size as well as loading of each individual bead. From larger beads (e.g., 220 μm) up to 5 nmol can be released in each stage. This is equivalent to 50 μM when released into a volume of 100 μl. In certain

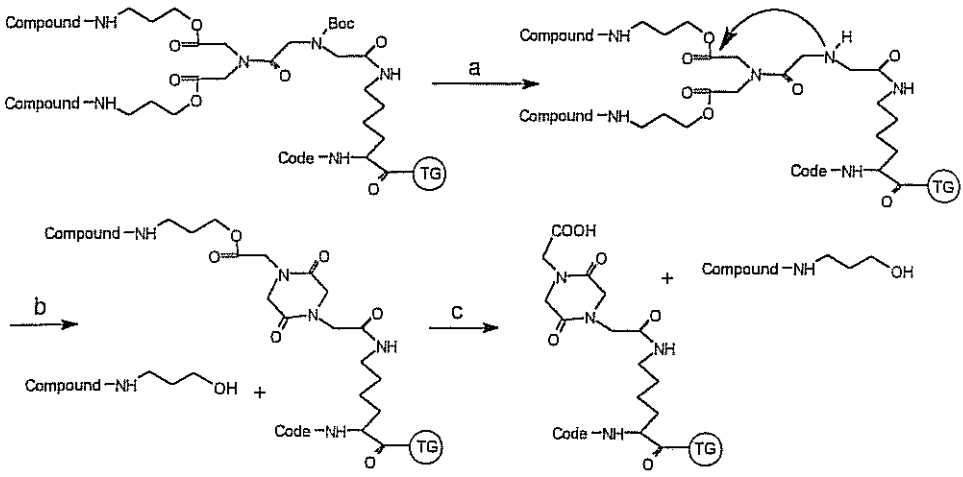

Figure 6-2. Scheme of double release from iminodiacetic acid-based doubly cleavable linker. Reagents: a, trifluoroacetic acid; b, aqueous buffer pH 8.5; c, 0.1% NaOH or gaseous ammonia.

specific applications, beads can also be immobilized on polyethylene sheets and/or agar [17, 23] and the ligand released for *in situ* biological assay.

6.6 Libraries of Organic Molecules

Construction and screening of peptide and peptide-like libraries limit the structural search to a very limited conformational space. Expansion of this space is possible by using libraries of nonpeptide, or low molecular weight organic compounds. The potential for small organic libraries is enormous. Thousands of building blocks are readily available, eliminating the limitations imposed by the 50 or so commercially available amino acids. For instance, a small organic trimer with 100 possible subunits in each coupling step will generate a complexity of 10^6 and the synthesis of such libraries is feasible using current synthetic methods. Ligands isolated from such libraries are more likely to be able to cross cellular membranes, resulting in a lead with characteristics closer to that of a therapeutic agent. An example of a ligand identified in a simple nonpeptide library constructed using subunits selected from readily available amino acids, aldehydes, and carboxylic acids is given in Fig. 6-3 [40]. We have used alkylation and acylation reactions for the construction of some of these libraries [42, 44]. The addition of amines to isocyanates [73], and Wittig reaction followed by Michael addition [21] have also been used by other researchers. Explosion of activities in this area resulted in the flood of publications. For recent

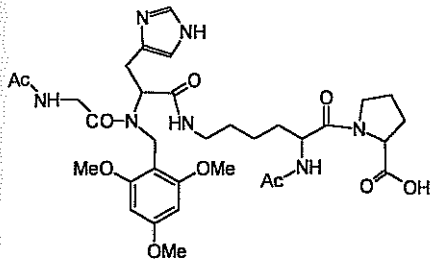

Figure 6-3. Streptavidin ligand selected from small nonpeptide library.

reviews, see e.g. [74–76]. The dynamic data base of papers in molecular diversity field can be found on Internet [77].

6.7 Scaffold Libraries

An alternative to the use of various organic reactions for linking building blocks containing different functional groups, is the application of well-developed techniques for amide bond formation, using large numbers of commercially available amines and carboxylic acids [37–39, 41, 43]. These buildings blocks can be attached to a great variety of "scaffolds", ranging from linear and flexible structures allowing the exploration of a wide conformational space, to cyclic and rigid structures, mapping a smaller space, but potentially providing higher affinity ligands (Fig. 6-4). Figure 6-5 shows structure inhibiting enzymatic activity of thrombin as an example of ligands found in these libraries [78]. Inhibitory activity of the depicted compound was at the level of 4 μM, which is equivalent to that found in the primary peptide libraries.

6.8 Structure Determination of Positive Reaction Compounds

Edman degradation is a standard method for the determination of peptide sequences. Sequencing of bead-bound peptides often requires modifications of the standard sequencing protocol. However, even optimized conditions will not allow separation of all amino acid derivatives, and in some cases, alternative methods, such as coding may become necessary. An average degradation cycle takes up to 40 minutes, making structure elucidation the rate limiting step in the screening process. However, not all positive compounds need to be sequenced individually. It may be advantageous to sequence multiple positive beads simultaneously to obtain an initial idea about structure–activity relationships [79] and to speed up the process signifi-

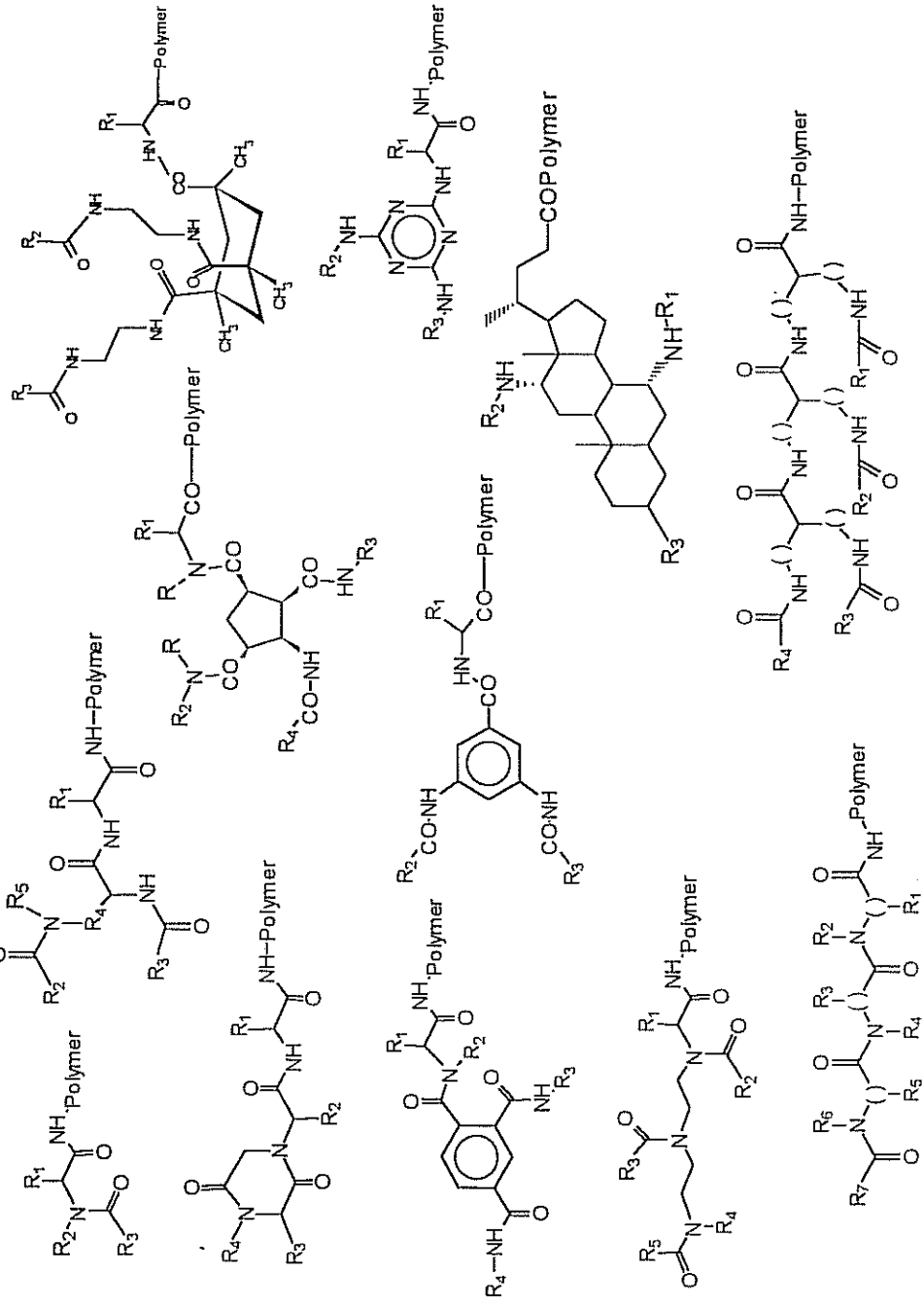

Figure 6-4. Structures of various "scaffold" libraries.

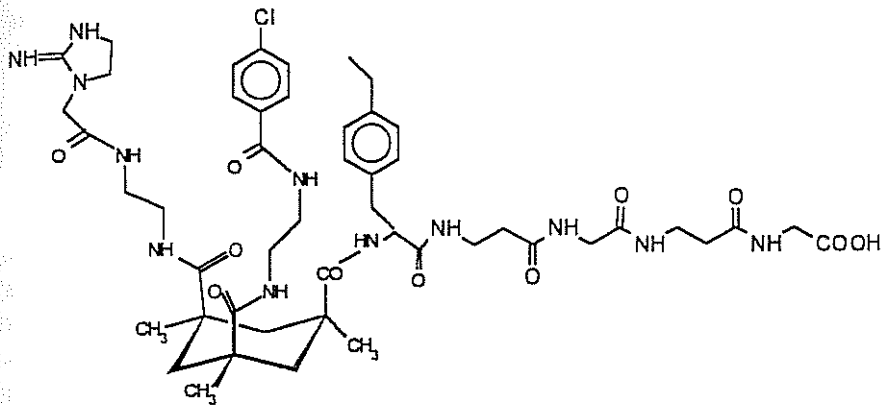

Figure 6-5. Thrombin inhibitor identified in scaffold library based on Kemp's triacid.

cantly. Figure 6-6 shows as an example the result of multiple sequencing of anti-β-endorphin antibody ligands. The motif YG_F_ is clearly visible.

For peptide and nonpeptide molecules other than oligonucleotides, the only viable alternative for direct structure elucidation is mass spectroscopy. This method can use the exchange of labile hydrogenes in the peptide molecule to significantly improve the speed and precision of structure determination [80]. Diminishing the number of possible compositions allows the application of an algorithm beginning from the composition of a full-length peptide [81] rather than from small fragments (see, e.g., [74]). Knowledge of fragmentation pathways is important to the success of mass

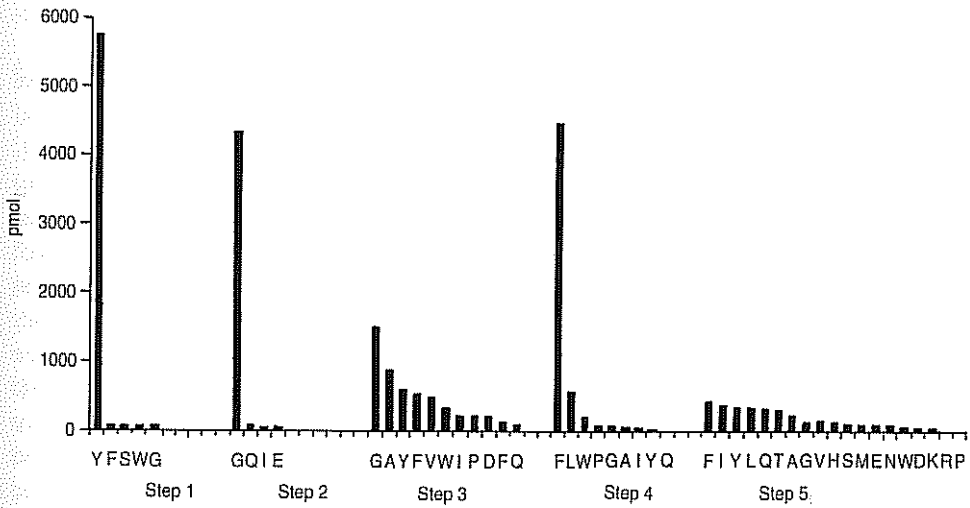

Figure 6-6. Result of multiple sequencing of 99 beads which expresses binding affinity to anti-β-endorphin antibodies (from [71]).

spectroscopy in structure elucidation; however, even without this knowledge it has been possible to determine structure of hits form a small nonpeptidic library [40].

6.9 Coding

Three main reasons for the introduction of coding techniques are: (i) structure identification of nonsequencable compounds; (ii) improvement of sensitivity; and (iii) increase of the speed and throughput of compound identification. The idea of encoding library compounds was derived from biological systems in which the composition of peptides/proteins is encoded by DNA. The phage technology, in which the structure of the peptide displayed on the phage surface is decoded by sequencing the phage DNA, takes advantage of this biological coding [1–4]. Coding of peptide libraries by nucleic acids was suggested by Brenner and Lerner [45, 46], and further developed by others [26]. Recently, coding by halogenated molecules followed by gas chromatographical analysis was published [27, 47]. This technique employs a "digital" code comprising separate sets of coding structures for each position of the library, with coding subunits within each set defining the identity of the specific compound subunit. A single step of analysis is needed to identify the test compound. A similar technique for structural elucidation was described using mass spectroscopy [28, 81]. In these cases, in each step of synthesis, a fraction of available amino groups (in the case of a peptide) is capped, and the "history" of synthesis is documented. Once a positive bead is isolated, the test compound is cleaved together with all capped intermediates. The resulting mixture is then injected into a mass spectrometer and the sequence of the test compound can be identified by reading the mass differences of individual molecules in the spectrum.

Methods using peptide molecules for coding were first described for the structure determination of test compounds identified from peptide libraries with unnatural amino acids [17] and nonpeptide chemical libraries or small organic libraries [44]. In these approaches, each step of building the nonsequencable structure is accompanied by the attachment of one or several amino acids onto an independent point on the solid-phase particle resulting in a linear coding molecule. This "peptidic tag" can then be sequenced by the application of standard techniques (Edman degradation or mass spectroscopy), and the structure of the nonpeptide molecule recalled. Alternatively, the peptide tag can be built in such a way that it can be cleaved in one step of Edman degradation, followed by HPLC analysis as a "bar code" [78]. Figure 6-7 illustrates the structure determination of a compound from the library with four randomizations coded by doublets of amino acid derivatives. N amino acids used as a doublet can code for a total of $N \times (N-1)/2$ structures (e.g., 10 amino acids can code for 45 building blocks). To have more amino acids for coding, we have prepared the set of side chain acylated diaminocarboxylic acids

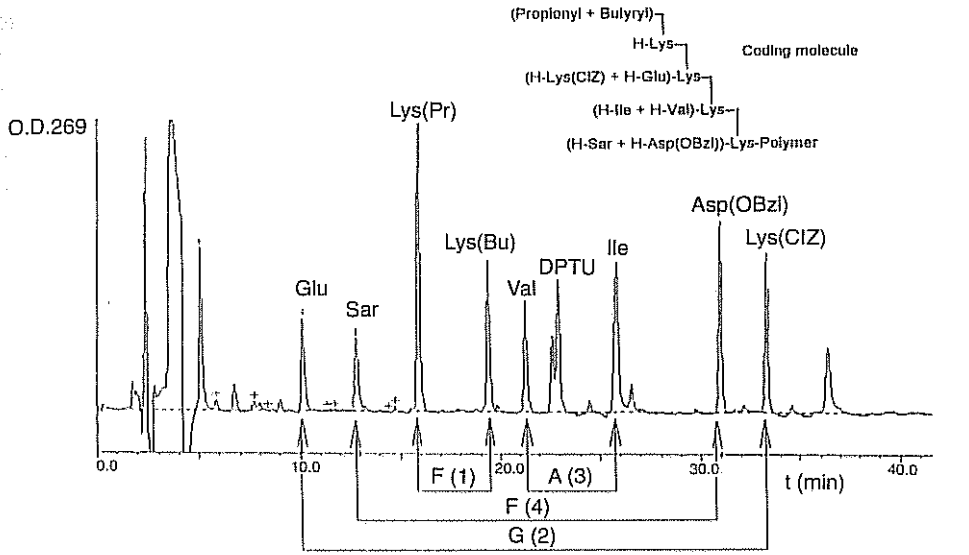

Figure 6-7. HPLC trace of bar code. Note that building block F is coded by a different amino acid doublet in position 1 (Lys(propionyl) Lys(Pr) and Lys(butyryl) Lys(Bu)) and 4 (Sar and Asp(OBzl)).

(Dap, Dab, Orn, Lys). The HPLC retention times of these acid derivatives (in the form of phenylisothiohydantoins) do not overlap with those of natural amino acid derivatives.

6.10 Elimination of Possible Interaction of Target Macromolecule with Coding Structure: Bead Shaving

Coding structure in on-bead screening may interfere with the interaction between the target molecule and the test compound. This problem can be addressed in at least four ways. One approach is to resynthesize both code and test structures from positive beads independently and determine their activity. Another is to use only a very small fraction of the material on the bead as the coding (~1 to 5%), or use a mixture of molecules for coding. This mixture, however, must provide unambiguous sequencing data. The fourth approach is to arrange the coding and screening structures in such a way that only the test compound is available for interaction with the target molecule. The interior of polyoxyethylene grafted polystyrene (TentaGel) beads is not available to macromolecular targets owing to the microporous structure of this polymer. This carrier can first be modified with a protected peptide sequence, which is then cleaved by a reagent that has access to the "surface" of the solid phase

support only, thus exposing a free amino group on the surface. This free amino group is subsequently protected with an orthogonally cleavable protecting group. In this way it is possible to synthesize the test compound exclusively on the bead surface and the coding structure exclusively in the bead interior. We have used enzymatic "shaving" to produce this selective surface modification, using chymotrypsin as the shaving agent and the sequence Boc-Ala-Gly-Val-Phe-Gly-βAla-Gly-TG as the substrate [50].

6.11 Is It Necessary To Have Full Representation in a Selectide Library?

Assuming the resins are distributed equally in each coupling step during the "split synthesis", the statistical distribution of individual species is based on Poisson distribution and the number of possible species actually synthesized is determined by the total number of beads present in the synthesis. For example, approximately 95% of all possible permutations will be present in a library if the number of beads used in the synthesis and screening equals three times the number of possible permutations. From our experience with peptide library screening, most of the biologic targets that we have studied have three to five contact residues necessary for specific molecular interaction. These contact residues can be separated by a variable number of non-essential residues. Because of this degeneracy, for most biological targets, a full representation of every species in a library is not needed for the determination of the general binding motif. For a motif with five contact residues, one would need a library of approximately 10^7 beads to ensure that 95% of all possible motifs (consisting of 20 eukaryotic amino acids) are included. For practical reasons, the upper limit for the synthesis and screening of an 80 μm bead library is probably 10^8 beads (approximately 30 g of resin). For routine screening, a 10^7 bead library can easily be handled by one person in a day. However, this does not imply that the "best" ligand can always be isolated from a limited library. Except in simple cases where the high affinity ligand is a short linear epitope (e.g., opiate receptor ligands, anti-β-endorphin monoclonal antibody epitope, YG_F), often the binding affinity of the initial leads isolated from a primary library screen, particularly for a ligand with a "discontinuous epitope", is relatively weak (>1 μM). To improve the binding affinity, one may use a sequential screening approach by synthesizing and screening a secondary library based on the primary motif. This can be repeated in several additional cycles, and perhaps with branching structures, until a higher affinity ligand is isolated. This sequential screening approach has been applied successfully to isolate high affinity ligands for an anti-insulin monoclonal antibody that recognizes a discontinuous epitope [36].

6.12 One-Bead–One-Motif Libraries ("Libraries of Libraries")

The number of expected positive beads in the library depends on the number of "critical residues" (or contact residues) in the peptide sequence, or critical pharmacophores in a nonpeptide structure, i.e., residues required for minimal observable binding. This number can be calculated according to the formula:

$$n = (x)(P_f)[S/(A_n)^{n_{crit}}]$$

In this equation n is the number of expected positive hits, x is the number of different motifs which exhibit binding, P_f is the "placement" factor, i.e., number of possible placements of each motif in the peptide chain, S is the number of beads screened, A_n is the number of amino acids (subunits) used for randomization, and n_{crit} is the number of critical residues. The number of positive hits depends on the number of beads tested, but it does not depend on the length of the library. Therefore, even a very incomplete library, e.g., a library of decapeptides, can provide a reasonable number of positive beads if only three or four residues in the sought-after peptide are critical for binding under the screening conditions used.

The individual sequence identified in the peptide library with incomplete representation of all permutations is less valuable than information describing the motif responsible for binding or other biological activity. We have designed a library which addresses this question [51, 52]. This library contains all possible motifs (e.g., all tripeptide motifs in a hexapeptide library) in the framework of a library of given length. At the same time, each individual bead contains a multiple of peptides (each with the same motif). In this arrangement all tripeptide motifs in up to a 15meric peptide can be synthesized and screened in a manageable library size (see Table 6-1). Synthesis of this library follows a simple scheme, given in Fig. 6-8. This library combines the principles of iterative library techniques [7–9, 83] with those of the one-bead–one-compound approach; however, it results in the screening of all possible motifs at the same time, rather than in consecutive dipeptide motifs – which may be too short [84] or not appropriately localized in the peptide chain to identify the ligands. Each bead of the library of libraries represents a unique entity the structure of which is determined only after its biological relevance is established.

6.13 The Selectide Process Versus Other Combinatorial Library Methodologies

As mentioned earlier, the four general combinatorial library methods are (i) biological libraries, (ii) iterative libraries, and (iii) one-bead–one-compound libraries, and (iv) affinity chromatography selection libraries. All these four methods have

Table 6-1. Numbers of beads (and weight of resin – 130 μm bead size) required for testing full representation of peptides of various lenght using (a) one-bead–one-peptide approach and (b) library of libraries approach with tripeptide motif

Length	One bead–one peptide (a)		Library of libraries (b)	
	Number of beads	Resin amount	Nuber of beads	Resin amount
3	8000	8 mg	8000	8 mg
4	160000	160 mg	32000	32 mg
5	3200000	3.2 g	80000	80 mg
6	64000000	64 g	160000	160 mg
7	1280000000	1.28 kg	280000	280 mg
8	25600000000	25.6 kg	448000	448 mg
9	512000000000	512 kg	672000	672 mg
10	10240000000000	10.2 t	960000	960 mg
11	204800000000000	204 t	1320000	1.32 g
12	4096000000000000		1760000	1.76 g
13	81920000000000000		2288000	2.29 g
14	1638000000000000000		2912000	2.91 g
15	32770000000000000000		3640000	3.64 g

both advantages and disadvantages. Comparisons between the four methods are summarized in Table 6-2. The main advantage of the biological libraries is that long peptides can be produced with this method, thus allowing one to incorporate big scaffolds such as immunoglobulin folds [85] or scorpion toxin folds into the library. On the other hand, only relatively short peptides (e.g., up to 20-mers) can be produced by the synthetic methods. Another possible advantage of the biological library is that perhaps up to 10^9 molecules can easily be produced by the biological method. However, because it is a biological system, only the 20 L-amino acids can be incorporated into the library. In contrast, the three latter synthetic methods allow one to incorporate L-amino acids, D-amino acids, unnatural amino acids, small organic nonpeptide moieties, carbohydrates, lipids, nucleic acids, various secondary structures and synthetic scaffolds into the libraries.

The major difference between the iterative process and the Selectide process is that the latter is addressable where each chemical species is physically separated, and the structure of the ligand is determined by chemical analysis of individual positive beads. In other words, similar to the biological library, the Selectide process uses a parallel approach where all the chemical species are screened concurrently in one step. The corollary is that all the multiple independent motifs present in the library can be discovered with one screen. This is particularly important for receptors that recognize a discontinuous epitope as multiple motifs are likely to be discovered. This will be illustrated by the anti-insulin monoclonal antibody system in the later section.

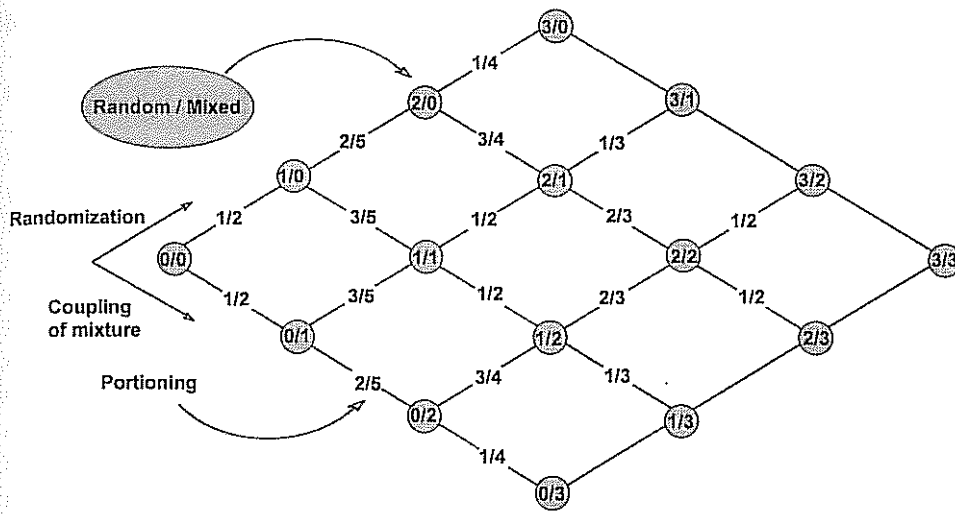

Figure 6-8. Scheme of the synthesis of "library of libraries" with a tripeptide motif in a hexapeptide frame. The polymeric carrier is split in the ratio $n_{Rr} : n_{Mr}$ (numbers located on the lines connecting circles denote the fraction of carrier undergoing the specified operation) (n_{Rr} = number of remaining randomization steps; n_{Mr} = number of remaining steps in which a mixture of amino acids is to be coupled) based on the status n_R/n_M (number located in the shaded circles) (n_R = number of randomizations performed on the carrier; n_M = number mixtures to be coupled). At the beginning of the synthesis $n_R/n_M = 0/0$, at the end $n_R/n_M = 3/3$. The lines pointing up designate a randomization step, the lines pointing down designate a mixture step. This scheme can be applied to any length of library and motif.

The iterative process, on the other hand, is a convergent approach, in which mixtures of compounds are synthesized and tested either on solid-phase or in solution in a multistep fashion. The result of the biological assay will determine the next synthetic step. This is straightforward if there is only one clearcut motif. However, if there were multiple motifs of similar activity, one has to decide which path to take for each of the subsequent steps. Even though remarkable results have been obtained with this method (see e.g., [11, 84, 86, 87]), this convergent approach may result in the possible omission of otherwise very important motifs. Since the standard iterative approach requires multiple synthetic and analysis steps which are time-consuming, a rapid positional scanning method has been developed [14, 15]. In this method, each amino acid position is fixed while the rest of the positions are randomized. With these standard mixtures (e.g., 120 mixtures for a hexapeptide), one can rapidly determine the binding motif. This method is very efficient for receptors with only one dominant linear binding motif. However, for receptors with multiple binding motifs, the results will be very complicated and likely uninterpretable.

Table 6-2. Comparison of combinatorial library methods

	Biological	Iterative process	Selectide process	Affinity selection
Number of species	10^9	10^6–10^8	10^6–10^8	10^6–10^8
Unnatural amino acids or small organics	No	Yes	Yes	Yes
Size of ligand	Small to very large	Small	Small	Small
Constrain structures	Simple disulfide or large scaffolds of proteins	Yes	Yes	Yes
Binding assay	Yes	Yes (pin and spot)	Yes	Yes
Solution assay	No	Yes	Yes	No
Approach	Parallel	Convergent	Parallel	Parallel
Structure determination	Addressable, DNA sequencing	Based on multi-step synthetic and assay algorithm	Addressable, Edman degradation, encoding mass spectroscopy	Summation of many possible solutions, Edman degradation
Solution motif	Multiple	Often one	Multiple	Often one
Biological bias	Yes	No	No	No

Although a solution-phase assay is preferred in many biologic assays, it also poses some important limitations. For instance, a signifcant number of ligands, particularly from the non-peptide libraries, may be insoluble or only very sparingly soluble, and their identification will be missed if a solution-phase assay is used. In this instance, an on-bead screening assay where the relatively insoluble ligand is coupled to a highly hydrophilic linker will be preferred. Often a solution-phase binding assay requires a radiolabeled-ligand as a tracer. In the case where no known ligand is available, the on-bead binding assay is preferred. Additionally, if a known ligand is available, the dual-color on-bead binding assay allows one to identify binders that bind to the ligand binding pocket or those that bind to the receptor surface outside the ligand binding pocket.

As mentioned above, all the four combinatorial library methods have advantages and disadvantages. The choice of which method to use will depend on specific applications, expertise, and resources available in individual laboratories. Sometimes it may be advantageous to combine some of these methods together as they are complementary to each other.

6.14 Examples of Application

6.14.1 Anti-β-Endorphin Monoclonal Antibody

Using an on-bead binding assay with an anti-β-endorphin monoclonal antibody (clone 3E7) that recognizes an *N*-terminal continuous epitope (YGGF), we were able to idenify ligands of various affinities from an all L-amino acid library [16, 34, 88]. The general motif YG_F/W is readily identified. We have also screened several D-amino acid containing libraries with the same antibody. The results are shown in Table 6-3. The "on-bead binding" screening process is rapid and usually takes less than a day. The rate limiting step is often the microsequencing. To speed up the structure determination step, we have pooled the positive beads (e.g., 30 beads) of some of our screen and subjected them to concurrent sequencing. The result is shown in Fig. 6-8, which demonstrates that with simple motifs and with limited critical residues that are relatively "in-frame", the multiple sequencing method works very well. This is quite analogous to the positional scanning method described by Houghten *et al.* [14, 15]. However, if multiple motifs were significantly "off-frame", both multiple sequencing or positional scanning methods will likely produce uninterpretable data. The multiple sequencing method not only works for short motifs but also for longer ligands with few "in-frame" critical residues. For example, anchor residues for the 9-mer of the MHC-Class I molecule binding peptides (see below) can readily be identified by multiple sequencing [89].

Table 6-3. Peptide ligands that interact specifically with anti-β-endorphin monoclonal antibody (clone 3-E7)

Library	Ligands isolated
XXXXX	YGGFA, YGGFT, YGGFQ, YGGFI, YGGFL
	YGGLS, YGGMV
	YGAFF, YGAFM, YGAFQ, YGAFT, YGALQ, YGALT, YGAWD
	YGNFF, YGVFA, YGVFI, YGQFV, YGVFE, YGVFQ
	YGFFQ, YGWFN, YGWWM, YGYWQ, YGWFH, YGWFQ, YGYWQ
	YGEAF, YGHAF, YGMGF, YGGGF, YGLGF, YGPGF
	YKGGF, YQGGF, YLGGF
	LYGGF, NYGGF, MYGGF, RYGLL
XxXxX	YGGfM, YGAfW, YGAfF
	YqGGF, YGAaF
	YGFGL, YGFGF, YGGGF
	IyGGF, YaNaW, YaQaW
xXxXx	yGGFa, yGGFv
xxxxx	wtGGy

X = all 18 L-amino acids plus glycine, no cysteine.
x = all 18 D-amino acids plus glycine, no cysteine.

6.14.2 Anti-Insulin Monoclonal Antibody

Unlike anti-β-endorphin monoclonal antibody, the anti-insulin monoclonal antibody recognizes a discontinuous epitope on an intact porcine or human insulin molecule. Multiple libraries have been screened systematically with this antibody. The results are summarized in Table 6-4. Immediately apparent is that multiple motifs can bind to this antibody. Importantly, the binding of these motifs to the monoclonal antibody was blocked by porcine or human insulin. When specific structures such as cyclic (disulfide bond) or type II β-turns (D-proline) structures were incorporated into the library, some totally new motifs were detected. It is interesting that, although multiple motifs were identified for the L-amino acid libraries, only one motif was identified for the two all-D-amino acid libraries (6-mer and 8-mer). The binding affinities of most of the ligands identified during the primary screen were relatively low (>10 μM). A sequential screening approach was then applied to one of these motifs __W__GF, identified from the primary screen (Table 6-5). Based on the motif of the primary screen, longer secondary libraries were synthesized and screened under higher stringency. This process was repeated several times until ligands of higer affinity were finally isolated. The binding affinity (IC_{50}) of the best ligand identified (SKQDIWGRGF) was 0.3 μM, five fold weaker than that of insulin, the native ligand.

Table 6-4. Peptide motifs indentified from the primary screen on an anti-insulin monoclonal antibody (Clone AE9D6)

Tetra	QNPR	Cyclic 7-mer	CW__GF__C
Penta	_QDPR	Cyclic 8-mer	C_F_W__GGC
	FNW__		C____HGVQC
	FDW__		
Hexa	__QDPR	Cyclic 9-mer	CQDI_Y____C
	FNW___		
	FDW___	"Turn-library"	_FQp_RP_.
	_W__GF		_FQp_IP_
Octa	___W__GF	All D library	___q_Gs_G
	______GF		
Nona	___GF__GF		
Deca	_____W__GF		
Quindeca	_FDW__________		
	__________W__GF		
	______W__GF____		

Table 6-5. Optimization by sequential screening with secondary and tertiary libraries

	Library	Peptide motif
1°	XXXXXX	_W__GF
2°	XXXWXXGF	___WKYGF, Q_IWG_GF
3°	XXXXWKYGF	NH_(G)WKYGF
	XXQXIWGXGF	S(R/K)Q(D/A)IWG_GF

6.14.3 MHC-Class I Molecule

Using papain-treated MHC-Class I molecules purified from the human lymphoblastoid JY cells, it was possible to rapidly identify the anchor residues for the binding peptides. The MHC-Class I molecule was first dissociated from its β_2-microglobulin by 5M potassium thiocyanate. Upon dialysis in the presence of excess β_2-microglobulin and a random nonapeptide library, the MHC-Class I molecule reassociated on the peptide bead with the correct anchor residues. Detection was accomplished by probing with an enzyme-linked antinative MHC-Class I molecule antibody (clone W6/32). The results are shown in Table 6-6 [90]. In one experiment, several beads were pooled and microsequencing was performed on multiple beads (multiple screening) as described earlier. The anchored residues identified for A2 and B7 were determined to be FL/M_____L/I and _PR_____L/I/V, respectively. These results corrobrate closely with those reported in the literature [91–93].

Table 6-6. Peptide ligands that interact specifically with MHC-Class I molecules

HLA-A2.1	HLA-B7
FLWEFPHDL	TPRFLNSPI
FLWAIMHTE	APRVVQMPL
FLLPSFAPD	LPRNVTFAV
FLWTLEGDV	APRGGMYHV
FMLGYDFYI	KPRGFVPMN
FMLDWFPTI	RPRPVSHMW
MMQDIDFYL	RPRGAYGDL
MLWEGFTYI	LPFKRGGSL
LLYDWDFGL	IPMGRRGGI

6.14.4 Releasable Assay Screening System

As mentioned earlier, the releasable assay screening system, although significantly slower than the on-bead binding screen, offers an important alternative since solution phase assay can be used. The two-stage releasable assay screening method has been applied successfully in identifying ligands for anti-β-endorphin monoclonal antibody (YGGF) and gpIIb-IIIa (CRGDC) [34].

6.14.5 Posttranslational Modification such as Protein Phosphorylation

To determine the phosphorylation site of cAMP-dependent protein kinase, we have modified our "on-bead binding assay" by actually covalently radiolabeling the positive bead with [γ^{32}P]ATP and the enzyme. Random penta- or heptapeptide libraries were first mixed with [γ^{32}P]ATP. The catalytic subunit of the cAMP-dependent protein kinase was then added to initiate the phosphorylation reaction. After incubation, the library was washed thoroughly and immobilized on a glass plate with 1.5% low-gelling temperature agarose. The immobilized beads were then exposed to an X-ray film. Beads at the area corresponding to the dark spot on the autoradiograph were isolated, the agar redissolved by heating, diluted with additional agarose, and re-immobilized on the glass plate as described above. The single beads corresponding to the dark spots were then isolated for microsequencing. Figure 6-9 shows the autoradiograph of a primary screen. The result of such a screen

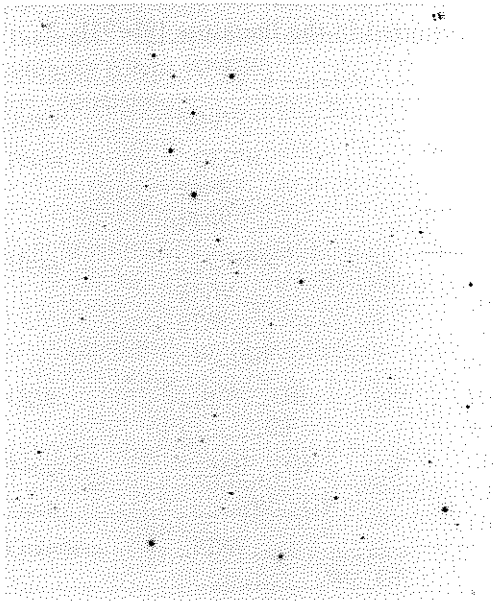

Figure 6-9. Autoradiograph of protein kinase screen.

is shown in Table 6-7. The motif _RR_S_ identified from such a random screen is exactly identical to that reported in the literature [94]. More recently, we have applied the same method and have identified a novel peptide substrate for $p60^{c\text{-}src}$ protein tyrosine kinase. We have shown that this peptide, YIYGSFK, is about sevenfold more efficient than the commonly used peptide substrate, cdc2(6–20) peptide, for tyrosine kinase [95]. Currently, work is being done on using this method to determine phosphorylation for other tyrosine kinases, many of which are without any known physiological substrate.

Table 6-7. Peptide substrate for cAMP dependent protein kinase

Library	Peptide
XXXXX	RRYSV
XXXXXXX	SQRRFST
	YRRTSLV
	IIRRKSE

6.14.6 Small Organic Dye Molecule as a Target

Using a small organic dye, indigo carmine (MW = 466.56 dalton) as a probe, an all L-amino acid heptapeptide and an all D-amino acid octapeptide library were screened. The beads that absorbed the dye and turned blue were isolated and microsequenced. The sequences obtained are summarized in Table 6-8 [55]. All the sequences (both from the all L- or all D-libraries) have the following motif: X(K/R)OOO(K/R)X where O = I, L, V, Y, F, or M, the relatively more hydrophobic amino acid. A series of three of these amino acids were flanked by two positively charged amino acids. This is not unexpected since indigo carmine, as shown in Fig. 6-10, is planar and has one sulfonic acid group at each end of the molecule. This simple experiment proves that small peptides that interact specifically with a small organic molecule can be isolated readily with this method.

Table 6-8. Peptide ligands that interact specifically with indigo carmine

Libraries	Peptide
XXXXXXX	YKVVYKL, LTKLVLK, VTKIIFK
xxxxxx	klilkf, wlikmk
xxxxxxxx	ikivyrfr, akwkwvyr
	ykvvyris, vkkmvikf

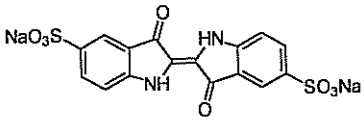

Figure 6-10. Chemical structure of indigo carmine.

6.14.7 Screening of Library of Libraries

The library of tripeptide motifs in the framework of a linear hexapeptide composed of 19 natural amino acids (Cys excluded) was screened against anti-β-endorphin and streptavidin. Peptides with the expected motifs YG_F and HPQ were identified as evidenced in Table 6-9 [51–52]. The illustrated results were obtained using only a small sample of the library (300000 beads) for screening – demonstrating substantial savings in the consumption of reagents needed for both synthesis and screening, compared with a complete library of unique hexapeptides which would be represented on 64 million beads.

Table 6-9. Ligands for streptavidin and anti-β-endorphin antibody identified in library of libraries

Streptavidin	Anti-β-endorphin
XXXHPQ	YGXFXX
XXXHPM	YXGFXX
XXHPQX	YGGXXX
XWXHPX	YGAXXX
WXXHPX	XGAFXX
WXXXPQ	XGGFXX

6.15 Perspective

Using a combinatorial library method based on the "one-bead-one-structure" concept and with an appropriate detection scheme, potentially one can isolate and identify a pure bead-bound compound with the desired physical, chemical, electrochemical, photochemical, biological, or enzymatic properties. Thus far, our efforts have been focused primarily on the discovery of compounds that bind specifically to various macromolecular targets such as antibodies, enzymes, MHC-Class I molecules, viral proteins and biologic receptors. The development of nonpeptide or small organic combinatorial libraries is particularly exciting as this will allow us to explore further diversity and at the same time discover drug leads that are smaller and more likely to be permeable to the cell membrane. There has been enormous in-

terest from both the pharmaceutical industry as well as academia in this area. Undoubtedly within the next five years, new coupling chemistries suitable for the library format will be developed. Since combinatorial library methods often generate a vast amount of data from each screen, it is particularly powerful when coupled with modern computational chemistry.

We are currently beginning to explore the possible application of the Selectide process to other areas as well. The identification of small peptides that bind specifically to an organic dye [55] or the studies of ligands to artificial receptors [19, 96] are milestones, as they indicate that we should be able to identify specifc binding pairs for the design of self-assembled supramolecular structures. Additionally, one may also apply this method to identify efficient binders for specific toxic waste. As mentioned earlier, the technology also offers great potential for the design of novel catalyst or "artifical enzymes". For example, one can envision that amino acids with cofactors such as FAD, NAD, or CoA can be attached to a side chain and incorporated into a random mono- or bicyclic peptide library with a constrained structure. By doing so, an individual peptide bead with a desired enzymatic activity may be isolated.

In the area of material science and electronics, one can envision that with the appropriate detection scheme, new materials with the desired physicochemical or electronic properties can be isolated. For instance, from a combinatorial library of various chromophores strung together, one may be able to isolate a compound with efficient photoelectric properties. Likewise, materials with the desired conductance properties could also be isolated.

It is evident from the above discussion that combinatorial library methodologies will allow us to rapidly explore a huge number of compounds concurrently. These have already proven to be extremely powerful tools for drug discovery as well as basic research. Undoubtedly in the next few years, we'll see more laboratories using these techniques to solve problems across a wide range of disciplines.

Acknowledgments

This work is supported by Selectide Corporation and NIH grants CA17094, CA57723, and CA13074. Kit S. Lam is a Scholar of the Leukemia Society of America.

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