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Development and Applications of Topologically Segregated Bilayer Beads in One-bead One-compound Combinatorial Libraries

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Abstract

Using a "split – mix" synthesis approach, "One-Bead One-Compound" (OBOC) combinatorial libraries can be generated such that each bead displays only one chemical entity. Tens of thousands to millions of compound-beads can be screened concurrently using a variety of biochemical and cell-based screening methods. Positive beads are then physically isolated for structure determination. Peptide beads or peptoid beads consisting of α-amino acids and with a free *N*-terminus can be routinely sequenced by an automatic microsequencer using Edman chemistry. Libraries with *N*-terminally blocked peptides, peptides with unsequenceable building blocks, or small molecules require encoding. To fully exploit the OBOC combinatorial library methods, we have developed topologically segregated bilayer beads. Such bilayer beads allow us to prepare library compound on the outer layer of each bead and the coding tags in the bead interior. In addition, we can use these bilayer beads to prepare OBOC combinatorial libraries that are down-substituted on the bead surface but fully substituted in the bead interior. This configuration enables one to screen at a much higher stringency and yet have enough peptides or coding tags retained in the bead interior for structure determination.

1 Introduction

In 1991, we first introduced the One-Bead One-Compound (OBOC) combinatorial library method in which tens of thousands to millions of compound beads could be rapidly prepared and screened concurrently for a specific biological property [1]. To prepare OBOC libraries, a "split – mix" synthesis method [1 – 3] is used such that each bead displays only one chemical entity. From peptide libraries created early in this laboratory, we were able to identify short linear peptides that bind to anti-β-endorphin antibody and streptavidin [1]. Since then, we and others have expanded the OBOC libraries to cyclic peptides [4 – 11], peptoids (*N*-substituted oligoglycine) [12 – 14], peptidomimetic and small-molecule libraries [15 – 19], and applied this powerful method to discover ligands against a number of different biological targets, such as protein kinase substrates and inhibitors [15, 20], protease substrates and inhibitors [21 – 24], cell surface recep tors [4 – 7, 25], artificial enzymes [26, 27], and various ligands for the preparation of affinity column media [28 – 30].

Most of the published work in OBOC combinatorial libraries involves on-bead screening, in which the library compounds remained tethered to individual bead during screening. Some of the screening assays include enzyme-linked colorimetric assays [31], fluorescent quench protease substrate assays [21, 22], radiolabeled protein kinase substrate assays [32], and on-bead whole-cell binding assays [4 – 7]. For fluorescent screening, an automatic fluorescent activated bead sorter (COPAS™ BIOBEAD, Union Biometrica, Inc, Somerville, MA) can be used to rapidly screen OBOC combinatorial libraries at a rate of 30000 – 50000 beads per hour [13, 33 – 36]. In addition to on-bead screening assays, we and others have also developed *in situ* releasable assays in which a portion of the library compounds are released from individual beads for solution-phase biological assays [37 – 39].

Positive compound beads identified during the screening assays are physically isolated for structure determination. For α-amino acid-containing peptides and peptoids with a free *N*-terminus, direct automatic Edman microsequencing can be used to determine the amino acid sequence. For peptides without a free *N*-terminus, branched

peptides, or peptides with one or more non-sequenceable building blocks, such as β- and γ-amino acids, alternative sequencing strategies are needed. In the last few years, we have developed simple, yet highly robust, methods to prepare topologically segregated bilayer beads, as well as several encoding methods to encode such "non-sequenceable" peptides and even small molecules. In our encoding systems, the library compounds reside on the outer layer of the bead and the coding tags remain in the bead interior. Such a configuration facilitates not only the encoding process but also allows us to minimize interference by the coding tags during screening. Furthermore, the bilayer configuration enables us to easily increase the screening stringency to identify high-affinity ligands, by down-substituting the chemical loading on the outer layer but retaining full (100%) substitution in the bead interior for decoding. In this article, we shall review these methods and also provide recent data on the application of some of these encoding techniques to OBOC combinatorial peptide and peptidomimetic libraries with non-sequenceable building blocks.

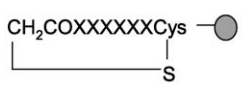
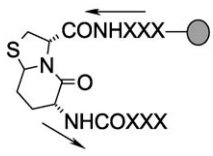
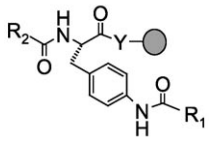
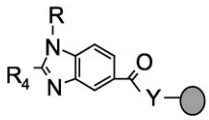
2 Non-sequenceable Libraries

For OBOC combinatorial peptide libraries that consist of natural amino acids, we routinely use automatic Edman microsequencing to determine the amino acid sequence of the positive beads. To sequence peptides by Edman chemistry, there are two basic requirements. First, the peptide consists of sequenceable amino acids, *i.e.*, α-amino acids. Second, the peptide has a free *N*-terminus. Edman chemistry can also be applied to peptoids that consist of *N*-alkylated oligoglycine, an α-amino acid. Such large numbers of unnatural amino acids are now commercially available, and the incorporation of these amino acids into library construction will greatly increase the diversity of peptide libraries. We therefore have developed sequencing protocols for determining the sequence of peptides containing many such unnatural α-amino acids [40]. However, peptides containing β- and γ-amino acids, β-turn mimetic amino acids, or other non-α-amino acid building blocks are not amenable for Edman chemistry. Similarly, branched peptides and cyclic peptides utilizing the *N*-terminal amino group for cyclization cannot be sequenced by Edman degradation. Peptides are useful for targeting extracellular macromolecules or cell surface receptors; however in general they are not good drug candidates for intracellular targets because they often cannot penetrate cell membranes. Moreover, peptides comprising of natural amino acids are susceptible to proteolysis. Therefore, there is a great need to develop peptidomimetic libraries, small-molecule libraries, peptide libraries comprising of unnatural amino acids, and peptide libraries with cyclic and branched structures. Table 1 shows a few examples of these desirable OBOC combinatorial libraries. These modifications not only greatly increase the diversity of the library but also render the molecule more resistant to proteolysis. However, chemical structures of compound beads from these libraries cannot be determined directly with Edman microsequencing. Instead, an alternative strategy such as chemical encoding is required. Chemical coding tags are added to the bead during the synthetic steps so that the synthetic history of each compound bead in the chemical library can be recorded. The coding tag can then be decoded by either Edman microsequencing or mass spectrometry (see below).

3 Methods for Generating Topologically Segregated Bilayer Beads

If both the library compounds and their corresponding coding tags co-exist at the bead surface where the interaction between target proteins and library compounds occurs, the coding tags may interfere with the screening. To eliminate such interference, the coding tags should be confined in the bead interior. We have previously reported generating topologically segregated bi-functional beads by using polyglutamic acid [41] or proteases [41, 42], to modi-

Table 1. Different types of non-sequenceable OBOC combinatorial libraries that can be encoded by bilayer bead approaches.

	N-Terminally blocked cyclic library	Library with non-sequenceable building blocks	Peptidomimetic library	Small-molecule library
Example	Cyclic thioether library	β-Turn library	(4-Amino)phenylalanine based peptidomimetic library	Benzoimidazole-based small-molecule library
Structure				

X: sequenceable amino acids; Y: sequenceable or non-sequenceable amino acids; R: alkyl or aryl.

fy the bead surface of TentaGel bead (Rapp Polymere, Tübingen, Germany). However, these methods are technically difficult to control and not reproducible from batch to batch.

We have recently developed a simple, inexpensive, and highly reproducible bi-phasic solvent approach to topologically segregate resin beads [43]. We called this method the "Partial Amine-Protection (PAP)" bilayer approach. In this method, the TentaGel resin bead is first swollen in water and the outer layer of the bead is exposed to organic solvent comprised of a mixture of dichloromethane (DCM) and diethyl ether, as well as an amine-derivatizing reagent (*e.g.*, *N*-(9-fluorenylmethoxycarbonyloxy)succinimide (Fmoc-OSu), *N*-(allyloxycarbonyloxysuccinimide (Alloc-OSu)). Meanwhile, the bead interior remains in water without any derivatizing reagent. As a result, derivatization is confined to the outer layer of the bead (Figure 1a). The reaction usually completes at room temperature in 30 min with vigorous shaking. Fmoc-OSu, Alloc-OSu and Bsmoc-OSu (*N*-(1,1-dioxobenzo[*b*]thiophene-2-ylmethyloxycarbonyloxy)succinimide) worked well but Fmoc-Cl (9-fluorenylmethyloxycarbonyl chloride), Boc-OSu (*N*-(*tert*-butoxycarbonyloxy)succinimide) and $(Boc)_2O$ (di-*tert*-butyl dicarbonate) did not yield satisfactory bilayer beads, probably because Fmoc-Cl is too reactive and reacts quickly with trace levels of water retained in organic phase. Boc-OSu and $(Boc)_2O$ are bulky and require longer reaction time, and as a result, the reagents diffuse into the bead interior prior to reacting with the bead outer layer. We have found that the optimal ratio between DCM and diethyl ether is 55:45 but can vary between 60:40 and 50:50. At such a ratio, the derivatizing reagents diffuse slowly into the bead interior. By adjusting the amount of derivatizing reagents used, the thickness of the outer layer can be controlled. The reason to choose DCM and diethyl ether is because they are immiscible with water and the bead swelling properties of DCM and diethyl ether mixture are comparable to that of water. Other water immiscible solvents such as toluene and chloroform have been tried but with unsatisfactory results. We have used the PAP bilayer method to prepare multi-layer beads. For example, three-layer TentaGel beads (Figure 2) were prepared by this bi-phasic protection method. TentaGel beads were first swollen in water, and the outer layer (~30%) was derivatized with Fmoc-OSu resulting in bilayer beads. These bilayer beads were then thoroughly washed, swollen in water, and 40% of the remaining amino-substitution was protected with Alloc-OSu. In order to visualize the three-layer structure, the beads were treated with piperidine to remove the Fmoc group in the outer layer, followed by derivatization of the whole bead with excess fluorescein Isothiocyanate (FITC). The three-layer beads (Figure 2) can be clearly seen under a confocal microscope (Zeiss LSM 510).

Recently, we reported an alternative approach to prepare bilayer beads [44]. We named this method the "Partial Alloc-Deprotection (PAD)" bilayer approach. In this method, the amino groups of the TentaGel beads are first protected with Alloc group and then thoroughly swollen in water, followed by deprotection with $Pd(PPh_3)_4/PhsiH_3$ in DCM for a pre-defined limited time. This procedure results in beads with a deprotected outer layer (free *N*-termini) and an Alloc-protected inner core. Figure 1b illustrated the method for the generation of bilayer beads by the PAD bilayer approach. The thickness of the outer layer (Alloc-deprotection percentage) of beads is dependent on

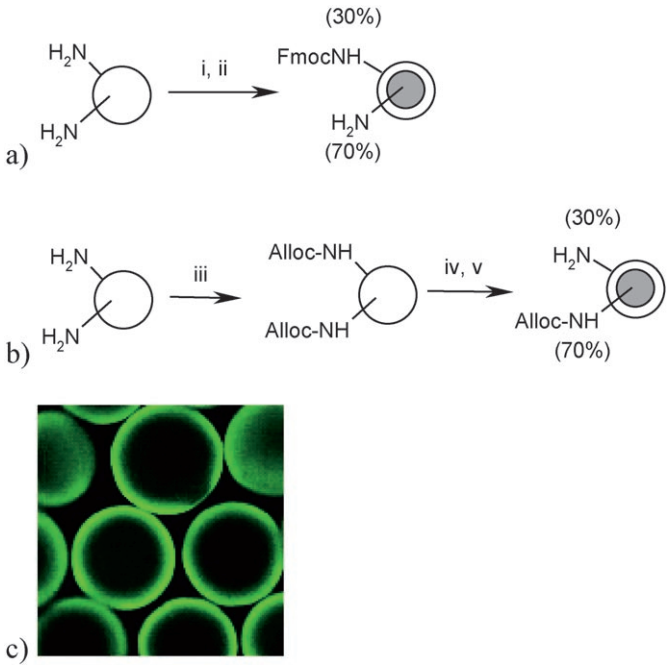


Figure 1. a) Preparation of bilayer bead using the PAP bilayer approach. b) Preparation of bilayer bead using the PAD bilayer approach; Reagents and conditions: i: swell in water for 5–24 h; ii: Fmoc-OSu (0.3 equiv. to the bead substitution) and DIEA (0.6 equiv.), 30 min; iii: Alloc-OSu (3 equiv.) and DIPEA (6 equiv.), 1 h; iv: swell in water for 5–24 h; v: $Pd(PPh_3)_4$ (0.24 equiv.), $PhSiH_3$ (20 equiv.) and DCM, 9 min. c) Confocal photomicrograph of bilayer beads prepared by the PAD bilayer approach. The outer layer was labeled with FITC.

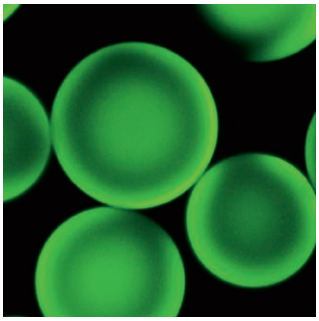


Figure 2. Confocal photomicrograph of three-layer TentaGel beads made by the PAP bilayer method.

the duration of deprotection. Figure 1c shows the photomicrograph of topologically segregated bilayer beads prepared by the PAD bilayer approach. For visualization under a confocal microscope, the outer layer of the beads was derivatized with FITC.

We have evaluated a number of different commercially available resins with different sizes, including TentaGel beads and PL-PEGA beads (Polymer Laboratories, Amherst, MA, USA) to prepare bilayer beads, and concluded that only the 90 μm (0.24 mmol/g) and 130 μm (0.26 mmol/g) TentaGel beads work. We have tried the 30 μm (0.27 mmol/g) TentaGel beads but were unable to generate any double layer. We have also found that in both PAP and PAD bilayer methods, the TentaGel beads need to be swollen in water for a minimum of five hours for reproducible results. In the PAD bilayer method, the degree of deprotection can be easily controlled by varying deprotection time [44]. Adding viscous solvents such as glycerol to the water in the bead-swelling step did not slow down the deprotection process. It is important to point out that for both methods the "bilayer step" can be employed at any stage of the library synthesis and can be repeated multiple times. As will be seen below, this flexibility allows us to prepare encoded OBOC combinatorial libraries with many different encoding schemes.

Farrer *et al.* have reported the generation of multi-layer beads using a chemical method similar to our PAP approach by employing Fmoc-chloride [45]. Concentric bead layers were produced by means of sequential protection and deprotection of the primary amines within the beads. However, instead of using a bi-phasic solvent system as in our PAP method, they used dimethylformamide (DMF) as a single-solvent system in their protection reactions, which could be difficult to control and reproduce. Using fluorescent and infrared (IR) microscopy, Rademann *et al.* studied the effects of bead size, diffusion and adsorption on the alkylation and acylation of resin beads [46]. Very recently, the same group reported a general algorithm that models the time and space dependence of chemical reactions on solid support such as beads [47]. Such studies may provide useful information that facilitates the development of better approaches for solid-phase reactions and for the construction of high-quality multilayer beads.

4 Chemical Encoding

4.1 A Peptide-based Encoding Method

Currently, there is no reliable method to directly determine the chemical structure of compound on one single bead (100 pmol) isolated from a huge diversity OBOC non-sequenceable combinatorial library, *e.g.*, 150000 members. Chemical encoding is ideal for OBOC non-sequenceable libraries. Therefore various chemical encoding methods, such as using fluorophenyl ether tags [48, 49], peptide tags [50, 51] and secondary amine tags [52, 53] have been developed. Several reviews on this subject have been published [20, 54–57]. Chemical tags are added to the bead during the synthetic steps so that the synthetic history of each compound bead in the chemical library can be recorded. These chemical codes can then be decoded by spectroscopic or chromatographic methods. However, these methods suffer several disadvantages. First, the coding structure (on the bead surface) may interfere with the screening assay involving the library compound. Second, these chemical encoding methods require that the chemistry of adding the tag and synthesizing the library be orthogonal, resulting in a nearly doubling the number of synthetic steps. Third, most of the encoding methods cannot encode a large number of compounds, *e.g.*, 100000. To solve these problems, we have recently developed a novel and robust peptide-based encoding strategy for OBOC non-sequenceable combinatorial libraries by using abovementioned topologically segregated bilayer beads [43]. In this method, the library compound and the encoding tags are "packed" into a very small volume such as a 90 μm diameter bead. Moreover, only the library molecule is on the bead surface, and the coding tags are in the bead interior (Figure 3). Therefore, the coding tags will not interfere with the screening assay. This encoding method is highly efficient as each of the building blocks is incorporated into the testing arm (bead surface) and coding peptide backbone (bead interior) simultaneously. Consequently, no additional synthetic steps are needed, and the amount of undesirable side products is minimized. After screening, the positive beads can be isolated, and the peptide coding tags, which consist of α-amino acids with side chains derivatized by the building blocks, can be directly and readily decoded by Edman microsequencing. Cleavage and retrieval of the coding tags are unnecessary.

4.2 An Encoding Method Based on Mass Spectrometry

We have successfully applied the peptide-based encoding method to the construction of different peptidomimetic and small-molecule libraries [43, 58]. One major limitation of this method is that Edman microsequencing is slow. In addition, the choices for scaffolds are limited to those having the same functional groups as the side chain of commercialized trifunctional amino acids. To further improve the OBOC technology, we developed a highly efficient encoding strategy based on mass spectrometry (MS) that is particularly suited to small-molecule libraries [59]. The decoding step with MS is simple and fast and has great potential for automation. In this method, the bilayer beads are again used to segregate the library compounds on outer layer from coding tags in the bead interior to avoid undesired interference during screening. Prior to the library synthesis, a cleavable linker is constructed in the inner core of the bilayer beads (Figure 4). The linker provides not only a cleavage site but also excellent ionization ability

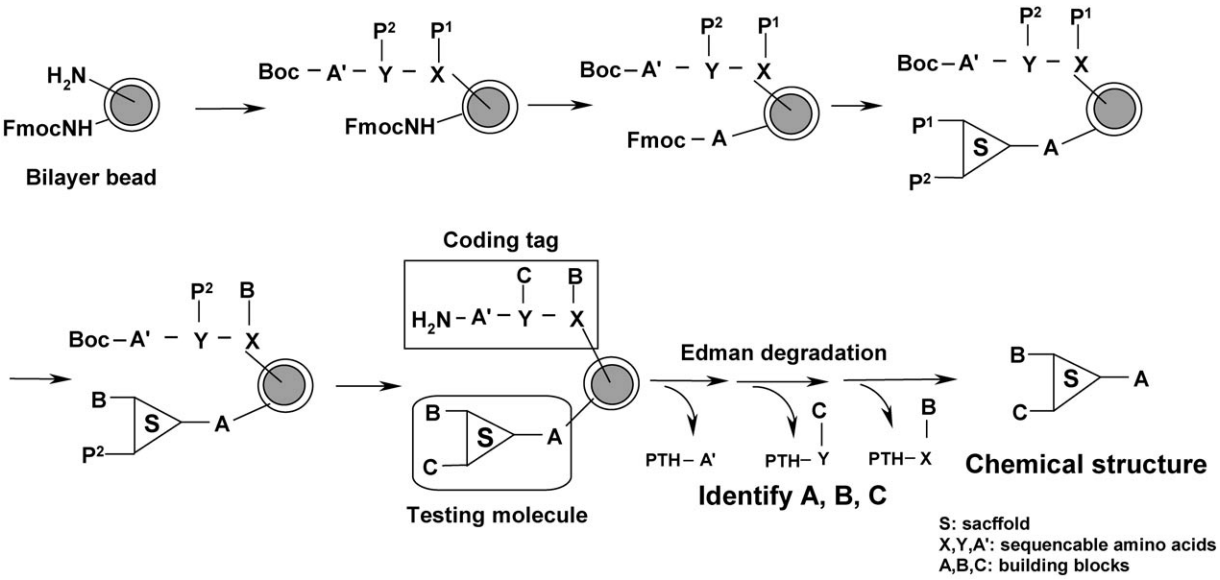


Figure 3. General encoding and decoding approach of peptide-encoded OBOC combinatorial peptidomimetic or small-molecule library (adapted from Ref. [43]).

for the coding tags. Coding blocks (C^1, C^2, C^3) chosen according to the functional groups (X, Y, Z) on the synthetic scaffold (S) are subsequently tethered to the linker. Each of the coding blocks contains a functional group (X′, Y′, Z′) that has identical or similar chemical reactivity as the one on the scaffold. During the library synthesis, the same chemical reactions occur on the scaffold and the coding blocks at the same time. Thus, the extra encoding reactions required by other methods are eliminated by combining them with the library synthesis. After biological screening, the positive beads are isolated and subjected to chemical cleavage. Only the coding tags are released from the beads and characterized by MS. The structures of building blocks (B^1, B^2, B^3) can be easily identified according to the molecular masses of the coding tags. This information enables the researcher to reconstruct the chemical structure of the active library compounds. In our study, we use Matrix-Assisted Laser Desorption/Ionization Fourier

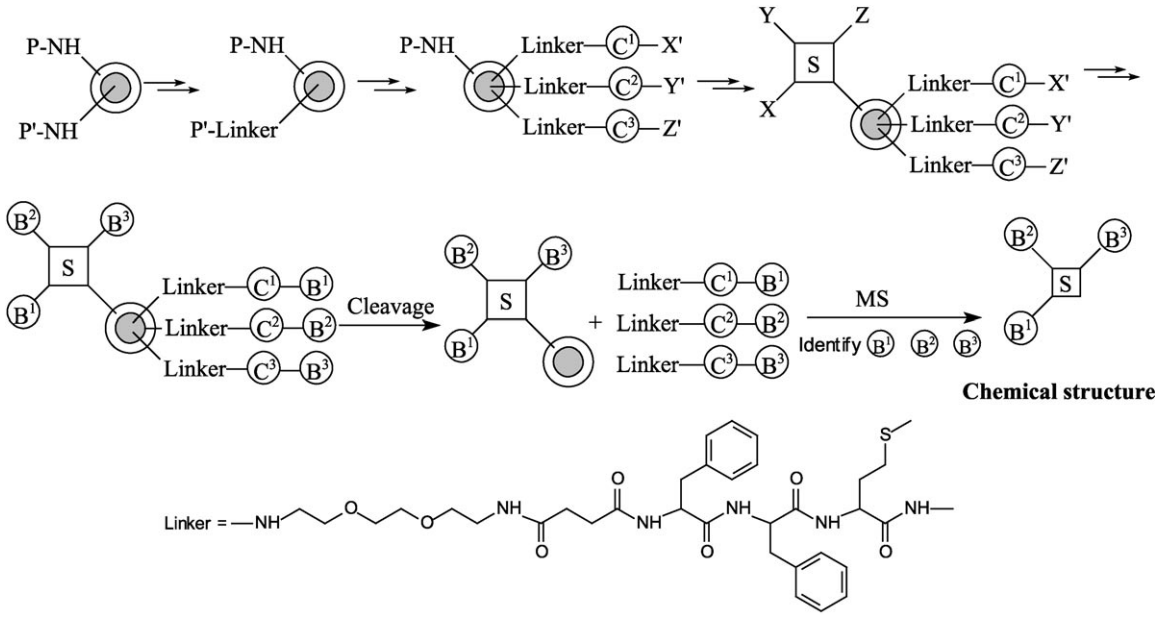


Figure 4. MS-based encoding strategy for OBOC combinatorial small-molecule combinatorial libraries; with the chemical structure of the methionine-based cleavable linker (adapted from Ref. [59]).

Transform Mass Spectrometry (MALDI-FTMS) for decoding due to its high accuracy, high sensitivity and high resolution. Considering a library using a scaffold with three diversity sites, if we use 50 different building blocks in each synthetic step, we will generate a library containing 125 000 compounds, while the number of coding tags will only be 150. Using the highly accurate MALDI-FTMS, the molecular masses of 150 coding tags can be easily discriminated without ambiguity.

This encoding method is highly efficient, fast and reliable. As the chemical reactions on the scaffold and the coding blocks are either identical or very similar, the quality of a specific coding tag on the decoding spectrum reflects the quality of the corresponding coupling reaction on the scaffold. This is very important and useful information. Currently, we can manually analyze 20 – 30 beads using MALDI-TOF MS in an hour. We envision that over 500 – 1000 beads can be analyzed in a single day with proper automation. Work is currently underway in our laboratory to fully automate the sample preparation and the laser desorption/ionization steps. Recently, this method has been further improved by using bromine/chlorine-containing coding tags. The resulting MALDI-FTMS isotope pattern of each tag clearly defines the component building blocks within each positive bead [60].

4.3 Ladder Synthesis Methods

Chait *et al.* originated the "ladder-sequencing" method for OBOC combinatorial peptide libraries with α-amino acids [61]. In this method, each positive peptide bead undergoes Edman degradation using a mixture of Phenyl Isothiocyanate (PITC, 95%) and Phenyl Isocyanate (PIC, 5%, as a terminating reagent) to generate a series of peptide ladders, which are then released for MS analysis. Youngquist *et al.* subsequently developed a "ladder-synthesis" method applicable for both sequenceable and non-sequenceable peptide bead-libraries [62]. In this method, a series of pep-

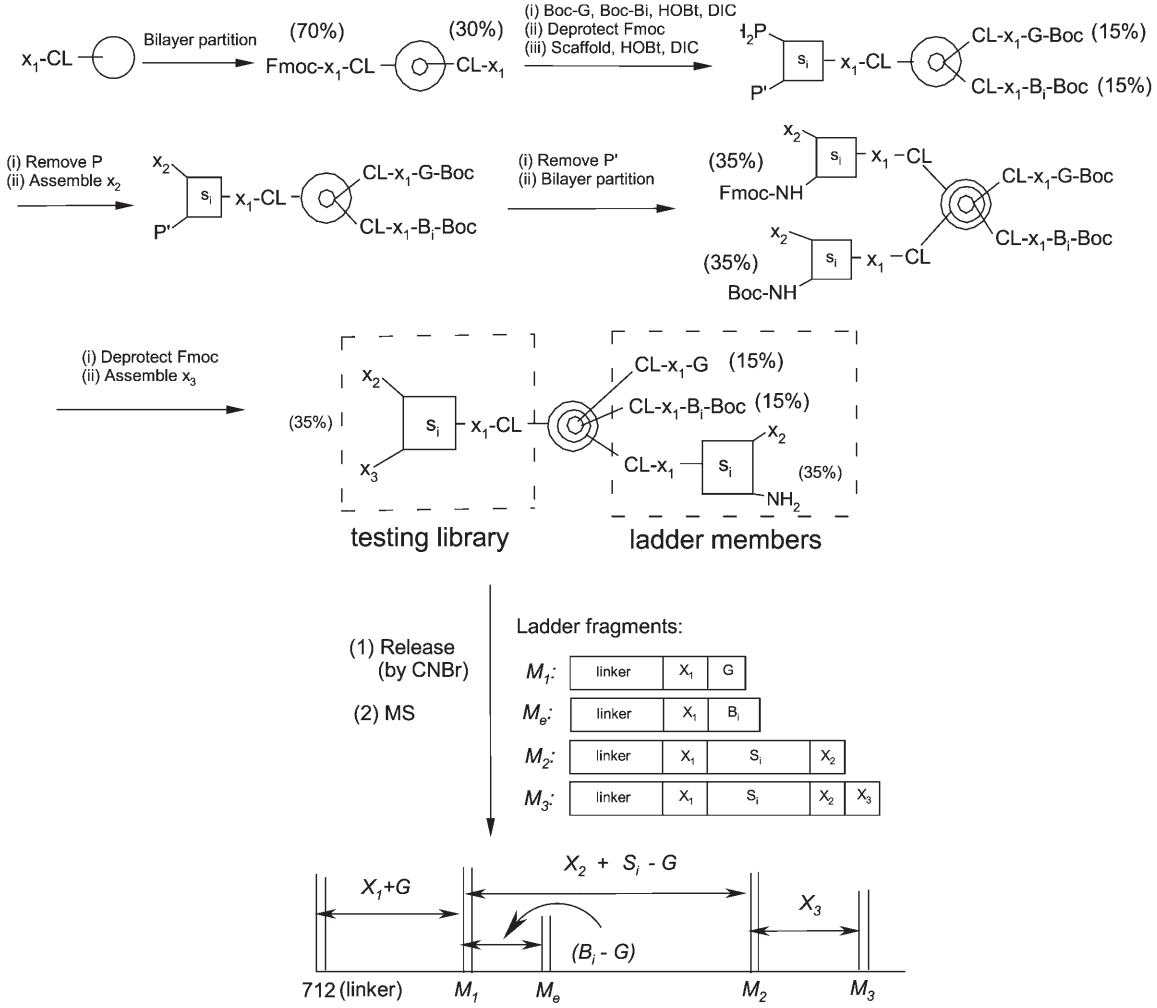


Figure 5. General synthetic scheme and MS-based decoding strategy of the scaffold-based OBOC combinatorial small-molecule combinatorial library based on the bilayer bead and ladder-synthesis concepts. 'CL' represents cleavable linker, and 'B_i' represents a coding amino acid for the scaffold (s_i) (adapted from Ref. [63]).

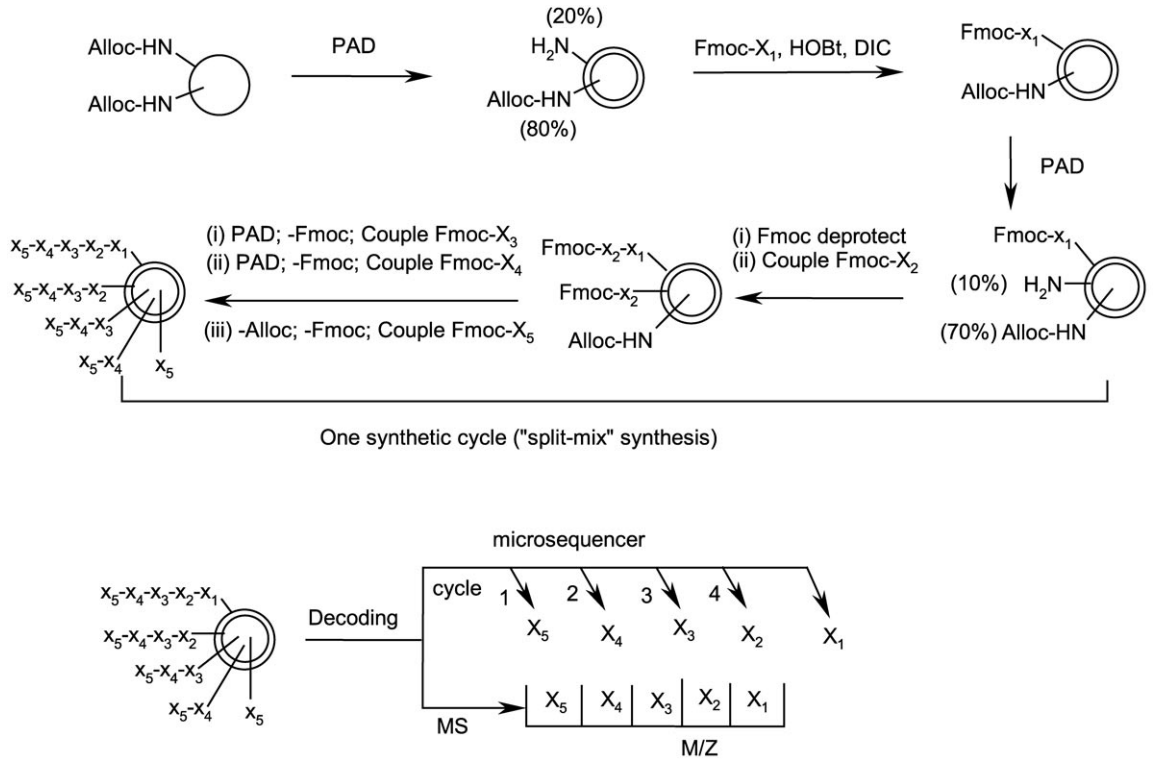


Figure 6. New ladder-synthesis method for OBOC combinatorial peptide library and MS-based decoding strategy (adapted from Ref. [44])

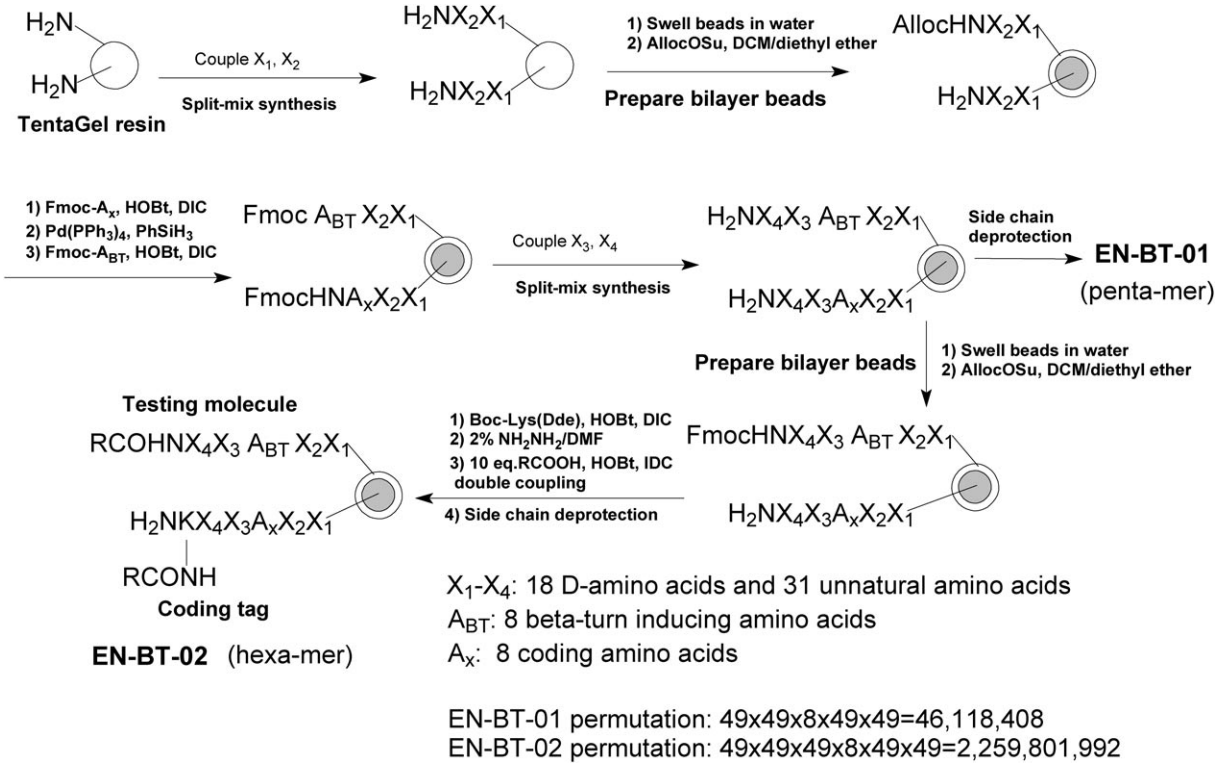


Figure 7. Synthetic and encoding approach for OBOC combinatorial β-turn peptide (EN-BT-01) and peptidomimetic (EN-BT-02) libraries.

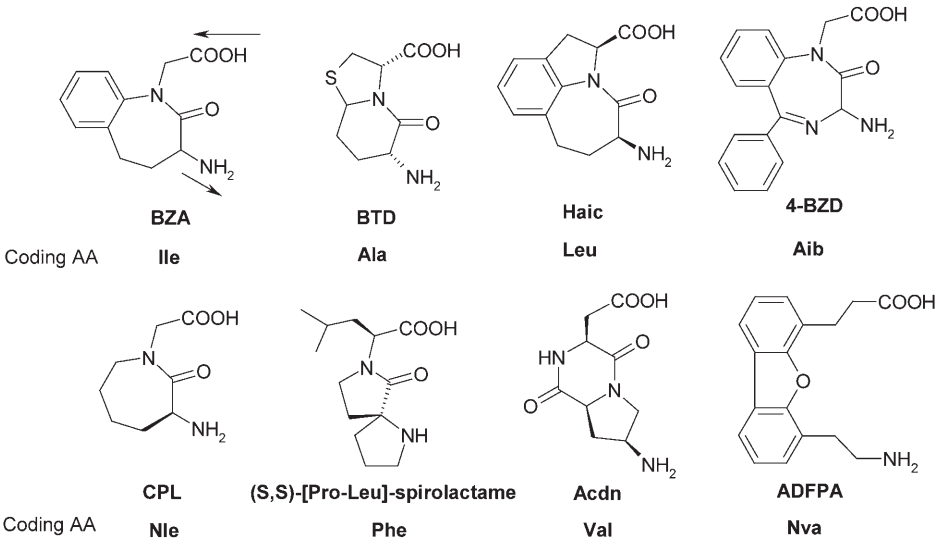


Figure 8. β-Turn inducing amino acids used for library synthesis and their corresponding coding amino acids.

tide ladders are generated during library construction, using a mixture of 95% Fmoc-amino acid and 5% acetyl amino acid as coupling agents. In both methods, the ladders are released from single bead and analyzed by MS. Based on the mass difference of each adjacent ladder, the sequence of the positive peptide can be determined. The major disadvantage of the ladder-synthesis method described by Youngquist is the presence of peptide ladders on the bead surface, which interferes with biological screening. Chait's ladder-sequencing method is restricted to libraries with sequenceable peptides or peptoids.

Recently we reported on the application of both ladder-synthesis and bilayer-bead concepts to encode OBOC small-molecule libraries [63]. Figure 5 shows the general synthetic scheme and MS-based decoding strategy of such scaffold-based small-molecule libraries. Prior to library synthesis, we assembled a Cleavable Linker (CL) containing four building blocks: methionine (efficient cleavage site by CNBr), arginine (easily protonated to generate good signal in mass spectrometry), 3-(4-bromophenyl)-β-alanine (to generate a characteristic isotopic doublet for mass peaks) and 2,2′-ethylenedioxy-bis(ethylamine) monosuccinamide (as a spacer, for improved solubility, and to produce a big mass shift for each compound beyond the noise region of matrix ions). The small-molecule library has four points of diversity (x_1, s_i, x_2, x_3) including the scaffold (s_i) itself. During the library synthesis, the above-mentioned PAP bilayer step was used twice to generate three layers, which permits the synthesis of the complete library compound on the outer layer and the other ladder members as well as the scaffold coding tag in the bead interior. Using the decoding strategy shown in Figure 5, MS analysis of the releasates from each bead allows us to unambiguously determine the chemical structure of the testing library compound on the outer layer of the beads. This encoding method is simple, reliable, and amendable for diverse small-molecule libraries.

As shown above, PAP bilayer approach to topologically segregate bilayer beads works well for the ladder synthesis of OBOC small-molecule libraries with three building blocks. However, for oligomeric libraries (*e.g.*, peptide,

Table 2. Forty-nine sequenceable unnatural and D-amino acids for construction of β-turn libraries.[a]

No.	Amino acid	No.	Amino acid	No.	Amino acid	No.	Amino acid
1	D-Asp	14	4-Pal	27	2-Thi	40	Tyr(diI)
2	Acpc	15	D-Ala	28	Dpr	41	Aic
3	D-Asn	16	D-3-Pal	29	D-Trp	42	Phe(3-Cl)
4	D-Ser	17	Acdt	30	Tyr(Me)	43	D-HoPhe
5	D-Gln	18	Ahch	31	Phg	44	Chg
6	D-Thr	19	D-Arg	32	D-Phe	45	Bpa
7	Hoser	20	Akch	33	D-Ile	46	D-Nal-2
8	Gly	21	D-Tyr	34	D-Lys	47	Ana
9	D-Glu	22	Aib	35	Tyr(diBr)	48	Phe(diCl)
10	HoCit	23	D-Pro	36	Nle	49	Cha
11	D-His	24	D-Met	37	4-Apc		
12	Hyp	25	D-Val	38	4-App		
13	Aad	26	Phe(4-CN)	39	Phe(4-Me)		

[a] Natural amino acids are designated by the standard three-letter code. Other abbreviations: Aad, α-aminohexanedioic acid; Acdt, 4-amino-4-carboxy-1,1-dioxo-tetrahydrothiopyran; Acpc, 1-aminocyclopropane-1-carboxylic acid; Ahch, 1-amino-1-(4-hydroxycyclohexyl) carboxylic acid; Aic, 2-aminoindane-2-carboxylic acid; Aib, α-aminoisobutyric acid; Akch, 1-amino-1-(4-ketocyclohexyl)carboxylic acid; 4-Apc, 1-amino-1-(4-piperidinyl)carboxylic acid; Ana, 2-amino-2-naphthylacetic acid; 4-App, 2-amino-3-(4-piperidinyl) propionic acid; Bpa, 4-benzoylphenylalanine; Bta, benzothienylalanine; Cha, cyclohexylalanine; Chg, α-cyclohexylglycine; Dpr, α,β-diaminopropionic acid; HoCit, Homocitrulline; D-HoPhe, D-homophenylalanine; HoSer, Homoserine; Hyp, hydroxy proline; D-Nal-2, D-3-(2-naphthyl)alanine; Nle, norleucine; D-3-Pal, D-3-(3-pyridyl)alanine; Phe(3-Cl), 3-chlorophenylalanine; Phe(4-CN), 4-cyanophenylalanine; Phe(diCl), 3,4-dichlorophenylalanine; Phe(4-Me), 4-methylphenylalanine; Phg, phenylglycine; Thi, 3-(2-thienyl)alanine; Tyr(Me), *O*-methyltyrosine; Tyr(diBr), 3,5-dibromotyrosine; Tyr(diI), 3,5-diiodotyrosine.

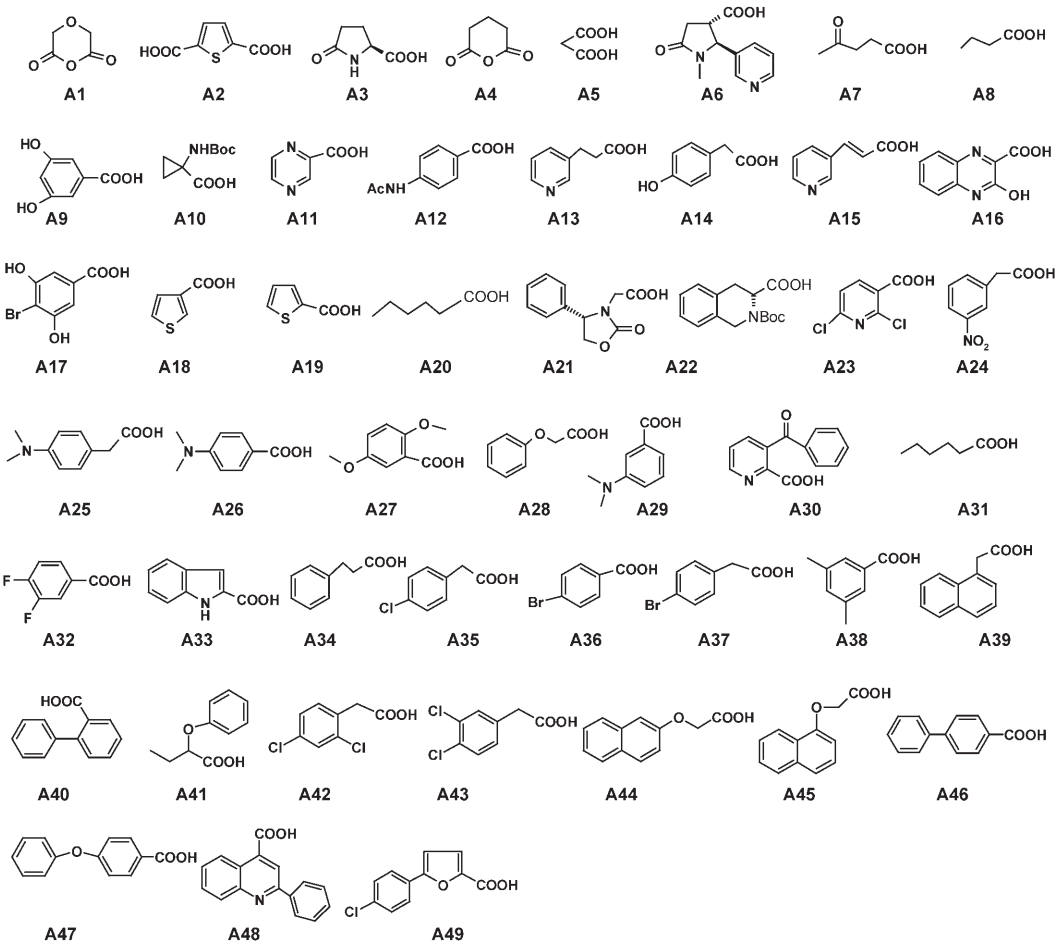
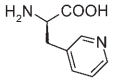
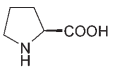
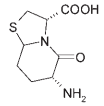
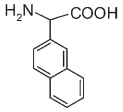
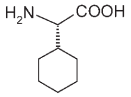
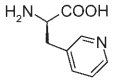
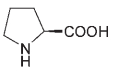
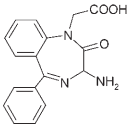
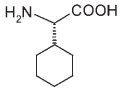
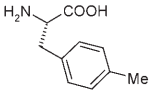
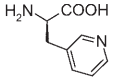
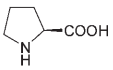
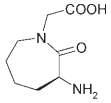
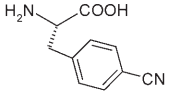
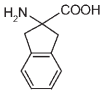
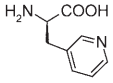
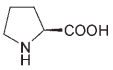
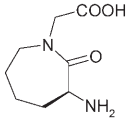
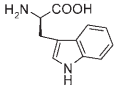
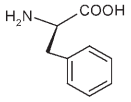
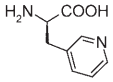
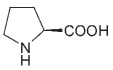
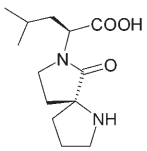
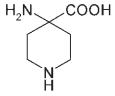
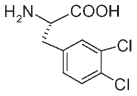
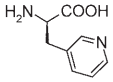
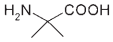
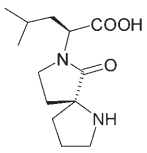
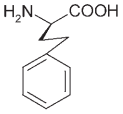
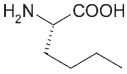
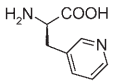
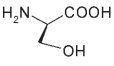
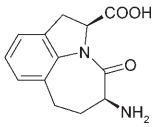
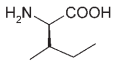
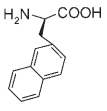


Figure 9. Chemical structure of carboxylic acids and anhydrides used to derivatize the *N*-terminal amine group in the EN-BT-02 library.

peptoid, oligocarbamate) with five or more repeating subunits, the PAD bilayer approach (see above) that was designed for this purpose, works much better. We have recently reported the use of PAD bilayer method for the ladder synthesis of an OBOC combinatorial pentapeptide library [44]. During library construction, the PAD bilayer step was employed prior to each Fmoc-amino acid coupling cycle (Figure 6). As a result, we were able to remove the Alloc protecting group, layer by layer, from the bead surface to the bead interior. By the end of library construction, each peptide bead carried a complete library compound ($X_5X_4X_3X_2X_1$-) on the outer layer, and four truncated ladder members ($X_5X_4X_3X_2$-, $X_5X_4X_3$-, X_5X_4-, and X_5-) in the bead interior. For the peptide libraries containing isobaric building blocks such as Leu/Ile and Lys/Gln, we have added an encoding step by using a small coding tag to encode one of the two isobaric building blocks. For decoding, the presence or absence of coding tag in a mass peak indicates the presence/or absence of isobaric block in the preceding position [44].

There are several advantages of the new ladder-synthesis approach. (i) In contrast to the conventional ladder-synthesis method that uses a mixture of two building blocks for coupling, this method only uses one single building block for coupling during each coupling step, therefore avoiding the problems caused by the different coupling rates of two different building blocks. (ii) All the truncated ladder members are confined to the bead interior, and only the full-length library compound is displayed on the bead surface. Consequently, the undesirable interference of ladder tags with the biological screening can be avoided. (iii) This method generates a reverse ladder enabling one to determine the subunit sequences (oligomers comprising of α-amino acid, β-amino acid, γ-amino acid or other non-sequenceable building blocks) by calculating mass differences between each two adjacent peaks in the mass spectrum. (iv) Finally, one unique feature of this reverse ladder, which refers to a ladder truncated at the carboxyl terminus therefore having the same *N*-terminus, is that the library bead is amenable to Edman microsequencing if the oligomer is a sequenceable peptide. As a result, one may section the positive bead in two halves. If the MALDI-TOF MS result obtained from one half is ambiguous, the other half can be sequenced by standard Edman microsequencing (Figure 6).

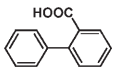
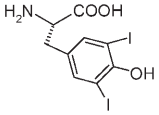
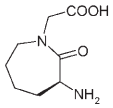
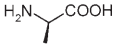
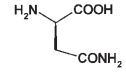
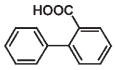
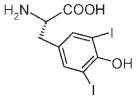
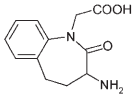
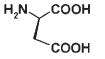
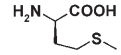
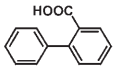
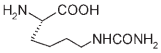
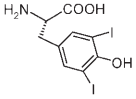
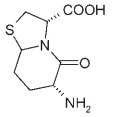
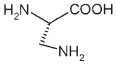
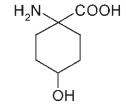
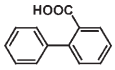
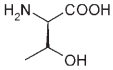
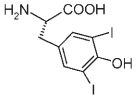
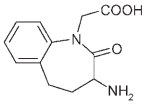
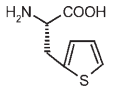
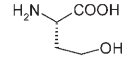
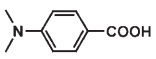
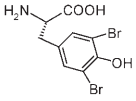
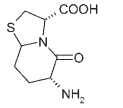
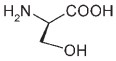
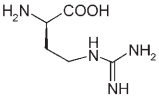
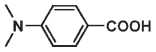
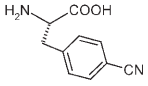
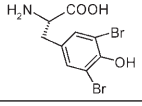
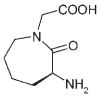
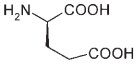
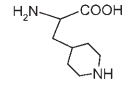
Table 3. β-Turn peptide ligands that bind to streptavidin.

No.	X_4	X_3	A_{BT}	X_2	X_1
1	D-3-Pal 	D-Pro 	BTD 	Ana 	Chg 
2	D-3-Pal 	D-Pro 	4-BZD 	Chg 	Phe(4-Me) 
3	D-3-Pal 	D-Pro 	CPL 	Phe(4-CN) 	Aic 
4	D-3-Pal 	D-Pro 	CPL 	D-Trp 	D-Phe 
5	D-3-Pal 	D-Pro 	(*S*,*S*)-[Pro-Leu]-spiro lactame 	4-Apc 	Phe(diCl) 
6	D-3-Pal 	Aib 	(*S*,*S*)-[Pro-Leu]-spiro lactame 	D-HoPhe 	Nle 
7	D-3-Pal 	D-Ser 	Haic 	D-Ile 	D-Nal-2 

5 Applications of Topologically Segregated Bilayer Beads in OBOC β-Turn Peptide and Peptidomimetic Libraries

β-Turn peptides that bind to proteins may interrupt β-sheet formation and block protein–protein interactions, providing an important source of compounds for drug development. We have employed the topologically segregated bilayer approach to prepare a β-turn penta-peptide library and a β-turn peptidomimetic library (Figure 7). These libraries use eight non-sequenceable β-turn-inducing amino acids (Figure 8) as scaffolds on the outer layer of the bead. These non-sequenceable amino acids are encoded with α-amino acids in the bead interior. Forty-nine amino acids including 18 D-amino acids and 31 unnatural amino acids are used at the positions X_1 to X_4 in the libra-

Table 4. β-Turn peptidomimetic ligands that bind to Avidin.

No.	RCOOH	X_4	X_3	A_{BT}	X_2	X_1
1	2-Biphenyl carboxylic acid 	Thi 	Tyr(diI) 	CPL 	D-Ala 	D-Asn 
2	2-Biphenyl carboxylic acid 	Thi 	Tyr(diI) 	BZA 	D-Asp 	D-Met 
3	2-Biphenyl carboxylic acid 	HoCit 	Tyr(diI) 	BTD 	Dpr 	Ahch 
4	2-Biphenyl carboxylic acid 	D-Thr 	Tyr(diI) 	BZA 	Thi 	HoSer 
5	4-(Dimethyl amino) benzoic acid 	Thi 	Tyr(diBr) 	BTD 	D-Ser 	D-Arg 
6	4-(Dimethyl amino) benzoic acid 	Phe(4-CN) 	Tyr(diBr) 	CPL 	D-Glu 	4-App 

ries (Table 2). These amino acids are sequenceable using our published sequencing protocol [40]. This greatly increases the structural diversity of the libraries.

The β-turn peptidomimetic library (EN-BT-02) is generated from the β-turn peptide library (EN-BT-01) by acylating the *N*-terminal amine group with 49 carboxylic acids (Figure 9), which are encoded by the corresponding sequenceable lysine derivatives (an α-amino acid derivative) [43]. The PAP bilayer step was used twice, during library construction, to segregate the coding tag from the testing molecule (Figure 7). Acylation of *N*-terminal amine (outer layer) and ε-amine of lysine (bead interior) occurred simultaneously. In both of these libraries, the β-turn peptides and peptidomimetics (30% bead substitution) were present on the outer layer of the bead, and the coding molecules (70% substitution) reside in the bead interior. Automatic Edman microsequencing was used to decode the chemical structure of the positive beads. This encoding method provides a general approach to synthesize peptide libraries with non-sequenceable building blocks (and even branched peptide) in certain positions and/or peptidomimetic libraries that are *N*-terminally blocked.

The β-turn peptide library (EN-BT-01, Figure 7) was screened with very low concentration of streptavidin (5 ng/mL). Seven potent ligands were identified (Table 3). All ligands contained D-3-pal in the X_4 position. Five ligands shared D-proline in the X_3 position. All seven ligands except #5 required hydrophobic amino acids in X_2 and X_1 po-

sitions. A β-turn peptidomimetic library with very similar structure (EN-BT-02, Figure 7) was screened against avidin with avidin-AP at 0.4 μg/mL. Six novel ligands were identified (Table 4). There was strong consensus among these ligands. All ligands had Tyr(diI) or Tyr(diBr) in the X_3 position. While the first four ligands (#1 – 4) preferred 2-biphenyl carboxylic acid as a derivatizing agent, ligands #5 and 6 shared 4-(dimethylamino)benzoic acid. Different β-turn-inducing amino acids were found in the A_{BT} position.

Although both streptavidin and avidin bind very strongly to biotin, it is not surprising to discover different sets of ligands for each of these proteins when screening two similar combinatorial libraries. In fact, over a decade ago, when we screened these two proteins with the same random pentapeptide library, we found that streptavidin bound to HPQ peptide and avidin bound to HPYPP peptide [31]. In our current experiment, for each of these two target proteins, there is high consensus among the different ligands even though the β-turn-inducing amino acid appears to be quite variable. This is probably because the β-turn-inducing amino acid itself is not very important as long as the β-turn conformation is preserved.

6 Increasing Screening Stringency by Down-substituting the Outer Layer of Each Bead

From our experience on using OBOC combinatorial libraries, even weak ligands (*e.g.*, high micromolar binding affinity) can be readily identified. This is not surprising as the local concentration of ligands on the surface of the TentaGel bead is rather high (estimated to be ~100 mM). Therefore, when screening focused libraries for high-affinity compounds, it is necessary to increase the stringency of the screening conditions. Common approaches for increasing the screening stringency are to lower the concentration of target protein (probe) or to incorporate soluble competing ligands in the screening buffer. A third approach is to lower the concentration of ligands on the bead surface by blocking a predetermined portion of the amino group within the bead. However, by doing so, there will be insufficient quantity of peptide or coding tags left in one single bead for structure determination. This problem can easily be solved by applying the bilayer bead concept, in which only the outer layer is down-substituted but the substitution in the bead interior remains 100% [64]. To validate this method, a model motif (p-Nle-D-I) -based focused library was synthesized (Figure 10) and screened against binding with live Jurkat cells, a T-leukemia cell line. Eighteen high-affinity peptide ligands were easily identified upon screening and therefore demonstrating the utility of the down-substitution method for high stringency of biological screening.

7 Perspectives

The OBOC combinatorial method has proven to be an invaluable tool for basic research and drug discovery. Both the library construction and screening steps are extremely fast, and the efficiency is unmatched by any high-throughput parallel synthesis and screening methods. In order to fully exploit this powerful technology, highly versatile and robust chemistry and encoding systems as well as reliable biochemical and cell-based screening assays adaptable to a variety of biological targets need to be developed. In this mini-review, we describe the development of the bilayer-bead concept in conjunction with several novel encoding schemes. Together, these methods greatly expand the versatility of the OBOC combinatorial library method. We can now develop OBOC combinatorial libraries for non-sequenceable peptides, oligomers with repeating subunits, peptidomimetic and small molecules. We have demonstrated that the bilayer-bead concept can also be applied to screening. Down-substituting the library compounds on the bead surface can greatly increase the screening stringency. Work is currently underway in our laboratory to exploit the versatility of the bilayer-bead concept by developing various novel biochemical and cell-based screening assays for OBOC combinatorial libraries.

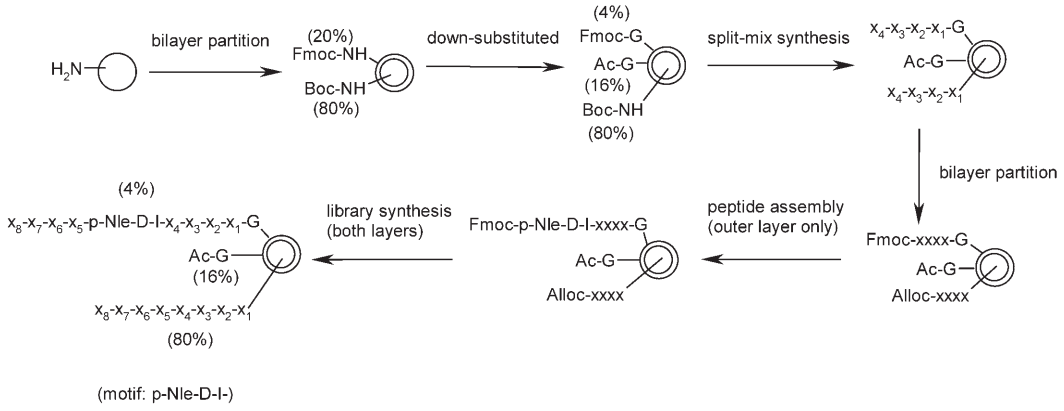


Figure 10. Synthesis of motif-based OBOC combinatorial peptide library using down-substitution method (adapted from Ref. [64]).

Acknowledgements

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