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Encoding Method for OBOC Small Molecule Libraries Using a Biphasic Approach for Ladder-Synthesis of Coding Tags

Xiaobing Wang,[†] Jinhua Zhang,[‡] Aimin Song,[†] Carlito B. Lebrilla,[‡] and Kit S. Lam*,[†]

Contribution from the Division of Hematology and Oncology, Department of Internal Medicine, UC Davis Medical Center, 4501 X Street, Sacramento, California 95817, and Department of Chemistry, University of California, Davis, California 95616

Received February 6, 2004; E-mail: Kit.lam@ucdmc.ucdavis.edu

Abstract: In the "one-bead one-compound" (OBOC) combinatorial library method, each compound bead displays only one compound entity. Hundreds of thousands to millions of compound beads can be synthesized rapidly and screened simultaneously. Positive compound beads are then isolated for structural analysis. To fully exploit the power of OBOC combinatorial small molecule libraries, a robust and high throughput encoding method is needed to decode the positive compound beads. In this paper, we report on the development of a novel encoding strategy that combines the concepts of ladder-synthesis and chemical encoding on bilayer beads. In these encoded libraries, small molecule compounds are displayed on the bead surface, and cleavable coding tags consisting of a series of truncated molecules reside in the bead interior. Such a library can be easily constructed using the biphasic approach (*J. Am. Chem. Soc.* **2002**, *124*, 7678) to topologically segregate the functionalities of the beads during library synthesis. The ladder members and coding tags are then released for MALDI-TOF-MS analysis. To simplify the interpretation of the mass spectra, we purposely add bromine into the cleavable linker so that the cleavage products generate a characteristic isotope fingerprint. The chemical structure of library compounds can be determined by analyzing the mass differences between adjacent peaks on the mass spectra. This encoding strategy also provides valuable information on the quality of the testing compound on the surface of the bead. To validate this methodology, a model OBOC small molecule library with 12,288 members was synthesized on TentaGel beads and screened against streptavidin. The chemical structures of the compound on each positive bead were unambiguously identified.

Introduction

The "one-bead one-compound" (OBOC) combinatorial library approach has been used extensively to facilitate ligand discovery. OBOC combinatorial libraries, containing hundreds of thousands to millions of compound beads, are prepared by a "split-mix" synthesis method[1] such that each bead displays only one single compound.[1a,d] The compound beads are then screened in parallel against diverse targets of interest and the positive beads isolated for structural analysis. Figure 1 summarizes the three published methods for determining the sequence of the peptide residing on one single bead. The synthesis and screening of OBOC libraries are extremely efficient, but the structural determination step is slow.[2] For example, only 3−4 peptides can be sequenced each day with an automatic protein microsequencer (Edman degradation chemistry). The "ladder-sequencing" method developed by Chait et al is much faster.[3] In this method, the positive peptide beads were subject to sequential Edman degradation using a mixture of PITC and PIC. The *N*-terminus of each bead-bound peptide was partially degraded, resulting in the generation of a series of peptide ladders, which were then analyzed by mass spectrometry. Based on the mass differences between adjacent peaks, the peptide sequence can be rapidly determined. This method, however, is limited to peptide or peptoid libraries with a free *N*-terminus[4] and cannot be applied to small molecule or peptidomimetic libraries. An alternative method termed "ladder-synthesis" described by Sepetov et al.[5a] and Youngquist et al.[5b] can overcome some of these limitations. In this method, a small portion of the peptides were *N*-terminally capped at each coupling cycle during library synthesis. The peptide ladders were then released and analyzed by mass spectrometry. Recently, Davies et al. described a similar ladder method by coupling methionine (5% of the total substitution) to the resin in each coupling step.[6] A ladder can then be generated by treating the beads with cyanogen bromide. These methods are suitable for encoding peptide or peptoid libraries

[†] UC Davis Medical Center.
[‡] University of California, Davis.

(1) (a) Lam, K. S.; Salmon, S. E.; Hersh, E. M.; Hruby, V. J.; Kazmierski, W. M.; Knapp, R. J. *Nature* **1991**, *354*, 82−84. (b) Furka, A.; Sebestyen, F.; Asgedom, M.; Dibo, G. *Int. J. Pept. Protein Res.* **1991**, *37*, 487−493. (c) Houghten, R. A.; Pinilla, C.; Blondelle, S. E.; Appel, J. R.; Dooley, C. T.; Cuervo, J. H. *Nature* **1991**, *354*, 84−86. (d) Lam, K. S.; Krchnak, V.; Lebl, M. *Chem. Rev.* **1997**, *97*, 411−448.
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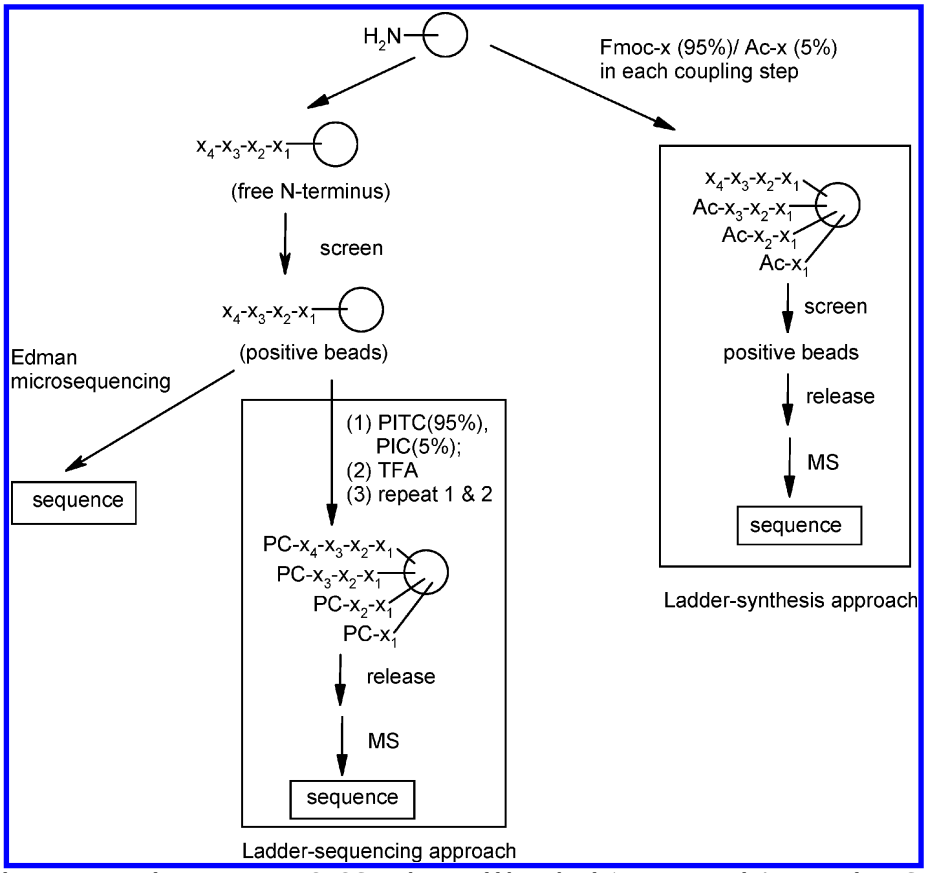

Figure 1. Methods for determining peptide sequence on an OBOC combinatorial library bead. (x = amino acid, Ac = acetyl, PITC = phenyl isothiocyanate, PIC = phenyl isocyanate, and PC = phenyl carbamyl.)

with a free or blocked *N*-terminus. Although in principle this ladder-synthesis concept can also be applied to encoding small molecule or peptidomimetic libraries, there has been no report in the literature exploiting this possibility. One major disadvantage of the ladder-synthesis method, as originally reported, is that the ladder members are displayed on the bead surface. Consequently, they may interact with the screening probe, complicating the interpretation of the screening results. Furthermore, during the capping step, the capping reagent tends to react with the bead surface, particularly if diffusion, rather than chemical reaction, is the rate-limiting step.

For chemical encoding, it would be ideal to confine the encoding tags (or ladder members) in the bead interior and reserve the outer layer of the bead for the construction of the library compound. This arrangement would eliminate the undesirable interference by the coding tag during library screening. Initially, we used proteases to generate such topologically segregated bifunctional beads.[7] However, this method is tedious and not very flexible. We subsequently developed a much more simple and robust approach to prepare the topologically segregated bifunctional beads. In this method, TentaGel beads are first swollen in water. After excess water is drained, a limiting amount of amino-protecting reagent, such as Fmoc-OSu dissolved in organic solvent, is added to the water-swollen beads. Under these conditions, only the outer layer of the TentaGel bead is derivatized.[8] This biphasic approach is easy and robust and can be applied during any of the library construction steps. We have successfully used this biphasic approach to develop two novel encoding methods for OBOC peptidomimetic and small molecule libraries.[8,9] In the present study, we now describe a third alternative chemical encoding method that takes advantages of the "bilayer bead" concept and the "ladder-synthesis" concept and applies them to small molecule bead library encoding. In this method, the library is constructed such that the library compound is on the outer layer of the bead and the sequentially truncated compounds reside in the bead interior. The full-length compound and the truncated molecules are analyzed by MALDI-TOF-MS after being released from the solid support. To demonstrate the utility and efficiency of this new encoding method, a model scaffold-based small molecule library with 12,288 members was prepared on beads and screened against streptavidin. Unique ligands with consensus motifs were identified.

Results and Discussion

Design of Cleavable Linker. Library beads for MS-based sequencing must include a cleavable linker that allows easy release of the library compounds from the beads. An ideal linker must meet the following criteria:[9] (i) selectively cleavable, (ii) compatible with the chemical conditions for library synthesis, (iii) facilitate sample manipulation and ionization for MS analysis, and (iv) facilitate peak identification. With these critical criteria in mind, we designed a new cleavable linker (CL)

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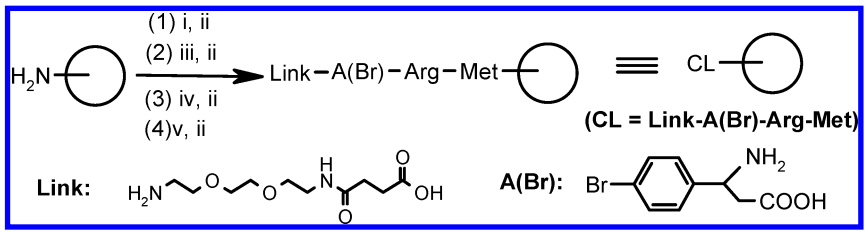
Scheme 1. Synthesis of a Cleavable Linker on Beads[a]



[a] Reagents and conditions: (i) Fmoc-Met-OH, HOBt, DIC; (ii) 25% piperidine, 10 min; (iii) Fmoc-Arg-OH, HOBt, DIC; (iv) *N*-Fmoc-3-(4-bromophenyl)-*β*-alanine, HOBt, DIC; (v) *N*-Fmoc-2,2′-ethylenedioxy-bis(ethylamine)monosuccinamide, HOBt, DIC.

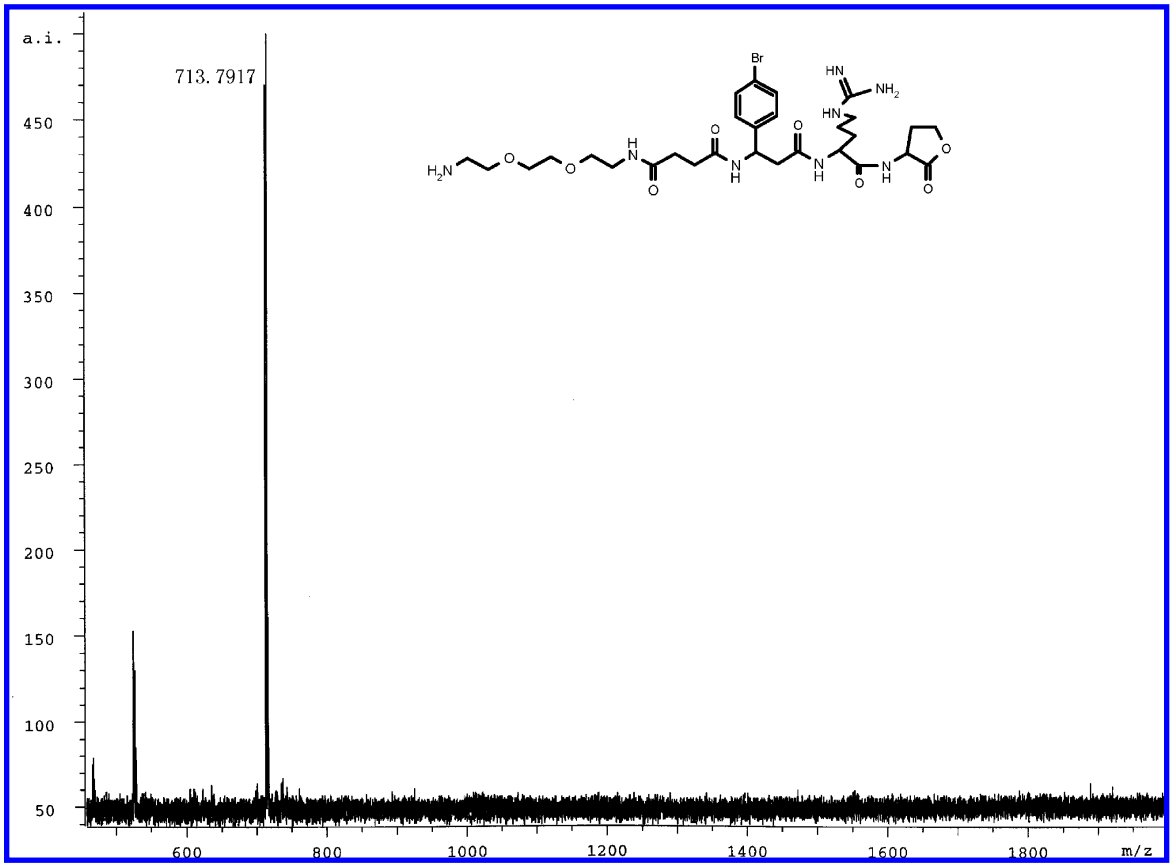


Figure 2. Mass spectrum of the cleavable linker released from a single bead. The calculated molecular mass (protonated): 713.2619.

composed of four different chemical components: methionine (Met), arginine (Arg), 3-(4-bromophenyl)-*β*-alanine (A(Br)), and 2,2′-ethylenedioxybis(ethylamine)monosuccinamide (Link). These four carefully selected linker components are sequentially assembled on TentaGel beads as shown in Scheme 1.

Rationales for using these linker components are as follows. Methionine can be chemo-selectively cleaved efficiently to form a stable homoserine lactone by cyanogen bromide.[10] Arginine will be protonated and produce an excellent signal in MS.[5b] The bromine-containing *β*-amino acid will generate a characteristic isotopic doublet for each ladder member in MS.[5a,11] Finally, the linear and highly hydrophilic PEG-like molecule will form an excellent spacer between the linker and the library compound. This spacer not only improves the solubility and extractability of the released chemicals from beads but also, when cleaved with CNBr, produces together with the other three linker components a mass shift of up to 712 Da which pushes all of the ladder member peaks away from the noise region of matrix ions.[12] Figure 2 illustrated the mass spectrum of the linker released from one single bead by cyanogen bromide. Significant background noise was detected in the region below 600 Da. The protonated peak of the linker appeared at 713.7917 (The M + 2 peak was not labeled; calculated molecular mass: 713.2619). Beyond the linker peak (>713 Da), the background is low.

General Synthetic Strategy of Scaffold-Based Small Molecule Combinatorial Library. The biphasic approach recently published by our group generates topologically segregated bilayer beads.[8] Typically, TentaGel beads with free amino groups are thoroughly swollen in water, and then the water-soaked beads are treated with a limited amount of an amine-protecting reagent such as Fmoc-OSu or Alloc-OSu in organic solvents (DCM/ether, 55:45) for a short time. Thus, the outer layer of beads is protected first. Subsequently, the interior of beads can be protected with a different protecting group such

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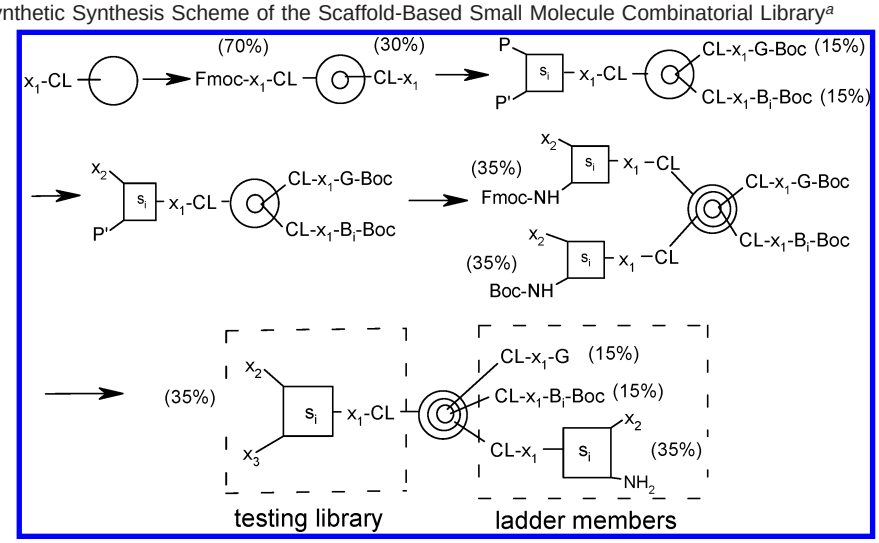
Scheme 2. General Synthetic Synthesis Scheme of the Scaffold-Based Small Molecule Combinatorial Library[a]



[a] B_i represents a coding amino acid for the scaffold (s_i).

as Boc. The thickness of the outer layer can be controlled by the amount of the first protecting reagent used. The feasibility of the bead segregation approach has been confirmed by staining the free amino group of the bead interior with bromophenol blue.[8,13] We have found that the biphasic bead partitioning approach can be employed at any point during the library synthesis. Combining this approach with the "ladder-synthesis" approach (Figure 1) permits highly diverse, encoded OBOC small molecule libraries to be easily synthesized.

Scheme 2 illustrates the general synthetic strategy of scaffold-based small molecule library. Including the scaffold itself, this small molecule library has four points of diversity (x_1, s_i, x_2, x_3). Initially, the beads are preassembled with the cleavable linker (Scheme 1) and then coupled with the first randomized residue (x_1). To generate the first ladder member in the inner core of beads, the beads are topologically derivatized with 0.7 equiv of Fmoc on the outer layer while the interior of beads remains underivatized. The segregated beads are then split into aliquots, followed by coupling with a 1:1 mixture of Boc-Gly-OH and a specific Boc-protected amino acid (B_i) to each aliquot. At this point, the first ladder member (x_1-G-Boc) and a coding tag (x_1-B_i-Boc) are simultaneously formed in the inner core of the beads. A specific B_i will be used to encode a specific scaffold (s_i) to be added in the following step. After Fmoc-deprotection of the outer layer, a specific trifunctional scaffold (s_i), with orthogonal P and P′ protecting groups, is coupled to the outer layer. The first protecting group P is then removed, and the second building block (x_2) is coupled to the second functional group of the scaffold. The second orthogonal protecting group P′ on the scaffold is then removed, exposing the third functional group on the scaffold. Prior to the coupling of the third building block (x_3), the third functional group on the scaffold needs to be partially blocked to form the second ladder member (x_1-s_i(x_2)-Boc) in the inner layer of the bead. This can easily be accomplished with the biphasic method as mentioned above. After deprotecting the Fmoc group of the third functional group of the scaffold on the outer layer of the beads, the last building block (x_3) is conjugated. After TFA treatment, all Boc groups are removed and each bead contains four components: the complete library compound on the outer layer, the second ladder member in the middle layer, and the first ladder member as well as the scaffold encoding block in the inner layer. Analysis of the mass spectra obtained from the releasates of each compound bead will enable us to unambiguously identify the chemical structure of the library compound on the outer layer of each bead.

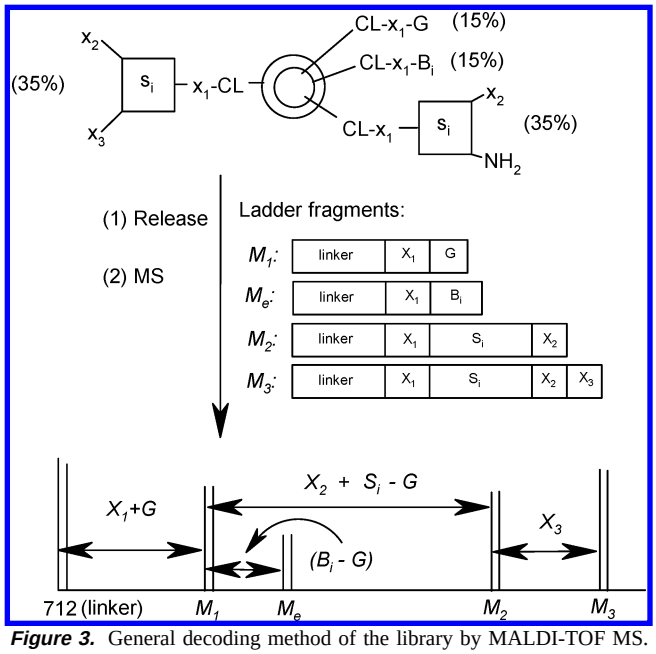


Figure 3. General decoding method of the library by MALDI-TOF MS. S_i and B_i represent the mass contribution of scaffold precursors and the coding amino acids, respectively. X_1, X_2, and X_3 represent the mass contribution from the three building blocks. G represents the mass contribution from glycine.

General Decoding Method of the Scaffold-Based Small Molecule Library. MALDI mass spectrometry has become a powerful technique for bead scale analysis due to its tolerance of complex heterogeneous mixtures and extremely high resolution.[14] The general decoding strategy of the library is illustrated in a hypothetical mass spectrum shown in Figure 3. Upon releasing compounds off a bead, three mass peaks of M_1, M_2,

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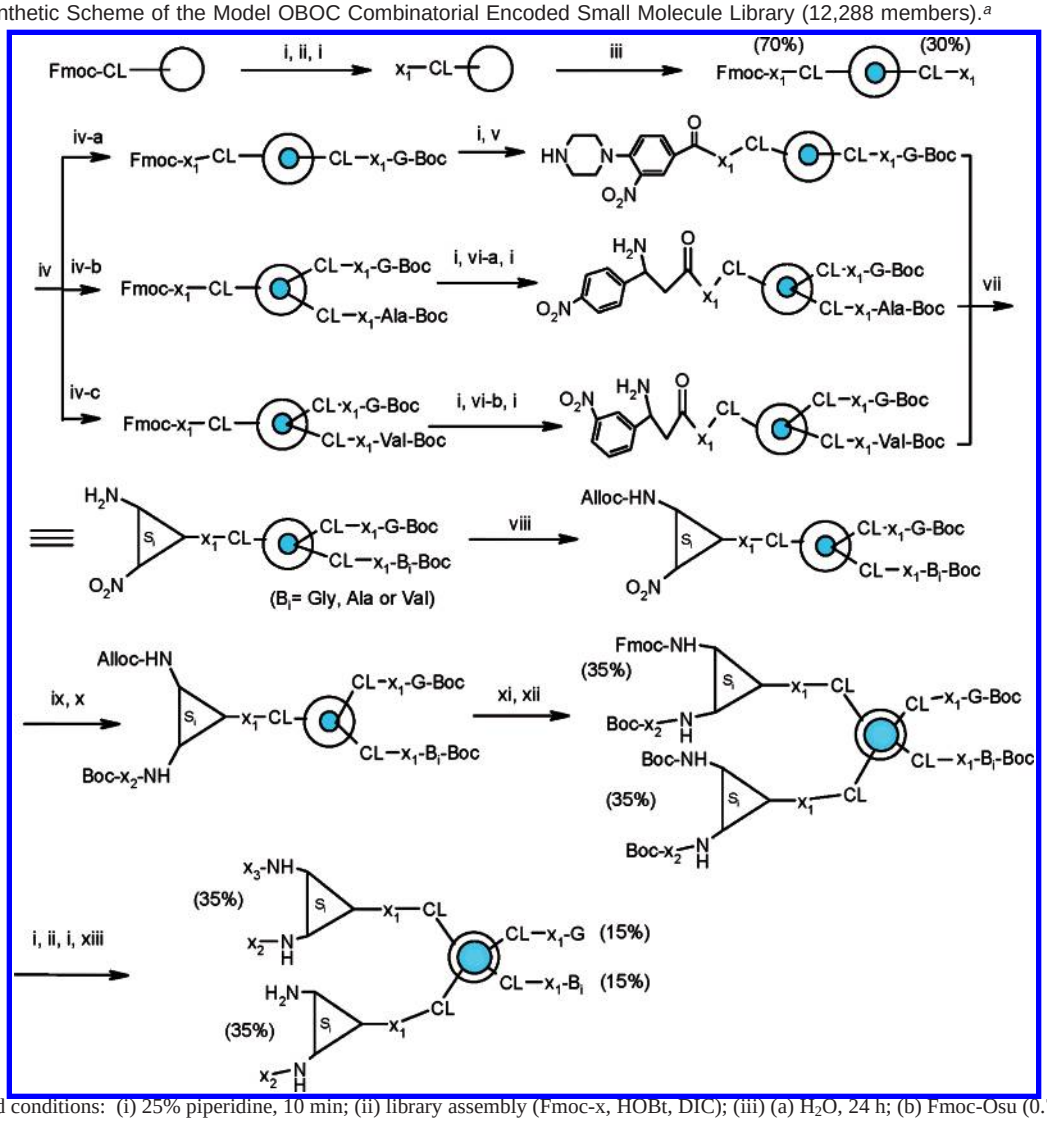
Scheme 3. Synthetic Scheme of the Model OBOC Combinatorial Encoded Small Molecule Library (12,288 members).[a]



[a] Reagents and conditions: (i) 25% piperidine, 10 min; (ii) library assembly (Fmoc-x, HOBt, DIC); (iii) (a) H_2O, 24 h; (b) Fmoc-Osu (0.7 equiv), DIPEA, DCM/ether (55:45, v/v), 30 min; (iv) split beads into 3 aliquots, and then each aliquot reacted with (a) Boc-Gly-OH, HOBt, DIC, (b) Boc-Gly-OH, Boc-Ala-OH (1.5:1.5), HOBt, DIC, or (c) Boc-Gly-OH, Boc-Val-OH (1.5:1.5), HOBt, DIC; (v) (a) 4-fluoro-3-nitrobenzoic acid (3 equiv), HOBt, DIC; (b) piperazine (10 equiv), DIPEA (10 equiv), overnight; (vi) (a) *N*-Fmoc-3-(4-nitrophenyl)-β-alanine (3 equiv), HOBt, DIC; (b) *N*-Fmoc-3-(3-nitrophenyl)-β-alanine (3 equiv), HOBt, DIC; (vii) combine all the beads together; (viii) Alloc-OSu (3 equiv), DIPEA; (ix) $SnCl_2 \cdot 2H_2O$ (2 M), 4 h; (x) library assembly (Boc-x, HOBt, DIC); (xi) $Pd(PPh_3)_4$ (0.24 equiv), $PhSiH_3$ (20 equiv), DCM/Ar, 30 min; (xii) (a) H_2O, 24 h; (b) Fmoc-OSu (0.35 equiv), DIPEA, DCM/ether, 30 min; (c) Boc_2O (3 equiv), DIPEA; (xiii) TFA-TIS-H_2O (95/2.5/2.5, v/v/v), 2.5 h.

and M_3 (for clarity, upper case italics depict molecular weights or peaks on mass spectra) corresponding to the library ladder family and a coding tag peak (M_e) can be obtained. The three peaks M_1, M_2, and M_3 appear in the order from the smallest mass value to the largest mass value. The coding peak (M_e) is located between the M_1 and M_2 because the molecular weight of coding amino acids (B_i) are higher than that of glycine (G) but much lower than those of the scaffolds. The coding peak is easy to identify due to the known mass difference between M_1 and M_e for all the coding amino acids.

In principle, the three residues (x_1, x_2, and x_3) can be directly determined by the following equations:

$$X_1 = (M_1 - 712) - G \quad (1)$$

$$X_2 = (M_2 - M_1) - S_i + G \quad (2)$$

$$X_3 = M_3 - M_2 \quad (3)$$

$$B_i = M_e - M_1 + G \quad (4)$$

Wherein, S_i and B_i represent the mass contribution of scaffold precursors and scaffold encoding tags, respectively.

In practice, X_1 and X_3 can be easily calculated by the standard ladder mass differences (eqs 1 and 3). However, for identifying X_2, B_i must be first determined (eq 4). B_i can then be used to determine S_i (through synthetic history), which in turn will enable one to calculate X_2 (eq 2).

Synthesis of Model Scaffold-Based Small Molecule Library. To demonstrate the utility of the above-mentioned encoding strategy, a model scaffold-based OBOC small molecule library was synthesized (Scheme 3). Sixteen amino acids with unique molecular weights and three trifunctional scaffolds as building blocks were used in the construction of this library. After coupling of the first building block (amino acid x_1), the beads were topographically segregated with the biphasic approach mentioned above. The outer layer was then protected with Fmoc, and the inner layer reacted with a mixture of Boc-Gly and an encoding Boc-amino acid (Boc-Ala or Boc-Val

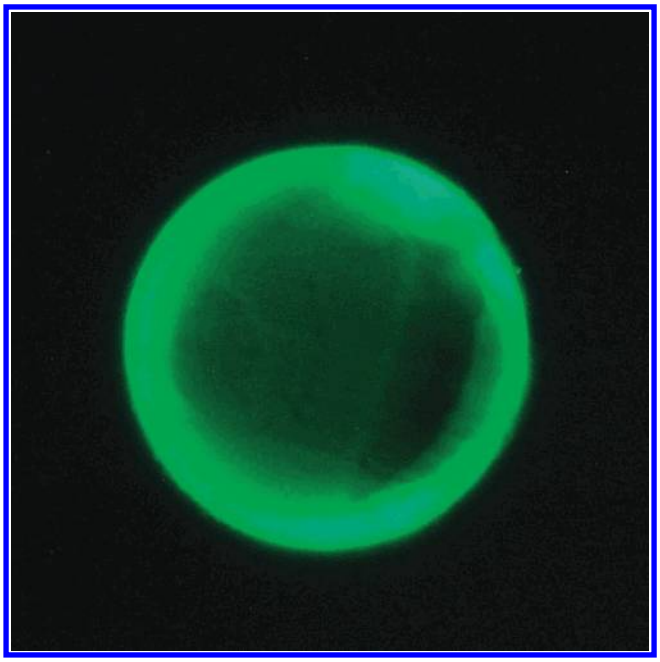


Figure 4. Photomicrograph of a bilayer half-sphere bead under fluorescent microscope. The bead interior was capped by a Boc group and appeared dark. The outer layer was labeled with FITC.

corresponding to the scaffolds 3-(4-nitrophenyl)-β-alanine and 3-(3-nitrophenyl)-β-alanine, respectively). The scaffold of 4-fluoro-3-nitrobenzoic acid was not encoded because the other two scaffolds were encoded by Ala and Val. The fluoro group of this scaffold can be readily displaced by a primary amine or a diamine to generate an amino group.[15] Here, we used piperazine to substitute the aryl fluorine. The aliphatic amines of the scaffolds were temporarily protected with Alloc, which could later be selectively removed using palladium chemistry.[16] The nitro groups on the scaffolds were then reduced with tin(II) chloride to form amines,[17] which then reacted with the second building block Boc-x_2. Upon Alloc deprotection,[16] the beads were washed and swollen in water to generate a biphasic condition again. The new outer layer was protected with 0.35 equiv of Fmoc-OSu, and the new interior was acylated by Boc_2O. Finally, the last residue was assembled to the scaffolds upon Fmoc deprotection of the outer layer. The total number of permutations in this model library was $16 \times 16 \times 16 \times 3 = 12{,}288$.

To demonstrate that the biphasic approach is still valid for the second bilayer segregation, a small amount of beads after the second bilayer segregation were labeled with FITC (a fluorescent probe) after Fmoc deprotection. A bead was sliced under a dissecting microscope with a scalpel. The half-sphere bead was then examined under a fluorescent microscope. Two distinct layers, with a fluorescent layer on the outside and a dark layer inside, were clearly visualized (Figure 4), demonstrating the validity of the approach.

MS Analysis of the Model Library. Since all ladder members are linked to the solid support with the cleavable linker, their molecular peaks must appear beyond 713 Da and must display isotopic doublets. In other words, peaks with masses less than 713 Da and those without doublet characteristics can be ignored. Considering the mass losses of glycine (17 Da loss, OH) and x_1 (18 Da loss, OH and H) in the peptide, the molecular weight of the first residue, X_1, can be calculated by the following equation:

$$X_1 = (M_1 - 712) - (G - 17) + 18 = M_1 - 752 \quad (5)$$

wherein, $G = 75$.

Subsequently, the coding peak can be used to identify the scaffold. This additional encoding step is particularly important if the scaffolds are isomers and have identical molecular mass, as in the current model library. For the encoded scaffolds, the coding peak (M_e) with a unique mass difference must appear beyond M_1. A mass difference ($M_e - M_1$) of 14 (difference between Ala and Gly) and 42 (difference between Val and Gly) unambiguously confirm that the scaffolds are 3-(4-nitrophenyl)-β-alanine and 3-(3-nitrophenyl)-β-alanine, respectively, even though these two scaffolds have identical molecular mass. In both cases, the molecular weight of the second residue, X_2, can be calculated by the following equation:

$$X_2 = (M_2 - M_1) - S_i + (G - 17) + 17 = (M_2 - M_1) - 87 \quad (6)$$

wherein, $S_i = 162$

If no coding peak is observed, the scaffold precursor must be 4-fluoro-3-nitrobenzoic acid. In this case, if $S_i = 203$, then X_2 can be calculated as follows:

$$X_2 = (M_2 - M_1) - 128 \quad (7)$$

The molecular weight of the third residue, X_3, can be determined by the mass difference between M_2 and M_3, which is independent of the scaffolds:

$$X_3 = (M_3 - M_2) + 18 \quad (8)$$

To validate the equations, two model compounds and their ladder members including coding tag were synthesized on beads. Each of these compound beads was treated with cyanogen bromide, and the releasates were analyzed by MS. The structures of the compounds and their spectra were shown in Figures 5 and 6. In Figure 5, as expected, the compound based on the scaffold of 3-(4-nitrophenyl)-β-alanine exactly matched the eqs 5, 6, and 8. Surprisingly, in Figure 6, the compound based on the scaffold of 4-(*N*-piperazinyl)-3-nitrobenzoic acid did not match the eqs 7 and 8. Further analysis by MS revealed that formylation occurred on the second ladder member of this compound during cyanogen bromide cleavage,[10] resulting in an additional mass of 28 Da. Therefore, for the library beads containing this scaffold, the eqs 7 and 8 must be modified as follows:

$$X_2 = (M_2 - M_1) - 128 - 28 = (M_2 - M_1) - 156 \quad (9)$$

$$X_3 = [(M_3 - M_2) + 18] + 28 = (M_3 - M_2) + 46 \quad (10)$$

In Figure 6, the peak at 1015.5105 Da was evidence of incomplete coupling because of the weak reactivity of the aniline. Some sodiated molecular peaks (M + Na) were also observed as small peaks with 22 Da additional masses. To confirm the validity of eqs 5, 6, 8, 9 and 10 for the library beads,

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(17) Stephensen, H.; Zaragoza, F. *Tetrahedron Lett.* **1999**, *40*, 5799−5802.

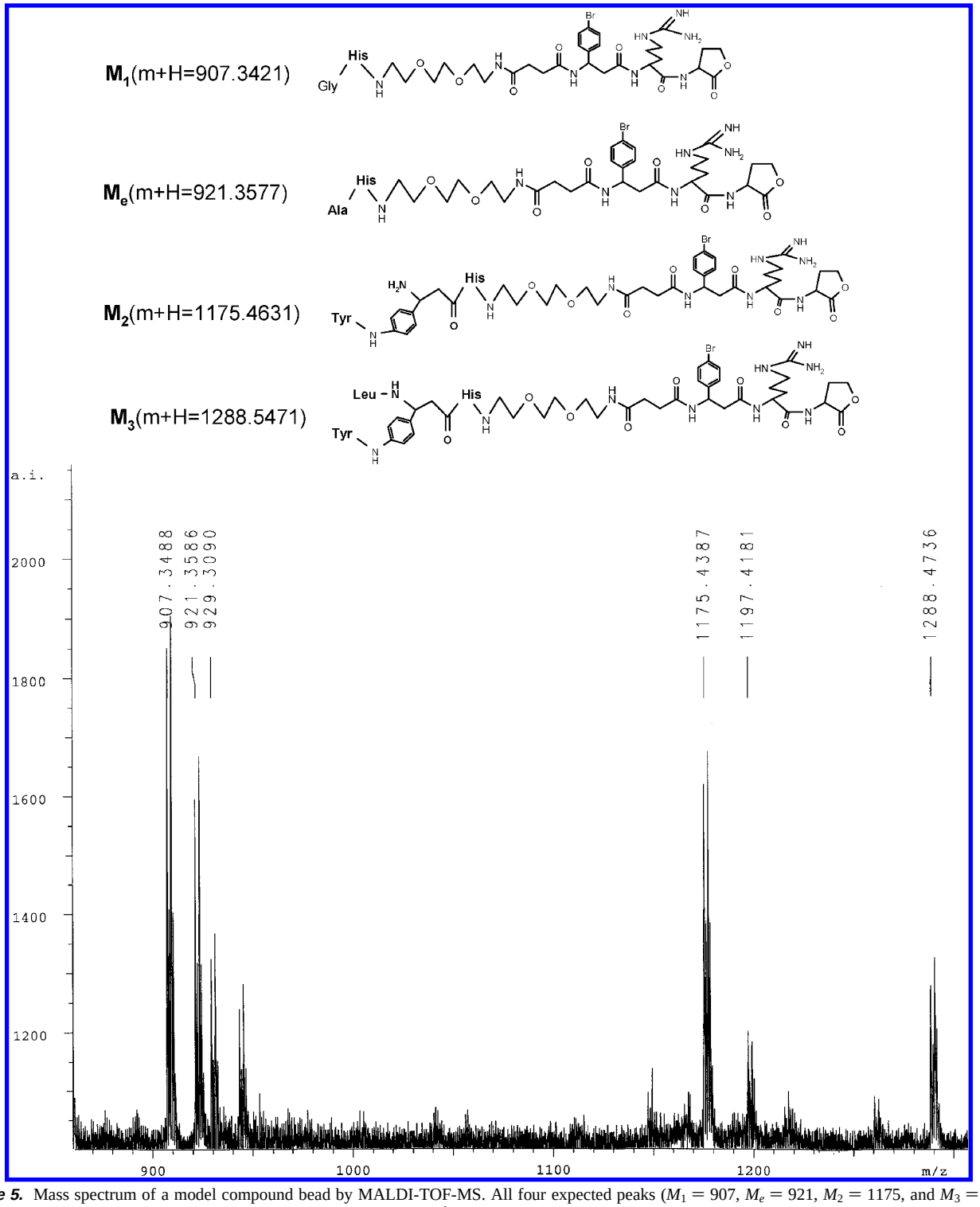


Figure 5. Mass spectrum of a model compound bead by MALDI-TOF-MS. All four expected peaks (M_1 = 907, M_e = 921, M_2 = 1175, and M_3 = 1288) were detected. x_1 = His, x_2 = Tyr, x_3 = Leu, s_i = 3-(4-nitrophenyl)-β-alanine.

20 library beads were randomly picked and sequenced. All beads were unambiguously identified using the above equations except one bead, which lost the third ladder member, probably due to low ionization. In some spectra, partial oxidation of Trp was observed as reported. However, we did not detect any bromination of Trp or Tyr.[5b] It should be noted that incomplete coupling of anilines to the second building block might result in two byproduct peaks, one located between M_1 and M_2 due to the loss of x_2, the other one located between M_2 and M_3 due to the further coupling of anilines with the third building block (x_3). It becomes evident that this encoding method will allow us to evaluate the quality of the compound on an individual bead.

Identification of Streptavidin Ligands. To test the utility of the model library, we screened the library against streptavidin-alkaline phosphatase with our standard enzyme-linked colorimetric assay[18] using a low concentration of streptavidin conjugate (1:50 000 of 1.5 mg/mL). Nineteen dark blue beads

(18) Lam, K. S.; Lebl, M. *ImmunoMethods* **1992**, *1*, 11−15.

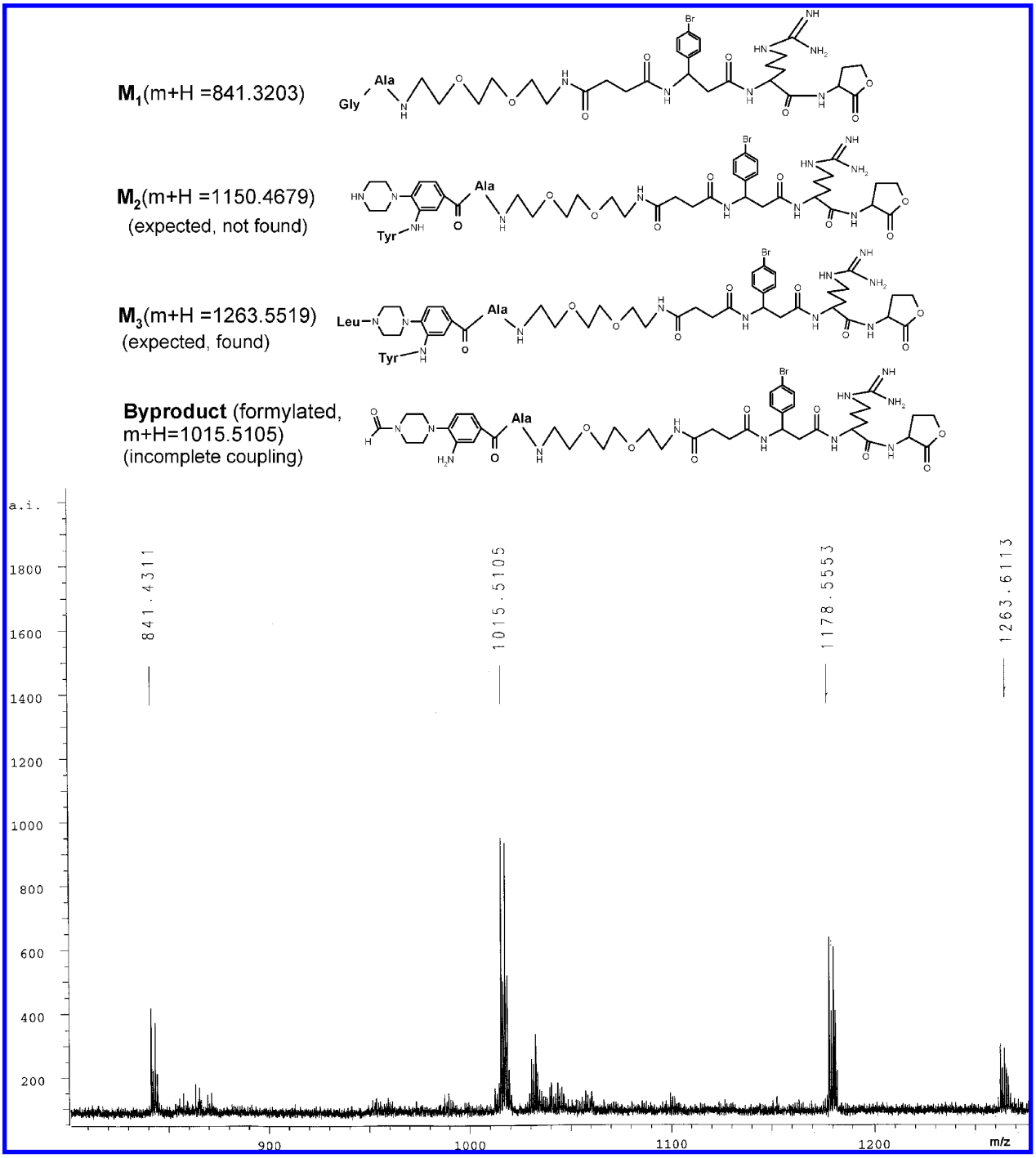


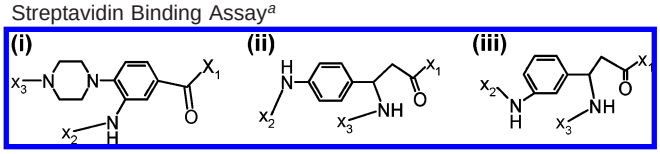
Figure 6. Mass spectrum of a model compound bead by MALDI-TOF-MS. Only one (M_1) of the four expected peaks were detected. x_1 = Ala, x_2 = Tyr, x_3 = Leu, s_i = 4-(*N*-piperazinyl)-3-nitrobenzoic acid. The mass of the actual peak of the second ladder member M_2 (1178.5553) is 28 Da larger than calculated mass (1150.4679) due to formylation during cyanogen bromide cleavage. The peak at 1015.5105 is due to incomplete coupling of aniline (deletion of Tyr, m + H = 987.4046) plus formylation. Some unlabeled smaller peaks following the high peaks are sodiated molecular peaks.

were selected and processed for MALDI-TOF-MS analysis. The results are shown in Table 1. Interestingly, the sequence of x_1= F (Phe), x_2=H (His), and x_3=H (His) are preferred for streptavidin binding. Some of these compounds were resynthesized, and their binding to streptavidin was confirmed.

Conclusions

In this report, we have developed a novel method to construct and encode scaffold-based OBOC peptidomimetic and small molecule libraries by combining the ladder synthesis mass decoding concepts[5] with our biphasic approach to encoding.[8,9] The method is simple, reproducible, and efficient. The simple biphasic approach for generating bilayer beads, during library synthesis, has greatly increased the versatility of the OBOC combinatorial library methods. Using this encoding method, the "full-length" combinatorial library compounds can be constructed on the outer layer of the bead, and the remaining ladder members and coding tags remain in the interior of the bead, thereby eliminating the potential interference of the ladder members and coding tags with the biological screening. During

Table 1. Chemical Structures of Positive Beads Isolated from Streptavidin Binding Assay[a]



entry	x_1	x_2	x_3	scaffold (s_i)
1	Y	L	L	i[c]
2	F	H	H	i[c]
3	W	H	H	i[c]
4[b]	G	H	H	i[c]
5	L	V	H	iii[d]
6	Y	F	H	iii[d]
7	F	A	F	ii[e]
8	F	N	N	ii[e]
9	F	H	P	ii[e]
10	F	H	H	ii[e]
11	F	T	F	ii[e]
12	F	T	S	ii[e]
13	F	H	T	ii[e]
14	F	D	H	ii[e]
15	W	H	N	ii[e]
16	W	E	P	ii[e]
17	Y	Y	P	ii[e]
18	Y	H	W	ii[e]

[a] The chemical structures above the table depict the scaffold structure (s_i) and the attachment sites for the amino acid building blocks (x_1, x_2, and x_3); the upper case letters are standard single letter symbols for eukaryotic amino acids. [b] This ligand appeared twice. [c] No coding peak observed. [d] A coding peak with 42 Da mass difference observed. [e] A coding peak with 14 Da mass difference observed.

library construction, the bilayer bead segregation can be performed at any point and at any desirable ratio using the biphasic approach. The new linker containing bromine with the characteristic doublet greatly facilitates the interpretation of the mass spectra. Although α-amino acids were used as building blocks in our model library, the same encoding strategy can easily be applied to libraries with other building blocks and scaffoldings. Since MALDI-TOF-MS is used as the analytical tool, the decoding step is rapid and, in principle, could be automated. Furthermore, unlike many other chemical encoding methods, this encoding approach provides useful information on the quality of the compound on each positive bead.

Experimental Section

Materials. TentaGel S NH_2 resin (0.26 mmol/g loading, 90 μm diameter) was purchased from Rapp Polymere GmbH (Tübingen, Germany). HOBt (1-hydroxybenzotriazole) and Fmoc-OSu were purchased from GL Biochem (Shanghai, China). DIC (1,3-diisopropylcarbodiimide), HATU (2-(1*H*-9-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) and 17 *N*-Fmoc-protected and *N*-Boc-protected L-amino acids excluding Cys, Ile, and Gln were purchased from Chem-Impex International (Wood Dale, IL) and Advanced ChemTech (Louisville, KY). DIPEA (*N*,*N*′-diisopropylethylamine), tin(II) chloride, piperazine, 4-fluoro-3-nitrobenzoic acid, all organic solvents, and other chemical reagents were purchased from Aldrich (Milwaukee, WI). *N*-Fmoc-3-(3-nitrophenyl)-*β*-alanine, *N*-Fmoc-3-(4-nitrophenyl)-*β*-alanine, and *N*-Fmoc-3-(4-bromophenyl)-*β*-alanine were purchased from InnovaChem (Tucson, AZ). *N*-Fmoc-2,2′-ethylenedioxy-bis(ethylamine)monosuccinamide was synthesized according to literature.[9] Fluorescein-5-isothiocyanate (FITC) was purchased from Molecular Probes (Engene, Oregon, USA). Streptavidin-alkaline phosphatase conjugate was purchased from Upstate Biotechnology (Lake Placid, NY). All solvents were used directly in the library synthesis without any purification unless otherwise noted.

General Methods. (1) For Fmoc deprotection, beads were incubated with 25% piperidine solution in DMF for 10 min twice and then thoroughly washed with MeOH, DCM, and DMF 3 times each. (2) For Alloc deprotection,[16] beads were shaken with 0.24 equiv of Pd(PPh_3)$_4$ and 20 equiv of $PhSiH_3$ in DCM for 30 min under argon and then washed with DCM and DMF 3 times each. The coupling completeness and deprotection were monitored by Kaiser or chloranil test. (3) For topological bilayer bead segregation,[8] beads were first swollen in water for 24 h. After the water was drained, Fmoc-OSu or Alloc-OSu at the desired amount dissolved in DCM/ether (55:45, v/v), and DIPEA (3 equiv) was rapidly added into the bead container and vigorously shaken for 30 min (for outer layer protection). Upon washing with DMF 3 times, the beads were then protected with the other protecting group such as Boc_2O (for interior protection). The resulting beads were washed with DMF 3 times and ready for further modification. (4) For the chemical release of compounds from the beads for MS analysis, the compound beads were thoroughly washed and then individually transferred into 200 μL polypropylene microcentrifuge tubes in ethanol under the microscope. Cyanogen bromide (20 mg/mL, 20 μL) in 70% formic acid was added into each tube and stored at room temperature overnight. All samples were lyophilized to dryness and subjected to MALDI-TOF-MS analysis. (5) For MALDI-TOF-MS analysis, samples were analyzed with a Bruker Biflex III MALDI-TOF mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany) equipped with a pulsed N_2 laser (337 nm), a delayed extraction ion source, and a reflectron. The peptide aliquot (0.5 μL) was mixed with an equal volume of matrix solution (a saturated solution of α-cyano-4-hydroxycinnamic acid in 0.1% TFA-acetonitrile (50:50) and applied to the target. The mass spectra were acquired in reflectron mode.

Synthesis of Cleavable Linker. TentaGel beads (0.05 g) were swollen in DMF overnight, and then successively coupled with a 3-fold excess of Fmoc-Met-OH, Fmoc-Arg-OH, Fmoc-3-(4-bromophenyl)-*β*-alanine, and Fmoc-2,2′-ethylenedioxy-bis(ethylamine)monosuccinamide with HOBt and DIC. Finally, after Fmoc deprotection, a single bead was isolated, treated with TFA/TIS/H_2O (95/2.5/2.5, v/v/v) for 2.5 h, thoroughly washed, and then released for MS detection.

Synthesis of Model Small Molecule Library. TentaGel beads (1.6 g) were first assembled with the cleavable linker. Upon Fmoc deprotection, the beads were evenly distributed into 16 aliquots followed by 16 Fmoc-L-amino acid couplings. The resulting beads were mixed and Fmoc deprotected. The beads were swollen in water for bilayer segregation. Fmoc-OSu (98 mg, 0.70 equiv) in DCM/ether (55:45, v/v) and 0.2 mL of DIPEA was used for the outer layer protection. The organic solvents were filtered, and the beads were washed and evenly split into three aliquots (aliquot A, B, C). For aliquot A, Boc-Gly-OH (100 mg, 4-fold excess) and HOBt/DIC were added for coupling with the beads. Upon Fmoc deprotection, 4-fluoro-3-nitrobenzoic acid (3 equiv) was coupled to the beads in the presence of HOBt and DIC followed by reaction with piperazine (10 equiv) and DIPEA (10 equiv) overnight; for aliquot B, a mixture of Boc-Gly-OH (1.5 equiv) and Boc-Ala-OH (1.5 equiv), HOBt, and DIC (3 equiv) were added for coupling with the beads. Upon Fmoc deprotection, Fmoc-3-(4-nitrophenyl)-*β*-alanine (3 equiv) was coupled to the beads followed by Fmoc deprotection; for aliquot C, a mixture of Boc-Gly-OH (1.5 equiv), Boc-Val-OH (1.5 equiv), and HOBt/DIC (3 equiv) were added for coupling with the beads. Upon Fmoc deprotection, Fmoc-3-(3-nitrophenyl)-*β*-alanine (3 equiv) was coupled followed by Fmoc deprotection. Three aliquots of beads were mixed and protected with Alloc-Osu (3 equiv) and DIPEA (6 equiv). The nitro groups on beads were reduced with $SnCl_2 \cdot 2H_2O$ (2 M, 40 mL) in DMF for 4 h. After washing, the beads were evenly distributed into 16 aliquots. The aliquots were coupled with 1 of the 16 amino acids. After Fmoc was removed, the beads were protected with Boc_2O (3 equiv) and DIPEA (6 equiv). Upon Alloc deprotection, the beads were topologically segregated again with Fmoc-OSu (0.35 equiv) for the outer layer protection and Boc_2O (3 equiv, excess) for the interior protection. Upon Fmoc deprotection, 0.05 mL of the beads was taken for slicing; the rest of the beads were equally split again into 16 aliquots followed by coupling with the amino acids.

After Fmoc removal, the beads were washed and dried followed by treatment with a cleavage cocktail of TFA/TIS/H_2O (95/2.5/2.5, v/v/v) for 2.5 h. After thorough washing, the beads were stored in 70% ethanol.

Synthesis of Model Compounds. Beads with a preassembled cleavable linker were used for model compound synthesis. Using the procedure as described above, two model compounds were ladder-synthesized on beads (0.1 g each). For compound **A**, the blocks for four residues (full-length compound and its ladder members including the coding tag) were the following: x_1=Fmoc-His (capping reagent: Boc-Gly), x_2 = Fmoc-Tyr, x_3 = Fmoc-Leu, s_i = Fmoc-3-(4-nitrophenyl)-β-alanine, B_i = Boc-Ala; for model compound **B**, the blocks for four residues were: x_1 = Fmoc-Ala, x_2 = Fmoc-Tyr, x_3 = Fmoc-Leu, s_i = 4-fluoro-3-nitrobenzoic acid (followed by the substitution of fluorine with piperazine). All beads were then treated with TFA/TIS/water (95:2.5:2.5) for 2.5 h. One bead of each compound was picked for cyanogen bromide cleavage.

Slicing of FITC-Labeled Beads. The beads taken from the above library after the second bilayer bead segregation step were treated with fluorescein-5-isothiocyanate (3 equiv) in the presence of DIPEA (6 equiv) for 5 h. After washing with DMF and MeOH 5 times each, one bead was retrieved and placed in a Petri dish. The bead was sliced with a scalpel under a dissecting microscope and then visualized under a fluorescent microscope.

Screening Against Streptavidin. The library beads (0.1 mL) were transferred into a 2 mL disposable polypropylene column and thoroughly washed with PBS and water. Using the standard enzyme-linked colorimetric assay,[18] the beads were incubated with streptavidin-alkaline phosphatase (1:50 000) for 1 h and then treated with BCIP solution for 1 h to develop color on the positive beads. The dark blue beads were collected under a microscope and washed with 6 M guanidine-HCl (pH 1.0). The blue beads were further washed with water, DMF, MeOH, and DCM. Finally, the positive beads were individually transferred into microcentrifuge tubes, and the compounds were released from the beads and analyzed by MALDI-TOF-MS.

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